Sample Preparation and Biopharmaceutical Analysis

by

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DECLARATION

I hereby certify that this material, which I now submit for assessment on the programme of study leading to the award of Doctor of Philosophy (PhD) is entirely my own work and has not been taken from the work of others save and to the extent that such work has been cited and acknowledged within the text of my work.

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ABSTRACT

In chapter 1, an overview is given of sample preparation methods and analytical techniques in use today. Each one is discussed, and relevant examples are given.

In chapter 2, the development of a method for the HPLC analysis of taurine in human plasma using acetonitrile precipitation and pre-column derivatisation with fluorescamine is presented. This procedure was found to be faster and easier to use than previous taurine assays.

In chapter 3, the evaluation of novel aspirin derivatives as prodrugs for transdermal aspirin delivery was made possible by an analytical procedure developed as part of this PhD. Following *in vitro* application of the derivative to skin, the perfusate samples in PBS could be taken and injected directly onto the HPLC system. In addition to quantifying aspirin (ASA) and its metabolite salicylic acid (SAL), the introduction of a gradient after ASA and SAL had eluted allowed determination of the parent compounds.

In chapter 4, the quantitation of ASA and SAL in human plasma using column-switching HPLC with on-line solid-phase extraction is discussed. Following a simple dilution step, the plasma was injected directly onto the system. On switching the 10-port valve, the sample containing the extracted ASA and SAL was sent to the analytical column for quantitation. The protocol was successfully applied to the investigation of levels of these compounds in healthy volunteers following an oral dose of aspirin.

In chapter 5, identification and qualitative analysis of a cachectic factor and its albumin-bound complex in the urine of cancer patients with cachexia is presented. This work was carried out using capillary electrophoresis.

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I love you very much

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"It is a great thing to make scientific discoveries of rare value,
but it is even greater to be willing to share these discoveries
and to encourage other workers in the same field of scientific research."

William J. Mayo, 1935

Table of Contents

Chapter 1 :

Introduction to Sample Preparation and Biopharmaceutical Analysis

Chapter 2 :

HPLC Analysis of Taurine in Human Plasma using Pre-Column Extraction and Derivatisation

Chapter 3 :

Evaluation of Aspirin Derivatives as Prodrugs for Aspirin and Determination of Aspirin and Salicylic Acid in Transdermal Perfusates

Chapter 4 :

Determination of Aspirin and Salicylic Acid in Human Plasma by Column-Switching Liquid Chromatography using on-line Solid Phase Extraction

Chapter 5 :

Identification and Qualitative Analysis of Protein-Mobilising Factors in the Urine of Cancer Patients with Cachexia

List of Tables

List of Figures

List of Appendices

Appendix	Appendix Title
A	Synthesis of ASA and Prodrugs
B	Use of Platelet Aggregation Profiler
C	Extraction Procedure for Aspirin
D	Presentations and Publications
E	Published Articles

List of Abbreviations

Ab	Antibody
ACN	Acetonitrile
Ag	Antigen
AGP	α1-acid-glycoprotein
AIDS	Acquired Immunodeficiency Syndrome
ASA	Acetylsalicylic Acid (Aspirin)
ASA-Ala	Aspirin Alanine
ASA-Gly	Aspirin Glycine
ASA-Iso	Aspirin Isosorbide Ester
ASA-Phe	Aspirin Phenyl Ester
ASA-PheAla	Aspirin Phenylalanine
ASA-Phe-NO_2	Aspirin Nitrophenyl Ester
BSA	Bovine Serum Albumin
CE	Capillary Electrophoresis
CGE	Capillary Gel Electrophoresis
CIA	Chemiluminescence Immunoassay
CIEF	Capillary Isoelectric Focussing
CITP	Capillary Isotachophoresis
CZE	Capillary Zone Electrophoresis
DAD	Diode Array Detector
Dimer	Aspirin Anhydride (Dimer of Aspirin)
DME	Dropping Mercury Electrode
ECD	Electron Capture Detector
EDTA	Ethylene Diamine Tetra-acetic Acid
EIA	Enzyme Immunoassay
ELISA	Enzyme Linked Immunosorbent Assay

EMIT	Enzyme Multiplied Immunoassay Technique
EOF	Electroosmotic Flow
EPA	Eicosapentaneoic acid
FIA	Fluorescence Immunoassay
FID	Flame Ionisation Detector
FMOC-Cl	9-Fluorenylmethyl chloroformate
FPD	Flame Photometric Detector
FT	Fourier Transform
GC	Gas Chromatography
GFC	Gel Filtration Chromatography
GPC	Gel Permeation Chromatography
HPLC	High Performance Liquid Chromatography
HSA	Human Serum Albumin
IAC	Immunoaffinity Chromatography
IR	Infra-Red
ISE	Ion-Selective Electrode
kDa	Kilodaltons
LC	Liquid Chromatography
LIF	Laser Induced Fluorescence
LMF	Lipid Mobilising Factor
LOD	Limit of Detection
LOQ	Limit of Quantitation
MAC	Murine Adenocarcinoma
MECC	Micellar Electrokinetic Capillary Chromatography

MI	Myocardial Infarction
MIA	Molecular Imprint Assay
MS	Mass Spectroscopy
MW	Molecular Weight
NMR	Nuclear Magnetic Resonance
NPD	Nitrogen/Phosphorous Detector
7-OHC	7-hydroxycoumarin
OPA	Ortho-phthalaldehyde
PBS	Phosphate Buffered Saline
PID	Photoionisation Detector
PMF	Protein Mobilising Factor
PMT	Photomultiplier Tube
PPP	Platelet-Poor Plasma
PRP	Platelet-Rich Plasma
RI	Refractive Index
RIA	Radioimmunoassay
RSD	Relative Standard Deviation
SAL	Salicylic Acid
SC	Stratum Corneum
SDS	Sodium Dodecyl Sulphate
SFC	Supercritical Fluid Chromatography
SFE	Supercritical Fluid Extraction
SPE	Solid Phase Extraction
TBAH	Tetra-Butyl Ammonium Hydroxide
TBAP	Tetra-Butyl Ammonium Phosphate

TCA	Trichloroacetic Acid
TCD	Thermal Conductivity Detector
THF	Tetrahydrofuran
TIA	Transient Ischaemic Attack
TLC	Thin Layer Chromatography
TNF-α	Tumour Necrosis Factor-α
UV	Ultraviolet
Vis	Visible

Chapter 1

Introduction to Sample Preparation and Biopharmaceutical Analysis

1.1 SAMPLE PREPARATION IN BIOPHARMACEUTICAL ANALYSIS

In any analytical procedure, special attention must be given to the sample pretreatment protocol since this is an integral part of the overall process. About two thirds of the total analysis time is spent on sample preparation, and 30% of all errors generated during the entire analytical procedure are introduced at this stage[1]. Contamination can be minimised during sample preparation if sufficient care is given to collecting, storing, transporting and pretreating the sample. However, these procedures are an aspect of analytical practice that is often neglected in the literature, particularly for biological samples.

1.1.1 COLLECTION OF BIOSAMPLES

In drug analysis, the most commonly sampled matrices are blood and urine[2]. Urine samples are usually collected in plastic containers and are often treated with a preservative such as boric acid; untreated urine will gradually lose carbon dioxide, thus becoming more alkaline with time. Calcium and phosphate precipitates may also form. Hence, any dipstick tests must be carried out on fresh urine in order to accurately record pH and other parameters that can change progressively. The most important factor to consider when collecting a urine sample is the volume of the sample, since often it is the amount of analyte excreted rather than its concentration that is required.

Blood is generally treated such that plasma or serum is the actual fluid analysed. Whole blood is not often analysed due to its higher viscosity and more complex nature when compared to serum or plasma. After a fresh blood sample has been taken and allowed to stand, a clot will form in about 20 to 30 minutes at room temperature (or longer at 4°C). The pale fluid which remains is serum and can be simply decanted from the clot which contains the cellular elements of blood i.e. erythrocytes, leukocytes and platelets. Alternatively, the tube can then be centrifuged to separate the clot and the serum from each other more definitively. Serum and plasma differ only in the fact that serum contains no fibrinogen, since this soluble element of blood is involved in the clotting process and is thus removed with the

cellular components. Plasma is collected in a different way. An anticoagulant is added to the collecting tube, which means that centrifugation can take place immediately, an advantage if time is an important factor. From a given volume of blood, more plasma can be obtained than serum. Care should be taken when handling whole blood such that hemolysis does not occur since this would preclude the harvesting of plasma or serum.

There are many variables involved in the collection of biological samples. The temperature of centrifugation can affect recovery of the drug from the whole blood into the plasma as illustrated by the fact that phenytoin levels increase by 10% when centrifugation takes place at 4°C instead of 24°C[3]. The type of anticoagulant used can introduce extra contamination into the plasma or can even affect the concentration of drug extracted. This has been shown in a study comparing the effect of using different anticoagulants (and even different brands) on the number and level of interferences in blank plasma[4]. Also, plastic tubes used for collection can release plasticisers or other components of the tube material into the matrix, which may interfere with the assay. The type of syringe is another consideration since cells can be ruptured during blood-taking if the needle gauge is too small. This is illustrated by the fact that taurine, an amino acid which is present in high concentrations in platelets, will be released into plasma giving a falsely elevated concentration if the platelets are disturbed by a narrow bore needle. Thus, a large bore butterfly syringe was used for taking blood samples so that no hemolysis occurred and the whole blood could then be prepared as platelet-poor and/or platelet-rich plasmas[5]. Although plasma is a very complex matrix, its composition is remarkably stable from person to person with pH and protein and salt concentrations remaining well controlled. However, lipid content can vary considerably with the timing and nature of food intake and this can require extra clean-up of the sample[2]. The time of day can affect the quality of the biological sample. Early morning blood collection is favoured to avoid diurnal variation, postural effects, postprandial changes and exercise effects[6]. For example, the total protein level can increase by as much as 9% while standing as opposed to supine, and analytes that are protein bound also increase in concentration if the person is in the erect position[6]. With so many factors affecting sample collection, analysts should make every effort to be present during the sampling or liase closely with the person collecting the samples so that any information relevant to the sample is known before analysis takes place.

1.1.2 STORAGE OF BIOSAMPLES

Another important factor in the sampling process is whether the samples are stable or not under the chosen storage conditions. Refrigeration of biological fluids is really only sufficient if the samples will be used in a matter of hours. If there is to be a significant time lapse between collection and analysis, and to ensure stability of biosamples generally, freezing is used to slow down degradation of the sample due to the action of organisms or enzymes. Freezing at –20°C or lower virtually eliminates microbial growth. Plastic containers are usually used since alterations in the concentration of the analyte can result due to adsorption onto glass. Jorgenson *et al.* circumvented the problem of adsorption of nitroglycerin and its metabolites onto glassware during the analysis of plasma by including triethylamine in the sample preparation step[7]. Adsorption onto glass was also a problem in the analysis of mitoxantrone in plasma, but when the glassware was silanised, the recovery from plasma increased from 50 to 85%[8]. However, adsorption onto denatured proteins or precipitated salts in stored samples can also occur.

Whether samples such as plasma or serum are treated to some extent (e.g. acidification) before freezing depends on the stability of the species of interest. For example, fluoride is often combined with an anticoagulant or added alone to blood samples to prevent the action of esterase enzymes[4]. If the analyte of interest is susceptible to breakdown by enzymes, preventing this action should be made a priority to ensure that the integrity of the sample is preserved. This is a factor that should be evaluated during validation of the method. Frozen blood samples should be carefully thawed just prior to analysis and the stability of the analyte through a number of freeze-thaw cycles should also be evaluated. The stability of the drug in the biofluid under different conditions of temperature, light and pH should also be assessed during development of a protocol for storage.

1.1.3 TRANSPORT OF BIOSAMPLES

Biological samples are generally packed on dry ice in well-insulated containers to protect from light, heat and other possible hazards.

1.1.4 PRETREATMENT OF BIOSAMPLES

Not all biopharmaceutical determinations require sample pretreatment, especially the cleaner matrices such as bile, cerebrospinal fluid or even urine. Generally however, some clean-up steps will be needed. There are two main approaches to sample preparation. The first is complete removal of all interferences followed by a rather non-selective separation/reaction/detection system. The second approach is partial removal of interfering compounds and/or preconcentration of the analyte followed by a more selective separation/reaction/detection system. Usually a combination of these two approaches is used. The main objectives are to extract as much as possible of the intact analyte from the matrix to the exclusion of the other matrix components. In practice, this is rarely achieved totally. The choice of sample handling procedure(s) depends on the available instrumentation and expertise, the time available, the type of matrix, the amount of sample, the concentration of analyte in the sample, the stability of the analyte, the binding of the analyte and whether qualitative or quantitative analysis is to be performed.

Many important aspects of the analysis will be influenced by these initial sample preparation techniques. For example, careful selection of solvent during extraction can save time in subsequent liquid chromatography where samples should be dissolved in the mobile phase or similar solvent in order to minimise chromatographic artifacts. Choice of the correct pore size for a dialysis membrane during sample processing can ensure that most of the endogenous interferences from a matrix are eliminated, thereby increasing the selectivity of the method. The concentration of the samples is an important parameter and must be controlled so that overloading does not occur during quantitation of the species of interest, or at the other extreme, that the method is capable of detecting the low levels required. In short, the handling of biofluids at this stage should succeed in extracting, concentrating and isolating the species to be analysed while removing as much endogenous material as possible.

There are a number of sample preparation methods in use which can be classified into four types : a) protein precipitation, b) liquid extraction techniques, c) solid-phase extraction techniques and d) membrane techniques.

1.1.4.1 Protein Precipitation

This is the simplest and oldest method for removing proteins from plasma and other biological media. It involves the addition of a water-miscible organic solvent, a solution of an inorganic salt or strong acid to a proteinaceous sample with a view to precipitating the proteins and isolating the supernatant. Following mixing and centrifugation, the supernatant containing the compound of interest is separated from the protein pellet and is analysed immediately or subjected to further clean-up.

The theory behind the use of water-miscible organic precipitating agents is that they lower the solubility of the proteins and hence the proteins precipitate out of the solution. As long as the analyte is very soluble in the organic precipitant, it will be released from the proteins into the supernatant. Sometimes a mixture of solvents is required to improve the recovery of the compound from the sample. The ratio of precipitant to sample is important since dilution at this stage will reduce the concentration of the analyte which may adversely affect the sensitivity of the analysis. Acetonitrile gives good recovery of most drugs in an efficient 1:1 or 1.5:1 ratio, while at least two volumes of ethanol are required to precipitate all plasma proteins[9], and a less powerful agent such as methanol may need a ratio of 3:1 to effect quantitative precipitation. Acetonitrile is hence the most efficient[10] and the most popular organic precipitant[11]. It is popular also because of its compatibility with subsequent chromatography eluents and also because it forms a loose floc which means that there is minimum coprecipitation of the analyte with the proteins.

The use of inorganic solutions in protein precipitation is advantageous because they can be added in small amounts causing less dilution of the sample and they generally give very clean extracts. Anionic precipitants such as strong acids e.g. perchloric and trichloroacetic acid, are commonly used at a concentration of 10 to 20% w/v in a ratio of as little as 1:10 with the sample. They work by forming insoluble protein salts with the positively charged proteins at low pH. A disadvantage of their use is that unless acidic samples are required for the assay, the samples will subsequently need to be either adjusted to a more neutral pH or extracted into a different solvent prior to analysis. Cationic protein precipitants can also be used and these consist of a solution of a heavy metal dissolved in a strong base such as zinc sulphate in sodium hydroxide. Inorganic agents can be used in combination with an organic precipitant in order to optimise recovery of an analyte – an example of this is the mixture of 10μl of 10% zinc sulphate and 100μl of methanol or acetonitrile used to extract drugs from a 100μl sample[12].

Some problems encountered when using protein precipitating agents are the possibility of further precipitation on standing (which can adversely affect the analytical instrument), and the fact that the supernatant usually still contains many interferences that are carried through the precipitation step i.e. this procedure is not very selective. These procedures are generally dilutional and hence are often not suitable for work requiring a low limit of detection.

1.1.4.2 Liquid Extraction Techniques

Liquid-Liquid Extraction

Liquid-liquid extraction is a classical method for separating a compound from a liquid matrix. It is a selective dissolution method that involves the transfer of the material of interest from the original solvent into a different immiscible solvent, leaving behind the rest of the sample. Usually an aqueous-based solution is extracted with an immiscible organic solvent e.g. dichloromethane or diethyl ether, although more unusual solvents are sometimes employed such as 1-chlorobutane, which was used to extract phenmetrazine from urine[13]. For liquid-liquid extraction, if the analyte is to be isolated in the non-polar phase, an organic solvent should be chosen that has less than 10% solubility in water, that is volatile for easy removal after extraction and that is compatible with the analytical technique to follow. The amount of solute that is extracted depends on the relative distribution between the two phases which in turn depends on the relative solubilities in the two layers. Thus, for a solute A partitioning between an aqueous and an organic solvent,

$$[A]_{org}/[A]_{aq} = K$$

where square brackets denote concentrations and K is the distribution or partition coefficient. Constant temperature and pressure are assumed and A must exist in the same form in both solvents. Equilibrium is achieved after a few minutes of vigorous shaking. If the value of K is very large, the transfer of solute into the organic phase will be easier and is considered to be quantitative. If $K < 1$, very little of the solute has been extracted. Since K is influenced by the type of organic solvent employed and the pH and ionic strength of the aqueous solution[14], these conditions must be optimised for maximum partition.

More universally applicable is the value D, the distribution constant, which takes into account species of the analyte that may be in different chemical states, some of which may be extracted and some of which may not.

$$D = c_A \text{ (org)} / c_A \text{ (aq)}$$

The value c_A in the equation above refers to the total concentration of all analyte species present in that phase. Repeated extractions (>2) with small portions of organic solvent recovers more analyte than extraction with one large aliquot. Continuous liquid extraction techniques are used when the sample volume is large, the value of D is low or the rate of extraction is slow.

The polarity of the extraction solvent is very important, and generally the more non-polar it is, the more selective the extraction procedure. There is usually a trade-off between choosing a solvent that is non-polar, where co-extraction of interferences is minimised and a solvent that is polar, where recovery of the compound is high. Some typical solvents used for liquid-liquid extraction are shown in Table 1.1, along with some of their relevant properties. For an acidic compound with a single pK_a, a pH of at least 1- 2 units lower than the pK_a value will maximise recovery by ensuring that it exists mainly in its unionised form. For a basic compound with a single pK_b, a pH of at least 1 - 2 units higher than the pK_b value will maximise recovery by ensuring that it exists mainly in its unionised form.

Table 1.1 : Order of Polarity of some Solvents used in Liquid-Liquid Extractions

Solvent	UV cut-off (nm)	Boiling Point (°C)	Viscosity (Cp @ 25°C)	
n-Hexane	190	69	0.30	**Least Polar**
Cyclohexane	200	81	0.90	
Carbon tetrachloride	265	77	0.90	
Toluene	285	111	0.55	
Benzene	280	80	0.60	
Methylene chloride	233	40	0.41	
Diethyl ether	220	35	0.24	
Tetrahydrofuran	212	67	0.46	
Chloroform	245	61	0.53	
Ethyl acetate	256	77	0.43	
Methanol	205	64	0.54	
Acetonitrile	190	82	0.34	**Most Polar**

'Salting out' is another solvent extraction technique. The addition of salts to an aqueous solution increases its ionic strength to such an extent that polar compounds can be isolated from the polar matrix. If a polar solvent e.g. n-propanol is added to an aqueous solution that has been saturated with salt, it will form a separate layer when they are mixed, although under normal circumstances n-propanol would be miscible with such a solution. 'Salting out' can also aid in the breaking up of an emulsion. Cimetidine was extracted from plasma into ethyl acetate using a salting out procedure with a saturated solution of potassium carbonate[15]. The extraction protocol resulted in recoveries of 79-81% and a limit of quantitation of 50ng/ml.

Microextraction is a form of liquid-liquid extraction where the volumes of organic solvents are greatly reduced and the analyte concentration in the organic phase is greatly increased. These extractions tend to take place in volumetric flasks using organic solvents with densities lower than that of water, so that the small volume of organic solvent accumulates in the narrow neck of the flask for easy withdrawal. However, this particular variation on liquid-liquid extraction is only suitable for analytes with high values of D.

Liquid-liquid extraction is a useful technique for extracting drugs from biological matrices such as plasma and urine. Table 1.2 lists some of the recent applications in the literature that use liquid-liquid extraction as a sample preparation procedure prior to analysis.

While liquid-liquid extractions are relatively easy to develop such that a high recovery of solute can be obtained, they are somewhat labour-intensive, difficult to automate and are susceptible to safety hazards due to the use of flammable and/or toxic solvents. Emulsions can sometimes be formed, evaporation can be slow and loss of analyte can occur at this stage. Disposal of solvents can be difficult, costly and can cause legal and environmental problems. Another drawback sometimes associated with solvent extraction is that of adsorption of the species of interest onto glassware. To overcome this, alkylsilylating agents have been used to treat the glass or amines have been included in the organic solvent. Addition of polyethylene glycol to dichloromethane prior to solvent evaporation was found to significantly improve the recovery of cortisol from urine and plasma[16].

Table 1.2 : Examples of Liquid-Liquid Extraction in Biopharmaceutical Analysis

Solvent used	Compound and Matrix	Comments	Reference
Acetonitrile-butyl chloride (1:4)	Docetaxel from human plasma	Recovery was 84.3%	17
Methanol	Irinotecan and its active metabolite from plasma	Recoveries of 87% and 90%, respectively	18
Toluene	Nisoldipine from plasma	Recovery of 94%; octanol gave a recovery of 90%	19
Diethyl ether	Fluoxetine from human plasma	Efficiency of extraction was 86 – 91%	20
Chloroform	Cocaine and 3 metabolites from rat plasma	Extraction efficiencies were 57 – 79% for the four compounds	21
Ethanol containing 1mg/ml ascorbic acid	Flavone from human serum	Recoveries over the range of the standard curve were 93 – 103%	22
Hexane	Lignocaine and bupivacaine from human plasma	Recovery of each of the compounds was greater than 95%	23
Dichloromethane containing 10% v/v isopropanol	Midazolam and its major metabolites	Recoveries were > 70%	24

Liquid-Solid Extraction

Sometimes the solute is present in a solid rather than a liquid matrix. In this case, the weighed solid, such as a tissue sample, can be extracted by bringing it into contact with the extracting solvent. The mixture is then shaken or stirred, the length of time depending on the rate of the dissolution. Shake flask methods work well when the analyte is very soluble in the extracting solvent and when the solid is porous to some extent. Ultrasonic agitation allows more intimate solid-liquid contact, and the warming of the sample during sonication can also aid the extraction process. Following this procedure, the undissolved solid material is removed by filtration and the filtrate analysed. For example, Gonzalez *et al.* used a solid-liquid extraction

protocol for the 99.3% extraction of clenbuterol from bovine liver[25]. The sample was first homogenised with barium hydroxide-barium chloride and then extracted by stirring at 37°C for 4 hours in t-butyl methyl ether before analysis.

An extension of the above process is when it is desirable to continually expose the sample to fresh extracting solvent so as to maximise the transfer of the solute to the liquid. In a soxhlet apparatus, this is made possible by placing the sample in a soxhlet thimble which receives the solvent after it has boiled, evaporated and condensed into it from above. Although a slow procedure, this type of extraction is very useful since fresh solvent is in continuous contact with the sample, the experiment can be left unattended and recoveries are very high.

Forced-flow leaching and homogenisation are two other established techniques that are now used to a lesser extent. Microwave-assisted solvent extraction is a more modern method which employs microwaves to heat solvents in contact with the solid sample.

Supercritical Fluid Extraction

Supercritical fluids combine the low densities and viscosities of gases with the solvating powers of liquids. The advantages of supercritical fluid extraction (SFE) as compared with other sample pretreatment techniques are due to the lower viscosities, (which makes for more rapid extractions), and the ability to manipulate the density (and hence polarity) by changing temperature or pressure.

SFE is more commonly used in an on-line mode, particularly in conjunction with supercritical fluid chromatography (SFC). SFE has also been coupled to gas chromatography (GC) by depressurising the supercritical fluid extract inside a conventional GC injector or by inserting the extract directly into the capillary column itself. It is technically a lot simpler than coupling SFE to high performance liquid chromatography (HPLC), due to the fact that supercritical fluids are more compatible with high temperature, non-polar, gaseous systems than high pressure, polar, liquid ones. Also, the types of compounds that are extracted well by SFE are the type of compounds that are best suited to GC analysis (volatile organic compounds), hence SFE is often used to extract non-polar analytes from solid samples. The most commonly used supercritical fluid, carbon dioxide (CO_2), is usually too non-polar to extract the majority of polar biopharmaceuticals efficiently. This is because its solubilising power lies somewhere between hexane and benzene, depending on the density. To circumvent this problem, organic modifiers can be added to the

supercritical fluid, but these modified solvents lose the advantages that make SFE an attractive alternative to other sample preparation procedures.

Temazepam has been successfully extracted from whole blood using SFE followed by HPLC[26]. Melengestrol acetate has been extracted from bovine fat tissue using a combination of SFE and SPE, giving an overall recovery of 99.4%[27]. This new procedure used a total of 12ml of organic solvent per fat tissue sample versus more than 1700ml in previous extraction procedures.

1.1.4.3 Solid-Phase Extraction Techniques

Solid-Phase Extraction

In its simplest form, solid-phase extraction (SPE) can be carried out by bulk mixing the SPE sorbent with the analyte sample and then desorbing the analyte from the sorbent prior to analysis. The first SPE applications involved the use of columns based on diatomaceous earth (celite). These columns were really sophisticated liquid-liquid columns in which the buffered aqueous fraction of a biological sample was adsorbed onto the diatomaceous earth particles forming a stationary liquid phase[28]. An organic solvent such as dichloromethane was passed through the column, which extracted the analyte from the immobilised liquid phase.

Conventional hydrophobic bonded phase extraction columns e.g. C18, and macroreticular resins e.g. polystyrene divinylbenzene, began to be used for the extraction of drugs of abuse in the 1980's. The strategy with these materials was to raise the pH to between 8 and 9 to deprotonate amines while attempting to extract the neutral and acidic compounds on the bonded hydrocarbon chains. However, there were a number of problems with these phases such as the fact that these columns extracted many interferents from the biological matrix as well as the analyte. There were difficulties too with the degree of ionisation of the compounds being extracted. Newer ion-exchange columns began to tackle this problem for charged molecules, but they were not suitable for neutral substances. It was only with the development of the phases available today that it has been possible to extract almost any molecule from almost any matrix, without necessarily requiring harsh pH conditions.

Both disposable and reusable precolumns filled with a variety of bonded phases can be used in conjunction with liquid chromatography (LC) or gas chromatography (GC) techniques. SPE is a very important technique in conjunction with high performance liquid chromatography (HPLC), especially since it is particularly suitable for the extraction of clinical samples[29,30]. These bonded phases

are mainly silica-based and Table 1.3 describes some of the sorbent types available and the samples for which each one is most appropriate.

Table 1.3 : Silica-Based Bonded-Phase Sorbents and their Characteristics

Sample Type /Extraction Type	Suitable Sorbent Type for SPE
Non-polar compounds e.g. drugs, steroids, and vitamins by reversed-phase extraction.	Octadecyl silane (C18)
Moderately polar compounds e.g. drugs by reversed-phase extraction	Octyl (C8)
Non-polar compounds by reversed-phase extraction.	Phenyl
Polar compounds e.g. amines, vitamins and phenols by normal phase extraction.	Cyanopropyl (CN)
Polar compounds e.g. drugs, amino acids, steroids and vitamins by adsorption.	Silica gel
Polar compounds e.g. proteins and peptides by normal phase extraction.	Diol (OH)
Polar compounds e.g. peptides, steroids and vitamins by normal phase extraction.	Aminopropyl (NH_2)
Amino acids by weak anion-exchange extraction.	Dimethylaminopropyl
Amino acids and catecholamines by strong cation-exchange extraction and reversed-phase extraction.	Sulphonic acid
Antibiotics and nucleotides by strong anion-exchange extraction.	Quaternary amines

Since the materials are similar to those used currently in LC techniques, except for the particle size, the same packing chemistries can be used to prepare any material in a form suitable for SPE. Sample pretreatment columns are extremely useful where large numbers of samples require analysis, thereby making reproducibility and unattended operation possible.

Although the most popular sorbent of choice for SPE, modified silica does have disadvantages when used in certain SPE applications. The hydrophobic nature of C18 and C8-bonded silicas requires a wetting step (usually with methanol) prior to use in order to condition the column. The column should not then be allowed to dry out to avoid the problem of low and/or variable recoveries. Silica is also limited to some extent by its sensitivity to pH. Very acidic solutions will hydrolyse the bonded phase while very basic solutions can dissolve the base silica. As an alternative to silica, some SPE methods employ polystyrene divinylbenzene phases. These sorbents are stable over a broader pH range. However, a wetting step is still required. Bouvier *et al.* used a new polymer-based sorbent of N-vinylpyrrolidone and divinylbenzene which they found overcame all of the limitations associated with silica sorbents when used for the SPE of a number of model compounds from serum[31]. The recoveries of the compounds were greater than 85% even when the cartridges were allowed to run dry for 10 minutes.

Residual surface silanols on silica-bonded phases can pose problems for basic or ionised compounds. Unreacted silanol groups that are ionised above pH 4 can interact with these compounds through an ion-exchange mechanism, which may prevent complete elution of the analyte. Secondary electrostatic interactions were eliminated in the extraction of basic drugs from cyanopropylsilica when a competing amine (2-aminoheptane) was added to the eluting solvent[32]. However, these secondary interactions due to the presence of the silanol groups are often useful. In fact, in many extractions, the secondary ion-exchange reaction is a vital component in the retention mechanism. Some of the newer SPE columns specifically exploit mixed-mode interactions such as the hydrophobic/cation exchange resin "Clean Screen DAU". This column has been used for the high recovery extraction of a variety of drug classes such as barbiturates and opiates from urine[33]. Patel *et al.* compared a polystyrene divinylbenzene sorbent that contained both C18 and sulphonic acid sites and a silica-based sorbent that also exhibited mixed-mode behaviour in a study where they investigated the isolation of amphetamines and barbiturates from urine using both types of SPE column[34]. They found the polymeric mixed-mode sorbents exhibited much higher reversed-phase and cation-exchange capabilities than the silica-based mixed-mode sorbents, making them more suitable for the isolation of compounds with high pK_a values. More unusual SPE cartridges such as those packed with graphitised carbon black have also been used to extract compounds of interest from biofluids. Kim *et al.* used 50mg carbon black columns (Carbopak B) to

selectively extract chloramphenicol from serum[35]. Recovery of the antibiotic using this process was 90.8%. From the wide array of columns and/or materials now available, a little method development usually yields a tailored extraction protocol that provides specific interactions for the analyte of interest. Applications of the use of SPE in biopharmaceutical analysis are shown in Table 1.4.

Table 1.4 : Examples of Solid-Phase Extraction in Biopharmaceutical Analysis

Sorbent	Compound and Matrix	Comments	Reference
C18	Ivermectin in bovine plasma	Columns were dry-packed and reusable. Limit of quantitation of 2 ng/ml by HPLC. 76% recovery	36
C2	Seven benzodiazepines in urine and plasma	Recoveries all greater than 90%. Recoveries also examined from C8, C18, CN, phenyl and cyclo-hexyl	37
C18	Amphetamine and metham-phetamine in human urine	Detection limits of 4 and 2 ng/ml, respectively, by HPLC	38
NH_2	Nisoldipine in plasma	Limit of quantitation of 0.1 ng/ml by GC. Bond Elut® NH_2 cartriges gave only 50% recovery. Baker® NH_2 cartridges gave 100%	19
C18	Serotonin in whole blood and platelet-rich plasma	Linear from 0.5-3000 ng/ml by HPLC. Recovery was 100%	39
C18	Doxorubicin and prochlor-perazine in human plasma	Limits of quantitation were 6.25 and 10.0 ng/ml, respectively, by HPLC. Recoveries were 78% and 89%, respectively	40
CN	Eight basic drugs from human plasma	Absolute recoveries were found to range from 82-99%	41
C18	Tilmicosin in bovine and porcine sera	Limit of detection 0.05 μg/ml by HPLC. Recoveries were 91-95% for bovine serum and 85-93% for porcine serum	42
C18	Morphine and its metab-olites in serum, blood, urine, cerebrospinal fluid and vitreous humor	Limit of quantitation for morphine was 1 ng/ml by HPLC. Recovery ranged from 85 to 98% for each analyte	43

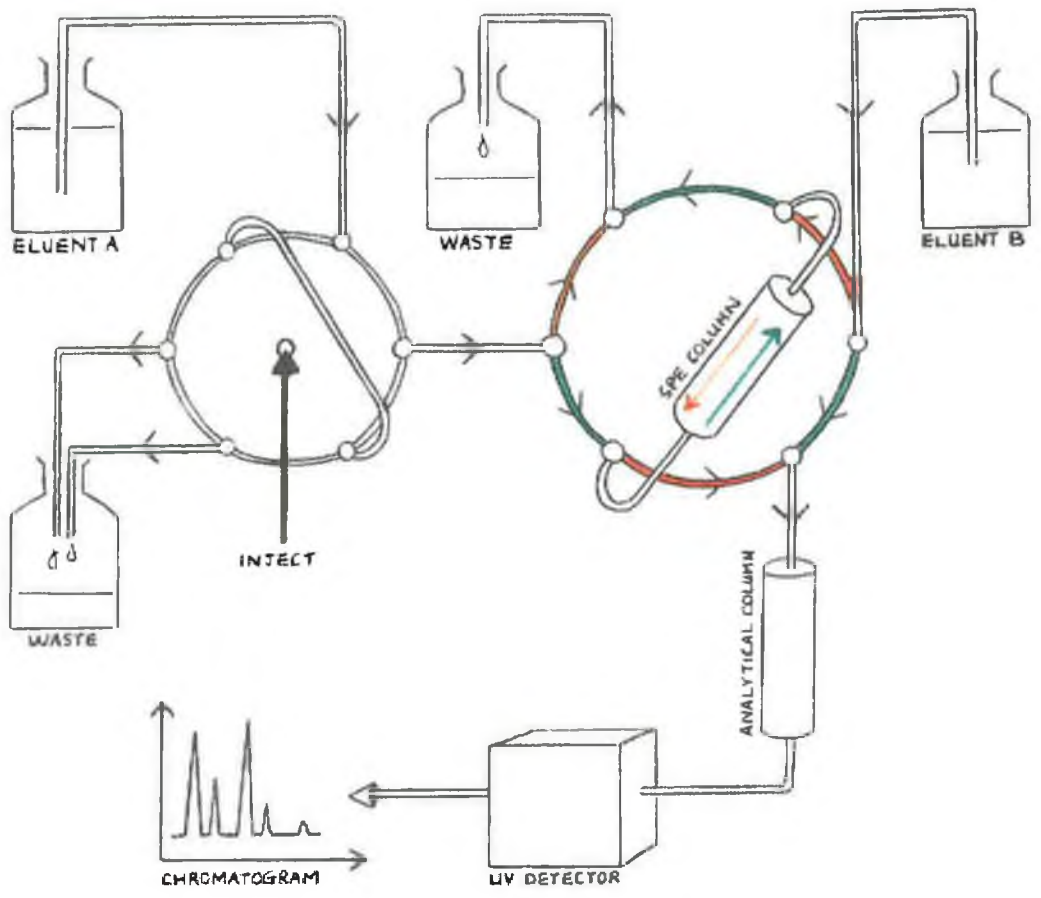
C8	Simvastatin and its active metabolite in human plasma	Limit of quantitation was 0.1ng/ml for both compounds by HPLC	[44]

Recently, as an alternative to precolumns, ultra-low mass sorbent bed cartridges and membrane extraction disks have been introduced which contain a suitable stationary phase e.g. silica-bonded C18. These allow a significant reduction in solvent usage and extraction times. One interesting application of membrane extraction disks incorporated 4mm size disks into the hub of a syringe needle for the extraction of organic compounds from human urine and serum[45]. Empore® disks have been used in the 96-well microtitre plate format for the extraction of pharmaceuticals from biofluids, allowing elution volumes to be reduced to as low as 30μl[46].

Although traditionally an off-line procedure, SPE has been used on-line with HPLC. With on-line SPE-HPLC, a column-switching device is used which requires a valve and usually an extra pump. The analyte should be less retained by the concentration column than by the analytical column, and the flow rate through the precolumn should be less than or equal to that through the analytical column. The wash solvent for the first column should be a weakly eluting solvent for the analyte to ensure its retention on the SPE column, and must be miscible with the mobile phase for the second column. A diagram of such a system, which uses a 10-port valve for sample introduction, and switching of the solvent flow between the columns, is shown in Figure 1.1.

The sample is loaded onto the loop with the valve in position 1. The valve is then turned to position 2, causing the sample to be swept onto the short SPE column, which will have been chosen to selectively retain the compound of interest, ideally to the exclusion of most other components of the matrix. The precolumn is flushed with a solvent that will wash any lingering interferents from the column, leaving the analyte unaffected. During this time, the mobile phase is equilibrating the analytical column. After a few minutes, the valve is switched back to position 1, whereupon the analyte(s) are desorbed and backflushed onto the analytical column for quantitation. In order to ensure that these three steps achieve their aim, it is necessary to optimise parameters such as pH, composition of solvents, length of time for loading, flushing and eluting, amount of sample etc.

Figure 1.1 : Diagram of Column-Switching SPE-HPLC System



Valve in Position 1 **Valve in Position 2**

The main advantages of SPE include the facts that trace enrichment is often possible when large volumes are loaded, and the elution volume is small. This has an obvious positive impact on the levels of sensitivity possible. The precolumns can be designed to suit any laboratory since they can be purchased commercially (and batch to batch reproducibility is continually improving) or prepared in-house (which allows rapid screening of a number of packings in a short time). There are now commercially available instruments that allow the use of disposable precolumns for column-switching such as the ASPEC® system from Gilson and the Prospekt® system from Spark-Holland. The Prospekt system is a fully automated on-line sample clean-up and injection instrument which has a number of advantages over conventional SPE. Firstly, the use of a high pressure pump means that the flow rate through the

cartridges is constant and the high back pressures do not alter this flow rate. Secondly, there is no carryover effect or contamination introduced because the entire process takes place in a closed system and the cartridges are only used once and are disposable. Thirdly, the entire analysis is considerably faster and less labour-intensive than manual manipulation of SPE cartridges. The Prospekt system has been used for the extraction of serotonin from whole-blood samples and platelet-rich plasma using C18 cartridges[39]. When compared to a previous method for serotonin, based on perchloric acid deproteinisation, good correlation was obtained. Reproducibility varied from 2-5%, depending on the matrix, and recovery was 100%.

Immunoaffinity Extraction

Antibodies have been used for many years in immunoassays but have only been employed for sample pretreatment in the past two decades. Immunoaffinity chromatography (IAC) is an excellent way of increasing the speed, sensitivity and selectivity of the clean-up process. It involves the binding of antibodies to a short precolumn that precedes the analytical apparatus in a setup similar to on-line SPE. With on-line IAC-HPLC, the immuno-precolumn is connected in series with the HPLC column, sometimes linked by a second precolumn which allows efficient refocusing prior to analysis as well as relieving some of the pressure build-up in the system. Many of the immunoaffinity matrices are preactivated and some are even prepackaged into columns or cartridges. This allows easy and efficient coupling of the antibodies. The use of commercially available immunocolumns has also been reported e.g. anti-aflatoxin IAC cartridges from Biocode (U.K.)[47]. The most important hyphenated technique to emerge is IAC-HPLC, but IAC has been used in other configurations such as IAC-GC-mass spectrometry [48,49].

There are also two very obvious advantages for using IAC in tandem with HPLC. The first reason is the specificity of the antibody for the analyte, and although this specificity is not absolute, that exhibited by the antibody-analyte interaction is far greater than any physiochemical system available. The second reason is the possibility of creating antibodies against any particular analyte of interest. There are other advantages such as the fact that due to selective preconcentration, there is a high probability of excluding false positive results in screening, reusable immunocolumns are an economical option and modification of existing HPLC instrumentation requires only a switching valve and an additional pump. Oestrogen has been extracted from plasma[50] and propranolol has been extracted from urine[51], both with reusable

columns, in just two examples of on-line IAC-HPLC. Alongside the advantages of immunoaffinity extraction, the increasing number of applications documented in the literature is evidence of its acceptance in the field of analysis.

However, IAC has disadvantages too. There can be problems in the raising of suitable antibodies, which takes time and can be expensive. Immobilisation procedures can be inefficient and difficult to optimise. Most importantly, non-specific binding can occur which would render the extraction less selective. However, as with other extraction techniques, the few disadvantages it has can be overcome by careful consideration and understanding of all the criteria involved. The three most important parameters during IAC method development are the choice of solid support, immobilisation procedure and desorption conditions.

Molecularly Imprinted Polymers

Molecular imprinting allows the creation of selective recognition sites in synthetic polymers, and as such these imprinted polymers are mimics of antibodies. The imprints are made by polymerising functional momoners in the presence of the 'print' molecule. Removal of the molecule from the polymer results in sites within the polymer network that are complementary to and have affinity for the original print molecule. These polymers can be incorporated into columns and used for the extraction of analytes from biological matrices. A molecular imprinted polymer to 7-hydroxycoumarin (7-OHC) has been prepared and used as an SPE sorbent for the extraction of 7-OHC from urine[52]. Extraction recovery was greater than 90%.

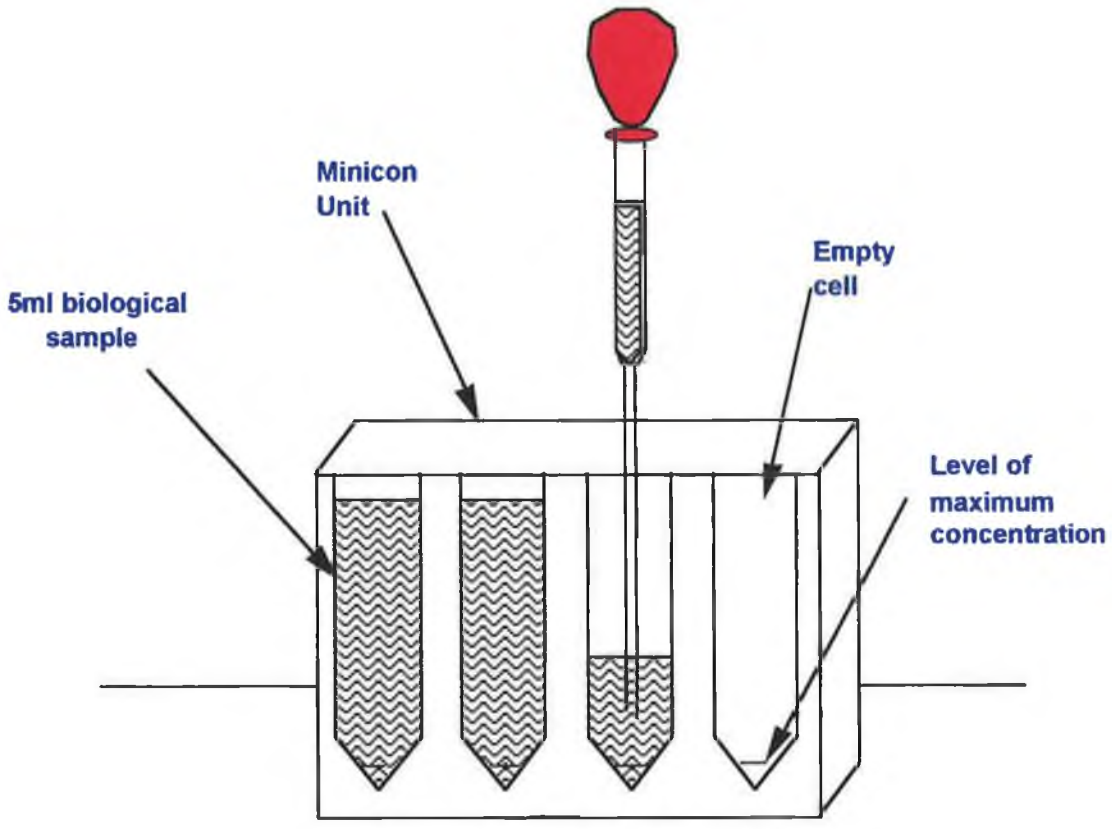
1.1.4.4 Membrane Techniques

Dialysis

With this sample preparation method, analytes are separated by their ability to diffuse through a membrane as a result of a concentration gradient. It is usually employed to eliminate small-sized species from a sample matrix. The relative size of the different molecules has the greatest effect on the permeation selectivity of the porous membrane. Dialysis is most often used for the desalting of protein solutions and in separating small molecules from large ones in biofluids such as plasma and serum. It is also possible to use dialysis on-line and an example of where this has been employed is for the enantioselective determination of oxprenolol in human plasma[53]. A cellulose acetate membrane was used for the dialysis step that was coupled on-line with chiral chromatography.

A recent development called the Minicon B15 (Figure 1.2) from Amicon is a static dialyser particularly suited to the isolation of large molecular weight species from urine samples. The aliquot of urine is transferred by pasteur pipette into one of the eight 5ml cells in the Minicon unit. The back of the clear cell has a membrane with a chosen molecular weight cutoff e.g.14 kDa. The sample is left standing in the unit for a period of time, during which, solvent and small molecular weight species leave the cell through the dialysis membrane. This device allows dialysis and concentration (up to a 100-fold i.e. 50 μl) to take place in about 2 hours. The resulting sample is very concentrated, contains few small molecules and can be directly analysed in many cases.

Figure 1.2 : Diagram of Minicon B15 Static Dialyser



Ultrafiltration

Like dialysis, ultrafiltration is a size-selective membrane-based method of sample preparation. However, this method is usually employed to eliminate large-sized

molecules from a sample matrix. The membrane pore size is chosen such that all molecules above a certain size will be retained on the surface of the membrane while smaller molecules, such as the drug to be determined, can pass through into the filtrate. Another difference between this and dialysis is that force is used to push the smaller molecules through the membrane using pressure or centrifugation. However, if the drug is protein-bound, recovery will not be quantitative since some will remain on the membrane with the proteins, though the extent of the protein binding may be lowered depending on how vigorous the ultrafiltration process is. Hence, this sample pretreatment step is very useful when free (non protein-bound) drug is to be measured and less so when total drug is to be measured since losses may be incurred. Wilkins *et al.* evaluated the percentage of protein-free and protein-bound methadone in the plasma of patients on methadone treatment using an Amicon MPS-1 ultrafiltration device[54]. Two independent procedures demonstrated that, following the ultrafiltration process, no proteins were measurable in the filtrate. In addition, they found that the ultrafiltration process functioned independently of the concentration of the methadone and of the volume of sample. Free L-tryptophan in plasma was also determined using this device[55].

Advantages of ultrafiltration include the fact that it is suitable for small volumes, and no dilution occurs during filtration. For example, Garcia-Capdevila *et al.* used ultrafiltration for sample preparation of human plasma and required only 250μl in order to determine imipenem with a limit of detection of 30 ng/ml and an inter-day precision of 6.26%[56]. However, the disadvantages that must be taken into account with this process include the possibility of non-specific binding of the drug to the membrane, which can be minimised or eliminated with some appropriate rinsing steps, and the previously mentioned problems if the analyte is protein-bound. Another consideration is the fact that if high shear is not maintained throughout the procedure, an impermeable layer of proteins will build up on the surface of the membrane slowing down the rate of filtration. Other conditions such as temperature and pressure require strict control for the method to be reproducible from sample to sample.

1.2 ANALYTICAL TECHNIQUES IN BIOPHARMACEUTICAL ANALYSIS

Analysis is the science of detection and determination, encompassing a large number of disciplines, techniques and species. There are many reasons why compounds need to be measured e.g. control of environmental pollutants, monitoring of industrial waste, quality control of drug substances in tablet formulations and flavourings in food to name but a few. Biopharmaceutical analysis is the determination of chemicals (usually drug compounds) in biological matrices such as whole blood, plasma, urine, or solid tissues such as in the case of post-mortem samples.

There is a large degree of freedom in the decision about which approach to take or even which instrumentation to use within a chosen technique. Before starting any development work, it is necessary that certain key items of information be available regarding the physiochemical properties of the substance to be analysed i.e. solubility, molecular size, functional groups, spectral properties and stability to name just a few. This allows for a logical process of deduction in order to arrive at the most suitable choice of technique. The method of taking the sample and the preparation of that sample prior to analysis is then chosen such that manual handling and manipulation time are minimised, while sensitivity and recovery of the compound from the matrix are maximised (as discussed in section 1.1). In order to achieve these aims, knowledge of the stability, metabolism and protein-binding (if applicable) of the substance in the biosample is required. An understanding of the expected levels of the compound and the nature of any interferences present are also necessary if the analyst is to develop the most suitable analytical protocols. After the preparation and analytical decisions have been made, further considerations include the extent of validation and the applications the assay will ultimately have.

The most commonly used instrumental techniques are those based on immunoassay, spectroscopy, electrochemistry and more importantly, chromatography and electrophoresis.

1.2.1 IMMUNOASSAY

The first competitive binding immunoassay was developed by Yalow and Berson in 1959 for the diagnosis of diabetes[57]. For their achievements in developing this new

application of analytical chemistry, they received the Nobel Prize for medicine in 1977. In general, immunoassays are competitive binding techniques where an unknown concentration of unlabelled analyte in a sample competes with a known concentration of labelled analyte for binding sites on an antibody raised against the analyte of interest. Labelled analyte is the compound with a 'tag' or 'label' attached to it or incorporated into it. The label can be an enzyme, a radioactive atom or a molecule capable of fluorescence. The importance of the label is the fact that it allows a means of specific detection. The basic principle of competitive immunoassays is shown below :

$$Ab + Ag + Ag^* = AbAg + AbAg^*$$

where Ab represents the antibody, Ag represents unlabelled analyte (antigen) and Ag^* the labelled analyte. Antibodies, which are immunoglobulin proteins, are raised by introducing the analyte into a laboratory animal, which elicits an immune response. However, very small molecular weight species (haptens) may not be sufficiently immunogenic. In these cases a larger molecule - drug-antigen complex - is created by linking the drug to a large protein molecule such as bovine serum albumin (BSA) or human serum albumin (HSA). An appropriate animal such as a sheep or a rabbit is then innoculated with this complex whereupon the animal develops antibodies against the foreign drug-antigen. If serum is obtained from the blood of the animal, it will contain a high concentration of the raised antibodies and is called antiserum. The antiserum may be further purified to isolate the antibodies or it may be used directly. The antibodies may be used as a solution or may be immobilised onto a solid phase e.g. they may be coated onto wells in microtiter plates or onto beads which will be subsequently used in immunoassays[58,59].

In the original 'heterogeneous' immunoassays, the bound and unbound fractions are separated after the reaction between the labelled drug, unlabelled drug and the antibody. Precipitation, extraction, electrophoresis or centrifugation are usually used to accomplish this. The labelled drug in the bound or unbound fractions (or both) is measured using a detection method which depends on the label used. Thus, the amount of drug present in the initial sample can be found by interpolation on a standard curve of fraction bound labelled antigen versus unlabelled antigen concentration. Newer, 'homogeneous' assays do not require separation of the bound

and unbound fractions, since the reaction itself causes a loss in activity of the measured entity and this can be related back to the concentration of the analyte.

The advantages of immunoassays are numerous. They are sensitive and selective (due to the specificity of the Ab-Ag interaction). They are inexpensive to execute once the antibody has been developed, they require only small amounts of reagents and they are rapid. Detection is now easy, especially with commercially available kits and automated readers. However, there can be disadvantages such as non-specific binding, insufficient washing (for heterogeneous assays), labelling difficulties, cross-reactivity and stability problems. Furthermore, antibodies are difficult to develop. Polyclonal antibodies are inhomogeneous in their binding affinities, while being easier to handle due to better stability and lower costs in their production. Monoclonal antibodies are more homogeneous, all being from the same clone, but are less stable and more expensive to raise due to the specialised techniques required for their preparation.

1.2.1.1 Radioimmunoassay

Radioimmunoassay (RIA) combines the sensitivity of radiochemistry with the specificity of immunology. This type of immunoassay was originally developed for the determination of hormones, and the first one to be applied was the insulin RIA developed by Yalow and Berson in 1962[60]. This assay was based on competition between the human insulin (in the serum under test) and the radioactive iodine-labelled insulin for binding sites on the antibody. The insulin-antibody complex was separated by paper chromatography. Free (unbound) insulin remained at the point of origin while the complexed (bound) insulin migrated with the globulins. The ratio of 'bound' to 'unbound' iodinated insulin decreased as the concentration of insulin in the sample increased. The principle of RIA remains the same today and is shown diagrammatically in Figure 1.3.

In this diagram it can be seen that when there are 5 units of compound in the sample, there are 3 units of bound radioactive compound to be measured. When the concentration of compound in the sample is doubled i.e. to 10 units, there are only 2 units of bound radioactive compound to be measured. In short, increasing the amount of analyte in the sample decreases the radioactivity.

Figure 1.3 : The Principles of Radioimmunoassay

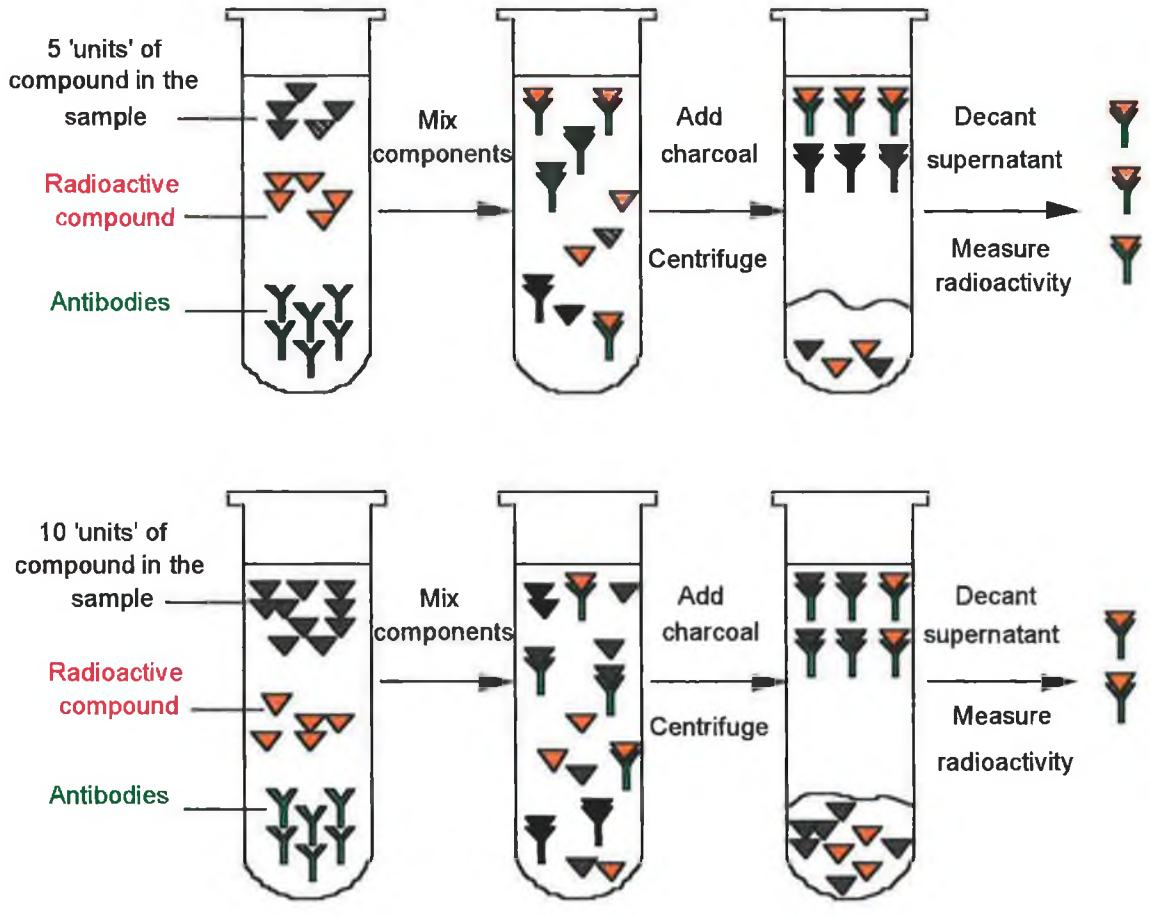


Figure 1.3 : In this diagram it can be seen that in radioimmunoassay, the concentration of analyte in the sample is inversely proportional to the measured radioactivity.

Radioisotopes, although they cause some difficulty with handling and disposal, are still used in immunoassay because they have the advantage of allowing detection of extremely low levels of analyte. RIA is classically used to determine hormones such as the thyroid-related hormones, digestive hormones, sex hormones and substances which require determination at very low concentrations such as vitamins and drugs. An example of a compound classically determined by RIA is digoxin. Until recent years, the only way of obtaining the required low limits of detection of this drug in biological fluids was by using RIA. Some of these RIA assays for digoxin are now available commercially e.g. the Ciba Corning Magic[61]. The emission of beta and gamma radiation can be readily measured by a scintillation

counter following separation of the bound and unbound fractions. Some disadvantages include the fact that the scintillation equipment is relatively expensive, the radiolabels have shorter shelf lives when compared to fluorescent reagents and there is, of course, the safety consideration when handling any radioactive substance.

1.2.1.2 Enzyme Immunoassay

With enzyme immunoassay (EIA), the binding of the Ab to the Ag is detected using an enzyme label. The enzyme acts on the colourless substrate to give a coloured product which is readily measured. The two principal types of EIA are enzyme-linked immunosorbent assay (ELISA) and enzyme multiplied immunoassay technique (EMIT). ELISA is a heterogeneous immunoassay while EMIT is a homogeneous immunoassay.

ELISA Immunoassays

An example of a competitive ELISA is where enzyme-labelled Ag competes with unlabelled Ag in a sample for binding to the immobilised Ab. The enzyme substrate is then added and after a suitable length of time, the enzyme activity is measured. The enzyme activity is inversely proportional to the unlabelled *Ag concentration* in the sample. Alternatively, the Ag can be immobilised onto the solid phase. Then enzyme-labelled Ab and unlabelled Ab compete for binding to the Ag. Following addition of substrate, the enzyme activity is inversely proportional to the *Ab concentration* in the sample. There are also variations of this type of assay where a second Ab is employed.

An example of a non-competitive ELISA is where the Ab is immobilised onto the solid phase. The sample containing Ag is added, followed by enzyme-labelled Ab. Because of the Ab-Ag-Ab effect, these assays are often called 'sandwich assays'. Enzyme substrate is then added and after a suitable length of time, the enzyme activity is measured. The enzyme activity is directly proportional to the unlabelled *Ag concentration* in the sample. Alternatively, the Ag can be immobilised and then Ab in the sample is added followed by a second Ab. The enzyme activity in this case is directly proportional to the *Ab concentration* in the sample.

ELISA techniques are very useful diagnostic indicators in certain disease states, for example ELISA can be used to confirm suspected cases of meningitis[62]. There is also a commercially available ELISA kit for assessing exocrine pancreatic function in children with cystic fibrosis[63]. An ELISA has been developed by Laurie *et*

al. for the determination of benzodiazepines in human urine[64]. Using only 10μl of urine, a limit of detection of 0.3μg/ml was obtained.

EMIT Immunoassays

In a competitive EMIT, the activity of the enzyme is modified upon immunochemical reaction and hence the change in activity can be related directly to the concentration of the Ag. Enzymes commonly used include isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase. These systems can be designed to enhance or diminish the enzyme activity. A number of non-competitive EMIT immunoassays are now available and these assays employ two different types of Ab, which react with different sites on the Ag, each labelled with a different enzyme. One enzyme produces the substrate for the other and significant enzyme activity is only detected when both enzymes are in close proximity to each other i.e. both of the Abs are bound close together to the Ag. EMIT is used mainly for the determination of drugs and metabolites in biofliuds, for example the quantitation of benzoylecgonine in serum without any sample preparation[65], cocaine in human urine[66] and vancomycin in human serum[67].

1.2.1.3 Fluorescence Immunoassays

The Ab or Ag can be labelled with fluorescent dyes such as fluorescein isothiocyanate or lissamine rhodamine B. These fluorescent agents are easier to handle than radioactive compounds. A fluorescence immunoassay (FIA) can be homogeneous or heterogeneous. The protein to be conjugated to the dye is usually a mixture of immunoglobulins which should be free of other serum protein fractions because albumin and other globulins label more readily than gamma globulins, and they would render the method non-specific. After labelling, unreacted dye is removed and the labelled protein is purified. As with the EIA technique, the activity (fluorescence) is often quenched upon reaction with the Ab and the decrease in fluorescence can be related to the concentration of the analyte. This immunoassay technique has many medical applications. For example, FIA can be used to rapidly diagnose oleander poisoning, using a serum digitoxin assay[68] and also to determine digoxin pharmacokinetics in patients with renal dysfunction[69]. The FIA for digoxin has been compared to EMIT, RIA and chemiluminescence immunoassay for digoxin in terms of cross-reactivity, accuracy and precision[61,70].

1.2.1.4 Chemiluminescence Immunoassays

The chemiluminescence immunoassay (CIA) is used mainly for the determination of steroids, steroid conjugates, hormones and certain drugs. One application of the use of an automated CIA is the determination of theophylline in serum and plasma[71]. The assay had a limit of quantitation of 0.2μg/ml and a range up to 40μg/ml. Two digitoxin immunoassays were compared in terms of their susceptability to cross reactivity with oleandrin[72]. The FIA exhibited high cross reactivity, while the CIA showed minimal cross reactivity.

1.2.1.5 Molecular Imprinting Assays

Although not an immunoassay, a molecular imprint assay (MIA) is worth mentioning in this section since these assays are in direct competition with immunoassays. In the same way as antibodies can be raised to any analyte, so too can molecular imprints be raised to any analyte. The main advantages of MIA are the greater speed and lower cost with which the imprints can be prepared. However, MIAs also display excellent stability when stored dry at ambient temperature for several years, showing no loss of recognition. Also important to note is that, unlike many immunoassays, no laboratory animals are used during the production of imprints. An MIA was developed for the quantitation of theophylline in serum and the assay yielded results comparable (correlation coefficient of 0.98) to those obtained using a well-established EMIT, during the analysis of 32 patient samples[73].

1.2.2 SPECTROMETRY

Both absorption and emission may be observed in each region of the electromagnetic spectrum but in practice, only absorption spectra are studied extensively. Some of the oldest and most popular of instrumental analysis techniques include ultraviolet and visible (UV-Vis) spectrometry, which is based on electronic energy changes, fluorescence spectrometry (a related technique), infrared (IR) spectrometry, which is based on vibrational and rotational energy changes and nuclear magnetic resonance (NMR) spectrometry, which is based on nuclear spin energy changes. Mass spectrometry (MS) is included in this section because it gives similar structural information to that of the other techniques even though the principle upon which it is

based is entirely different. Spectrometric methods do not involve a separation step and they are generally quick and easy to use. Certain drugs can be determined in this way especially if they are present in high concentrations in the sample. The type of spectrometric technique used depends on a number of factors such as whether the analyte is coloured or can be converted into a coloured derivative (see examples in Table 1.5), whether it contains functional groups that absorb in the appropriate region of the spectrum, and the sensitivity required. All of these spectrometric techniques can be adapted for use as detectors following LC or GC, and their application in this field will be discussed in section 1.2.4 (Chromatography).

1.2.2.1 UV-Vis and Fluorescence Spectrometry

The reason that UV-Vis absorption is useful is that no two molecular absorption spectra are exactly alike. The absorbance versus wavelength plot is a simple but characteristic spectrum of the molecule. The wavelength at which absorbance is maximum is called the λ_{max}, and this wavelength is the one at which the spectrometer is set when absorbance readings are taken for quantitative analysis. The usual procedure is to construct a plot of absorbance versus concentration; a straight line is obtained within the limits of the Beer-Lambert Law, and from this, the unknown concentrations can be calculated by interpolation. Benzene absorbs strongly at 200nm with a weaker band at 255nm. As conjugation and substitution onto the benzene molecule increase, the wavelengths of absorption are shifted (usually to longer wavelengths). Most inorganic, organic and biochemical substances can therefore be determined either directly or after formation of an absorbing derivative or complex.

A spectrometer consists of a) a source of continuous radiation, b) a monochromator for selecting a narrow band of wavelengths from the source spectrum, c) a detector and d) a recorder for data output. A typical UV-Vis spectrometer will have both a tungsten lamp and a deuterium lamp for the visible region and the UV region, respectively. A photomultiplier tube (PMT) is the most common detector for the UV-Vis region. It consists of a series of electrodes, each amplifying the signal of the previous one. The final amplification factor is at least 10^6. Spectrometers that use PMTs are generally called spectrophotometers. Although not a suitable technique for all compounds, UV-Vis is useful in certain cases, some of which are described in Table 1.5. The use of the UV-Vis spectrophotometer as a detector following HPLC separation is discussed in section 1.2.4.4.3.

Table 1.5 : Examples of UV-Vis Spectrometry and Fluorimetry in Biopharmaceutical Analysis

Analyte	Method	Detection	Reference
Uric acid in serum	Proteins precipitated with tungstic acid. Supernatant oxidised with alkaline phosphotungstate	Measure absorbance @ 680nm (blue reduction product)	74
Creatinine in serum	Proteins precipitated with trichloroacetic acid and supernatant reacted with alkaline picrate solution	Measure absorbance @ 490nm (coloured product)	74
Proteins in serum	Alkaline copper (II) tartrate (biuret reagent) formed complex with proteins in 30 mins.	Measure absorbance @ 550nm	74
Barbiturates in serum	Serum sample extracted with chloroform and back extracted into NaOH	Measure UV absorbance of the alkaline solution @ 252nm	74
Salicylate in serum	Complex formed with acidic ferric nitrate	Measure absorbance @ 540nm	75
P-aminophenol in human urine	After enzymatic hydrolysis, free compound was reacted with resorcinol in presence of manganese ions	Measure absorbance @ 550nm (indophenol dye)	76
Paracetamol in plasma	Proteins precipitated with methanol and the supernatant incubated with aryl acrylamidase for 2 min. Then o-cresol and ammoniacal copper sulphate were added	Measure absorbance @ 615nm	77
Glucose in serum	1,1'-dimethylferricinium ($DMFe^+$) is reduced to DMFe in presence of glucose in maleate buffer pH 6.5	Measure absorbance @ 650nm	78
Norfloxacin in urine	Aluminium (III) ion	Measure fluorescence	79

Fluorescence spectrometry (fluorimetry) is also a spectrophotometric technique and it utilises the ability of some substances to exhibit fluorescence. This phenomenon occurs when a compound is irradiated with light (such as UV) and following the absorption process, emits light of a longer wavelength. Only those molecules that absorb radiation, usually UV radiation, can fluoresce, and of those that

do absorb, only 5-10% will fluoresce. The molecular requirements for fluorescence are not clearly defined but many fluorescent molecules contain rigid, planar, conjugated systems. The emitted radiation may be in the UV region of the spectrum (especially if the compound absorbs at less than 300nm) but it is more often in the Vis region. This emitted radiation is measured and is related to the concentration. Samples that are not naturally fluorescent can be labelled with a fluorescing agent, and this considerably expands the applications base for this measurement technique. The use of the fluorimeter as a detector in HPLC separations is discussed in section 1.2.4.4.3.

With the spectrofluorimeter, it is necessary to separate the emitted radiation from the incident radiation and this is most easily done by measuring fluorescence at right angles to the incident radiation. A usual source of radiation is a mercury vapour lamp. Two monochromators are used – one to select the wavelength of excitation and one to select the wavelength of fluorescence. Commercial instruments usually adjust the spectra to correct for variations in the source intensity with wavelength and for variations in detector response. When compared to absorption spectrometry, fluorescence has advantages; it suffers much less from interferences and it is more sensitive. This is because in absorption spectrometry, the difference between two finite signals is measured and the sensitivity is governed by the ability of the instrument to distinguish between these two. In fluorescence, the difference between zero and a finite number is measured, so the sensitivity is governed by the intensity of the source and the sensitivity and stability of the detector. Fluorescence has a third advantage and that is the fact that this technique has a greater linear range for concentration (10^3–fold would not be uncommon). However, disadvantages with fluorescence include the problem of quenching and the fact that the numbers of chemical species that exhibit native fluorescence are limited.

1.2.2.2 Infrared Spectrometry

IR spectrometry is based on the measurement of energy changes due to the rotational and vibrational energy transitions of the molecules. The absorbance versus wavelength plot is a molecular fingerprint of the molecule. However, unlike UV-Vis, IR spectra give more qualitative than quantitative information. Hence, this technique is a very important identification test for a known compound as well as a method that yields valuable structural information about a new compound. Quantitative analysis in IR spectrometry uses the Beer-Lambert Law but reading the spectrum can pose difficulties if the peak is not fully resolved from neighbouring peaks and if the

baseline is not adequately defined. Finding a suitable, transparent solvent for use in quantitative IR can also be difficult. The slit width must be kept constant if quantitative measurements are to be made, as band intensities are highly sensitive to changes.

Newer infrared spectrometers which employ an interferometer and have no monochromator are called Fourier-Transform (FT) spectrometers. These instruments can record IR spectra with much greater speed and sensitivity than before. This is because instead of scanning a spectrum over a range of wavenumbers, an interferometer allows simultaneous collection of all data points in the form of an interferogram. This interferogram is then mathematically transformed into a conventional spectrum. A full-range FT-IR spectrum can now be recorded in less than one second and by accumulating multiple scans, greater sensitivity is also obtained. Quantitative analysis of FT-IR interferograms is discussed in an article by Mattu *et al.*[80]. Due to its relative imprecision and insensitivity when compared to UV-Vis, there are few applications of its use in the literature for biopharmaceutical analysis. However, like UV-Vis, this technique has bioanalytical applications as a detector for chromatographic separations.

1.2.2.3 Nuclear Magnetic Resonance Spectrometry

NMR uses electromagnetic radiation in the radio wave region of the spectrum involving nuclear spin energy transitions, which occur in a magnetic field. Absorption of radiation in this technique can be observed for those nuclei which spin about their own axes. The instrumentation is expensive and complex, with moderate to poor sensitivity. There is a limited range of solvents available since they must be deuterated or tritiated. Most of the applications of this spectrometric technique are in fact qualitative or as a detector for chromatographic separations.

1.2.2.4 Mass Spectrometry

MS uses a high-energy beam of electrons to cause destruction and/or fragmentation of the molecules in a sample. The fragments (ions) pass through a magnetic field which separates them according to their mass and charge. A detector determines the mass-to-charge ratio for each ion and plots this parameter versus the quantity of that particular ion. The essential components of a mass spectrometer include a sample inlet system, an ionisation source, an acceleration chamber, an analyser, a detector and a recorder. Numerous methods of sample ionisation are available of which the most important are

a) electron impact, b) chemical ionisation and c) fast atom bombardment. Depending on the type of ionisation method used, the extent of ionisation of the compound will be lesser or greater which will correspond to a less or more complicated fragmentation pattern. As well as being a very important qualitative tool, MS is used as a very sensitive quantitative instrument and finds special applications as a detector for LC, GC and CE.

1.2.3 ELECTROCHEMISTRY

If a solution forms part of an electrochemical cell, the voltage, current and resistance of that system are all determined by the chemical composition of the solution. By measuring one or more of these electrical properties under controlled conditions, both qualitative and quantitative information can be obtained. This section includes analytical techniques which are based on electrical measurements of such solutions. One of these parameters is quantified and related to the concentration of the species under analysis. Electrolytic methods include some of the most accurate, as well as most sensitive, instrumental techniques.

1.2.3.1 Potentiometry

This is the most widely used electroanalytical mode of detection and it utilises the galvanic cell concept. There is no net current flow. Potentiometric techniques include pH measurement, ion-selective electrode (ISE) measurement and potentiometric titrations. The Nernst equation forms the basis for measuring the potential (voltage) of one of a pair of immersed electrodes. The unchanging reference is a portable electrode designed to give a constant potential e.g. the saturated calomel electrode or the silver-silver chloride electrode. The working electrode is the electrode of analytical significance and examples of such electrodes include the pH glass electrode and the fluoride-selective electrode.

An ISE responds quickly and logarithmically to the *activity* of an ion. The errors associated with ISEs, which can be large, are however independent of concentration. ISEs are most useful when the concentration of an ion is required at the point of sample collection, due to their portability. They are also very important in cases where the solution to be measured is cloudy or coloured i.e. where spectrometric techniques may fail, and in flowing systems or kinetic studies where

constant monitoring is needed. A number of sensors incorporating potentiometric detection have been developed for the determination of drugs and biomolecules in biological matrices. For example, Hassan *et al.* determined uric acid in human urine and serum samples using a miniaturised graphite sensor[81]. Response times were 5-10 seconds, the minimum detectable concentration was 8μg/ml and recovery was > 97%.

Potentiometric titrations can also be carried out using such electrodes but these have no real applications in biopharmaceutical analysis.

1.2.3.2 Voltammetry/Polarography

Voltammetry and polarography are current-voltage techniques and are essentially electrolysis on a microscale. The principle of these techniques is based on applying a voltage (or current) to an electrode and measuring the resultant current flow (or change in potential) through the system. The current is directly proportional to the concentration of the electroactive species. The resultant waveforms can be quite complex.

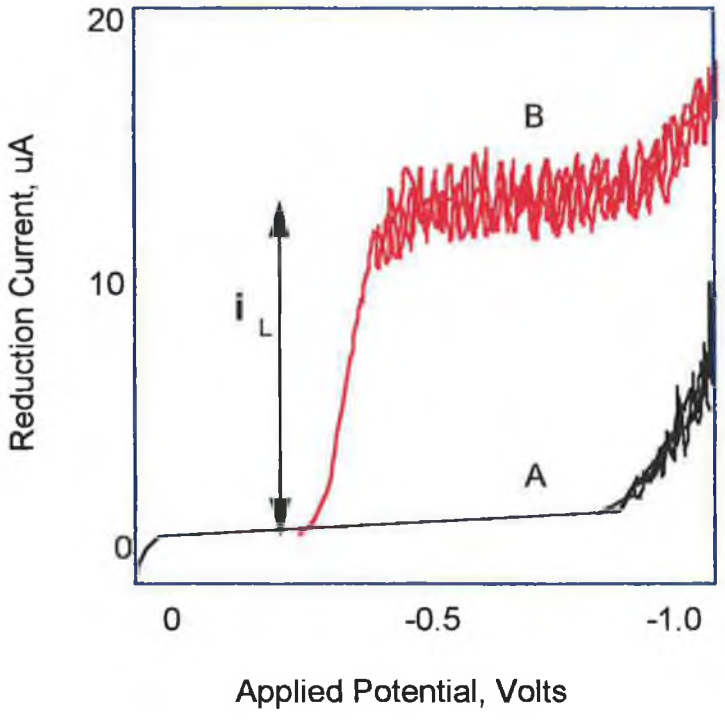
Voltammetry

In voltammetric techniques, current-versus-voltage curves are recorded as a gradually changing voltage is applied to the cell. To obtain *diffusion-controlled* currents, the solution is unstirred and the temperature kept constant. A high concentration of electrochemically inert background electrolyte is added to suppress migration of the analyte of interest towards the electrodes by electrostatic attraction. The cell typically comprises of a working micro-electrode e.g. carbon or platinum (readily polarisable) and a reference electrode e.g. calomel (non-polarisable). The potential of the working electrode is slowly scanned and the resulting current is recorded as a function of the applied potential.

The voltammogram produced has a number of key parameters such as diffusion current, the background current and the half-wave potential. The diffusion current is the current due directly to the presence of the analyte of interest, the background current is the current that would be observed if there was no analyte present in solution and the half-wave potential is the potential at which the increase in current is at half its full value. Measurement of the limiting current (i_L) forms the basis of quantitative analysis. This value i_L is calculated by subtracting the background current (A) from the diffusion current (B), as can be seen in the typical

voltammogram in Figure 1.4 below. Reproducibility can be a problem with this technique due to a build-up of contamination on the electrodes.

Figure 1.4 : Diagram of a Typical Voltammogram



Variations on voltammetric methods include linear sweep voltammetry which is carried out on an electrode with a constant and unchanging surface e.g. a mercury pool, or on an electrode with a periodically renewed surface. The rate of scanning must be fast. Stripping voltammetry is suitable for materials that can be accumulated *within* the electrode (as an amalgam) or *on* the electrode (surface). This technique is essentially an electrochemical preconcentration step wherein the analyte of interest is concentrated from a larger volume into a suitable electrode volume, hence it can have a very sensitive limit of detection of 10^{-9} M. Applications of voltammetry include the analysis of thyroxine in urine (with a detection limit of 2.7 ng/ml)[82], clenbuterol in bovine urine (with a detection limit of 1.02×10^{-9} M)[83] and doxazosin in urine[84].

Polarography

When the working electrode used is the dropping mercury electrode (DME), the technique is called polarography. All the above voltammetric methods can be carried

out using the DME or hanging DME. This non-stationary electrode gives more reproducible waveforms due to the constantly renewed electrode surface i.e. a new drop each time. As the mercury drop forms and falls, the current increases and decreases. The current-versus-voltage curves are now called polarograms. A typical limit of detection would be of the order of 10^{-6} M.

Modern polarographic techniques include sampled DC polarography, pulse polarography and differential pulse polarography. These are more sensitive than classical polarography because of the nature of the potential-time characteristic or the mode of current measurement (or both). Pulse polarography, instead of a continuously changing voltage, applies a single rectangular voltage pulse to the electrode during the last quarter of its life. In this way, the period at the beginning of the drop life, when changes in charging current are greatest, is avoided. The current is also measured in a very short time. Differential pulse polarography limits the contribution from the charging current even further. It does so by applying a 40-60 milliseconds duration of pulses during the last quarter of the drop life, but these pulses have constant amplitude and are superimposed on a slowly increasing linear voltage ramp. There are two current-measuring periods. This method can have a detection limit of 10^{-8} M. Two examples of the bioanalytical applications of polarography are the quantitation of cinnamic acid in urine (detection limit 0.1μg/ml)[85], nicardipine in blood and urine (with no sample clean-up)[86] and ceftriaxone in aqueous humour and serum (linear range 0.02 to 1300 ng/ml)[87].

1.2.4 CHROMATOGRAPHY

1.2.4.1 Principles of Chromatography

During a chromatographic separation, components of a mixture are continually moving back and forth between two phases. The nature of the two phases and the kind of interaction the solute molecules have with them can be varied and this gives rise to the different types of chromatography. One of the phases is a moving phase (mobile phase) and the other is a non-moving phase (stationary phase). The mobile phase can be a gas or a liquid and the stationary phase can be a liquid or a solid. The separation is achieved because compounds differ in their distribution between the mobile and the stationary phases. The distribution varies because molecules have different polarities,

charge and/or size. Chromatography can be broadly divided into two categories - planar chromatography and column chromatography. The planar methods utilise a thin sheet of stationary phase material and the mobile phase moves across the sheet. Column methods use a cylindrical tube to contain the stationary phase and the mobile phase moves through this tube either by gravity, with the use of a high-pressure pump or by gas pressure.

1.2.4.1.1 History of Chromatography

The word 'chromatography' was first used by Tswett in 1903 when he described the separation of coloured plant pigments through a column filled with calcium carbonate[88]. Coloured bands were formed as the pigments migrated at different rates. The next milestone was in 1941 when Martin and Synge developed the theory of partition chromatography between a stationary liquid and a flowing liquid[89]. Martin, along with A.T. James, was also responsible for the first application of GC in 1952[90]. Soon afterwards, the first commercial GC instruments became available. It was Giddings who provided the 'unifying theory' in 1965 which allowed comparisons of all types of chromatography[91] and this rejuvenated interest in LC which led to the design of the first commercially available LC instrument in 1969. Although LC was slower to be developed as a technique, it is now more popular and more widely applicable than GC.

Modern chromatographic techniques are more complex than those in the early part of the century and are often used to quantify substances such as drugs to very low levels. Chromatography allows greater versatility, speed and applicability than many other analytical techniques, particularly in view of the possibilities offered by modern instrumentation.

1.2.4.1.2 Theory of Chromatography

Efficiency

The ideal chromatographic process is one where all components of a mixture form narrow bands that are completely resolved from one another. The narrowness (width) of a band peak is a measure of the efficiency of the process. The separation efficiency of a column can be expressed in terms of the number of theoretical plates in the column. A theoretical plate can be thought of as representing a single equilibrium step. For high efficiency, a large number of theoretical plates is therefore necessary. Efficiency N, also called plate number, is related to retention time and peak width

measured in terms of the standard deviation, assuming an ideally shaped gaussian peak i.e.

$$N = 16\left(\frac{t_r}{w}\right)^2 \quad \text{or} \quad N = 5.54\left(\frac{t_r}{w_{1/2}}\right)^2$$

where w is the baseline peak width and $w_{1/2}$ is the peak width measured at half of the peak height and t_r is the retention time for the compound. In order to compare efficiencies for different solutes on a column or different columns for a solute, the same formula for N must be used throughout.

An alternative means of quoting efficiency is in terms of plate height H. This value is the length of the column divided by N. In order to avoid the use of long columns, the value H should be as small (short) as possible. Values of N may be thousands with the corresponding values of H being very tiny (<1mm). N and H are inversely related by the following equation, where L is length of the column

$$H = L/N$$

Ultimately, the width of a peak is determined by the total amount of *diffusion* occurring during movement of the solute through the system and on the rate of *mass transfer* between the two phases. Both of these effects are interdependent and complex because they consist of a number of contributions from different sources. Because they are kinetic effects, their influence on efficiency is determined by the rate at which the mobile phase travels through the system. Attempts to define efficiency in terms of diffusion and mass transfer effects are numerous but the most useful is that by van Deemter[92], from which the following simplified equation can be derived for efficiency in terms of plate height H

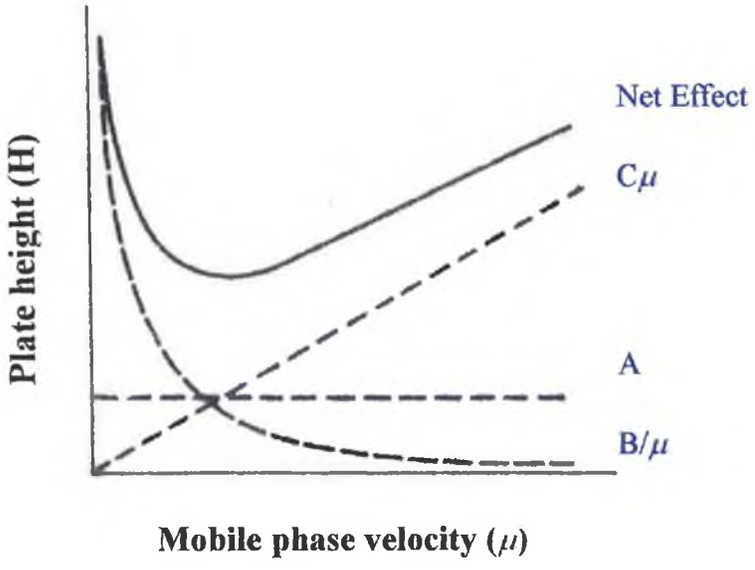
$$H = A + B/\mu + C\mu$$

where μ is the mean linear flow rate of the mobile phase, and A, B and C are terms involving diffusion and mass transfer. A is the 'eddy diffusion' term which accounts for the different portions of the mobile phase (and hence the solute) travelling

different total distances because of the various routes taken around the particles of stationary phase. This effect is minimised by reducing particle size but increases with length of column. B/μ is the 'molecular diffusion' term and relates to diffusion of solute molecules within the mobile phase caused by local concentration gradients. Diffusion within the stationary phase also contributes to this term, which is significant only at low flow rates and increases with column length. Cμ is the 'mass transfer' term and arises because of the finite time taken for solute molecules to move between two phases. Consequently, a true equilibrium situation is never established as the solute moves through the system, and the concentration profiles spread out. This effect is minimised with small particle sizes and thin coatings of stationary phase but increases with flow rate and length of column.

Experimental values of H when plotted against the rate of flow of mobile phase for a given solute and set of conditions, produces a hyperbolic curve (Figure 1.5) showing an optimum flow rate for maximum efficiency.

Figure 1.5 : Illustration of the van Deemter Equation



This position is dependent on the solute, and each solute in a mixture will have a different optimal flow rate. Hence, the most efficient flow rate for a particular sample is a matter of compromise. The equation also indicates that the highest efficiencies are achieved with stationary phases of small particle size (small values of A and Cμ) and

thin coatings of liquid (small value of $C\mu$). Although this equation applies strictly speaking to packed column GC, similar equations have been derived for capillary GC and HPLC.

Capacity Factor

A solute can be characterised by the length of time required it takes for it to appear (as a peak) after injection onto the column. This is known as the retention time t_r. The mobile phase solvent front will take a length of time t_m to appear, which is the time it would take an unretained solute to appear as a peak. The difference $t_r - t_m$ is the adjusted retention time t_r'. The capacity factor k' can be defined in terms of retention by

$$k' = \frac{t_r'}{t_m}$$

If $k' = 0$, then $t_r = t_m$ and the solute is not retained by the column at all. A large value of k' favours good separation of a solute. However, large capacity factors also mean increased elution time, so there is a compromise between separation efficiency and separation time. Ideal values of k' for most separations is between 1 and 10. A change in the value of k' over time indicates degradation of the stationary phase.

Resolution

Resolution is the ability to resolve the peaks of components with similar t_r values. Resolution R_s is a measure of the degree of separation of two solutes and is given by the expression

$$R_s = \frac{N_2}{4} \cdot \frac{(\alpha-1)}{\alpha} \cdot \frac{k'_2}{1+k'_2}$$

N_2 is the plate number measured for the second solute, k'_2 is its capacity factor and α is the separation factor defined as k'_2 / k'_1. An R_s value of 0 indicates that the two solutes are completely unresolved, and this would occur if α was 1 i.e. $k'_2 = k'_1$ or if $k'_2 = 0$ i.e. if solute two eluted in the solvent front. Improvement in the resolution of two solutes can be achieved by increasing the magnitude of N, k' or α. A doubling in

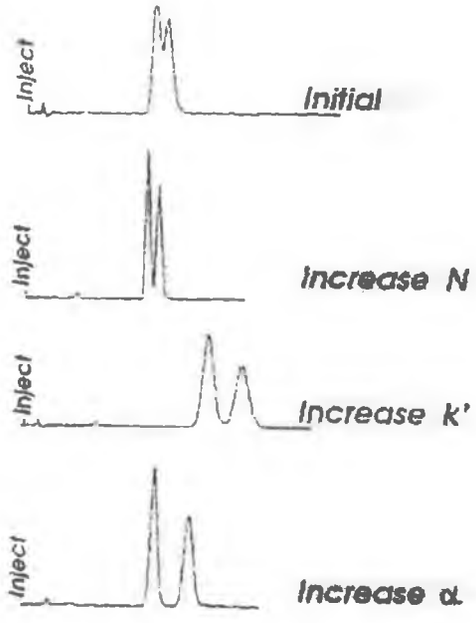
R_s would require a fourfold increase in the value of N. Very significant improvements in R_s can also be achieved by changing one or both of the stationary and mobile phases since this can have a large effect on α. The chromatographic effects that modification of N, k' and α would have under a typical set of conditions are illustrated in Figure 1.6.

In practice, R_s is measured from a chromatogram by relating the peak-to-peak separation to the average peak width. This is expressed by the equation

$$R_s = \Delta t_r / w$$

where Δt_r is the separation of the peak maxima and w is the average width of the peaks involved. An R_s value of 1.5 or more indicates less than 0.1% contamination.

Figure 1.6 : The Effects of Changing N, k' and α on a Particular Set of Chromatographic Conditions



1.2.4.1.3 Retention Mechanisms in Chromatography

The physiochemical processes that can occur during chromatography are based on one or some combination of the following types of retention: adsorption, partition,

ion-exchange and size exclusion. A general classification of the various retention mechanisms of chromatography are given in Table 1.6.

Table 1.6 : General Classification of Retention Mechanisms

Property of Molecule	Retention Mechanism
Adsorption	Adsorption
Solubility	Partition
Ion-exchange	Ion-exchange
Size and shape	Size exclusion

Adsorption

In adsorption, there is competition between the solvent (liquid or gas) and the solid adsorbent for the solute molecules. The relative polarities of solute and stationary phase determine the rate of movement of that solute through the column or across the surface. Separations where this is the predominant sorption process depend on polarity differences between solute molecules in a mixture. The more polar a molecule, the more tenaciously it will be adsorbed by a polar surface. During the process, there is competition for adsorption sites between solute molecules and those of the mobile phase. Solute and solvent molecules are continually adsorbed and desorbed as the mobile phase travels through the system. Solutes of low polarity spend proportionately more time in the mobile phase than those that are highly polar. Consequently, the components of a mixture are eluted in order of increasing polarity. Silica gel or alumina are typical polar phases. In silica, the adsorption sites are the oxygen atoms and silanol groups (Si-OH) which readily form H bonds with polar molecules. Adsorption chromatography is often called normal-phase chromatography because its most common application involves the use of polar stationary phases and non-polar mobile phases.

Partition

Partition is a process that occurs when there is a liquid coated or bonded onto the solid support (silica gel is the most common support). This coated or bonded liquid forms the stationary phase. The mobile phase is immiscible with this stationary phase.

Movement of the solute is determined solely by its relative solubility in the two phases if the mobile phase is a liquid, and by its relative volatility if the mobile phase is a gas. Solutes move through the system at rates dependent on their solubilities in the stationary versus the mobile phase. Coated liquid phase partition chromatography is rarely used since coated stationary phases are often unstable and are liable to bleed during the separation. The stationary phase is now typically composed of long chain alkyl groups chemically bonded to the solid support. These chains behave like liquids.

Ion-Exchange

Ion-exchange interactions occur when the stationary phase is a support with fixed charged groups and mobile counter ions which can readily exchange with solute and other ions in the mobile phase. In cation exchange columns, cations in the sample are exchanged for cations on the resin. In anion exchange columns, anions in the sample are exchanged for anions on the resin. Hence, only charged components can be separated by this retention mechanism. Ion-exchange columns can be silica bonded phases with anionic (e.g. tetraalkylammonium) or cationic groups (e.g. sulphonic acid) attached, polymeric gels or ion-exchange resins.

Size-Exclusion

The size exclusion mechanism effects separation because of variations in the extent to which the solute molecules can diffuse through an inert but porous stationary phase. The stationary phase is normally a gel-type structure which has a fixed pore size through which molecules greater than the predetermined size are excluded from accessing the gel network. Small molecules are trapped in the pores while the larger molecules cannot enter the pores and pass around the gel. Hence, large molecules elute first and the smallest ones elute last.

1.2.4.1.4 Identification and Quantitation in Chromatography

In chromatography, there are two main aims a) to determine the nature of the analytes in a sample (qualitative analysis) and b) to determine the concentration of each analyte (quantitative analysis). Qualitative analysis of peaks can be aided by the use of retention data i.e. t_r, t_r' or k' values are generally used for this. Spiking (standard addition) experiments are used to further identify the components then thought to be present in the sample.

The first step in quantitative analysis is measurement of the peaks obtained (heights, areas or densities as appropriate). This is usually achieved by electronic devices which employ analog-to-digital conversion of the signals. The next data manipulation step is calculation which can be carried out in a number of ways: normalisation, external standard method, internal standard method and standard addition method. The most commonly used of these calculations are the external and internal standard methods.

In the external standard method, standard solutions containing the solutes of interest are prepared at a number of different concentrations. These solutions are analysed and the peak response versus concentration plotted as a calibration curve. From this calibration plot, the unknown concentrations of solutes in the sample can be determined. Standard solutions can be run just prior to the samples or can be used to 'bracket' the samples so as to minimise any chromatographic effects that may have occurred over the course of the run. Some researchers also use quality control standards which are interspersed with the samples for extra control compensation for any changes in the chromatographic separation.

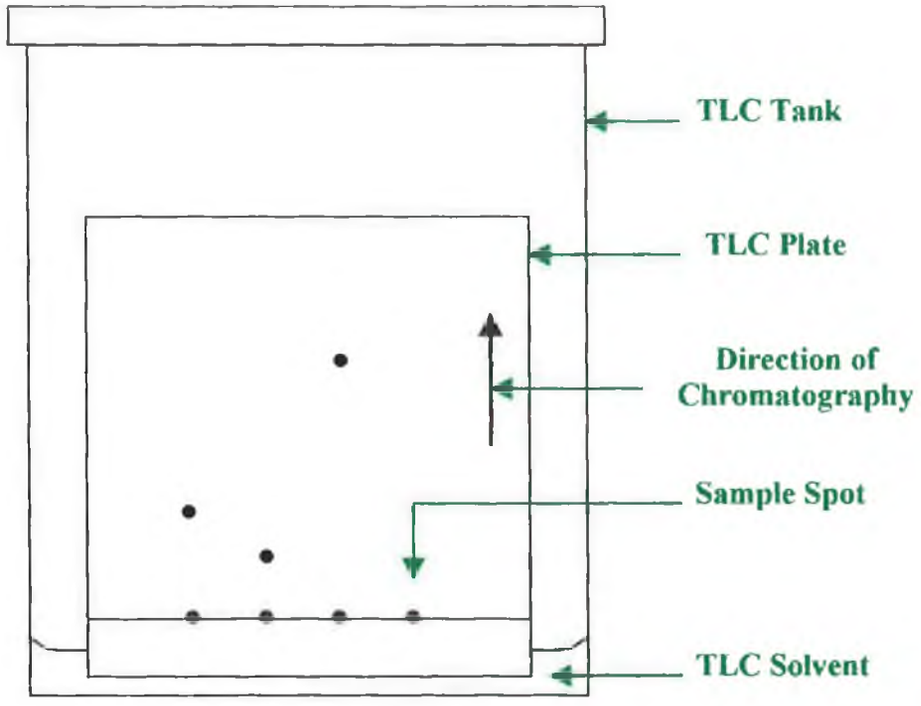
Internal standards are used to correct for errors in sample preparation and handling, for variation in instrument response and to help determine solute recoveries. The internal standard is usually a compound chemically similar to the analyte, and in general, the more its chemical structure resembles that of the compound under test, the better the control of variation achieved. Both samples and standards have a particular concentration of the internal standard added to them. It is essential that the internal standard and the analyte have similar responses to in the detection scheme employed. All samples and standards receive the same treatment from addition of the internal standard right through to detection. Internal standards must be carefully chosen to minimise the introduction of further errors. Calibration curves are constructed using the standard solutions, by plotting the 'ratio of the detector response for each solute relative to that of the internal standard' against the 'solute concentration'. The peak area ratio for each component in the sample is then calculated, and from these values, the amount of each constituent in the sample can be quantified.

1.2.4.2 Thin Layer Chromatography

Thin layer chromatography (TLC) is a planar chromatographic method since the stationary phase is a thin layer of material spread across a glass or metal plate. These

plates can be purchased commercially or prepared in-house. The most common stationary phase used in TLC is silica gel. The mobile phase is always a liquid. The mixture to be separated and known substances (standards) are applied as spots near to one end of the plate (approx. 2cm from the bottom edge). Following application, the plate is placed in a developing chamber with the bottom edge down and the mobile phase level below the level of the sample spots. A diagram of a TLC plate in a TLC tank is shown in Figure 1.7.

Figure 1.7 : TLC Apparatus



The solvent moves up the plate by capillary action. The samples are carried up the plate at different rates depending on their interaction with the stationary and mobile phases. This interaction is determined by any of the four types of retention processes, the most common mechanisms involved in TLC being adsorption and partition.

Two-dimensional chromatography can also be carried out by removing the plate and allowing it to dry after the first separation has occurred. A second edge of the plate, at right angles to the original line of migration, is then dipped into a different solvent and chromatography occurs again. This second TLC can separate two or more components that coeluted in the first system.

1.2.4.2.1 Detection in TLC

When chromatography has finished, the plate can be developed in a number of ways if the spots are not visible i.e. coloured. The most common means of developing the plate is by using special plates that have been impregnated with a fluorescent dye (Silica F-254 plates). Under UV light, the spots show up dark against the fluorescent green background. Fluorescence detection can also be used. If the molecules of interest are not naturally fluorescent, they can be derivatised prior to examination under a spectrofluorometer. Cephalosporins have been determined in serum and urine using fluorescent detection[93]. Two derivatising agents were compared - fluorescamine and o-phthalaldehyde - in this work. Iodine staining, spray reagents e.g. ninhydrin, and ashing techniques are also used in order to visualise the chromatography. TLC is often used for drug screening in cases of toxicity or poisoning because it is simple and rapid. A positive result is usually confirmed by another technique. TLC has important applications in the analysis of metabolites in new compounds.

1.2.4.2.2 Identification and Quantitation in TLC

In TLC, retardation factors (R_f values), as opposed to t_r , t_r' or k' values, can be calculated for each component on the plate in order to mathematically define their positions. Quantitative analysis can be carried out by using a scanning densitometer which measures the absorbance of UV-Vis light at the spot location.

1.2.4.3 Gas Chromatography

Gas chromatography (GC) has very widespread use, especially for organic materials. The technique is rapid, simple and can handle very complex mixtures and very small sample sizes. GC is so-called because the mobile phase is a gas, typically purified helium or nitrogen. This gas flows from a compressed gas cylinder through the column containing the stationary phase where the separation takes place. The GC column is housed in the oven which is at elevated temperatures. Hence, the analytes should be volatile and thermally stable at the operating temperature. It is important to maintain a constant high temperature during the chromatographic run to prevent condensation of the sample components in the system. The components in the sample migrate at different rates because of differences in boiling point, solubility or adsorption. The continuous gas flow elutes the components from the column into the detector, where they are quantified. Samples are introduced into the gas flow via an

injection port located at the top of the column. The injection chamber is heated to a very high temperature, and a small volume of injected liquid (typically 0.1 - 3.0μl) is flash vapourised and carried onto the column. To prevent overloading of narrow capillary columns, split injectors have been developed where only a fraction of the liquid from the syringe (2% or less) is passed to the column. The remaining portion is vented to air. As previously mentioned, precise control of temperature is vital in GC, so the role of the oven is crucial. This is because retention times are increased at lower temperatures, yielding improved separations, but at the expense of increasing the analysis time. Both isothermal and gradient-controlled temperature programmes are possible.

1.2.4.3.1 Columns in GC

There are two types of columns used for GC. The older, less widely used type are packed with the sorbent whereas the newer columns are of an open tubular design where the sorbent is coated onto the walls of the column. Packed columns in GC are usually no longer than 6-7 metres and are made from stainless steel or glass. The liquid stationary phase is adsorbed onto the surface of an inert and finely divided support, such as diatomaceous earth or Teflon, before the phase is packed into the GC column. The open tubular columns can be a hundred metres in length and are composed of fused silica, usually with an imide coating on the outside surface. These capillary columns have largely replaced packed columns due to their superior resolving power arising from increased length and decreased internal diameter. The actual stationary phase can be any of hundreds of different liquids. The selection of an appropriate stationary phase depends largely on trial and error but some chromatographers refer to various tables of constants and indices as a starting point. The Kovats retention index and the McReynolds constants[94] are two examples where phases are grouped according to their retention properties.

1.2.4.3.2 Detectors in GC

Flame Ionisation Detector

There are many different detectors available for GC but the most commonly used one is the Flame Ionisation Detector (FID). It detects all flammable organic compounds, irrespective of functional groups present. It is insensitive to inorganic gases and water. The lack of response to water is an asset since most bioanalytical samples will contain

some amount of water. It has high sensitivity and the widest linear range (10^7) of any detector in common use, but it does destroy the sample.

Thermal Conductivity Detector

The Thermal Conductivity Detector (TCD) is the most universal of all GC detectors but it has only moderate sensitivity when compared to other detectors for GC. It works on the principle that gases eluting from the column have different conductivities from that of the carrier gas. It does not destroy the sample.

Electron Capture Detector

The Electron Capture Detector (ECD) is especially useful for large halogenated hydrocarbon molecules for which it is extremely sensitive, selective and non-destructive. Thus it finds special application in the analysis of biomedical samples.

Nitrogen/Phosphorous Detector

The Nitrogen/Phosphorous Detector (NPD), which is a slight modification on the FID, is the most sensitive of all GC detectors but is only selective for phosphorous and nitrogen-containing compounds.

Flame Photometric Detector

In the Flame Photometric Detector (FPD), a flame photometer aspirates the column effluent into a hydrogen flame. This detector is selective and sensitive for sulphur and phosphorous-containing compounds.

Electrolytic Conductivity Detector

The Electrolytic Conductivity Detector (Hall) has excellent selectivity and sensitivity, giving a peak only for the compounds that produce ions in the reaction chamber. The conversion to ions is effected by chemically oxidising or reducing the components with a reaction gas in a small reaction chamber made of nickel which is positioned between the column and the cell.

Photoionisation Detector

The Photoionisation Detector (PID) is selective and sensitive for aromatic hydrocarbons and inorganics. It is a non-destructive detector that involves the ionisation of the eluting components by UV light.

1.2.4.3.3 GC-MS and GC-IR

Two very important analytical techniques employing GC are GC-MS and GC-IR. With complex mixtures or mixtures containing unknown compounds, both of these hyphenated techniques can give remarkable structural and molecular weight information. Commercially available instruments allow the direct transfer of the gas effluent from the GC to the mass spectrometer. The MS is an extremely sensitive and selective, if somewhat costly, detector. Table 1.7 below gives some recent bioanalytical examples of GC-MS.

Table 1.7 : Examples of GC-MS in Biopharmaceutical Analysis

Compound & Matrix	Comments	Reference
Clenbuterol in bovine liver	Trimethylsilyl derivatives were prepared. The quantitation limits were in the low ng/ml range	25
Phenmetrazine in urine	Perfluorooctanoyl chloride derivatives were prepared. The linear range of the assay was 1-100 μg/ml with a detection limit of 0.5μg/ml	13
Antiepileptics in serum i.e. lamotrigine, carbamazepine and carbamazepine epoxide	A reference spectrum for pure lamotrigine was published for the first time	95
Homocysteine in human plasma	The propoxycarbonyl propyl ester derivative was prepared and the ion selectively monitored	96
Metabolites of pyrethroids in human urine	The calibration graphs for the five most important metabolites were linear from 0.5 to 200ng/ml and detection limits were 0.3 to 0.5ng/ml for each	97
Mandelic acids in human urine	The chlorodifluoromethyl-1,2-dioxolan-5-one derivatives were prepared and the ions monitored selectively	98

The effluent gas from a GC can also be fed into a heated infrared gas cell as part of a GC-IR configuration. The vapour phase FT-IR spectra of the components can then be recorded. These spectra are, however, slightly different from the corresponding liquid or solid phase spectra but with the availability of spectral libraries, this no longer presents a real problem. Compared to GC-MS, GC-IR is much

less sensitive. Also, if sample size is a consideration, a mass spectrum can be recorded from as little as 10^{-10}g of sample while an IR spectrum requires at least 10^{-6}g.

1.2.4.3.4 Derivatisation in GC

Some non-volatile, polar or thermally sensitive compounds can be derivatised to enhance their volatility and stability prior to GC analysis. Compounds containing functional groups such as hydroxyl, carboxyl and amino can be readily reacted with appropriate reagents to convert these polar groups into less polar methyl, trimethylsilyl or trifluoroacetyl derivatives of greater volatility. Fatty acids, carbohydrates, phenols, amino acids and other compounds of biological interest are the most frequently derivatised, although liquid chromatography is very often the preferred technique for these types of compound. Some examples of the use of derivatising agents in GC can be seen in Table 1.7.

Alkylsilylating Reagents

Alkylsilyl derivatives are easily prepared and are suitable for polar molecules containing protonic functional groups. Alkylsilylating agents are the most versatile and universally applicable GC derivatising agents since the reactions occur instantaneously at room temperature. No artifacts or byproducts are formed.

Haloalkylacylating Reagents

Haloalkylacyl derivatives are easily detected by ECD due to the presence of the halogen atom(s). These derivatives are, however, just as suited to detection by FID. These compounds are volatile and produce early-eluting peaks of good shape.

Other Reagents

Esterification of acidic groups is another form of derivatisation, but these reactions can be slow. Straightforward alkylation replaces a H atom with an alkyl or aryl group. There are also bifunctional derivatising agents which can be utilised in very specific situations.

1.2.4.3.5 Identification and Quantitation in GC

Identification and quantitation in chromatography in general has been discussed in section 1.2.4.1.4. However, particular to GC, retention time (t_r) or adjusted retention time (t_r') is used to identify peaks for qualitative analysis. The hyphenated techniques

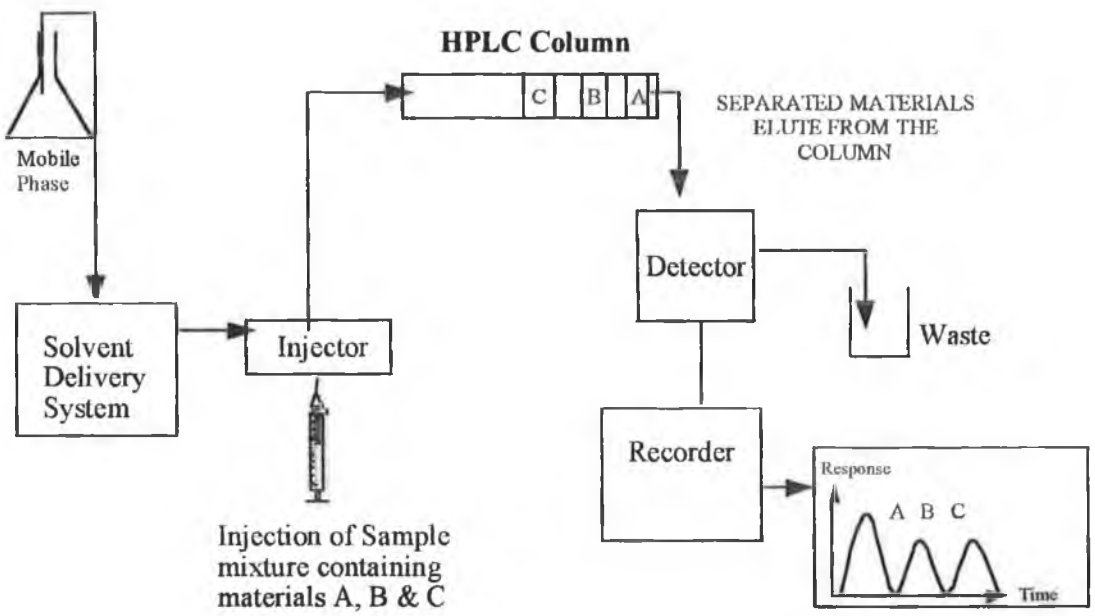
GC-MS and GC-IR can also be used to give structural information on the compounds of interest. Quantitative measurement in GC is possible in a number of ways. Internal standards are often employed to compensate for variations in physical parameters, such as flow rate. The ratio of the area or height of the standard or analyte to that of the internal standard is used to prepare the calibration curve and hence determine the unknown concentration. An alternative method is to use standard additions, especially for occasional samples. External standards are not usually used in GC as the technique is quite susceptible to instrumental variations.

1.2.4.4 High Performance Liquid Chromatography

HPLC has its origins in classical column chromatography. Mixtures of non-volatile components are separated by passage of the sample through a column containing a stationary solid, by means of the pressurised flow of a liquid phase. The compounds migrate at different rates due to differences in solubility, adsorption, size or charge and they are detected as they elute from the column. Complex mixtures can be resolved in very short times if conditions are optimised. The layout of a typical HPLC system is shown in Figure 1.8.

The sample is most commonly introduced into an *injector* from which a fixed, accurate loop volume is injected into the high-pressure system. Autosamplers involve the use of a loop injector coupled to a robotic needle which draws measured aliquots from the sample vials. Some automated systems allow extraction and/or derivatisation to take place prior to injection. The sample, after injection, is swept along the *analytical column* and thence into the *detector* and on to the *recorder*, (or integrator or computer). The choice of column for a particular analysis is the most important step in developing a suitable assay and there are many types of columns available with different dimensions, packings and hence characteristics. To prolong the life of a HPLC column, the mobile phase is always of analytical grade, is prefiltered and degassed before use, and in-line filters and a guard column are often employed to prevent build-up of contamination. With isocratic elution, the same *mobile phase* is used throughout the run whereas with gradient elution, the mobile phase composition can be changed during the run. A gradient programmer can draw from at least two solvent reservoirs at once and hence is capable of altering the mobile phase that is delivered to the HPLC *solvent delivery system*. The very high pressures (often 4000 – 6000 p.s.i.) are usually delivered by special pulsation-free pumps.

Figure 1.8 : Layout of a Typical HPLC System



1.2.4.4.1 Modes of HPLC

HPLC may be classified into modes, which mainly involve one or more of the retention mechanisms shown in Table 1.6. These are normal-phase, reversed-phase, ion-exchange, affinity, size exclusion and chiral chromatography. Consideration of the properties of the sample will aid in the choice of a suitable HPLC mode. If the compound is ionic or ionisable, ion-pair or ion-exchange chromatography should be tried. If the sample is soluble only in non-polar solvents, normal phase chromatography is a likely candidate. Size exclusion chromatography is preferred if the compound has a molecular weight of greater than 2000 and if separation of enantiomers is required, chiral LC should be used.

Normal-Phase Chromatography

The oldest type of chromatography is normal phase (based on adsorption retention mechanism) and it involves the use of a non-polar mobile phase e.g. hexane, and a polar stationary phase e.g. silica. Normal phase HPLC is used mainly for water-insoluble samples, and it can be a powerful technique for isomer separation.

Reversed-Phase Chromatography

In reversed-phase chromatography, the situation is the opposite to that in normal-phase chromatography; the stationary phases are non-polar and the mobile phases are polar. Solutes are usually eluted in order of increasing hydrophobicity (decreasing polarity) and their elution can be speeded up by increasing the organic content of the mobile phase. Reversed-phase chromatography is more versatile and more generally applicable than any other type of chromatography, and this is due in part to the readily available array of suitable stationary phases. This technique is based heavily on the partition retention mechanism, but there are small contributions from adsorption and ion-exchange interactions. The stationary phase is usually a silica-bonded material (such as C8 or C18-modified silica and other examples previously described in Table 1.3) or it may be a resin such as styrene divinylbenzene copolymer. The mobile phase for reversed phase HPLC is usually a mixture of polar organic solvent(s) and an aqueous buffer or water. This mode is preferred for neutral or non-ionised compounds that are soluble in organic solvent/water mixtures. Because of the toxicity and environmental implications of using organic solvents, such as are required in normal phase HPLC, reversed phase HPLC is favoured when at all possible.

Two subtypes of reversed phase HPLC are (i) ion-pair HPLC and (ii) ion-suppression HPLC. Ion-pair HPLC is used when the molecule of interest is ionisable or ionic. It utilises reversed-phase sorbents. Ion-pair HPLC is especially suitable for the simultaneous separation of acidic, basic and neutral components in a mixture e.g. by using a mobile phase containing an ion-pairing agent for cations at pH 2.5, the ionisation of the acidic component is suppressed, the neutral component is unaffected and the basic component forms a neutral ion pair. Water/organic solvent mixtures that are pH buffered, and include an ion-pair reagent such as tetrabutyl ammonium hydroxide, are employed as mobile phases. The presence of the ion-pair reagent increases retention and improves selectivity. However, ion-pair mechanisms are complex and hence, development of ion-pair separations can be difficult. Method development using a step-wise approach for ion-pair HPLC has been discussed[99].

Ion-suppression HPLC is used for the separation of weak acids and weak bases. In this technique, a buffer of appropriate pH is added to the mobile phase to render the analyte neutral. Acidic buffers are used to separate weak acids and alkaline buffers are used to separate weak bases[100]. As a result of the added buffer, samples are eluted as sharper zones than they would have been in a similar mobile phase without the addition of a modifier[101].

Ion-Exchange Chromatography

Ion-exchange HPLC is usually used for mixtures of organic or inorganic ions, for example amino acids. The mobile phase is mainly pH-buffered water, and may contain additional neutral salts to adjust the ionic strength of the mobile phase. The column has positive or negative binding sites on its surface at which ions are exchanged with the analyte molecules in the mobile phase. In order to change selectivity, two parameters are very important i.e. pH and salt type.

Affinity Chromatography

Affinity chromatography is based on a special case of adsorption retention mechanism. This technique is based on the 'lock and key' mechanism prevalent in biological systems. It can be used to separate any molecule that is capable of recognising, binding to and forming a dissociable complex with another species e.g. antibody and antigen. When antibodies and antigens are involved, this technique is known as immunoaffinity chromatography (IAC). The use of IAC in sample extraction has been discussed in section 1.1.4.3. The separation mechanism is very specific, but can be time-consuming and expensive when compared to other types of chromatography. An affinity ligand that is specific to one type of biological molecule is covalently bonded to an inert support material. The sample mixture containing the biomolecule of interest is applied to the column and washed through and only the molecule that can bind to the affinity ligand will be retained by the column. The adsorbed species is eluted after the rest of the sample by changing the composition of the mobile phase.

Size Exclusion Chromatography

Another mode of HPLC is size exclusion HPLC which is divided into two subtypes : gel permeation chromatography (GPC) and gel filtration chromatography (GFC). GPC uses non-polar organic solvents such as chloroform and toluene, and rigid gel stationary phases to analyse large organic molecules (often polymers). GFC uses water-based eluents as mobile phases and soft gels e.g. sephadex, as stationary phases to measure naturally occurring polymers such as proteins and nucleic acids.

Chiral Chromatography

Chiral chromatography is based on the separation of stereoisomers. This technique has developed because of the importance of enantiomeric purity of certain drugs.

Some molecules are chiral and as such can exist in one of two 3-dimensional enantiomeric forms. Since enantiomers often have different bioavailability, pharmacological effects and rates of absorption and elimination[102,103], the ability to discriminate between the R and S forms of the molecule can be of vital importance. There are three approaches to chiral discrimination in chromatography a) to form diastereomers from the enantiomers by derivatisation, b) to use a chiral stationary phase and c) to use a chiral mobile phase.

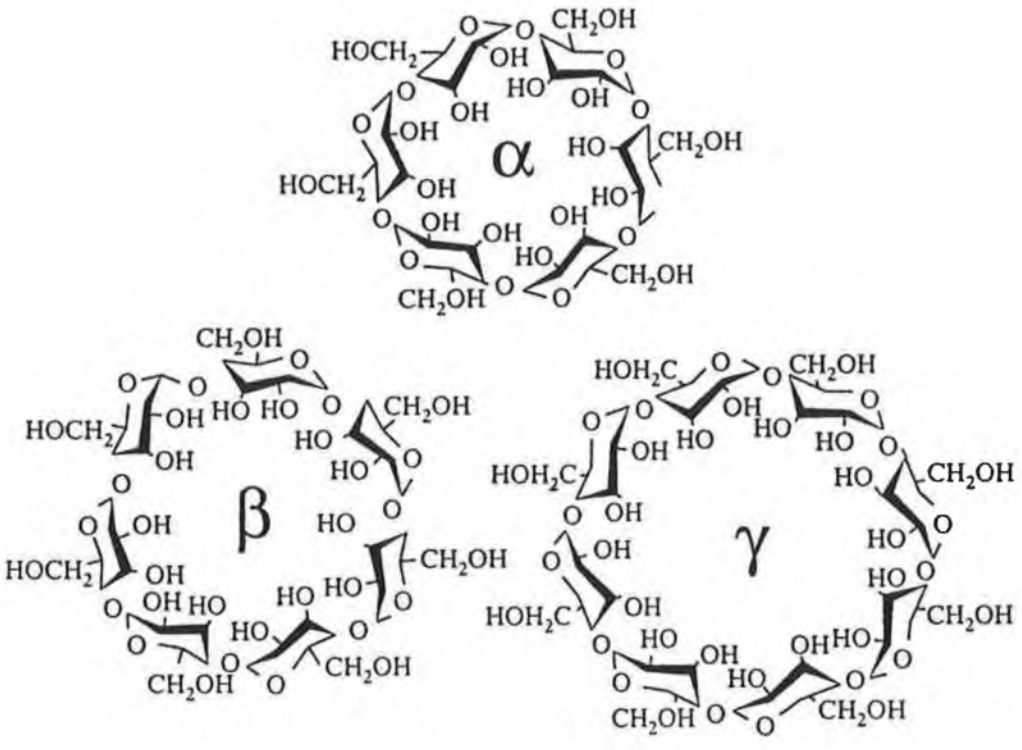
If the first approach is used, diastereomers are structural isomers of each other and can now be separated using normal techniques. The determination of warfarin enantiomers in the plasma of stroke patients was made possible by their derivatisation to diastereoisomeric esters followed by HPLC[104]. The second method involves the use of special chiral stationary phases which work by causing the transient formation of diastereomeric complexes, which are then retained to different extents on the column. There are a number of commercially available stationary phases for chiral chromatography such as ligand-exchange phases, protein phases, cycoldextrin phases and Pirkle phases. Ceccato *et al.* used a Chiralcel OD-R column which contained cellulose tris (3,5-dimethylphenylcarbamate) coated on silica as chiral selector for the enantioselective determination of oxprenolol in human plasma[53]. The third approach involves the incorporation of special reagents into the mobile phase. These chiral mobile phases may be used in normal or reversed phase HPLC and usually cause separation of the enantiomers by the dynamic formation of diastereoisomers. Cyclodextrins are typical additives for this purpose and the chemical structures of three cyclodextrins are shown in Figure 1.9.

1.2.4.4.2 Columns in HPLC

Silica in HPLC Columns

Silica is the basic starting material for the majority of HPLC stationary phases. It is a very polar phase which has a large number of silanol (Si-OH) and siloxane (Si-O-Si) groups on its surface. As discussed in section 1.1.4.3, these residual surface silanols, when ionised, can interact with basic or ionic compounds through a mixed retention mechanism which causes problems such as peak tailing. The presence of this unknown variable in chromatography makes it difficult to obtain reproducible results. Since the pH range in which silica can be used is generally restricted to between 2 and 7 (as silica dissolves in basic solvents), supression of these interactions simply by raising the pH to greater than 7 or 8 is not feasible.

Figure 1.9 : The Structures of α, β and γ-Cyclodextrins



Polymers in HPLC Columns

The pH stability considerations have led to the development of polymeric reversed-phase columns. These columns are stable over the pH range 1-13 or 14. The base material is usually polystyrene divinylbenzene copolymers with C18 functionality. Low flow rates and higher temperatures are usually required to achieve maximum efficiency on these phases. The retention characteristics of these materials can be quite different to that of silica-based reversed-phase materials because of the extensive network of π-orbitals.

Bonded Phases

Because of the inherent problems associated with pure silica, it has been modified in many ways to produce silica-bonded phases. This serves to reduce, though not eliminate, these undesirable secondary interactions that affect chromatographic separations. The greater the extent to which the silica surface is bonded, the lower the number of residual silanols. Further treatment of the bonded phase with 'end-capping' agents such as trimethylchlorosilane can eliminate these silanols to an even greater

extent. The one way to virtually remove silanol effects is to add an amine to the mobile phase or to use a buffer containing a silanol masking agent e.g. ammonium acetate. Base-deactivated columns are available and these columns have been exhaustively end-capped. To circumvent the limited pH range that silica columns have, some manufacturers have coated the silica surface with a polymer before bonding the alkyl chains to it. These columns are stable from pH 2 to 13.

The first bonded phases were produced in 1969 by Halasz *et al.* who reacted silica with alcohols[105] and amines[106]. Subsequently, materials of greater hydrolytic stability have been manufactured by reacting silica with alkylsilanes, some of which are now described.

C18 columns are the most widely used of all silica-bonded phases and consist of $-(CH_2)_{17}-CH_3$ groups chemically bonded to silica, which renders the surface very hydrophobic. These C18 columns, also known as ODS columns, are the most commonly used in HPLC and are supplied by most manufacturers. They are used in reversed-phase separations. It is worth noting however that large differences can exist between different brands of the same type of column, even if the same type of bonded phase chemistry has been used.

C8 columns consist of $-(CH_2)_7-CH_3$ groups chemically bonded to silica. Like ODS, it is hydrophobic in nature and hence non-polar. It is therefore used in reversed-phase chromatography. Generally, C8 packings show similar selectivity to C18 but with reduced retention for many types of compounds. It has been used for the separation of low to medium polarity analytes and has been found to provide better selectivity than C18 for HPLC analysis of the benzodiazepines[24,107,108].

Phenyl columns consist of $-(CH_2)_3-C_6H_5$ groups chemically bonded to silica. In general, phenyl columns exhibit similar retention characteristics to C8 columns but their selectivity, especially for aromatic compounds, may be different due to the possibility of π-π interactions. Phenyl columns have been used in HPLC for the separation of polar aromatics and polycyclic aromatic hydrocarbons.

Cyano columns have $-(CH_2)_3-CN$ groups chemically bonded to silica. It is a polar bonded phase and is used extensively in normal phase HPLC in addition to reversed phase. In the latter mode, it is less retentive than ODS and often shows different selectivity. It is usually used for the analysis of medium polarity compounds. The cyano analytical column has been found to be very successful for the separation of the β-blocker class of drugs[109].

NH_2 bonded phase consists of $-(CH_2)_3-NH_2$ groups chemically bonded to silica. It is a very polar material which is used extensively in normal phase HPLC as a replacement for silica. It has been used in this mode for the HPLC analysis of a number of drugs including vancomycin[110]. It is also used as a reversed phase material.

1.2.4.4.3 Detectors in HPLC

There is a wide selection of detectors available for use in HPLC, the most common of which are compared and contrasted in terms of their typical specifications, relative to each other, in Table 1.8.

Table 1.8 : Typical Specifications for some Detectors used in HPLC

Detector type	Limit of Determination (μg/ml)	Maximum linear range	Gradient compatible?
UV-Vis	10^{-2}	10^{5}	Yes
Fluorescence	10^{-5}	10^{3}	Yes
Refractive index	10^{-1}	10^{4}	No
Conductivity	10^{-2}	10^{4}	No
Amperometric	10^{-4}	10^{6}	No

UV-Vis Detector

The most widely used of LC detectors is the UV-Vis spectrophotometer. A spectrophotometer measures the ratio of the radiant power from two beams (generally, one that has passed through the sample and one that acts as a reference beam). The two beams can be measured separately or simultaneously in a single-beam or double-beam instrument, respectively. The spectrophotometer contains a flow cell through which the absorbance of the column effluent is monitored continually. Sensitivity depends on how strongly the sample absorbs light at a particular wavelength and the availability of a mobile phase that is transparent at the monitoring wavelength. While UV-Vis is reasonably sensitive and universal, it is not suitable for all compounds such as those with poor or no chromophores.

Diode array UV-Vis spectrophotometers provide a means of acquiring a full-range spectrum in usually less than one second. The optics are reversed in the sense

that the monochromator disperses the radiation onto the array of photodiodes *after* it has passed through the sample, unlike ordinary spectrophotometers. This facilitates the continuous monitoring of a wide spectral range. The diode array detector (DAD) optics yield detection limits and linear ranges that can equal or even exceed that of single or multiple wavelength detectors. The advantages of the DAD are the possibility of rapid spectral scanning of each component as it elutes from the column, thereby facilitating identification, 3-D images of the peaks allowing detection of coeluting components, peak purity calculations, concurrent with quantitative analysis at the wavelength of choice, for each species under assay.

Fluorescence Detector

Fluorescence detection is a very selective technique and hence interferences from other compounds in a mixture are less of a problem than with other more universal detection methods. It is also more sensitive than UV-Vis detection by virtue of the fact that the signal is measured against a dark background. There are many available reagents that can react with non-fluorescing compounds to form fluorescent derivatives that may then be detected using a fluorimeter.

Refractive Index Detector

Refractive index (RI) is another characteristic physical property that can be exploited in detection since it is rare to find two substances that have the same RI value. The RI detector is a 'bulk property' detector as opposed to UV-Vis and fluorescence detection which are 'solute property' detectors. A solute present in the mobile phase changes the RI of the eluent and this is measured by a refractometer. The column effluent is passed through a flow cell in the refractometer and is compared to a reference flow cell containing only mobile phase. Although this detector is more universal than any other used in HPLC, it suffers from a lack of sensitivity, it cannot be used with gradient systems and it is strongly affected by temperature.

Conductivity Detector

The conductivity detector is a 'bulk property' electrochemical detector, reasonably universal in application but generally has a limited dynamic range. The conductivity detector is based on the principle of resistance and has a simple and robust design, consisting of a small chamber containing two electrodes. A potential is applied and the resistance of the low-volume cell is measured using a Wheatstone bridge circuit.

These detectors have a means of offsetting background conductivity signals. They are especially suitable when used in conjunction with ion-exchange chromatography. Solutions of ionic compounds have low electrical resistance and hence have high conductivity values, which makes this type of detection measurement very feasible.

Amperometric Detector

A second type of electrochemical detector is the amperometric 'solute property' detector. These detection systems can afford excellent selectivity since organic functional groups electrolyse only at specific values of the applied potential. The amperometric detector has broad applicability for both ionic and molecular components as long as they can be oxidised or reduced at low voltages The two most popular designs are the thin-layer and wall-jet cell, with the three electrode configuration. The three electrodes (reference, auxiliary and working) are usually embedded in the wall of the flow stream. The chromatogram is recorded by measuring the detector cell current at a fixed potential as the sample is eluted from the column. The resulting current is directly proportional to the concentration of electroactive species in accordance with Faraday's Law. HPLC with amperometric detection was the technique of choice for the determination of polyglucose metabolites in plasma, which was linear in the range 0.1-10μg/ml[111]. A carbon fibre flow-through amperometric detector cell has been developed and used to determine nifedipine, nicardipine and pindolol in plasma, with a limit of detection of 15ng/ml for each compound[112]. Terbutaline and salbutamol have also been quantified in plasma using the same design of cell, yielding the excellent limits of detection of 0.8 and 1.0 ng/ml, respectively[113,114]. The sensitivity is generally greater than with UV-Vis but is less than with fluorescence. A disadvantage is the fact that the indicator electrode requires frequent cleaning due to a build-up of products from the electrochemical reaction.

1.2.4.4.4 LC-MS and LC-IR

MS and IR can be employed in the hyphenated mode as detectors and/or spectral libraries to aid in structural elucidation as well as being capable of quantitative analysis. It is easier to combine HPLC with IR since this mode of detection is normally used for liquids. LC-IR allows an IR spectrum to be taken of each component as it elutes from the column. This technique is especially suited for non-aqueous systems. The interfacing of LC and MS is slightly more difficult since MS is normally operated under conditions of low pressure and high vacuum. Interfacing is

most commonly achieved using thermospray ionisation, where the column effluent is converted into a fine mist spray and the analyte molecules are ionised by a dissolved salt, before being introduced into the MS. Some recent LC-MS and LC-MS-MS applications in biopharmaceutical analysis are given in Table 1.9.

Table 1.9 : Recent Biopharmaceutical Applications of LC-MS and LC-MS-MS

Compound and Matrix	Technique and Comments	Reference
Tamsulosin hydrochloride in human plasma and urine	LC-MS-MS used. The method was validated in the range 0.5–50 ng/ml in plasma and 1-100 ng/ml in urine	115
A series of new AIDS drugs i.e. pronucleotides in cells	LC-MS used. The method was used to quantify levels and unambiguously determine structures of the breakdown products. The use of triethyl-ammonium acetate as an ion-pairing agent in the HPLC facilitated interfacing to the MS	116
Buspirone and identification of its metabolites in bile, liver and urine	Both LC-MS and LC-MS-MS used. The authors developed a metabolite structure database	117
Identification of impurities in paclitaxel	Both LC-MS and LC-MS-MS used	117

MS is a universal but destructive detector. The MS bombards the substance under investigation with an electron beam and quantitatively records the result as a spectrum of positive fragments. MS for HPLC can be performed using a single-quadrupole (LC-MS) or a triple-quadrupole (LC-MS-MS). LC-MS-MS allows selective ion monitoring for more accurate qualitative and quantitative work.

1.2.4.4.5 Derivatisation in HPLC

Derivatisation is sometimes used in HPLC to introduce a chromophore into a molecule, hence enabling its detection by UV-Vis or fluorescence. It can also be used to improve the response of an analyte to other kinds of detector. Samples can be derivatised before being separated on the analytical column (pre-column derivatisation) or after being separated on the analytical column and before reaching

the detector (post-column derivatisation). However, derivatisation introduces an extra step into the protocol which can give rise to errors and should only be used if other detectors are not suitable and/or if significant improvements in sensitivity and selectivity are to be gained. Table 1.10 gives some examples of derivatising agents and the compounds for which they are most suitable.

Table 1.10 : Some Derivatising Agents used in HPLC

Type of Detection Required	Derivatising Reagent	Type of Compound	Reference
UV-Vis	Benzoyl chloride	Alcohols	118
	3,5-dinitrobenzoyl chloride	Amines	119
	2,4-dinitrophenylhydrazine	Aldehydes & ketones	120
	Benzyl bromide	Carboxylic acids	121
Fluorescence	1-anthroylcyanide	Primary and secondary alcohols	122
	o-phthalaldehyde	Primary amines & amino acids	123
	Dansyl hydrazine	Aldehydes & ketones	124
	1-bromoacetylpyrene	Carboxylic acids	44
Electrochemical	N-(4-anilinophenyl)maleimide	Sulphahydryl Compounds	125
	o-phthalaldehyde	Primary amines & amino acids	126

1.2.4.4.6 Identification and Quantitation in HPLC

This has been previously discussed in general terms in section 1.2.4.1.4 (Identification and Quantitation in Chromatography). However, particular to HPLC is the fact that t_r, t_r' or k' can be used for qualitative identification of components, and external or internal standards are usually used for the purpose of calibration and calculation. As in GC, the hyphenated techniques such as LC-MS and LC-IR can also be used to give structural information on the compounds of interest. Quantitation in chromatography, particularly in HPLC, is further explored in section 1.3 on validation.

1.2.4.5 Supercritical Fluid Chromatography

Supercritical Fluid Chromatography (SFC) is performed at pressures and temperatures above the critical values for the mobile phase. A supercritical fluid is neither a liquid or gas but has properties intermediate between the two. It can be formed from a conventional liquid by raising the temperature, or from a conventional gas by increasing the pressure. The density of supercritical fluids can be conveniently varied from gas-like to liquid-like by adjusting the temperature and pressure. In this way a compromise can be found between favourable chromatographic conditions and eluotropic strength. Supercritical carbon dioxide is a typical eluent for SFC with a critical temperature of 304K and a critical pressure of 72.8atm. It is safe, is compatible with a number of detectors, it is readily available, relatively inert and inexpensive. Most importantly, however, it has favourable critical properties and can be stored and handled as a liquid. CO_2, however, is not a good solvent for polar and high molecular weight solutes and it is not transparent in the mid-IR range. In these cases, alternative SFC mobile phases are ammonia, nitrogen oxide and alkanes. Ammonia however, is corrosive, which makes its use somewhat prohibitive. Sometimes modifiers such as methanol can be used to alter the base eluent properties of supercritical CO_2 to render it more polar, but care must be taken since this can interfere dramatically with detection, especially when using an FID.

The stationary phase, as in HPLC, determines the selectivity for the compound(s) of interest. Both open tubular and packed columns can be used in SFC. Typical phases are polysiloxanes (which have high temperature stability and high diffusion coefficients for solutes) and carbowax. For packed column SFC, rapid, efficient analysis can be obtained on columns packed with 3 – 10 μm particles. Typical phases include modified silicas, porous-bonded phases and polymer-bonded phases. Simon *et al.* used a diol-bonded silica column, a mobile phase consisting of ethanol-water-methanesulphonic acid (97.5:2.4:0.1, v/v/v) to determine the urinary metabolites of styrene[127]. Because of the limited number of supercritical fluids and the disadvantages associated with them such as those mentioned for ammonia, SFC has not expanded as much as would have been expected for a technique with so much to offer analytically. The technique is also limited to the analysis of those compounds that are soluble in supercritical fluids. Hence SFC is not a widely used form of chromatography but for the analytes to which it is suited, it offers the benefits of both HPLC and GC. This is because since both volatile and non-volatile solutes can be

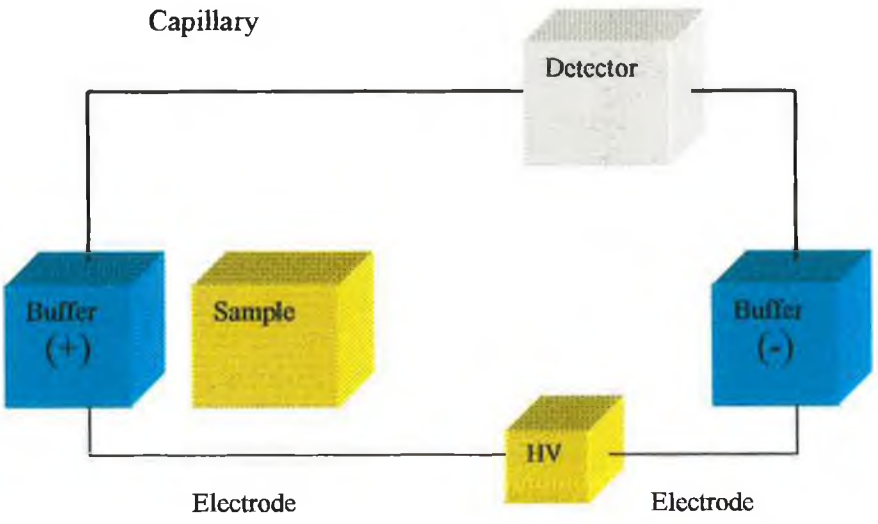
determined, rapid analysis is possible with high resolution, and a wider range of detection modes is available than in HPLC or GC alone.

1.2.5 CAPILLARY ELECTROPHORESIS

1.2.5.1 Principles of Capillary Electrophoresis

Capillary electrophoresis (CE) is a technique combining the separation principles of electrophoresis and the instrumentation and design concepts of chromatography. Charged species are separated on the basis of their different velocities in an electric field. Under the influence of an external potential, each ion moves towards the electrode of opposite charge. CE is performed in a system based on that shown in Figure 1.10.

Figure 1.10 : Layout of a Typical CE System



A narrow-bore, fused silica *capillary* is used for the separation and the ends of which are placed in *buffer reservoirs*. These reservoirs of electrolyte also usually contain the *electrodes* which are used to make contact between the high voltage (*HV*) power supply and capillary. The *sample* is introduced into the capillary by placing one end of the capillary into the sample vial for just long enough so that a very small volume

(usually a few nanolitres) can be introduced into the capillary, usually by hydrodynamic means. After replacing the capillary into the buffer reservoir, the high voltage is applied and the electrophoretic separation performed. After migrating from the injector some way into the capillary, the solutes are visualised by the *detector* through a window in the wall of the capillary.

1.2.5.1.1 History of Capillary Electrophoresis

In the 1930s, it was discovered that by placing a mixture of macromolecules between buffer solutions in a tube and applying an electric field, sample components migrated in a direction and at a rate determined by their charge and mobility. Electrophoresis has traditionally been performed in anti-convective media such as polyacrylamide gel, cellulose acetate or paper because of thermal diffusion and convection limitations, and has been mainly applied to the size-dependant separation of biological macromolecules such as nucleic acids and proteins. In 1967, Hjerten began to investigate the use of narrow-bore capillaries rather than gel media[128]. These capillaries were only available as small as one millimetre in bore size. Later, capillaries of approximately 200μm internal diameter composed of glass and teflon were reported[129]. The capillaries used by Jorgenson and Lukacs in the early 1980s were even more narrow (75μm)[130,131]. The theory for CE was developed when the relationships between operational parameters and separation quality were described, and when the potential of high performance capillary electrophoresis as a technique was demonstrated. Since the late 1980s, numerous books[132,133] and reviews have been written, and commercial instruments are now in relatively widespread use.

1.2.5.1.2 Theory of Capillary Electrophoresis

Mobility

The migration of a solute in CE depends on the mobility (μ) of that species. Electrophoretic mobility (μ_e) can be described in terms of velocity (v) and applied electric field (E),

$$\mu_e = v / E$$

The electric field (in volts/cm) is a function of the applied voltage and capillary length. Mobility is a constant for that ion in a particular medium under the

electrophoretic conditions. Mobility can also be expressed in terms of physical parameters,

$$\mu_e = \frac{q}{6\pi\eta r}$$

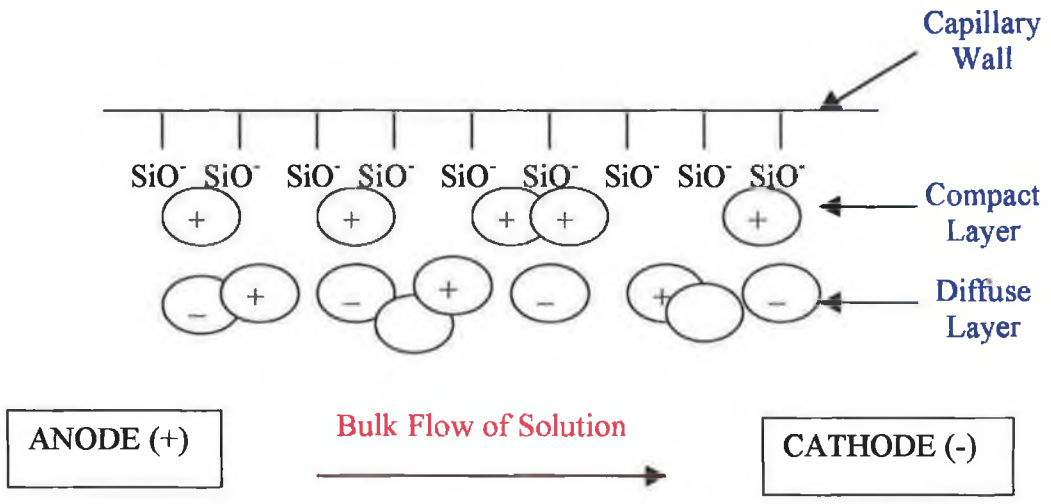
where q is the charge on the ion, η is the viscosity of the electrolyte and r is the radius of the ion. It is evident from this equation that small, highly charged species have high mobilities while large, minimally charged species have low mobilities. The electrophoretic mobility found in standard tables is usually different to that determined experimentally. The latter is called effective mobility (μ_{eff}) and is often highly dependent on factors such as pH and composition of the electrolyte buffer as well as temperature. In fact, mobility is found to increase by approximately 2% for each degree rise in temperature[134]. In the presence of electroosmotic flow (discussed next), the effective mobility can be described as the sum of the electrophoretic mobility and the mobility of the electroosmotic flow,

$$\mu_{eff} = \mu_e + \mu_{EOF}$$

Electroosmotic Flow

Another important parameter in CE operation is electroosmotic flow (EOF) which describes the 'bulk flow' of liquid in the capillary and is a consequence of the surface charge on the interior capillary wall. The EOF results from the dissociation of acidic silanol groups at the capillary wall in contact with the electrolyte buffer. Hydrated cations in the electrolyte solution are attracted to the negatively charged silanol groups and become arranged into two layers (Figure 1.11). One layer is tightly bound to the wall by electrostatic forces (compact layer) and the other is more loosely bound (diffuse layer). When an electric field is applied, the predominantly negatively charged diffuse layer breaks away and moves towards the cathode, dragging with it the bulk solution of the electrolyte as a result of viscous drag.

Figure 1.11 : Electrical Double-Layer at the Capillary Wall



Thus, EOF can influence the mobility of a solute in the capillary. It can accelerate the migration of a solute if flowing in the same direction, whereas it can increase the resolution of a mixture of solutes if flowing in the opposite direction. Because the EOF arises from ionisation of acidic silanol groups at the surface of the capillary wall, the magnitude of the EOF will increase with increasing pH. It will also increase with increasing applied voltage. However, EOF is found to decrease with increasing ionic strength of the buffer.

Under normal conditions, the power supply is positively charged at the anode and the EOF flows from the anode via the detector to the cathode, such that cations in a mixture will migrate first, followed by neutral molecules followed by anions. However, both the direction and magnitude of the EOF can be controlled by varying parameters such as pH. To change the direction of the EOF, for example, if a mixture of anions was to be separated, the polarity of the electrodes can be reversed such that the cathode is at the injector end of the capillary. To take advantage of the EOF in this situation, the charge on the capillary wall must be reversed also. This is achieved by the addition of cationic surfactants to the electrolyte solution which coat the capillary wall, thus giving it a positive charge. The charged diffuse layer now flows towards the detector from the *cathode* to the *anode* in the same direction as the analytes. To reduce or suppress the EOF in capillaries, the ionic strength of the buffer may be increased or CE carried out at pH extremes. Alternatively, the surface of the capillary may be chemically derivatised to eliminate charged attraction sites or a chemical

reagent e.g. a surfactant, can be added to dynamically reduce solute-capillary interactions.

Efficiency and Resolution

Efficiency (N) in CE describes peak broadening as a function of migration time. High voltages, fast migration times and species with low diffusion coefficients result in the most efficient separations. N is also affected by injection length. For small injection lengths, the peak widths are determined by diffusion, while with large injection lengths, the peak widths are related to the variance of the injection length. The shape of the peaks is determined by the concentration of the analytes. Unless the concentrations of the compounds are at least two orders of magnitude lower than the ionic strength of the buffer, slower moving analytes will exhibit tailing while faster moving analytes will exhibit fronting. N can also be affected by a phenomenon called Joule heating which is the heat generation that occurs when analyte and buffer molecules collide as a result of the conduction of electric currents. It can cause non-uniform temperature gradients and local changes in viscosity, both of which lead to zone broadening. It is minimised when capillaries are shorter than 90cm and the internal diameter is less than 80μm.

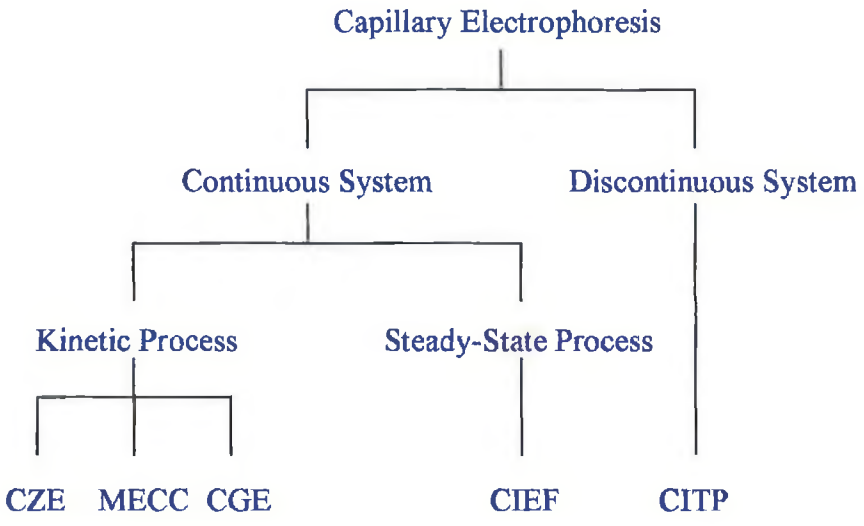
Resolution (R) is dependent primarily on the efficiency of the system and to a certain extent on the differences in the migration times of the analytes of interest. This is in contrast to chromatography, where separation and resolution are primarily driven by selectivity. Resolution is affected by the speed and direction of the EOF, the mobility of the analytes of interest and to a lesser degree, the applied voltage.

1.2.5.2 Modes of CE

As in HPLC, there are a number of modes of CE, which make it very versatile (see Figure 1.12). There are two main types of CE systems – continuous and discontinuous. In the continuous CE modes, there is further subdivision into those modes that have a constant electrolyte composition throughout the analysis (kinetic process) and those modes where the composition of the electrolyte is not constant over the length of the capillary (steady-state process). The final array of modes are capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MECC), capillary gel electrophoresis (CGE), capillary isoelectric focussing (CIEF) and capillary isotachophoresis (CITP), all exploiting different separation mechanisms.

For the most part, the different modes are accessed simply by altering the electrolyte buffer composition.

Figure 1.12 : Modes of CE According to the Nature of the Electrolyte System



CZE

CZE is fundamentally the simplest form of CE, mainly because the capillary is filled only with buffer. The separation mechanism is based on the charge-to-mass ratio of an analyte in an electric field. Because the composition of the electrolyte buffer is constant, the electric field and the effective mobilities of the solutes will remain constant, and the sample components will migrate with mutually different but constant velocities. Both anions and cations can be separated due to the EOF. Neutral species do not migrate and elute together with the EOF. In CZE, selectivity is easily altered by changing the composition of the buffer or its pH, or by the use of additives in the buffer. Many of the applications of CZE are in the area of bioscience, including the analysis of glycoproteins such as erythropoietin[135], and peptides[136,137], as well as small molecular weight species such as cefixime or cefotaxime drugs[138] and cephalosporin antibiotics.

Chiral CE is considered to be a variation of CZE, with the additional separation mechanism based on chiral selectivity. When compared to chiral HPLC, chiral CE is less complex, not necessarily requiring specialised capillaries and it provides higher efficiencies, thereby allowing quantitation of trace levels of enantiomers, which is difficult in HPLC. As with chiral HPLC, the same three

approaches apply (see section 1.2.4.4.1), the most commonly taken approach being the addition of chiral agents to the buffer e.g. cyclodextrins.

MECC

MECC is widely used since it can separate mixtures containing both neutral and charged solutes. This is achieved by the use of a surfactant in the running buffer. The individual surfactant molecules will aggregate to form micelles if the concentration is above the critical micelle concentration, which for example is 8 to 9 mM for sodium dodecyl sulphate (SDS). Micelles are essentially spherical with the hydrophobic tails of the surfactant molecules oriented towards the centre, and the charged heads oriented towards the buffer. It is this interaction between the charged micelle and the solutes (neutral or charged) that effects the separation. The presence of micelles allows neutral components to be separated by a partitioning mechanism of the solute between the micelles and the buffer. There are many applications of MECC for the determination of drugs such as cefotaxime[138], amikacin[139], paclitaxel[140] and glipizide and glyburide[141].

CGE

CGE is principally employed for the separation of larger biomolecules such as proteins, DNA and nucleotides. This technique operates using both electrostatic and size exclusion mechanisms. The 'gel' capillary contains either a real gel, such as crosslinked polyacrylamide or more commonly, a polymer network which acts as a molecular sieve. Thus, larger molecules are hindered to a greater extent than smaller ones as they migrate through the capillary.

CIEF

CIEF is a technique whereby a pH gradient is formed in the capillary (using ampholytes i.e. zwitterionic molecules) in order to separate peptides and proteins on the basis of their isoelectric points. This separation mechanism is quite different from the mechanisms encountered in the previous CE modes. A basic solution is added to the reservoir at the cathode and an acidic solution to the reservoir at the anode. The electric field is applied, and the mixture of ampholytes and solute in the capillary migrate along the capillary until they reach a pH which coincides with their pI value. After this 'focussing' has taken place, the narrow zones are mobilised and passed through a detector. Larger sample volumes can be used when compared to other CE

modes, but special coated capillaries are usually required to reduce the EOF, which otherwise would interfere with the focussing.

CITP

In CITP, a combination of two buffers is used to create a state whereby all separated zones move at the same velocity. The zones remain sandwiched between leading and terminating buffers. If a group of anions were to be separated, a leading buffer with a higher electrophoretic mobility and a terminating buffer with a lower electrophoretic mobility than that of the solutes would be chosen. Either anions or cations, but not both, can be separated in one CITP experiment. Amiloride and β-blockers were separated by cationic CITP yielding quantitation limits of 32-46ng/ml depending on the drug[142].

1.2.5.3 Capillaries in CE

Fused silica is the most commonly used material for capillaries in CE. It is electrically inert, flexible and inexpensive. In a similar way to GC capillaries, the silica is coated with a protective layer of polyimide to make it stronger. To create the optical window, a small section of this polyimide material is removed, leaving only the silica glass which is UV-Vis transparent. Another material used for capillaries is teflon. However, teflon exhibits a number of disadvantages including inhomogeneous inner diameters and significantly higher EOF values than silica. Short capillaries yield shorter analysis times, but longer capillaries yield superior resolution. Most commonly, 50 to 75cm effective lengths of capillary are used. In order to maintain a high quality of electrophoresis, conditioning of the capillary must be carried out prior to starting CE and in between each injection thereafter. The precise procedure will depend on the nature of the sample, but frequently a one or two minute wash is used to restore the characteristics of the capillary wall and thus maintain the EOF. Temperature and voltage must also be carefully controlled.

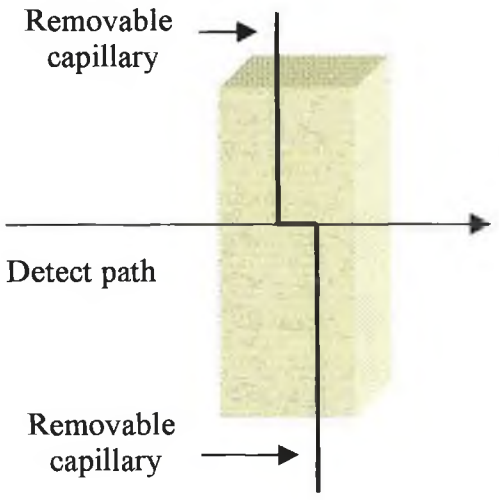
1.2.5.4 Detectors in CE

Detectors that are suitable for HPLC are usually suitable for CE. The most widely used detection method, as with HPLC, is that of UV-Vis.

UV-Vis Detector

With fused silica capillaries, wavelengths as low as 190nm can be scanned. This has useful applications in the analysis of compounds such as proteins that have no strong chromophores but which can be adequately detected at these wavelengths. At these low wavelengths, it is necessary to use buffers with low background absorbance such as phosphate or borate. It is important to note that since the detection window is in the capillary itself, there is no band broadening as a result of dead-volume or mixing effects. However, the short pathlength is the factor that limits sensitivity in CE. Special capillaries have been developed to extend the optical pathway without increasing the overall capillary area. One such design is the 'bubble cell' which has an expanded region inside the capillary. Sensitivity is improved 3 to 5-fold without increasing the band broadening. Another design is called the Z-cell which offers an improvement of an order of magnitude or more in the level of sensitivity. A schematic diagram of such a cell is shown below in Figure 1.13.

Figure 1.13 : Diagram of a Z-Cell for CE



The diode array detector (DAD) can greatly simplify the analysis of electrophoretic data by making possible the rapid scanning of novel compounds so that the optimum wavelength for each analyte can be chosen rapidly. DAD then allows the monitoring of different components of a sample, at their different absorbance maxima simultaneously. Peak purity can be assessed, identities of peaks can be confirmed and concentrations can be determined.

Laser-Induced Fluorescence

The next most commonly used mode of detection is fluorescence. Fluorescence detectors have the great advantage over UV-Vis that sensitivity is comparatively independent of pathlength. However, few commercial HPLC fluorescence detectors can be modified for efficient use with small capillaries. Consequently, most fluorescence detectors in CE are custom-built and because of superior focusing capabilities and reduced stray light levels, lasers have been employed as the excitation source for fluorescence detectors used with CE. The use of laser-induced fluorescence (LIF) detectors is a relatively new technique with sensitivity so great (10^{-14} – 10^{-16} M) that single molecule detection is now possible. Zolpidem was determined in urine without extraction using CE-LIF[143]. The separation took only 10 minutes and the limit of detection was 2ng/ml.

As few molecules exhibit natural fluorescence, derivatisation is usually required prior to analysis. In the determination of amikacin in human plasma by CE with fluorescent detection, the derivatisation reagent used was 1-methoxy-carbonylindolizine-3,5-dicarbaldehyde[139]. MECC was the mode of choice, and the overall method yielded a limit of detection of 0.5μg amikacin per ml of plasma.

Other Detectors

Other detection systems include electrochemical detection such as amperometry, conductivity and voltammetry as well as RI detection.

1.2.5.5 CE-MS

The use of MS with CE is especially suitable since the liquid flow rate is compatible with conventional mass spectrometers. There are three main ways to interface CE with MS i.e. electrospray ionisation, ion spray and continuous-flow fast atom bombardment. The enantiomers of terbutaline and ephedrine have been determined by CE coupled with ion spray MS detection[144]. A comparison of this mode of detection with UV detection was carried out to demonstrate the selectivity and sensitivity advantages of the MS as a detector for CE.

1.3 VALIDATION IN BIOPHARMACEUTICAL ANALYSIS

In sections 1.1 and 1.2, sample preparation and analytical procedures have been discussed. However, the choice of techniques to use and the subsequent development of applicable methodology do not guarantee a reliable and effective assay. Just as important in the overall scheme of method development is validation of the entire protocol. Before an analytical method can be routinely implemented, it must first be shown to fulfil certain performance criteria. When this has been documented, the method is said to be validated. However, controversy remains as to exactly what parameters should be measured, what the acceptable limits are for these parameters and what must be done to show that the parameters meet the performance criteria. Most regulatory bodies, such as the Food and Drug Administration, have mandated a code of Good Laboratory Practice for clinical and non-clinical laboratory studies. However, guidelines for validation are constantly in a state of flux as methods and techniques change and improve.

The following twelve parameters, for assessing the validity of a method, are discussed in the context of chromatographic techniques, but in theory they can be applied to any analytical procedure. It should be noted that validation protocols are tailored for each individual assay, and hence criteria for validating one method may be different from those used to validate another method. Few validations will incorporate every parameter since each analytical technique will have different applications and hence different validation requirements. However, each parameter should be carefully considered even if it is not to be evaluated in the final validation protocol.

1.3.1 CALIBRATION

The heart of a chromatographic method is the calibration procedure used to calculate the analyte(s) to be being measured. The calibration model should be described when reporting on a validation. There are a number of options of which the most common are linear regression[148] (weighted and unweighted), average by amount and multilevel calibration; more obscure calibration routines such as quadratic and cubic have been reported[145]. Five to seven concentrations should be used to define the standard curve[146].

1.3.2 SELECTIVITY

Selectivity is a measure of the extent to which the method can determine a particular compound in the matrix being analysed without interference from matrix components. It is recommended that the selectivity of the method be established with respect to endogenous substances, metabolite(s) and known degradation products[147]. If a method is completely selective for a component or a group of components, it can be considered to be specific. Specific methods are very rare.

1.3.3 RANGE

Quantitative analyses should have the working range of the method stated. It is within this range that the precision and accuracy are calculated to be acceptable.

1.3.4 LINEARITY

Linearity is determined by analysing samples of varying concentration and establishing the concentration-response relationship. It is very desirable that a method is linear over a substantial range, but it is not an absolute requirement. Linearity has been discussed in detail by Karnes *et al.* [148]

1.3.5 LIMIT OF DETECTION

The limit of detection (LOD) is determined experimentally for each analyte in a method. This is achieved by repeatedly analysing a blank matrix and one containing analyte at a concentration that produces a response equivalent to the mean blank response plus three standard deviations. The usefulness of this parameter depends on whether the results close to this value are likely to be important.

1.3.6 LIMIT OF QUANTITATION

The limit of quantitation (LOQ) is the lowest concentration of an analyte that can be measured with an acceptable level of precision and accuracy. An acceptable level is in general considered to be 10-20% relative standard deviation[145]. The LOQ value must

be determined by experiment and should be measured using at least five samples (of analyte in matrix)[149]. The LOQ is usually, but not always, the lowest point on the calibration curve.

1.3.7 RUGGEDNESS

Also known as robustness, this parameter attempts to measure variations that are introduced when a method is set up under other conditions, which may alter the performance of the method. Such influences can most easily be tested for by using experimental design to elucidate the factors that are most crucial for successful execution of the method. In HPLC for example, such tests could comprise the influence of type and concentration of modifiers and additives in the mobile phase, pH of the mobile phase, temperature, concentration of buffer etc.

1.3.8 ACCURACY

Accuracy, according to the Washington Conference Report on Bioanalytical Method Validation[149], is the "closeness of determined value to the true value. Generally, recovery of added analyte over an appropriate range of concentrations is taken as an indication of accuracy. Whenever possible, the concentration range chosen should bracket the concentration of interest". Accuracy is probably the most difficult parameter to validate. The influences of sampling and sample preparation must be considered, together with interferences in the separation and detection systems. Reference materials truly representing the unknown sample are not always available and complex biological matrices are difficult to mimic. The analyte can be added to liquid samples and recovery studies may give an indication of method accuracy. The ideal situation is to compare the method with a totally independent method, even with respect to sample treatment. Accuracy should be evaluated at both high and low concentrations. Accuracy is affected by a combination of two different kinds of experimental error – systematic and random. These are discussed in detail in a 1994 report[150] which analysed the 'Washington Conference Report on Bioanalytical Method Validation'.

1.3.9 PRECISION

Precision is "the closeness of agreement between independent test results obtained under prescribed conditions"[151]. In other words, precision is a measure of the degree to which independent test results agree with each other, and is expressed in terms of standard deviation. According to the Eurachem/WELAC document on Accreditation for Chemical laboratories[152], when measurements have been performed on the same material under the same experimental conditions, with the same analyst, using the same instrumentation (in the same laboratory) within a reasonable time frame (usually the same 24-hour period), this is described as repeatability or intra-assay. When measurements have been performed on the same material under the same experimental conditions, but with a different analyst or different instrumentation or during a time frame of days or weeks, this is described as reproducibility or inter-assay. The same definitions of repeatability and reproducibility are given by ISO[151]. At a minimum, three concentrations representing the entire range of the calibration curve should be studied – one near the LOQ, one near the centre and one near the upper boundary of the standard curve[149].

1.3.10 SENSITIVITY

Sensitivity is the difference in analyte concentration corresponding to the smallest difference in the response of the method that is discernable. This parameter can be determined from the slope of the calibration curve or experimentally by measuring closely related analyte concentrations over the entire range of the assay.

1.3.11 STABILITY

The stability of the analyte in the matrix during the collection process and the sample storage period should be assessed, preferably prior to sample analysis. Assessment of stability during storage can be accomplished by analysing spiked samples immediately and on subsequent days for the anticipated storage period[146]. The influence of freeze/thaw cycles should also be studied[149].

1.3.12 RECOVERY

High recovery of analyte(s) from the matrix is desirable, but it may sometimes be necessary to intentionally sacrifice high recovery in order to achieve better selectivity[147]. Relative recovery is defined as the response of the analyte measured from the matrix as a percentage of that measured from pure solvent[146]. Absolute recovery is defined as the response of a processed spiked matrix standard expressed as a percentage of the response of pure standard which has not been subjected to sample pretreatment[146].

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Chapter 2

HPLC Analysis of Taurine in Human Plasma using Pre-Column Extraction and Derivatisation

2.1 INTRODUCTION

2.1.1 Biological Background

Although taurine was discovered more than 160 years ago, it is only in the past two decades that the biochemical importance of this molecule has really been investigated. Initial interest in taurine was generated when it was found that during both World Wars, naval doctors had administered taurine to their soldiers to enhance their night vision and relieve fatigue. Taisho Pharmaceutical Company in Japan heard about this and began production and sale of "taurine extract" in 1949. Taurine, believed to be an all-round medicine, was used to cure everything from pneumonia to bedsores. With research beginning to show that taurine was useful for general health, Lipovitan D, a mixture of taurine and B vitamins, was developed and sold for the first time in 1962. Today there are many taurine products sold in all forms. Perhaps the most recent taurine products are the new high energy drinks which are generally a combination of taurine with caffeine and/or vitamins. A typical 250ml can contains 1000mg of taurine and 80mg of caffeine. Manufacturers claim that their drinks "stimulate and revitalise the body and brain, and enhance mental and physical performance".

Taurine ($NH_2CH_2CH_2SO_3H$), which is a naturally-occurring ß-sulphonated amino acid, has many other important and beneficial effects on the human body. This molecule has been implicated in many physiological functions[1], pharmacological actions and pathological conditions; it also has a role in bile salt metabolism and has bacteriocidal and anti-inflammatory properties. Among the physiological roles attributed to taurine are membrane stabilisation[2], antioxidation[3,4], neuromodulation[5] and regulation of calcium homeostasis[6]. Taurine has also recently been associated with blood thinning abilities –taurine was found to augment the anti-aggregative effect of aspirin on the blood[7] and to actually reverse blood clotting when given as a supplement to diabetics[8]. Some of these unique properties of taurine may be explained by the fact that, unusually, it is a free circulating amino acid that is not incorporated into proteins[9]. It is also different from the more commonly known amino acids insofar as it is a sulphonic rather than a carboxylic amino acid, and it is a ß-amino acid rather than an α-amino acid. Taurine, also known as 2-aminoethanesulphonic acid, has a molecular weight of 125.2 and has two pKa values (at 25°C) of 1.5 and 9.1. It is the most abundant amino acid in the human body, and a 70kg person can contain up to 70g of

taurine[10]. Most of our taurine supply comes directly or indirectly from animal protein in our diet, which is why vegetarians may have lower levels of taurine.

Taurine concentrations are extraordinarily high in the developing brain and fall sharply thereafter. Other amino acids present in the brain show an opposite developmental pattern being higher in adult brain than in growing brain. However, this demand for taurine is combined in humans with an apparent inability to synthesise it in sufficient quantities to fulfil our needs. The major, if not the sole, source of taurine in infants is breast milk, hence babies weaned on milk formulae can be deprived of this source of taurine. This deprivation results in a number of biochemical abnormalities, but no obvious clinical ones. Research has shown that supplementing premature infant's food intake with 0.05g taurine per kg body weight brings their taurine levels as high as those in breastfed babies[11]. Although there is no compelling evidence to show that taurine deficiency in humans does any real harm, such evidence does exist for other mammals including cats and monkeys. Insufficient taurine in cats is associated with retinal degeneration and blindness[12] and with cardiomyopathy[13], a condition that is reversible as soon as the diet is supplemented with taurine[14]. This is now so important that manufacturers of catfood supplement their products with taurine. Monkeys fed a taurine-deficient diet experience suppressed growth and development[15].

Although intracellular taurine concentration in humans is controlled within narrow limits[16], concentrations in blood are altered in certain disease states, for example, plasma levels of taurine alter in trauma[17], sepsis[18] and cancer[19]. There is also evidence to suggest that the level of taurine in plasma may be a useful indicator of myocardial infarction[20]. Taurine levels are significantly raised in the bronchoalveolar lavage fluid of asthmatic patients[21] and in the cerebrospinal fluid of patients with migraine[22]. Changes in the concentration of taurine in the plasma of patients with major depression have also been found[23,24]. Taurine levels are decreased in the neutrophils of patients with chronic psoriasis[25], in the cerebrospinal fluid of patients with Parkinson's disease[26] and Alzheimer's disease[27], in the plasma of patients undergoing long-term parenteral nutrition[16], and in the plasma and muscle of uremic patients[28,29,30]. As the diagnostic role of taurine in various disease states becomes more widely recognised, the need for simple, rapid assays for routine plasma taurine analysis has become increasingly important.

2.1.2 Analytical Background

Amino acids of interest can mainly be found in biological fluids such as blood and urine. The clinical interest is not focussed so much on the total amount of free amino acids in these samples but in the changes in their concentration that can be of diagnostic significance. The typical concentrations of some amino acids in human plasma are given in Table 2.1

Table 2.1 : Levels of Amino Acids in Human Plasma

Amino Acid	CONCENTRATION (μg/ml)				
	Ref. [31]	Ref. [32] (n=8)*		Ref. [32] (n=76)+	
	Range	Mean	Range	Mean	Range
Alanine	25.7 - 53.7	30.7	22.2 – 44.7	29.9	18.7 – 58.9
Arginine	11.0 - 35.8	14.3	8.6 – 26.3	13.0	3.7 – 24.0
Asparagine		5.7		5.8	5.4 – 6.5
Aspartic acid	5.8	2.2	0.0 – 7.2	1.0	0.0 – 3.2
Citrulline	3.8 - 5.9	5.3	2.1 – 9.7	5.0	
Cystine	8.9 - 20.5	17.7	11.5 – 33.7	10.5	2.0 – 20.2
Glutamine	26.8 - 88.8	83.0	60.7 – 101.5	83.0	
Glutamic acid	6.3 - 61.9	8.6	2.5 – 17.3	8.5	2.1 – 28.2
Glycine	12.5 - 22.9	17.4	10.8 – 36.6	17.8	9.0 – 41.6
Histidine	10.6 - 17.8	12.4	9.7 – 14.5	11.5	4.9 – 16.6
Isoleucine	9.8 - 22.2	7.1	4.6 – 11.5	8.3	4.8 – 12.8
Leucine	12.3 - 25.9	13.2	9.3 – 17.8	14.5	9.8 – 23.0
Lysine	21.1 - 37.9	25.4	21.1 – 30.9	22.4	12.1 – 34.8
Methionine	4.6 - 14.8	3.2	2.3 – 3.9	3.4	0.9 – 5.9
Phenylalanine	7.4 - 20.2	9.5	6.3 – 19.2	8.8	6.1 – 14.5
Proline	23.6	27.1	12.8 – 51.4	21.2	11.7 – 38.7
Serine		11.8	6.8 – 20.3	12.1	7.7 – 17.6
Taurine		*8.3*	*5.7 – 17.3*	*7.9*	*3.4 – 21.0*
Threonine	11.2 - 29.2	19.4	12.2 – 29.3	15.4	9.4 – 23.0
Tryptophan	6.6 - 15.0	9.8	5.1 – 14.9		
Tyrosine	7.4 - 22.2	9.1	6.5 – 11.3	9.4	3.9 – 15.8
Valine	21.5 - 35.1	19.9	13.6 – 26.6	25.0	16.5 – 37.1

*** Data from one laboratory.**
+ Data from 9 laboratories.

The classic procedures for the separation and determination of amino acids have been ion-exchange chromatography using the ninhydrin reaction, and certain TLC systems which have worked most successfully as semi-quantitative methods. The

answer to the quantitative analysis of amino acids has been given by HPLC, which allows rapid and sensitive determinations to be made, even in difficult matrices such as blood. When HPLC results for taurine in human plasma have been compared with those from an Amino Acid Analyser, the concentrations correlate very well, which is not the case with some other amino acids[33].

Most amino acids have low extinction coefficients and therefore they are usually derivatised before being determined by HPLC with fluorescence detection. Some of the derivatising agents that have been used to allow the quantitation of taurine in biological matrices for HPLC analysis are given in Table 2.2.

Table 2.2 : Derivatising Agents used in the HPLC Analysis of Taurine

Derivatising Agent	Reference
Ortho-phthalaldehyde	Kisrakoi, 1988 [34] Patrizio, 1989 [35]
Ortho-phthalaldehyde / thiol	Hirshberger, 1985 [36] Haller, 1987 [37] Eslami, 1987 [38] Hirai, 1987 [39] Anderson, 1988 [40] Porter, 1988 [41] Waterfield, 1994 [42]
Dansyl chloride	Wiedmeier, 1982 [43] Biondi, 1987 [44] Saller, 1989 [45] Amiss, 1990 [46]
Dabsyl chloride	Stocchi, 1994 [47]
Phenylisothiocyanate	Lippincott, 1988 [48]
9-fluorenylmethyl-chloroformate	Mant, 1992 [49]
3,5-dinitrobenzoyl chloride	Masuoka, 1994 [50]
1-naphthyl isocyanate	Neidle, 1989 [51]
Thiamine	Yokoyama, 1991 [52]
Fluorescamine	Shihabi, 1979 [53] Sakai, 1992 [54]

The most widely used derivatising agent for taurine has been ortho-phthalaldehyde (OPA), which usually requires the presence of a thiol such as 2-mercaptoethanol to ensure that derivatisation occurs rapidly and in order to stabilise the derivatives. OPA is not fluorescent itself but it produces high yields of highly fluorescent derivatives. Although the OPA derivative of taurine has been determined in

HPLC by both UV absorbance[39,41] and fluorescence[36,38,40], there are stability problems associated with the adducts formed[55]; within only a few minutes of their formation, fluorescence begins to diminish. Hirschberger *et al.* documented this problem when they noticed that the fluorescence yield of their taurine derivative decreased dramatically when solutions were injected four minutes after derivatisation compared to one or two minutes after derivatisation[36]. Anderson *et al.* improved the half-life of OPA-derivatised taurine to 17 minutes, when the thiol used in the reaction was 3-mercaptopropionic acid[40]. Dansyl chloride has also been used to determine taurine by forming the fluorescent derivative[43,45,46], but this derivatisation reaction typically suffers from many side products, requires quenching of the reaction and can have a long reaction time e.g. four hours[43]. Taurine has also been analysed by measuring the strong absorbance of its dabsyl chloride derivative in the visible region[47], which is very stable. However, this reaction requires high temperatures (70°C) and is sensitive to the presence of salts which can have detrimental effects on the reaction yield.

Other derivatising agents for amino acids include phenylisothiocyanate (Edman's reagent)[48] which allows taurine to be monitored in the UV region (240-255nm). In this derivatisation, the pH needs to be carefully controlled to prevent conversion of the phenylthiocarbamyl derivatives to phenylthiohydantoin derivatives. This reaction is also markedly affected by the presence of salts, divalent cations and buffers. The reagent 9-fluorenylmethyl chloroformate (FMOC-Cl) forms a very stable, fluorescent carbamate derivative with taurine[49]. Disadvantages of the use of this compound include the fact that it is itself fluorescent and it also forms hydrolysis products that in turn are fluorescent. Hence, an extraction step is usually required prior to HPLC analysis. The HPLC analysis of taurine in biological samples has also been carried out using 3,5-dinitrobenzoyl chloride[50], 1-naphthyl isocyanate[51] and thiamine[52].

The objectives of this work were to develop an analytical HPLC procedure for the determination of taurine in human platelet-rich plasma. This was to be done using a derivatisation reaction so that taurine could be detected by its resultant absorbance in the UV-Vis region of the spectrum.

Two derivatising agents for taurine were investigated in this project. The first, hypochlorite, has been previously employed to quantify taurine in neutrophils[56]. The reason for using this reaction was based on the knowledge that taurine is known to undergo reaction with native HOCl *in vivo*, during the respiratory burst, resulting in chlorinated derivatives[3]. When previously used, the derivatisation procedure was

carried out at room temperature, and taurine was reacted with sodium hypochlorite to form the taurine derivative (monochlorotaurine) which has maximum absorbance at 254nm. The β-chloramine derivative, once formed, has been reported to be more stable than α-amino acid chloramines[57], which is a clear analytical advantage and the reason it was selected as the preferred derivatising agent for investigation.

The second derivatising agent, fluorescamine (fluram), was first introduced for the determination of primary amines and amino acids in 1972[58]. Fluorescamine and its hydrolysis products are not fluorescent and excess fluorescamine is concomitantly destroyed with a half-life of milliseconds. The fluorophors have been found to be stable for several hours. This reagent has been used for the quantitation of taurine using fluorescence detection[53,54]. Fluorescamine was selected for investigation because its reaction with primary amines proceeds instantaneously at ambient temperature in alkaline solution, the reagent and its major hydrolysis products do not interfere, and the derivatives are stable.

2.2 REAGENTS AND MATERIALS

2.2.1 Equipment

The high performance liquid chromatograph was equipped with a Waters (Milford, MA, USA) 510 dual-piston pump, a Waters 486 tunable absorbance detector and a Waters 746 data module. The Rheodyne (Cotati, CA, USA) injection port was fitted with a 20μl loop. A C-8 guard column (10mm X 4.6mm) was fitted prior to the Bondclone® (Phenomenex, Torrance, CA, USA) C-18 10μm stainless steel analytical column (300mm X 3.9mm).

2.2.2 Reagents and Chemicals

Taurine (99%) and tetra-butyl ammonium hydroxide (TBAH), a 40% solution in water, were obtained from SIGMA (Poole, Dorset, UK). All other amino acids, sodium hypochlorite (NaOCl, a solution with 14% available chlorine), dipotassium hydrogen phosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4), perchloric acid (70%) and di-sodium tetraborate were obtained from BDH Chemicals (Poole, Dorset, UK). Acetonitrile, methanol, tetrahydrofuran and all other solvents were HPLC grade and were purchased from Labscan (Dublin, Ireland). Super purity

acetonitrile from Romil Chemicals (Loughborough, UK) was used for the deproteinisation step. Trichloroacetic acid, phosphoric acid (85% wt., 99.999%) and fluorescamine (98%) were obtained from the Aldrich Chemical Company (Poole, Dorset, UK). Boric acid was from Merck (Darmstadt, Germany). Water was deionised using an Elgastat Purification system.

2.2.3 Other Materials

Hypersep® C18 cartridges (200mg, 3ml) were received as a gift from Shandon Scientific (Runcorn, England). IST (Mid-Glamorgan, UK) kindly donated anion (SCX) and cation (PRS) exchange columns (200mg, 3ml). Microcon-3® (MW 3000 cutoff) concentrators and Micropure® (0.22μm filter) separator inserts were purchased from Amicon Ltd. (Stonehouse, UK).

2.3 HYPOCHLORITE DERIVATISATION OF TAURINE

2.3.1 EXPERIMENTAL

2.3.1.1 Blood Collection

Blood samples were taken from fasting volunteers into glass tubes containing 10% sodium citrate as anti-coagulant. Large bore butterfly syringes were used so as to minimise cell damage, since this can result in platelet ruption and hence give rise to falsely elevated levels of basal taurine present in the plasma. Platelet-rich plasma (PRP) was obtained by centrifugation for 5 min. @ 170*g* (1000rpm) at room temperature. Platelet-poor plasma (PPP) was obtained by centrifugation for 15 min. @ 1500*g* (3000rpm), also at room temperature. Care was taken during pipetting so as not to disturb the buffy coat layer.

2.3.1.2 Preparation of Reagents for HOCl Derivatisation

For the hypochlorite derivatisation reaction, a 1M stock solution of NaOCl was prepared on a weekly basis and it was stored at 4°C, away from sunlight when not in use. The required standard solutions were prepared daily by dilution of the stock

solution. The 50 or 100mM phosphate buffer was prepared by making a solution of K_2HPO_4 in purified water and adjusting the pH to 6.5 with phosphoric acid. A 0.5M solution of tetra-butyl ammonium phosphate (TBAP) was prepared by mixing 8ml TBAH with 10ml water, adjusting the mixture to pH 6.5 with a few drops of phosphoric acid and making the volume to 25ml in a volumetric flask.

2.3.1.3 Procedure for Hypochlorite Derivatisation

This derivatisation reaction is shown in Figure 2.1. The sodium hypochlorite solution in water becomes a solution of hypochlorous acid (HOCl). As the derivatisation of taurine by HOCl is pH-dependent, the pH of the reacting mixture was maintained at ≥ 6.5, to ensure formation of the monochlorinated derivative and prevent formation of the dichlorinated derivative[57]. The 0.01M taurine solution was mixed with 0.02M NaOCl solution in equal proportions by volume and was diluted as appropriate to the required concentrations in order to form the aqueous standard solutions of monochlorotaurine for investigation by spectrophotometry. In each case, the HOCl was in excess and the derivatisations were carried out in stoppered flasks to minimise evaporation of reagents.

Figure 2.1 : Derivatisation of Taurine by Hypochlorous Acid

Taurine **Hypochlorous acid** **Monochlorotaurine**

The aqueous standard solutions of monochlorotaurine for HPLC analysis were prepared by mixing 0 to 40 μg/ml solutions of taurine (0 to 0.32 mM) with 1 mM NaOCl. This procedure was called Method 1 and was found to compare favourably to the preparation of standards by two other methods (in terms of the linearity of the standard curve). Method 2 involved the preparation of a stock solution of monochlorotaurine using 0.001M taurine and 0.001M NaOCl, and subsequent dilution to obtain the correct concentrations of the other standards. Method 3 involved the

preparation of a stock solution of monochlorotaurine using 0.01M taurine and 0.01M NaOCl, and subsequent dilution to obtain the correct concentrations of the other standards. These results are displayed graphically in Figure 2.2, showing that for three different preparation methods for aqueous standards of monochlorotaurine, each gave similar peak height results by HPLC and each were linear with concentration for the concentration range examined. Since normal levels of taurine in human platelet-rich plasma are 7.7 to 19.1 μg/ml (from Table 2.3, references 24 and 40), this concentration range was expected to be suitable for the analysis of samples.

Figure 2.2 : Standard Curves of Aqueous Standards of Monochlorotaurine Prepared in Three Different Ways

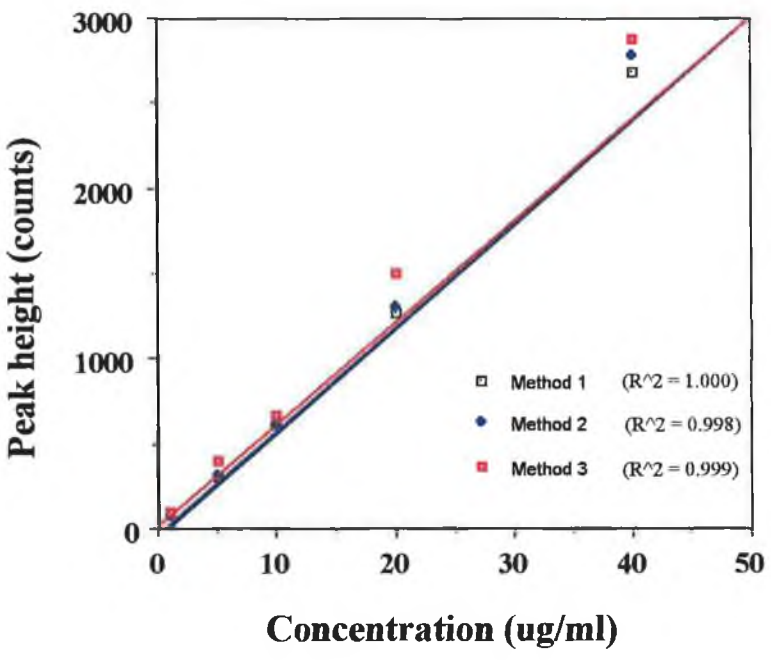


Fig. 2.2 : Method 1 monochlorotaurine standards were prepared by mixing 0 to 40 μg/ml solutions of taurine (0 to 0.32 mM) with 1 mM NaOCl. Methods 2 and 3 involved the preparation of a stock solution of monochlorotaurine using 0.001M reactants for Method 2 and 0.01M reactants for Method 3, respectively, and subsequent dilution of the stock solution to obtain the correct concentrations of the working standards.

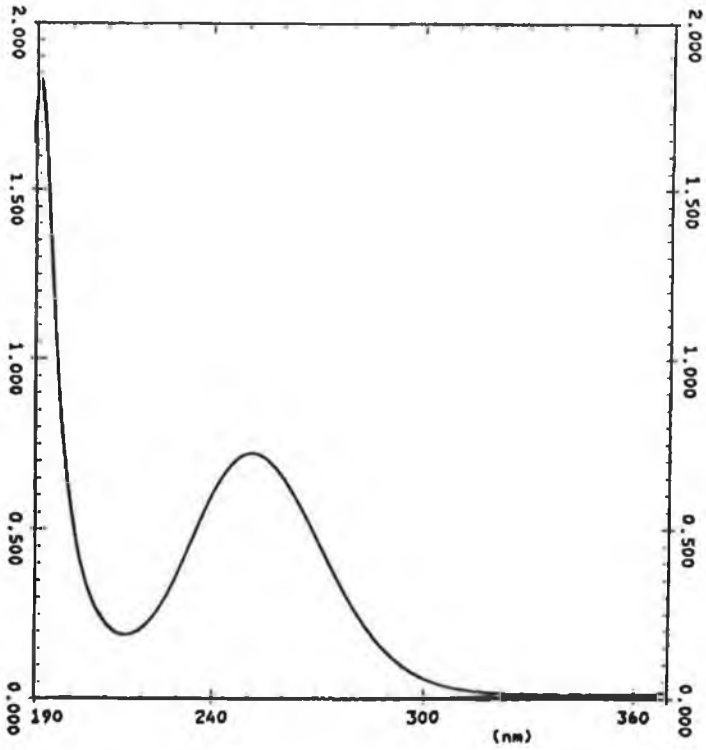
The plasma standards were prepared by mixing 500μl of platelet-poor plasma (PPP) and 10μl of taurine solution on a vortex mixer for 10 seconds. Acetonitrile (500μl) was added to deproteinise the sample. Following centrifugation, the supernatant was added to 500μl of 1mM NaOCl solution for the derivatisation

reaction. The taurine derivative is formed almost instantaneously at room temperature. Samples were usually analysed on the HPLC system within 2 hours.

2.3.1.4 HPLC Conditions for Monochlorotaurine

An ion-pair HPLC method was employed. The flow rate of the eluent was 1ml/min, the system pressure was approximately 1100 p.s.i. and all measurements were made at ambient temperature. The chart speed was 0.5 cm/min and the attenuation was 2. Taurine was reacted with the hypochlorite (in excess) at room temperature to form the monochlorinated derivative. UV detection was carried out at 254nm, the maximum absorbance wavelength for the monochlorotaurine derivative (Figure 2.3). Taurine itself does not absorb in the UV region and hypochlorite absorbs at 295-302nm. The mobile phase consisted of TBAP, acetonitrile and phosphate buffer (pH 6.5) in the proportions 1:5:94, v/v/v[56]. After mixing the phosphate buffer and the acetonitrile, the ion pair reagent, TBAP, was incorporated into the mobile phase and the mixture was adjusted to pH 6.5 with phosphoric acid. It was then filtered under vacuum through a 0.45μm Millipore filter and sonicated for 20 minutes. Under these HPLC conditions, the peak for the derivative appeared at 9.0± 0.2 min.

Figure 2.3 : UV Absorbance Spectrum of Monochlorotaurine Derivative



2.3.2 RESULTS AND DISCUSSION

2.3.2.1 Optimisation of Blood Collection Procedure

Blood levels of taurine have been extensively determined and reported (see Tables 2.1 and 2.3). However, the values obtained vary considerably depending on a number of factors. One of the major factors influencing the measurement of taurine concentration is the type of blood analysed – whole blood, serum, platelet-rich plasma (PRP) or platelet-poor plasma (PPP). The treatment of blood to yield whole blood or serum is well established but the treatment of blood to yield PRP or PPP is less well established. This is exemplified in Table 2.3 where certain criteria for the isolation of PPP and PRP such as centrifugation speeds, temperatures and anticoagulants can vary dramatically from one report to the next, or details may be omitted entirely. The extent of centrifugation is probably the most important variable in the isolation of PRP, since the more gentle the centrifugal force, the more platelets will remain in the plasma. And since platelets contain relatively high concentrations of taurine, their number in plasma will affect the concentration of taurine in the PRP. Platelet count in blood can vary from 100,000 to 500,000 per ml of plasma with factors such as sex, age[59], diet[60], health and sample treatment contributing to these numbers. Many reports do not state which type of plasma (PPP or PRP) was examined and simply use the term 'plasma'. For some biomolecules, this lack of distinction would make no differences to the final results, but taurine levels in PRP are of the order of 3-4 times higher than in PPP[24], the primary reason for measuring taurine in PRP as opposed to PPP. Another reason for measuring taurine specifically in PRP is that any changes in concentration, due to various disease states, will be more dramatic than in PPP. Thus, this distinction between the two types of plasma is important in the analysis of taurine and should be documented in the preparation procedure.

Table 2.3 : Comparison of Previous Methods for the Determination of Taurine in Blood

Reference	Derivatising Agent and Detection Mode	Blood Collection and Preparation	Taurine Results (μg/ml)
Tachiki, 1977 [24]	OPA/mercaptoethanol Fluorescence	Blood collected in EDTA vacutainer 7ml glass tubes from antecubital vein. Blood centrifuged @ 1000g at 4°C for 15min. to isolate PRP. PRP spun @ 12,100g at 4°C for 30min. to isolate PPP. Plasma proteins precipitated with trichloroacetic acid and kept on ice. Supernatant isolated by centrifugation and extracted with diethyl ether (3 times) before being subjected to cation-exchange. Extract dried and reconstituted.	*Human PRP* : 17.7±1.4 *Human PPP* : 5.6±0.5
Hirschberger, 1985 [36]	OPA/mercaptoethanol Fluorescence	Blood collected in heparinised tubes. Blood centrifuged @ 2000g for 10min to isolate plasma. Plasma proteins precipitated with sulphosalicylic acid. Supernatant neutralised and diluted.	*Human 'plasma'* : 5.0±0.9
Eslami, 1987 [38]	OPA/ethanethiol Fluorescence	Blood collected in EDTA tubes. Blood centrifuged @ 1800rpm for 5min to isolate plasma. Plasma proteins precipitated with methanol. Sample mixed and centrifuged to isolate supernatant.	*Human 'plasma'* : 14.0±4.9
Hirai, 1987 [39]	OPA/mercaptoethanol UV (350nm)	No blood collection information. Proteins precipitated with perchloric acid. Supernatant subjected to cation-exchange and extract diluted with water.	*Rat whole blood* : 41.9±4.9
Anderson, 1988 [40]	OPA/mercapto-propionic acid Fluorescence	Blood collected in EDTA tubes. Blood centrifuged @ 300g at 10°C for 10min. to isolate PRP. PRP spun @ 800g at 10°C for 15min. to isolate PPP. Plasma proteins precipitated with perchloric acid and kept on ice. Supernatant isolated by centrifugation and subjected to mixed-bed ion-exchange.	*Human whole blood* : 25.1±15.4 *Human PRP* : 11.3±3.6 *Human PPP* : 4.9±1.5
Porter, 1988 [41]	OPA/mercaptoethanol UV (340nm)	Blood collected in heparinised syringes. Blood centrifuged @ 1400g for 10min to isolate plasma. Plasma proteins precipitated with picric acid or by boiling for 15 min. (no recovery differences). Supernatant isolated by centrifugation and subjected	*Rat 'plasma'* : 12.6±1.6 *Chick 'plasma'* : 13.5±0.04

		to dual-bed ion-exchange. Extract dried and reconstituted.	
Amiss, 1990 [46]	Dansyl chloride Fluorescence	Blood collected in chilled, heparinised syringes. Blood centrifuged @ 2677g to isolate plasma. Plasma mixed with organic solution, vortexed and ultrafiltered (Centricon-10) for 15min. @ 2677g. Whole blood subjected to 2 freeze-thaw cycles to release taurine from cells.	*Feline 'plasma'* : 3.0±0.3 Although whole blood preparation given, no results shown. Quoted normal levels to be 10.3±4.2 [13]
Sakai, 1992 [54]	Fluorescamine Fluorescence	Blood collected in heparinised tubes. No isolation information. Plasma deproteinised with trichloroacetic acid, extracted with diethyl ether (3 times), evaporated to dryness and redissolved in water.	*Yellowtail fish 'plasma'* : 125±54 *Beef cattle 'plasma'* : 5.6±1.4 *Dairy cattle 'plasma'* : 2.2±0.7 *Chicken 'plasma'* : 20.0±9.6
Stocchi, 1994 [47]	Dabsyl chloride Visible (436nm)	Blood collected in heparinised tubes. Blood centrifuged @ 3000rpm at 4°C for 10min to isolate plasma. Plasma pretreated by (i) proteins precipitated with perchloric acid, and the supernatant obtained by centrifugation and nuetralisation or (ii) ultrafiltration (CF-50) @ 2500rpm for 10 min.	*Human 'plasma'* : 4.8±0.7 by extraction (i) 5.3±0.5 by extraction (ii)
Mosuoka, 1994 [50]	3,5-dinitrobenzoyl chloride UV (254nm)	No blood collection information. Blood deproteinised by Folin-Wu method[61] and the supernatant was isolated by centrifugation.	*Human blood* : 19.9±2.4 *Rat blood* : 49.4±5.6
Waterfield, 1994 [42]	OPA/mercaptoethanol Fluorescence/UV	Serum obtained directly from rats (abdominal aorta) and added to serum microtainers. Serum centrifuged @ 13,000g for 1 min., deproteinised with sulphosalicylic acid for 5 min. at 4°C, spun again and the supernatant subjected to dual-bed ion-exchange.	Although serum preparation method given, no results shown.
This work, 1995	Fluorescamine UV (385nm)	Blood collected in sodium citrate glass tubes. Large bore butterfly syringes used to minimise cell rupture. Blood centrifuged @ 170g for 5 min. to isolate PRP. PRP was spun @ 1500g for 15 min. to isolate PPP. Plasma deproteinised with acetonitrile, vortex-mixed and centrifuged to isolate supernatant.	*Human PRP* : 16.8±1.8

It was found in this study that the platelets could be removed from plasma with minimum rupture if the samples are collected with the large bore butterfly syringes. This procedure ensures that platelet taurine in PPP is minimised. However, it should be noted that there is always a basal level of taurine present in human plasma. Other workers have circumvented this problem and obtained taurine-free plasma from kittens raised on a taurine-free diet. Feline plasma contains naturally low concentrations of taurine (see Table 2.3). In order to isolate PRP in this work, blood samples were taken and subjected to gentle centrifugation for 5 min. @ 170*g* (1000rpm) at room temperature. If PPP was required, more vigorous centrifugation was necessary (15 min. @ 1500*g* (3000rpm)).

2.3.2.2 Optimisation of Sample Preparation Procedure

As can be seen from Table 2.3, the manner in which blood has been treated prior to the determination of taurine therein are numerous. Hence, a number of sample preparation procedures were investigated in this work. The results of these are summarised in Table 2.5 at the end of this section. Each procedure was evaluated using plasma samples spiked with taurine, unless otherwise specified. Recovery and/or general performance of the different methods were assessed by comparing the results of the extracted, derivatised plasma sample with a derivatised, aqueous sample of the same concentration that had not been subjected to the extraction procedure. HPLC was used to determine the quantitative results.

Protein Precipitation

The starting point for the plasma extraction was the boiling method used previously for neutrophils in conjunction with HOCl derivatisation[56]. This procedure had been used to precipitate proteins present in the sample and was used because taurine is known to be stable to boiling. Boiling was carried out by mixing 300µl of plasma with 700µl of water and boiling the mixture for 20 min. in an open tube. Subsequent centrifugation allowed the separation of the (cloudy) supernatant. This method of sample preparation was found to give poor reproducibility and to introduce interferences.

Perchloric acid proved to be an efficient deproteinisation agent, especially since only 50µl were required to achieve removal of most of the proteinaceous material from

a 500μl plasma sample. However, it was subsequently difficult to increase the pH reproducibly.

Methanol was required in a 3:1 ratio to sample in order to effect complete precipitation of proteins. This was found to have too great a dilutional effect and so was not employed.

Deproteinisation by acetonitrile proved to be the most reproducible method of sample preparation and was the easiest to execute. A ratio of between 1 and 1.5 to 1 of acetonitrile to sample was required.

Solid-Phase Extraction

Clean up with reversed-phase SPE C18 cartridges was examined next. The Hypersep® cartridges (200mg, 3ml) were solvated with 2ml of methanol, washed with 5ml of water and then the 300μl sample in 10% trichloroacetic acid (TCA) was applied to the column. It was allowed to equilibrate for 5 min. and then the cartridge was washed with 5% TCA. When an aqueous solution of taurine was applied to the C18 cartridges, the resulting extracts contained variable concentrations of taurine and hence recovery was poor and irreproducible. When derivatised taurine was applied to the C18 cartridges, various solvents were examined as to their ability to selectively elute monochlorotaurine from the cartridge. These varied in pH from phosphate buffer pH 2.2, mobile phase of pH 6.5, water and phosphate buffer pH 9.2. These results are listed in Table 2.4 overleaf, from which it was concluded that the phosphate buffer of pH 9.2 was the best eluting solvent for aqueous samples, giving a recovery of the derivative of 93%. However, when plasma samples were subjected to this procedure, the results were spurious and irreproducible.

Anion and cation exchange SPE columns were also investigated, the aim being to selectively retain or to selectively elute the taurine by exploiting its charged moieties. A general procedure for the extraction of amino acids from plasma using cation exchange (PRS columns) was used. This was unsuccessful, probably because buffering the sample to 2 pH units below the pKa of the acidic group was required, which in the case of taurine is impossible : with a pKa of 1.5, it was only possible to lower the pH to approximately 1 using 0.1M HCl.

Table 2.4 : Extraction Recoveries of Monochlorotaurine from C18 Cartridges in Different Eluents

Desorbing Eluent	Recovery in 1ml
Phosphate buffer, pH 9.2	93%
Phosphate buffer, pH 2.2	13%
Mobile phase eluent*	86%
Water	75%

*** The mobile phase consisted of TBAP, acetonitrile and phosphate buffer (pH 6.5) in the proportions 1:5:94, v/v/v. See experimental section.**

The anion exchange procedure (on SAX columns) was easier to carry out for taurine in the plasma samples, with application of the sample in pH 11 borate buffer, but there was still little or no retention of the taurine on the extraction columns. Overall, it was not possible to retain the taurine on the columns, from aqueous samples, under any circumstances and in fact, extra impurities were introduced during this procedure. It has previously been reported that taurine is not well retained on either cation or anion exchange columns[62].

Ultrafiltration

The next investigations centred on ultrafiltration as a method of sample preparation. Microcon-3® concentrators with a molecular cut-off of 3000 were used with or without Micropure® separator inserts with a 0.22μm polysulfone membrane for removal of macromolecules prior to derivatisation and analysis. An extraction recovery of approximately 70% was achieved when both eppendorf and insert were used together. The centrifugation time was found to be very long (approximately 140 min. @ 5800g) and although there was a concentrating effect, few of the smaller molecular weight interferents were removed. This method was found to be better than boiling, but not as efficient as precipitation with acetonitrile.

A combination of ultrafiltration and SPE was also investigated. Platelet-free plasma samples were spiked with taurine and each sample was centrifuged in a Microcon® eppendorf tube alone, or one containing a Micropure® 0.22μm separator. The filtrates were derivatised with NaOCl and applied to Hypersep® SPE cartridges.

For desorption of monochlorotaurine, phosphate buffer pH 9.2, which had given the highest recovery (93%), was used. The combination of Microcon®, Micropure® and SPE gave cleaner extracts than using SPE alone, but took an unreasonable length of time to complete. Also, the clean-up achieved using this procedure was still inadequate. The eluates were again compared to samples that did not undergo any extraction procedure.

Table 2.5 : Summary of Methods Investigated for Extraction of Taurine

Method of Extraction	Results/Comments
Boiling deproteinisation	Poor clean-up of plasma. Interfering peaks introduced.
Perchloric acid deproteinisation	Efficient agent for removal of proteins. Difficult to raise pH subsequently.
Methanol deproteinisation	Required in a volume ratio of 3:1 with plasma – too dilutional.
Acetonitrile deproteinisation	Required in a volume ratio of at least 1:1 with plasma. Satisfactory, reproducible clean-up.
C18 SPE	Good recovery of monochlorotaurine from aqueous samples (up to 93%). Poor and irreproducible recovery from plasma samples.
Anion and cation exchange	Little or no retention of taurine on the ion-exchange columns. Interferents introduced.
Microcon concentrator® and Micropure® filter	Approx. 70% extraction recovery of taurine from plasma. Long centrifugation time (140 min.)

2.3.2.3 Optimisation of HOCl Derivatisation Procedure

HOCl is well known for its potent oxidising abilities, and it also forms secondary chlorinating agents with amines. Chlorination of amino acids by HOCl is a pH-dependent reaction, and is first order with respect to both the amino acid and the HOCl. This reaction occurs naturally in the body and is suggested to act as a protective mechanism to remove the highly toxic endogenous hypochlorite[10,63]. The pH for the NaOCl derivatisation step was kept above 6.5 because above pH 6.5, amino acids form monochloroamines (the required derivative) and below pH 6.5, they form dichloramines[57]. The monochloroamines which are formed from α-amino acids at

above pH 6.5 are reported to be unstable e.g. the monochloramines of lysine and leucine decompose within 5 minutes[57], however the monochloramine of taurine (a ß-amino acid), has been reported to be more stable, rendering this method of derivatisation quite selective. Stapleton *et al.* reported that monochlorotaurine was stable for 8 days in aqueous solution[3]. Taurine has even been seen to abstract chlorine from the monochloroamine of another amino acid to form its own derivative. The only reported conditions under which monochlorotaurine was expected to be unstable were a) excessive heat (temperature is greater than 56°C), b) excessive light and c) excessive acid (pH < 3.5), in which case the dichloroamine may be formed[57].

Although the reaction between HOCl and amines is believed to occur by a mechanism whereby OCl^- is the attacking nucleophile in the reaction[57], there is a more recent theory in circulation which puts forward Cl^+ as an attacking electrophilic species[64].

Problems with the NaOCl derivatisation procedure arose when the method (which had been previously used for taurine in neutrophils[56]) was applied to both aqueous and plasma samples. While linearity could be shown in aqueous standards over the concentration range 0 - 40 µg/ml under general conditions, this was not consistently so. Under identical experimental conditions, following the same protocol, the monochloramine of taurine would sometimes break down. Linearity was not obtained for samples in a plasma matrix. Even under strict reaction conditions, the procedure was very irreproducible. A number of parameters were investigated in an effort to determine the reason for this lack of reproducibility :

(1) Was NaOCl in too high a concentration relative to taurine? The exact strength of the NaOCl was calculated by titration with thiosulphate so that molarities were accurate and various molar excesses of NaOCl were reacted with taurine, but no differences were seen in the stability of the reaction,

(2) Was the pH of the reaction changing as it progressed? To examine the effect of pH, a buffer was added to maintain consistent pH values, but no differences were seen in the stability of the reaction,

(3) TLC was carried out on the taurine standards, the reagent and the reaction mixture to ensure that the reaction was occurring,

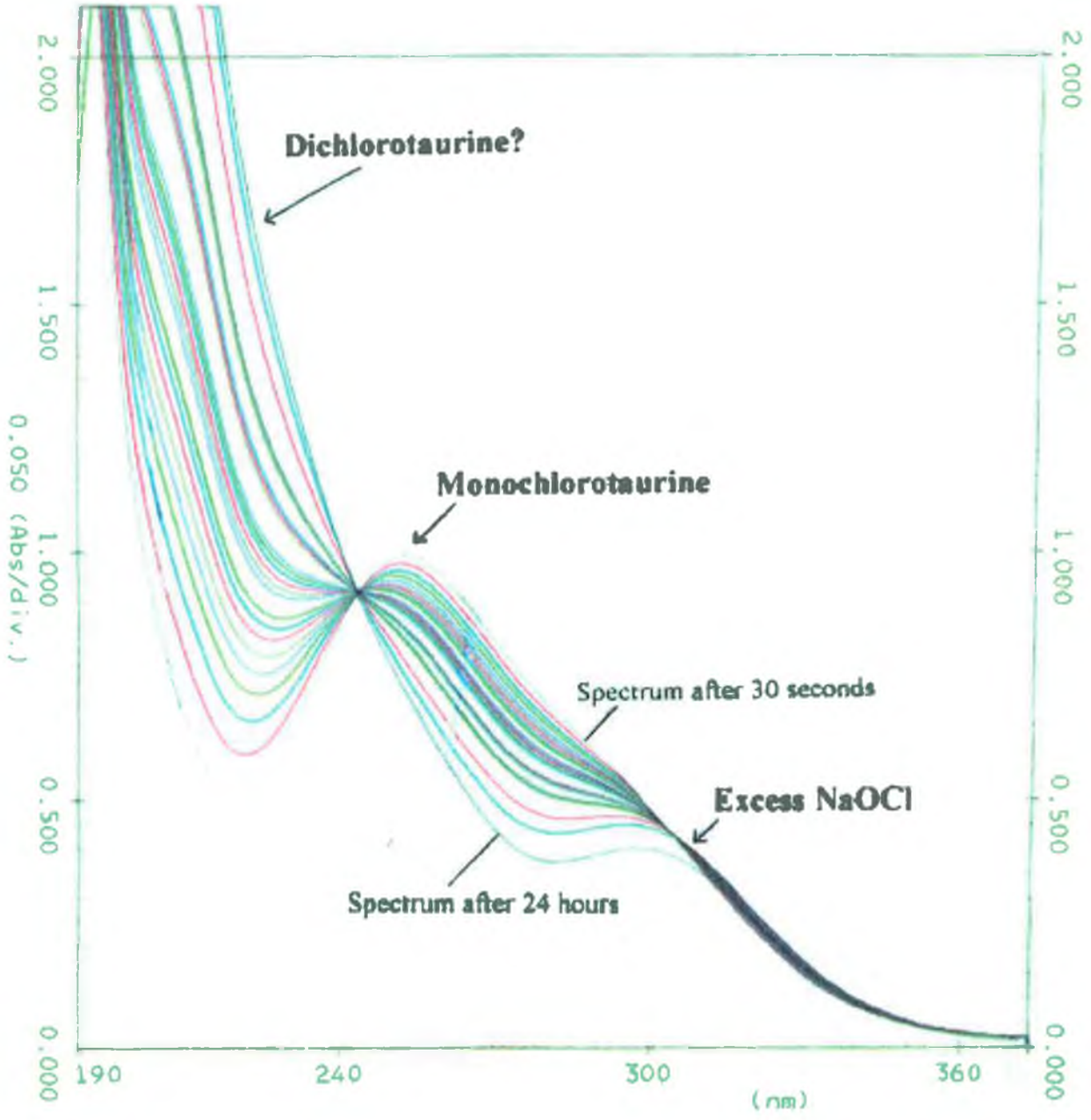
(4) All HPLC parameters were checked and the samples rerun to eliminate instrumental error.

However, investigation and optimisation of these parameters failed to render the method more reproducible, so spectrophotometry was employed to examine the absorbance spectra of the samples before, during and after derivatisation. It was found that although the monochloramine was being formed, on certain occasions its concentration decreased over time and this coincided with the increase in the absorbance of another interfering compound with maximum absorbance at 210nm (Figure 2.4).

Although previous work on monochlorotaurine has shown it to be stable under aqueous conditions, it was found not to be stable in this work, and hence not suitable as a derivatisation reagent for taurine. The derivative was found to be even more unstable in the presence of salts, buffers or solvents such as acetonitrile. Hence, even if the destabilising factor(s) could have been identified for the aqueous samples, there would have been further problems when the assay was attempted in plasma, where the use of salts, buffers and solvents would have been unavoidable.

It is postulated that in fact the monochlorotaurine *was* breaking down to give dichlorotaurine, even under non-acidic conditions ($pH \geq 6.5$). It was thought that two monochlorotaurine molecules were dismutating to give one dichlorotaurine and one original taurine molecule, or that the monochlorotaurine could be breaking down by deamination, decarboxylation and dechlorination to give its respective aldehyde, as this reaction is known to occur spontaneously for the unstable α-amino acid monochloroamines[57]. Thus, under the conditions of this experiment, the monochloramine was unfortunately not as stable as expected.

Figure 2.4 : Spectra of Monochlorotaurine Derivative taken over 24 hours



When this reagent had been employed previously for quantitation of taurine, the matrix was less complex and cleaner than plasma i.e. neutrophils. In neutrophils, the levels of taurine are 22mM (2.75 mg/ml), which is a 1000-fold greater than the levels expected in platelet-free plasma of 10 - 100 μM (1.25 - 12.5 μg/ml)[10]. Hence, degradation of the derivative may not have been as noticeable in the neutrophil experiments.

2.4 FLUORESCAMINE DERIVATISATION OF TAURINE

2.4.1 EXPERIMENTAL

2.4.1.1 Blood Collection

This was carried out in the same way as described in section 2.3.1.1.

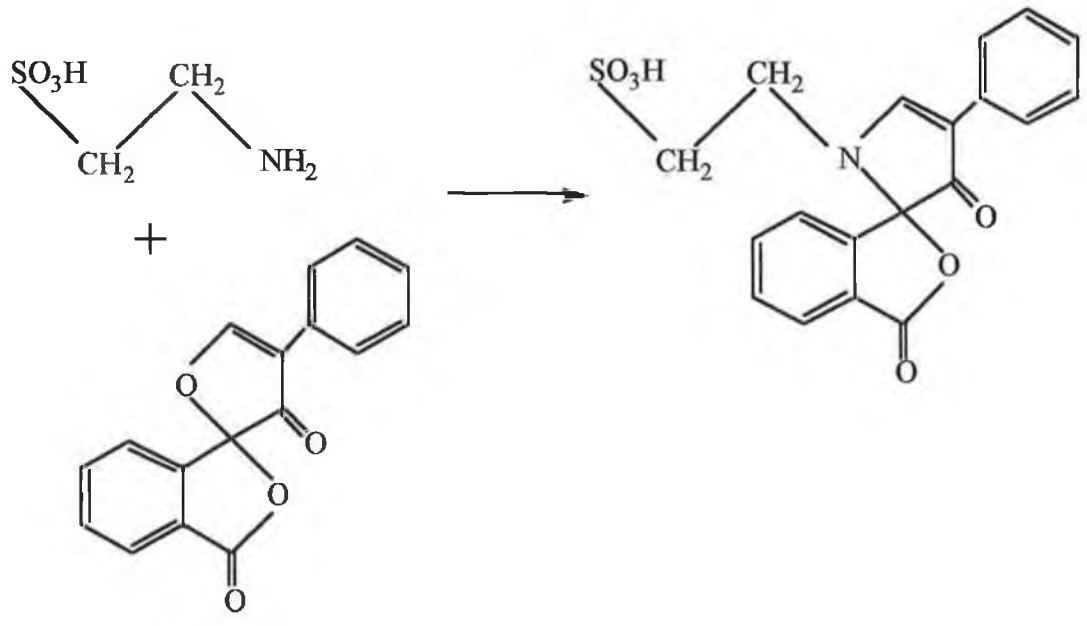
2.4.1.2 Preparation of Reagents for Fluorescamine Derivatisation

For the fluorescamine derivatisation reaction, standard fluorescamine solutions were prepared in acetonitrile and kept at room temperature. Borate buffer was prepared by adjusting 100mM disodium tetraborate solution to pH 9.2 with 100mM boric acid. Fresh 15mM phosphate buffer was made weekly by dissolving potassium dihydrogen phosphate in water and adjusting to pH 3.5 with phosphoric acid.

2.4.1.3 Procedure for Fluorescamine Derivatisation

The derivatisation reaction is shown in Figure 2.5. The reaction mixture was adjusted to pH 9, the optimum pH for the reaction to take place. For preparation of spiked samples, 5µl of taurine standard solution was added to 95µl of water or plasma to give standards with 0, 5, 10, 20 and 30 µg/ml added taurine. Again the standards were prepared in a range that was expected to be suitable for the analysis of samples. Each sample (100µl) was treated with 150µl of acetonitrile, vortex mixed and centrifuged for 10 min. @ 5800*g*. The supernatant was added to 50µl of borate buffer (100mM, pH 9.2) to adjust the pH to 9. Then 50µl of fluorescamine in acetonitrile (5mM) was added and the solution was immediately vortexed. Rapid addition and mixing was required for optimal results, as although the taurine derivative is formed almost instantaneously at room temperature, fluorescamine begins to break down in water within a few seconds. Samples were usually analysed on the HPLC system within 6 hours.

Figure 2.5 : Derivatisation of Taurine by Fluorescamine

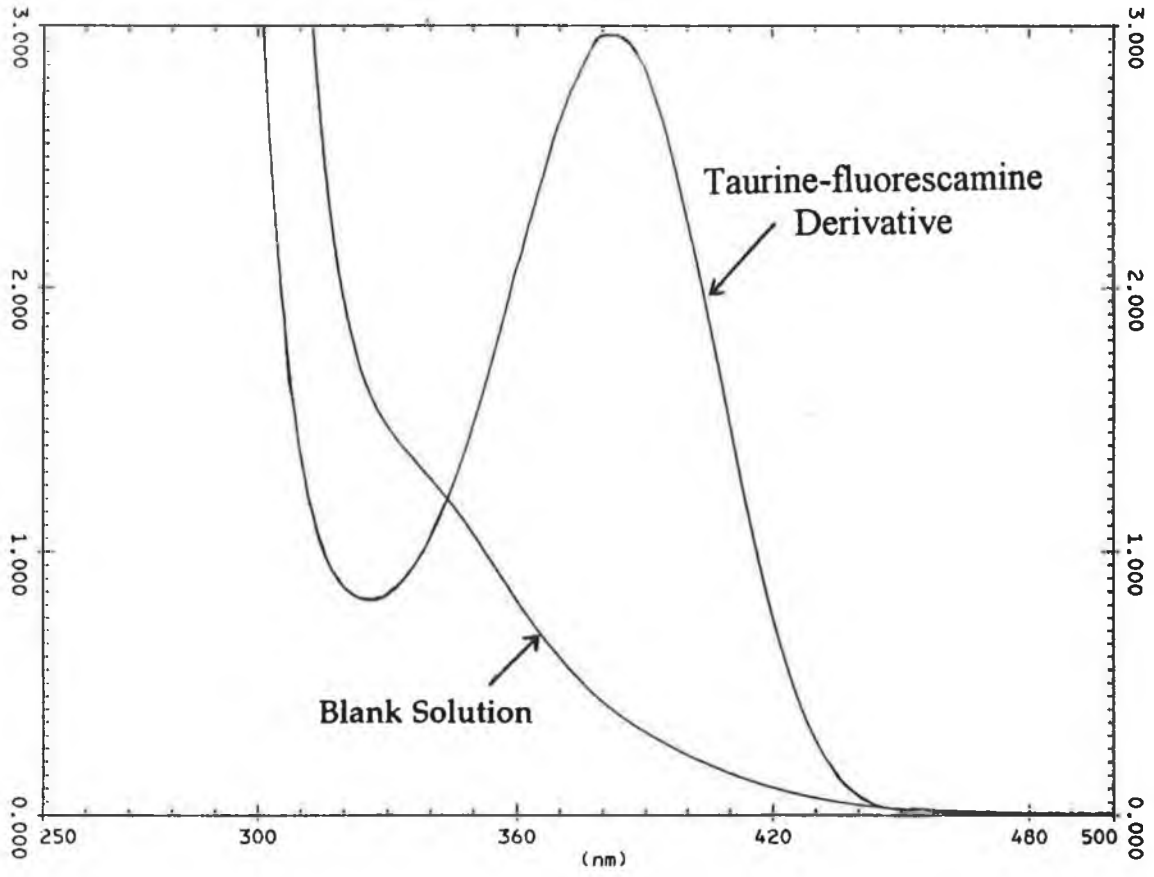


Taurine **Fluorescamine** **Fluorescent derivative**

2.4.1.4 HPLC Conditions for Fluorescamine Derivative

The flow rate of the eluent was 1ml/min; the system pressure was approximately 1100 p.s.i. and all measurements were made at ambient temperature. UV detection was carried out at 385nm, the maximum absorbance wavelength for the taurine derivative (Fig. 2.6). The mobile phase eluent finally consisted of tetrahydrofuran, acetonitrile and phosphate buffer (pH 3.5) in the proportions 4:24:72, v/v/v. After mixing, the mobile phase was filtered under vacuum through a 0.45μm Millipore filter and sonicated for 20 minutes. Under these HPLC conditions, the peak for the derivative eluted at 9.0± 0.3 min.

Fig. 2.6 : UV Absorbance Spectrum of Taurine-Fluorescamine Derivative



2.4.2 RESULTS AND DISCUSSION

2.4.2.1 Optimisation of Blood Collection Procedure

This was previously discussed in section 2.3.2.1.

2.4.2.2 Optimisation of Sample Preparation Procedure

Although this was previously discussed in section 2.3.2.2, some further investigations were carried out using SPE. Aqueous taurine was applied directly to the cartridge, eluted with solution A (acetonitrile-phosphate buffer (pH 2.5) 30:70 v/v), and subsequently derivatised with fluorescamine, which yielded a recovery of 57-58%. When borate buffer pH 9.2 was used instead of solution A, a recovery of 56% was obtained. Recovery was raised to 71% when the eluting solution was comprised of acetonitrile-water, 35:65, v/v.

When taurine was derivatised with fluorescamine prior to being applied to the cartridges and eluted with a volume of 1.2 ml of solution A, recovery was 53-57%.

With plasma samples, however, the above results could not be reproduced and recoveries were spurious.

2.4.2.3 Optimisation of Fluorescamine Derivatisation Procedure

The pH of the reaction between fluorescamine and primary amines must be ≥ 8.5, and is optimum at pH 9 for amino acids. Fluorescamine was dissolved in acetonitrile because of its compatibility with the deproteinised supernatant and its suitability as a non-hydroxylic but water-miscible solvent. Such a solution of fluorescamine is stable for 12 weeks[65]. Immediate mixing was essential for maximum response because the reagent itself breaks down (by hydrolysis) in a matter of seconds.

2.4.2.4 Development of HPLC Conditions for Fluorescamine Reaction

As a starting point, the mobile phase used in the work of Sakai *et al.* was tried i.e. 23% acetonitrile in acidic 15mM phosphate buffer[54]. However, initial HPLC experiments with the taurine fluorescamine derivative in aqueous solution, using this mobile phase, resulted in very late elution of the taurine peak. The acetonitrile content was therefore increased and the mobile phase was initially optimised with a composition of acetonitrile:phosphate buffer (pH 2.5, 15mM) [35:65]. While this gave good chromatography for aqueous standards, in plasma samples taurine was found to co-elute with other components. Methanol was used as an organic modifier at varying ratios instead of acetonitrile to try to resolve the taurine derivatives from other closely-eluting peaks, but with little success. However, the use of tetrahydrofuran altered the selectivity in a way that improved the resolution between taurine and other endogenous compounds. Only a small percentage was required and mobile phases containing 3, 3.5, 4 and 5% tetrahydrofuran were investigated. The optimal composition was tetrahydrofuran:acetonitrile:phosphate buffer (15mM) [4:24:72].

A variety of pH's were examined - 1.6, 2.5, 2.8, 3.5 and 4.0, and at pH 3.5, taurine was adequately separated from other closely-eluting amino acid derivatives such as alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, serine, threonine and valine. Sakai *et al.* found that acidic conditions were required to separate the taurine fluorescamine derivative from other derivatives[54]. In fact, after trying three mobile phases of different pH (2.7, 2.1 and 1.9), only the most acidic eluent allowed the derivatives to be resolved under their assay conditions.

A number of absorbance wavelengths were investigated before it was found that 385nm was optimal (Figure 2.6). The concentrations and volumes of the acetonitrile (for protein precipitation), borate buffer (for pH control during derivatisation) and sample (or standard) were modified a number of times before the optimum conditions for the analysis were identified.

2.4.2.5 Calibration and Calculation

The spiked samples of PPP were quantified by external standardisation. The slope and intercept of the calibration graphs were determined by unweighted linear regression of the taurine peak height versus the concentration of taurine added.

2.4.2.6 Precision

Precision was defined in terms of the variability between batches (reproducibility) and within batches (repeatability). Reproducibility was assessed in four replicate runs on four consecutive days, covering the concentration range 0-30 μg/ml. Repeatability was assessed by running, in quadruplicate, standards at each concentration level in one day of the validation study in the same concentration range. The precision of the method was described by the mean relative standard deviation (RSD), and this was found for taurine when the peak heights were interpolated as unknowns on the regression lines. For reproducibility, the interpolations were based on the four regression lines generated from the four replicate runs and for repeatability, the interpolations were based on a single regression line generated from the quadruplicate run. Because human plasma contains a basal level of taurine, platelet-poor unspiked plasma shows a small peak in the chromatogram that corresponds to taurine. An unspiked platelet-poor sample was run with each calibration curve and the height of the taurine peak was subtracted from the taurine peak in each of the spiked samples. Precision data, calculated on the basis of the subtracted results, are presented in Table 2.6, and they demonstrate that the reproducibility (mean RSD = 3.87%) and repeatability (mean RSD = 5.56%) of the methods are within accepted values for clinical analyses. These results were compared with data from unsubtracted values (i.e. where the peak in the blank sample was not subtracted from the spiked standards) and it was found that the mean precision values were higher (4.08% and 6.75% for reproducibility and repeatability, respectively) and there was greater variability in precision amongst individual values.

2.4.2.7 Linearity and Accuracy

The correlation coefficient of the regression line for the mean intra-assay values was 0.9998 (subtracted data) and 0.9985 (unsubtracted data) for taurine. Accuracy (presented in Table 2.6), as defined as the percentage difference between the amount added and the amount found by back calculation, was usually less than 5% with mean values of 4.38% and 2.75% for intra-day and inter-day analyses, respectively.

Table 2.6 : Precision and Accuracy Data

Inter-assay (reproducibility)

Amount added (μg/ml), n=4	Mean Amount found (μg/ml)	RSD (%)	Accuracy(%)
5	4.82 ± 0.26	5.33	3.56
10	10.22 ± 0.58	5.69	2.21
20	20.00 ± 0.69	3.43	0.02
30	29.95 ± 0.31	1.05	0.15

y = 347.74 (±19.85)x - 179.11(±125.78) r = 0.9995 ± 0.0002

Intra-assay (repeatability)

Amount added (μg/ml), n=4	Mean Amount found (μg/ml)	RSD (%)	Accuracy(%)
5(n=3)	4.56 ± 0.41	9.01	8.87
10	10.30 ± 0.66	6.38	3.03
20	20.50 ± 0.59	2.90	2.50
30	29.63 ± 1.17	3.95	1.21

y = 350.28x - 192.77 r = 0.9998

2.4.2.8 Recovery

Recovery may be calculated in absolute or relative terms. In the calculation of absolute recovery, peak response of an extracted standard is compared with that of unextracted standards that are prepared to the same theoretical concentration as the extracts.

Relative recovery is calculated by comparing peak responses of extracted matrix standards against those of extracted aqueous standards. This procedure was used to account for the presence of acetonitrile in the sample to be injected. Using this method, the relative recovery of taurine from plasma was found to be 89.7%.

2.4.2.9 Limits of Detection and Quantitation

The limit of quantitation was found to be 5μg/ml taurine in plasma samples. The limit of detection was found to be 1μg/ml taurine in plasma samples.

2.4.2.10 Selectivity

A number of amino acids are present in both platelet-poor and platelet-rich plasma and the ten amino acids thought most likely to interfere were subjected to the same extraction and separation conditions. Alanine, glutamine, threonine and valine were chosen because it can be seen from Table 2.1 that they are the amino acids with the highest concentration in human plasma. Proline (although of high concentration) does not derivatise with fluorescamine because it is a secondary amine. Some of the other amino acids with typically high concentrations in human plasma are arginine, glutamic acid, glycine and serine, so these too were investigated (Table 2.1). Asparagine and aspartic acid were subjected to derivatisation and HPLC analysis because other authors have investigated the presence of one or both of these amino acids in the plasma of humans, even though their concentrations in plasma tend to be low[38,47,50]. Hypotaurine, homotaurine and phosphoserine were not included in these experiments because it has been reported that these substances are not present in human plasma by Hirschberger *et al.*[36], Eslami *et al.*[38] and Tachiki *et al.*[24]. By adjustment of the mobile phase pH and aqueous-to-organic ratio, it was possible to resolve these compounds from the taurine peak. In fact, it is expected that the method described could be applied to the simultaneous determination of taurine and other amino acids.

2.4.2.11 Stability

Stability of the taurine fluorophor was assessed in aqueous solution initially. Three concentrations of taurine (1, 10 and 50 μg/ml) were derivatised using the standard protocol i.e. 100μl sample mixed with 150μl acetonitrile, 50μl 100mM borate buffer and 50μl 5mM fluorescamine solution in acetonitrile. The final mixtures were injected onto the HPLC and the peak heights obtained. The samples were also run 60 hours

later. The results are shown below in Table 2.7. This agrees with work carried out by Guzman *et al.* when they used fluorescamine as a derivatising agent for the determination of amino acids and peptides by CE using UV detection[66]. They found the fluorescamine derivative of arginine to increase its UV response (by about 5%) when kept at ambient temperature for a period of 24 hours. As can be seen from Table 2.7, a small increase in absorbance can be seen to occur over time for the lower concentrations. However, when one considers that this small effect is over a time frame of 2.5 days, the derivative can be considered to be relatively stable.

Table 2.7 : Stability Data for Aqueous Taurine Fluorescamine Derivative

Concentration (μg/ml)	Peak height within 1 hour	Peak height after 60 hours
1	1051	1165 (111%)
10	9227	8805 (95%)
50	43411	43744 (101%)

When a 5μg/ml sample of taurine in plasma was derivatised and left for 24 hours at room temperature, the absorbance was found to increase by 10%. In order to minimise any small changes in absorbance due to further reaction over time, all samples derivatised using fluorescamine were analysed by HPLC within 6 hours of preparation.

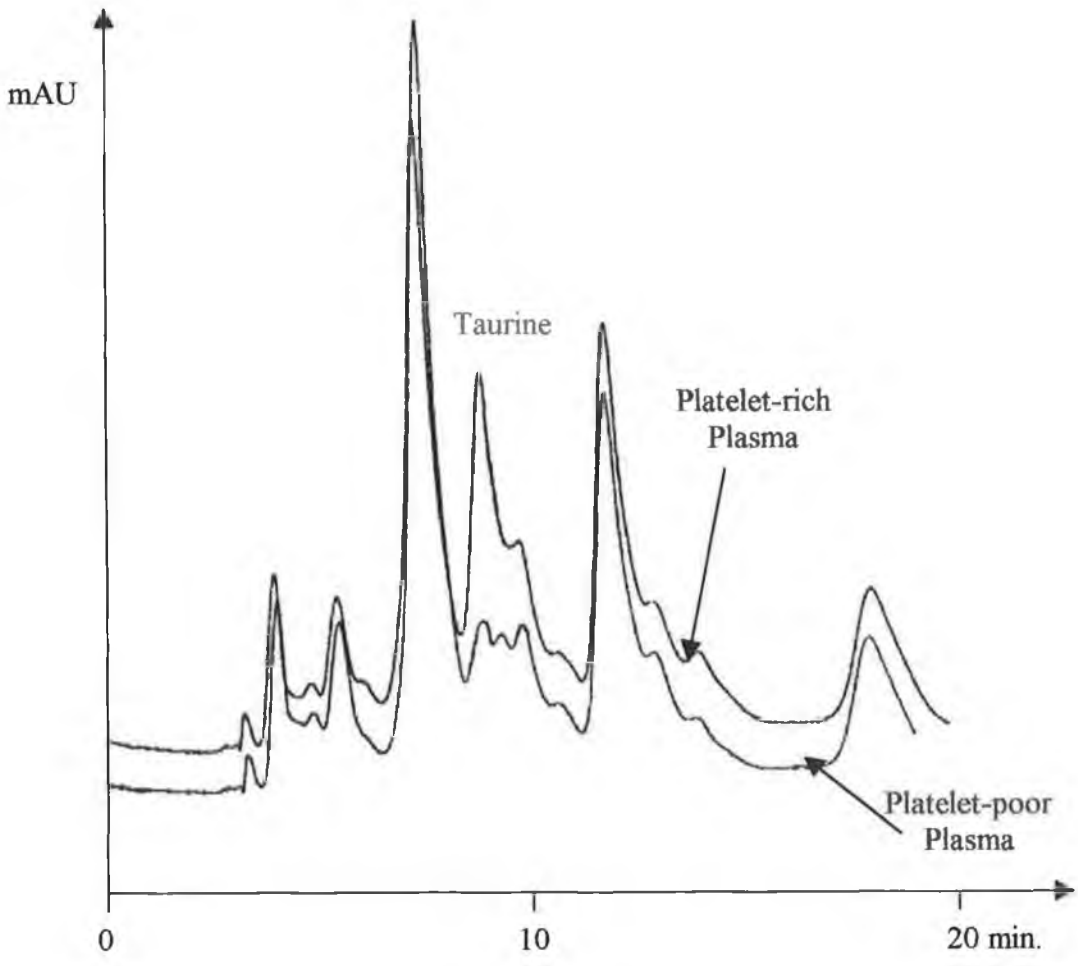
2.4.2.12 Quantitative Determination of Taurine in Volunteer Samples

Table 2.3 highlights some of the difficulties in taurine analysis in plasma, when the range of concentrations under seemingly similar experimental conditions are compared. It is obvious from Table 2.1 that the normal range of concentration of taurine in plasma is quite wide. A more accurate 'normal' taurine concentration range in PPP can be obtained by averaging the specific taurine levels obtained in publications where the aim of the work was to determine taurine, and where the plasma was centrifuged to such an extent that most of the platelets would have been eliminated. This yields a range 3.4 – 7.3 μg/ml based on the PPP concentrations found in five different reports (references 24,40,67,68 and 69). A more accurate 'normal' taurine concentration range in PRP can be obtained by averaging the specific taurine levels obtained in publications where the plasma was very gently centrifuged such that most of the platelets will have remained in the plasma. This yields a range 7.7 – 19.1 μg/ml based on the concentrations from two different reports (references 24 and 40). Hence,

looking at the results in Table 2.3 in light of these 'normal' ranges for taurine concentration, it is probable that the plasma in the work of Eslami *et al.*[38] is PRP, while the plasmas in the work of Hirschberger *et al.*[36] and Stocchi *et al.*[47] are likely to be PPP.

Samples of PRP from three different sources were determined in this project by interpolation of the peak heights on a calibration curve (0-30 μg/ml). Results from the individual in Fig. 2.7 gave a value of 15.0 μg/ml in PRP, while values from two pooled PRP samples were calculated to be 16.4 and 18.5μg/ml. All three results are within the expected range calculated above for PRP. It can be seen in Figure 2.7 that taurine is indeed at a higher concentration in PRP compared to PPP from the same volunteer.

Figure 2.7 : HPLC Chromatograms showing PPP and PRP from a Volunteer



Column : Bondclone C18, 10μm (300 x 3.9 mm i.d.). Mobile phase THF:ACN: Phosphate buffer (15mM, pH 3.5) [4:24:72]. Sample preparation as described in text.

2.5 CONCLUSIONS

Hypochlorite was investigated as a reagent for the pre-column derivatisation of taurine prior to HPLC analysis of the derivative. Although it had previously been used successfully for the quantitation of taurine in neutrophils, it was found to be unsuitable for the quantitation of taurine in this project. The successfully formed monochlorinated taurine derivative was unstable, breaking down to give what was thought to be the dichlorinated taurine derivative.

Fluorescamine was investigated and was found to be a useful reagent for the pre-column derivatisation of taurine allowing the estimation of taurine levels in platelet-rich plasma. Other analyses have difficult and labour-intensive sample preparation and some involve derivatisation procedures that require quenching and/or the presence of co-solvents in order for the reaction to take place. Many are prone to interference from the derivatising agent itself and there are discrepancies in the stability of derivatives.

The method presented here, for the determination of taurine in platelet-rich plasma, is simple, rapid and efficient, and a result is obtainable within one hour of taking blood from a patient. With the ever-increasing need for the quantitation of taurine in human plasma for medical purposes, the simplicity of this assay will be of benefit to clinical research. It could also aid in further elucidation of the complex biochemical roles and beneficial effects that taurine has in the human body.

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Chapter 3

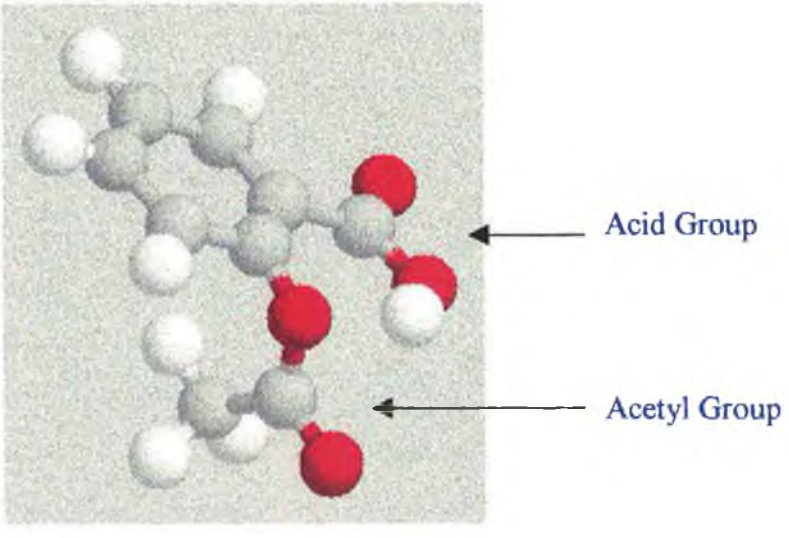
Evaluation of Aspirin Derivatives as Prodrugs for Aspirin and Determination of Aspirin and Salicylic Acid in Transdermal Perfusates

3.1 INTRODUCTION

3.1.1 Historical Background

Acetylsalicylic acid (ASA), more commonly known as aspirin, is the commercially synthesized form of a naturally occurring compound called salicin, which is found in the bark of the willow tree. It was discovered about 200 B.C. by the Greek physician Hippocrates when he found that chewing on willow bark could relieve pain and fever. He gave it to women to ease their labour pains. It was not until 1897 that the ASA molecule was chemically synthesised (Figure 3.1). This was achieved by Felix Hoffmann, a chemist who had been seeking a pain-relieving medication for his father's debilitating rheumatism. Not only did the drug powder ease his father's pain and inflammation, but when it was marketed as 'aspirin' by Bayer two years later, it quickly became the world's most popular pain reliever.

Figure 3.1 : Aspirin Molecule



In 1900, Bayer® aspirin was the first drug ever to be marketed in tablet form. During its first decade, ASA was found by physicians worldwide to be so effective as a medicine, it became popularly used for a range of ailments from headaches, fever, inflammation, pleurisy and tonsillitis to tuberculosis, gonorrhea and gout. Affordable for practically everyone, ASA quickly became a household necessity for safe and effective pain relief. Tens of thousands of people took ASA during several major influenza epidemics in the early 20th century.

3.1.2 Pharmacological Background

Although ASA was a proven pain reliever, no one really knew how it worked until more than 70 years after Hoffmann's discovery. In 1971, British pharmacologist John Vane discovered that the anti-inflammatory properties of ASA result from its ability to inhibit the enzyme that produces prostaglandins which promote inflammation and therefore cause pain. Vane received the Nobel Prize for Medicine in 1982 for this medical research breakthrough[1,2,3]. More recent research has shown that the enzyme involved – cyclooxygenase – contains a very small tunnel into which arachidonate (the precursor) must go to be converted into prostaglandin[4]. ASA attaches its acetyl group inside the tunnel, where it acts like a gate, blocking the arachidonate from entering the 'active site' of the enzyme.

3.1.3 Biological Background

Today, ASA is the most widely used oral medication in the world. In America alone, 80 billion ASA tablets are taken each year[5]. It cures our headaches, reduces fever and alleviates muscle pain and inflammation. However, according to Bayer, the uses of ASA are for prevention of heart disease (37.6%), arthritis (23.3%), headache (13.8%), body ache (12.2%) and other pain uses (13.1%). As can be seen from these statistics, the use of ASA in cardiovascular disease is its most important medical application and this is discussed in more detail in section 3.1.4. However, ASA also has important potential in other medical conditions. Some researchers have reported that ASA may reduce the risk of many common cancers e.g. colon and colorectal cancer[6,7], oesophageal and stomach cancer[8] and ovarian cancer[9]. Research is also ongoing to explore possible links between ASA and prevention of other diseases including diabetes[10,11,12], migraine headaches[13,14,15] and Alzheimer's disease[16,17]. The use of ASA, in conjunction with heparin, has recently been shown to dramatically increase the number of live births in pregnant women suffering from recurrent miscarriages[18].

The National Library of Medicine in the US has logged more than 23,000 scientific papers on ASA. Experts say yearly ASA consumption could rise even higher in its second century as researchers uncover new applications. "Aspirin now has a whole host of possibilities that were never envisioned," says Charles Hennekens, chief of the Physicians Health Study, one of the ongoing research projects that has examined ASA use closely. Unlike other pain relievers, many of which have

been getting poor publicity due to their side-effects e.g. acetaminophen, ASA's benefits continue to significantly outweigh any risks.

3.1.4 Aspirin and the Heart

Nearly five decades of research now link ASA to the prevention of stroke and heart attacks. This is ironic when one considers Bayer advertisements from the 1920s where the slogan "aspirin does not affect the heart" was used to show how safe ASA was as a drug (Figure 3.2). Nowadays, ASA is used to save lives *because* of its effect on the heart.

Figure 3.2 : Bayer Advertisement for Aspirin in the 1920s

In the UK, about 25,000 people die each year from acute myocardial infarction, other heart disease or stroke, events that are largely due to platelet activation and thrombosis[19]. In the United States, the number of deaths due to these events is 900,000[20] annually. Because aspirin (ASA) inhibits platelet aggregation in

blood by blocking cyclooxygenase activity[21,22], much research has focussed on the use of ASA to protect the heart against cardiovascular disease, especially in 'high risk' groups[20]. Many clinical trials and collaborative studies have been undertaken in order to better understand the beneficial effects of taking ASA, the optimum doses of ASA to administer and the possible side-effects of such a regime.

The first experiment to examine the protective effect of aspirin on the heart was conducted in 1948 by Dr. Lawrence Craven, a general practitioner from California. He noticed that aspirin increased bleeding in tonsillectomy patients, suggesting possible anti-clotting properties. At his suggestion, 400 of his male patients took aspirin for two years and not one of them had a heart attack. By 1956, Dr. Craven reported that among 8,000 men who faithfully took one or two aspirin tablets daily, not one experienced a heart attack. Craven could only publicize his reports through interviews with the Los Angeles Times, because most medical journals refused to publish his findings.

Since then, there have been many trials carried out to investigate both primary and secondary prevention of cardiovascular disease. The main clinical trial investigating ASA's primary prevention of heart attack was The Physicians' Health Study who published their findings in 1989[23]. This study had focused on 22,000 healthy American male physicians aged 50 and older, who took either 325mg of ASA or a placebo. Regular aspirin administration reduced the incidence of heart attack by 44%. The study only lasted for four versus its intended eight years in an effort to allow those taking the placebo to switch to aspirin therapy given its significant positive impact on heart attack prevention. After the trial, 99% of doctors on the aspirin regimen stated that they wished to continue taking the drug.

Some of the main studies that have demonstrated the secondary benefits of taking ASA include : The UK Transient Ischaemic Attack (UK-TIA)[24] Aspirin Trial, The Dutch TIA Trial Study Group[25], The 1994 Antiplatelet Trialists' Collaboration[26] and The International Stroke Trial (IST)[27]. In the UK-TIA Trial, two ASA doses were investigated (1200mg and 300mg) in the prevention of major stroke, myocardial infarction (MI) or vascular death in patients with previous TIA[24]. Both doses reduced the incidence of these events to a similar extent (15%). The incidence of gastrointestinal side effects was significantly lower in those patients receiving the lower dose of aspirin. The Dutch TIA Trial investigated lower doses of ASA (30 mg and 283 mg) in patients after TIA or minor stroke with a view to preventing

subsequent nonfatal stroke, nonfatal MI or death from vascular causes[25]. The results showed that the reduction of these events was similar in both low and medium dose groups (14.7% versus 15.2%). Minor bleeding complications and gastric discomfort occurred less often with the lower doses of aspirin (30mg). The 1994 Antiplatelet Trialists' Collaboration analysed results of randomised trials of antiplatelet therapy among more than 54,000 high risk patients with prior evidence of cardiovascular disease[26]. ASA therapy was found to reduce the risk of subsequent vascular events by one quarter. The largest ever trial carried out on acute stroke was the IST which reported its findings in 1997. IST examined the early use of ASA, heparin or both following ischaemic stroke[27]. The absolute number of recurrent ischeamic strokes within 14 days dropped by 1.1% and the combined endpoint of death or non-fatal recurrent stroke was also reduced by 1.1%.

Intervention therapy has been reported by The Second International Study of Infarct Survival (ISIS-2) in 1988[28]. The results of the study showed that in patients who took one-half tablet (162.5mg) of ASA no later than 24 hours after the onset of a heart attack, and for one month thereafter, there was a reduction in the risk of death from a heart attack by 23%. The combination of ASA and streptokinase (a clot-dissolving drug) reduced mortality from heart attack or stroke by up to 50 percent.

In the US alone, it is estimated that as many as 10,000 additional lives could be saved annually by broader use of ASA in appropriate people who are at risk of heart attack and stroke[29]. Many of the USA's 1.25 million heart attacks each year[5] could thus be prevented, providing additional years of healthy, productive living. If ASA was regularly used after a transient ischaemic attack (TIA) or minor stroke, thousands of strokes could also be prevented. Overall, the potential cost savings for health care systems and economy are enormous, with a simple regimen of ASA likely to reduce lost work time and hospital-related costs. Worldwide, experts believe that as many as 100,000 deaths could be prevented each year through the regular use of low-dose ASA in people at high risk of vascular disease.

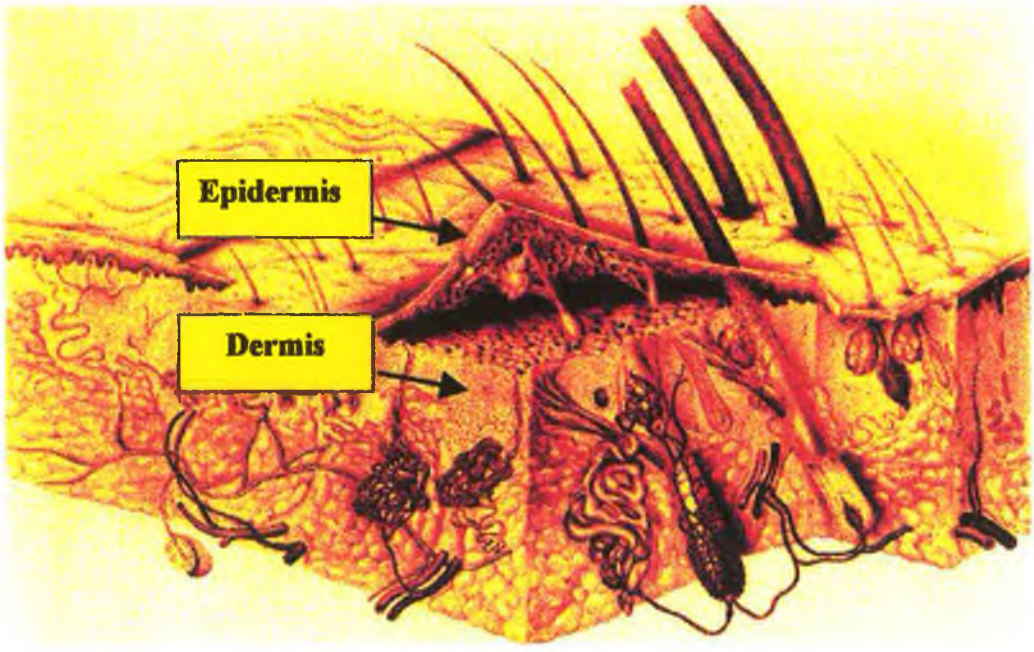
3.1.5 The Skin as a Novel Route for Aspirin Delivery

As discussed in the previous sections, there is strong evidence to support the fact that a regime of long term, low-dose ASA can reduce the occurrence, the reoccurrence and risk of death from cardiovascular and cerebrovascular events[24,25,26,27,28,30,31,32,33]. A typical daily dose of ASA for prevention of heart disease would be 75mg (although

doses that range from 30 – 325mg are prescribed). ASA is usually given orally but there are some problems associated with this route of administration such as gastro-intestinal bleeding and peptic ulceration[25,34,35]. Hence, the transdermal route has been investigated for continuous low-dose ASA administration.

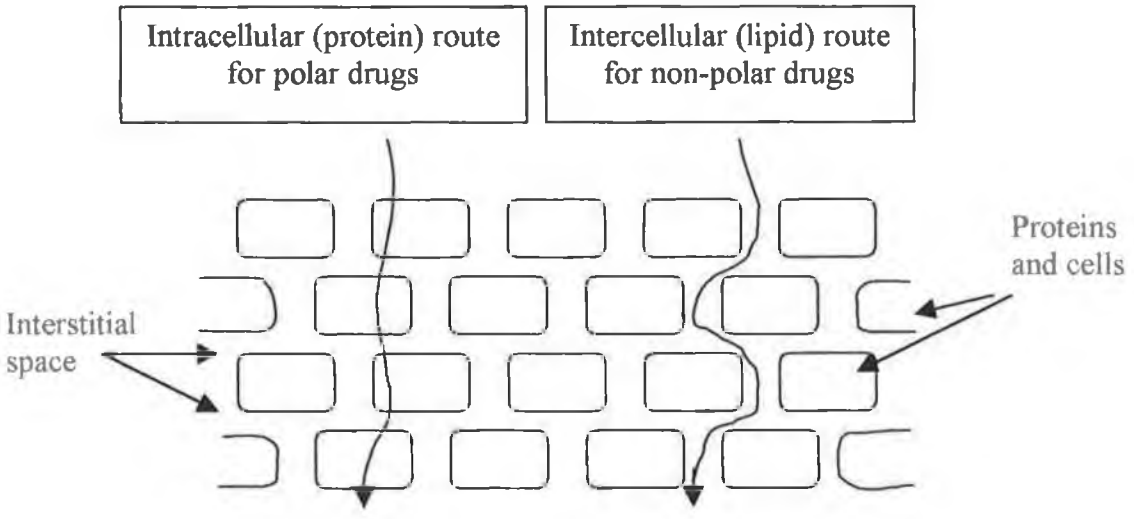
The delivery of compounds across the skin is not a new concept. There are established transdermal patches for drugs such as nicotine[36,37,38] and hormones such as oestrodiol[39,40], to name but a few. This means of delivering a drug can allow many of the problems of oral absorption to be overcome. Since the stomach and intestine are essentially bypassed with transdermal therapy, pH changes, food intake and intestinal transit time are no longer important considerations. The constant and controlled delivery of the drug eliminates pulse entry into the circulation, a phenomenon associated with its own side effects. Another advantage of the topical approach is that in the event of any allergic reaction, absorption of the medication can be immediately terminated by simple removal of the patch. This non-invasive therapy can also improve patient comfort and compliance, especially where a drug is to be taken long term i.e. decades.

Figure 3.3 : Diagram of a Cross-Section of the Skin



The skin is the largest organ of the human body, covering an area of approximately 15-20 square feet and weighing several kilograms. Our skin protects us against becoming dehydrated and prevents us from being poisoned with toxic compounds from the environment. The skin is a very complex structure (see Figure 3.3) comprising of two main tissue layers, the epidermis (40-150μm) and the dermis (2–5mm). The outermost layer of the epidermis is the stratum corneum (SC), which is approximately 17μm thick in human skin[41], and is considered to be essentially impermeable since it behaves like a passive diffusion barrier with no evidence of metabolic transport processes. The SC consists of approximately 40% proteins, 40% water and 20% lipids and a schematic diagram of it is shown in Figure 3.4.

Figure 3.4 : A Diagrammatic Representation of the Stratum Corneum



The biopharmaceutics of topical preparations are very complex because of the many physiochemical principles involved in penetrating the SC. Of the many factors that can enhance or impede drug penetration through the SC, the most important are drug concentration (C) and drug partition coefficient (P), measured between skin and vehicle. P is more commonly expressed as log P, which can be approximated by experimentally determining the distribution of a compound between oil and water. Substances that are very polar will have low values of log P and hence poor permeation through skin, since the main pathway of transport for water-soluble molecules is transcellular i.e. through cells, proteins and cell walls, which is difficult (see Figure 3.4). Hence, the more non-polar drugs can penetrate the skin more readily

since they have higher log P values and can travel intercellularly i.e. between the cells and proteins, which is less difficult.

Penetration of compounds through the skin can be predicted using a form of Fick's law. The penetration flux (J, mol m^{-2} s^{-1}) of a low molecular weight, non-electrolyte drug is directly proportional to C and P as shown below :

$$J = \frac{PD}{\delta} . \Delta C$$

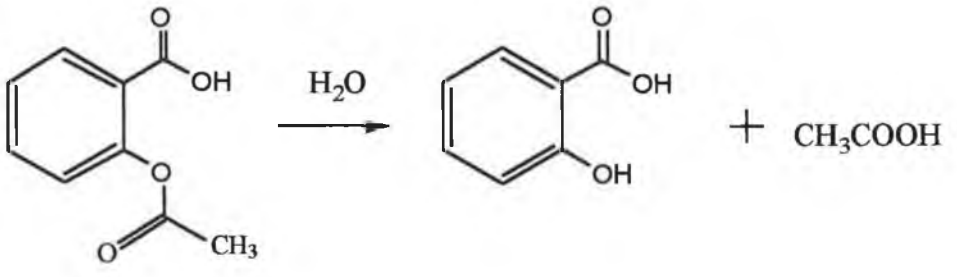
D = diffusion coefficient of drug (m^2 s^{-1})
δ = thickness of stratum corneum (m)
ΔC = difference in drug concentration between skin and vehicle (mol m^{-3})

The value of D is determined by factors such as size, shape and charge of the molecule. The ideal drug for transdermal delivery should also be small (< 1000 Da) with a short biological half-life[42]. A parabolic relationship between log P and percentage absorption into skin for a series of salicylates and other non-steroidal anti-inflammatory drugs applied topically has been reported, with the optimum log P found to be 2.4[43]. Log P values for homologous series of compounds can also be correlated with their retention on a HPLC column.

The vehicle in which the drug is applied to the skin strongly influences the rate and extent of absorption. There are a number of types of formulations used : aqueous lotions, ointments and aqueous creams are just a few. Some vehicles leave a greasy, occluding film on the skin which hydrates the surface of the skin (occluded skin may absorb up to 5-6 times its dry weight of water). These occlusive films can increase penetration of low molecular weight species 2-5 fold[44]. If the vehicle is changed so that the drug is less soluble in it, P will increase and hence penetration will increase too. To aid dissolution of the drug in the formulation, solubilisers or enhancers such as ethanol are often used. These also allow a higher concentration of drug to be applied to the skin at one time. There is a compromise between using a vehicle that *is* a good solvent for the drug, which allows a high concentration to be applied to the skin, which increases penetration flux, and a vehicle which is *not* a good solvent for the drug which promotes transfer of the drug from the formulation to the skin, thereby increasing penetration flux. The optimum is often found at the level where the drug is *just* dissolved in the vehicle.

The skin has previously been investigated as a route for continuous low-dose aspirin administration. ASA itself is a polar molecule and at first sight is not an attractive molecule for transdermal delivery. However, only 30-40mg/day ASA is required for >90% inhibition of platelet cyclooxygenase[45]. Delivery of small amounts of ASA continuously is a more effective way of inactivating cyclooxygenase[46]. ASA does cross the skin but in doing so, it is rapidly hydrolysed to its principal metabolite salicylic acid (SAL) during transport as seen in Figure 3.5. When the transdermal absorption of a range of drugs was examined by Beckett *et al.* in 1972, it was found that over a period of 120 hours, only 22% of ASA was absorbed and 23% of SAL was absorbed[47]. However, a more recent study showed that ASA applied to the skin surface in ethanol and propylene glycol selectively inhibited cyclooxygenase in platelets[48]. This work showed that even at a low rate of transport, pharmacological effects are seen. Unfortunately, skin reactions were noted in 50% of the subjects at the 750mg/day dose and this was thought to be due to the presence of SAL on the skin surface, a known irritant. In 1996, McAdam *et al.*[49] reported that ASA in a transdermal patch, at a lower dose, was also found to induce marked suppression of platelet cyclooxygenase in spite of the fact that the bioavailability of ASA was again calculated to be low (only 20%). This was further evidence for the fact that the therapeutic effects of ASA are present at very low doses.

Figure 3.5 : Hydrolysis of ASA to SAL



Aspirin (ASA) **Salicylic Acid (SAL)**

It was suggested by McAdam *et al.*[49] in the conclusion of their report on the transdermal patch that "it may be possible to improve the delivery of aspirin through the skin using stable analogues of aspirin." Many researchers over the past two

decades have prepared and evaluated prodrugs of ASA with a view to improving the delivery of ASA across the skin[50]. Central to the prodrug design is *in vivo* reconversion to ASA. For ASA, most prodrug design has centred on masking the ionisable carboxylic group (which slows down its transdermal delivery). Hence, the ASA derivatives formed are more non-polar than ASA, aiding their transdermal permeability, and once in the top layers of skin, they are hydrolysed to yield ASA.

3.1.6 Analytical Background

While much HPLC methodology has been reported in the literature which can determine ASA and SAL simultaneously, most of the examples refer to analysis in biological fluids (such as plasma and whole blood)[51,52,53], food[54,55] or tablet formulations[56,57]. Many of the reported methods involve protein precipitation and/or solvent extraction. However, this study required the development of a number of HPLC methods, requiring analysis of ASA and SAL in different matrices, for different reasons in different experiments.

The aim of this study was to examine a number of possible prodrugs for ASA. The compounds investigated in this study were to be derivatives of ASA which were predicted to release the more polar ASA by their hydrolysis on or within the skin. The role of the prodrug for ASA is to a) break down on or in the skin to give ASA, b) to do this at a continuous rate (after the lag time has passed) and c) not to pass into the systemic circulation itself. By doing this, small amounts of ASA should be continually arriving into the circulation, thus achieving the desired pharmacological effect. Because there is less ASA on the surface of the skin, however, there should be less SAL produced on the surface and this would serve to reduce or even eliminate skin reactions. It was also the objectives of this work to develop analytical methodology that could reliably determine the concentrations of ASA and SAL in the transdermal perfusates following the application of the various derivatives to the skin surface.

3.2 REAGENTS AND MATERIALS

3.2.1 Equipment

The high performance liquid chromatograph was equipped with Waters (Millford, MA, USA) models 501 single-piston and 510 dual-piston pumps, a Waters Model 486 tunable absorbance detector, a Waters model 680 gradient controller and a Waters model 746 data module. The Rheodyne injection port (Cotati, CA, USA) was fitted with a 20μl loop. All experiments were carried out and all measurements were made at ambient temperature. The UV-Vis recording spectrophotometer was a UV-2100 model from Shimadzu (Kyoto, Japan). The Franz cell apparatus for the transdermal experiments is described later in the text.

3.2.2 Reagents and Chemicals

Aspirin was supplied by SIGMA Chemical Co. (Dorset, England) and salicylic acid by BDH (Poole, England). ASA and the aspirin derivatives were prepared in-house and the protocols for their synthesis are given in Appendix A. The structures and full names of the derivatives (ASA-Gly, ASA-Ala, ASA-PheAla, ASA-Phe, ASA-Phe-NO_2, Dimer and ASA-Iso) are shown in Figure 3.9. Acetonitrile, tetrahydrofuran, methanol, diethyl ether and water were HPLC grade and were purchased from Labscan (Dublin, Ireland). Toluene was purchased from Romil Ltd. (Cambridge, England). Analytical grade orthophosphoric acid (OPA), sodium hydroxide and polyethylene glycol (MW 6000) came from BDH (Poole, England). High purity ethanol (>99.8%), sulphuric acid (selectipur 96%) and acetic acid (glacial 100%) were purchased from Merck (Darmstadt, Germany). Phosphate buffered saline (PBS) sachets were obtained from Sigma Diagnostics (St. Louis, MO, USA).

3.2.3 Other Materials

Two different HPLC analytical columns were used in this work : an Alphabond® C18 column (10μm, 300 x 3.9mm) from Alltech (Lancashire, U.K.) and a Nucleosil® C8 column (5μm, 250 x 4.6mm) from Hichrom (Berkshire, U.K.). Silica gel (F-254) TLC plates were purchased from Merck (Darmstadt, Germany).

3.3 IDENTIFICATION OF AN IMPURITY IN ASPIRIN

3.3.1 EXPERIMENTAL

3.3.1.1 Preparation of Standards for TLC

The stock standard solutions were prepared in acetonitrile. For TLC analysis, the stock standard solutions were further diluted in acetonitrile.

3.3.1.2 Preparation of Standards for HPLC

The standard solutions were prepared in acetonitrile to 5mg/ml. For HPLC analysis, the stock standard solutions were further diluted in the mobile phase.

3.3.1.3 Preparation of Samples for TLC

All samples were dissolved in acetonitrile or tetrahydrofuran as appropriate.

3.3.1.4 Preparation of Samples for HPLC

All samples were prepared in mobile phase prior to injection.

3.3.1.5 TLC Conditions

The eluent was a mixture of toluene-ether-acetic acid-methanol [120:60:18:1, v/v/v/v]. The TLC chamber was equilibrated for at least one hour with eluent before the plates were inserted. Unless otherwise stated, 10μl of sample or standard was applied to the plate using a micropipette.

3.3.1.6 HPLC (Method 1) Conditions

The final HPLC conditions for this work were as follows: Alphabond® C18 analytical column, mobile phase of tetrahydrofuran-water-acetic acid [30:70:0.05, v/v/v, pH 3.5*], flow rate of 1ml/min, UV detection at 225nm. The injection volume was 20μl. The choice of C18 column and the pH of the mobile phase was based on the fact that almost all previous HPLC methods for the analysis of ASA and/or SAL employed this type of column and acidic working pH (2-3).

3.3.2 RESULTS AND DISCUSSION

3.3.2.1 Development of HPLC Conditions for Method 1

A number of eluents were investigated, using an Alphabond® C18 analytical column (10μm, 300 x 3.9mm) and a UV detection wavelength of 230nm. At a flow rate of 0.8ml/min, various combinations of acetonitrile or tetrahydrofuran and water with acetic acid or phosphate buffer were equilibrated on the HPLC system and standard solutions of ASA and SAL were injected. The mobile phase was maintained at an acidic pH (2.0 to 3.5) and this was achieved using acetic acid. Although ASA and SAL are weak acids with pKa values of 3.5 and 3.0[58], respectively, their peak shapes were unaffected by having the pH at or close to these values, where the molecules would be ionised to some extent (by 50% at the pKa). It was found that ASA and SAL were not well resolved on the Alphabond® column when acetonitrile was the organic modifier used in the eluent, so tetrahydrofuran was used instead. Under the final conditions of HPLC method 1, the retention times for ASA, SAL and dimer were 6.4±0.1, 10.8±0.2 and 24.0±0.5 min., respectively. A typical example of a chromatogram is shown in Figure 3.6.

3.3.2.2 Identification of the Impurity in ASA by TLC

The ASA was prepared in-house from salicylic acid and acetic anhydride. Some problems arose with regard to the purity of this ASA, whereupon a number of analytical procedures were carried out in order to ascertain the identity and quantity of the impurity. Hydrolysis of ASA with sodium hydroxide and subsequent titration of the excess NaOH with sulphuric acid showed the in-house ASA to be 90% pure with respect to Sigma ASA. Samples for TLC were dissolved in acetonitrile to minimise any on-plate hydrolysis. The presence of the impurity could be clearly seen in the ASA samples which were investigated by TLC, especially when compared to Sigma ASA (see Figure 3.7). This impurity was identified as being the dimer of ASA (aspirin anhydride), the structure of which is shown in Figure 3.9. This impurity hydrolyses to release ASA and SAL, and this can be seen to occur on the TLC plate shown in Figure 3.7. Other possible impurities such as acetylsalicylsalicylic acid and salicylsalicylic acid were not found to be present.

Figure 3.6 : Typical Chromatogram using HPLC Method 1

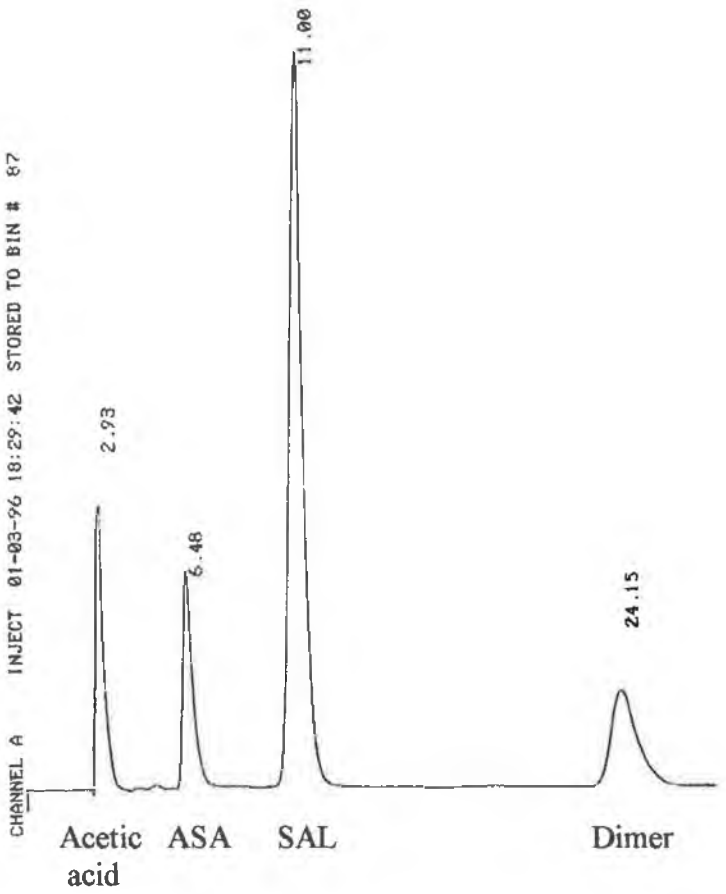
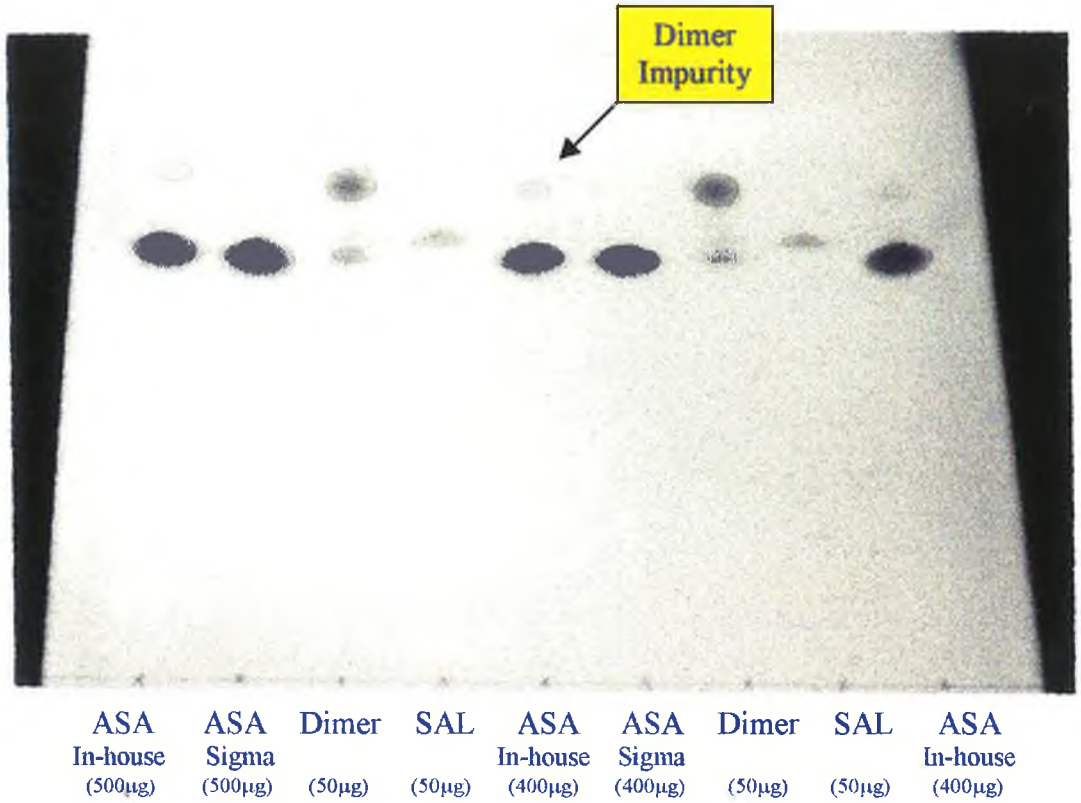


Figure 3.6 : HPLC Method 1 using Alphabond® C18 analytical column, mobile phase of tetrahydrofuran-water-acetic acid [30:70:0.05, v/v/v, pH 3.5*]. Other experimental conditions described in text. The vertical axis measures absorbance in mAU and horizontal axis measures time in minutes. This is the case for all chromatograms in this chapter.

3.3.2.3 Identification of the Impurity in ASA by HPLC

Further evidence for the identity of the impurity was obtained when a standard of the aspirin anhydride was found to have the same retention time as the impurity in the in-house ASA by HPLC. Figure 3.8 shows two chromatograms : a) Sigma ASA (100μg/ml) and b) In-house ASA (100μg/ml). The presence of the dimer impurity can clearly be seen.

Figure 3.7 : Identification of Dimer Impurity in ASA by TLC



3.3.3 CONCLUSIONS

It was discovered that the dimer impurity found in the in-house ASA is a known condensation byproduct of the synthesis of ASA. Determination of this impurity is of great importance since it has been established that allergic reactions can occur with ASA and that it is substances like the dimer that are thought to be responsible for 'aspirin allergy'. The in-house protocol for the preparation of ASA was modified so as to minimise production of the aspirin anhydride and subsequent batches of ASA were more than 99% pure.

Figure 3.8 : Chromatograms of Sigma ASA versus In-house ASA

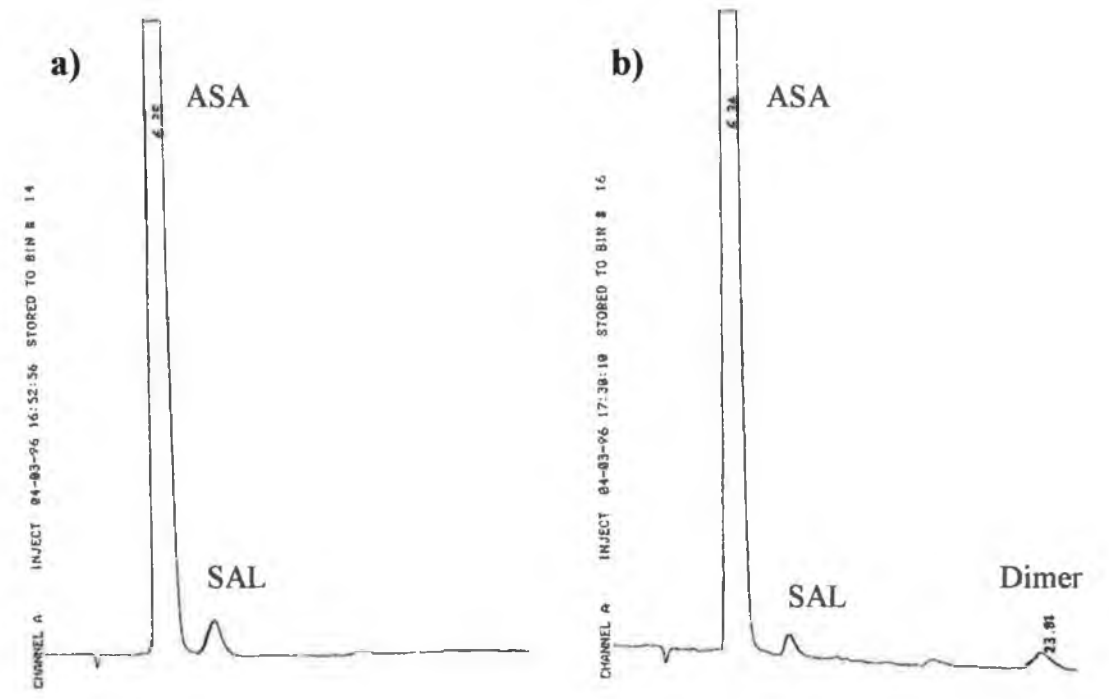


Figure 3.8 : the two chromatograms are a) Sigma ASA (100μg/ml) and b) in-house ASA (100μg/ml). The presence of the dimer impurity can clearly be seen. HPLC Method 1 was carried out using an Alphabond® C18 analytical column and mobile phase of tetrahydrofuran-water-acetic acid [30:70:0.05, v/v/v, pH 3.5*]. Other experimental conditions described in text. The vertical axis measures absorbance in mAU and horizontal axis measures time in minutes.

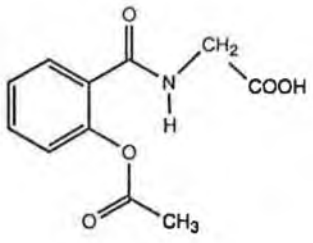
3.4 SCREENING OF DERIVATIVES BY HPLC AND TLC

3.4.1 EXPERIMENTAL

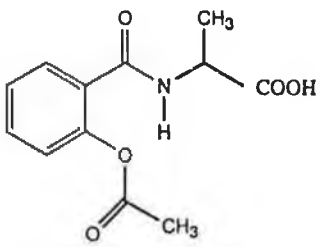
3.4.1.1 Synthesis of Aspirin Derivatives

The following compounds were prepared in-house by derivatising ASA with various amino acids and esters : aspirin glycine (ASA-Gly), aspirin alanine (ASA-Ala), aspirin phenylalanine (ASA-PheAla), aspirin phenyl ester (ASA-Phe), aspirin nitrophenyl ester (ASA-Phe-NO_2), aspirin anhydride (dimer) and aspirin isosorbide ester (ASA-Iso). Their structures are given in Figure 3.9.

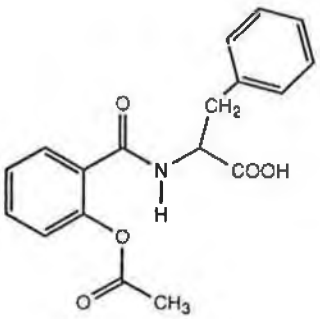
Figure 3.9 : Structures of the Derivatives for Screening



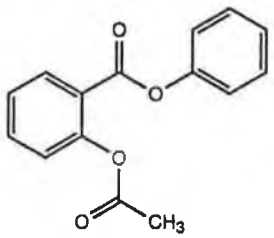
Aspirin Glycine (ASA-Gly)



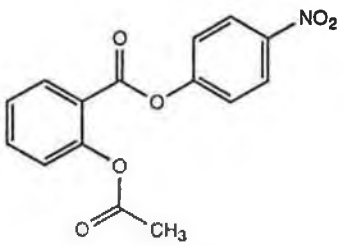
Aspirin Alanine (ASA-Ala)



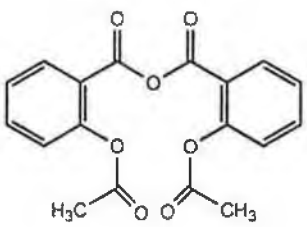
Aspirin Phenylalanine (ASA-PheAla)



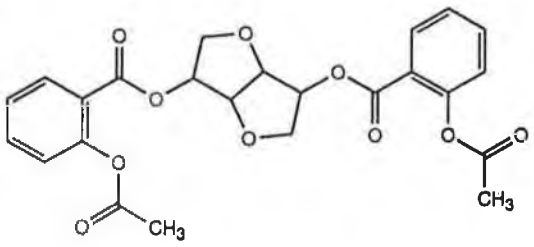
Aspirin Phenyl Ester (ASA-Phe)



Aspirin Nitrophenyl Ester (ASA-Phe-NO_2)



Aspirin Anhydride (Dimer)



Aspirin Isosorbide Ester (ASA-Iso)

3.4.1.2 Preparation of Standards for TLC

The stock standard solutions were prepared in tetrahydrofuran. For TLC analysis, the working standards were diluted from the stock standard solutions in tetrahydrofuran.

3.4.1.3 Preparation of Standards for HPLC

The stock standard solutions were prepared in acetonitrile. For HPLC analysis, the working standard solutions were diluted from the stock standard solutions in mobile phase.

3.4.1.4 Preparation of Samples for TLC

Sample solutions of the derivatives were prepared by dissolving each of them in tetrahydrofuran to a concentration of 10 or 20mg/ml for TLC analysis.

3.4.1.5 Preparation of Samples for HPLC

For purity assessment, sample solutions were prepared by diluting the stock 10mg/ml TLC standard of derivative in mobile phase, as required, to a concentration of 100μg/ml for HPLC analysis. Preparation of the platelet aggregation samples involved diluting the extracts in mobile phase. Preparation of the derivative samples for stability in aqueous solution was carried out by dissolving both ASA-Gly and ASA-Ala in PBS.

3.4.1.6 TLC Conditions

The TLC chamber was equilibrated for at least one hour with eluent before the plates were inserted. Unless otherwise stated, 10μl (200μg) of sample or standard was applied to the plate using a micropipette. The plates were chromatographed in TLC eluent (toluene-ether-acetic acid-methanol [120:60:18:1, v/v/v/v]).

3.4.1.7 HPLC (Method 2) Conditions

The final HPLC conditions for this section of the work were as follows : Alphabond® C18 analytical column, mobile phase of tetrahydrofuran-water-acetic acid [25:75:0.05, v/v/v, pH 3.5*], flow rate of 1ml/min, UV detection at 225nm. The injection volume was 20μl. The reasons for changing the method are explained in section 3.4.2.2.

3.4.2 RESULTS AND DISCUSSION

3.4.2.1 Rationale for Synthesis of the Derivatives

The seven derivatives of ASA synthesised in this part of the project were prepared because they are more non-polar structures than the ASA molecule alone. Also, they were predicted to release ASA on hydrolysis of the derivatising bond (ester or amide). Being more non-polar than ASA, the partition coefficient of the derivative would be higher than ASA, hence increasing penetration into the SC barrier layer of the skin. Once in the top layer of the skin, the derivatives should release ASA into the dermis of the skin and thence into the circulation. However, it was required to investigate the suitability of the prepared derivatives from two perspectives a) in terms of the derivative's ability to inhibit platelet aggregation *in vitro* and b) in terms of the derivative's ability to actually break down to give ASA *in vitro*. Any derivative that did inhibit platelet aggregation via its production of ASA on hydrolysis, that was also of sufficient purity, was considered a suitable candidate for subsequent transdermal testing.

3.4.2.2 Development of HPLC Conditions for Method 2

Although the same column was used as in Method 1, the mobile phase composition was modified slightly to allow better separation of the early-eluting amino acid derivatives and their hydrolysis products from ASA and SAL. Under the final conditions of HPLC method 2, the retention times for ASA and SAL were 7.4±0.2 and 13.6±0.3 min., respectively.

3.4.2.3 Platelet Aggregation Studies - Biological Results

An instrument called a Platelet Aggregation Profiler (model PAP-4) was used and these experiments carried out in the Department of Pharmacology, The Royal College of Surgeons. A description of how the PAP-4 works can be seen in Appendix B. Blank readings were made using plasma free of platelets. All platelet-rich plasma (PRP) samples were maintained at 37°C while stirring. Negative and positive controls were run in conjunction with the derivative samples. The results of this work are given in Table 3.1.

3.4.2.4 Platelet Aggregation Studies – HPLC

Derivatives that gave a positive result in the platelet aggregation studies i.e. they caused the desired pharmacological effect, were examined by HPLC to confirm the presence of ASA. Samples at various time points : 30 and 60 min. (and sometimes 90 min.) were extracted from plasma. The extraction was carried out based on a procedure from reference 59 and is described in Appendix C. Samples were then reconstituted in mobile phase prior to injection. ASA itself was subjected to the same tests for purposes of comparison. The overall results are given in Table 3.1.

Table 3.1 : Summary of Results from the Screening of Prodrug Candidates

Derivative	TLC Results	HPLC Results	Biological Results
ASA-Ala	Quite pure by TLC. Hydrolyses to SAL-Ala	Elutes at ≈4 min. Breaks down to give SAL-Ala	No inhibition of platelet aggregation
ASA-Gly	Quite pure by TLC. Hydrolyses to SAL-Gly	Elutes at 2.5 min. Breaks down to give SAL-Gly. >96% pure	No inhibition of platelet aggregation
ASA-PheAla	Impure (many extra components)	Elutes at ≈33 min. Impure with many extra peaks, no ASA or SAL present	No results
ASA-Iso	No results	Poor chromophore but at high concentrations, could be seen to elute at ≈40 min. >95% pure	Inhibits platelet aggregation. ASA seen in aggregation sample
ASA-Phe	No results	Elutes at ≈40 min. Appears not to break down. >99% pure	Inhibits platelet aggregation. ASA seen in aggregation sample
ASA-Phe-NO_2	No results	Elutes at ≈70 min. >99% pure	Inhibits platelet aggregation. ASA seen in aggregation sample
Dimer	No results	Elutes at ≈37 min. Pure but unstable	Inhibits platelet aggregation. ASA seen in aggregation sample

3.4.2.5 Breakdown of Amino Acid Derivatives by TLC

From 10mg/ml solutions of ASA-Ala and ASA-Gly in tetrahydrofuran, aliquots were spotted onto TLC plates each day over seven days, and until the 7th day, no impurities were seen (under UV light at 254nm) to have formed in the tetrahydrofuran solution. On the 7th day, a degradation product was seen to appear just above the ASA-Ala spot, indicating that the new product was more polar than the precursor. On the 7th day, a few degradation products were seen above the ASA-Gly spot. In the case of both derivatives, their degradation products (hydrolysis products) did not coincide with ASA or SAL standards. Samples left on the plate for a number of hours prior to chromatography, and subsequently examined under UV light, also revealed the presence of the degradation products.

The bond between ASA and the amino acid moiety in each of the two derivatives did not cleave (as had been hoped) to give ASA and the associated amino acid, enhanced by the fact that no ASA was seen on hydrolysis of either compound by TLC. Using a different TLC procedure, no free alanine or glycine were seen. The principal hydrolysis product for each of ASA-Ala and ASA-Gly was postulated to be due to straightforward hydrolysis of the ASA moiety on the compound to SAL. In other words, the acetyl group on the ASA molecule was hydrolysed in preference to the amide bond. SAL-Ala and SAL-Gly standards were obtained and run by TLC to give further evidence for this finding, and these standards did, in fact, correspond to the hydrolysis products on the TLC plate.

TLC analysis was also carried out on ASA-PheAla but this derivative was found to be very impure. This may have resulted from problems during synthesis. A summary of the TLC results for the three amino acid derivatives is given in Table 3.1.

3.4.2.6 Breakdown of Amino Acid Derivatives by HPLC

ASA-Gly and ASA-Ala standards were made to 100μg/ml in PBS, pH 7.2 and their stability monitored by HPLC over a number of days by repeated injection. ASA-Ala in PBS, pH 7.2 broke down slowly to give the hydrolysis product SAL-Ala, as opposed to ASA. After 24 hours, this approximated to an overall breakdown of 10%. ASA-Gly in PBS, pH 7.2, gave a similar hydrolysis profile, degrading by 10% in 24 hours to give SAL-Gly. These results agreed with those seen by TLC. The formation of SAL-Gly and SAL-Ala over time in PBS solutions, due to the hydrolysis of ASA-Gly and ASA-Ala, respectively, is shown graphically in Figure 3.10.

Although ASA-Gly (which elutes at 2.5 min. by HPLC) and ASA-Ala (which elutes at ≈4 min. by HPLC) were predicted to be more non-polar than ASA via the amide bond, in fact they were found to elute earlier on the chromatograms. This indicated that relative to the other four derivatives (ASA-Phe, ASA-Phe-NO_2, dimer and ASA-Iso), these amide derivatives were more polar and therefore less likely to cross the skin more efficiently than ASA alone.

Figure 3.10 : The Formation of SAL-Gly and SAL-Ala over Time in PBS Solution, due to the Hydrolysis of ASA-Gly and ASA-Ala, respectively

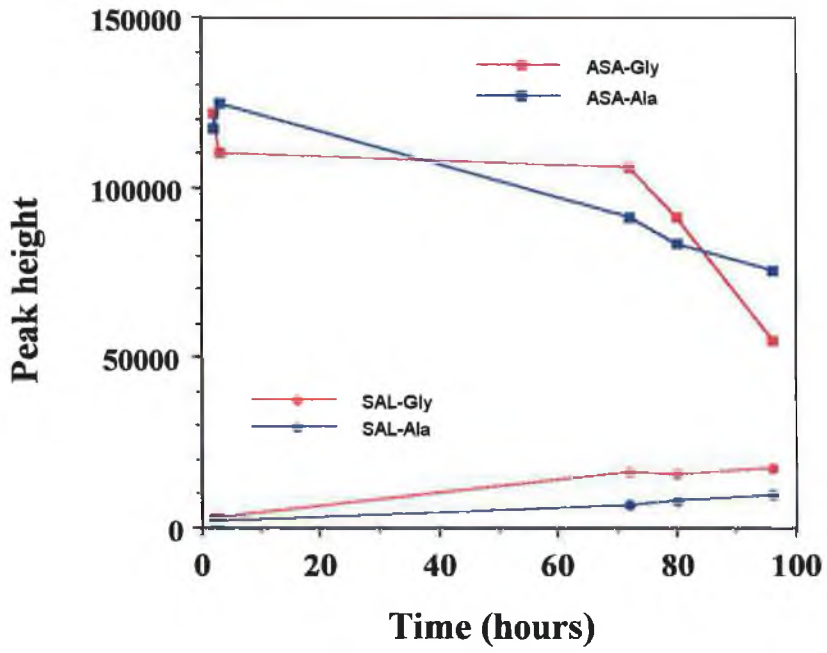
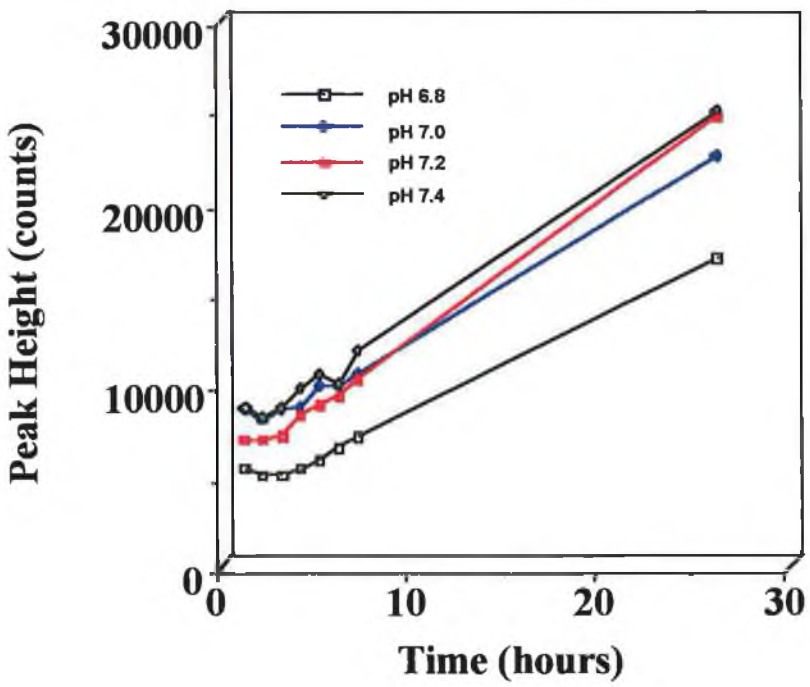


Figure 3.10 : the molecular weight of the compounds has been taken into account with the peak height in order to compensate for the quantity balance.

To investigate the effect of pH on the hydrolysis of ASA-Gly, the derivative was hydrolysed in PBS solutions with four different pH values. The results can be seen in Figure 3.11, and a HPLC chromatogram overlay showing the formation of SAL-Gly with time is shown in Figure 3.12. It was apparent that over the pH range examined, the rate of breakdown of ASA-Gly to SAL-Gly remained relatively constant.

HPLC analysis was also carried out on the ASA-PheAla derivative but it the derivative was found again to be very impure. This may have resulted from problems during synthesis. A summary of the HPLC results for the three amino derivatives is given in Table 3.1.

Figure 3.11 : Breakdown of ASA-Gly to SAL-Gly over Time in Different pH Solutions of PBS



3.4.2.7 HPLC Results for the Other Four Derivatives

The ASA-Iso derivative was found to have a poor UV response, but could be seen by HPLC (at high concentrations) to elute at approximately 40min. The ASA-Phe derivative had a similar retention time of 40min. The ASA-Phe-NO_2 eluted very late by HPLC indicating that the molecule was very non-polar. The dimer, although pure (IR, NMR), was found to break down quickly in aqueous solution to give ASA and thence, SAL.

Figure 3.12 : HPLC Chromatogram showing Increase in SAL-Gly with Time

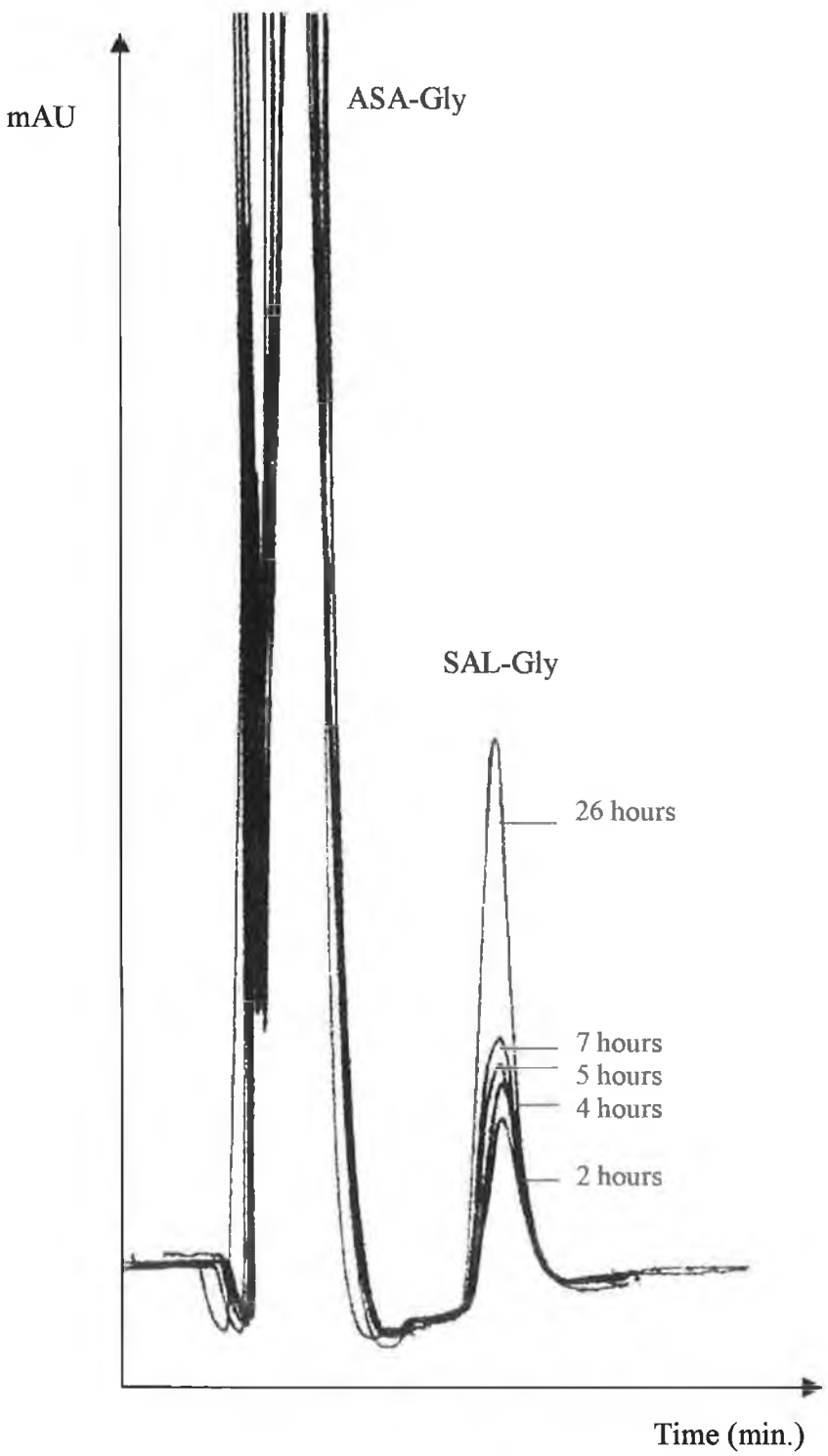


Figure 3.12 : HPLC Method 2 using Alphabond® C18 analytical column, mobile phase of tetrahydrofuran-water-acetic acid [25:75:0.05, v/v/v, pH 3.5*]. Other experimental conditions described in text.

3.2.5 CONCLUSIONS

At the end of this section, three of the derivatives were eliminated from further investigation. These were ASA-Ala, ASA-Gly and ASA-PheAla. The first two did not have the required pharmacological effect i.e. did not prevent platelet aggregation. It was also shown by TLC and HPLC that these compounds were hydrolysing to give SAL-Ala and SAL-Gly, as opposed to ASA. This explained the inability of these derivatives to elicit the desired biological affects. The ASA-PheAla was eliminated due to problems during synthesis, which resulted in production of a very impure derivative. Due to these high levels of impurities, further investigation was not carried out. The remaining four of the derivatives were brought forward to the next stage of investigation since they had the desired biological effect via their production of ASA and they were of high purity.

3.5 EVALUATION OF DERIVATIVES AS PRODRUGS FOR ASPIRIN AND DETERMINATION OF ASA AND SAL IN TRANSDERMAL SAMPLES

3.5.1 EXPERIMENTAL

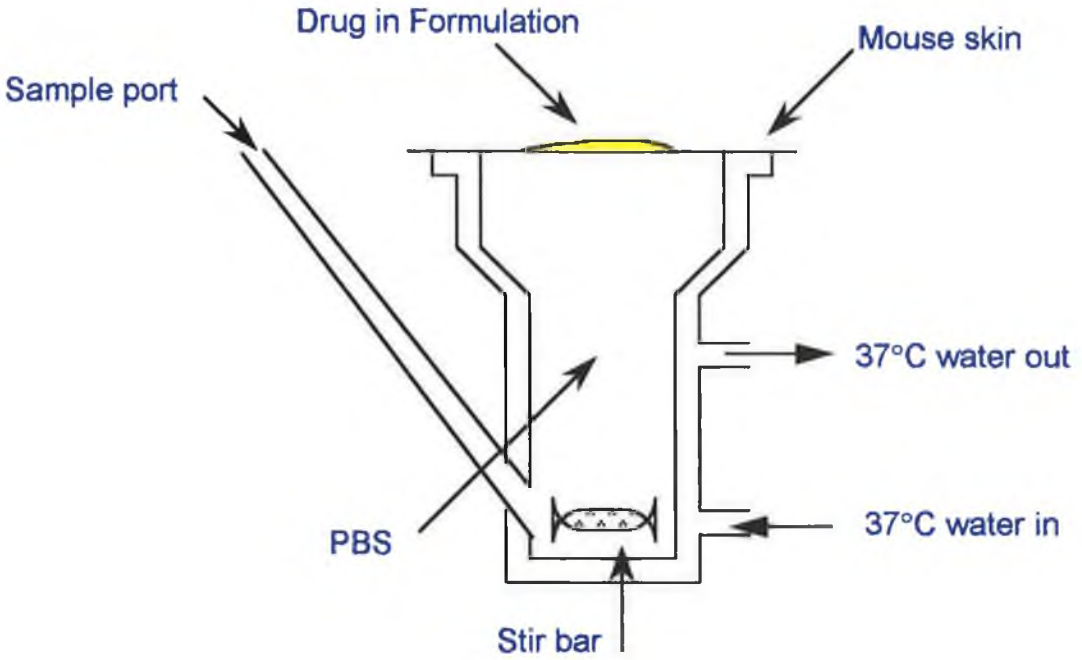
3.5.1.1 Preparation of Skin for Franz Cell Experiments

Sha/Sha hairless male mice aged 60±5 days were sacrificed in a carbon dioxide chamber. The skins were immediately removed and because they were to be used fresh, were not subjected to any pretreatment such as presoaking or chemical preservation.

3.5.1.2 Franz Cell Apparatus

The *in vitro* transdermal experiments were performed on the fresh sha/sha mouse skins directly in contact with a 22ml Franz cell reservoir of PBS at physiological pH and 37°C. A diagram of the Franz cell is shown in Figure 3.13 below.

Figure 3.13 : Diagram of a Franz Cell for Transdermal Experiments



3.5.1.3 Preparation of Samples for Application to Skin

The prodrug being examined (20mg) was diluted in 250µl ethanol, mixed with 250µl of 15% polyethylene glycol (PEG) and topically applied to a 5cm^2 area of skin.

3.5.1.4 Preparation of Standards for HPLC

Stock solutions of ASA, SAL, ASA-Phe, ASA-Phe-NO_2, dimer and ASA-Iso were prepared by dissolving each of the compounds in acetonitrile to a concentration of 1mg/ml. The structures of the four derivatives can be seen in Figure 3.9. Working standards were prepared on a daily basis by dilution of the stock solutions with PBS. The ASA and SAL standards were mixed together for the validation procedure and had final concentrations of 0.2, 0.5, 1.0, 2.0 and 5.0µg/ml. The standard solutions of the derivatives were prepared individually in PBS to a concentration of 2µg/ml as required.

3.5.1.5 Preparation of Samples for HPLC

Aliquots were taken from the buffer vessel at 0, 2, 4 and 6 hours, injected directly onto the HPLC system and analysed by comparison with external standards, also in PBS (pH 7.35). Blank skin perfusates containing only the vehicle components i.e. ethanol and PEG, were run in parallel with the drug perfusates at all times to account for any formulation effects. At the end of the transdermal experiment for each prodrug, a sample from the top of the skin was taken by dissolving the remaining drug in 1ml of acetonitrile. A 1 in 1000 dilution with PBS was usually required for these samples prior to analysis.

3.5.1.6 HPLC (Method 3) Conditions

The final HPLC conditions for this work were as follows : Nucleosil® C8 analytical column, flow rate of 1ml/min, UV detection at 225nm and an injection volume of 20μl. Eluent A was a mixture of water-acetonitrile-OPA [650:350:2, v/v/v], the aqueous component of which had a pH of 2.0. The entire mixture had a pH of 2.5* when the acetonitrile was added. Eluent B was a mixture of acetonitrile-OPA [1000:2, v/v]. Both eluents were filtered (0.45μm filter) under vacuum and sonicated for 20 to 30 minutes to remove dissolved gases. The solvent program used was: time 0 to 6.5min. - 100% A, time 10.5 to 19.0min. - 70% A/30% B, time 22.0 to 28.0min. - 100% A, employing a linear gradient ramp.

3.5.2 RESULTS AND DISCUSSION

3.5.2.1 Rationale for Choice of Animal Model

Animal models are often used for *in vitro* dermal evaluation of drugs since human skin is usually not readily available. Hairless mouse skin was used in this work since it is reported to be similar to human skin in the absorption of anti-inflammatory steroids[60] and short chain alcohols[61]. Hairless mouse skin was found to be usefully predictive of human skin in terms of permeability of compounds[62]. Some papers dispute the wisdom of directly applying murine data to man[63,64] but it has been shown that for a range of drugs, even the most permeable of animal skins such as rabbit and mouse are often well within an order of magnitude of values for human skin[41].

3.5.2.2 Rationale for Choice of Formulation

The derivatives ASA-Phe, ASA-Phe-NO_2, dimer and ASA-Iso, shown in Figure 3.9 were selected for this section of the project because they were found to inhibit platelet cyclooxygenase *in vitro* in the platelet aggregation studies in section 3.4. Because of their hydrophobicity, special formulations are required to permit their application to the skin. A number of possible formulations were tried including the following : paraffin oil, aqueous cream BP (Uniphar® and ROWA®) and PEG, each with and without ethanol as an enhancer. There were a number of interferences present in paraffin oil and aqueous cream, some that even coeluted with ASA by HPLC. Hence, it was necessary to change the formulation and the final vehicle was a mixture of 250µl ethanol and 250µl 15% PEG. This formulation did not interfere with the HPLC analysis.

3.5.2.3 Development of HPLC Conditions for Method 3

The HPLC method was again modified for this part of the project. The reason for this was that it was required to run many samples and time was now a limiting factor. It was sought to reduce the retention times for ASA and SAL, and to sharpen the peaks for the late-eluting derivatives. This was achieved by using a Nucleosil® C8 column (5µm, 250 x 4.6mm) instead of an Alphabond® C18 column. Eluent A was optimised to a final mixture of water-acetonitrile-OPA [650:350:2, v/v/v]. The aqueous component of eluent A had a pH of 2.0 and the mixture was pH 2.5* when the acetonitrile was added (ASA is most stable at this pH value[65]). Eluent B was a mixture of acetonitrile-OPA [1000:2, v/v]. Both eluents were filtered (0.45µm filter) under vacuum and sonicated for 20 to 30 minutes to remove dissolved gases. Under the final conditions of HPLC method 3, the retention times for ASA and SAL were 6.7±0.1 min. and 9.0±0.3 min., respectively. The solvent program developed was: time 0 to 6.5 min. - 100% A, time 10.5 to 19.0 min. - 70% A/30% B, time 22.0 to 28.0 min. - 100% A, employing a linear gradient ramp. The retention times for the prodrugs using the gradient were as follows : dimer 18.4±0.2 min., ASA-Iso 19.1±0.3 min., ASA-Phe 20.2±0.3 min. and ASA-Phe-NO_2 20.6±0.3 min.

3.5.2.4 Calibration and Calculation

Calibration was based on regression analysis of peak areas versus concentration (peak areas were found to correlate better than peak heights with concentration). Calibration

curves were constructed for 0.2, 0.5, 1.0, 2.0 and 5.0μg/ml ASA and SAL. This concentration range was suitable for the detection of these compounds in the PBS buffer after transdermal experiments. Since the transdermal release of ASA and/or SAL was the most important criterion and since none of the intact parent drugs penetrated the skin intact, full validation was carried out only on the analysis of ASA and SAL in the PBS.

3.5.2.5 Precision

Precision was defined in terms of the inter-day variability (reproducibility) and intra-day variability (repeatability). Inter-assay reproducibility was assessed in four replicate runs on four consecutive days, on the concentration points in the range 0.2 to 5.0μg/ml. Intra-assay repeatability was determined by running, in quadruplicate, standards at each concentration level on day two of the validation study. The precision of the method was described by the mean relative standard deviation (RSD) of the recovered amounts, determined by interpolation of the peak areas on the regression lines. Precision data are presented in Table 3.2, and they demonstrate that the reproducibility (mean RSDs = 1.51% and 1.30% for ASA and SAL, respectively) was slightly lower (better) than the repeatability (mean RSDs = 1.49% and 2.51% for ASA and SAL, respectively). However all of the precision values are well within accepted values for bioanalyses.

3.5.2.6 Linearity and Accuracy

The correlation coefficient of the regression line for the mean intra-assay values was 0.9999 for both ASA and SAL. Accuracy (presented in Table 3.2), as defined as the percentage difference between the nominal amount and the amount found, gave mean inter-day values of 0.81% and 1.67% for ASA and SAL, respectively, and mean intra-day values of 0.28% and 1.87% for ASA and SAL, respectively.

3.5.2.7 Limit of Detection

The limit of detection was found to be 0.05μg/ml for both compounds which corresponded to a peak which was three times the standard deviation of the baseline noise.

Table 3.2 : Precision and Accuracy Data

Inter-assay (reproducibility)

Amount added (μg/ml) n=4	Amount found(μg/ml) ASA	SAL	RSD (%) ASA	SAL	Accuracy(%) ASA	SAL
0.200	0.194	0.212	3.46	2.60	3.00	6.00
0.500	0.499	0.503	2.20	1.24	0.20	0.60
1.000	1.004	0.994	1.18	0.89	0.40	0.60
2.000	2.008	1.979	0.62	1.67	0.40	1.05
5.000	4.997	5.006	0.11	0.12	0.06	0.12

Intra-assay (repeatability)

Amount added (μg/ml) n=4	Amount found(μg/ml) ASA	SAL	RSD (%) ASA	SAL	Accuracy(%) ASA	SAL
0.200	0.199	0.215	1.67	2.18	0.50	7.50
0.500	0.498	0.498	0.99	3.28	0.40	0.40
1.000	1.004	0.990	2.24	2.14	0.40	1.00
2.000	2.002	1.993	1.72	2.26	0.10	0.35
5.000	4.999	5.005	0.83	2.71	0.02	0.10

For ASA : $y = 2711 + 121230x$ For SAL : $y = -2574 + 109060x$

3.5.2.8 Quantitative Determination of ASA and SAL in Perfusate Samples

No ASA was found in the perfusates for prodrugs ASA-Iso, ASA-Phe or ASA-Phe-NO_2, but SAL was found in these samples. The presence of SAL in the perfusates would appear to indicate that the prodrugs did in fact break down in the skin, and that the rate of release of ASA was less than the rate of its subsequent hydrolysis to SAL. The poor penetration of the skin could have been due to the preferential solubility of the prodrugs in the formulation as opposed to the skin.

The dimer (an anhydride) was significantly more susceptible to hydrolysis than the ester prodrugs, yielding ASA (and hence SAL) in the perfusate samples. This could be explained by the fact that anhydrides undergo hydrolysis more readily than

esters. Figure 3.14 shows a chromatogram of a) a blank perfusate and b) a perfusate sample taken two hours after application of the dimer prodrug.

Figure 3.14 : Chromatograms of Perfusate Samples

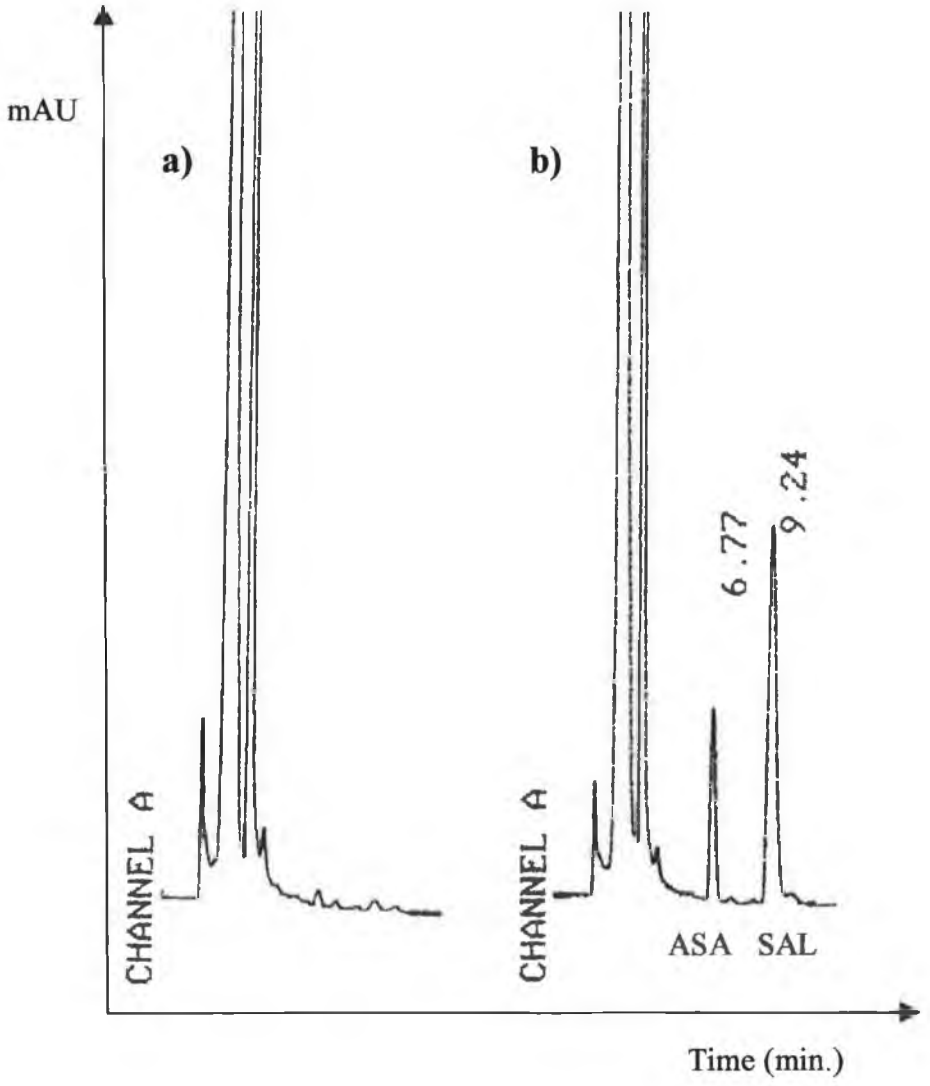


Figure 3.14 : Chromatograms of a) Blank perfusate sample and b) Perfusate sample taken two hours after topical application of dimer prodrug to the skin. Concentrations of ASA and SAL in b) are 0.72μg/ml and 2.99μg/ml, respectively. Chromatographic conditions as per text.

Figures 3.15 and 3.16 show the transdermal results for both ASA and SAL in terms of their flux i.e. μg of compound that traversed the skin per square centimetre of skin onto which the prodrug was applied. It can be seen that the dimer delivered double the flux of ASA (Figure 3.15) and less SAL (Figure 3.16) when compared to the case where ASA was applied to the skin alone.

Figure 3.15 : Transdermal Fluxes (μg/cm^2) of ASA due to Prodrugs and ASA

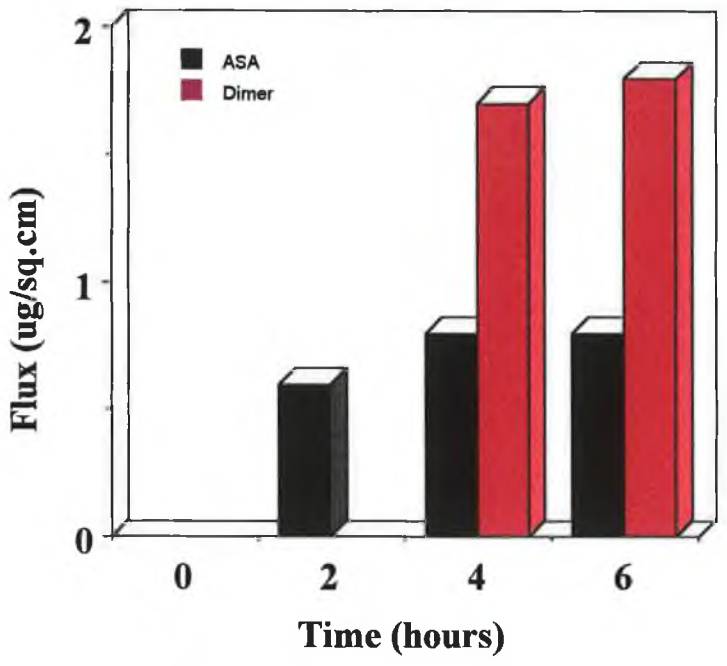
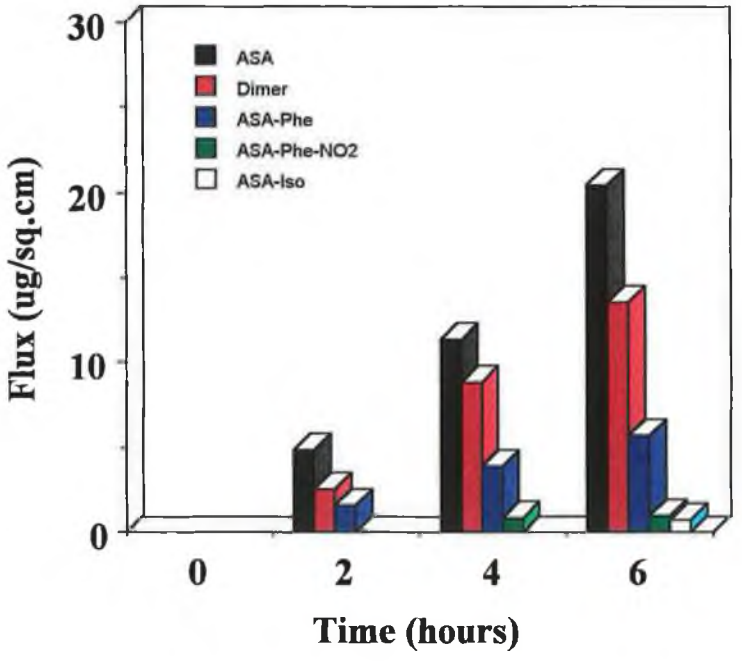


Figure 3.16 : Transdermal Fluxes (μg/cm^2) of SAL due to Prodrugs and ASA



3.5.2.9 Qualitative Analyses of Prodrug Transport and Breakdown on Skin Surface

At no time did any of the intact prodrugs traverse the mouse skins. This was a promising result which was confirmed by gradient HPLC analysis of the perfusates, by comparing the samples to external standards of the prodrugs at a minimum concentration of 2μg/ml. This concentration would correspond to less than 0.25% of the prodrug crossing the skin intact. The samples taken from the top of the skin at the end of the transdermal experiment for ASA-Iso, ASA-Phe and ASA-Phe-NO_2 contained only the parent compound in each case. This indicated that these derivatives were not hydrolysed on the surface of the skin. Only the dimer was hydrolysed on the surface of the skin to yield ASA.

3.5.2.10 Correlation of Log P values and Retention Times by HPLC

The Log P values were calculated by a program called ACD Log P (Advanced Chemistry Development Inc., Toronto, Canada). The values obtained for these compounds correlated (with the exception of the dimer) with their retention order on the reversed-phase HPLC system, as can be clearly seen in Table 3.3. According to the calculated log P values of the test compounds, it was predicted that they would permeate the skin in the order ASA-Phe, ASA-Phe-NO_2, ASA-Iso, dimer and ASA.

Table 3.3 : Log P Values and Retention times for the Test Compounds

Compound	Retention Time (mins)	Log P
ASA	6.7±0.1	1.19
SAL	9.0±0.3	2.06
Dimer	18.4±0.2	1.33
ASA-Iso	19.1±0.3	2.19
ASA-Phe	20.2±0.3	2.74
ASA-Phe-NO_2	20.6±0.3	2.92

However, no correlation was observed between Log P values and transdermal delivery of ASA in the transdermal samples. Log P values are usually only found to correlate when homologous series of compounds are used such as steroids, alcohols or a series of salicylates, with only the substituent groups differing on the parent molecule. Also, when Fick's law is taken into account (See section 3.1.5), P is only one of a number of important physiochemical factors that influence drug penetration through a membrane.

3.6 OVERALL CONCLUSIONS

In part one of this project, an impurity present in the in-house aspirin was identified using a combination of HPLC and TLC, and as a result, it was possible to modify the protocol for the preparation of aspirin such that formation of dimer impurity during synthesis would be minimised.

Using the aspirin prepared in-house, a number of derivatives were prepared and these were subjected to a general screening procedure in part two of the project to assess their suitability as prodrugs for aspirin. Using HPLC and TLC, two of the amino acid derivatives of aspirin (ASA-Gly and ASA-Ala) were found to hydrolyse to salicylic acid derivatives rather than releasing aspirin as hoped. Because of this, they did not have the desired pharmacological effect on platelets. A third amino acid derivative was found to be very impure and was not investigated further. The other four of the compounds investigated were found to have the desirable biological effects i.e. inhibition of platelet aggregation. They were also of sufficient purity to be investigated transdermally.

In part three of the project, the four prodrugs with the desired inhibitive effect on platelet aggregation were subjected to transdermal assessment across mouse skin. They were compared to aspirin alone in terms of their ability to undergo hydrolysis and release aspirin into the receptor fluid of the Franz Cell. Samples were removed periodically and analysed for the presence of aspirin and salicylic acid using a HPLC method. Evidence of hydrolysis of the ester compounds to aspirin was seen, but it was not at a level sufficient to warrant further investigation of these compounds as aspirin prodrugs. Only the dimer prodrug released enough aspirin over time to warrant further investigation as a prodrug for an Aspirin Patch. In comparison to aspirin alone, this

compound produced double the concentration of aspirin and less salicylic acid over a six hour time frame. Therefore, the dimer is considered a suitable candidate for further investigation, and is currently undergoing more transdermal studies. Log P values for the derivatives and aspirin and salicylic acid were found to have some correlation with their retention order by HPLC. However, there was no correlation between log P and transdermal penetration of the derivatives.

This HPLC method was optimised and validated for the determination of aspirin and salicylic acid produced by these prodrugs in transdermal samples. Samples could be directly injected and the analysis time was only ten minutes for determination of aspirin and salicylic acid. The assay was reproducible with inter-day RSD values not exceeding 3.46% and 2.60% for aspirin and salicylic acid, respectively. The method was linear over the concentration range 0.2-5.0 μg/ml and had a limit of detection of 0.05 μg/ml for both analytes. The method could also be modified to run a gradient after salicylic acid had eluted to determine if the non-polar derivatives were present.

3.7 REFERENCES

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Chapter 4

Determination of Aspirin and Salicylic Acid in Human Plasma by Column-Switching Liquid Chromatography using on-line Solid Phase Extraction

4.1 INTRODUCTION

4.1.1 Biological Background

The history, pharmacology and biology of aspirin (ASA) have been discussed in the previous chapter. In summary, ASA has a myriad of medical applications in diseases such as arthritis and cancer, but the most important application of ASA is in heart disease. Recent studies have shown that ASA can a) prevent heart attacks and strokes among individuals who have previously suffered such events[1,2,3,4], b) save lives of heart attack victims if initiated in the hours just after the onset of the attack[5] and c) prevent an initial heart attack when taken regularly by apparently healthy people with no past history of heart disease[6].

4.1.2 Analytical Background

The plasma half-life of aspirin is only about 20 minutes because it is so readily hydrolysed to salicylic acid (SAL)[7,8], its principal metabolite. An analytical method that measures ASA in biological fluids should therefore be capable of measuring SAL also. Such assays are required for many reasons - in cases of aspirin poisoning, intolerance reactions, side-effects and for metabolic and pharmacokinetic studies. Low level determination of the compounds is usually achieved using HPLC methods, many of which have been described in a review by Kwong in 1987[8]. Some details of these and more recent methods are listed in Table 4.3. The mobile phases usually contain methanol or acetonitrile and have a low pH (normally 2.5 - 3.0), and UV detection tends to be in the range 225 - 240nm.

Since 1980, HPLC methodology for ASA and SAL has only really changed in terms of applications; room for improvement lies mainly with the sample preparation step, as always, the bottleneck in the protocol. Until now, assays have required sample clean-up procedures such as protein precipitation or solvent extraction. Usual methods of deproteinisation involve the addition of an organic solvent such as acetonitrile[9,10,11], ethanol[12], or a strong acid such as perchloric acid[13,14]. Following centrifugation, the supernatant is usually dried and reconstituted in mobile phase before injection. The solvent extraction procedure is usually effected with ether[15], dichloromethane[16,17,18], chloroform[19] or hexane[20] and the organic extract evaporated and the residue reconstituted in mobile phase. Nieder *et al.*[21] used a mixture of hexane and ether to

effect their extraction. Shen *et al.* [22] and Buskin *et al.* [23] also used a mixture of hexane and ether but in order to avoid the evaporation step, the compounds were back-extracted into phosphate buffer before injection. This is because during evaporation, loss of ASA and/or SAL are known to occur due to sublimation[8,10,12,16,19,20,21,22,23]. Another problem encountered in many of these methods is poor and/or variable recovery of the compounds because of losses during the protein precipitation procedure (which can be due to protein binding of the drug) or during the solvent extraction step. The use of internal standards has gone some way towards reducing the variability of recoveries, but it is worth remembering that their presence can also adversely affect the results of analysis because of the added variability in the extraction recovery of the internal standard itself [24].

The aim of this project was to develop an analytical method for the quantitation of ASA and its principal metabolite SAL in human plasma with an alternative sample preparation protocol to the manual methodologies used in previous assays (described above). It was hoped to achieve this using a column-switching HPLC system with on-line solid-phase extraction, since switching devices permit the off-line multistep methods for sample pretreatment to be transformed into on-line single-step procedures. It was also noted that this technique had not been employed previously for these compounds in plasma.

Column-switching is just one of a number of techniques encompassed by multidimensional liquid chromatography[25]. These methods are characterised by the use of more than one column interfaced with each other by a valve which allows the 'switching' of fractions between the columns. The valve can be controlled manually or automatically but must possess two features i.e. it must be capable of high pressure operation without deterioration and it must provide a low dead volume in order to avoid significant peak broadening. Column-switching has been commonly employed for trace enrichment and sample clean-up. In both cases, the compounds of interest are retained on the precolumn while interferences flow to waste. The analytes are then transferred to the analytical column for separation and quantitation. Trace enrichment is used when the sample is too dilute to be detected. In this case, the precolumn is called a concentrator column. The concentrator column usually contains the same stationary phase as the analytical column and is often simply a guard column. Sample clean-up is used when separation of the analytes of interest from endogenous interferences in the matrix is required prior to analysis. In this case, the precolumn is

usually referred to as a solid-phase extraction (SPE) column. The particle size of the SPE column is usually large (10-40μm) compared to that in the analytical column in order to prevent clogging problems. The only previous work that had been reported employing column-switching HPLC for ASA and SAL was by Cockaerts *et al.* in 1986. They analysed the compounds in a tablet formulation using two C8 columns (10cm and 25cm) in series.

On-line sample extraction on short precolumns is finding increasing use in the determination of drugs in biological fluids[26,27,28]. Some examples are described below in Table 4.1.

Table 4.1 : Examples of Column-Switching HPLC using on-line Solid-Phase Extraction in Biopharmaceutical Analysis

Matrix	Compound	Sorbent and Comments	Reference
Plasma	Ivermectin	A 25 x 2.1mm SPE with Corasil C18 material. The LOQ was 2 ng/ml by HPLC. Recovery was 76% from plasma	29
Urine	Diuretics and probenecid	20 x 2.1mm SPE was used. Hypersil ODS material (30μm) was found to give better retention than SynChropak material (30-70 μm). Recoveries of the 10 cited drugs were >92% from urine	30
	Amphetamines	20 x 2.1mm SPE with Hypersil ODS(30μm) material. Following clean-up, in-line deriv-atisation was carried out. When the optimal conditions were found, recoveries of the 2 analytes from urine were 86 and 96%	31
Serum	Pantoprazole enantiomers	A 10 x 6mm SPE with Lichroprep RP-2, 25-40μm particles was used. The LOQ for each enantiomer in 150μl serum was 0.1 μg/ml. Recoveries ranged from 93-117% and from 94-119% from serum for the (+) and (-) enantiomers, respectively	32

These SPE-HPLC column-switching systems allow circumvention of the problems usually associated with direct injection of untreated plasma, serum or urine

onto reversed-phase columns i.e. adsorption of proteins on the solid phase, precipitation of the proteins by components of the mobile phase and shortening of the useful lifetime of the analytical column. The use of the SPE precolumn serves the dual function of acting as a guard column as well as effecting a preseparation of the analytes from the biological matrix. This system, if developed, would allow the direct injection of the plasma sample, sample clean-up and analysis to be performed on-line in the same closed system, and a result to be obtained quickly. This would improve therapeutic monitoring of ASA and SAL following oral administration of aspirin tablets, especially in cases of intolerance reactions or unwanted side-effects.

4.2 REAGENTS AND MATERIALS

4.2.1 Equipment

The high performance liquid chromatograph was equipped with Waters (Millford, MA, USA) models 501 single-piston and 510 dual-piston pumps, a Waters Model 486 tuneable absorbance detector, a Waters model 680 gradient controller and a Waters model 746 data module. The Rheodyne injection port (Cotati, CA, USA) was fitted with a 20µl loop. The VICI AG 10-port switching valve (Valco Europe, Schenkon, Switzerland) was fitted with a 200µl loop. A Hypersil® C-8 guard column (30µm, 10 x 4.0mm) was fitted prior to the Nucleosil® C-8 analytical column (5µm, 250 x 4.6mm). The solid-phase extraction (SPE) column was a biocompatible peek cartridge system (10 x 4.3mm) from Anachem (Luton, Bedfordshire, England). The cartridge was dry-packed with Hypersil® C-18 material (30µm) from Shandon HPLC (Runcorn, Cheshire, England).

4.2.2 Reagents and Chemicals

Aspirin was supplied by SIGMA Chemical Co. (Dorset, England), as were all the drugs used in the interference study. Aspirin tablets (300mg) for the volunteer study were from Bayer Ltd. (Dun Laoghaire, Dublin, Ireland). Salicylic acid was supplied by BDH (Poole, England). Acetonitrile, methanol and water were HPLC grade and were purchased from Labscan (Dublin, Ireland). Acetic acid (glacial 100%) was purchased

from Merck (Darmstadt, Germany). High purity (99.999%) orthophosphoric acid (OPA) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA).

4.2.3 Other Materials

Plastic blood collection tubes (monovettes) were donated by Sarstedt Ltd. (Wexford, Ireland). Monovettes containing four types of anticoagulant were obtained : Li-heparin, EDTA, fluoride/EDTA and citrate. Monovette needles and adapters, also from Sarstedt Ltd.,were used in conjunction with the above collection tubes.

4.3 EXPERIMENTAL

4.3.1 Blood Collection and Storage

Blood samples were collected into chilled plastic tubes containing a fluoride/EDTA mixture. The chilled blood samples were centrifuged @ 1500g for 10 min. to harvest the plasma. Samples were frozen at –30°C until required if there was to be a time lapse before analysis. Both fresh and thawed plasma were kept on ice at all times.

4.3.2 Preparation of Standards for HPLC

Stock solutions of ASA and SAL were prepared by dissolving each of the compounds (50mg) in acetonitrile (50ml) to a concentration of 1mg/ml. In acetonitrile, the stock solutions were found to be stable for at least ten weeks when kept at room temperature. Aqueous working standards were made on a daily basis by dilution of the stock solutions in eluent A (wash solvent, see section 4.3.4) to give the concentrations required. Eluent A was used for dilution because ASA and SAL, with pKa values of 3.5 and 3.0, respectively[33], are stable at this pH (2.5). Common ASA and SAL plasma standards were prepared by spiking blank plasma with working standard solutions, and had final concentrations of 0.1, 0.25, 0.8, 2.0 and 5.0μg/ml for ASA and 0.25, 0.75, 2.4, 6.0 and 15.0μg/ml for SAL. These concentrations were chosen because they covered the expected range of the volunteer study samples. The drugs for the interference study were prepared individually as required and each had a final concentration of approximately 1μg/ml.

4.3.3 Preparation of Samples for HPLC

OPA (250μl of 0.2M solution) was added to 250μl of the chilled plasma sample within 10 minutes of centrifugation (if fresh) or within 10 minutes of thawing (if frozen) in order to minimise enzymatic hydrolysis of ASA to SAL. The samples were vortex-mixed for 20 seconds. Direct injection was usually possible at this stage but some of the volunteer's plasma samples, especially after eating, appeared cloudy after vortexing, so a short centrifugation step (5800g for 3 minutes) was required. In order to treat all samples equally, this centrifugation step was introduced as part of the protocol. Following centrifugation, the sample was injected directly onto the HPLC. Samples that fell outside the standard curve concentration ranges were diluted appropriately and brought on scale for quantitation.

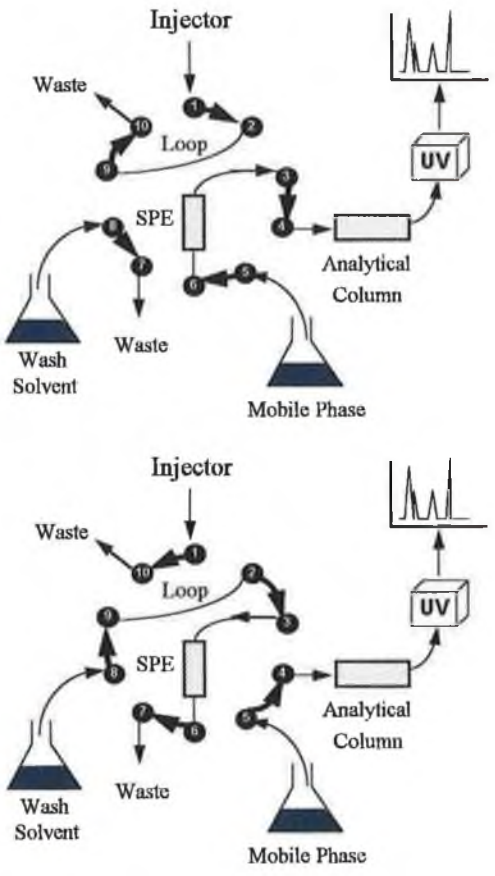
4.3.4 HPLC Conditions

Eluent B was a mixture of water-methanol-acetonitrile-OPA [650:200:150:1, v/v/v/v] with a resultant pH of 2.6*. This mobile phase was based on the final HPLC method used in chapter 3. The same column (Nucleosil C8) was also employed. Eluent A was a mixture of water-OPA [1000:1, v/v] and had a pH of 2.5 Both eluents were filtered (0.45μm filter) under vacuum and sonicated for 20 to 30 minutes to remove dissolved gases. The flow rates of eluent A and eluent B through the columns were each 1ml/min, UV detection was at 225nm and all measurements were made at ambient temperature. Under these conditions, the retention times of ASA and SAL were 11.5±0.2 and 15.6±0.3 minutes, respectively.

4.3.5 Column-Switching Conditions

The 200μl of sample was loaded onto the loop with the valve in position 1 (Figure 4.1). The valve was then turned to position 2, injecting the sample onto the extraction column (Figure 4.1). The wash solvent was eluent A and this was allowed to flow through the C18 extraction column for two minutes. During this time, the analytes of interest would remain on the extraction column while endogenous components flowed to waste. After two minutes, the valve was switched back to position 1, whereupon the ASA and SAL were backflushed onto the analytical column by mobile phase (eluent B).

Figure 4.1 : Diagram of Column-Switching Apparatus



Valve in Position 1

Valve in Position 2

Figure 4.1 : Sample is loaded onto loop with valve in position 1. The valve is then switched to position 2 so the sample is swept onto the SPE column while the analytical column is equilibrating. Finally, the valve is switched back to position 1 so the sample is desorbed from SPE column in backflush mode and carried onto analytical column for quantitation. Next sample is loaded onto loop.

4.4 RESULTS AND DISCUSSION

4.4.1 Optimisation of Blood Collection

Since the nature of the anticoagulant used in conjunction with the blood collection procedure can have a dramatic effect on the number of interfering peaks, it was decided to investigate four different anticoagulant types. Previous work has shown that the levels of plasma interferents following on-line SPE (using a C18 precolumn)

can be quite different when different anticoagulants and/or brands are used[34]. Hence, for this study, blood was taken from two volunteers using a butterfly syringe connected to each of four types of blood collection tube (Li-heparin, EDTA, fluoride/EDTA and citrate). All eight blank plasma samples were treated as per the protocol and analysed on the LC system. There was very little difference between the plasma profiles obtained, but the fluoride/EDTA mixture appeared to give a slightly cleaner baseline in the case of both volunteers and so it was chosen as the anticoagulant for this method. A significant advantage of the fluoride anticoagulant is that it inhibits the action of esterases, and hence the *in vitro* hydrolysis of ASA. A number of workers have used fluoride (in the form of NaF or KF salts) in conjunction with blood collection for this reason[12,13,14,16,17,19,20,21,23]. In fact, Rumble *et al.*[14] compared three commonly used esterase inhibitors ie. ecothiopate, physostigmine and fluoride and found that KF inhibited ASA hydrolysis to the greatest extent. Nieder *et al.*[21] also found KF to be the most effective inhibitor when they conducted a similar study.

4.4.2 Optimisation of Sample Dilution

While the use of acidic diluent or water made no difference to aqueous standards, it was critical to the recovery of both ASA and SAL from plasma samples. It was especially important to use an acidic diluent for the recovery of SAL. SAL is highly protein bound in plasma (80-90%)[35,36] and the acid caused the liberation of the SAL molecules from their binding sites. In determining the optimum concentration for the diluting acid, three concentrations were investigated : 0.02M, 0.2M and 2M OPA. The 0.2M acid gave the highest recoveries (>90%) for both drugs. The lower concentration of OPA gave low recoveries of SAL (not strong enough to free SAL from proteins) while the higher concentration was high enough to cause a small amount of protein precipitation, which was not a desirable effect.

The ratio in which the 0.2M OPA was added to the plasma sample also had an impact on recovery of the analytes. Obviously, the lowest possible dilutional effect is always required so that sensitivity can be maximised. Three ratios of OPA to plasma were examined i.e. 1:1, 2:1 and 3:1. When OPA was added in a ratio of 2:1 or 1:1 to the plasma sample, the highest recoveries for both ASA and SAL were obtained. Hence, the 1:1 ratio of OPA to plasma was used throughout the rest of this work. These results are illustrated in Figure 4.2 below.

Figure 4.2 : Effect of Ratio of OPA to Plasma on Recoveries of ASA and SAL

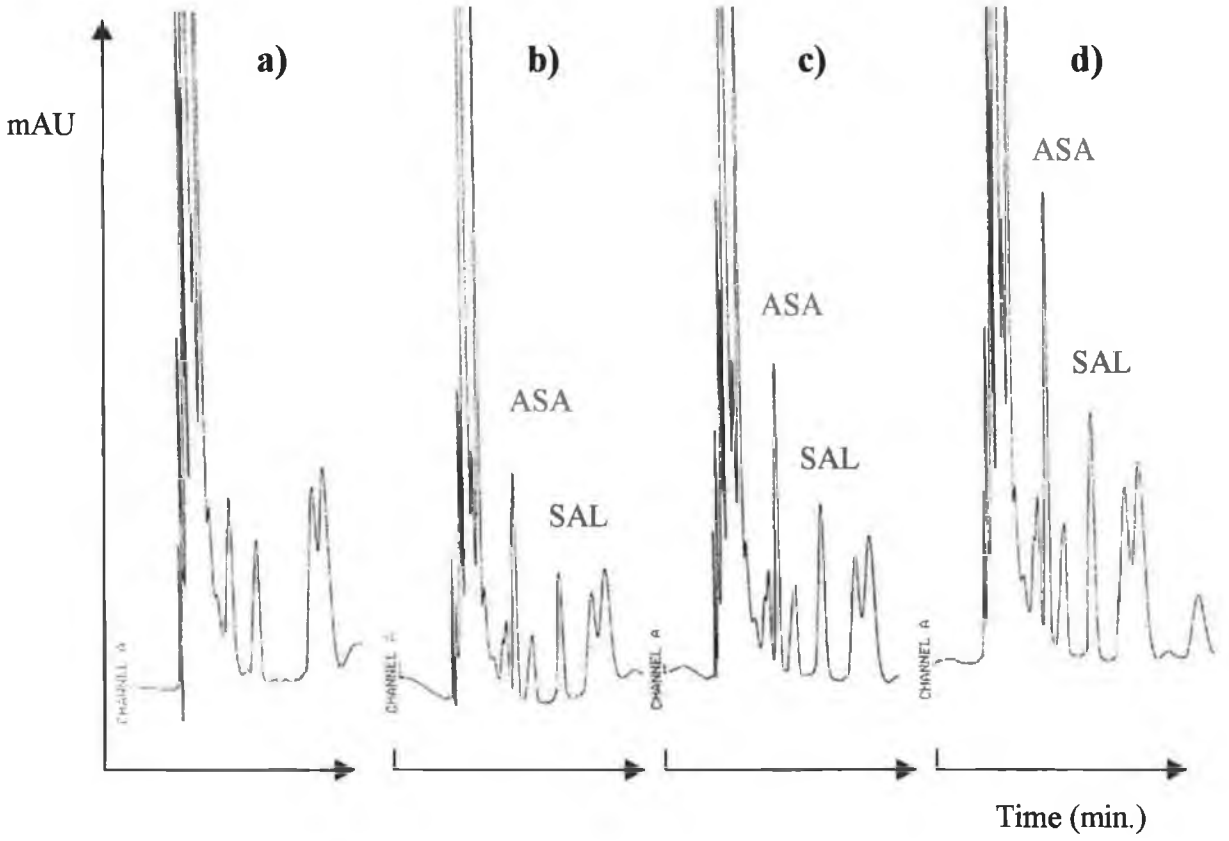


Figure 4.2 : Chromatogram a) blank plasma, b) plasma diluted 1:3 with OPA, yielding recoveries of ASA and SAL of 85% and 93%, respectively, c) plasma diluted 1:2 with OPA, yielding recoveries of ASA and SAL of 94% and 100%, respectively and d) plasma diluted 1:1 with OPA, yielding recoveries of ASA and SAL of 94% and 100%, respectively. Each of the time axes represents 20 minutes from beginning (injection) to end of the arrow.

4.4.3 Development of HPLC Conditions

Initially, a mobile phase with the composition of water-acetonitrile-acetic acid [700:300:0.5, v/v/v] was employed. The pH of this eluent was 3.5*. However, under these conditions, the peak for SAL had a very poor shape and was not well resolved from the ASA peak. The first change made to the mobile phase was the substitution of acetic acid for OPA, and using 1ml of the acid per 1000ml of eluent to maintain the pH at 2.6*. This improved the baseline but not the chromatography. The presence of methanol in the mobile phase improved the peak shape of SAL, which had been tailing badly. The presence of 20% methanol was found to be the optimum amount with respect to its effect on the SAL peak. The amount of acetonitrile was adjusted in order

to elute both ASA and SAL in a reasonable time frame. The final mobile phase composition was a mixture of water-methanol-acetonitrile-OPA [650:200:150:1, v/v/v/v] with a resultant pH of 2.6*. This solution was referred to as eluent B. The flow rate of eluent B through the analytical column was 1ml/min. UV detection was at 225nm, although a number of other wavelengths were investigated i.e. 210, 230, 235 and 270nm. Under these conditions, the retention times of ASA and SAL were 11.5±0.2 and 15.6±0.3 minutes, respectively.

4.4.4 Development of Column-Switching Conditions

4.4.4.1 Choice of Packing Material for SPE Column

A number of packing materials such as cyanopropyl, phenyl, C1, C8 and C18 (see section 1.1.4.3) were examined in terms of their retention and selectivity for ASA and SAL on the extraction column. The C18 phase was found to be the most retentive for the compounds.

4.4.4.2 Choice of Wash Solvent

When water alone was used as eluent for the SPE column, the subsequent recoveries of ASA and SAL in aqueous solution were found to be very low (66-79% and 9-10%, respectively). When eluent A i.e. water-OPA [1000:1, v/v] was used for this step, the recoveries of ASA and SAL improved to 99-100% and 98-100%, respectively (Figure 4.3). This is probably because both ASA and SAL, as weak acids, will interact with the hydrophobic SPE column to a greater extent in an acidic environment.

4.4.4.3 Choice of Dimensions of SPE Column

Two dimensions of cartridge were assessed : 10 x 4.3mm and 10 x 2.0mm. The smaller column had approximately 37% the capacity of the larger column for a sample of ASA and SAL containing 0.2μg/ml of each (see Figure 4.4). This was not adequate for the column-switching SPE-HPLC system, so the larger of the two sizes was used for the remainder of the work.

Figure 4.3 : Effect of Acidic Wash Solvent on Recovery of ASA and SAL

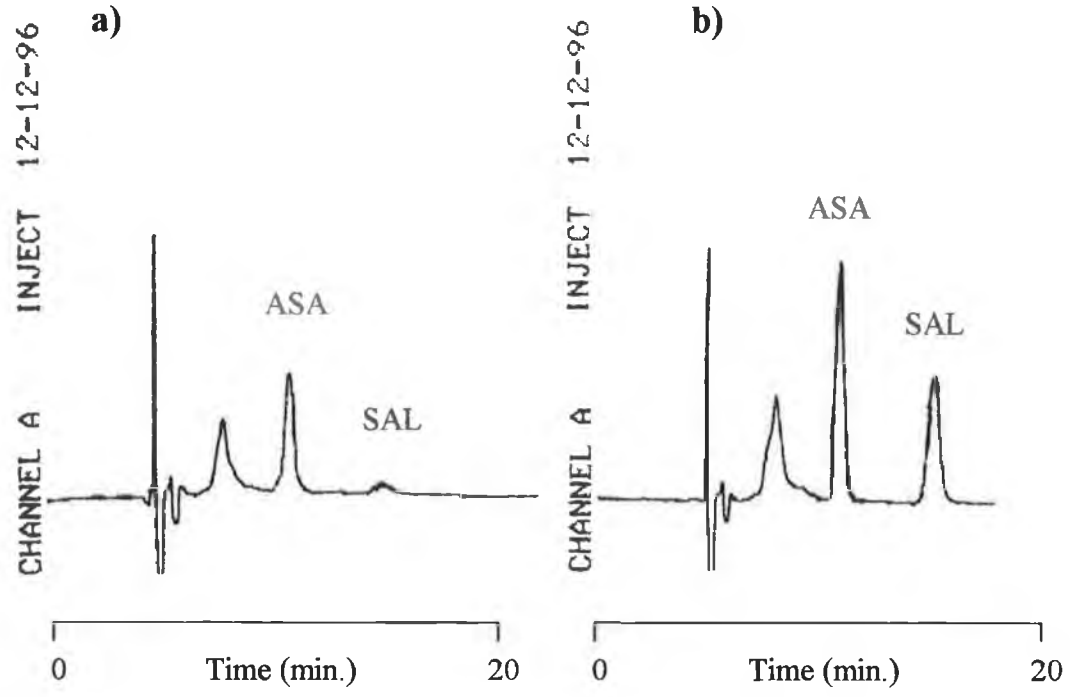


Figure 4.3 : Chromatogram a) shows the poor recoveries of a 2μg/ml sample of ASA and SAL when a water wash was used during the 2 min. SPE step, and b) shows the same sample when an acidic wash solvent was used (water-OPA, 1000:1, v/v) with a pH of 2.5.

4.4.4.4 Choice of Injection Loop Size

Two sizes of injection loop were investigated in this project. Initial experiments were carried out on the 20μl loop but since a larger sample volume was required, for the purposes of increasing sensitivity 10-fold (in theory), a 200μl loop was evaluated under the same conditions. To investigate if there would in fact be a 10-fold increase in sensitivity, a 2μg/ml aqueous sample of ASA and SAL was injected onto the 20μl loop (chromatogram a) and the peak areas compared with those obtained by injection of a 0.2μg/ml sample (chromatogram b) and a 2μg/ml sample onto a 200μl loop (chromatogram c). The peak area results from Figure 4.5 showed that the recoveries of ASA and SAL in chromatogram b were 93.6% and 84.5%, respectively when compared to chromatogram a. It was found from the peak areas in chromatogram c that the use of the 200μl loop afforded sensitivity increases by a factor of 8.85 for ASA and 8.20 for SAL, a little less than the theoretical increase expected. This can be

explained due to a small amount of band broadening evident in the peak shape, which would not be unexpected when changing from a small volume 20µl loop to a large volume 200µl loop.

Figure 4.4 : Effect of Type and Dimensions of SPE Column on Recovery of ASA and SAL from Plasma

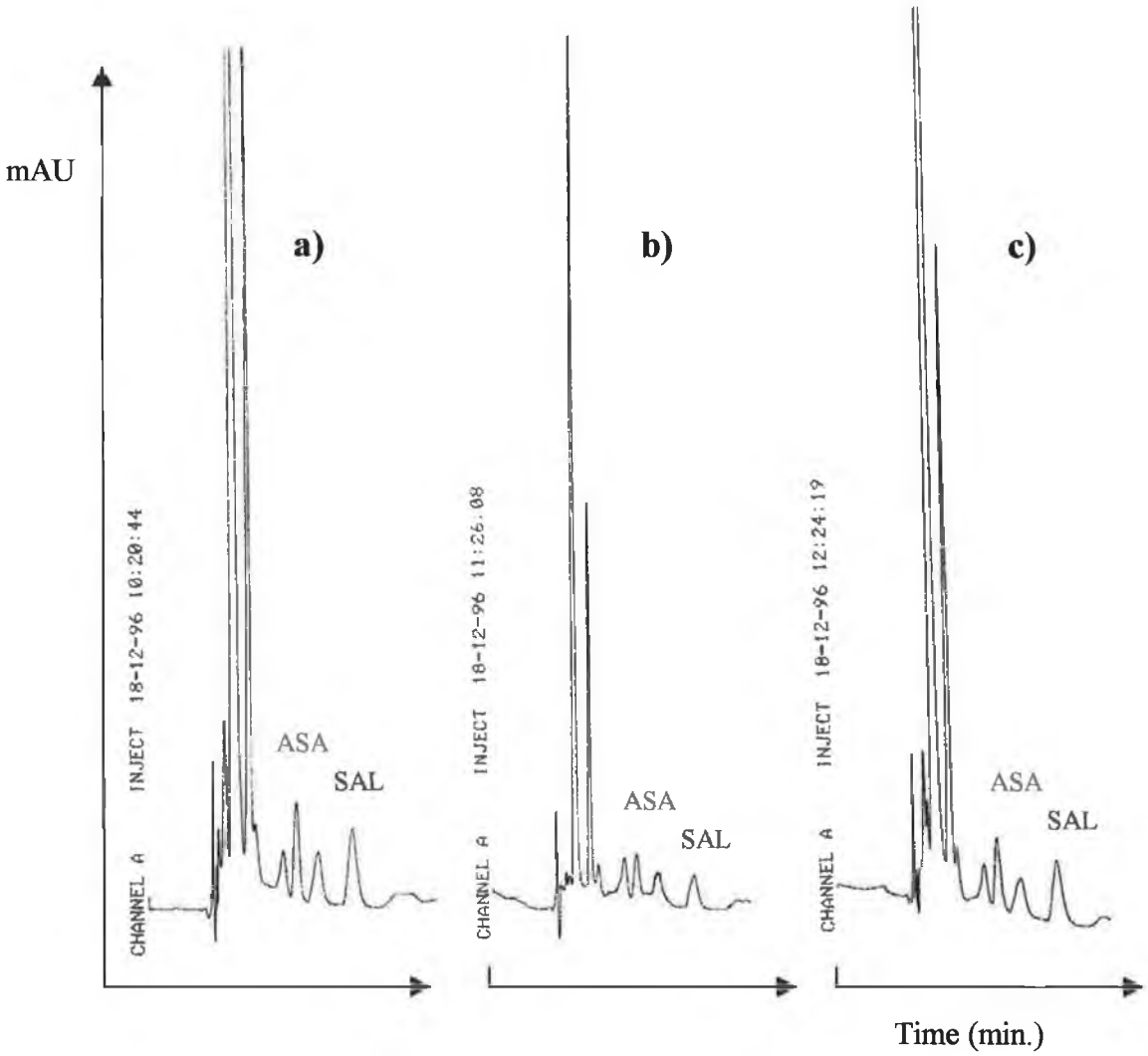


Figure 4.4 : Chromatogram a) shows the good recoveries of a 0.2µg/ml sample of ASA and SAL when the self-pack C18 cartridge was used (10 x 4.3mm) for the 2 min. SPE step, b) shows the same sample when the self-pack C18 cartridge was used (10 x 2mm) with 37% recoveries and c) shows the same sample when a commercial C18 cartridge was used (10 x 4mm) with 65% recoveries of the two analytes. Each of the time axes represents 20 minutes from beginning (injection) to end of the arrow.

Figure 4.5 : Effect of Loop Volume on Sensitivity

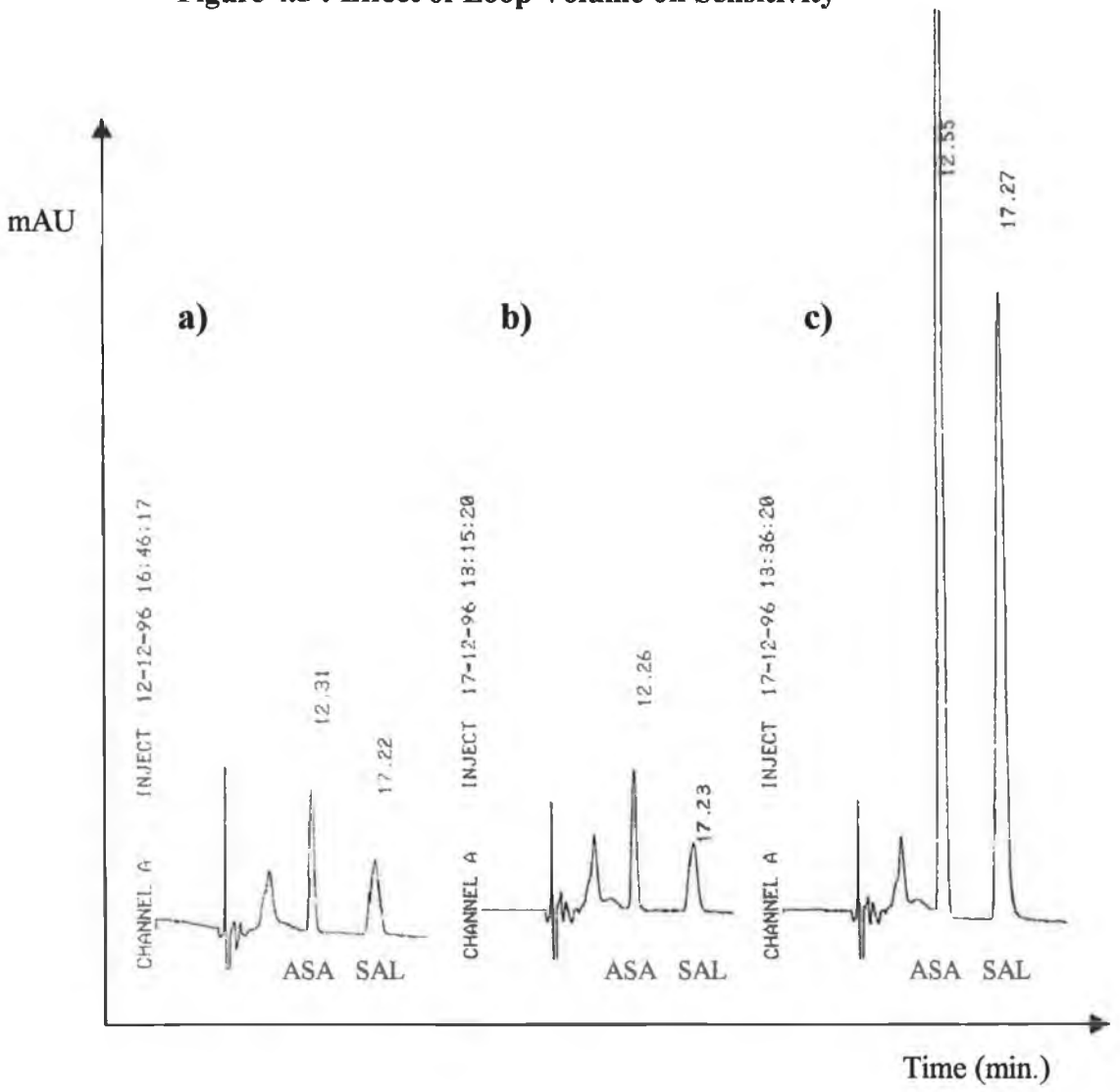


Figure 4.5 : The chromatograms show injections of a) 2μg/ml sample of ASA and SAL using a 20μl loop, b) 0.2μg/ml sample using a 200μl loop and c) 2μg/ml sample using a 200μl loop. Results described *in* text.

4.4.5 Calibration and Calculation

Spiked plasma samples were used for calibration, validation and quantitation. Calibration was based on unweighted linear regression analysis of concentration versus peak areas (which were found to correlate better than peak heights with concentration). Calibration curves were linear in the concentration ranges 0.10-5.00 μg/ml for ASA and 0.25-15.00 μg/ml for SAL with all r values > 0.999.

4.4.6 Precision

Precision was defined in terms of the variability between batches (inter-assay) and within batches (intra-assay). Inter-assay variation (reproducibility) was assessed in four replicate runs on four consecutive days, covering the concentration range 0.1 to 5.0μg/ml for ASA and 0.25 to 15.0μg/ml for SAL. Intra-assay variability (repeatability) was determined in quadruplicate on the fourth day of the study. The precision of the method was described by the mean RSD of the recovered amounts, determined by interpolation of the peak areas on the regression lines. For reproducibility, the interpolations were based on the four individual regression lines generated from the four replicate runs. For repeatability, the interpolations were based on a single regression line based on the mean peak areas of the quadruplicate run. Precision data (Table 4.2) demonstrates that the reproducibility (mean RSDs = 3.83% and 4.76% for ASA and SAL, respectively) and repeatability (mean RSDs = 2.58% and 2.56% for ASA and SAL, respectively) are well within accepted values for clinical analyses.

4.4.7 Accuracy

Accuracy (presented in Table 4.2), as defined as the percentage difference between the nominal amount and the amount found, by back-calculation, gave mean between-batch values of 2.21% and 1.27% for ASA and SAL, respectively and mean within-batch values of 3.06% and 2.57% for ASA and SAL, respectively.

Table 4.2 : Precision and Accuracy Data

Intra-assay (repeatability) n=4

For ASA : y = -11914 + 513530x

Amount added ASA (μg/ml)	Amount found ASA (μg/ml)	RSD (%)	Accuracy (%)
0.10	0.091±0.004	4.28	9.00
0.25	0.235±0.005	2.29	6.00
0.80	0.799±0.023	2.90	0.13
2.00	2.003±0.037	1.85	0.15
5.00	5.000±0.078	1.56	0.00

For SAL : y = 69807 + 458990x

Amount added SAL (µg/ml)	Amount found SAL (µg/ml)	RSD (%)	Accuracy (%)
0.25	0.239±0.006	2.58	4.40
0.75	0.714±0.020	2.85	4.80
2.40	2.446±0.075	3.07	1.92
6.00	6.088±0.122	2.01	1.47
15.0	14.960±0.339	2.26	0.27

*Inter-assay (reproducibility) n=4 , * n=3*

Amount added ASA (µg/ml)	Amount found ASA (µg/ml)	RSD (%)	Accuracy (%)
0.10	0.093±0.006	6.74	7.00
0.25	0.254±0.018	7.11	1.60
0.80	0.789±0.025	3.18	1.38
2.00	1.982±0.036	1.80	0.90
5.00	5.009±0.016	0.31	0.18

Amount added SAL (µg/ml)	Amount found SAL (µg/ml)	RSD (%)	Accuracy (%)
0.25	0.238±0.024	10.12*	4.80*
0.75	0.746±0.041	5.55*	0.53*
2.40	2.412±0.109	4.52	0.50
6.00	5.972±0.186	3.11	0.47
15.0	15.008±0.077	0.51	0.05

4.4.8 Limits of Detection and Quantitation

The limit of quantitation (LOQ) in plasma was found to be 100ng/ml for each of the analytes. It can be seen in Table 4.3 that this LOQ for ASA is superior to the LOQs for previous assays in all but two cases. Brandon *et al.* obtained an LOQ of 25ng/ml for ASA but the LOQ for SAL in the assay was 0.5μg/ml[15]. A similar situation arose for Buskin *et al.* with an LOQ of 50ng/ml reported for ASA and 0.5μg/ml for SAL[23]. Certain validation parameters were carried out at the LOQ in the same way as for the calibration curve. The reproducibility at the LOQ (mean RSDs = 4.0% and 14.3% for ASA and SAL, respectively) and repeatability at the LOQ (mean RSDs = 18.4% and 11.7% for ASA and SAL, respectively) were acceptable. Accuracy results were expressed as relative recovery values at this concentration i.e. against aqueous spiked samples at the same concentration. Inter-day recoveries at the LOQ were found to be 99.3% and 84.4% for ASA and SAL, respectively, and intra-day recoveries were found to be 116.2% and 91.9% for ASA and SAL, respectively. The limit of detection (LOD) in plasma was found to be 50ng/ml for each of the analytes. In aqueous samples, the LOD values were 10 and 20ng/ml for ASA and SAL, respectively.

4.4.9 Recovery

Both absolute and relative recoveries were obtained for this method. Absolute recovery is defined as the response of a processed, spiked matrix standard expressed as a percentage of the response of pure standard which had not been pretreated[24]. Two concentrations of each compound were chosen for examination (0.5 and 5.0μg/ml) and the absolute recoveries were calculated by comparing the response in the spiked plasma samples to the response in aqueous samples injected directly onto the analytical column i.e. without SPE and column-switching. Absolute recoveries for ASA were found to be 99 and 100% at 0.5μg/ml and 5.0μg/ml, respectively; for SAL these results were 104 and 101%, respectively. Relative recovery was defined as the response of extracted analyte measured from matrix i.e. plasma, as a percentage of analyte extracted from water[24]. The relative recovery for this study was calculated for both analytes at both concentrations in two different plasmas on two different days. Relative recoveries for ASA were calculated to be 101 and 94% at 0.5μg/ml and 5.0μg/ml, respectively; for SAL these results were 88 and 90%, respectively.

Table 4.3 : HPLC Assays for Aspirin and/or Salicylic Acid in Plasma

Reference & Year	Sample Preparation	Linear range (µg/ml)	LOQ or LOD (µg/ml)	Recovery	Internal Standard
This work 1997	Direct injection	0.10 - 5 (ASA) 0.25 - 15 (SAL)	0.1 (LOQ ASA) 0.1 (LOQ SAL)	99-100% ASA 101-104% SAL	No
[9] 1996	ACN deproteinisation	0.1 - 200 (SAL)	0.2 (LOQ SAL)	> 85% SAL	Yes
[10] 1996	ACN deproteinisation Aqueous fraction separated with NaCl	0.2 - 20 (ASA) 0.5 - 50 (SAL)	0.1 (LOQ ASA) 0.1 (LOQ SAL)	106.8% ASA 121.7% SAL	Yes
[12] 1996	Ethanol deproteinisation Supernatant diluted in mobile phase	4 - 4000 (SAL)	0.5 (LOD SAL)	96.2% SAL	Yes
[16] 1992	Dichloromethane extraction	20 - 400 (ASA) 100 - 500 (SAL)	0.02 (LOD ASA) 0.02 (LOD SAL)	100.7% ASA 80.1% SAL	Yes
[22] 1990	Ethyl ether/hexane extraction Back extracted into phosphate buffer	0.05 - 200 (ASA) 0.10 - 200 (SAL)	0.05 (LOD ASA) 0.10 (LOD SAL)	92 - 100%	Yes
[20] 1987	Hexane extraction	0.1 - 20 (ASA) 0.1 - 20 (SAL)	0.1 (LOD ASA) 0.1 (LOD SAL)	27±3% ASA 54±2% SAL	Yes

[15] 1985	Ethyl ether extraction	0.025 - 10 (ASA) 0.5 – 70 (SAL)	0.025 (LOQ ASA) 0.5 (LOQ SAL)	96.4-100.3% ASA 76.7 – 91.2% SAL	Yes
[17] 1984	Dichloromethane extraction	0.2 - 100 (ASA) 0.2 - 100 (SAL)	0.2 (LOQ ASA) 0.2 (LOQ SAL)	92% ASA 85% SAL	Yes
[13] 1984	Perchloric acid-methanol deproteinisation	1 - 500 (ASA) 1 - 500 (SAL)	0.3 (LOD ASA) 0.2 (LOD SAL)	80-86% ASA 80-86% SAL	Yes
[19] 1983	Ethyl ether/hexane extraction	0.1 - 10 (ASA) 0.1 - 40 (SAL)	0.05 (LOD ASA) 0.05 (LOD SAL)	93-95% ASA 88-89% SAL	No
[19] 1982	Ethyl ether/hexane extraction Back extracted into phosphate buffer	0.05 - 10 (ASA) 0.5 - 100 (SAL)	0.05 (LOQ ASA) 0.5 (LOQ SAL)	98.6-107.7% ASA 95.6-98% SAL	Yes
[14] 1981	Perchloric acid-methanol deproteinisation	0.5 - 200 (ASA) 0.5 - 200 (SAL)	0.1 (LOD ASA) 0.1 (LOD SAL)		Yes
[19] 1980	Chloroform extraction	0.5 - 20 (ASA) 0.5 - 100 (SAL)	0.5 (LOD ASA) 0.5 (LOD SAL)	> 95% ASA 70% SAL	Yes
[11] 1980	ACN deproteinisation Supernatant used for injection	2 - 30 (ASA) 5 - 100 (SAL)	2.0 (LOQ ASA) 5.0 (LOQ SAL)	98 - 107% ASA 93 - 116% SAL	No
[18] 1980	Dichloromethane extraction	2 - 100 (ASA) 1 - 150 (SAL)	1.0 (LOD SAL)		Yes

When looking at previous work in Table 4.3, it would appear that no one assay obtained recoveries that were as quantitative for both analytes as those reported in this work. This is probably best explained by the fact that there is little sample loss in the column-switching on-line SPE LC system since once the sample is injected and the SPE conditions have been optimised to retain the analytes, the entire sample volume remains in the closed system for extraction and analysis. This is in sharp contrast with manual extraction procedures where losses can occur in many areas e.g. liquids adsorbing onto glassware, sample lost during transfer from vessel to vessel, losses due to sublimation, losses during evaporation and reconstitution of the sample; losses are also possible during protein precipitation if coprecipitation of any of the analytes occurs.

4.4.10 Selectivity

According to Karnes *et al.*[24] and Shah *et al.*[37], the matrix should not interfere with the analysis of the compounds and this should be demonstrated in six different sources. Hence, plasma from six different individuals was examined for endogenous interferences and in no case were any interferences found that coeluted with ASA. In some sources of plasma, a small endogenous peak was found that had the same retention time as SAL. However, its response always corresponded to a concentration of less than the LOD of SAL. A number of commonly coadministered and/or available drugs were also investigated for possible coelution with either ASA or SAL. These are listed in Table 4.4. Only xylazine (which has mainly veterinary uses) and prazosin were found to interfere.

4.4.11 Stability

Kees *et al.*[10] found that a 1mg/ml solution of ASA in acetonitrile was stable for at least one month (at 4°C). This study found that the same concentration of ASA in acetonitrile was stable for at least 10 weeks at room temperature *or* in the fridge.

Various aspects of the sample handling procedures prior to extraction of the plasma samples and standards were also investigated with a view to minimising degradation due to *in vitro* hydrolysis of ASA. Four plasma samples (each containing 2μg/ml ASA) were subjected to different treatments. Sample 1 was acidified 1:1 with 0.2M OPA and held on ice prior to injection, sample 2 was acidified 1:1 with acid and maintained at room temperature, sample 3 was diluted 1:1 with water and held on ice,

Table 4.4 : Interference Testing

Drugs Tested for Interference	
Barbitone	Imipramine
Butobarbitone	Nitrazepam
Caffeine	Phenytoin
8-chlorotheophylline	Pindolol
Clonazepam	Prazosin
Cocaine	Propranolol
Diazepam	Quinidine
Flurazepam	Theophylline
Furosemide	Xylazine
Hydralazine	

sample 4 was diluted with water and held at room temperature. Upon analysis three hours later, it can be seen from the chromatograms in Figure 4.6 that sample 1 had the smallest peak for SAL, indicating the least hydrolysis. The amount of degradation (as evidenced by the hydrolysis of ASA to SAL) increased from sample 1 to 4, showing that acidifying the sample enhances the stability to a greater extent than cooling on ice. In conjunction with fluoride in the anticoagulant to inhibit enzymatic decomposition of ASA, all samples were acidified and kept chilled in order to minimise hydrolysis of ASA, hence ensuring sample integrity. Stability of ASA and SAL in the spiked, acidified samples was then investigated during storage. The results of this work concluded that the levels of ASA and SAL in these samples remained constant when stored at room temperature for up to 24 hours, at 4°C for up to 48 hours and at -30°C through two freeze-thaw cycles. After these lengths of time, the levels of endogenous interferences had increased to such an extent that quantitation of the ASA and SAL peaks was not possible.

Figure 4.6 : Effect of Handling Conditions on Stability of ASA in Plasma

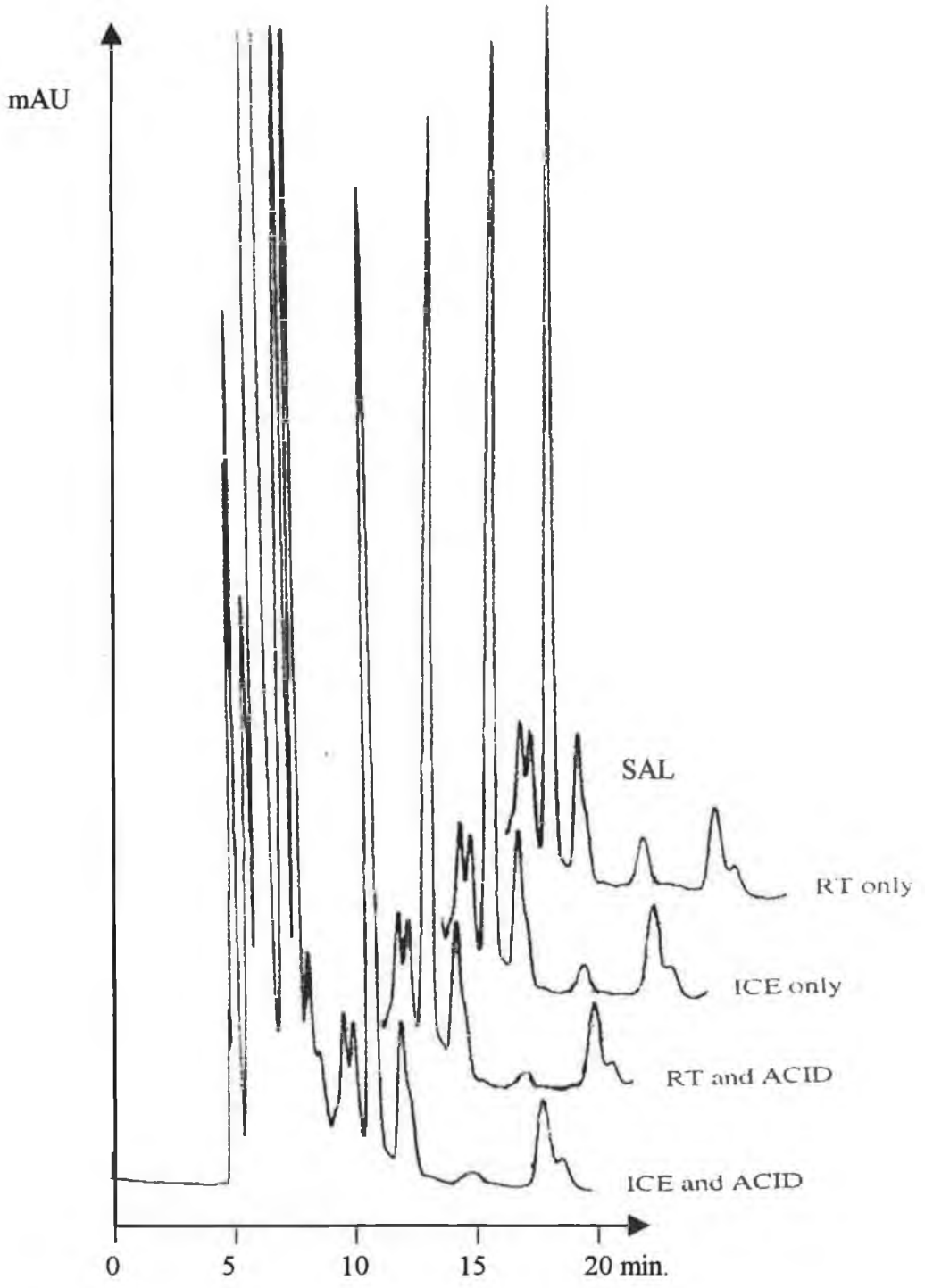


Figure 4.6 : Chromatograms showing the effect of handling conditions on the level of hydrolysis of ASA to SAL. Sample 1 : plasma acidified 1:1 with OPA and held on ice, Sample 2 : plasma acidified 1:1 with acid and held at room temperature, Sample 3 : plasma diluted 1:1 with water and held on ice, Sample 4 : plasma diluted 1:1 with water and left at room temperature. Chromatographic conditions as per text.

4.4.12 Ruggedness

Using an aqueous solution containing 2μg/ml of both analytes, a number of parameters were examined to demonstrate the robustness of the method. Normal retention times for ASA and SAL were 11.5±0.2 and 15.6±0.3 minutes, respectively. Changes of ±10% to the flow rate through the extraction column had no effect on the peak areas obtained. Increasing the flow rate by 10% through the analytical column had the effect of decreasing the peak areas by 10% as would be expected, and decreasing the flow rate by 10% had the effect of increasing the peak areas by the same amount, also as expected. The washtime of the sample through the SPE column was incremented by ±5% and ±10% and this was shown not to affect the peak areas. Changes of ±5% of the acetonitrile content of the mobile phase had more of an effect on the retention times (±0.4 mins for ASA and ±0.6 mins for SAL) than the same changes in the methanol content. However, neither significantly affected the size of the peak areas. To adjust the normal pH of the mobile phase by 0.1 pH unit, it was necessary to increase or decrease the volume of OPA by 20%. The retention time for SAL, as it is more pH sensitive, moved slightly i.e. +0.1 mins at pH 2.5* and -0.3mins at pH 2.7*.

When the C18 extraction column was changed after approximately 50 plasma injections, the new C18 column yielded no difference in the recoveries of both components. Guard column changeover also had no effect on the chromatography.

4.4.13 Quantitative Analyses of Samples

One healthy male volunteer was given 600mg aspirin (in the form of two tablets) 45 minutes after he had eaten a meal. Blood samples were taken just before the administration of ASA and then at a number of time points over seven hours, and subjected to extraction and analysis. Some of the resultant chromatograms are shown in Figure 4.7.

The results were plotted on a concentration-time curve (Figure 4.8) and from this, the peak plasma concentrations of ASA and SAL were determined to be 6.2 and 27.1 μg/ml, respectively. The times taken to reach these peak concentrations were 33 and 60 min., respectively. These results are in general agreement with those found in the literature, as can be seen in Table 4.5, the only difference being that SAL reaches its peak concentration faster than would be expected. However, the fact that rates of metabolism vary widely among individuals probably accounts for this discrepancy.

Figure 4.7 : Chromatograms of Plasma from a Volunteer before and after Administration of 600mg ASA

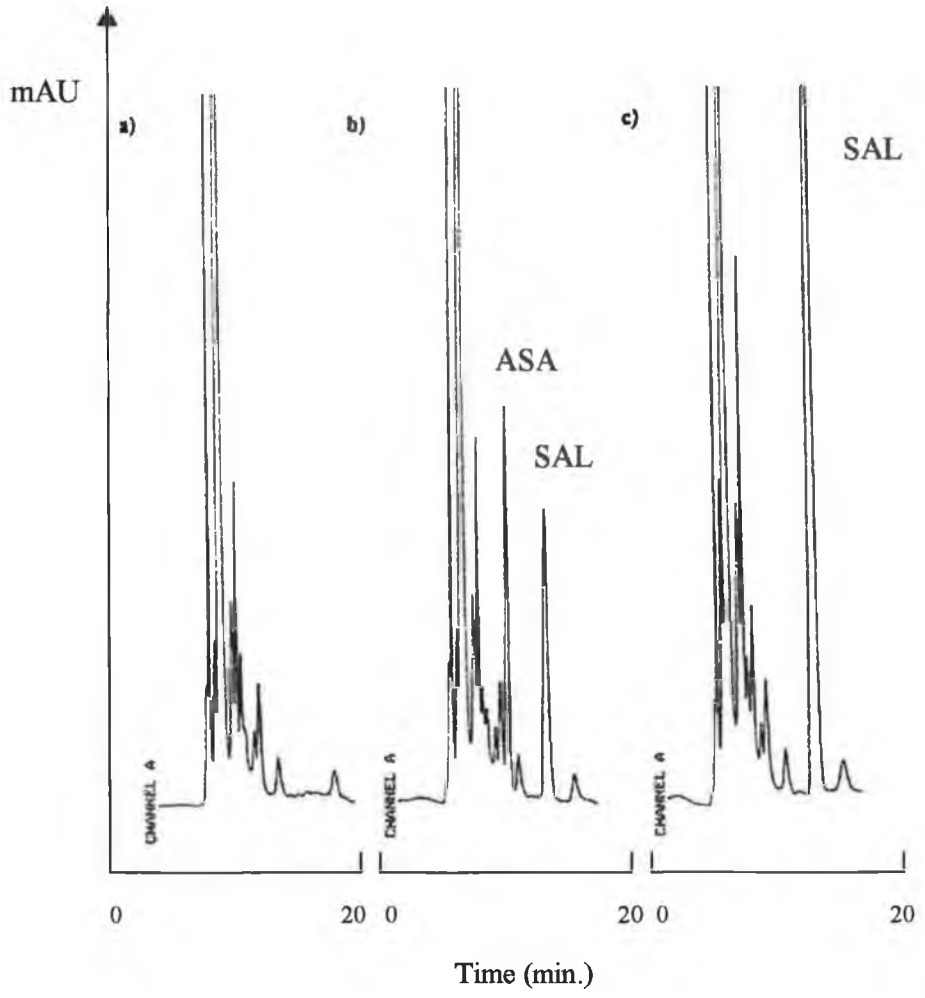
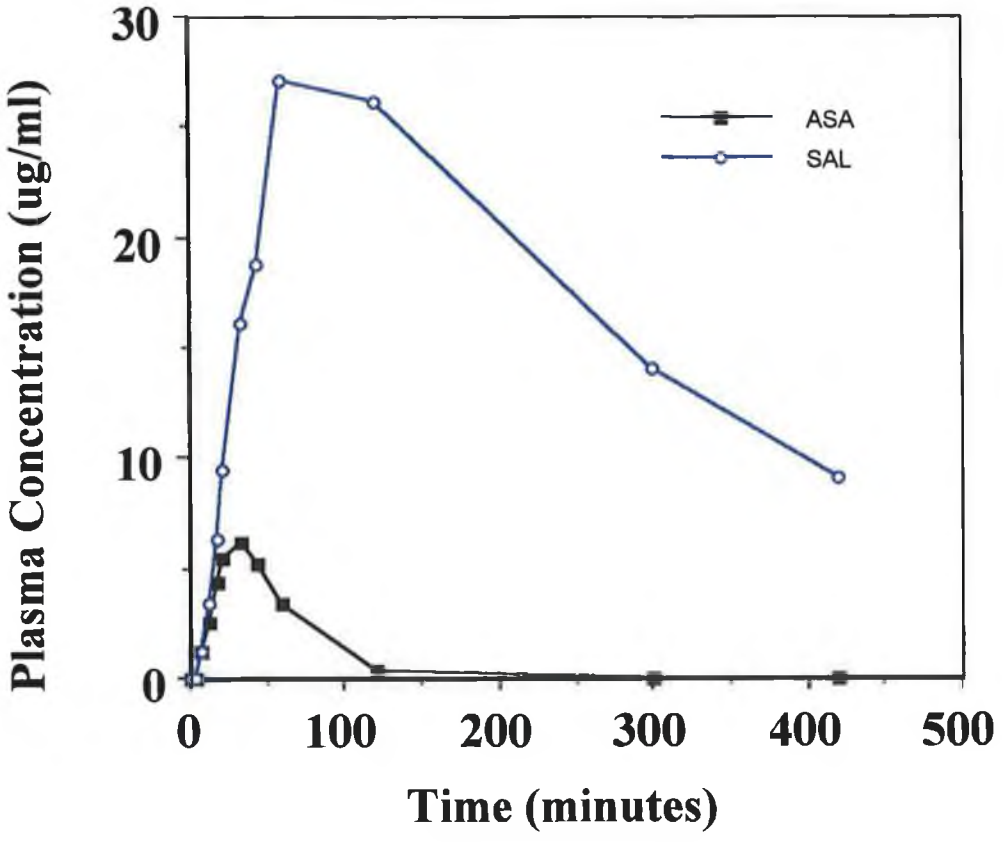


Figure 4.7 : a) Plasma from volunteer immediately prior to administration of ASA b) Plasma sample taken 13 mins after administration of ASA, concentration of ASA = 2.55µg/ml and concentration of SAL = 3.43µg/ml c) Plasma sample taken 5 hours after administration of ASA, concentration of SAL = 13.98µg/ml. Chromatographic conditions as per text.

Table 4.5 : Results of Concentration-Time Curves from other Studies

Reference & year	Amount of ASA given	ASA Peak Concentration (μg/ml)	SAL Peak Concentration (μg/ml)	Time to reach ASA Peak Conc. (mins)	Time to reach SAL Peak Conc. (mins)
This work	600mg	6	27	33	60
[10] 1996	500mg	5	30	30	130
[15] 1985	600mg	8	40	30-40	90-100
[19] 1982	650mg	8	40	20	80
[19] 1980	600mg	14	45	22	80

Figure 4.8 : Concentration-Time Curve of ASA and SAL in a Male Volunteer following Oral Administration of 600mg ASA



4.5 CONCLUSIONS

A new column-switching method has been developed for the analysis of aspirin and salicylic acid in plasma by on-line SPE in conjunction with HPLC. Only 300μl of plasma is required for analysis and the method could be easily automated via the 10-port switching valve. The procedure is easier to execute and requires less sample handling than methods currently described in the literature. The method is linear over the concentration range 0.10-5.00 μg/ml for aspirin and 0.25-15.00 μg/ml for salicylic acid. Both compounds have a limit of quantitation of 0.1μg/ml and a limit of detection of 0.05 μg/ml. Total analysis time is less than 20 minutes. Extensive stability work has been carried out and precautions taken to minimise *in vitro* hydrolysis of aspirin to salicylic acid ensures the integrity of the plasma sample. Validation of the method shows it to be reproducible and repeatable. Excellent recoveries from plasma obviate the need for an internal standard. It has been successfully applied to the investigation of the levels of aspirin and salicylic acid in a healthy, non-fasting volunteer following a 600mg oral dose of aspirin.

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Chapter 5

Identification and Qualitative Analysis of Protein-Mobilising Factors in the Urine of Cancer Patients with Cachexia

5.1 INTRODUCTION

5.1.1 Biological Background

The term cachexia is derived from the Greek words *kakos* meaning 'bad' and *hexis* meaning 'condition'. Cachexia is an important manifestation of clinical disease most commonly seen in patients with cancer and AIDS. In cancer patients, it is characterised by anorexia, weight loss, anaemia and muscle weakness. Although most commonly associated with the final stages of cancer, cachexia may present itself even before the other signs and symptoms of malignancy appear[1]. This condition is more prevalent in patients with certain types of tumours, especially solid tumours such as those of the pancreas, stomach, colon and lung.

Cachexia is a wasting condition in which the patient has a marked and progressive loss of body mass, especially cardiac and respiratory muscle[2] and adipose tissue. The weight loss that occurs in cancer patients with cachexia is very different to the type of weight loss that occurs in cancer patients or otherwise healthy subjects with anorexia. In starvation or anorexia, more than three quarters of the weight loss arises from fat losses and only a small amount from muscle, due to the fact that the body tries to conserve its muscle mass[3]. However, in cancer cachexia, there is equal loss from both fat and muscle[4]. Although cancer patients typically have an elevated resting energy expenditure[5,6] and a lower nutrient intake[7], these reasons alone do not account for the extent of the weight loss[8,9]. In fact, loss of both muscle and adipose tissue has been reported to occur before the fall in food intake[10]. The fact that cancer patients have been reported to have increased resting expenditure even before the onset of weight loss[5] suggests that this elevated metabolic rate may be a contributing factor rather than a consequence of the condition. Protein synthesis is also known to be reduced in cachexia[11] but it is in fact, the accelerated protein breakdown which is largely responsible for the significant muscle wasting[12].

Cachectic cancer patients exhibit a reduced response to chemotherapy[13] and a decreased survival rate[14]. The degree of weight loss is inversely proportional to life span[14]; moreover, this progressive loss of host cell body mass may be the actual cause of death in up to two thirds of cancer patients[15]. Patients who do not lose weight survive approximately twice as long as patients with the same type of cancer who are

not cachectic[13]. A 30% loss of body mass is generally considered to be fatal but there have been rare cases where patients have survived with up to 50% weight loss[14].

This wasting seems to be mediated by a circulatory catabolic factor (which in some respects is believed to act similarly to a hormone) produced by the tumour. Thus, in theory, reversal of cachexia should be achievable by blocking the action of this factor. Several candidate compounds have been proposed as the factor that triggers cachexia in cancer patients based on observations of the unusual metabolic occurrences before and during weight loss in cachexia.

One compound that has been implicated in signalling the onset of cancer cachexia is tumour necrosis factor-α[16] (TNF-α), since it has been detected at high levels in certain groups of cancer patients[17]. However, several studies failed to detect *any* increase in TNF-α in cachectic cancer patients[18]. Also, in animal models, it was found that the weight loss was more likely to be due to toxic effects[19]. Interleukins one[20] and six[21] have also been suggested as the elusive cachectic factors but while evidence shows them to be involved, they are not believed to be causative. Several clinical studies have reported the increased mobilisation of fatty acids before weight loss begins, suggesting the production of lipid-mobilising factors (LMFs) by the tumour[22]. This mobilisation of fats may be an important factor contributing to tumour growth and some fatty acids have been found to directly stimulate tumour growth *in vivo*[23] and *in vitro*[24]. The first indications that the LMF might cause cachexia were seen when serum from mice bearing a lymphoma was injected into healthy mice, producing massive fat loss[25]. Other studies showed that the level of LMF in the sera of cancer patients was proportional to the extent of weight loss[26] and was then reduced in patients responding to chemotherapy[27]. Most studies provide evidence that the LMF is an acidic protein but there are many variations in its reported molecular weight (6kDa[28] to 75kDa[29]).

Recently there has been a great deal of evidence to support the fact that the material is actually a protein-mobilising factor (PMF). This product was first seen in 1991 in human serum when a study of cancer patients with weight loss displayed PMFs *in vitro*; the factor was not found in healthy subjects[30]. The PMF has also been found in the serum of animals bearing the MAC16 tumour with weight loss[31]; the factor was not present in the serum of mice bearing the similar but non cachexia-inducing MAC13 tumour[32]. In 1996, splenocytes from mice bearing the MAC16 tumour were fused with mouse myeloma cells to produce hybridomas, which were

used to produce monoclonal antibodies against the PMF[33]. The PMF material was purified from both murine tumour and human urine sources by use of affinity chromatography using the antibody, and appeared to be of identical composition in both cases (24kDa, acidic material). The conservation in structure of the PMF between murine and human sources suggests that production of a PMF may be important in the growth and survival of some tumours. Intravenous administration to mice of affinity-purified material from the tumour (30μg protein) or the 24kDa material (30ng) produced rapid weight loss, which could be prevented by prior administration of the monoclonal antibody[34]. Furthermore, it was reported by Todorov *et al.* that material of molecular weight (MW) 24kDa had been isolated from the urine of patients with cancer cachexia (if their weight loss was greater than 1.5kg per month), but not from the urine of healthy subjects, patients with weight loss due to trauma, or patients with cancer but no cachexia[34]. Thus, the evidence that the cachectic factor is a PMF has been compelling.

The structural features of this PMF as elucidated thus far are as follows. This factor is believed to have a total mass of 24kDa and consists of a central polypeptide chain (~2kDa) of unique amino acid sequence[34]. The polypeptide chain is phosphorylated, and is also extensively glycosylated at both asparagine and serine residues[35], giving it a total MW of 4kDa. There are two sulphated oligosaccharide chains attached to the central polypeptide, one is O-linked (MW 6kDa) and one is N-linked (MW 10kDa). These carbohydrate chains are also sulphated. It was also shown that the N- and O-linked sulphated oligosaccharide chains were both the antigenic and the biological determinants. The PMF also binds strongly to albumin producing a species of ~ 69kDa[33]. Because the PMF did not appear to contain glycosaminoglycan chains attached to either serine or asparagine residues, it was therefore deemed to be a sulphated glycoprotein rather than a proteoglycan. The fact that the cachectic factor is a glycoprotein is quite significant because these proteins are known to play a role in cellular adhesion and contact inhibition of cell growth in culture. They are also responsible for cellular recognition of foreign tissue and may be involved in tumour-specific antigenic activity.

As distinct from anorexia, attempts to halt or reverse cachexia by nutritional repletion have not been very successful. It has been found that it is not possible to reverse the physical composition changes seen in patients with cancer cachexia by the provision of extra calories or by dietary counselling[36]. Limited benefits have been

seen with the use of two groups of agents a) those stimulating food intake e.g. megestrol acetate[37] or hydrazine sulphate[38], and b) those directly inhibiting the PMF e.g. eicosapentaenoic acid (EPA)[39]. However, while megestrol acetate has been shown to improve appetite and food intake leading to weight gain in some cancer patients, studies have also shown that host weight gain has been associated with a doubling of the weight of the tumour[40]. Hence there is optimism that the second approach may have better success[41], and EPA has recently been initiated in clinical trials for patients with pancreatic cancer.

Weight loss in cachexia is not only associated with psychological distress and a lower quality of life, but through its serious effects on the cardiac and respiratory muscle, it can be the direct cause of death of the patient. Hence, any treatment regimen for the cachectic patient will have enormous benefits in terms of their general well-being, their response to chemotherapy and their overall survival time. In addition, since some tumours may depend on the products from host tissue catabolism for survival, such therapies may also have anti-tumour effects. However, more information about its structure, the circulating levels of it in the body and its exact role in mediating cachexia is required.

The aim of this project was to determine the presence of the PMF in the urine of cancer patients with cachexia using capillary electrophoresis (CE). The examination of human urine by CE for changes in levels of certain proteins is used as a diagnostic indicator for many disease states. Some of these assays include the monitoring of oestrogens for pregnancy and reproductive disease[42], glycosidase enzymes for renal disorders[43] and imidodipeptides in prolidase deficiency[44].

5.1.2 Analytical Background

Analysis of glycoproteins in biological matrices can be achieved by CE, which lends itself more favourably to this task when compared to the technique of HPLC. This is because proteins have many charged moieties enabling their separation in the electric field provided by CE. However, because proteins tend to adhere to a variety of surfaces, especially silica, this is an important consideration when using CE for protein analysis. If precautions are not taken, proteins can build up on the capillary wall causing rapid degradation of the CE separation. There are two approaches to avoiding this problem.

The first approach involves surface modification of capillaries for the prevention of these interactions. Chemically coating the surface converts it to a more neutral and less adsorptive one. Strege *et al.* resolved five model proteins with a wide range of ionic and hydrophobic character with high migration time reproducibility in C18-derivatised capillaries[45]. The coated capillaries were found to exhibit greater precision than bare silica capillaries in this work. Polymeric coatings generally demonstrate greater stability than non-polymeric coatings, hence many of the chemical derivatisation reactions of capillaries for CE over the past few years incorporate polymer chemistry[46]. Poly(acrylamide)-coated capillaries were used to simultaneously separate acidic and basic proteins with high resolution[47]. Gilges *et al.* used poly(vinylalcohol) to both permanently and dynamically coat silica capillaries in their analysis of highly charged proteins. They used a thermal immobilisation process for the permanent coating[48]. Other types of polymer coatings employed in the determination of proteins by CE are hydrogels which swell in aqueous environments, eliminating the interaction of large molecules with the surface[49], and hydrophilic polymers e.g. dextran, which are reported to have excellent stability in the presence of detergents and at extremes of pH[50]. However, there are disadvantages associated with the use of coated capillaries such as batch-to-batch variability[50,51] and poor stability over wide pH ranges. Irreproducibility of coated capillaries is thought to be due to the differences among silanisation reagents, the presence of water in the reagents which can facilitate unwanted polymerisation, and undesirable polymerisations taking place, catalysed by the byproducts of silanisation[51].

The second approach in minimising protein interactions with the silica surface involves using particular buffers and/or the addition of a modifier to the run buffer for CE analysis. CZE was used for the separation of insulin and its deamidation products in an untreated capillary by using zwitterions and acetonitrile in the migration buffer[52]. Under these conditions, reproducibility of the runs was high and rinsing of the capillary with base between runs was unnecessary. Hydroxypropylmethyl cellulose was incorporated into an acidic phosphate buffer for the determination of histone variants and their derivatives on a fused silica capillary[53]. The presence of the additive eliminated interactions of the basic proteins with the capillary wall. Some examples of relevant protocols that employed ordinary fused silica specifically for glycoproteins are given in Table 5.1 below. In these cases, borate buffer is usually used for glycoproteins since it can dynamically interact with the diols of the

carbohydrate groups, affording improved separating ability[54,55,56]. The presence of carbohydrate groups on the PMF being investigated in this project meant that the use of borate buffer would aid in its detection by CE methods.

Table 5.1 : Some Methods for the Analysis of Glycoproteins by CE using Uncoated Fused Silica Capillaries

Analyte & Matrix	Run Buffer	Comments	Reference
Glycoproteins in ovalbumin mixture	Borate buffer with 1mM putrescine	5 major protein peaks resolved in 30 min.	Landers, 1992 [55]
Ribonuclease B glycoforms	Borate buffer	5 glycoform populations found	Rudd, 1992 [56]
Plasminogen activator, fetuin and α1-acid-glycoprotein	Tricine buffer (pH 8.2) with 1.25mM putrescine	High resolution of the glycoforms was obtained within 20 min.	Taverna, 1992 [58]
Glycoforms of recombinant erythropoietin	Putrescine and urea added to run buffer	The additives caused the EOF to decrease and the resolution to increase	Watson, 1993 [59]
Peptide map of erythropoietin	Phosphate buffer (pH 2.5) with 100mM heptane-sulphonic acid	The heptane-sulphonic acid also functioned as an ion-pair reagent	Rush, 1993 [57]

Sometimes a modifier is added to the buffer to enhance separation of glycoproteins, the most popular of which is putrescine (1,4-diaminobutane). Putrescine has been used in the run buffer to improve the resolution of a mixture of glycoproteins in ovalbumin[55], the carbohydrate moieties of glycoproteins[58] and the glycoforms of recombinant erythropoietin[59]. In between injections, rinsing of the uncoated capillary with strong base and/or water is carried out to ensure that there is no build-up of adsorbing protein. Some of the advantages of the use of bare silica capillaries includes the fact that batch-to-batch reproducibility is excellent, the surface is stable over a wide pH range and if blockage of the capillary does occur, aggressive cleaning with strong acids and bases is possible, while this is not the case with coated capillaries.

The objectives of this project were to identify and qualitatively determine the presence of a the PMF (and potentially its albumin-bound complex) in the urine of

cachectic cancer patients and to show it not to be present in the urine samples of healthy subjects or cancer patients who were not cachectic. This was to be achieved using the technique of CE.

5.2 REAGENTS AND MATERIALS

5.2.1 Equipment

Two capillary electrophoresis instruments were used in the course of this work. The first was from Applied Biosystems (Foster City, CA, USA), model 270A-HT and the second was from Hewlett-Packard (Palo Alto, CA, USA), model HPCE 3D with built-in diode-array detector. The high sensitivity optical cell assembly (Z-cell) for the ABI CE system was obtained from LC Packings Inc. (San Francisco, CA, USA) and the extended light path capillary (bubble cell capillary) for the HPCE was purchased from Hewlett-Packard as above.

5.2.2 Reagents and Chemicals

All chemicals, unless otherwise stated, were analytical grade and were obtained from the manufacturer at the highest available purity. Sodium hydroxide (NaOH), sodium tetraborate and boric acid were purchased from Sigma (St. Louis, MO, USA). Ammonium sulphate ($(NH_4)_2SO_4$), analytical grade, was purchased from Mallinckrodt (Holland). The model protein α1-acid-glycoprotein (AGP) of MW 44kDa and pI 2.7 was obtained at 99% purity from Sigma (cat. no. G3643, 25mg bovine AGP). All other chemicals were of analytical reagent grade. Distilled water was used throughout this work.

5.2.3 Other Materials

Uncoated fused silica was purchased from Polymicro Technologies (Phoenix, AZ, USA). The detection window was created by burning off a small section of the polyimide coating, and the excess residue was then gently wiped away with methanol. Minicon® B15 concentrators were obtained from Amicon (Beverly, MA, USA). Dialysis tubing with 12-14kDa exclusion size, ¾ inch diameter, cat. no. 15961-022, was obtained from GIBCO BRL (Life Technologies, MD, USA). The urine dipsticks

used were Bayer Multistix SG from Bayer Corporation, Diagnostics Division (Elkhart, IN, USA).

5.3 EXPERIMENTAL

5.3.1 Urine Collection

Spot urine samples were collected from patients who were on a routine check-up at the Lombardi Cancer Centre in Georgetown University, Washington DC. This study was endorsed by the Bioethics Committee. Under conditions of informed consent, urine samples were collected in 200ml plastic urine cups, labelled with relevant patient details (time of collection was referred to simply as 'morning' or 'afternoon') and were immediately placed on ice. No preservatives were used.

5.3.2 Preparation of Reagents

Stock borate buffer (100mM, pH 9.2) was prepared on a twice-weekly basis by dissolving sodium tetraborate in distilled water. It was diluted as appropriate for use. The borate buffer solution and all other aqueous solutions were filtered under vacuum through a 0.22μm filter and subjected to sonication for 20 minutes before use. The 80% w/v $(NH_4)_2SO_4$ was prepared by dissolving 8g in 10ml of aqueous sample unless otherwise stated. Solutions were stored at 4°C when not in use.

5.3.3 Preparation of Urine Samples

Within one hour of collection, the urine samples were subjected to dipstick analysis to qualitatively examine levels of various compounds in the urine i.e. glucose, ketone, blood, protein, urobilinogen, bilirubin, specific gravity, pH and nitrite. The most relevant of these measurements to this project were protein, blood and bilirubin. All results were recorded on the patient 'chart information' form (see Figure 5.1). Each of the samples were divided into two portions and labelled accordingly. One was kept at 4°C (to be used on the same day) and one was frozen at –30°C. Each urine sample (fresh or thawed from frozen) was centrifuged for 10 min. @ 2000rpm at 4°C to sediment any particulate matter, although fresh samples were usually free of such sediment. For the concentration and dialysis step, 5ml of urine was transferred into

the rinsed cell of a minicon® B15 unit using a 9 inch pasteur pipette. The cell was tapped gently to remove any air bubbles. The 5ml sample was left standing until it had been concentrated 50 times to 100µl (this usually took 1 to 2 hours), whereupon it was removed by pasteur pipette and transferred into a 1.5ml eppendorf tube. This sample could be frozen at –30°C if it was not to be used immediately, or diluted as appropriate with water whereupon it was now ready for CE analysis. The frozen extracts were stable for at least three weeks. The minicon® unit was stored at 2-4°C in the fridge until further required.

Figure 5.1 : Typical Chart Information Form

CHART INFORMATION

DETAILS TAKEN BY : Gillian Mc Mahon DATE : 3/10/97

PATIENT NAME : _____ ID NO : _____

DATE OF BIRTH : 29/3/31 ETHNIC ORIGIN : Caucasion

WEIGHT : see below HEIGHT : 5 ft BMI : —

Diagnosis : Colon Cancer Liver mets When diagnosis was made: 1996

Doctor Attending: Dr. Marshall

Treatment History: 5 FU / Leucovorin
Paxil 20mg

Current Therapy: Procardia 30 mg
Campostar

Adverse Reactions : Penicillin and Sulfa drugs

Smoke/Drink : Smoked for 15 years (not now) . Does not drink alcohol

WEIGHT CHANGE : Sept. 12 1997 180 lb Jul 31 1997 191 lb
Intentional loss of 10 lb

Urine Dipstick :

Glucose	⊖	Bilirubin	⊖
Ketone	⊖	Specific Gravity	1.01
Blood	Trace	pH	5.0
Protein	Trace		
Urobilinogen	0.2	Nitrite	⊖

5.3.4 CE Conditions for ABI System

The final run conditions used a Z-cell extended light-path capillary (57cm fused silica, effective length 36cm). The other conditions were as follows: the electrolyte buffer was borate buffer (50mM, pH 9.2), the voltage was 13-15kV (current 60-85μA) for 50 minutes, the injection was by pressure for 5 seconds, the UV wavelength monitored was 200nm, and the rinse schedule between injections consisted of 2 minutes of NaOH (0.1M), 2 minutes of water and 2 minutes of electrolyte buffer. The capillary was conditioned each morning by flushing with air for 5 minutes, with NaOH (0.1M) for 10 minutes, with water for 10 minutes and with borate buffer for 10 minutes. Each evening, the capillary was rinsed with water for 20 minutes and stored as such overnight.

5.3.5 CE Conditions for HP System

The final run conditions used a bubble cell extended light-path capillary (64.5cm fused silica, effective length 56cm) which is shown in Figure 5.2. The other conditions were as follows: the electrolyte buffer was borate buffer (60mM, pH 9.2), the voltage was set at 24kV for 35 minutes, injection was by pressure (50mbar) for 10 seconds, the UV monitoring wavelength was 200nm and the rinse schedule consisted of 2 minutes of NaOH (0.2M) followed by 2 minutes of water and 2 minutes of electrolyte buffer. Conditioning of the capillary each morning was carried out by flushing for 5 minutes with air, for 10 minutes with NaOH (0.2M), for 10 minutes with water and finally for 10 minutes with borate buffer. Each evening, the capillary was rinsed with water for 20 minutes and stored as such overnight.

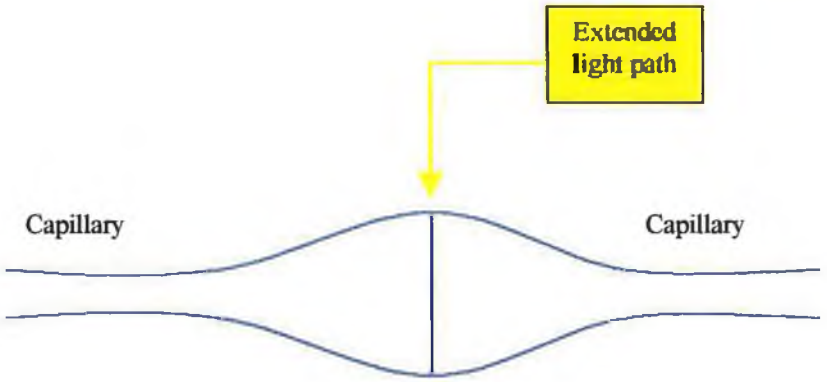
5.4 RESULTS AND DISCUSSION

5.4.1 Optimisation of Urine Collection Procedure

In the early stages of the project, 24-hour urine collection was considered. However, owing to the difficulties associated with this protocol such as patient compliance and the handling and storage of large volumes of urine, it was decided to take spot urine samples. Also of importance at this stage was the fact that it was not known if any qualitative differences would be seen between the cachectic and non-cachectic urines,

so no purpose would have been served by collecting large volumes of urine for analysis.

Figure 5.2 : Cross-sectional Diagram of Bubble Cell for HP CE System



Because the urine samples were random specimens, there were many variables that could affect the nature of the urine sample. Some of these included times of day, void volume, age and sex of the patient, drug therapy and type of cancer. As much information as possible was obtained from the patients' charts at the time of receiving the samples, so that it could be used later if required. As previously mentioned, the exact time of day was not recorded and samples were simply referred to as 'morning' or 'afternoon', depending on whether they were provided during morning or afternoon clinic, respectively.

Samples were kept on ice until analysis to conserve their integrity and in order to treat all samples in the same way and eliminate temperature differences.

5.4.2 Development of CE Conditions for ABI System

The development of the analytical procedure were based on the analysis of the model protein AGP, since no standard PMF was available. AGP has a similar molecular weight, pI and general characteristics as the PMF structure reputedly has. Starting conditions were based on the typical conditions that other authors had used in their analysis of proteins or glycoproteins in urine. Since most CE analysis of glycoproteins has taken place successfully on bare silica capillaries, these were employed in this

assay. The total length of the silica capillary (with Z-cell) was 57cm but the effective length was 36cm.

The background electrolyte was a borate buffer for the reason that it complexes with the diol groups of carbohydrates affording better separation. The other advantage of borate is its high pH (pH 9.2) which limits adsorption of the proteins to the wall of the capillary by keeping both the wall and the proteins negatively charged, thus limiting their mutual interaction because of charge repulsion. It has been used by many authors for the analysis of glycoproteins or proteins[55,56,60,61]. The borate buffer was prepared to 50mM concentration since the lower ionic strength (25mM) gave very early-eluting peaks, which although sharp had low peak areas, while higher ionic strength buffer (75 or 100mM) gave very late-eluting peaks, which were very broad in shape. The pH was maintained at 9.2 for a number of reasons. Lowering the pH of the borate buffer (by addition of boric acid, 50mM) reduced the current quite significantly causing an immediate and dramatic loss of resolution. Higher pH, with NaOH, did not result in any significant gain (or loss) of separating ability and hence was not used.

The UV wavelength used for most protein separations by CE is 200nm[60,62], and proved to give optimum response for the urine samples in this work when compared to 214nm and 280nm. The 200nm wavelength was also found to give maximum response for the model protein AGP.

The voltage was set at 15kV initially but for later samples such as the minicon® extracts, it was necessary to lower the voltage to 13kV. This was because it was recommended that the current should not exceed 100μA under the experimental conditions in use.

The rinse schedule was based on similar schedules seen in the literature[60,62] and it consisted of 2 minutes of NaOH (0.1M), 2 minutes of water and 2 minutes of run buffer. Injection was by pressure for 5 seconds.

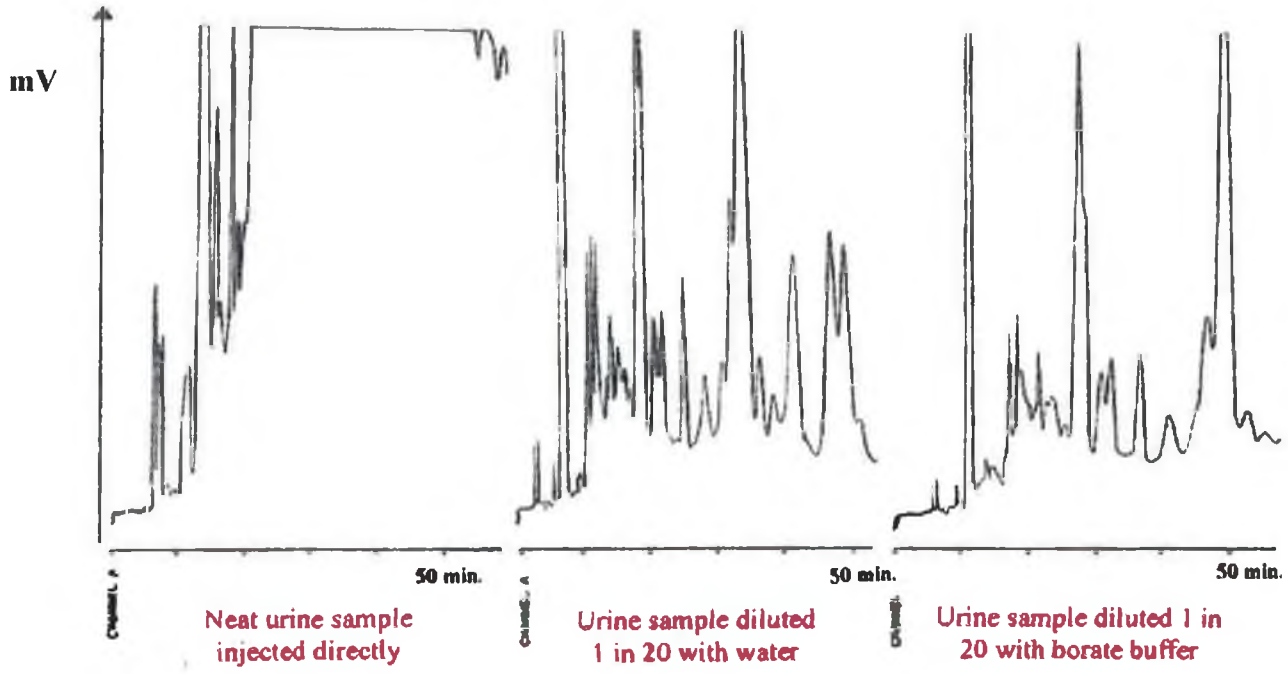
5.4.3 Optimisation of Sample Preparation Procedure

Generally for urine analysis, sample preparation may or may not be required, depending on the substance under test, though direct injection of urine has been possible in many applications where compounds in urine have been detected. Various metabolites in urine were determined by direct injection by Jellum *et al.*[62]. The high ionic strength borate buffer (300mM) they used appeared to dissolve any precipitates

present. Creatinine and calcium and other inorganic cations in urine were also determined following direct injection (after a simple 1:50 dilution in buffer)[63]. Ideally, direct injection or injection following a simple dilution would have been the procedure of choice. However, it was not possible to inject untreated urine, or to inject a urine sample following a simple dilution step with water or run buffer. This was because, as it can be seen from Figure 5.3, there were too many compounds present in these samples. So it was decided that some clean-up of the sample was required. A number of sample preparation procedures were hence investigated in this work.

The first and simplest procedure investigated was to subject the urine samples to centrifugation for 10min. @ 3000g at 4°C to sediment any particulate matter. Capillary electrophoresis of the diluted supernatant yielded electropherograms very similar to those obtained in Figure 5.3.

Figure 5.3 : Electropherograms of Urine Samples subjected to different Sample Preparation Procedures



Precipitation with 80% w/v $(NH_4)_2SO_4$ was also tried. This is a common biological sample preparation step, and had been used by Todorov *et al.* when isolating the PMF from the urine of cancer patients[34]. This procedure was carried out by adding 8g of $(NH_4)_2SO_4$ directly to 10ml of fresh urine (80% w/v solution) and stirring for 24 hours at 4°C. The precipitated protein was recovered and dissolved in 1ml of 25mM borate buffer and injected onto the CE system. However, the resulting electropherograms were very poor, possibly due to the very high ionic strength of the sample at this stage.

Dialysis of the urine samples was carried out with a view to removing small molecular weight species from the matrix. The urine sample (10ml) was placed into dialysis tubing with a MW cut-off of 12-14 kDa and stirred in a large water-filled container at 4°C for 4 days. The water in the container was changed every 6 hours where possible. Since the cachectic factor, if present, would have a MW of 24kDa, this meant that it should remain in the dialysis tubing. Samples were removed from the tubing after 1,2 and 4 days and injected directly onto the CE system. Results in Figure 5.4 shows that the extent of dialysis had a large effect on the removal of compounds from the urine. Although the procedure did clean up the samples, the length of time required meant that this procedure was prohibitively lengthy.

Also investigated was the combination of $(NH_4)_2SO_4$ precipitation and dialysis. The protein pellet which remained after 24 hours of precipitation with $(NH_4)_2SO_4$ was resuspended in 1ml of water and dialysed as described above. It was envisaged that the small molecular weight species in the pellet would be removed by dialysis, leaving only larger molecular weight molecules such as the PMF. The resultant electropherograms showed a conglomerate of peaks with very poor definition. It was realised that this technique would involve the introduction of large errors due to extensive manual handling of the sample and losses during the dialysis step.

The final sample preparation protocol examined was the use of minicon® B15 static dialyser units. A diagram of such a unit is shown in Figure 5.5. These units had been used very successfully by Jenkins *et al.* for the quantitation of human urine proteins such as the Bence Jones protein[60]. When used, it was found to eliminate almost all of the small molecular weight species in the urine samples, simplifying the identification of the larger molecules present.

Figure 5.4 : Electropherograms of Urine Samples following Dialysis

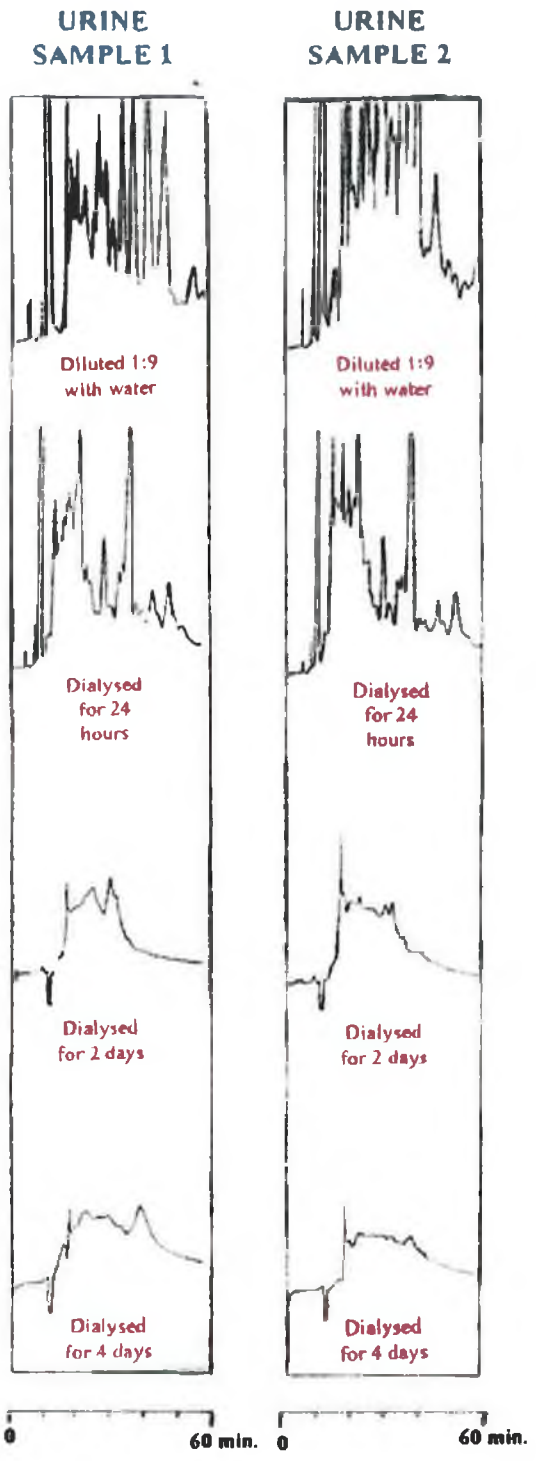
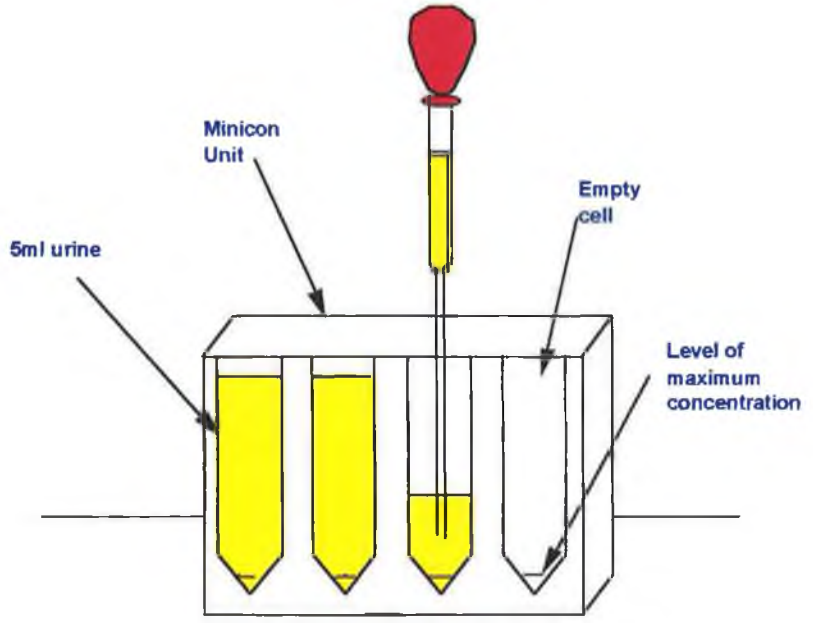


Figure 5.4 : The units of the vertical axis in each case are millivolts (mV) and all electropherograms are to the same scale.

Figure 5.5 : Diagram of Minicon B15 Static Dialyser



The 5ml urine sample was centrifuged for 10 min. @ 3000g at 4°C to sediment any particulate matter and the supernatant was added to the minicon® B15 cell using a pasteur pipette. Prior to this, the cell was rinsed with deionised water according to the manufacturer's instructions. The 5ml sample was left standing until its level reached the 50x mark on the cell, where it was now of an approximate volume of 100µl (this usually took 1 to 2 hours). The extract was then removed by pasteur pipette and diluted as appropriate with water i.e. 40µl in 200µl (if concentrated urine) and 80µl in 200µl (if dilute urine).

The decision as to whether urine was 'concentrated' or 'dilute' was based on visual inspection of the colour and turbidity as well as taking into account the total void volume (if available). The darker, more opaque samples, which usually came from smaller void volumes were found to be more concentrated than the paler, clearer samples, which usually came from larger void volumes. Distinguishing between 'concentrated' and 'dilute' samples by a dilution factor of two resulted in all electropherograms being comparable in terms of the five most important marker peaks.

The resulting electropherograms (Figure 5.6) obtained for the urine samples using the ABI CE, after employing the minicon® static dialyser unit, showed much

improved peak profiles and there was a definite pattern seen in all the samples subjected to this procedure. Since this procedure could both dialyse and concentrate the urine in less than two hours of unattended operation, it proved to be the most efficient sample preparation procedure. It was also found, on investigation, that a minicon® cell could be used twice for the same sample i.e. two separate 5ml portions of a urine sample could be concentrated without any loss of efficiency (see section 5.4.6).

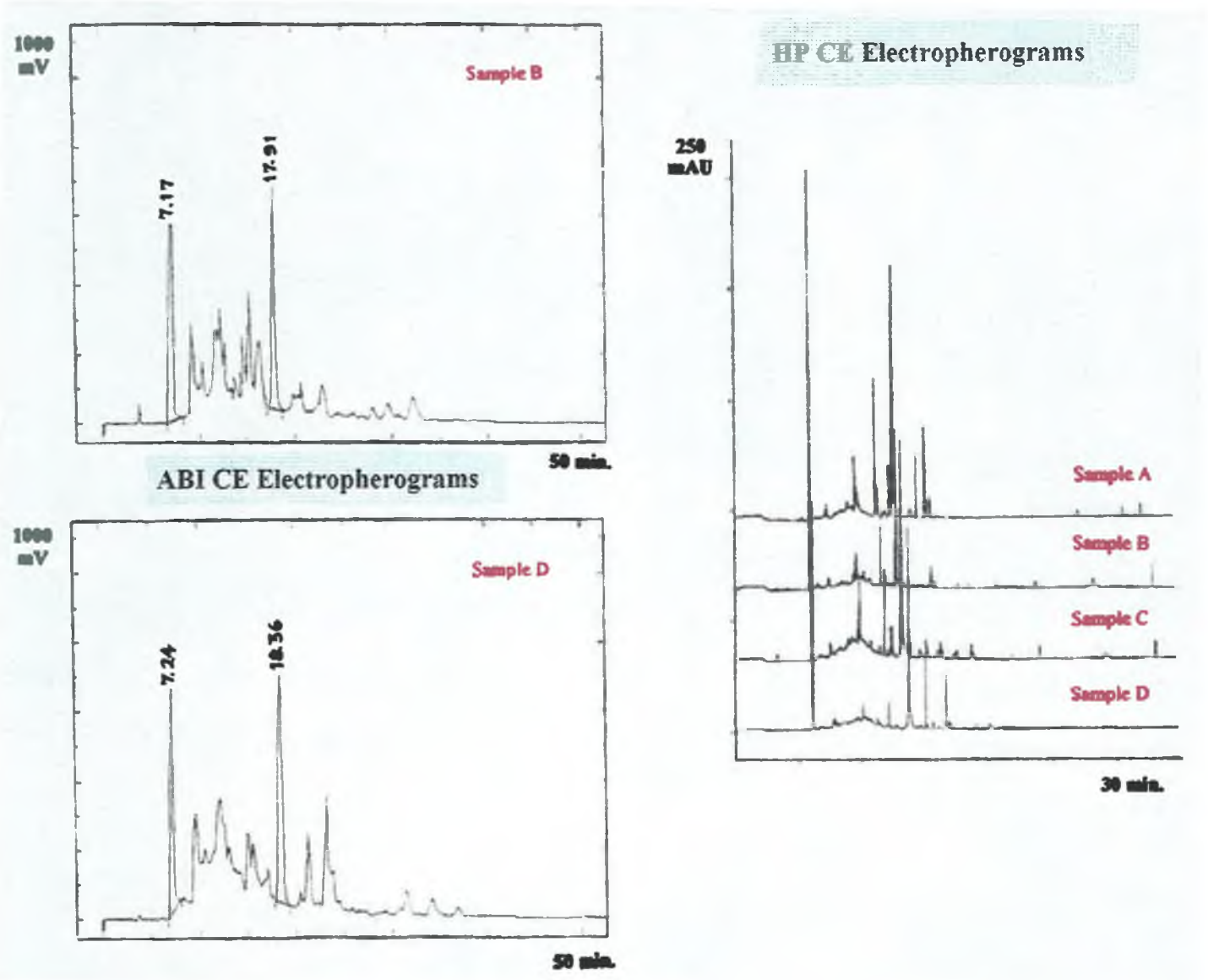
5.4.4 Development of CE Conditions for HP System

During method development, the higher specification HP CE became available for use and so the ABI CE conditions were modified such that similar profiles of the urine samples could be obtained on the HP instrument. Initial run conditions used for this system were as follows : the capillary was bare fused silica with bubble cell (64.5cm x 50μm i.d.), the electrolyte was borate buffer (50mM, pH 9.2), the voltage was 24kV (65μA) with a 40 minute run time, the injection was by pressure (50mbar) for 5 seconds, the UV monitoring wavelength was 200nm, and the rinse schedule consisted of 2 minutes of NaOH (0.1M), 2 minutes of water followed by 2 minutes of run buffer, also as before. The total length of the silica capillary was 64.5cm but the effective length was 56cm. The remaining parameters were as close as possible to those used with the ABI CE system (with Z-cell) and the conditions produced electropherogram profiles similar to those obtained with the other instrument (see Figure 5.6).

There was no Z-cell available for the HP CE instrument but there was a bubble cell capillary, so this was used instead. Although the Z-cell is reported to give 10-fold improvement in sensitivity, while the bubble cell is reported to give only 3 to 5-fold improvement, no major loss in response was noted in method transfer from the ABI CE to the HP CE. In fact, the peaks obtained were sharper, and resolution was improved.

After some initial experiments on patient samples, it was found that the migration times of the peaks were drifting quite significantly over the course of a sequence run. This was thought to be possibly due to buffer strength and/or buffer replenishment problems. Hence, the buffer vials were replenished periodically (every four hours during an overnight run) and borate buffer was prepared to the slightly higher ionic strength of 60mM, pH 9.2. The pressure injection was increased to 10

Figure 5.6 : Comparison of Urine Profiles from ABI CE and HP CE Instruments under similar Operating Conditions



seconds from 5 seconds and 0.2M NaOH was used instead of 0.1M, to ensure the capillary was cleared of any residual adsorbing protein in between injections. The combination of these changes to the method had the effect of increasing the retention times for the various peaks in the urine profiles under investigation. These conditions also served to improve separation between the peaks in the characteristic peak profile.

5.4.5 Identification of Cachectic Factors in Patient Samples by CE

5.4.5.1 Profiling of Urine Samples from Non-Cachectic Subjects

A number of urine samples from healthy subjects (n=7) and cancer patients with no cachexia (n=13) were run by CE. There were no apparent differences between the urine sample profiles of the two groups (Figure 5.7). Characteristic profiles resulted, with each sample containing the five expected peaks (in various proportions). Peaks 1,2,3,4 and 5 had migration times of 8±1,11±1,16±1,23±2 and 30±2 minutes, respectively. These peaks later became useful as markers because each of these components had a different UV spectrum which facilitated peak tracking when migration times drifted or if additional peaks were found to be present in samples. In samples where the later-eluting peaks were found to have drifting migration times, the time of their analysis usually corresponded to a change in temperature in the laboratory. For example, during the night, the temperature (on occasion) was found to drop by two or three degrees.

5.4.5.2 Rationale for Profiling Urine Samples from Cachectic Subjects

Although no pure standard of the PMF was available, it was decided to examine urine samples from cachectic cancer patients with the expectation that an extra compound, corresponding to the cachectic factor, would be found in the latter group of urine samples. The rationale for this was twofold. Firstly, there was the previous compelling evidence for the presence of the PMF in urine of cachectic cancer patients, hence there was a good possibility of resolving the factor in the urine of this group of cancer patients diagnosed to have cachexia[34]. Secondly, the small molecular weight species had been removed from the samples and there are few proteins eluted in urine, especially of MW > 15kDa, so it was expected that if the PMF (MW ~ 24kDa) was present, it would be clearly visible.

Figure 5.7 : Electropherograms of Urine Samples from Healthy Subjects and Non-Cachectic Cancer Patients

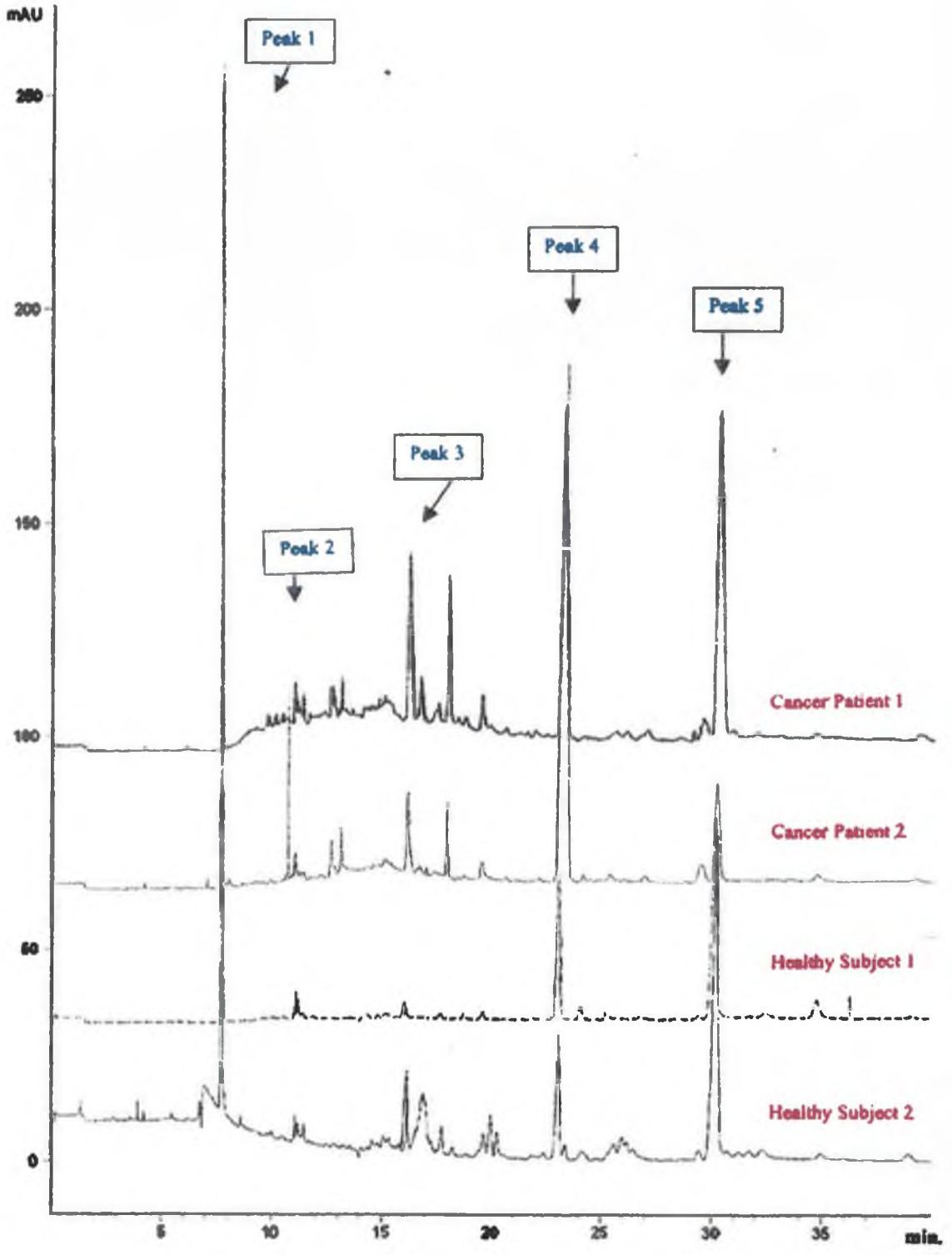


Figure 5.7 : Overlay of urine samples from healthy subjects and cancer patients with no cachexia. Peaks 1,2,3,4 and 5 appear to be characteristic to all urine samples from normal subjects and cancer patients with no cachexia. CE conditions as per text.

5.4.5.3 Profiling of Urine Samples from Cachectic Subjects

First Time Analysis of Samples

The urine samples of eight cancer patients who had been diagnosed as cachectic were obtained and subjected to analysis by CE under the same conditions as were used for the urine from non-cachectic patients. Urine from healthy individuals was run in conjunction with the cachexia samples for the purposes of comparison. Relevant information from their chart information forms has been tabulated in Table 5.2. It is obvious that, in the case of each patient, there had been relatively recent weight loss prior to the taking of the urine specimens.

Table 5.2 : Information on the Cancer Patients Diagnosed with Cachexia

Patient ID Number	Age	Ethnic Origin	Height (inches)	Weight Information (most recent weight taken)	Cancer Type
1	64	Caucasian	68.25	186-182lb over previous 2 months (182lb in Sept. 97) lost 10-15lb since beginning chemotherapy in April 97	Prostate and bone cancer
2	51	Caucasian	70	127.5lb (had lost weight prior to visit)	Esophageal cancer
3	45	Caucasian	69	202-164lb over previous 9 months (164lb in June 97)	Colon, liver and lymph nodes cancer
4	86	Caucasian	67	142-130lb over previous 7 months (130lb in Sept. 97)	Stomach and liver cancer
5	73	African	72	170-159lb over previous 6 months (159lb in Sept. 97) (153lb in Oct. 97)	Pancreatic cancer
6	77	Caucasian	67.5 - 68	160-131lb over previous 11 months (131lb in Sept. 97)	Prostate cancer
7	44	Caucasian	62	93-82lb over previous 12 months (82lb in Oct. 97)	Gastric and ovary cancer
8	63	Caucasian	64	148-134lb over previous 10 months (140lb in Sept. 97)	Pancreatic and liver cancer

Of this particular group of individuals (patients 1-8 in Table 5.2), it was found that there were two extra peaks in the electropherograms of 6 of the 8 patients, thought to be the cachectic factor (PMF) and its albumin-bound complex. These were referred to as cachectic factors 1 and 2 and had migration times of 13±1 and 25±3 minutes, respectively. These cachectic factors were present in the urine of the patients highlighted in Table 5.2, and examples of these electropherograms can be seen in Figure 5.8. The factors were thus present in 75% of the cachectic cancer patients investigated and were never seen in the urine of healthy subjects or in the urine of patients with cancer but no cachexia. The positive samples were reanalysed a number of times to confirm these findings. In each case, the electropherograms were identical.

Repeat Analysis of Samples

In order to check that these results were genuine in the patients who tested positive i.e. cachectic patients 1,2,4,5,6 and 8, it was decided to take repeat urine samples where possible. Four of the patients (2,4,5 and 6) that had tested positive on the first occasion agreed to provide a second specimen two weeks later. These samples were obtained as before, prepared using the minicon® cells and run under the usual CE conditions. These new samples (referred to as sample B) were run in conjunction with the previous positive urine sample (sample A) that had been donated by the patient. In the case of patients 2 and 6, the urine profiles obtained were identical in almost every respect, with the cachectic factors appearing in both samples A and B. However, in the case of patients 4 and 5, although the profiles were identical in all other respects, the factors were absent from sample B. This is illustrated in Figure 5.9.

Figure 5.8 : Electropherograms of Urine Samples from Healthy Subjects and Cachectic Cancer Patients

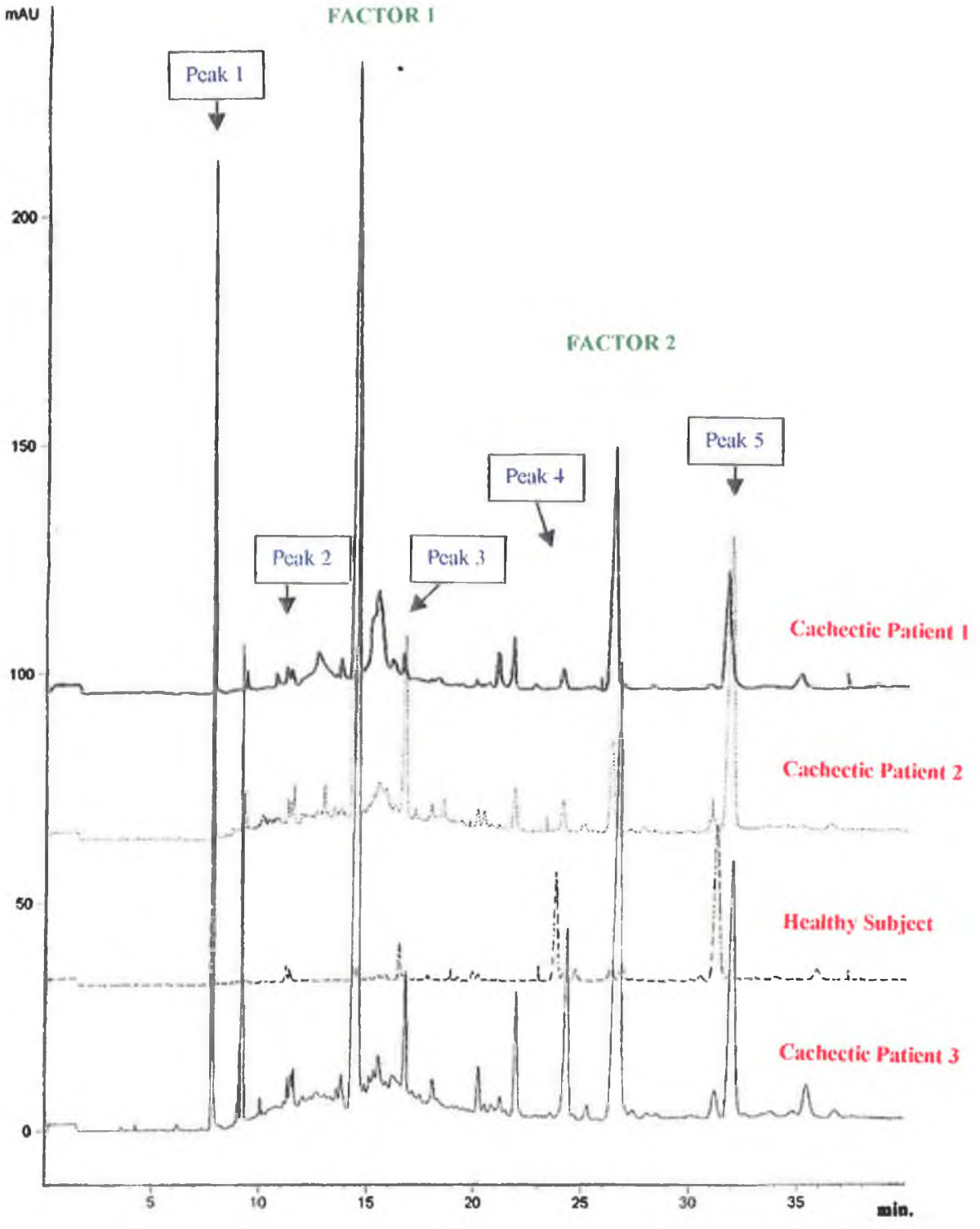


Figure 5.8 : Overlay of urine samples from healthy subjects and cancer patients with cachexia. Peaks 1,2,3,4 and 5 appear to be characteristic to all urine samples from normal subjects and cancer patients with cachexia. However, the two peaks marked 'Factor 1' and 'Factor 2' are particular to the urine samples from cancer patients with cachexia.

Figure 5.9 : Electropherograms of Repeat Urine Samples from Cachectic Cancer Patients

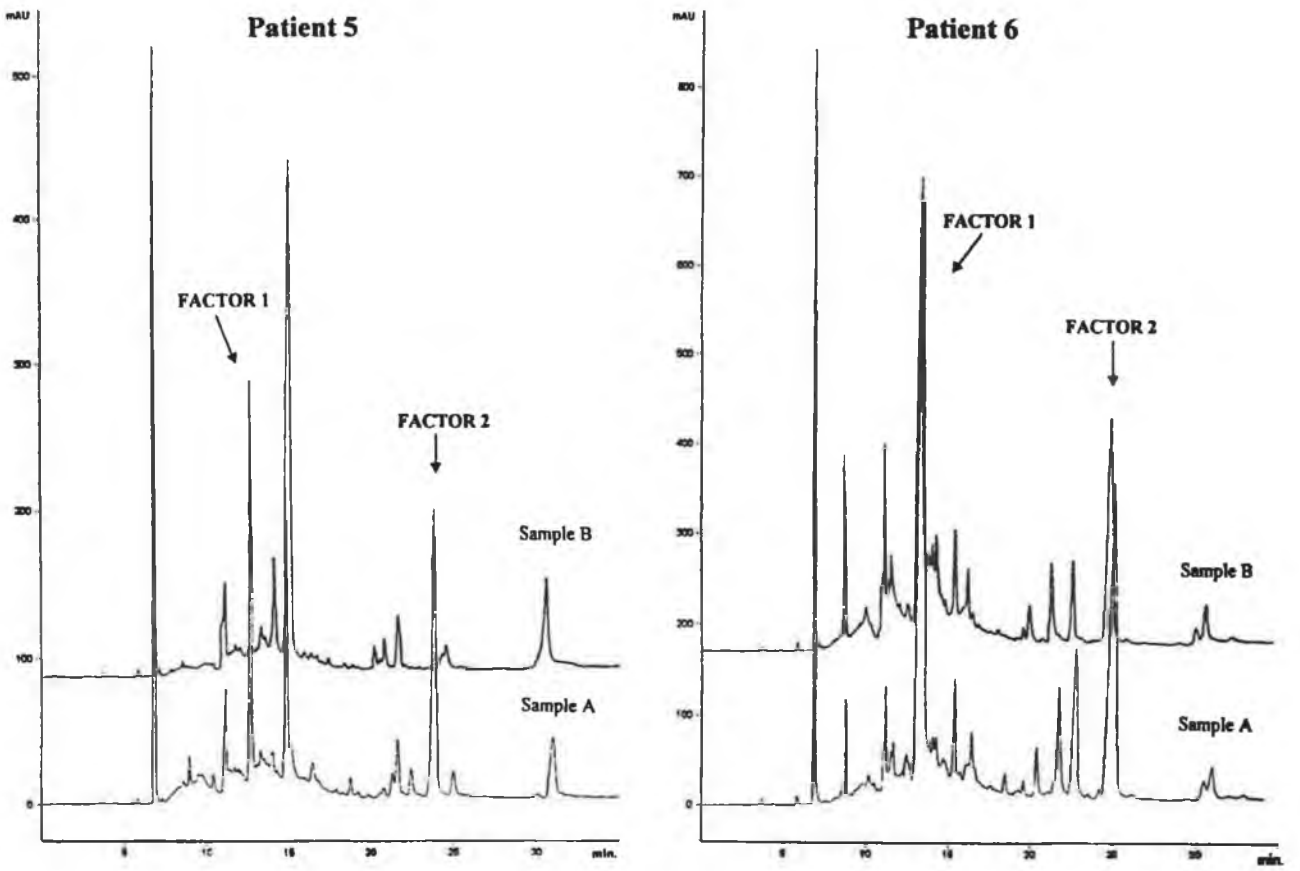


Figure 5.9 : Overlay of urine samples A and B from cachectic cancer patients. Sample A was taken two weeks prior to Sample B. Factor 1 and Factor 2 were evident in both samples for patient 6. However, the factors were only present in Sample A for patient 5.

5.4.6 Reusability of the Minicon® B15 Cells

A urine sample (from cachectic patient 8) was subjected to the sample preparation procedure using the minicon® B15 cell to dialyse and concentrate the sample. A few days later, a second aliquot of the urine sample, which had been frozen during that time, was concentrated as before in the *same* minicon® cell that had been used for the sample on the first occasion and that had been stored at 4°C. When both samples were injected onto the HPCE instrument and run under the usual CE conditions, the results showed that interferences were removed to the same extent on both occasions. In addition, the peaks were usually of the same height and comparable area, showing that this sample preparation technique was very reproducible. Examples are shown in Figure 5.10.

5.4.7 Identification of Cachectic Factors in Patient Samples by their UV Spectra

The UV spectra of the cachectic factor and its albumin-bound complex were identical, but they differed from the other typical components of urine (see Figure 5.11). This allowed the cachectic factor peaks to be located on CE electropherograms (using the PDA detector) even when migration times had changed slightly due to external factors.

5.4.8 Identification of Cachectic Factors in Patient Samples by Immunoblotting

To confirm that the factors seen in these electropherograms were the same as those seen by Todorov *et al.*[34,35], a sample of antibody to the PMF was obtained from the UK. When Western Blotting of positive urine samples (patients 2 and 6) was carried out, the immunoblots of the samples revealed two bands as expected – corresponding to the PMF and the albumin-bound PMF. When Western Blotting of negative urine samples (healthy subjects and non-cachectic cancer patients) was carried out, no bands appeared on the immunoblots. This evidence suggested that the two peaks seen in 75% of the cachectic cancer samples investigated were the PMF and its albumin-bound complex, as seen by Todorov *et al.* in the urine of cachectic cancer patients in 1996[34].

Figure 5.10 : Overlaid Electropherograms of the same Urine Sample prepared on two different days using the same Minicon® Cell

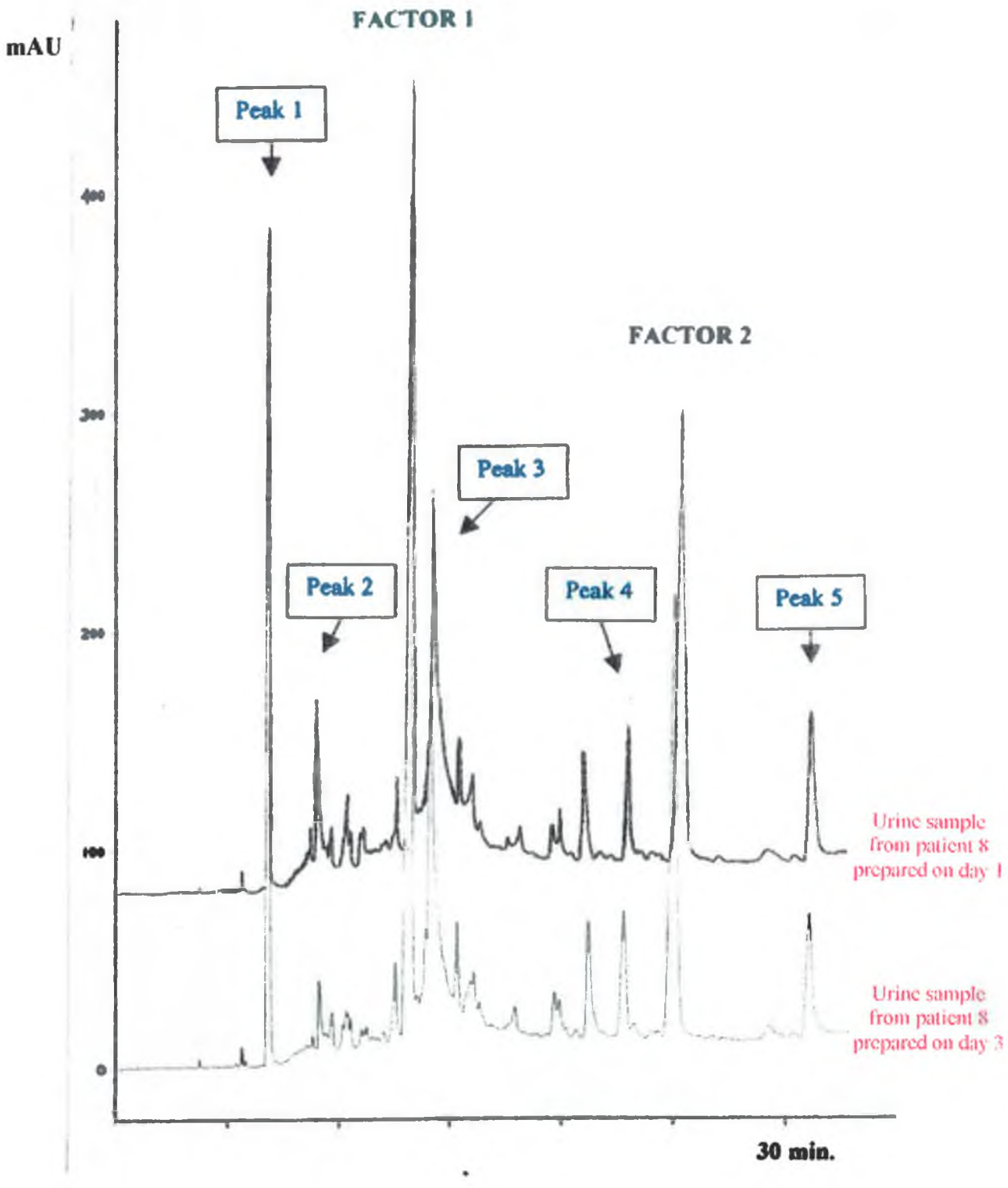


Figure 5.10 : Overlay of urine sample (from cachectic patient 8) prepared on different days using the same minicon® extraction cell. Peaks 1,2,3,4 and 5 along with cachectic factors 1 and 2 are clearly visible.

Figure 5.11 : UV Spectra of the Cachectic Factors

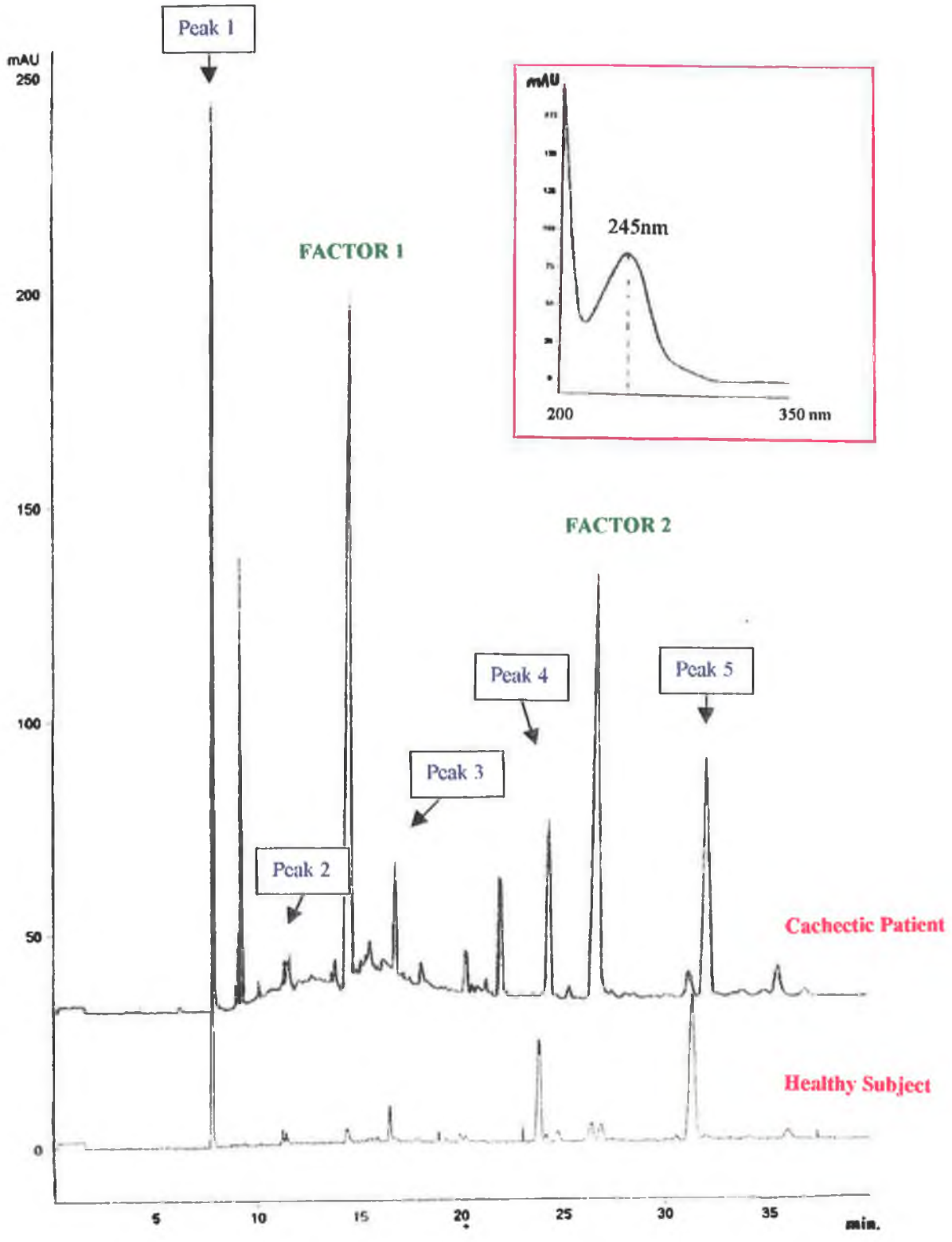


Figure 5.11 : Overlay of urine samples from a healthy subject and a cancer patient with cachexia. The inset diagram contains overlaid UV spectra from Factor 1 and Factor 2.

5.4.9 Diagnosis and Prevention of Cachexia

Because there are no definitive clinical criteria for the diagnosis of cachexia, diagnosis by physicians of cachexia in cancer patients is still subject to error. This is due in part to the fact that the body weight of most cancer patients will rise and fall during their therapy. Some weight loss may have non-cachectic causative factors such as lack of appetite, nausea, apathy or changing body mass dynamics while on chemotherapy. However, cachexia is more certainly the cause if there is a continuous decline in body mass and an inability to replace lost weight.

There is, as yet, no cure for cachexia and current therapies, at best, serve only to slow the progress of the disease. However, diagnosis of cachexia is important in terms of treating the underlying cancer. Chemotherapy may be altered in light of the fact that a patient is diagnosed as being cachectic.

Should a real relationship be found between clinical cachexia and the presence of the cachectic factors in the urine, as is indicated in this work, there is potential for a) a more objective method of diagnosing cancer cachexia (and possibly its severity), b) prediction of onset of cachexia, c) elucidation of the structure of the PMF, and ultimately d) prevention of cachexia in cancer by inhibition of the PMF.

5.4.10 Further Work

This project is being continued in Georgetown University Medical Centre, Washington DC in order to develop a quantitative assay by CE for the measurement of these factors in urine. There is a proposal to carry out a large-scale clinical study in conjunction with the Lombardi Cancer Centre, Washington DC over two years. Analysis of the urine of healthy subjects, non-cachectic and cachectic cancer patients will be undertaken to prove an association between the presence of these factors in the urine and the presence of cachexia. The patients' weights would be monitored carefully over time and the presence or absence of the cachectic factors in their urine, in addition to their concentrations would be followed. Immunoblotting techniques would be used to verify these results. There is hope that the levels may be correlated with the severity of the cachexia. This may prove to be an excellent approach for the prediction, objective diagnosis and monitoring of cachexia in cancer patients.

In parallel with this, CE-MS is also being carried out in Hewlett-Packard Laboratories in Palo Alto, California with a view to elucidating the full structure of this PMF. Once the structure is known, pure standards can be produced/isolated and

research could begin into discovering more about this molecule's exact role in mediating cachexia. Attempts to inhibit the action of this PMF in cancer patients may then be possible, and a therapy for treatment of cachexia could be developed.

5.5 CONCLUSIONS

A random urine sample was collected from normal subjects, cancer patients and cancer patients with cachexia at a general oncology clinic. These samples were concentrated (50x), dialysed to remove small molecular weight species (< 15kDa) and further diluted in water. HPCE analysis of the samples revealed the presence of two peaks in the electropherogram (in 75% of the samples) uniquely associated with the presence of carcinoma cachexia. These factors were not present in the urine of the healthy subjects or cancer patients who were not experiencing weight loss. The urine samples that gave positive results for the factor were from patients with different cancers, undergoing a range of therapies and who were of both sexes and all ages. The urine samples containing the cachectic factor peaks were subjected to immunoblot analysis using antibodies raised against PMF. Analysis of the immunoblots revealed the presence of the PMF factor and its albumin-bound complex by the presence of two bands of the correct molecular weight. Strong evidence is emerging to suggest that the factors reported by Todorov *et al.*[34] and the factors detected in this study by CE are identical. Ultimately, an assay would need to be developed to quantify PMF levels in the urine of patients and to correlate these levels with clinical status. Further investigation into the role and structure of the PMF may be one way to approach the difficult task of preventing cachexia.

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CONCLUSIONS AND SUMMARY

Sample preparation is often the most time-consuming step in any analytical protocol since manual handling of the sample is usually involved. While advances in instrumentation continue to reduce analysis times, the proportional length of time required for sample pretreatment becomes relatively greater, especially if the analyte is to be determined in a biological fluid. The main aim of the work described in this thesis was to develop analytical methodologies involving HPLC and CE which would involve the minimum number of manipulations to be performed on small biological samples and which could be rapidly executed while taking into account the selectivity and sensitivity requirements of the method. The methods selected presented particular challenges in terms of the sampling, pretreatment, analysis and validation steps. Before embarking on the development of these biopharmaceutical protocols for biological samples, certain criteria were considered. These included the chemical behaviour and metabolism of the compound, the nature of any interferences which could be carried through sample preparation and which would thus have some bearing on the analytical techniques selected, and the extent of validation required.

Four discrete problem areas with medical applications were carefully chosen on the basis that they were complex in terms of their analytical requirements. The target analytes (drugs or amino acids) were usually in difficult matrices such as plasma and urine, and were quantified successfully by HPLC or CE. All aspects of the processing of the sample were investigated, from blood collection through injection, separation, detection and data handling. A variety of sample preparation techniques were tried in each case before electing the optimum for the analyte(s) in question, bearing in mind the chosen instrumental technique.

The general approach to analytical method development in each problem area was carried out based on the following key stages :

- A literature survey was carried out to identify previous work, and a proposed methodology was devised that would represent significant improvements over prior methods
- The procedures for taking the biological samples were established
- The pretreatment of the samples was investigated with respect to reducing endogenous interferences, while ensuring the integrity and recovery of the analyte(s)

- Derivatisation, if required for increased selectivity or sensitivity, was introduced at this stage
- The analytical separation method for the determination of the compound(s) of interest was developed by varying one parameter at a time
- The overall method was subjected to statistical evaluation and validated according to conventional ICH parameters

In the first experimental work, which was based on the diagnostically important amino acid taurine, a method was required for its determination in human platelet-rich plasma. A number of sample pretreatment procedures were systematically investigated and these included ultrafiltration, ion-exchange extraction, and protein precipitation with strong acids such as perchloric acid. Ultimately, acetonitrile precipitation was shown to give the best recovery. Fluorescamine was shown to be a successful agent for the derivatisation of taurine whereas hypochlorite derivatisation (used in previous assays for amino acids) was found to yield an unstable derivative.

Novel aspirin derivatives were evaluated as prodrugs for transdermal aspirin (ASA) delivery using direct injection and reversed-phase HPLC. However, before the prodrugs were evaluated in terms of their ability to permeate the skin, a number of other tests were carried out on each compound. These were purity testing and degradation profiling (for which both TLC and HPLC were employed) as well as the effect that these prodrugs had on platelets. Of the initial seven derivatives, only four showed sufficient potential to warrant the final transdermal experiments. Following *in vitro* application of the derivative to mouse skins, samples from the PBS-filled wells of the Franz Cell apparatus could be injected directly onto the isocratic HPLC system. This allowed quantitation of ASA and its metabolite salicylic acid (SAL) and was followed by a gradient to elute the parent compounds in the same run. The validated method was used to examine levels of ASA and SAL released transdermally by the prodrugs, demonstrating that certain candidate compound(s) were appropriate for further pharmacological testing.

Sample pretreatment protocols in published methods for the determination of ASA and SAL in plasma are usually very labour-intensive involving solvent extraction and protein precipitation. In this work, column-switching HPLC with on-line solid-phase extraction (SPE) was for the first time shown to be a valuable approach for the determination of ASA and SAL in human plasma. It was shown to be essential to optimise the dimensions, the particle size and the type of material in the extraction

column for direct injection of plasma onto the system. This semi-automated method was extensively validated. Excellent recoveries of the analytes from the plasma (better than all previous HPLC methods for these compounds) obviated the need for an internal standard. The sampling procedure i.e. type of anticoagulant, addition of esterase inhibitor etc., was also optimised and stability testing was carried out to ensure the integrity of the sample. The method was found to be fast, reproducible and applicable to studies of aspirin metabolism in healthy volunteers.

The final area involved the identification of a glycoprotein, which is known to be a protein-mobilising factor (PMF), associated with cancer cachexia. Although previouly detected in serum and urine by immunoblotting, the structure of this cachectic factor has not been fully elucidated (although its molecular weight has been reported to be 24 kDa) and it had not been investigated by any separation techniques. A number of urine sample pretreatment procedures were investigated prior to analysis by CE e.g. dialysis, ammonium sulphate precipitation and simple dilution, before deciding on the use of a static dialyser unit. Although there was no standard PMF with which to carry out spiking experiments, the PMF (if present) would be easily detected as a molecule with molecular weight greater than 15 kDa (the filter cut-off point). In fact, few proteins normally appear in urine and when investigated by CE, the PMF (plus a compound believed to be its albumin-bound complex) were seen as two extra peaks in the urine samples of cancer patients with cachexia. These results were confirmed by immunoblotting. It was also shown that these factors were absent from the urine of healthy subjects and cancer patients who were not experiencing weight loss. The factor was found in patients of both sexes with different types of cancer, on a range of therapies. The results of this work have prompted further research into the screening of cancer patients with a view to predicting, monitoring and possibly preventing cachexia. In addition, research is ongoing to determine the structure of the PMF and to prepare a pure standard for use in a quantitative method for the cachectic factor(s).

This thesis has described the development of analytical methodology for a number of compounds which are diagnostically important. These ranged from the endogenous amino acid taurine to aspirin and prodrugs of aspirin. This research succeeded in finding ways of reducing the time required to prepare the biological samples by developing simple, rapid and reproducible sample preparation methods. All of these methods were applied to clinical samples, demonstrating that careful optimisation of the sample preparation process, followed by optimisation of the analytical measurement procedure, is necessary for high quality results in bioanalysis.

Appendix A

Preparation of Aspirin

50g (0.36 mol) of dry salicylic acid and 70ml (0.74 mol, 2.03 eq) of acetic anhydride were mixed together in an Erlenmyer flask. To this was added 40 drops of concentrated sulphuric acid. The mixture was stirred with a spatula and at the same time warmed for 20 minutes at 50-60°C in a water bath. First, a solubilisation of the salicylic acid occurred, then a precipitate of aspirin appeared. The precipitate was filtered, dissolved in the minimum of boiling ethanol. Water was poured drop by drop into the solution until the precipitate of aspirin appeared. The mixture was filtered and recrystallised from a mixture of ethanol/water (30/70). The yield was 93%, the melting point was 132°C (literature value 134°C) and both NMR and IR showed the material to be pure aspirin.

Preparation of Aspirin Glycine

600mg (3.33 mmol) of aspirin were dissolved in 7 ml of dry tetrahydrofuran under nitrogen. The solution was cooled to -5°C, and 0.442 ml (3.33 mmol) of freshly distilled triethylamine was added. After 5 minutes, 0.323 ml of ethylchloroformate was added and a precipitate of triethylammonium chloride appeared. A solution of 374.6mg (5 mmol) glycine in 2.5 ml of NaOH (2M) was added drop by drop to the mixture. After stirring for 15 minutes, the temperature rose slowly to 20°C. The tetrahydrofuran was removed under pressure and the aqueous solution was acidified with HCl until it reached pH 2-3. The mixture was extracted with ethyl acetate (3 x 10 ml), the organic layers were combined and washed with a saturated ammonium chloride solution. The organic layer was dried with Na_2SO_4, filtered and the solvent removed under pressure. A very dense slightly yellow oil was obtained which precipitated overnight. The solid was filtered off and washed with chloroform (10 ml). 173 mg of the compound was obtained as a white powder with a yield of 22% and a melting point of 151°C. IR and microanalysis proved the identity of the product and showed it to be pure.

Preparation of Aspirin Alanine

Aspirin (900 mg, 5 mmol) was dissolved in dry tetrahydrofuran (10 ml) under nitrogen and distilled triethylamine (0.7 ml, 5 mmol) was added to the solution. It was kept at 0°C and ethylchloroformate (0.5 ml, 5 mmol) was added. A precipitate appeared immediately. Alanine (623 mg, 7 mmol)was dissolved in a 0.63M NaOH solution and added drop by drop to the mixture which was stirred at 0°C for 1 hour and then left overnight at 20°C. The mixture was acidified with a solution of 5% HCl (20 ml) and extracted with ethyl acetate (20 ml). The organic layer was washed with brine (10 ml), dried over magnesium sulphate, filtered and the solvent was removed. The oil was purified by precipitation in petroleum ether and crystallised in a mixture of ethyl acetate and petroleum ether. The white crystals were washed with diethyl ether to eliminate any residual aspirin. 488 mg of white powder were obtained with a yield of 39% and a melting point of 115°C. IR and microanalysis showed that the product obtained was as expected and pure.

Preparation of Aspirin Phenylalanine

Aspirin (1.8 mg, 10 mmol) was dissolved in dry tetrahydrofuran (40 ml) under nitrogen and distilled triethylamine (1.4ml,10 mmol) was added to the solution. It was cold at 0°C and the ethylchloroformate (1ml, 10 mmol) was added. A precipitate appeared immediately. Phenylalanine (1.81 g, 11 mmol) was dissolved in a 1M NaOH solution (10 ml) and added drop by drop to the mixture which was stirred at 0°C for 1 hour and then left overnight at 20°C. The mixture was acidified with a solution of 5% HCl (20 ml) and extracted with ethyl acetate (20 ml). The organic layer was washed with brine (10 ml), dried over magnesium sulphate, filtered and the solvent was removed. The oil was purified by chromatography on silica gel column using ethyl acetate/petroleum ether (75/25) as eluent. The product was not the phenylalanine amide of aspirin but the amide of salicylic acid. The derivative at this point (0.7 g) was dissolved in pyridine (2 ml) and ethyl acetate (0.5 ml) under nitrogen. The mixture was cold at 0°C and the acetylchloride (0.21 ml) was added drop by drop to the solution and stirred for 30 minutes at 0°C and 1 hour at room temperature. The

mixture was then poured into a solution of 5% HCl (5 ml) and extracted with ethyl acetate (10 ml). The organic layer was washed with brine, dried over magnesium sulphate, filtered and the solvent was removed. The oil was purified by chromatography on an alumina (basic) gel using ethyl acetate/petroleum ether (3/1). The resultant oil gave the correct microanalysis.

Preparation of Aspirin Anhydride (Dimer)

Aspirin (1.8g, 10mmol) was dissolved in dry tetrahydrofuran (20ml) and dicyclohexylcarbodiimide (1.05g, 5mmol), 2:1 ratio was added at 0°C under nitrogen. The mixture became cloudy. After 3 hours at room temperature, the dicyclohexylurea was filtered off and the filtrate evaporated down in vacuo. The residue was taken up in ethyl acetate and filtered free of any further precipitated urea. The solvent was removed under reduced pressure. The product crystallised from diethyl ether. 38% of white crystals were obtained (MP 71-72°C)

Preparation of Aspirin Isosorbide Ester

Aspirin (3.6g, 20 mmol) and urea (0.06g) were dissolved in dry dichloromethane (10 ml) under nitrogen. Thionyl chloride (2.9 ml, 40 mmol) was added and the mixture was refluxed for 4 hours at 30-40°C. Then the excess thionyl chloride was distilled off, giving the crude acetylsalicylchloride as an orange oil which was used immediately without further purification. This oil was added slowly to the isosorbide (2.92g, 20 mmol) dissolved in dry pyridine (15 ml) at 0°C under nitrogen. The mixture was refluxed for 3 hours, then acidified with 5% HCl and extracted with dichloromethane. The organic phase was washed with brine, dried over magnesium sulphate, filtered and concentrated in vacuo. Crystallisation from ethyl acetate gave 2.7g (42%) of white crystals mp 166-7°C.

Preparation of Aspirin Phenyl Ester

Polyphosphoric ester (PPE) was prepared by boiling phosphorous pentoxide (25g) in chloroform (25ml) containing diethyl ether (50ml) until the solution became clear (8 hours). The solution was filtered to remove the phosphorous chloride which did not react and the solvents were evaporated. The viscous colourless syrup was used directly.

Aspirin (1.8g, 10 mmol) and phenol (0.94g, 10 mmol) were dissolved in dry chloroform (50 ml) and excess of PPE (24.9g, 150 mmol) was added under nitrogen. The mixture was stirred for 15 hours, then poured into a saturated sodium hydrogenocarbonate solution (60 ml) and extracted with dichloromethane (40 ml). The organic layer was washed with a saturated hydrogeno carbonate solution and then with water, dried over magnesium sulphate, filtered and the solvent was removed under reduced pressure. Column chromatography of the oil on silica gel with a 85/15 cyclohexane/ethyl acetate solvent mixture yielded a clear liquid. Crystallisation from diethyl ether gave 0.74g (29%) of white crystals mp 99°C. Both IR and microanalysis showed the material to be the expected product and to be pure.

Preparation of Aspirin Nitrophenyl Ester

Aspirin (1.8g, 10 mmol) and 4-nitrophenol (1.39g, 10 mmol) were dissolved in dry chloroform (50ml) and excess of PPE (24.9g, 150 mmol) was added under nitrogen. The mixture was stirred for 24 hours, then poured into a saturated sodium hydrogenocarbonate solution (50 ml) and extracted with dichloromethane (50 ml). The organic layer was washed with a saturated hydrogeno carbonate solution and then with water, dried over magnesium sulphate, filtered and the solvent was removed under reduced pressure. The yellow oil was recrystallised from ethyl acetate yielding 0.95g (31%) of white crystals mp 136-8°C. IR and microanalysis were carried out.

Appendix B

Use of Platelet Aggregation Profiler

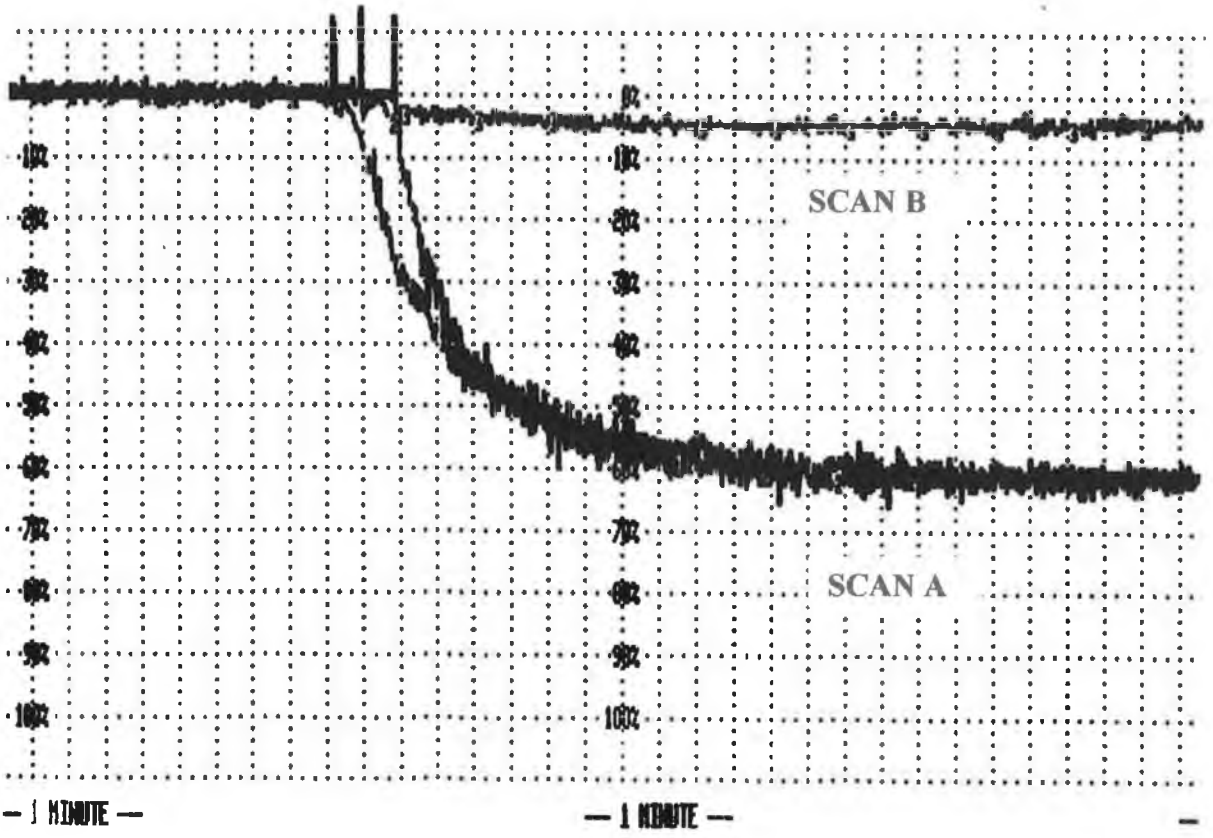
Sample Collection
Whole blood sample taken into sodium citrate anticoagulant (10%).

Sample Pretreatment
Sample centrifuged at 1000 rpm for 10 minutes to yield platelet-rich plasma (PRP). Platelet-poor plasma (PPP), which was to be used as a blank, was obtained by centrifuging at 3000 rpm for 10 minutes.

Background Correction Measurement
The platelet aggregation profiler (model 4, Biodata Corporation, Horseham, PA, USA) was first blanked. This was carried out by adding 500µl PPP to a glass tube, and taking an absorbance scan while the sample was kept at 37°C. This sample was regarded as 100% aggregation for blanking purposes, equivalent to all platelets having clumped together and fallen out of solution, yielding an essentially clear sample. This blank measurement is used to account for the differences in plasma composition for each blood donor.

Standard Measurement
The PRP sample was then measured by placing 500µl PRP in a glass tube, and allowing the sample to stir at 37°C. After two minutes, a platelet agonist such as arachidonic acid was added. Due to the presence of cyclooxygenase in platelets, clotting would occur within a few minutes and the clots would drop out of solution, leaving a clear solution behind. The extent of aggregation is measured on the vertical axis and is inversely proportional to absorbance of the sample. An example of such an absorbance profile is shown below in scan A where it can be seen that 60% aggregation of the sample has occurred within approximately one minute.

Sample Measurement
A sample of PRP containing the drug under investigation e.g. aspirin or prodrug of aspirin, was added to the glass tube, and was allowed to stir at 37°C. After two minutes, a platelet agonist such as arachidonic acid was added. If the drug under test was an inhibitor of platelet aggregation via its blocking of cyclooxygenase activity, clotting would not occur and a profile such as scan B below would result. In scan B, it can be seen that essentially no aggregation of the sample has occurred.


SCAN B
SCAN A
— 1 MINUTE —
— 1 MINUTE —

Appendix C

Procedure for Extraction of ASA from Plasma

Taken from reference 59 at the end of Chapter 3 :
Japanese Pharmacology and Therapeutics, **16(1)**, 17-25 (1988).

1ml plasma was placed in a stoppered test tube with 2ml water and 7ml dichloroethylene and shaken for 5 minutes.

0.5g sodium hydrogen carbonate was added and the mixture was again shaken for 10 minutes and centrifuged at 3500 rpm for 10 minutes.

The organic layer was transferred to another test tube.

To 3ml of the aqueous layer was carefully added 0.5ml 6M HCl.

7ml dichloroethylene and 2g anhydrous sodium sulphate were added, the tube shaken for 15 minutes and centrifuged at 3500 rpm for 10 minutes.

3ml of the organic layer was evaporated to dryness and combined with the previous organic layer, evaporated to dryness and reconstituted as required.

Appendix D

PRESENTATIONS

1. Research Day, Royal College of Surgeons, Dublin, Ireland. 19th April 1995. Poster presentation (a).

2. The 47th Irish Universities Colloquium, St. Patrick's College, Maynooth, Ireland. 7th-9th June 1995. Poster presentation (a).

3. The 32nd "R & D Topics in Analytical Chemistry" Meeting, University of Hull, Hull, England. 10th-11th July 1995. Poster presentation (a).

4. Eirchrom 1995, Regional Technical College, Tallaght, Dublin, Ireland. 5th-6th Sept. 1995. Poster presentation (b).

5. The 5th International Symposium on Drug Analysis, Catholic University of Leuven, Leuven, Belgium. 12th-15th Sept. 1995. Poster presentation (b).

6. Research Day, Royal College of Surgeons, Dublin, Ireland. 10th April 1996. Oral presentation (a) and poster.
   Awarded the RCSI Sheppard Trust Prize for best poster communication with oral summary by a postgraduate student.

7. The 48th Irish Universities Colloquium, University of Limerick, Limerick, Ireland. 5th-7th June 1996. Poster presentation (c).

8. The 33rd "R & D Topics in Analytical Chemistry" Meeting, Nottingham Trent University, Nottingham, England. 22nd-23rd July 1996. Poster presentation (d).

9. Research day, Royal College of Surgeons, Dublin, Ireland. 2nd April 1997. Oral presentation (b).
   Awarded the RCSI Council Prize for best oral communication by a postgraduate student.

10. HPLC '97, National Convention Centre, Birmingham, England. 22nd-26th June 1997. Oral presentation (c).

11. The 49th Irish Universities Colloquium, Dublin City University, Dublin, Ireland. 25th-27th June 1997. Oral presentation (c).

Poster Presentation Titles

(a) HPLC Analysis of Taurine in Human Plasma using Pre-column Extraction and Derivatisation.

(b) Development of a HPLC Method for the Analysis of Taurine in Human Plasma using Pre-column Derivatisation.

(c) Analysis of Aspirin and Novel Aspirin Analogues by HPLC.

(d) Determination of Aspirin and its Metabolites in Plasma by Column-Switching Liquid Chromatography using on-line Solid-Phase Extraction.

Oral Presentation Titles

(a) Determination of Drugs by Column-Switching Liquid Chromatography using On-Line Solid-Phase Extraction.

(b) Analysis of Aspirin and Salicylic Acid in Plasma by Column-Switching Liquid Chromatography using On-Line Solid-Phase Extraction.

(c) Design of a Column-Switching Liquid Chromatography System using On-Line Solid-Phase Extraction for the Determination of Aspirin and Salicylic Acid in Plasma.

PUBLICATIONS

ARTICLES

1. "High Performance Liquid Chromatographic Determination of Taurine in Human Plasma using Pre-Column Extraction and Derivatisation"
   Gillian P. Mc Mahon, Richard O'Kennedy and Mary T. Kelly,
   Journal of Pharmaceutical and Biomedical Analysis **14**, 1287-1294 (1996).

2. "Determination of Aspirin and Salicylic Acid in Human Plasma by Column-Switching Liquid Chromatography using On-Line Solid-Phase Extraction"
   Gillian P. Mc Mahon and Mary T. Kelly,
   Analytical Chemistry **70(2)**, 409-14 (1998).

3. "Determination of Aspirin and Salicylic Acid in Transdermal Perfusates"
   Gillian P. Mc Mahon, Shane J. O'Connor, Desmond J. Fitzgerald, Sylvie Le Roy and Mary T. Kelly,
   Journal of Chromatography B Biomedical Applications **707(1-2)**, 322-7 (1998).

4. "How safe are your hands?"
   Gillian P. Mc Mahon,
   Health & Safety Magazine (National Irish Safety Organisation) **February**, 36-37 (1995).

PUBLICATIONS

PRINTED ABSTRACTS

1. "Determination of Drugs by Column-Switching Liquid Chromatography using On-Line Solid-Phase Extraction"
   Gillian P. Mc Mahon and Mary T. Kelly,
   Journal of the Irish Colleges of Physicians & Surgeons **25(2)**, 133 (1996).

2. "Design of a Column-Switching Liquid Chromatography System using On-Line Solid-Phase Extraction for the Determination of Aspirin and Salicylic Acid in Human Plasma"
   Gillian P. Mc Mahon and Mary T. Kelly,
   Journal of the Irish Colleges of Physicians & Surgeons **26(4)**, 302 (1997).

3. "Design of a Column-Switching Liquid Chromatography System using On-Line Solid-Phase Extraction for the Determination of Aspirin and Salicylic Acid in Human Plasma"
   Gillian P. Mc Mahon and Mary T. Kelly,
   Irish Chemical News **11(2)**, 59 (1997).

Appendix E

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High-performance liquid chromatographic determination of taurine in human plasma using pre-column extraction and derivatization[1]

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ELSEVIER

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High-performance liquid chromatographic determination of taurine in human plasma using pre-column extraction and derivatization[1]

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Received for review 13 September 1995; revised manuscript received 13 November 1995

Abstract

Plasma samples (100 μl) were treated with 150 μl of acetonitrile and centrifuged at 5800*g* for 10 min and 50 μl of 10 mM borate buffer (pH 9.2) were added to the supernatant solution. This was followed by the addition of a 50 μl aliquot of 5 mM fluorescamine in acetonitrile and immediate vortex mixing. A 20 μl sample was injected on to a reversed-phase HPLC system using a Bondclone C-18 10 μm analytical column (300 mm × 3.9 mm). The mobile phase was tetrahydrofuran–acetonitrile–phosphate buffer (15 mM, pH 3.5) (4:24:72, v/v/v). The taurine derivative was detected by measuring the UV absorbance of 385 nm. Platelet-poor plasma samples were spiked with known amounts of taurine and inter- and intra-assay calibration curves were obtained. The method was applied to the determination of taurine in platelet-rich plasma.

Keywords: Derivatization; Fluorescamine; Human plasma; Reversed-phase chromatography; Taurine

1. Introduction

Taurine is a naturally occuring β-sulphonated amino acid that is not incorporated into proteins, but found free or in some simple peptides [1]. It has been implicated in many physiological functions, pharmacological actions [2] and pathological conditions. Among the physiological roles attributed to taurine are membrane stabilization [3], antioxidation [4,5], neuromodulation [6] and regulation of calcium homeostasis [7]. Although intracellular taurine concentration is stringently controlled [8], plasma levels are altered during trauma [9], sepsis [10] and cancer [11]. Recent evidence suggests that the level of taurine in plasma may be a useful indicator of myocardial infarction [12]. As the role of taurine in various disease states becomes more widely recognized, the need for a simple, rapid assay for routine plasma taurine estimation becomes more important.

* Corresponding author.

[1] Presented at the Fifth International Symposium on Drug Analysis, September 1995, Leuven, Belgium.

SSDI 0731-7085(95)01697-X

Recent HPLC methods involving derivatization for the determination of taurine have employed *ortho*-phthalaldehyde (OPA), which requires the presence of a thiol such as mercaptoethanol to ensure that derivatization occurs instantaneously. Although taurine has been determined in this way with both ultraviolet (UV) absorbance [13] and fluorescence detection [14], there are stability problems associated with this reagent. Dansyl chloride has also been used to determine taurine [15], but this derivatization forms many side products, requires quenching and has a long reaction time. Taurine has been determined by derivatization with dansyl chloride, forming a derivative that absorbs in the visible region [16], but the reaction requires high temperatures and the presence of salts can have detrimental effects on the reaction yield.

Other available derivatizing agents for amino acids include phenyl isothiocyanate, but the yield can be adversely affected by the presence of salts, divalent cations and buffers [17]. 9-Fluorenylmethyl chloroformate forms stable derivatives, but hydrolysis products of the reagent interfere unless removed prior to analysis [17].

Fluorescamine was first introduced for the determination of primary amines and amino acids in 1972 [18] and has been used for the quantitation of taurine (Fig. 1) using fluorescence detection [19]. In this work, UV absorption at 385 nm was chosen because whereas the fluorescence intensity may decrease over a few hours, the absorbance remains unchanged for up to 1 week. Fluorescamine was selected because its reaction with primary amines proceeds instantaneously at ambient temperature in alkaline medium, the reagent and its major hydrolysis products do not interfere with UV detection and the derivatives are stable.

2. Experimental

2.1. Chemicals

Taurine (99%) and fluorescamine (98%) were obtained from the Aldrich Chemical (Gillingham, Dorset, UK). All other amino acids were obtained from BDH Chemicals (Poole, Dorset, UK), as were perchloric acid (70%), disodium tetraborate and potassium dihydrogenphosphate. Boric acid was purchased from Merck (Darmstadt, Germany). Acetonitrile, methanol and tetrahydrofuran were of HPLC grade and were purchased from Labscan (Dublin, Ireland). Super-purity acetonitrile from Romil Chemicals (Loughborough, UK) was used for the deproteinization step. Water was deionized using an Elgastat purification system.

2.2. Materials

Hypersep C-18 cartridges (200 mg, 3 ml) were received as a gift from Shandon Scientific (Runcorn, UK). Anion- (SCX) and cation- (PRS) exchange columns (200 mg, 3 ml) were kindly donated by IST (Mid-Glamorgan, UK). Microcon-3 concentrators and Micropure separator inserts were purchased from Amicon (Stonehouse, UK).

2.3. Preparation of reagents and standard solutions

Taurine standard solutions were prepared daily from a stock aqueous solution of 1 mg ml^{-1} that was prepared on a weekly basis. Fluorescamine solution (5 mM) was prepared in acetonitrile and kept at room temperature. Such a solution is stable for 12 weeks [20]. Borate buffer was prepared by adjusting 100 mM disodium tetraborate solution to pH 9.2 with 10 mM boric acid. The 15 mM phosphate buffer was made up each week by dissolving potassium dihydrogenphosphate in water and adjusting the pH to 3.5 with phosphoric acid. The mobile phase was tetrahydrofuran–acetonitrile–phosphate buffer (pH 3.5) (4:24:72, v/v/v). After mixing, the pH of the mobile phase was

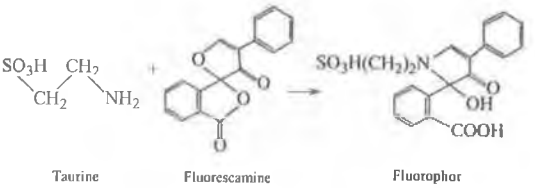

Fig. 1. Derivatization of taurine with fluorescamine.

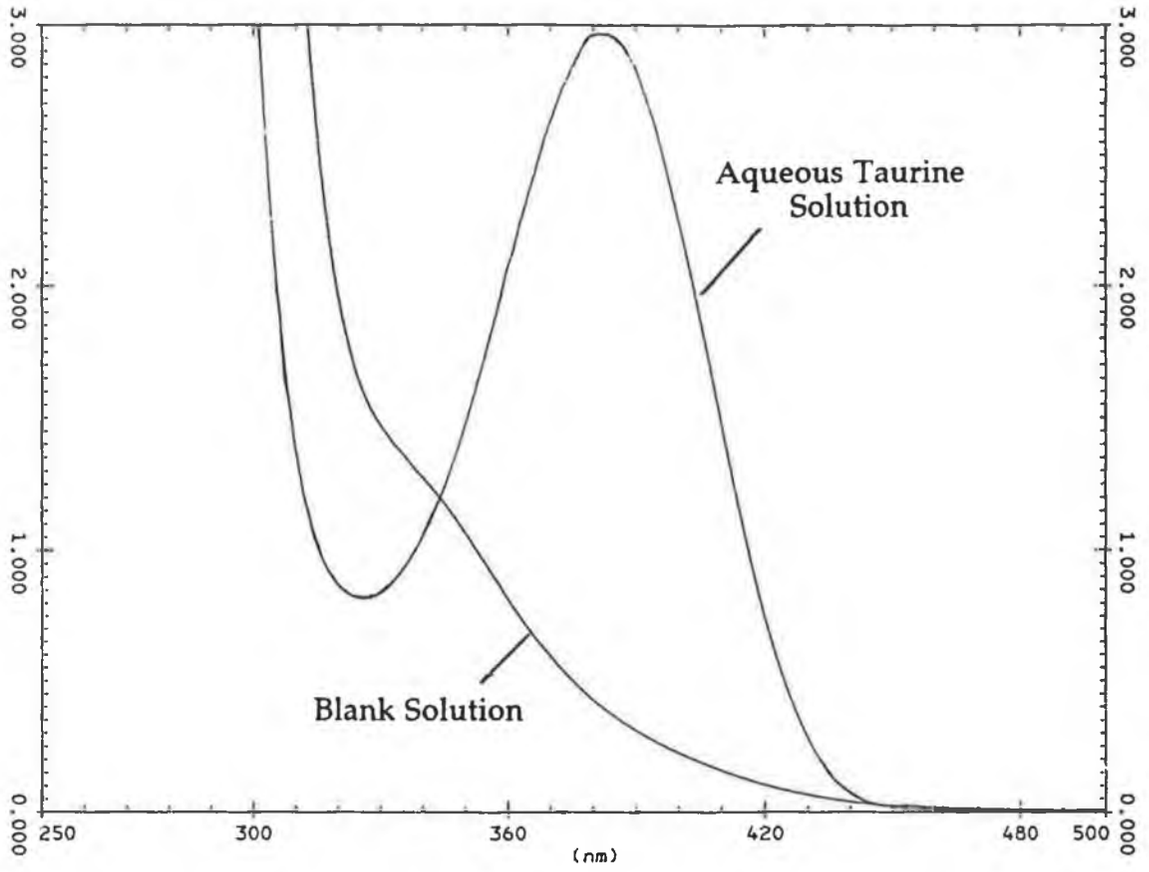

Fig. 2. UV absorbance spectrum of taurine derivative.

assessed, then it was filtered under vacuum through a 0.45 μm Millipore filter and sonicated for 20 min.

2.4. HPLC system

The high-performance liquid chromatograph was equipped with a Waters (Milliford, MA, USA) model 510 dual-piston pump, a Waters Model 486 tuneable absorbance detector and a Waters model 746 data module. The Rheodyne injection port (Cotati, CA, USA) was fitted with a 20 μl loop. A C-8 guard column was fitted prior to the Bondclone C-18 10 μm stainless-steel analytical column (300 mm × 3.9 mm i.d.). The flow rate of the eluent was 1 ml min^{-1}, the system pressure was approximately 1100 psi and all measurements were made at ambient temperature. UV detection was carried out at 385 nm, the maximum absorbance wavelength for the taurine derivative (Fig. 2).

2.5. Sample preparation

Blood samples were taken from fasting volunteers in glass tubes containing sodium citrate as anti-coagulant. Large-bore butterfly syringes were used so as to minimize cell damage, since this can result in platelet ruption and hence give rise to falsely elevated levels of basal taurine present in the plasma. Platelet-rich plasma (PRP) was obtained by centrifugation for 5 min at 170g at room temperature. Platelet-poor plasma (PPP) was obtained by centrifugation for 15 min at 1500g at room temperature. Care was taken during pipetting so as not to disturb the buffy coat layer.

For the preparation of spiked platelet-poor samples, 5 μl of the taurine standard solution were added to 95 μl of plasma to give standards with 0, 5, 10, 20 and 30 μg ml^{-1} of taurine added. Each sample (100 μl) was treated with 150 μl of super-purity acetonitrile, vortex mixed and centrifuged for 10 min at 5800*g*. Borate buffer (50 μl, 100 mM, pH 9.2) was added to adjust the supernatant to approximately pH 9. Then 50 μl of fluorescamine in acetonitrile (5 mM) were added and the solution was immediately vortex mixed. Samples were analysed on the HPLC system within 6 h.

3. Results and discussion

3.1. Optimization of protocol

3.1.1. Optimization of sample preparation

Initial experiments centred on ultrafiltration as a method of sample preparation. Microcon-3 concentrators with a molecular mass cut-off of 3000 were used in conjunction with Micropure separator inserts for removal of large molecules prior to derivatization and analysis. The centrifugation time was found to be very long (appproximately 140 min) and, although there was a concentrating effect, few of the lower molecular mass interferents were removed. Clean-up with reversed-phase solid-phase extraction cartridges resulted in a large dilution factor and poor clean-up and recovery. With the anion- and cation-exchange columns, the aim was selectively to retain or selectively to elute the taurine. It was not possible to retain the taurine on the columns under any circumstances and, in fact, extra impurities were introduced. Perchloric acid proved to be an efficient deproteinization agent, but it was subsequently difficult to raise the pH reproducibly prior to derivatization. Boiling was found to give poor reproducibility and methanol was required in a 3:1 ratio to the sample in order to effect complete precipitation of proteins. Deproteinization by acetonitrile proved to be the most facile and reproducible method of sample preparation and was the easiest to execute.

3.1.2. Optimization of reaction conditions

The pH at which the derivatization of taurine takes place is crucial to the reaction. The pH must be $\geqslant 8.5$, and the optimum is 9. Fluorescamine was dissolved in acetonitrile because of its compatibility with the deproteinized supernatant and its suitability as a non-hydroxylic but water-miscible solvent. Immediate mixing is essential for maximum response.

3.1.3. Optimization of mobile phase

The original mobile phase consisted of acetonitrile and phosphate buffer. Originally, this was optimized with the composition acetonitrile–phosphate buffer (pH 2.5, 15 mM) (35:65, v/v). Although this gave good chromatography for aqueous standards, in plasma samples taurine coeluted with other components. Methanol was used as an organic modifier at various ratios with little success, but tetrahydrofuran changed the selectivity, which improved the resolution between taurine and other endogenous compounds. Hence the final composition was tetrahydrofuran–acetonitrile–phosphate buffer (15 mM) (4:24:72, v/v/v). A variety of pH values were examined, 1.6, 2.5, 2.8, 3.5 and 4.0, and at pH 3.5 taurine was adequately separated from other closely eluting amino acid derivatives such as alanine, arginine, asparagine, aspartic acid, glutamic acid, glutamine, glycine, serine, threonine and valine.

3.2. Quantitative analyses of standards

3.2.1. Calibration and calculation

The spiked samples of platelet-poor plasma were quantified by external standardization. The slope and intercept of the calibration graphs were determined by unweighted linear regression of the taurine peak height versus the concentration of taurine added.

3.2.2. Precision

Precision was defined in terms of the variability between batches (inter-assay) and within batches (intra-assay). Inter-assay variation was assessed singly in four replicate runs covering the concentration range 5–30 μm ml^{-1}. Intra-assay variability was determined in quadruplicate in the same

Table 1
Precision data

Amount added (μg/ml)	Mean amount found ± SD (μg/ml)	RSD (%)	Difference between added and found (%)
Intra-assay (repeatability)			
5 ($n=3$)	4.56 ± 0.41	9.01	−8.87
10	10.30 ± 0.66	6.38	−3.03
20	20.50 ± 0.59	2.90	−2.50
30	29.63 ± 1.17	3.95	+1.21
		Mean: 5.56	Mean: 4.38
$y=350.28x-192.77$			
$r=0.9998$			
Inter-assay (reproducibility)			
5	4.82 ± 0.26	5.33	+3.56
10	10.22 ± 0.58	5.69	−2.21
20	20.00 ± 0.69	3.43	−0.02
30	29.95 ± 0.31	1.05	+0.15
		Mean: 3.87	Mean: 2.75
$y=347.74\ (\pm 19.85)x-179.11\ (\pm 125.78)$			
$r=0.9995 \pm 0.0002$			

concentration range. The precision of the method was described by the mean relative standard derivation (RSD), and this was found for taurine when the peak heights were interpolated as unknowns on the regression lines. For inter-assay variation (reproducibility), the interpolations were based on the four regression lines generated from the four replicate runs, and for intra-assay variation (repeatability), the interpolations were based on a single regression line generated from the quadruplicate run. Because human plasma contains a basal level of taurine, platelet-poor unspiked plasma shows a small peak in the chromatogram that was assumed to correspond to taurine. An unspiked platelet-poor sample was run with each calibration and the height of the taurine peak was subtracted from the taurine peak in each of the spiked samples. Precision data, calculated on the basis of the subtracted results, are presented in Table 1, and they demonstrate that the reproducibility (mean RSD = 3.87%) and repeatability (mean RSD = 5.56%) of the methods are within accepted values for clinical analyses. These results were compared with data from unsubtracted values (i.e. where the peak in the blank sample was not subtracted from the spiked standards), and it was found that the overall precision values were higher (4.08% and 6.75% for reproducibility and repeatability, respectively) and there was greater variability in precision amongst individual values.

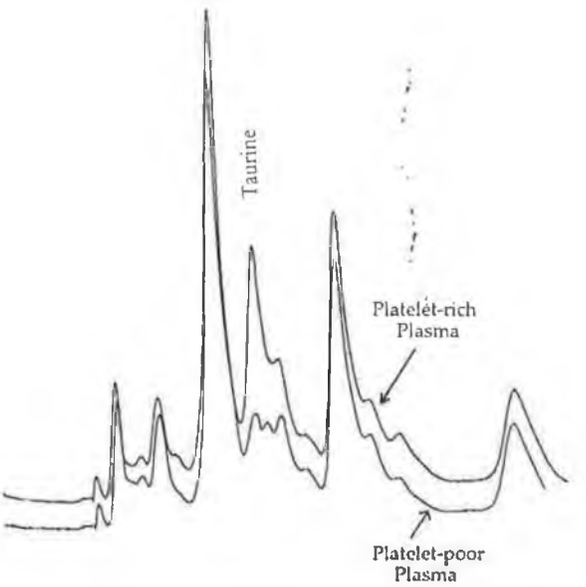

Fig. 3. Platelet-poor and platelet-rich plasma from one volunteer. Column, Bondclone C-18, 10 μm, 300 × 3.9 mm i.d.). Mobile phase, tetrahydrofuran–acetonitrile–phosphate buffer (15 mM, pH 3.5) (4:24:72). Sample preparation as described in text.

Table 2
Comparison of previous methods and results

Ref.	Derivatizating agent/ detection method	Method of collecting and treating blood	Taurine levels found ($\mu g/ml^{-1}$)
[21]	OPA/fluorescence	*Human:* Antecubital vein, vacutainer 7 ml glass tubes with EDTA. PRP: 1000*g*, 15 min, 4°C PPP: 12 100*g*, 30 min. 4°C	PPP: 5.6 ± 0.5 PRP: 16–19 (PC: 200–450 000 per ml plasma)
[22]	OPA/fluorescence	*Human:* Method not quoted	Quoted: 7.4 ± 1.5
[23]	OPA/fluorescence	*Human:* Heparinized tubes, 2000*g*, 10 min	5.0 ± 0.9
[24]	OPA/UV	*Rat:* Whole blood	41.9 ± 4.9
[13]	OPA/UV	*Rat:* Inferior vena cava using heparinized syringes *Chick:* Cardiac puncture, 1400*g*, 10 min	12.6 ± 1.6 13.5 ± 0.04
[25]	Dansyl chloride/ fluorescence	*Feline:* Chilled heparinized syringes, 2677*g*	3.0 ± 0.3

3.2.3. Linearity and accuracy

The correlation coefficient of the regression line for the mean intra-assay values was 0.9998 (subtracted data) and 0.9985 (unsubtracted data). Accuracy (presented in Table 1), as defined as the percentage difference between the amount added and the amount found by back-calculation, was usually less than 5% with mean values of 4.38% and 2.75% for within-batch and between-batch analyses, respectively.

3.2.4. Recovery

Recovery may be calculated in absolute or relative terms. In the calculation of absolute recovery, the peak response of an extracted standard is compared with that of unextracted standards that are prepared to the same theoretical concentration as the extracts. Relative recovery is calculated by comparing peak responses of extracted matrix standards against those of extracted aqueous standards. This procedure was used to account for the presence of acetonitrile in the sample to be injected. Using this method, the relative recovery of taurine from plasma was found to be 89.7%.

3.2.5. Limit of quantitation

The limit of quantitation was found to be 5 μg ml^{-1} taurine in plasma samples.

3.2.6. Selectivity

Taurine is adequately separated from endogenous plasma components, as can be seen in Fig. 3. A number of amino acids are inherently present in both platelet-poor and platelet-rich plasma and the ten thought most probable to interfere (as mentioned in Section 3.1.3) were subjected to the same extraction and separation conditions and, by adjustment of the mobile phase pH and aqueous-to-organic ratio, it was possible to resolve these compounds from the taurine peak. In fact, it is expected that the method described could be applied to the simultaneous determination of taurine and other amino acids.

3.3. Quantitative analyses of samples

The main difference between platelet-poor and platelet-rich plasma from the same volunteer is the height of the taurine peak (Fig. 3). The level of taurine in PRP is usually of the order of four times greater than in PPP [21], but this depends not only on the number of platelets present but also on how the sample was taken and treated. The role of platelets should be considered, since the platelet count can vary from 100 000 to 500 000 per ml of plasma with factors such as age, sex, health and sample treatment playing a part.

In the literature, it is often unclear whether or not platelets are present in plasma samples being analysed for taurine because protocols for blood collection may not be described or may often omit certain procedural steps (Table 2). Different syringes and collecting vials for the blood samples are used, different g-forces and temperatures are common during the centrifugation step and pipetting of the supernatant can cause cell rupture if sufficient care is not taken.

It was found that the platelets could be removed from plasma with minimum rupture if the samples are collected with the large-bore butterfly syringes. This procedure ensures that taurine in platelet-poor plasma is minimized. However, it should be noted that there is always a basal level of taurine present in human plasma. Other workers have circumvented this problem and obtained taurine-free, platelet-free plasma by using plasma from kittens raised on a taurine-free diet. Feline plasma naturally contains low concentrations of taurine (see Table 2).

Samples of PRP from three different sources were determined by interpolation of the peak heights as on a calibration curve (5–30 μg ml^{-1}). Results from the individual in Fig. 3 gave a value of 15.0 μg ml^{-1}, while values from two pooled PRP samples were calculated to be 16.4 and 18.5 μg ml^{-1}. All three results are within the expected range according to literature values.

4. Conclusions

Fluorescamine can be used for the pre-column derivatization of taurine and allows the estimation of taurine levels in platelet-rich plasma. Other analyses have difficult and labour-intensive sample preparation and some involve derivatization procedures that require quenching and/or the presence of co-solvents in order for the reaction to take place. Many are prone to interference from the derivatizing agent itself and there are discrepancies in the stability of derivatives. The method presented here offers a simple, efficient and rapid method for the determination of taurine in platelet-rich plasma, with comparable sensitivity to fluorescence methods, where a result is feasible within 1 h of taking blood from a patient. With escalating work into the quantitation of taurine in human plasma for medical purposes, the simplicity of this assay will be of great benefit to clinical research.

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Short communication

Determination of aspirin and salicylic acid in transdermal perfusates

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JOURNAL OF CHROMATOGRAPHY B

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Short communication

ı and salicylic acid in transdermal perfusates

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(HPLC) method has been developed for the simultaneous determination of
usates. The compounds were separated on a C_8 Nucleosil column (5 μm,
a mixture of water–acetonitrile–orthophosphoric acid (650:350:2, v/v/v) and
ples were in phosphate-buffered saline (PBS) and could be injected directly
ducible with inter-day R.S.D. values of no greater than 3.46 and 2.60% for
hod was linear over the concentration range 0.2–5.0 μg/ml and had a limit of
or certain samples, it was necessary to ensure that no transmembrane leakage
ases, a gradient was introduced by increasing the acetonitrile content of the
. The method has been applied to the determination of aspirin and salicylic
ıe compounds to mouse skin samples. © 1998 Elsevier Science B.V.

Keywords:

1. Introduc

Aspirin (A
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investigated as a route for continuous low-dose ASA administration. ASA itself is polar and can cross the skin without difficulty, but it is rapidly hydrolysed to salicylic acid (SAL) during transport. A 1993 study showed that ASA applied directly to the skin surface selectively inhibited the activity of cyclooxygenase in platelets [6]. ASA in a transdermal patch, at a lower dose, was also found to induce marked suppression of platelet cyclooxygenase, but the bioavailability of ASA was calculated to be only 20% [7]. Since ASA is rapidly hydrolysed to SAL, studies have focused on the synthesis of stable aspirin analogues and derivatives, the idea being that these molecules would traverse the skin efficiently, yielding ASA subcutaneously. The structure and polarity

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of the derivative are major determinants of transdermal permeability. The compounds investigated in this study were esters or anhydrides which were predicted to release the more polar ASA by their hydrolysis within the skin. While much HPLC methodology has been reported in the literature which can determine both ASA and SAL, most of the samples are in biological fluids (such as plasma and whole blood) [8–10], food [11,12] or tablet formulations [13,14]. Many of these involve protein precipitation and/or solvent extraction. However, this study required analysis of the compounds of interest in a different matrix and direct injection of the samples. It was also necessary to have a method which could be readily adapted to ensure that no transdermal leakage of intact prodrugs occurred. This paper reports the development and implementation of such a HPLC method for the analysis of ASA and SAL in transdermal perfusates.

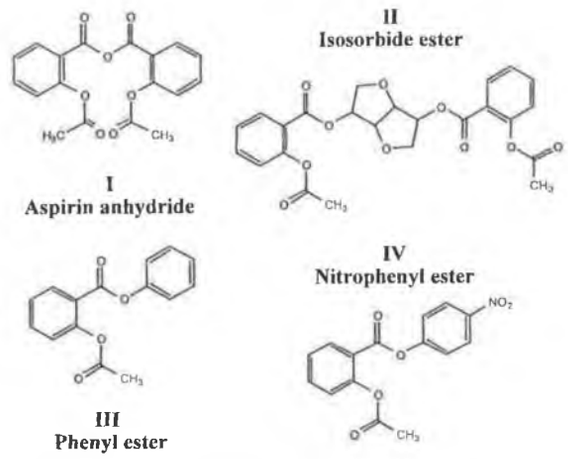

Fig. 1. Structures and names of the four aspirin prodrugs.

2. Experimental

2.1. Equipment

The high-performance liquid chromatograph was equipped with Waters (Millford, MA, USA) Models 501 single-piston and 510 dual-piston pumps, a Waters Model 486 tunable absorbance detector, a Waters Model 680 gradient controller and a Waters Model 746 data module. The Rheodyne injection port (Cotati, CA, USA) was fitted with a 20 μl loop. A Hypersil C_8 guard column (30 μm, 10×4.0 mm) was fitted prior to the Nucleosil C_8 analytical column (5 μm, 250×4.6 mm). The flow-rate of the eluent was 1 ml/min, UV detection was at 225 nm, attenuation was set to 32, chart speed was 0.5 cm/min and all measurements were made at ambient temperature.

2.2. Reagents and chemicals

Aspirin was supplied by Sigma Chemical Co. (Dorset, UK) and salicylic acid by BDH (Poole, UK). The four aspirin derivatives (**I**, **II**, **III** and **IV**) were prepared in-house (see Fig. 1). Acetonitrile and water were both HPLC grade and were purchased from Labscan (Dublin, Ireland). Analytical grade orthophosphoric acid (OPA) and polyethylene glycol (M_r 6000) came from BDH. Phosphate-buffered saline (PBS) sachets were obtained from Sigma Diagnostics (St. Louis, MO, USA). High purity ethanol (>99.8%) was purchased from Merck (Darmstadt, Germany).

2.3. Mobile phase

Eluent A was a mixture of water–acetonitrile–OPA (650:350:2, v/v/v). The aqueous component of eluent A had a pH of 2.0 and the mixture was pH 2.5* when the acetonitrile was added (ASA is most stable at this pH value) [15]. Eluent B was a mixture of acetonitrile–OPA (1000:2, v/v). Both eluents were filtered (0.45 μm filter) under vacuum and sonicated for 20 to 30 min to remove dissolved gases. The solvent program used was: time 0 to 6.5 min – 100% A, time 10.5 to 19.0 min – 70% A/30% B, time 22.0 to 28.0 min – 100% A, employing a linear gradient ramp.

2.4. Standard preparation

Stock solutions of ASA, SAL, **I**, **II**, **III** and **IV** were prepared by dissolving each of the compounds in acetonitrile to a concentration of 1 mg/ml. Working standards were prepared on a daily basis by dilution of the stock solutions with PBS. The ASA

and SAL standards were mixed together for the validation procedure and had final concentrations of 0.2, 0.5, 1.0, 2.0 and 5.0 μg/ml. The standard solutions of the derivatives were prepared individually in PBS down to a concentration of 2 μg/ml as required.

2.5. Sample preparation

The in vitro transdermal experiments were performed on fresh sha/sha mouse skins directly in contact with a 22 ml Franz cell reservoir of PBS at physiological pH and 37°C. Twenty mg of the prodrug being examined was diluted in 250 μl ethanol, mixed with 250 μl of 15% poly(ethylene glycol) (PEG) and topically applied to the 5 cm^2 area of skin. Aliquots were taken from the buffer vessel at 0, 2, 4 and 6 h, injected directly onto the HPLC system and analysed by comparison with external standards of the same matrix composition. Blank skin perfusates containing only formulation components, i.e. ethanol and PEG were run in parallel with the drug perfusates at all times to eliminate any formulation effects. At the end of the transdermal experiment for each prodrug, a sample from the top of the skin was taken by dissolving the remaining drug in 1 ml of acetonitrile. A 1 in 1000 dilution with PBS was usually required for these samples prior to analysis.

3. Results and discussion

3.1. Optimisation of protocol

The derivatives shown in Fig. 1 were selected for this study because they were found to inhibit platelet cyclooxygenase in vitro. Because of their hydrophobicity, special formulations have to be used in order to apply them to the skin. A number of possible formulations were tried and tested before deciding on 250 μl ethanol, mixed with 250 μl 15% PEG. When this was used for dissolution of the compounds, there was no interference from the formulation on the HPLC.

3.2. Validation of the method

3.2.1. Calibration

Calibration was based on regression analysis of concentration versus peak areas (which were found to correlate better than peak heights with concentration). Calibration curves were linear in the concentration range necessary for the detection of the compounds in the PBS buffer, i.e. 0.2 to 5.0 μg/ml. Since the release of ASA and/or SAL was the most important criterion and since none of the parent drugs permeated the skin intact, full validation was carried out only on the analysis of ASA and SAL in the PBS.

3.2.2. Precision

Precision was defined in terms of the inter-day variability (reproducibility) and intra-day variability (repeatability). Inter-assay reproducibility was assessed in four replicate runs on four consecutive days, covering the concentration range 0.2 to 5.0 μg/ml. Intra-assay repeatability was determined in quadruplicate on day two of the study in the same concentration range. The precision of the method was described by the mean relative standard deviation (R.S.D.) of the recovered amounts, determined by interpolation of the peak areas on the regression lines. Precision data are presented in Table 1, and

Table 1
Precision and accuracy data[a]

Amount added (μg/ml)	Amount found (μg/ml)		R.S.D. (%)		Accuracy (%)	
	ASA	SAL	ASA	SAL	ASA	SAL
Inter-assay (reproducibility)						
0.200	0.194	0.212	3.46	2.60	3.00	6.00
0.500	0.499	0.503	2.20	1.24	0.20	0.60
1.000	1.004	0.994	1.18	0.89	0.40	0.60
2.000	2.008	1.979	0.62	1.67	0.40	1.05
5.000	4.997	5.006	0.11	0.12	0.06	0.12
Intra-assay (repeatability)						
0.200	0.199	0.215	1.67	2.18	0.50	7.50
0.500	0.498	0.498	0.99	3.28	0.40	0.40
1.000	1.004	0.990	2.24	2.14	0.40	1.00
2.000	2.002	1.993	1.72	2.26	0.10	0.35
5.000	4.999	5.005	0.83	2.71	0.02	0.10

[a] $n=4$.

they demonstrate that the reproducibility (mean R.S.D.=1.51 and 1.30% for ASA and SAL, respectively) and repeatability (mean R.S.D.=1.49 and 2.51% for ASA and SAL, respectively) are well within accepted values for bioanalyses.

3.2.3. Linearity and accuracy

The correlation coefficient of the regression line for the mean intra-assay values was 0.9999 for both ASA and SAL. Accuracy (presented in Table 1), defined as the percentage difference between the nominal amount and the amount found, gave mean inter-day values of 0.81 and 1.67% for ASA and SAL, respectively, and mean intra-day values of 0.28 and 1.87% for ASA and SAL, respectively.

3.2.4. Limit of detection

The limit of detection was found to be 0.05 μg/ml for both compounds which corresponded to a peak which was three times the standard deviation of the baseline noise.

3.3. Quantitative analyses of ASA and SAL in perfusate samples

The retention times for ASA and SAL were 6.7±0.1 and 9.0±0.3 min, respectively. No ASA was found in the perfusates for prodrugs **II**, **III** or **IV**, but SAL was found in these samples. The presence of SAL in the perfusates would appear to indicate that the prodrugs did in fact break down in the skin, and that the rate of release of ASA was less than the rate of its subsequent hydrolysis to SAL. The poor permeation of the skin was probably due to the preferential solubility of the prodrugs in the formulation as opposed to the skin. Prodrug **I** (an anhydride) was significantly more susceptible to hydrolysis than the ester prodrugs, yielding ASA (and hence SAL) in the perfusate samples. Fig. 2 shows a chromatogram of (a) a blank perfusate and

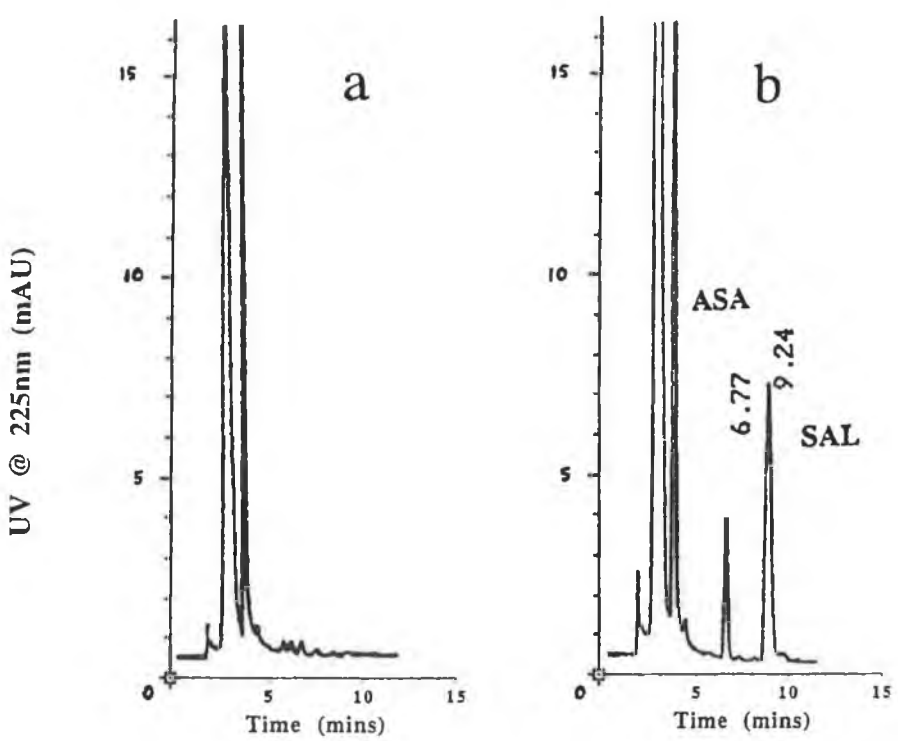

Fig. 2. Chromatograms of perfusate samples.

(b) a perfusate sample taken two hours after application of prodrug **I**. Fig. 3a,b show the transdermal results for both ASA and SAL in terms of their flux, i.e. micrograms of compound that traversed the skin per square centimetre of skin onto which the prodrug was applied. It can be seen that prodrug **I** delivered double the flux of ASA (Fig. 3a) and less SAL (Fig. 3b) when compared to the case where ASA was applied to the skin alone. The reason that prodrug **I** breaks down more readily than the other prodrugs could be explained by the fact that anhydride structures undergo hydrolysis more readily than ester structures.

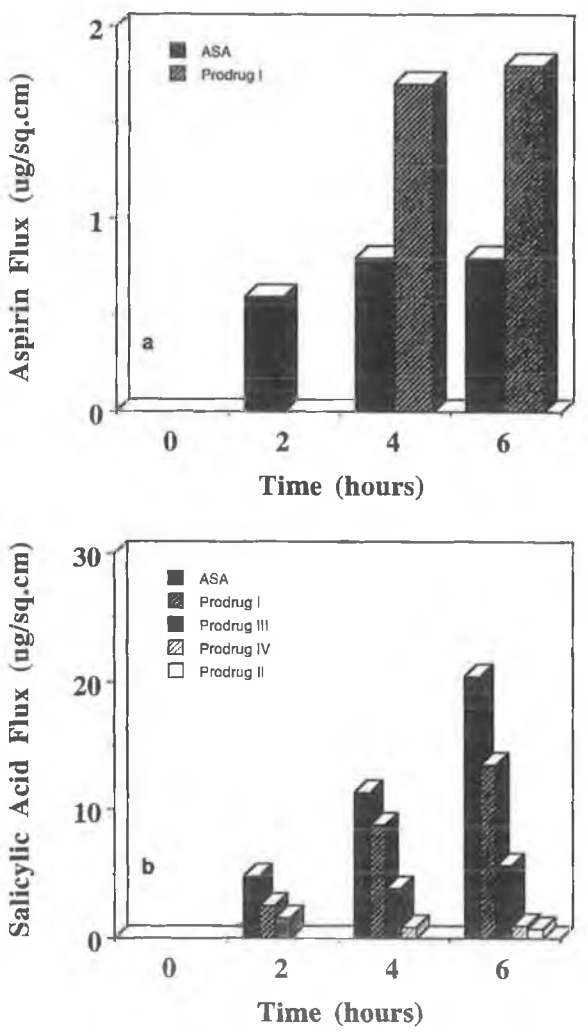

Fig. 3. (a) Transdermal fluxes ($\mu g/cm^2$) of ASA due to prodrug **I** and ASA itself. (b) Transdermal fluxes ($\mu g/cm^2$) of SAL due to prodrugs and ASA itself.

3.4. Qualitative analyses of prodrug transport and breakdown on skin surface

The retention times for the prodrugs using the gradient were as follows: prodrug **I** 18.4±0.2 min, prodrug **II** 19.1±0.3 min, prodrug **III** 20.2±0.3 min and prodrug **IV** 20.6±0.3 min. At no time did any of the prodrugs leak across the mouse skins intact. This was confirmed by gradient HPLC analysis of the perfusates by comparing the samples to external standards of the prodrugs down to a concentration of 2 μg/ml. This concentration would correspond to a transmembrane leakage of less than 0.25% of the prodrug. The top-of-skin samples for prodrugs **II**, **III** and **IV** contained only the corresponding parent compound. This indicated that these derivatives were not hydrolysed on the surface of the skin. Only prodrug **I** was hydrolysed on the surface of the skin to yield ASA.

4. Conclusion

The HPLC method presented was optimised and validated for the determination of ASA and SAL produced by prodrugs in transdermal samples. Samples could be directly injected and the analysis time was only ten minutes for determination of ASA and SAL. The method was easily adapted to allow determination of the nonpolar parent compounds using gradient elution. Using the method, four prodrugs were compared in terms of their ability to deliver ASA percutaneously. Evidence of hydrolysis of the ester compounds to ASA was seen, but it was not at a level sufficient to warrant further investigation of these compounds as aspirin prodrugs. The anhydride, prodrug **I**, did release significant amounts of ASA over time. In comparison to ASA alone, this compound produced more ASA and less SAL over the six hour time frame. Prodrug **I** is considered a suitable candidate for further investigation, and is currently undergoing more transdermal studies.

Acknowledgements

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Determination of Aspirin and Salicylic Acid in Human Plasma by Column-Switching Liquid Chromatography Using On-Line Solid-Phase Extraction

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Determination of Aspirin and Salicylic Acid in Human Plasma by Column-Switching Liquid Chromatography Using On-Line Solid-Phase Extraction

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A column-switching liquid chromatographic method is described for the simultaneous determination of aspirin and salicylic acid in human plasma. Blood samples are taken into chilled tubes containing a fluoride anticoagulant, and the plasma is isolated by centrifugation. Following a simple acidification step, a 200 μL aliquot of the sample is injected directly onto the HPLC system. The C-18 extraction column is washed with acidified water for 2 min, after which time the compounds are removed by back-flushing directly onto the analytical column (C-8 Nucleosil, 5 μm, 250 mm × 4.6 mm). The flow rate through both columns is 1 mL/min, and the analytes are quantified by measurement of their UV absorbance at 225 nm. The mobile phase is a mixture of water−methanol−acetonitrile−orthophosphoric acid (650:200:150:1 v/v/v/v). The method is linear in the concentration ranges 0.10−5.00 μg/mL for aspirin and 0.25−15.00 μg/mL for salicylic acid. Both compounds have a limit of quantitation of 0.10 μg/mL and a limit of detection of 0.04 μg/mL. Extensive stability tests have been carried out, and validation studies reveal the method to be reproducible and repeatable. Excellent recoveries from plasma obviate the need for an internal standard. The procedure is easier to execute and requires less sample handling than methods currently described in the literature. It has been successfully applied to the investigation of the levels of aspirin and salicylic acid in a healthy, nonfasting volunteer following a 600 mg oral dose of aspirin.

Aspirin (ASA) is the oldest known prescription drug, and each year in the United States alone, 80 billion aspirin tablets are taken.[1] ASA reduces pain, inflammation, and fever and also inhibits platelet aggregation in blood by blocking cyclooxygenase activity.[2,3] The plasma half-life of aspirin is only about 20 min because it is readily hydrolyzed to salicylic acid (SAL),[3,4] its principal metabolite. An analytical method that measures ASA in biological fluids should, therefore, be capable of measuring SAL also. Such assays are required for many reasons—in cases of aspirin poisoning, intolerance reactions, and side-effects and for metabolic and pharmacokinetic studies. Low-level determination of these compounds is usually carried out using HPLC methods, many of which have been described in a review by Kwong in 1987.[4] The mobile phases usually contain methanol or acetonitrile and have a low pH (normally 2.5−3.0), with UV detection in the range 225−240 nm. Small improvements in validation results (e.g., sensitivity) have been made over the years, but since 1980, HPLC methodology for ASA and SAL has only really changed in terms of applications. Scope for improvement lies mainly in the sample preparation step, the bottleneck in most analytical protocols. Assays for ASA and SAL have required sample cleanup procedures such as protein precipitation[5−11] or solvent extraction.[12−20] Usual methods of deproteinization involve the addition of an organic solvent such as acetonitrile,[5,7,11] ethanol,[8] or a strong acid such as perchloric acid.[6,9,10] Following centrifugation, the supernatant is usually dried and the residue reconstituted in mobile phase before injection. The solvent extraction procedure is usually effected with diethyl ether,[15] dichloromethane,[12,16,20] chloroform,[19] or hexane,[14] the organic extract evaporated, and the residue

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reconstituted in mobile phase. Nieder and Jaeger[17] used a mixture of hexane and ether to effect the extraction. Shen et al.[13] and Buskin et al.[18] also used a mixture of hexane and ether, but in order to avoid the evaporation step, they back-extracted the compounds into phosphate buffer before injection. This is because, during evaporation, loss of ASA and/or SAL is known to occur due to sublimation.[4,7,8,12-14,17-19] Another problem encountered in many of these methods is poor and/or variable recovery of the compounds because of losses during the protein precipitation procedure (which can be due to protein binding of the drug) or during the solvent extraction step. The use of internal standards can reduce the variability of recoveries, but it can also adversely affect results of analysis because of the added variability in the extraction recovery of the internal standard itself.[21] When column-switching is used in conjunction with HPLC, on-line cleanup of the biological sample is possible. A number of drugs have been analyzed in plasma employing this technique, such as bupivacaine,[22] finasteride,[23] dimiracetam,[24] carbapenem antibiotics,[25] midazolam,[26] fluconazole,[27] and quinidine.[28] However, ASA and SAL have not previously been quantified in plasma using column-switching. The method presented in this paper involves a simple, one-step, on-line solid-phase extraction system which gives good precision, accuracy, and recovery without the use of an internal standard. The method is economical, lends itself to automation, and also ensures the integrity of the plasma sample by taking precautions to minimize in vitro hydrolysis of ASA to SAL.

EXPERIMENTAL SECTION

Apparatus and Chromatography. The high-performance liquid chromatograph was equipped with Waters (Milford, MA) Models 501 single-piston and 510 dual-piston pumps, a Waters Model 486 tunable absorbance detector, a Waters Model 680 gradient controller, and a Waters Model 746 data module. The VICI AG 10-port switching valve (Valco Europe, Schenkon, Switzerland) was fitted with a 200 μL loop. A Hypersil C-8 guard column (30 μm, 10 mm × 4.0 mm) was fitted prior to the Nucleosil C-8 analytical column (5 μm, 250 mm × 4.6 mm). The solid-phase extraction (SPE) column was a biocompatible PEEK cartridge system (10 mm × 4.3 mm) from Anachem (Luton, Bedfordshire, England). The cartridge was dry-packed with Hypersil C-18 material (30 μm) from Shandon HPLC (Runcorn, Cheshire, England). Eluent A was a mixture of water−OPA (1000:1 v/v) and had a pH of 2.5. Eluent B was a mixture of water−methanol−acetonitrile−OPA (650:200:150:1 v/v/v/v) with a pH* of 2.6. Both eluents were filtered (0.45 μm filter) under vacuum and sonicated for 20−30 min to remove dissolved gases. The flow rates of eluent A and eluent B through the columns were each 1 mL/min, UV detection was at 225 nm, and all measurements were made at ambient temperature. Under these conditions, the retention times of ASA and SAL were 11.5 ± 0.2 and 15.6 ± 0.3 min (n = 40), respectively.

Reagents and Chemicals. Aspirin was supplied by Sigma Chemical Co. (Dorset, England), as were all the drugs used in the interference study. Aspirin tablets (300 mg) for the volunteer study were from Bayer Ltd. (Dun Laoghaire, Dublin, Ireland). Salicylic acid was supplied by BDH (Poole, England). Acetonitrile, methanol, and water were HPLC grade and were purchased from Labscan (Dublin, Ireland). High-purity (99.999%) orthophosphoric acid (OPA) was obtained from Aldrich Chemical Co. (Milwaukee, WI).

Standards Preparation. Stock solutions of ASA and SAL were prepared by dissolving each of the compounds in acetonitrile to a concentration of 1 mg/mL. In acetonitrile, the stock solutions were found to be stable for at least 10 weeks when kept at room temperature. Working standards were prepared on a daily basis by dilution of the stock solutions with eluent A. This is because ASA and SAL, having pKa values of 3.5 and 3.0, respectively,[29] are stable at this pH. The ASA and SAL plasma standards were prepared together by spiking blank plasma with working standard solutions and had final concentrations of 0.1, 0.25, 0.8, 2.0, and 5.0 μg/mL for ASA and 0.25, 0.75, 2.4, 6.0, and 15.0 μg/mL for SAL. These concentrations were chosen because they covered the expected range of the volunteer study samples. The drugs for the interference study were prepared individually as required, and each had a final concentration of approximately 1 μg/mL.

Sample Collection and Storage. Blood samples were collected into chilled plastic collection tubes containing a mixture of fluoride and EDTA salts (2.7 mL Monovette Catalog No. 05.1073.100, Sarstedt Ltd., Wexford, Ireland). The chilled blood samples were centrifuged at 1500g for 10 min to harvest the plasma. If there was to be a time interval of more than a few minutes before analysis, the samples were frozen at −30 °C until required. Both fresh and thawed plasma were kept on ice at all times.

Sample Preparation. Two hundred fifty microliters of 0.2 M OPA was added to 250 μL of the chilled plasma sample within 10 min of centrifugation (if fresh) or within 10 min of thawing (if frozen) in order to minimize enzymatic hydrolysis of ASA to SAL. The mixture was vortexed for 20 s. Direct injection was usually possible at this stage, but some of the volunteer plasma samples, especially those obtained after the volunteer had eaten, appeared cloudy after vortex mixing, so a short centrifugation step (5800g for 3 min) was required. In order to treat all samples equally, this centrifugation step was introduced as part of the protocol for all samples.

Column-Switching. Two hundred microliters of the sample was loaded onto the loop with the valve in position 2 (Figure 1b). The valve was then turned to position 1, injecting the sample onto the extraction column (Figure 1a). During this time, the analytes of interest were retained on the extraction column, while endogenous components were removed to waste by the wash solvent. After 2 min, the valve was switched back to position 2, whereupon the ASA and SAL were back-flushed onto the analytical column for final separation and quantitation (Figure 1b).

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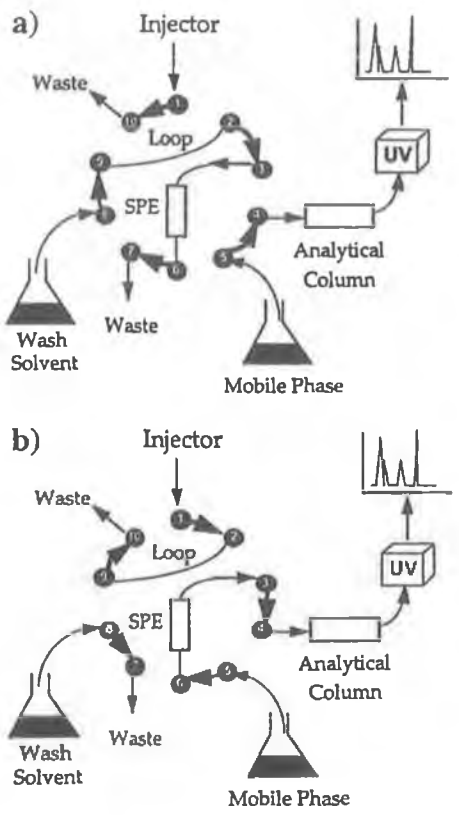

Figure 1. Diagram of column-switching apparatus. (a) Sample is loaded onto the SPE column for analytical column equilibrating. (b) Sample is desorbed from SPE column in back-flush mode and carried onto the analytical column for quantitation. Next sample is loaded onto loop.

RESULTS AND DISCUSSION

Optimization of Blood Collection and Sample Treatment. Since the nature of the anticoagulant used in conjunction with the blood collection procedure can have a dramatic effect on the number of interfering peaks in chromatograms of plasma extracts, it was decided to investigate four different anticoagulant types. Previous work has shown that the levels of plasma interferences following on-line SPE (using a C-18 precolumn) can be quite different when different anticoagulants and/or brands are used.[30] Hence, for this study, blood was taken from two volunteers using a butterfly syringe connected to each of four types of blood collection tubes (Li-heparin, EDTA, fluoride/EDTA, and citrate), and all eight blank plasma samples were subjected to the on-line SPE method and LC analysis. There was very little difference between the plasma profiles obtained, but the fluoride/EDTA mixture appeared to give a slightly cleaner baseline in the blank plasma chromatograms of both volunteers, and so it was chosen as the anticoagulant for this method. A significant advantage of the fluoride anticoagulant is that it inhibits the action of esterases, which catalyse in vitro hydrolysis of ASA, and for this reason a number of workers have used fluoride (in the form of NaF or KF salts) during the blood collection procedure.[6,8–10,12,14,16–19] In fact, Rumble et al.[10] compared three commonly used esterase inhibitors, i.e., ecothiopate, physostigmine, and fluoride, and found that KF inhibited the hydrolysis of ASA to the greatest extent. Nieder and Jaeger[17] also found KF to be the most effective inhibitor when they conducted a similar study.

In determining the optimum concentration for the diluting acid, three concentrations were investigated: 0.02, 0.2, and 2 M OPA. The 0.2 M acid gave the highest recoveries (90%) for both drugs when added in a ratio of 1:1 to the plasma sample.

Optimization of Analytical System. A number of packing materials, such as cyano, phenyl, C-1, C-8 and C-18, were examined in terms of their retentivities for ASA and SAL on the extraction column. The C-18 phase was found to be the most retentive for the compounds. Once this type of packing had been chosen, two dimensions of the cartridge were examined: 10 mm × 4.3 mm and 10 mm × 2.0 mm. The smaller column had approximately 37% the capacity of the larger column for an aqueous sample containing 0.2 μg/mL of both ASA and SAL, and a commercially available prepacked C-18 column (10 mm × 4.0 mm) gave recoveries of only 65% when compared to the larger extraction column. Therefore, a 10 mm × 4.3 mm PEEK cartridge packed with C-18 material was used throughout this work.

Stability. Kees et al.[7] found that a 1 mg/mL solution of ASA in acetonitrile was stable for at least 1 month (at 4 °C). In this study, it was found that the same concentration of ASA in acetonitrile was stable for at least 10 weeks either at room temperature (~20 °C) *or* under refrigeration (4 °C). Various aspects of the handling of the plasma samples and standards were also investigated in terms of minimizing degradation due to in vitro hydrolysis of ASA. Four plasma samples (each containing 2 μg/mL ASA) were subjected to different treatments. Sample 1 was acidified 1:1 with 0.2 M OPA and held on ice prior to injection; sample 2 was acidified 1:1 with acid and left at room temperature; sample 3 was diluted 1:1 with water and held on ice; sample 4 was diluted with water and maintained at room temperature. Upon analysis 3 h later, it can be seen from the chromatograms in Figure 2 that the amount of degradation (as evidenced by the hydrolysis of ASA to SAL) increased from sample 1 to 4, showing that acidity the sample is more important than temperature in terms of the stability of ASA. As a result of these findings, all samples were acidified and kept chilled to minimize hydrolysis of ASA. The effect of prolonged storage on the stability of spiked, acidified samples of ASA and SAL was then determined. The results of this experiment showed that the levels of ASA and SAL in these samples remained constant when the samples were stored at room temperature for up to 24 h, at 4 °C for up to 48 h, and at −30 °C through two freeze−thaw cycles. After these periods of time, and after more than two freeze−thaw cycles, the levels of endogenous interferences had increased to such an extent that quantitation of the ASA and SAL was adversely affected.

Quantitative Analyses of Standards. *Calibration.* Aliquots of drug-free plasma spiked to known concentrations of ASA and SAL were used for calibration, validation, and quantitation. Calibration was based on unweighted linear regression analysis of concentration versus peak areas (which were found to correlate better than peak heights with concentration). Calibration curves were linear in the concentration ranges 0.10−5.00 μg/mL for ASA and 0.25−15.00 μg/mL for SAL, with all values of correlation coefficient (r) greater than 0.999.

Precision. Precision was determined from measurements of the variability in results between batches (interassay) and within

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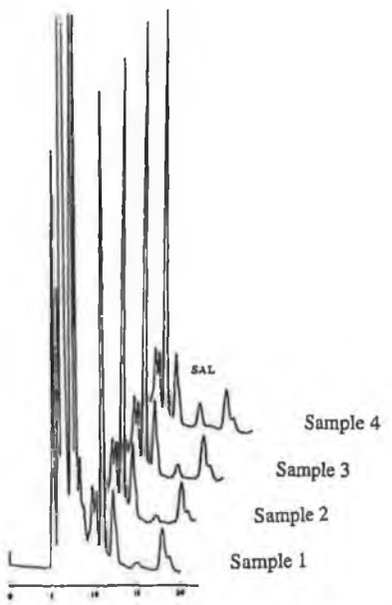

Figure 2. Effect of handling conditions on stability of ASA in plasma. Chromatograms showing the effect of handling conditions on the level of hydrolysis of ASA to SAL. Sample 1, plasma acidified 1:1 with OPA and held on ice; sample 2, plasma acidified 1:1 with acid and left at room temperature; sample 3, plasma diluted 1:1 with water and held on ice; sample 4, plasma diluted 1:1 with water and left at room temperature. Chromatographic conditions are as per text.

batches (intraassay) of spiked plasma calibration standards. It was measured as the mean relative standard deviation (RSD) of recovered concentrations (amounts found), determined by interpolation of the response values as unknowns on the regression lines. Interassay variation (reproducibility) was assessed in four replicate runs on four consecutive days in the concentration range 0.1–5.0 μg/mL for ASA and 0.25–15.0 μg/mL for SAL. The amounts found were calculated from the four individual regression lines generated from the four replicate runs. Intraassay variability (repeatability) was determined in quadruplicate on the fourth day of the study in the same concentration range. In this case, the amounts found were calculated from a single regression line based on the mean peak areas of the quadruplicate run.

Precision data are presented in Table 1, and they demonstrate that the reproducibility (mean RSD = 3.83% and 4.76% for ASA and SAL, respectively) and repeatability (mean RSD = 2.58% and 2.56% for ASA and SAL, respectively) are well within accepted values for clinical analyses.

Accuracy. Accuracy (presented in Table 1) was defined as the percentage difference between the nominal amounts and the amounts found and was calculated as described above. The mean between-batch accuracy values were 2.21% and 1.27% for ASA and SAL, respectively, while the corresponding mean within-batch values were 3.06% and 2.57%.

Recovery. Both absolute and relative recoveries were obtained for this method. Absolute recovery was defined as the response of a processed, spiked matrix standard expressed as a percentage of the response of a pure standard in mobile phase which had not been pretreated.[21] Two concentrations of each compound were chosen for examination (0.5 and 5.0 μg/mL), and the absolute recoveries were calculated by comparing the response in the spiked plasma samples to the response in aqueous samples injected directly onto the analytical column, i.e., without SPE and column-switching. Absolute recoveries for ASA were found to be 99% and 100% at 0.5 and 5.0 μg/mL, respectively; for SAL these results were 104% and 101%, respectively.

Table 1. Precision and Accuracy Data

amount added (μg/mL)	Intra assay (Repeatability), $n = 4$ amount found (±SD) (μg/mL)	RSD (%)	accuracy (%)
	Aspirin (ASA)		
0.10	0.091 ± 0.004	4.28	9.00
0.25	0.235 ± 0.005	2.29	6.00
0.80	0.799 ± 0.023	2.90	0.13
2.00	2.003 ± 0.037	1.85	0.15
5.00	5.000 ± 0.078	1.56	0.00
	Salicylic Acid (SAL)		
0.25	0.239 ± 0.006	2.58	4.40
0.75	0.714 ± 0.020	2.85	4.80
2.40	2.446 ± 0.075	0.07	1.92
6.00	6.088 ± 0.122	2.01	1.47
15.0	14.960 ± 0.339	2.26	0.27

amount added (μg/mL)	Interassay (Reproducibility), $n = 4$[a] amount found (±SD) (μg/mL)	RSD (%)	accuracy (%)
	Aspirin (ASA)		
0.10	0.093 ± 0.006	6.74	7.00
0.25	0.254 ± 0.018	7.11	1.60
0.80	0.789 ± 0.025	3.18	1.38
2.00	1.982 ± 0.036	1.80	0.90
5.00	5.009 ±0.016	0.31	0.18
	Salicylic Acid (SAL)		
0.25	0.238 ± 0.024	10.12*	4.80*
0.75	0.746 ± 0.041	5.55*	0.53*
2.40	2.412 ± 0.109	4.52	0.50
6.00	5.972 ± 0.186	3.11	0.47
15.0	15.008 ± 0.077	0.51	0.05

[a] Asterisk indicates $n = 3$.

Relative recovery was defined as the analyte response measured from matrix, i.e., plasma, as a percentage of that measured in water.[21] The relative recovery for this study was calculated for both analytes at the two concentrations in two different plasma sources on two different days, and for ASA these were determined to be 101% and 94% at 0.5 and 5.0 μg/mL, respectively; for SAL these results were 88% and 90%, respectively. The fact that the absolute recovery values appear to be slightly greater that the relative recovery values may be accounted for by the fact that, with direct injection, the compounds are loaded onto the column in the mobile phase, whereas with column switching, the compounds are loaded in 100% aqueous solution. For relative recovery, by comparing aqueous and plasma standards, differences arising from variation in the method of sample introduction are eliminated, and what is demonstrated is the extent to which the matrix affects recovery. Examination of the relative recovery values reveals that the recovery of SAL from the matrix is less than that for ASA, which may be accounted for by the fact that SAL is highly protein bound.

Sensitivity. The limit of quantitation (LOQ) was taken as the lowest point on the calibration curve and was, therefore, 100 ng/mL for both ASA and SAL. Certain validation parameters were carried out at this concentration in the same manner as for the calibration curve. The mean interday RSDs at the LOQ were 4.0%

Table 2. Drugs Used in Interference Study

barbitone	imipramine
butobarbitone	nitrazepam
caffeine	phenytoin
8-chlorotheophylline	pindolol
clonazepam	prazosin
cocaine	propranolol
diazepam	quinidine
flurazepam	theophylline
furosemide	xylazine
hydralazine	

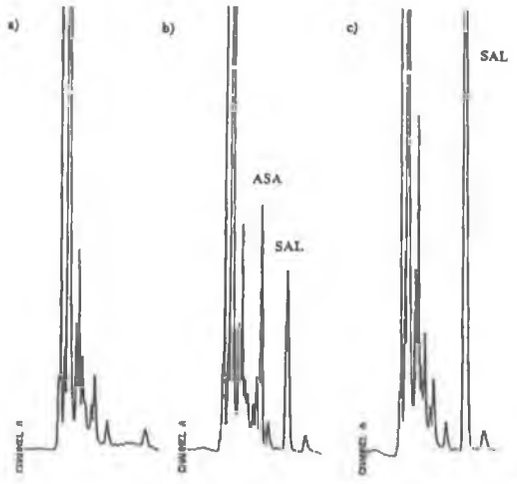

Figure 3. Chromatograms of plasma from a volunteer before and after administration of 600 mg of ASA. (a) Plasma from volunteer immediately prior to administration of ASA. (b) Plasma sample taken 13 min after administration of ASA, concentration of ASA = 2.55 μg/mL and concentration of SAL = 3.43 μg/mL. (c) Plasma sample taken 5 h after administration of ASA, concentration of SAL = 13.98 μg/mL. Chromatographic conditions are as per text.

and 14.3% for ASA and SAL, respectively; the corresponding intraday values were 18.4% and 11.7%. These values are within accepted limits for biopharmaceutical analyses. Interday relative recovery values (against aqueous spiked standards) were found to be 99.3% and 84.4% for ASA and SAL, respectively, and the corresponding intraday values were 116.2% and 91.9%. The limit of detection (LOD) in plasma was found to be 40 ng/mL for each of the analytes. In aqueous samples, the LOD values were 10 and 20 ng/mL for ASA and SAL, respectively.

Selectivity. According to Karnes et al.[21] and Shah et al.,[31] the matrix should not interfere with the analysis of the compounds, and this should be demonstrated in six different sources. Hence, drug-free plasma from six different individuals was examined for endogenous interferences, and in no case were any peaks found that coeluted with ASA. In some sources of plasma, a small

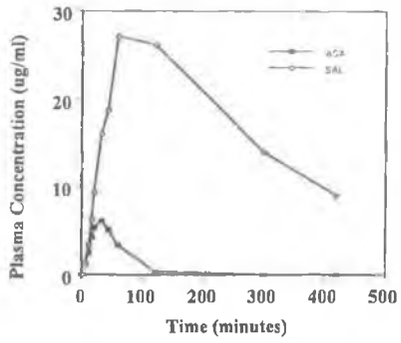

Figure 4. Concentration–time curve of ASA and SAL in a male volunteer following oral administration of 600 mg of ASA.

endogenous peak was found that had the same retention time as SAL, but its response always corresponded to a concentration of less than the LOD of SAL. A number of commonly coadministered and/or available drugs—listed in Table 2—were also investigated for possible coelution with either ASA or SAL, and of these, only xylazine (which has mainly veterinary uses) and prazosin were found to interfere.

Ruggedness. Using a solution containing 2 μg/mL of both analytes, a number of parameters were examined to demonstrate the robustness of the method. Normal retention times for ASA and SAL were 11.5 ± 0.2 and 15.6 ± 0.3 min, respectively. Changes of ±10% in the flow rate of the wash solvent through the extraction column had no effect on the peak areas, whereas by increasing the flow rate by 10% through the analytical column, peak areas were decreased by 10%, and decreasing the flow rate by 10% increased the peak areas by the same amount. The wash time of the sample through the SPE column was incremented by ±5% and ±10%, but this was found not to affect the peak areas. Changes of ±5% of the acetonitrile content of the mobile phase had more of an effect on the retention times (±0.4 min for ASA and ±0.6 min for SAL) than the same changes in the methanol content. However, neither affected the size of the peak areas. The pH* of the mobile phase following the addition of the organic components was 2.6. By changing the pH* by ±0.1, the retention time for SAL, being more pH-sensitive, was more strongly affected than that of ASA (+0.1 min at pH* 2.5 and −0.3 min at pH* 2.7).

When the C-18 extraction column was changed after approximately 50 plasma injections, the new C-18 column yielded no difference in the recoveries of both components. Changing the guard column also had no effect on the chromatography.

Quantitative Analyses of Samples. A healthy male volunteer was given 600 mg of aspirin (in the form of two tablets) 45 min after he had eaten a meal. Blood samples taken immediately prior to the administration of ASA and at a number of time points over

Table 3. Results of Concentration-Time Curves from Other Studies

ref, year	amount of ASA given (mg)	ASA peak concentration (μg/mL)	SAL peak concentration (μg/mL)	time to reach ASA peak concn (min)	time to reach SAL peak concn (min)
this paper, 1997	600	6	27	33	60
ref 7, 1996	500	5	30	30	130
ref 15, 1985	600	8	40	30–40	90–100
ref 18, 1982	650	8	40	20	80
ref 19, 1980	600	14	45	22	80

7 h were subjected to extraction and analysis. A sample chromatogram is shown in Figure 3. The results were plotted on a concentration–time curve (Figure 4), and from this, the peak plasma concentrations were determined to be 6.2 (after 33 min) and 27.1 μg/mL (after 60 min) for ASA and SAL, respectively. The times taken to reach these peak concentrations were 33 and 60 min, respectively. These results are in general agreement with those found in the literature, and the only difference was that, in this study, SAL reaches its peak concentration faster than would be expected from previously published results, examples of which may be seen in Table 3. It is expected, however, that this discrepancy arises from the fact that rates of metabolism vary widely among individuals.

CONCLUSION

A new column-switching method has been developed for the analysis of ASA and SAL in plasma by on-line SPE in conjunction with HPLC. Only 300 μL of plasma is required for analysis, and the method could be easily automated via the 10-port switching valve. The HPLC method presented employs a simple and economical method of sample preparation, requiring little handling by the analyst, and is sufficiently reproducible not to require an internal standard. The assay is easier and more rapid, more stable, and more robust than current methodology.

(31) Shah, V. P.; Midha, K. K.; Dighe, S.; et al. *Pharm. Res.* **1992**, *9* (4), 588–92.

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