ANALYSIS OF PHARMACEUTICALS AND BIOMOLECULES USING HPLC COUPLED TO ICP-MS AND ESI-MS

by

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Abstract

Analysis of pharmaceuticals and biomolecules using HPLC coupled to ICP-MS and ESI-MS

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The work described within this thesis explores the use of HPLC coupled with ICP-MS and ESI-MS in order to develop novel methods which overcome specific analytical challenges in the pharmaceutical industry.

A membrane desolvation interface has been evaluated for coupling high performance liquid chromatography (HPLC) with inductively coupled plasma mass spectrometry (ICP-MS). Desolvation of the sample prior to reaching the plasma was shown to facilitate a versatile coupling of the two instrumental techniques, enabling chromatographic eluents containing up to 100 % organic to be used. This interface also allowed gradient elution to be used with ICP-MS.

Tris(2,4,6-trimethoxyphenyl)phosphonium propylamine bromide (TMPP) was used for the derivatisation of maleic, fumaric, sorbic and salicylic acids to facilitate determination by HPLC-electrospray ionisation tandem mass spectrometry (ESI-MS/MS) in positive ion mode. Improvements in detection limits post-derivatisation were achieved, and this method was successfully used for the determination of sorbic acid in a sample of Panadol^{TM}.

HPLC coupled with sector field inductively coupled plasma mass spectrometry (SF-ICP-MS) has been used for the determination of maleic, sorbic and fumaric acids after derivatisation with TMPP. This allowed $^{31}P^+$ selective detection to be performed for these compounds, which are normally undetectable by ICP-MS. Optimal reagent conditions for the derivatisation of 0.1 mM maleic acid were: 1 mM TMPP; 10 mM 2-chloro-1-methylpyridinium iodide (CMPI); 11 mM triethylamine. The efficiency of the derivatisation reaction was estimated to be between 10–20%. Detection limits, estimated as 3 times baseline noise, were 0.046 nmol for TMPP and 0.25 nmol for derivatised maleic acid, for a 5 μL injection.

Following on from this, a novel derivatising reagent, tris(3,5-dibromo-2,4,6-trimethoxyphenyl) phosphonium propylamine bromide (BrTMPP), was synthesised and subsequently characterised by proton NMR spectroscopy and ESI-MS. This was utilised to derivatise maleic acid, with a 9-fold increase in sensitivity gained when analysed by bromine selective detection as apposed to phosphorus selective ICP-MS. This derivatising reagent (BrTMPP) was also utilised to determine the degree of phosphorylation on phosphorylated peptides. A phosphorus containing carboxylic acid was successfully derivatised and the correct Br:P ratio was determined for this compound by ICP-MS. However, phosphorylated peptides were not successfully derivatised by BrTMPP. A combination of UV and phosphorus selective ICP-MS was also used to distinguish between phosphorylated and un-phosphorylated peptides after HPLC separation.

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Authors Declaration

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other university award.

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An advanced programme of study was undertaken including MSc accredited short courses in: Research Methods; Research Skills; Laboratory Based Teaching Methods and Practice. Relevant scientific seminars and conferences were regularly attended at which work was usually presented and several papers were prepared for publication. The research undertaken was regularly disseminated to GlaxoSmithKline and fellow research students at the University of Plymouth through oral presentations and research seminars. The author was also a Researcher in Residence at Ridgeway School, Plymouth, for the academic year 2003-2004. The author also took part in the UK GRAD programme in 2003.

Publications

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Oral Presentations

"Derivatisation of Carboxylic acids to enhance detection by Liquid Chromatography coupled to Electrospray Mass Spectrometry and Inductively Coupled Plasma Mass Spectrometry" A.J. Cartwright, E. H. Evans, P. Jones and J-C. Wolff. Paper presented at the 16th International Mass Spectrometry Conference, 31st Aug – 5th Sept 2003, Edinburgh, UK.

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"Determination of the degree of phosphorylation using LC-ICP-MS and LC-ESI-MS" A.J. Cartwright, E. H. Evans, P. Jones and J-C. Wolff. Paper presented at the Analytical Research Forum, 18th – 20th July 2005, Plymouth, UK.

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"Screening of Pharmaceuticals for Sulfur and other Heteroatoms using LC Coupled to High Resolution Sector Field ICP-MS" A.J. Cartwright, E. H. Evans, P.

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"Liquid Chromatography Coupled to High Resolution SF-ICP-MS and Electrospray Mass Spectrometry for the Determination of Phosphorylation Sites in Tryptic Digests of Peptides" A.J. Cartwright, E. H. Evans, P. Jones and J-C. Wolff. Paper presented at the European Winter conference on Plasma Spectrochemistry, 12th – 17th Jan 2003, Garmisch-Partenkirchen, Germany.

"Liquid Chromatography Coupled to Inductively Coupled Plasma Mass Spectrometry for the Determination of Phosphorylation Sites in Tryptic Digests of Peptides" A.J. Cartwright, E. H. Evans, P. Jones and J-C. Wolff. Paper presented at the European Analytical Science Conference, 25th March 2003, Stevenage UK.

"Derivatisation of Carboxylic Acids to Enhance Detection by Liquid Chromatography Coupled to Electrospray Mass Spectrometry and Inductively Coupled Plasma Mass Spectrometry" A.J. Cartwright, E. H. Evans, P. Jones and J-C. Wolff. Paper presented at the Analytical Research Forum, 21st – 23rd June 2003, Sunderland, UK.

"Synthesis of a halogenated phosphonium derivatising reagent to enhance detection of carboxylic acids using electrospray mass spectrometry and inductively coupled plasma mass spectrometry" A.J. Cartwright, E. H. Evans, P. Jones and J-C. Wolff. Paper presented at the Western Region of the Analytical Divisions 50th Anniversary, 14th July 2004, Plymouth, UK.

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Chapter One

Introduction

1.1 Analytical Challenges in the Pharmaceutical Industry

1.1.1 Research and Development of New Drugs

The development of our understanding of disease processes and the resulting discovery of new drugs for their treatment has improved the quality of life throughout the world. It is estimated the world-wide expenditures for ethical pharmaceuticals reached over $300,000,000,000 in 2000[1]. In the US during 2000, sales of prescription drugs amounted to $112,000,000,000. According to the Pharmaceutical Research and Manufacturer's Association[1], $25,600,000,000 was invested during 2000 on the research and development for the next generation of pharmaceuticals. Unfortunately, even with this level of effort dedicated to the eradication of disease, it is estimated that only 1/3 of all diseases known can be causally treated and cured.

The process of bringing a new medicine to market has evolved over the past 25 years. Increased focus on more complex diseases and the production of more sophisticated drug delivery systems have increased the time needed for drug research and development. Over the same period of time, increased legislation has led to longer clinical trial periods resulting in extended times for a drug to reach the market. It is estimated that the time taken to develop a new drug from the laboratory to FDA approval is between ten to fifteen years. For every new drug that is approved as a new medicine, it is estimated that approximately 5,000 fail.

This, together with the cost implications of the whole process, makes the development of a new drug a risky business. For these reasons, it is invaluable to a company that the process of research and development achieves as many new medicines in the shortest amount of time possible. This enables the manufacturing company to achieve the required sales to return the cost outlay whilst the drug is still under patent. Hence, much research is now being performed within pharmaceutical laboratories to improve and reduce the time taken to analyse samples to yield more confidence in the result in less time. This analysis can be anything from the initial drug candidates produced through combinatorial chemistry, to the biological fluids extracted throughout the phase I-III trials.

1.1.2 Protection of Intellectual Property Rights

Strong patent protection for pharmaceuticals drives medical progress by providing economic incentives for innovation. Without international respect for pharmaceutical patents, medical innovation would suffer. In fact, a 1988 study of 12 industries estimated that 65 percent of pharmaceutical products would not have been introduced without adequate patent protection[2]. Without intellectual property protection in the pharmaceutical industry, nearly two-thirds of the important medicines available today (chemotherapy drugs, clot-busters that save the lives of heart attack patients, AIDS medicines, drugs that save the lives of premature babies and many others) would not have been developed.

The underlying reason why pharmaceutical progress is dependent on intellectual property protection is the staggering cost of drug development. It costs an average of $500 million to develop a new medicine[1]. Perhaps in no other industry can an invention that costs so much to discover and develop be copied or reverse-

engineered so inexpensively, at a small fraction of the innovator's research and development costs. Without strong patent protection, pharmaceutical companies could not attract the investment needed to conduct this expensive, high-risk research.

Pharmaceutical manufacturers are facing increased risks related to counterfeit drug activities, including serious health risks, brand reputation risk, and adverse economic impact. Globalisation has sparked increased intellectual property rights (IPR) infringement activity. Consequently, IPR protection has become an urgent supply chain focus for multinational pharmaceutical companies producing and distributing medicines for global markets.

IPR infringement takes the form of counterfeit goods (unauthorised use of a registered trademark or tradename), pirated goods (unauthorised use of copyright) and black market goods (genuine goods manufactured in a foreign country bearing a trademark and imported without the consent of the trademark owner). Such illicit activity costs the pharmaceutical companies billions of dollars in lost revenue and creates the potential for unquantifiable harm to the company brand and reputation. The availability of counterfeit medicines also increases risks to public health.

Pharmaceutical products fall prey to IPR infringement because consumers are attracted to discounted prices, which provide criminals with financial incentives. Growing price differentials between the developed countries is causing the incidence of IPR infringement to increase.

To protect their brand name and company profile, pharmaceutical companies are taking IPR infringement very seriously. A major problem in controlling IPR infringement is identifying it in the first place. The easiest way to detect an

infringement can occur on the packaging of the counterfeit goods. This can include spelling mistakes on the packaging, or even on the material itself.

Many of the other methods of identifying infringement are to analyse the suspect material itself. These could be as simple as confirming that the active ingredient is that stated on the packaging to identifying fingerprints of excipients and impurities, to prove the material is not genuine. This process can be very specialised, and is highly secretive. For this reason, many pharmaceutical companies now have dedicated groups whose main responsibility is to confirm whether IPR has been infringed or not.

1.1.3 Quality assurance and regulatory requirements

Anyone working within the pharmaceutical industry will be aware that a great amount of their everyday work requires conformance of quality with standards dictated by various regulatory authorities[3]. These quality control procedures are essential to ensure the integrity of the product which will eventually be used by the patient. At all stages through drug production, from raw material testing through to testing of the final product, quality procedures must be adhered to.

The pharmaceutical industry has some of the most stringent quality assurance procedures to conform to. This is because the general public do not have the required equipment to test their product; so much trust is put onto the manufacturers of the product they are using. When the medicine is given to patients it must have been appropriately manufactured, tested and packaged to assure that[3]:

- It is the correct product.

- It is the correct strength/dosage.

- It has not degraded.

- It is free from harmful impurities and micro-organisms.

- It has not been contaminated.

- It is correctly labelled.

- It is properly sealed in a suitable container.

To assure the quality of the product, quality must be built into each stage of the manufacturing process. Any factor that may have an effect on the quality of the final medicinal product must be controlled. The philosophy of quality assurance is that batch to batch consistency should be maintained by reducing variability of all supporting processes[3]. Hence, if these procedures control all of these factors and trained personnel follow these procedures, then a product consistently meeting its predetermined specification should be produced.

According to the current International Conference on Harmonisation (ICH) guidelines, impurities and degradation products of pharmaceutical drug substances that exceed the threshold of 0.1% mass fraction relative to the active pharmaceutical ingredient (m/m) must be identified and qualified by appropriate toxicological studies[4]. This requirement has presented more challenges for pharmaceutical companies because methods have been developed for known target impurities, but many impurities may have been overlooked by the limitations of the analytical methods previously adopted. However, the introduction of Liquid Chromatography-Mass Spectrometry has resulted in increased confidence that no significant impurity has escaped attention.

1.2 Mass Spectrometry

Mass spectrometry is now widely accepted as a crucial analytical tool for the analysis of organic molecules. Although it is regarded as a spectroscopic technique, it does not rely on the interaction with electromagnetic radiation for the analysis. Rather, it is a micro-chemical technique relying on the production of characteristic ions, followed by separation and detection of those ions[3]. Due to its operation, mass spectrometry is a destructive technique, unlike other techniques such as nuclear magnetic resonance (NMR), infrared (IR) and Raman/UV spectroscopies. However, mass spectrometry is so sensitive that molecular weight and structural information can be provided on very small samples (attomolar (10^{-15} molar) amounts).

Mass spectrometry is used in a variety of ways. It can be interfaced with a wide variety of separation techniques (such as gas chromatography (GC), high performance liquid chromatography (HPLC) or capillary electrophoresis (CE) to name the most common ones) to give on-line analysis of mixtures. Its sensitivity allows detection, molecular weight determination and structural elucidation of minor components eluting from a column. As a quantitative technique it can be used as an assay tool, including the on-line monitoring for the optimisation of yields and minimisation of impurities[3].

The first mass spectrometer dates back to the work of J. J. Thompson in 1912, but the instrument that serves as a model for more recent mass spectrometers was built in 1932[5]. The ionisation source of the mass spectrometer produces charged particles of the analyte molecule. The source can be chosen to yield varying degrees of fragmentation, i.e. ranging from complete atomisation and ionisation to the formation of molecular ions. The mass spectrometer then sorts these ions out

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according to their mass to charge ratio (m/z). The mass spectrum is a record of the relative number of ions of different m/z which is characteristic of the analyte compound and its isomers[6].

Functionally, all mass spectrometers perform three basic tasks:

- Create gaseous ion fragments from the sample;

- Separate these ions according to their mass to charge ratio;

- Measure the relative abundance of ions at each mass.

To date, there is no universal design and configuration of mass spectrometer able to meet all analytical requirements[7]. For this reason, many different mass analysers and ionisation sources have been developed, with those most frequently used discussed in this introduction.

1.2.1 Mass analysers

The function of the mass analyser is to separate the ions produced in the ion source according to their mass/charge ratios. These ions are transported from the ion source to the analyser by a series of electrostatic lenses which accelerate and focus the ion beam.

1.2.1.1 Magnetic sector mass analysers

The magnetic sector analyser incorporates a magnetic field that causes the ions to be deflected along curved paths. As ions enter the magnetic sector analyser, they are subjected to a magnetic field applied parallel to the slits but perpendicular to the ion beam. This causes the ions to deviate from their initial path and curve in a circular fashion. A stable, controllable magnetic field (H) separates the

components of the ion beam according to momentum. This causes the ion beam to separate spatially and each ion has a unique radius of curvature or trajectory (R) according to its m/z. Only ions with a single m/z value will posses the correct trajectory that focuses the ion on the exit slit of the detector. By changing the magnetic field strength, ions with differing m/z values are focussed at the detector slit. To obtain a complete mass spectrum from a magnetic sector analyser, either the accelerating voltage (V) or the magnetic field strength (H) is varied. Each m/z ion from light to heavy is then focussed sequentially onto the detector, producing the mass spectrum.

1.2.1.2 Double focusing sector mass analysers

In a single-focusing magnetic sector instrument there is a lack of uniformity of ion energies, since the accelerating potential experienced by an ion depends on where in the source it is formed[7]. The result is peak broadening and low to moderate mass resolution.

Double focusing magnetic/electrostatic sector instruments use magnetic and electrical fields to disperse ions according to their momentum and translational energy[6]. An electrostatic deflection field is incorporated between the ion source and the mass analyser. Ions are accelerated out of the source and are forced into a narrow beam by a set of slits. In these devices, the electric sector acts as an energy analyser, while the magnetic sector acts as a mass analyser[8]. With such instruments, resolving powers of the order of 10^5 can be achieved.

1.2.1.3 Time of flight mass analysers

Time of flight (TOF) mass analysers operate in a pulsed mode rather than a continuous mode, so the ions are formed in the source as a discrete ion packet. These ion packets are then accelerated and introduced into a region that contains no external field[8]. This is known as the drift tube and is 30-100 cm long. The main principle behind TOF analysis is that if all ions with different masses are all accelerated to the same kinetic energy, with each ion acquiring a characteristic velocity depending upon its m/z ratio[7]. As a result, ions of different mass travel down the flight tube at different speeds, thereby separating spatially along the flight tube, with the lighter, faster ions reaching the detector up to 30 μs before the heavier ions.

1.2.1.4 Quadrupole mass analysers

The quadrupole mass analyser is formed by four parallel electrically conducting rods arranged in a square geometry[6]. Opposite pairs of rods are connected electrically. A voltage made up of two components is applied to the rods; the first component is a standard DC potential, whereas the second is an alternating radiofrequency (RF) component[8]. The result is the formation of an oscillating hyperbolic field in the area between the rods[6]. As ions pass through the z-axis of the quadrupole analyser, they experience traverse motion in the x- and y-planes and thus oscillate. If the ratio between the DC voltage and the RF field is kept constant, but both parameters are varied together, the ions of different m/z values can be analysed and a mass scan can be made[8].

The quadrupole mass analyser has certain advantages over magnetic sector instruments[8]. These are:

- It is relatively cheap to build

- It is smaller and lighter

- It is more robust

- More accuracy is achieved in computer control of rod voltages rather than magnetic fields

- Scanning is very fast

1.2.1.5 Ion traps

An ion trap is generally used in multi-stage mass spectrometry to help in characterisation studies. The ion trap can store ions for an extended period of time using electric and/or magnetic fields[8]. The most common traps consist of a central doughnut-shaped ring electrode and a pair of end-cap electrodes. A variable RF voltage is applied to the ring electrode, with the two end-cap electrodes connected to earth. Ions entering the trap through a hole in the end cap begin to oscillate[6]. The RF voltage is then increased causing ions of increasing m/z to destabilise and leave the trap, where they are detected.

1.2.2 Ionisation sources

The ion source is that part of the mass spectrometer that ionises the material under analysis. The ions are then transported by magnetic or electrical fields to the mass analyser. Techniques for ionisation have been key to determining what types of samples can be analysed by mass spectrometry. The function of the ionisation source is to produce an ion, or ions representative of the analyte molecule. The most common process of ionisation is the removal of an electron which produces a positively charged molecular radical ion[6].

$$M \longrightarrow M^{\cdot +} + e^-$$

Some ionisation sources also break down the analyte molecule into fragment ions, or in some cases totally dissociate the analyte molecule into its constituent atoms and ions. The way the ions are produced and the physical from in which the sample is presented to the ionisation source are the main differences between most ionisation sources. As there are many different ways to ionise samples, only the most frequently used techniques are discussed.

1.2.2.1 Electron ionisation

Electron ionisation (EI, formerly known as electron impact) is an ionisation technique widely used in mass spectrometry. In an EI source, electrons are produced through thermionic emissions by heating a wire filament that has an electric current running through it[9]. The electrons are accelerated through the ionisation space towards an anode. In the ionisation space, they interact with analyte molecules in the gas phase, causing them to ionise to a radical ion, and frequently cause numerous cleavage reactions that give rise to fragment ions, which can convey structural information about the analyte.

The efficiency of ionisation and production of fragment ions depends strongly on the chemistry of the analyte and the energy of the electrons. At low energies (around 20 eV), the interactions between the electrons and the analyte molecules do not transfer enough energy to cause ionisation[9]. At around 70 eV, the de Broglie wavelength of the electrons matches the length of typical bonds in organic molecules (about 0.14 nm), and energy transfer to organic analyte molecules is maximised, leading to the strongest possible ionisation and fragmentation. Under these conditions, about 1 in 1000 analyte molecules in the source are ionised. At

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higher energies, the de Broglie wavelength of the electrons becomes shorter than the bond lengths in typical analytes; the molecules then become "transparent" to the electrons, and ionisation efficiency decreases.

1.2.2.2 Chemical ionisation

As electron ionisation often leads to fragmentation of the molecular ion, chemical ionisation (CI) is a detection technique that produces ions with little excess energy, generating a spectrum in which the molecular species is easily recognised. Chemical ionisation consists of producing ions through a collision of the analyte molecule with a large excess of a reagent gas present in the source[9]. Reactions between ions and molecules normally take one of the following forms[6]:

- Proton transfer $\qquad$ $M + XH^+ \longrightarrow MH^+ + X$

- Charge exchange $\qquad$ $M + X^+ \longrightarrow M^+ + X$

- Electrophilic addition $\qquad$ $M + X^+ \longrightarrow MX^+$

- Anionic abstraction $\qquad$ $MH + X^+ \longrightarrow M^+ + HX$

Proton transfer is by far the most common CI process. The occurrence of such a reaction is related to the proton affinities of the analytes and reagent ion. If the proton affinity of the analyte is greater than that of the reagent ion, the proton transfer will occur. Typical reagent gases used for proton transfer CI are hydrogen, methane, isobutene and ammonia with the proton affinity increasing $H < CH_4 < C_4H_{10} < NH_3$[10].

1.2.2.3 Fast atom bombardment

Fast atom bombardment (FAB) is an ionisation technique in which an analyte and liquid matrix mixture is bombarded by an ~8KeV particle beam of usually an inert

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gas such as argon. This gas is ionised by a hot filament-type ion source, then accelerated in an electrostatic field, and focussed into a beam that bombards the sample. This Ar^+ provides high kinetic energy as well as undergoing charge exchange with the solvent. Common matrices used in FAB include glycerol and 3-nitrobenzyl alcohol (3-NBA). FABS is a relatively soft ionization technique and produces primarily quasimolecular ions such as $[M+H]^+$ and $[M-H]^-$ ions.

1.2.2.4 Electrospray ionisation mass spectrometry (ESI-MS)

The phenomena of electrospray has been known for hundreds of years, but it was not until the early parts of the 20th century that its significance to science was fully understood[11]. Some 30 years later, the pioneering experiments by Malcolm Dole *et al.*[12, 13] demonstrated the use of electrospray to ionise intact chemical species and the technique of electrospray ionisation (ESI) was invented. A further 20 years elapsed until work in the laboratory of John Fenn demonstrated the use of ESI for the ionisation of high mass biologically important compounds and their subsequent analysis by mass spectrometry[14-16]. This work won John Fenn* a share of the 2002 Nobel Prize for Chemistry - the 4th time a Nobel Prize has been awarded to mass spectrometry pioneers. In the original papers from the late 1980's, Fenn and his co-workers successfully demonstrated the basic experimental principles and methodologies of the ESI technique, including soft ionisation of involatile and thermally labile compounds, multiple charging of proteins and intact ionisation of complexes. Since that time, LC/ESI-MS has developed rapidly in applications, interface designs, in MS instrumentation and in computer software for analysis.

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* The prize was awarded jointly to John B. Fenn and Koichi Tanaka "for their development of soft desorption ionisation methods for mass spectrometric analyses of biological macromolecules"

The LC/ESI-MS literature is now extensive, with many reviews, special journal issues and books having been published[17-22].

The ESI source has undergone continued development since the earliest examples, but the general arrangement has remained basically the same (Figure 1.1). The analyte is introduced into the source in solution either from a syringe pump or as the eluent flow from liquid chromatography. The analyte solution flow passes through the electrospray needle which has a high potential difference (with respect to the counter electrode) applied to it (typically in the range from 3 to 5 kV)[9, 23]. This forces the spraying of charged droplets from the needle with a surface charge of the same polarity as the charge on the needle. The droplets are repelled from the needle towards the source sampling cone or heated capillary on the counter electrode. As the droplets traverse the space between the needle tip and the cone, solvent evaporation occurs, assisted by a flow of warm nitrogen gas, (known as the drying gas) which passes across the front of the source. As solvent evaporation occurs the droplets shrink until they reach the point that the surface tension can no longer sustain the charge (the Rayleigh limit) at which point a "Coulombic explosion" occurs and the droplets are ripped apart. This produces smaller droplets that can repeat, and eventually solvent free, charged sample ions are released from the droplets[24]. These charged analyte molecules (they are not strictly ions) can be singly or multiply charged. This is a very soft method of ionisation because very little residual energy is retained by the analyte upon ionisation, and this is why ESI-MS is such an important technique in biological studies, where the analyst often requires that non-covalent molecule-protein or protein-protein interactions are representatively transferred into the gas-phase. The major disadvantage of the technique is that very little (usually no) fragmentation is produced (although often this is desirable). For structural

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elucidation studies, this leads to the requirement for tandem mass spectrometry where the analyte molecules can be fragmented.
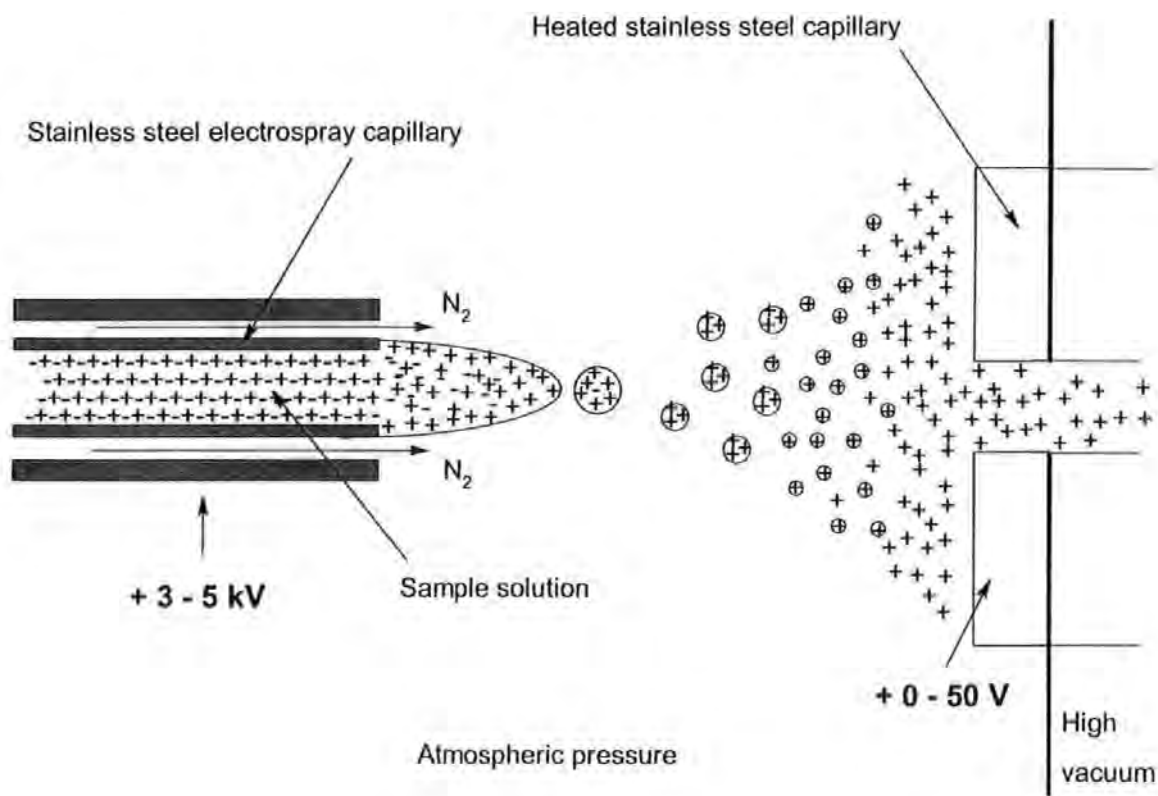
Figure 1.1   Simplified diagram[†] of the Finnigan Matt LCQ™ electrospray ionisation source with droplet and gas phase ion production.

The advantages and disadvantages of ESI interfaces for production of gas-phase ions are subjective and often somewhat dependent on whether an existing, non-MS based chromatographic method is being converted to a method based on LC with ESI-MS detection, or whether a new method is being developed for analytes that were not previously detectable by other methods. The following is a guide to some of the main advantages and disadvantages of ESI[25]:

[†] Reproduced with permission from Dr. Paul McCormack [(25)]

Advantages:

- Suitable for non-volatile, polar and thermally unstable compounds.

- ESI-MS is sensitive (fmol or lower, dependent on analyte).

- Positive and negative ions can be produced.

- Soft ionization produces mainly protonated molecules (or cation adduct ions) in positive ion mode and deprotonated molecules in negative ion mode allowing accurate molecular weights to be determined.

- Optimum chromatographic resolution is not required if analytes that co-elute have different m/z and do not cause ion suppression of one or other analyte.

- Structural information can be determined from protonated molecules by collision induced dissociation (CID), collision activated dissociation (CAD) or resonance induced dissociation and multistage mass spectrometry (MS^n).

- Multiply charged ions can be produced, allowing ionised high molecular weight molecules to be within the mass to charge (m/z) range of most mass analysers.

- Method is compatible with flow rates from nL min^{-1} to 1-2 mL min^{-1}.

- Method is concentration and mass sensitive. Flow splitting of LC eluents prior to ESI interface therefore does not reduce sensitivity (excluding peak broadening effects), thereby allowing conventional chromatography columns (4.6 mm diameter) with 1 - 2 mL min^{-1} flow rates to be used. The diverted eluent can be directed to other detectors for complementary data.

- ESI is, or can be made, compatible with many LC techniques such as: reversed phase (RPLC), normal phase (NPLC), supercritical fluid (SFC), size exclusion (SEC), ion-exchange (IEC), affinity (AC), ion-pair (IPC),

hydrophobic interaction (HIC), capillary electrophoresis (CE) and other electro-chromatography techniques.

Disadvantages:

- ESI is not compatible with high ionic strength solutions or high concentrations of involatile buffers/mobile phase modifiers used in many traditional chromatographic methods. ESI normally requires modification of such methods to allow the use of volatile reagents.

- Many traditional chromatographic phases and methods require the analyte to be non-ionised, whereas ESI requires the analyte to be ionised in solution. Post column addition of acid/base to eluent may be required to adjust solution pH.

- High purity solvents, reagents and chromatographic phases are required in order to reduce background chemical noise.

- Soft ionization produces mainly protonated (or deprotonated) molecules. CID methods (in source or tandem instruments (MS/MS or MS^n)) are then required to obtain structural information.

1.2.2.5 Atmospheric pressure chemical ionisation

Atmospheric pressure chemical ionisation (APCI) is a form of chemical ionisation which takes place at atmospheric pressure. The primary ions are produced by corona discharges on a solvent spray. The APCI technique is mainly applied to polar and ionic compounds with moderate molecular weights up to about 1500 Da and generally giving mono-charged ions[9]. This ionisation technique allows for the high flow rates typical of standard bore HPLC to be used directly, often without diverting the larger fraction of volume to waste. This flow is directed into a

pneumatic nebuliser where it is converted into a fine aerosol by a high-speed nitrogen beam. After desolvation, these compounds are carried along a corona discharge where ionisation occurs.

As the ionisation of the substrate occurs at atmospheric pressure with a high collision frequency, this method of ionisation is very efficient. In addition, the high frequency of collisions serves to thermalise the reactant species. The result is ionisation yielding predominantly ions of molecular species with few fragmentations.

1.2.2.6 Matrix-assisted laser desorption ionisation

The ionisation is triggered by a laser beam, normally a nitrogen-laser. A matrix is used to protect the biomolecule from being destroyed by direct laser beam. The matrix consists of crystallised molecules, of which the three most commonly used are 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), α-cyano-4-hydroxycinnamic acid (alpha-cyano or alpha-matrix) and 2,5-dihydroxybenzoic acid (DHB). A solution of one of these molecules is made, in a mixture of highly purified water and an organic compound (normally acetonitrile or ethanol).

The matrix-solution is then mixed with the analyte molecule of interest (e.g. protein-sample). The organic compound ACN allows for the hydrophobic proteins in the sample to dissolve into the solution, while the water allows for hydrophilic proteins to do the same. This solution is spotted onto a MALDI plate (usually a metal plate designed for this purpose). The solvents vaporise, leaving only the re-crystallised matrix, but now with proteins spread throughout the crystals. The matrix and the analyte are said to be co-crystallised in a MALDI spot.

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The laser is fired at the crystals in the MALDI spot. The spot absorbs the laser energy and it is thought that primarily the matrix is ionised by this event. The matrix components are then thought to transfer part of their charge to the analyte (e.g. a protein), thus ionising the protein while still protecting it from the disruptive energy of the laser. Ions observed after this process are quasi-molecular ions that are ionised by the addition of a proton to $[M+H]^+$, or another cation such as sodium $[M+Na]^+$, or the removal of a proton $[M-H]^-$. MALDI generally produces singly-charged ions, but doubly-charged ions such as $[M+2H]^{2+}$ have been observed as well.

1.2.2.7 Inductively coupled plasma mass spectrometry

Atmospheric-pressure inductively coupled plasmas (ICPs) are flame-like electrical discharges that have transformed the practice of elemental and isotopic ratio analysis[26]. The argon ICP has now become the most widely used atomic spectroscopy source in routine analytical chemistry[26]. Much work on the development of the ICP source and the practical implications were originally addressed in the 1960's, with ICP-AES first explored in 1964[27]. It was a further ten years before ICP-MS was developed by Gray[28]. Further developments in the use of ICPs as an ion source for mass spectrometry were made by Date and Gray[29, 30] and Houk and Fassel[31] in the 1980's.

The ICP is generated by coupling energy from a radio frequency generator into a suitable gas via a magnetic field surrounding a water-cooled, three turn copper coil. The gas flows through a quartz torch which consists of three concentric tubes, and is mounted axially in the copper load coil (Figure 1.2). The generator operates at a frequency of 27 or 40 MHz to yield typical output powers of 1 to 2 kW. To

initiate the plasma, the gas streams are supplied with free electrons via a tesla coil. This produces a potential large enough to overcome the dielectric resistance of the gas, thus the electrons accelerate in the electric and magnetic fields associated with the load coil and ionise the gas in the field[32]. A stable, self sustaining plasma is maintained as long as the gas flows in a symmetrical pattern and the magnetic field strength is sufficiently high[26]. Importantly, the largest current flow occurs in the periphery of the plasma which gives the ICP a distinctive annular configuration. This ring type structure ensures the efficient introduction of sample aerosol into the central channel of the plasma resulting in the efficient desolvation, vaporisation, atomisation, excitation and ionisation of the sample. The ionisation conditions within the plasma lead to highly efficient ionisation of most periodic table elements, with almost all being singly charged.

Liquid sample introduction is the most common way of introducing a sample into the plasma. Typical introduction systems consist of a nebuliser (e.g. concentric) and spray chamber (e.g. cyclonic). The process of nebulisation produces a fine aerosol of the liquid sample, with the spray chamber (which can be cooled) acting as a desolvation mechanism to remove the larger droplets. The nebuliser gas flow carries the finer droplets into the plasma where these undergo desolvation, vaporisation, atomisation, excitation and ionisation.

The ICP is an extremely efficient ion source, thus the interfacing of this with mass spectrometry yields a highly sensitive and specific elemental detector. The problems related to the coupling of the two techniques stem from the fact that an ICP operates at atmospheric pressure, whereas a mass spectrometer requires a high vacuum. These problems were overcome by using a small cooled orifice to transfer the sampled plasma into the high vacuum system[31]. The size of this orifice

is designed to ensure a continuous flow of analyte through the vacuum to the mass spectrometer. Ions pass from the central channel of the plasma through the sampling cone (a cone with a small orifice, 1.0 mm) and enter into a low vacuum region called the transfer/expansion chamber. A small percentage of these ions then pass through a second cone (skimmer, 0.7 mm) into a second, high vacuum region, where the ion beam is focused by a series of ion lenses. This focused ion beam passes to the mass spectrometer where the ions are separated by their mass to charge ratio and then detected by an electron multiplier or other similar detector.
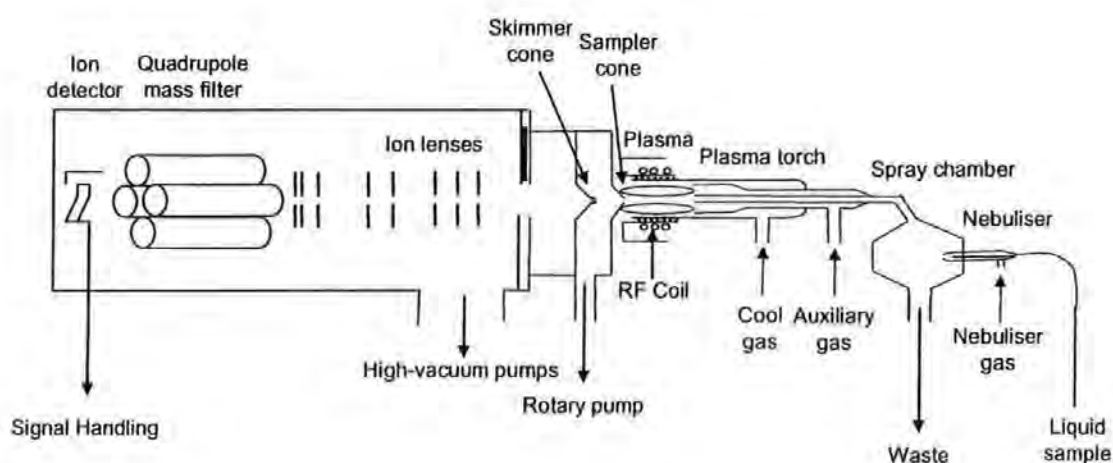
Figure 1.2   Schematic of a quadrupole ICP-MS instrument

1.2.3 Qualitative Mass Spectrometry

Initially organic mass spectrometry was primarily a tool for analysing unknown compounds to provide clues to their structures[33]. Now, the mass spectrometer can be used qualitatively to provide the analyst with much more information about the sample than just the molecular weight information. Structural elucidation is required in all areas of drug development. Within chemical development it is needed to identify impurities which may arise throughout the synthesis.

1.2.3.1 Determination of molecular weight

Although this procedure sounds simple, this process is full of potential pitfalls to catch the analyst out. Due to the soft ionisation procedure used, the molecules remain generally intact, but may also pick up other cluster ions/adduct ions to form clusters. The most commonly observed are those with sodium ($M + Na^+$), potassium ($M + K^+$) and ammonium ($M + NH_4^+$). When these clusters are formed, the mass of the analyte is not the mass observed, but the mass of the analyte plus the cluster ion. Analysts must be careful to identify these clusters.

1.2.3.2 Collisionally induced decomposition and MS^n

The generation of an ion related to the intact molecule is useful, but on its own tells us little about the structure. In order to study the structure of this ion, we need to look at the various components making up the ion. This involves the fragmentation of the molecule using a collision gas in a collision cell, or an input of RF voltage to generate fragmentation. These fragmentations can be controlled by the amount of collision gas or voltage to enable different fragments to be formed. These fragments can then be fragmented again to yield multi-stage fragmentation (MS^n). Together this information can be interpreted to provide information about the functional groups present, thus aiding the analyst with structural information.

1.2.3.3 Accurate mass measurement

High resolution instruments, including double focusing and Fourier transform ion cyclotron resonance (FTICR) mass spectrometers are capable in principle of measuring the mass of an ion with sufficient accuracy to allow the determination of its elemental composition[9]. This is possible because each element has a slightly

different characteristic mass defect, so the accurate mass measurement that shows the total mass defect provides an identification of its elemental composition. However, the accuracy requirements in measuring mass and thus the resolution requirements increase very rapidly as the mass increases[9]. For example, $C_{24}H_{19}N$ and $C_{21}H_{23}S$ with masses of 321.1517 and 321.1551 m/z respectively, require an accuracy of 11 ppm for their distinction.

1.2.4 Quantitative mass spectrometry

Mass spectrometers are specific detectors and the substantial reduction in noise allows sensitive detection and quantitation of analytes even in complex matrices such as biofluids or formulated tablets[3]. The degree of specificity of quantitative analysis depends on how the spectrometer is used and even more so on the signal that is used during the correlation[9]. For example, the ion current can be used as a signal to determine the concentration of a compound that is studied as long as there are no interferences with other substances.

In mass spectrometry, the limit of detection depends considerably on the abundance of the ionic species that is measured. These can therefore be dependent on the modification of ionisation conditions, the mode of operation (positive or negative ion mode) and the ionisation technique. For some compounds, sensitivities (detection limits) in the range of low pg mL^{-1} are achievable.

The errors in measurement arising due to the mass spectrometer are numerous, such as the variation in source conditions and instability of the mass scale. For statistical reasons, every measurement of a signal intensity carries a minimal

intrinsic error[9]. This error is inversely proportional to the square root of the number of ions detected for that signal. Thus, in order to optimise the reproducibility or the precision of the measurements, a maximum number of ions must be detected for every ionic species. These errors can be reduced by using an internal standard, or using an isotope dilution method.

To perform quantitative measurements using mass spectrometry, generally a standard of the compound of interest is needed. This can be introduced into the mass spectrometer at varying concentrations to produce a calibration curve for that specific compound. The use of an internal standard can improve the calibration, e.g. it can compensate for the effects of variation in small injection volumes and ionisation phenomena. The major drawback of this method is the fact that a specific standard of the analyte of interest must be used. The one exception to this is when ICP-MS detection is employed. As this is an element selective detection, then standards containing that specific element can be used. For example, if dichlorobenzene was to be quantified, a calibration curve could be produced using any standard with chlorine within the molecule.

1.3 High Performance Liquid Chromatography – Electrospray Ionisation Mass Spectrometry

1.3.1 High Performance Liquid Chromatography (HPLC)

The general principle of chromatography has been known for many years and involves two immiscible phases, one stationary and the other mobile which are brought into contact. A sample is introduced into the mobile phase and solutes in the sample undergo a repeated series of partitions between the mobile and stationary phases. When these phases are properly chosen, the solutes present in the sample separate into distinct bands in the mobile phase and emerge in order of increasing interaction with the stationary phase. Chromatography has many applications from purification to separation of mixtures and can be used with on or off-line detection techniques for qualitative and quantitative analysis.

Prior to the 1970's, few reliable chromatographic methods were commercially available to the laboratory scientist. During the 1970's, most chemical separations were performed using a variety of techniques, including open-column chromatography, paper chromatography, and thin-layer chromatography. However, these chromatographic techniques were not ideally suited for quantification of compounds and resolution between similar compounds. During this time, pressure liquid chromatography began to be used to decrease flow-through time, thus reducing purification times of compounds being isolated by column chromatography.

High pressure liquid chromatography (as it was known then) was developed in the mid-1970's and quickly improved with the development of column packing materials and the additional convenience of on-line detectors. In the late 1970's,

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new methods including reversed phase liquid chromatography allowed for improved separation between very similar compounds and it is this technique which has been used throughout the experimental chapters of this thesis so a brief description of this follows.

1.3.1.1 Reversed phase HPLC

Reversed phase chromatography is used to describe the state in which the stationary phase is less polar than the mobile phase[34]. Chemically bonded octadecylsilane (ODS), an *n*-alkane with 18 carbon atoms, is the most frequently used stationary phase. Shorter alkyl groups including ethyl (C-2), Octyl (C-8) and also cyclohexyl and phenyl groups provide other alternatives to aid chromatographic separation of compounds. The mobile phase (eluent) generally consists of mixtures of water or aqueous buffer solutions with various water miscible solvents, e.g. methanol and acetonitrile. The type of mobile phase used may have a big effect on the retention. It can promote or suppress ionisation of the analyte molecules, and it can also shield an accessible residual silanol or any other active adsorption centres on the adsorbent surface.

In reversed phase chromatography partition occurs between the bonded organic phase and mobile liquid phase. Let us consider a separation of a two component mixture dissolved in the eluent. Assume that component A has the same interaction with the adsorbent surface as the eluent, and component B has strong interaction with the stationary phase. On injection onto the column, these components will be forced through by the flow of the mobile phase. Molecules of the component A will interact with the adsorbent surface and retain on it in the

same way as eluent molecules. Thus, as an average result, component A will move through the column with the same speed as the eluent.

Molecules of component B, being adsorbed on the stationary phase due to their strong excessive interactions will interact with it much longer. Thus, they will move through the column slower than the eluent flow. This will result in two distinct separated peaks. The first peak to elute will be from component A, which has the same interaction with the stationary phase as the eluent. The second peak to elute will be compound B. This elutes later because it had been interacting with the stationary phase, and has thus been slower to move through the column.

Usually a relatively narrow band is injected (5 - 20 μL injection volume). During the run, the original chromatographic band will be spread due to the non-even flows around and inside the porous particles, slow adsorption kinetics, longitudinal diffusion, and other factors. These processes together produce so called band broadening of the chromatographic zone. In general, the longer the component is retained on the column, the broader its zone (peak on the chromatogram).

Separation performance depends on both component retention and band broadening. Band broadening is, in general, a kinetic parameter, dependent on the adsorbent particle size, porosity, pore size, column size, shape, and packing performance. On the other hand, retention does not depend on the above mentioned parameters, but rather reflects molecular surface interactions and depends on the total adsorbent surface. With the continuous improvement of column packing materials, these band broadening effects are becoming less apparent with newer columns.

The retention of the analyte depends on it's affinity to the mobile phase and stationary phase, so extensive method development, requiring changing the stationary and mobile phases is often necessary to enable adequate resolution between analytes. As a general rule for reversed phase chromatography, polar substances interact better with the mobile phase and elute first, as the hydrophobic character of the analyte increases, retention increases[34].

1.3.2 HPLC coupled to mass spectrometry

The need for more definitive identification of HPLC eluates than that provided by detectors such as UV/Vis and refractive index lead to the evolution of mass spectrometry as a HPLC detector. Initially, the combination of the two techniques involved the isolation of fractions as they eluted from the HPLC column, followed by the removal of the mobile phase, generally by evaporation, followed by mass spectrometry detection[35]. This obviously has inherent disadvantages due to loss of analyte during fraction collection, mixed spectra from unresolved peaks, thermal decomposition of analytes with evaporation, and the ionisation technique needs to be chosen very carefully[35]. Direct coupling of HPLC to mass spectrometry alleviates many of these problems, but can suffer from incompatibility between the two techniques. The major problems are the need to remove much of the HPLC mobile phase before entry into the mass spectrometer, and the requirement for the formation of volatilised ions from involatile and highly polar analytes. This has lead to a number of interfaces being developed, including direct introduction, moving-belt, thermospray, atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI). Some of the earlier interfaces did not lend themselves to routine analysis, but the introduction of APCI/ESI systems in the 1990's has pushed the boundaries of LC-MS and the technique has become

widespread with hundreds of applications for both quantitative and qualitative analyses.

1.4 Liquid chromatography with Mass spectrometry in the pharmaceutical industry

There are many applications of LC-MS within the pharmaceutical industry such as impurity identification, characterisation, analysis of waste effluents and clinical trial metabolites. These can be brought together into two categories namely qualitative and quantitative analysis. LC-MS has high sensitivity, thereby reducing the need for time-consuming preparative chromatography, and thus enabling impurities to be detected at the 0.1% area ratio level (relative to the main compound) online. Small molecules (less than 150 Da) are not easily amenable to trace analysis by LC-MS, due to the high background signal resulting from mobile phase impurities, ion adducts[4] and the large signal of the main peak. Wolff et al.[36] reported the use of a signal actuated switching valve for diverting the main matrix compound to waste, hence preventing it from entering the mass spectrometer and ensuring that the large chemical background resulting from the tailing of the main component did not affect later eluting impurities. This improved the detection limit for the trace impurities eluting after the drug substance by a factor of 4-5. The increase in sensitivity was dependent on the type of ion source which was used to interface the mass spectrometer. The limit of detection was improved by a factor of 10 with a line of sight type source but only by a factor of 5 with the orthogonal source.

The introduction of new instrumentation including tandem mass spectrometry (MS^n) and high resolution instruments has resulted in large advances in qualitative analysis. Tandem mass spectrometry involves at least two stages of mass

analysis, usually with a dissociation process or chemical reaction that causes a change in the mass or charge of an ion[9]. In the most common MS/MS experiments the first mass analysis isolates a precursor ion which then undergoes fragmentation to yield product ions. These product ions are subjected to a second mass analysis and then detected. This can be repeated 'n' times to yield MS^n experiments. At each stage, the analyte molecule and product ions are further fragmented leading to data where functional groups have been removed. By piecing together this data, and using knowledge of the possible components of the injected sample, a tentative structure can be deduced, which will need to be confirmed by other spectroscopic techniques such as IR, Raman and NMR.

Accurate mass measurement of small molecules is used to determine the elemental formulae of an unknown compound. The higher the accuracy, the less the ambiguity in the result[37]. Accurate mass measurements can be performed using a variety of mass spectrometers. Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) offers the highest mass resolution and mass accuracy of any mass spectrometer with a mass resolving power in excess of 1×10^6 and mass accuracy of 1 ppm. A number of reports have demonstrated that high accuracy mass measurements can be recorded using quadrupole-TOF mass spectrometers. Wolff *et al.*[38] achieved mass measurement accuracy to within 2 mDa of the theoretical values for the identification of impurities in drug substances. The high level of mass accuracy obtained using these instruments enables a molecular formula to be obtained, which can then be used in conjunction with libraries and knowledge of the possible contents of the sample, to determine the structure of the compound.

1.5 Liquid Chromatography Inductively Coupled Plasma Mass Spectrometry (LC-ICP-MS)

1.5.1 Chromatography coupled to ICP-MS

Inductively coupled plasma mass spectrometry (ICP-MS) is now recognised as one of the most useful and powerful techniques for element selective detection of trace elements in chromatographic eluents[39]. The sensitivity and selectivity of this method of detection enables the analysis of both metals and non-metals in a wide variety of samples. The use of ICP-MS as a chromatographic detector was initially described in the late 1980's and, since that time, the versatility of the detector has been realised for many chromatographic applications. Many papers which describe ICP-MS as a chromatographic detector are specifically aimed at speciation analysis, which is used to determine the chemical form of an element in a sample.

Various separation techniques have been utilised for both multi-element and elemental speciation analyses including liquid chromatography, gas chromatography, supercritical fluid chromatography and capillary electrophoresis. Many papers have been published within the last 15 years utilising these separation techniques with ICP-MS detection, and a comprehensive review has been published by Sutton *et al.*[39]

Traditional detectors for LC, such as UV/Vis absorbance and refractive index detectors, lack the sensitivity that is required for the determination of elements in environmental and biological samples[40]. To some degree, ICP-MS overcomes this, and can offer improvements in sensitivity up to three orders of magnitude

depending on the element under study. ICP-MS is an element selective detector so the mass spectrometer is usually set to monitor the isotopic signal of an element or several elements either sequentially or simultaneously, thus the resulting chromatograms only show peaks for the element/isotope of interest. This is a distinct advantage over detectors such as UV/Vis absorbance, which yield chromatograms of all species absorbing at the specified wavelength.

Mobile phase flow rates used in most chromatographic separations are of the order of 0.1 to 1 mL min^{-1} [39]. These are comparable to the direct aspiration rates of standard ICP-MS nebulisers. Thus, conventional pneumatic nebulisation with concentric and cross-flow nebulisers may be used along with single or double-pass spray chambers. To connect the LC outlet to the ICP-MS sample introduction system, a simple piece of inert tubing of PEEK or PTFE may be used. The internal diameter and length of this tubing should be kept to a minimum to ensure that minimal peak broadening is experienced.

Conventional nebulisers and sample introduction systems are relatively inefficient because only 1-3% of the actual sample enters the plasma[39]. Hence, an increase in this efficiency would improve the sensitivity of the technique and lower the limits of detection. Nebulisers with higher transport efficiencies have been described for LC-ICP-MS such as the Micro Mist[41] and the Direct injection high efficiency nebuliser[42].

Reversed-phase chromatography coupled with ICP-MS has now become widely established for the analysis of environmental samples and general analytical samples. Atomic spectroscopy methods generally determine the total

concentration or amount of the element(s) of interest. If more information about the speciation of the element(s) is required then a coupled technique is necessary. By coupling reversed phase liquid chromatography (RPLC) with ICP-MS these speciation analyses are possible with excellent sensitivity and selectivity.

One issue to consider when coupling reversed phase HPLC with ICP-MS is that the introduction of organic solvents changes the sensitivity of the ICP-MS and deposition of unburnt carbon on the sampler and skimmer cones can cause blocking[43]. These problems can be overcome to a certain degree by the addition of a small amount of oxygen to the nebuliser gas flow and operating the plasma at higher powers. The underlying problem is that organic solvents have a higher vapour pressure which leads to an increased solvent loading on the plasma [44]. An increased solvent load reduces the stability of the plasma and adversely affects the electron number density, ionisation and excitation temperatures of the plasma [45]. Thus, the chromatography required for the separation could involve the use of eluents which will extinguish the plasma and limit the versatility of ICP-MS as an HPLC detector. This problem is discussed in more detail in Chapter Two.

1.5.2 Applications of HPLC-ICP-MS in the pharmaceutical industry

Axelsson et al.[46] reported the use of LC-ICP-MS as a generic detector for structurally non-correlated compounds with common elements like phosphorus and iodine. The use of this element selective detection resulted in improved quantification of tested 'unknowns' compared to UV and organic mass spectrometry. They reported that the dynamic range was found to exceed 2.5 orders of magnitude. This application of LC-ICP-MS to pharmaceutical drugs and

formulations has shown that impurities can be quantified below the 0.1 mol-% level.

For identification and quantification of impurities in pharmaceutical products, new methods with high sensitivity and ease for calibration are required. Usually HPLC/ESI-MS is applied for this purpose, but quantification is hampered because the ionisation efficiency depends on the chemical structure and is therefore restricted to those compounds where standards are available. Busker *et al.*[10] described how HPLC/ICP-MS could be used as an element specific method for quantitative phosphorus determination even without standards for those compounds. This is possible because phosphorus sensitivity is independent of the specific chemical structure, so other phosphorus compounds with similar retention times can be used for quantification. This is one of the major advantages of ICP-MS as a quantitative detector for HPLC.

Traditionally, ICP-MS has been used to determine low levels of trace elements in environmental samples. Due to its high sensitivity, selectivity and sample throughput, ICP-MS is able to determine most elements in the periodic table simultaneously in the range of pg L^{-1} to µg L^{-1} for a wide range of samples[40]. In the pharmaceutical industry, metal contamination of bulk drug substances and their intermediates may be introduced in many ways, including raw materials, reagents, solvents, catalysts and plumbing[47]. For regulatory approval, these metals must be monitored and their concentrations established for both process intermediates and final drug substances. Traditionally the pharmacopoeial methods involve the precipitation of metal sulphides, followed by visual comparison of the colour. This method suffers from some obvious limitations. The technique of choice to determine the metal content of pharmaceuticals has become ICP optical emission

spectroscopy. Similarly, the introduction of commercial ICP-MS instrumentation with published methods will lead to the use of ICP-MS analysis in many pharmaceutical quality control laboratories. The ability of the technique to determine many elements in a single analysis enables many samples to be analysed, and makes a less laborious analysis. Lewen et al.[48] have developed and utilised an ICP-MS method to specifically detect and quantify 14 different metals in a single run for nearly 60 different pharmaceutically important sample matrices. The described method also demonstrates superior recoveries when compared to the corresponding pharmacopoeial methods. An alternative method has been published by Wang et al.[47] who have used ICP-MS to determine and quantify up to 69 elements simultaneously for pharmaceutical samples.

Other pharmaceutical applications have recently been developed utilising ICP-MS, but these have not been as wide spread as those for high-throughput trace element analysis. Kannamkumarath et al.[49] utilised the element selectivity of ICP-MS with iodine selective detection to compare its limits of detection against UV to determine levothyroxine and its degradation products after HPLC separation. ICP-MS detection improved the detection of these compounds by 175-375 times when compared with UV. Lam et al.[50] utilised laser ablation (LA) technology to analyse pharmaceutical tablets by ICP-MS. This removes the need for complicated and laborious sample dissolution, giving results in minutes. The limits of detection achieved were 40 $\mu g\ g^{-1}$ for Al and 6 $\mu g\ g^{-1}$ for Mg.

1.6 Liquid Chromatography with ICP-MS and ESI-MS detection

The detection and identification of impurities/metabolites structurally related to a drug substance are of utmost importance. Conventional methods of analysis to

detect, track, quantify and/or identify drug substance/or related impurities often use mass spectrometry with atmospheric pressure ionisation (API-MS)[51, 52]. However, this approach is not foolproof because some impurities are poorly ionised, or co-elute with the main peak, making them hard to observe. A significant number of drug substances contain heteroatoms, (e.g. sulphur, bromine) for which a sensitive element selective detector such as ICP-MS is ideally suited.

Neither inductively coupled plasma-mass spectrometry nor electrospray alone will be adequate for the identification of unknown elemental species at trace levels [53]. However, the two techniques can be used in parallel in a synergistic way to provide both selective elemental analysis and molecular identification to detect and characterise previously unidentified compounds[54].

Corcoran et al.[55] applied HPLC linked to ICP-MS and orthogonal time-of-flight mass spectrometry (oa-TOFMS) for the identification of phase I and II urinary metabolites of diclofenac. The metabolites were separated by reversed-phase HPLC monitored with a UV diode array detector (UV-DAD) after which, 90% of the eluent was passed to an ICP-MS source, and the remainder going to the oa-TOF mass spectrometer. Compounds containing ^{35}Cl, ^{37}Cl and ^{32}S were detected specifically using ICP-MS, and then identified by oa-TOF. A previously unreported in vivo metabolite, an N-acetylcysteinyl conjugate of diclofenac, was detected in this way and was further characterised by oa-TOF. This was the first application of the combination of HPLC/UV-DAD/ICP-MS/oa-TOFMS for the identification of the metabolic fate of chlorinated xenobiotics by direct biofluid analysis.

Evans et al.[56] described the use of liquid chromatography coupled to sector field inductively plasma mass spectrometry (SF-ICP-MS) for the specific detection of sulphur-containing compounds in cimetidine drug substance. Here, structurally related impurities below the 0.1% mass fraction level (relative to the main drug substance) could easily be detected. Further analysis using electrospray mass spectrometry (ESI-MS) confirmed the structure of most of the impurities, thus highlighting the benefit of these complementary techniques in drug analysis. The limit of detection by SF-ICP-MS for cimetidine in solution was ~4-20 ng g^{-1} at a resolution of 6000, but this was blank limited. At this resolution the main isotope of sulphur (^{32}S, abundance 95.018%), was resolved from the serious interference of the polyatomic ion $^{16}O^{16}O^{+}$, which would not be possible without the high-resolution capabilities of the instrument used.

Whereas ICP-MS detection is very sensitive, it does not provide structural information of molecules so the characterisation of unknowns is very difficult. In comparison, electrospray mass spectrometry does provide structural information, but does suffer in the fact that ionisation is compound dependent. The combination of both these detection techniques running in parallel enables the analyst to both identify the components within the mixture, which contain the element of interest, and also determine the molecular mass of the compound, and thus aid in the structural identification of the compound.

1.7   Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic Resonance (NMR) spectroscopy is used, in this study, to characterise synthesised derivatising reagents. NMR is an isotope specific technique based on the principle that many nuclei have a magnetic moment, or a non-zero spin quantum number. Of these, isotopes having a ground state nuclear

spin, I, of ½ are the easiest to study. In a magnetic field, such nuclei have a lower energy when aligned with the field than when opposed to the field. The energy difference between the aligned and opposed states corresponds to radio frequencies in the electromagnetic spectrum, hence the nuclei are able to absorb and re-emit radio waves. The precise resonance frequency of the individual nucleus is dependent on the effective magnetic field at that nucleus, and is affected by electron shielding, which is in turn dependent on the chemical environment. This frequency dependence, measured as the chemical shift (δ, ppm), makes NMR spectroscopy a powerful analytical tool. Since the value of the chemical shift is proportional to the magnetic field strength the resonance frequency is ratioed to the spectrometer frequency to normalise it. As such, chemical shifts measured using different spectrometers can be compared directly.

NMR spectroscopy is primarily employed for the structural elucidation of organic molecules. The sample (typically 1 mg) is placed in a constant, homogenous magnetic field which induces individual nuclei within the molecule to align with or against the direction of the field. A pulse of radio frequency (RF) energies is applied, thereby effecting a change in the orientation of the NMR sensitive isotopes within the magnetic field. Following this 'resonance' state the energised isotopes 'relax' back to their original orientation. It is these energy changes that are measured to give an NMR spectrum, which can then be enhanced by repeated cycles of RF pulsing/relaxation to improve sensitivity. Data is acquired as a resonance frequency versus time spectrum, which is subsequently processed to a resonance frequency versus amplitude spectrum by means of a Fourier transform algorithm.

The most common isotope studied by NMR spectroscopy is the proton (^1H), as it is the most receptive and abundant, followed by ^{13}C. However there are over 20

isotopes in the periodic table having $I = \frac{1}{2}$ which are thus relatively straightforward to study. An important feature of NMR spectroscopy is that solvents employed should ideally not contain any of the isotope under study. Thus, deuterated solvents are employed for use in ^1H NMR. The use of deuterated solvents also allows the magnetic field around the sample to be built homogeneously, and, as deuterated solvents typically contain up to 0.1% ^1H, provide a reference point from the residual protonated solvent.

A further feature of NMR spectroscopy is that other NMR active isotopes in the molecule(s) under study will also induce changes in the spectrum due to magnetic interactions, or coupling, between these isotopes and the isotope under study. Every hydrogen atom in a molecule spins, and moving charged particles generate their own magnetic field. The protons on neighbouring carbons (carbons adjacent to the carbon whose protons are generating the NMR signal) will generate magnetic fields whose magnetic moments will interact with the magnetic moment of the external magnetic field. This results in the splitting of the NMR signal. We can determine how many times the signal will split by adding up all the neighbouring protons that are non-equivalent to the protons whose NMR signal is being generated. It turns out that the number of ways the signal is split can be determined by the so called "n+1" rule where n = number of non-equivalent neighbouring protons. For example, if we consider the compound propane, $CH_3CH_2CH_3$ then there will be two different electronic environments. The two methyl groups on each end will generate one signal and the CH_2 will generate a second NMR signal at a different chemical shift. For the methyl signal, there are only a total of 2 neighbouring protons (those on the CH_2) so according to the n+1 rule the signal will be split three ways called a triplet. For the CH_2 signal there are a total of 6 neighbouring protons (three from each neighbouring methyl) so that

signal should be split 6+1 or 7 ways called a septet. Neighbouring protons that are equivalent to the protons whose signal is being generated will not split the signal. So for example, 1,2-dichloroethane will only have one signal since the CH_2 protons are equivalent (homotopic) and the signal will be a singlet not having been split.

1.8 Aims of this study

The work described within this thesis is directed towards developing LC-ICP-MS and LC-ESI-MS methods, to investigate the capability of these techniques and how they can be used simultaneously to provide complimentary information. The initial development work is to focus on developing a suitable interface to couple HPLC with ICP-MS detection, overcoming some of the incompatibility problems between these two techniques.

Utilising this HPLC-ICP-MS interface, both ICP-MS and ESI-MS methods are to be developed to analyse some organic acids after HPLC separation. By derivatising these acids with a phosphorus containing derivatising reagent, any advantages in ESI-MS detection are to be investigated. This derivatisation may also enable phosphorus selective ICP-MS detection to be utilised to detect these derivatised acids.

By utilising this derivatisation technology, a novel brominated derivatising reagent is to be synthesised. Any improvement in detection by ICP-MS utilising bromine selective detection is to be investigated, with also phosphorus and bromine ratios obtained with ICP-MS detection to be calculated. This has the possibility to determine the degree of phosphorylation of peptides, which is also to be examined.

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Chapter Two

Interfacing High Performance Liquid Chromatography with Inductively Coupled Plasma Mass Spectrometry

2.1 Introduction

Inductively Coupled Plasma Mass Spectrometry is a convenient and sensitive detector for interfacing with HPLC. The ICP ion source is at atmospheric pressure, so an elaborate interface to reduce the pressure prior to introducing the sample is not required. The use of a mass spectrometer as a detector, in combination with the efficient ionisation of the ICP offers lower detection limits than optical emission or atomic absorption detectors. However, a major limitation of HPLC-ICP-MS is the low tolerance of the plasma for organic solvents used in HPLC mobile phases, thus, the adaptability of ICP-MS as an element selective detector for speciation analysis is determined by the composition and flow rate of the eluent, which in turn is dependent on the chromatography utilised. To alter the chromatographic separations, it is often necessary to modify the organic solvents or dissolved salts in the mobile phase. The ICP is intolerant to large amounts of either of these, so mobile phase selection must be carefully considered. The most critical consideration when coupling ICP-MS to HPLC is to maximise analyte transport but minimise solvent transport to the plasma.

The underlying problem is that organic solvents have a higher vapour pressure than water which leads to an increased solvent loading on the plasma[44]. This reduces the stability of the plasma because it adversely affects the electron

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number density as well as the ionisation and excitation temperatures[45] and also causes a change in the capacitance of the plasma resulting in a higher reflected power[32]. If the reflected power increases too much, then the RF tuning circuit will be unable to cope and the power supply will be shut down. Tuning the capacitance circuits in the torch box can help compensate for small amounts of solvents, but not entirely. Further problems are encountered with organic solvents and gradient elution[57]. As the eluent composition changes (to an increased organic content in reversed phase chromatography) the nature of the plasma changes, meaning that the ion lens settings and nebuliser gas flows that gave an optimal signal at the start of a gradient may not be optimal at the end of an elution gradient. This results in varying sensitivity over a chromatographic run leading to sloping baselines, calibration and quantification problems.

Another problem is that the solvent vapour is atomised but not oxidised[32], and the carbon produced can build up on the sampler and skimmer cones, causing a drop in sensitivity when the cone orifices become blocked. Any carbon travelling through the cones will also build up on the ion lenses causing a further drop in sensitivity[43]. These problems can be overcome to a certain degree by the addition of a small amount of oxygen to the nebuliser gas flow, which oxidises the carbon to CO_2 and CO[58].

Ideally, only aqueous mobile phases would be utilised with HPLC separations employing ICP-MS detection[41]. However, for the majority of reversed phase separations, the use of 100% aqueous solvents will not result in the required separation resolution. One possible solution is to reduce the solvent load on the plasma by reducing the mobile phase flow rates, combined with smaller bore columns. For example, if a standard-bore column (4.6 mm id) was changed to a

micro-bore column (1-2 mm id), typical flow rates would decrease from around 1 mL min^{-1} to 100 µL min^{-1} while maintaining the same linear velocity of the eluent through the column. The disadvantage is that high efficiency nebulisers are required to maintain the limits of detection due to the smaller sample volumes which are used. Conventional nebulisers and sample introduction systems are relatively inefficient because only 1-3% of the actual sample enters the plasma[39]. An increase in this efficiency leads to improved sensitivity of the technique and lower limits of detection. Nebulisers with higher transport efficiencies such as the direct injection nebuliser (DIN) and the direct injection high efficiency nebuliser (DIHEN) have been described for LC-ICP-MS[41, 42]. An alternative approach is to produce a desolvated aerosol, i.e. removal of the solvent to produce a dry analyte particle, which can be introduced into the plasma[44]. This has been achieved in a number of ways.

2.1.1 Methods of aerosol desolvation

In order to remove the organic solvent from the carrier stream all of the solvent present must be in the vapour phase, leaving the remaining analyte molecules as dry particles. This has been attempted using a number of methods in combination with pneumatic, ultrasonic and thermospray nebulisation[32].

The most common method has been the utilisation of a heated spray chamber and condenser arrangement in series. Heated spray chamber desolvators have been used sporadically in atomic spectroscopy for more than 30 years in order to increase the proportion of sample that is conveyed to the plasma or flame to improve sensitivity[59]. Initial studies exhibited problems of drift, instability, long wash-out times and sample carry-over, as these earlier models used ohmic

electrical heaters placed outside the heated chamber where nebulisation was taking place. Since then the technology has been modified, with Luo et al.[60] describing a high efficiency nebuliser coupled with a heated tube and two-stage condenser which removed 97% of aqueous solvents and 94% of methanolic solvents. The analyte mass transport efficiency was 24%, leading to detection limits an order of magnitude lower for most of the elements under investigation.

Eastgate et al.[59] compared the effects of heating the spray chamber, both radiatively or conductively. Measurements of sensitivity, stability and sample carryover indicated that radiant heating was superior, giving better short-term stability and sharply reduced wash-out times and carryover effects.

Ultrasonic nebulisers (USNs) have been used since the initial introduction of ICP's by Wendt and Fassel[61], who used one to generate an aerosol into their ICP configurations. The ultrasonic nebuliser utilises a piezoelectric transducer to produce ultrasonic waves that pass through the solution and produce an aerosol at the surface of the solution[62]. This aerosol is then volatilised in a heated tube, followed by a condenser cooled to approximately 0 °C, to produce desolvated analyte particles.

The thermospray nebuliser was initially developed as an interface between LC and MS[63]. Here the liquid begins to boil near the outlet of the capillary and is converted to an aerosol by the expanding solution vapour. These nebulisers provide aerosols with finer droplets than pneumatic nebulisers, and produce higher solvent vapour loads on the plasma. Therefore desolvation is necessary and is achieved by heating the aerosol sufficiently to evaporate the solvent from the aerosol[63].

Probably the most promising approach to remove the solvent vapour is to use a membrane desolvation device. A membrane desolvator used at the interface between the spray chamber and torch may be used to remove close to 100% of organic solvent[44]. Within a membrane desolvator, the sample vapour enters a heated membrane and a counter-current flow of sweep gas (usually argon) is used to remove solvent vapours that diffuse across the membrane. The theory is that the solvent molecules are so small that they diffuse through the membrane, whereas the larger analyte molecules are too large and are not removed by the membrane. The removal of the solvent vapour, and thus the sensitivity of the instrument, is dependent on optimising the sweep gas flow. An internal schematic of a Membrane desolvation system is shown in Figure 2.1.

The Cetac Aridus™ is a commercially available membrane desolvator which incorporates a low-flow, fluoropolymer nebuliser with a normal aspiration rate of approximately 60 µL min⁻¹, enabling successful analysis with less than 1 mL of sample. Not only do these membrane desolvators effectively remove sample solvent vapour, they markedly reduce interferences caused by oxide and hydride molecules in the mass spectrum. It is claimed that the Aridus™ system can effectively reduce the interferences of ArH^+ at m/z 39 and ArO^+ at m/z 56 by factors of 700 and 175, respectively[64].
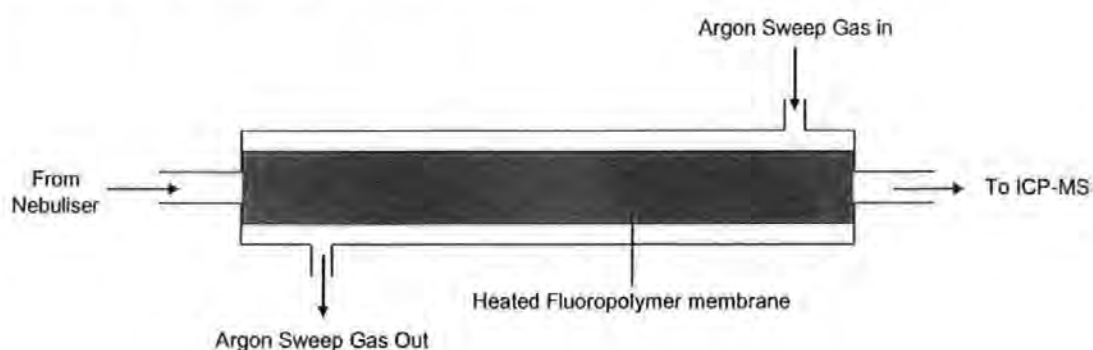
Figure 2.1   Schematic cross-section diagram of a membrane desolvator

2.1.2 Aims of this Chapter

Due to the incompatibility problems which are associated with the introduction of organic solvents into the ICP-MS, a procedure was required to enable effective coupling of HPLC operating with gradient elution. In order to identify the most suitable approach both low flow nebulisation and membrane desolvation were examined. The effects of different nebulisers, including low-flow nebulisers, and their effect on sample uptake and signal intensity with varying nebuliser gas flow rates and varying organic solvent content was investigated. A range of desolvation membranes were examined to investigate their utility with regard to desolvation of various mobile phases.

2.2 Experimental

2.2.1 Chemicals and reagents

HPLC grade acetonitrile and methanol were obtained from Fisher Scientific UK Ltd. (Loughborough, Leicestershire, UK). High purity water (18.2 MΩ) was obtained using an Elga Maxima water purifying system. BDH Aristar grade Indium atomic spectroscopy standard was purchased from VWR International (Poole, Dorset, UK). 2,4-dichlorobenzoic acid and 2,4-dichlorobenzaldehde were purchased from Sigma-Aldrich (Poole, Dorset, UK). Lamotrigine drug substance was supplied by GlaxoSmithKline (Stevenage, Hertfordshire, UK).

2.2.2 Nebuliser experiments with ICP-MS analyses

All experiments were performed using a sector-field inductively coupled plasma mass spectrometer (SF-ICP-MS, Thermo Elemental Axiom, Winsford, U.K.), using a mass resolution setting of 400. Operating conditions are shown in Table 2.1.

Table 2.1   Operating conditions for SF-ICP-MS

ICP	
Nebuliser gas flow/Lmin^{-1}	Range of flows (see specific experiment)
Auxiliary gas flow/Lmin^{-1}	0.85
Coolant gas flow/Lmin^{-1}	14.0
Nebuliser	Micromist (50 and 200 µL min^{-1}) and glass concentric (1 mL min^{-1}) (all Glass Expansion, Switzerland)
Spray Chamber	Jacketed quartz cinnabar, cooled to variable temperatures (Glass Expansion, Switzerland)
Torch	Quartz Fassel-type with quartz bonnet, with platinum shield
Interface	
Sampling cone	Nickel 1 mm i.d.
Skimmer cone	Nickel, 0.7 mm i.d.
Scanning	
Resolution	400
Slit setting	Source, 1000; collector, 1000
Masses monitored	Indium, (scanning between 114.401 and 115.407 m/z)
Dwell time/ms	50
Points	20

2.2.3 Nebuliser experiments

To examine the natural uptake rates of the nebulisers, a HP1050 modular chromatography system (Agilent Technologies, Stockport, U.K.) was used at a flow rate of 1.0 mL min^{-1} to deliver the liquids. A chromatographic T-piece (PEEK, Fisher Scientific, Loughborough, UK) was used before the nebuliser, to enable the nebuliser to uptake at its natural rate (Figure 2.2). The un-nebulised flow was directed to waste. A flow rate of 1 mL min^{-1} was chosen as this represents the standard flow rates used in "normal" bore chromatography with 4.6 mm i.d. columns.
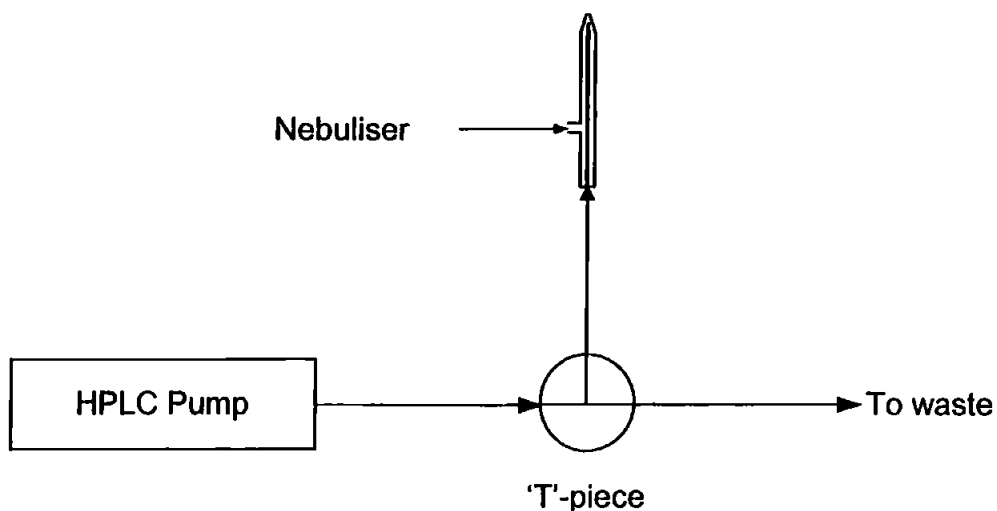
Figure 2.2   'T'-piece arrangement showing the nebuliser self aspiration set-up

2.2.4 Membrane desolvator experiments

Two different membrane desolvators were evaluated. A Universal Interface Model B desolvation device (Vestec Corporation, Houston, USA) and an Aridus™ sample introduction system (Cetac Technologies, Omaha, NE, USA). The sweep gas was optimised daily at approximately 2.0 L min^{-1} to ensure the maximum signal for ^{115}In^{+} was achieved. The Vestec desolvator was connected by simply inserting the end of the tubing from the HPLC column into the sample uptake inlet of the nebuliser, and the output from the spray chamber was introduced into the desolvator. The Aridus™ desolvator incorporates a low-flow (60 µL min^{-1}) nebuliser and spray chamber, so this was connected by using a 'T'-piece post column, with the nebuliser self aspirating (as shown in Figure 2.2). The dry aerosol exiting the desolvator was then transferred to the ICP torch via 1 m of 0.25 in. i.d. Tygon tubing.

2.2.5 Lamotrigine analysis

All samples were dissolved in methanol to contain 1 mg mL^{-1} of the compound in solution. 20 µL of this sample was injected using a HP 1090 chromatography system (Agilent Technologies, Stockport, U.K.). The HPLC column was a Nova-Pak C18 3.9 x 150 mm column (Waters, Elstree, Hertfordshire, UK). The elution conditions used are shown in Table 2.2. The HPLC waste outlet (after the UV detector) was connected to a 1 mL min^{-1} conikal nebuliser (Glass Expansion, Switzerland) via 2 m of 0.17 in. i.d. PTFE tubing. The nebuliser sprayed into a jacketed cyclonic spray chamber (Glass Expansion, Switzerland) cooled to 5°C. After the spray chamber, the outlet was connected to the Vestec membrane desolvator using 1 m of 0.25 in. i.d. Tygon tubing. After desolvation, the dry aerosol was transferred to the ICP-MS torch via 1 m of 0.25 in. i.d. Tygon tubing. All experiments were performed using a quadrupole inductively coupled plasma mass spectrometer (Thermo Elemental PQ3, Winsford, U.K.). The operating conditions were optimised daily to give the maximum signal for $^{35}Cl^{+}$. Typical operating conditions are shown in Table 2.3.

Table 2.2   Gradient elution profile used for Lamotrigine analysis

Time /minutes	% 0.05M Ammonium Acetate in DDW	% Acetonitrile
0	100	0
30	5	95
35	5	95
50 (#)	100	0

This involves a step from 5:95 % to 100:0% 0.05M Ammonium acetate:acetonitrile, not a gradient.

Table 2.3   Operating conditions for quadrupole-ICP-MS

ICP	
Nebuliser gas flow/Lmin^{-1}	1.10
Auxiliary gas flow/Lmin^{-1}	0.85
Coolant gas flow/Lmin^{-1}	14.0
RF Forward Power/W	1450
Nebuliser	Glass Concentric (Glass Expansion, Switzerland)
Spray Chamber	Jacketed quartz cyclonic, cooled to 5 °C
Torch	Quartz Fassel-type without shield
Interface	
Sampling cone	Nickel 1 mm i.d.
Skimmer cone	Nickel, 0.7 mm i.d.
Time Resolved Analysis	
Masses monitored	Single ion monitoring for $^{35}Cl^+$ at m/z 35
Dwell time/ms	500

2.3 Results and Discussion

2.3.1 Nebuliser uptake rates

Initial investigations were focused on measuring the aspiration rates of various nebulisers for different organic solvent compositions. Three different nebulisers were examined, a 1 mL min^{-1} glass concentric, a 200 µL min^{-1} Micromist and a 50 µL min^{-1} Micromist (all from Glass Expansion, Vevey, Switzerland). In each case the spray chamber was cooled to 5°C, and all connections were made using identical tubing, and the nebulisers were allowed to self aspirate via a 'T'-piece. The uptake rate of each nebuliser was determined for solutions with different compositions in order to establish the effect of this on aspiration rate. Methanol and acetonitrile were chosen as the organic solvents because they are the two most commonly used solvents in reversed phase chromatography. The aspiration rates were calculated by weighing the amount collected from the 'T'-piece waste over a 20 minute period and subtracting that from the total flow. By taking into account the density of the solutions, the amount aspirated in µL min^{-1} was calculated. The nebuliser gas flow was kept constant at 0.90 L min^{-1} for all nebulisers. The results obtained are shown in Figure 2.3, Figure 2.4 and Figure 2.5. For all nebulisers, the amount of nebulised solvent increased with increasing organic content. All the nebulisers tested self-aspirated 100 % aqueous solution at a much lower rate than that specified by the manufacturer. In practice, this increasing uptake rate would affect the nebulisation efficiency, solvent loading and sample transport of the analyte to the plasma, which in turn would result in changing sensitivity and increasing reflected power, with the plasma eventually being extinguished if the solvent loading increased sufficiently.
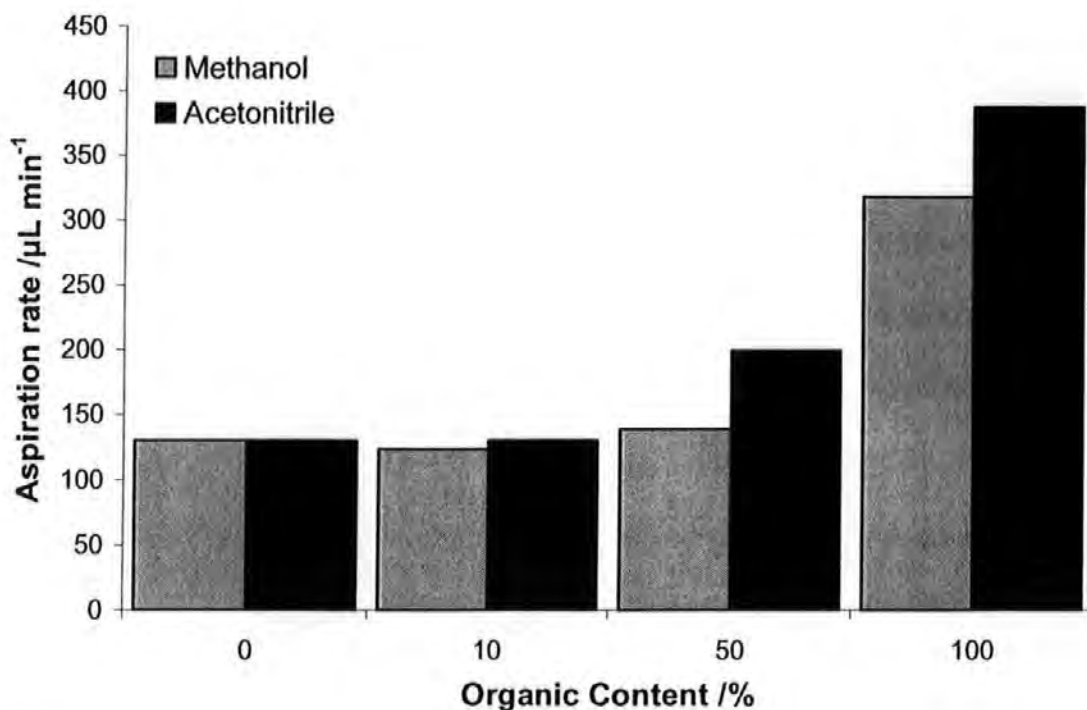
Figure 2.3 Self-aspiration rates for the 1 mL min^{-1} glass concentric nebuliser with varying organic solutions
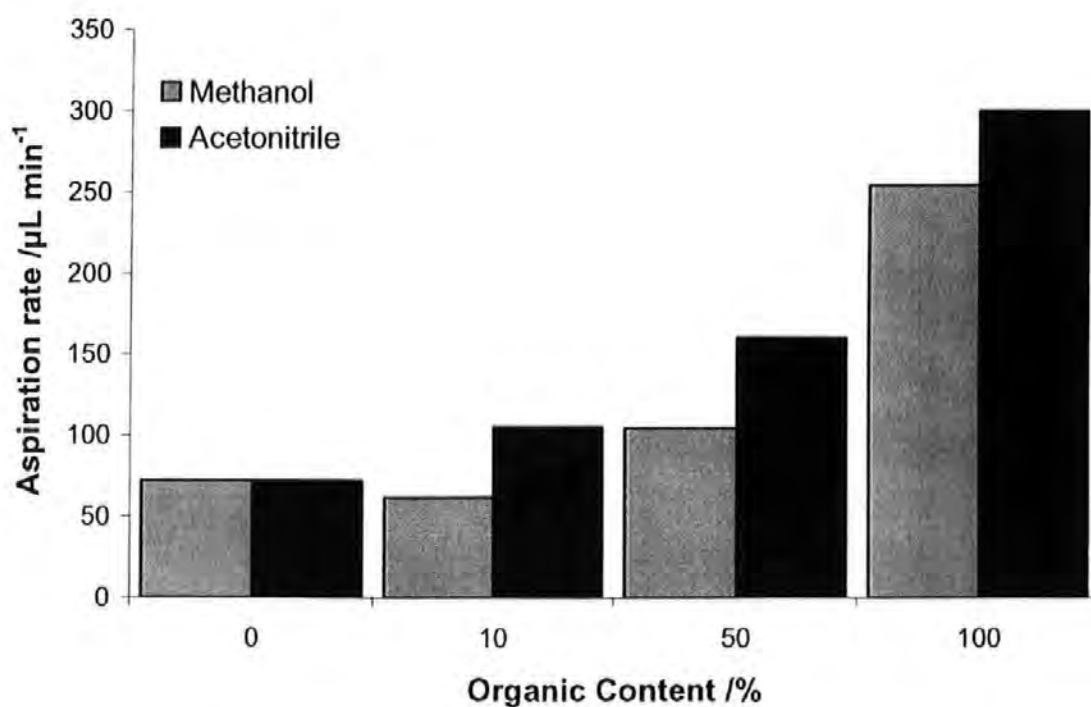
Figure 2.4 Self-aspiration rates for the 200 µL min^{-1} Micromist nebuliser with varying organic solutions
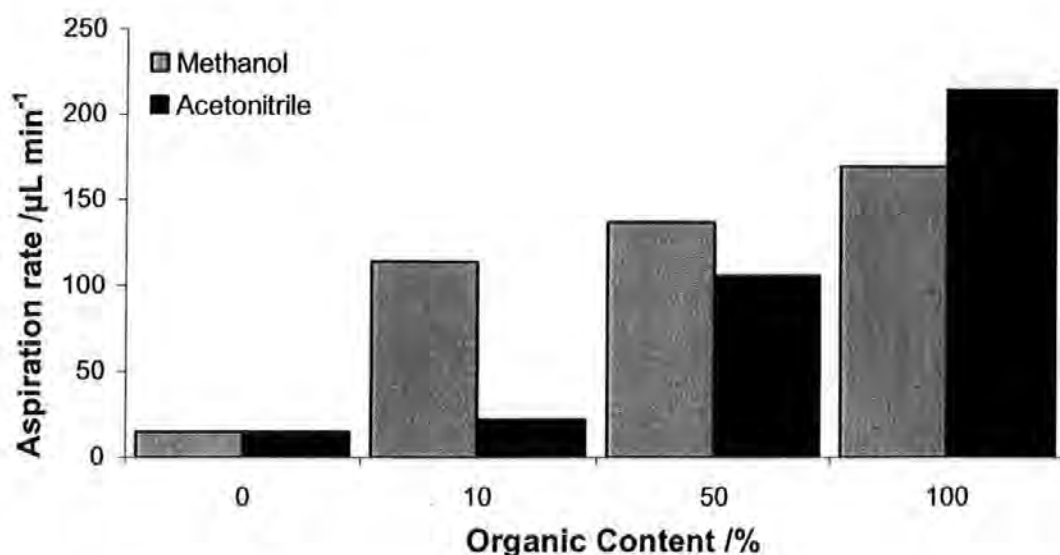
Figure 2.5   Self-aspiration rates for the 50 µL min⁻¹ Micromist nebuliser with varying organic solutions

2.3.2 Effect of nebuliser gas flow and organic content on sensitivity

These experiments were designed to establish how the $^{115}In^+$ signal was affected by nebuliser gas flow rate and the proportion of acetonitrile in the solvent. All three nebulisers detailed in the previous section were studied spraying into a Cinnabar spray chamber cooled to 5°C. Due to the high aspiration rate of the 1 mL min⁻¹ concentric nebuliser the reflected power increased above the threshold of 50 W and the plasma extinguished when 20% volume fraction acetonitrile was introduced. Acetonitrile volume fractions greater than this are often used in chromatographic separations, so this nebuliser would not be suitable for these applications, and was not studied further. Both the 200 and 50 µL min⁻¹ nebulisers were examined by self-aspirating from a 1 mL min⁻¹ stream of eluent pumped from the HPLC pump. The effect of the nebuliser flow rate and acetonitrile volume fraction on the $^{115}In^+$ signal for the 200 µL min⁻¹ nebuliser is shown in Figure 2.6. As can be seen, for the 100% aqueous solution, the nebuliser flow rate had a very

large influence on the signal intensity. Thus, if the nebuliser gas was not optimised, large variations in signal intensity would be observed. The other plots for varying acetonitrile volume fractions exhibited similar trends, with an optimal nebuliser flow rate which was different for each of the solutions. For the 100 % aqueous solution, the optimal nebuliser flow rate for maximum signal was 1.0 L min^{-1}, whereas for the 90 % water/10% acetonitrile volume fraction solution, the optimum was at a nebuliser gas flow of 0.8 L min^{-1}. A decrease in signal intensity with increasing acetonitrile volume fraction was also observed, with the signal decreasing from 600,000 cps for 100% aqueous, to 100,000 cps for the 10% acetonitrile solution. Thus, if a gradient elution was used during a chromatographic run the nebuliser gas flow would require re-optimisation corresponding to the acetonitrile volume fraction at the exact time at which the peak of interest eluted. When solutions containing an organic solvent were aspirated two signal maxima at different nebuliser flow rates were observed. It is also worth noting that, at the higher acetonitrile volume fractions (30 and 40 %), the stability of the plasma deteriorated and there was a considerable amount of carbon build up on the sampler and skimmer cones. This may have contributed to the decrease in signal with increasing organic content. Also, if solutions containing more than 30% acetonitrile are aspirated for more than an hour, there is the possibility that too much carbon build up could lead to the cone orifices blocking. These results suggest that this nebuliser was not ideally suited for chromatographic analysis due to the large variation in signal intensity observed for different organic solvent compositions.
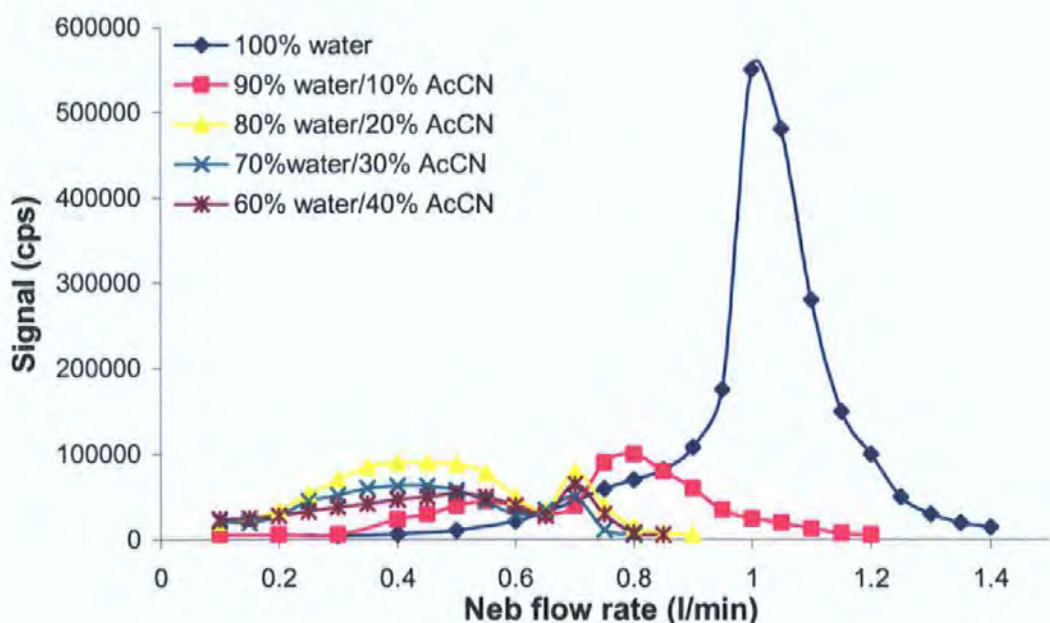
Figure 2.6 The effect of nebuliser gas flow rate and acetonitrile content on $^{115}In^+$ signal for a Micromist nebuliser (200 µL min^{-1}) with self aspiration

Similar plots for the 50 µL min^{-1} nebuliser are shown in Figure 2.7. In this case optimal signal intensity for the 20, 30 and 40 % acetonitrile volume fraction solutions was observed at a nebuliser argon gas flow of 0.40 L min^{-1}. However, for the 10 % acetonitrile solution, the optimum was at 0.85 L min^{-1} and for the 100 % aqueous solution, the optimum was at 0.95 L min^{-1}. This demonstrates that, if the nebuliser gas flow was optimised for 100 % aqueous, then a large decrease in signal would be observed during a gradient chromatographic run wherein the volume fraction of acetonitrile was increased. If the acetonitrile volume fraction at which the peak of interest eluted was known, then the optimum nebuliser gas flow rate could be chosen and utilised. With this nebuliser, the largest signal intensity was achieved with the higher acetonitrile volume fractions, probably due to increased nebuliser efficiency at higher acetonitrile volume fractions. There was also a distinct trend in the optimum signal from 100% aqueous to 60% aqueous

with the changeover occurring at 90% aqueous. At 80% aqueous and lower, a stable optimum signal is achieved at a nebuliser gas flow of 0.40 L min^{-1}. It is also worth noting that, at 40 % acetonitrile, there was visible carbon build up on the sampler cone, but this was not as extensive as with the 200 µL min^{-1} nebuliser. However, if this volume fraction of acetonitrile was used for long periods, or if solutions containing more than 40 % acetonitrile were nebulised, then the carbon build up caused blocking of the cone orifices, the reflected power eventually increased over the threshold and the plasma extinguished. There is also a double maxima observed with the organic containing solutions. This can be explained by there being an optimum sample transport efficiency, and also a separate optimum solvent loading of the plasma[65]. As these two variables were examined simultaneously, two peak maxima were observed.
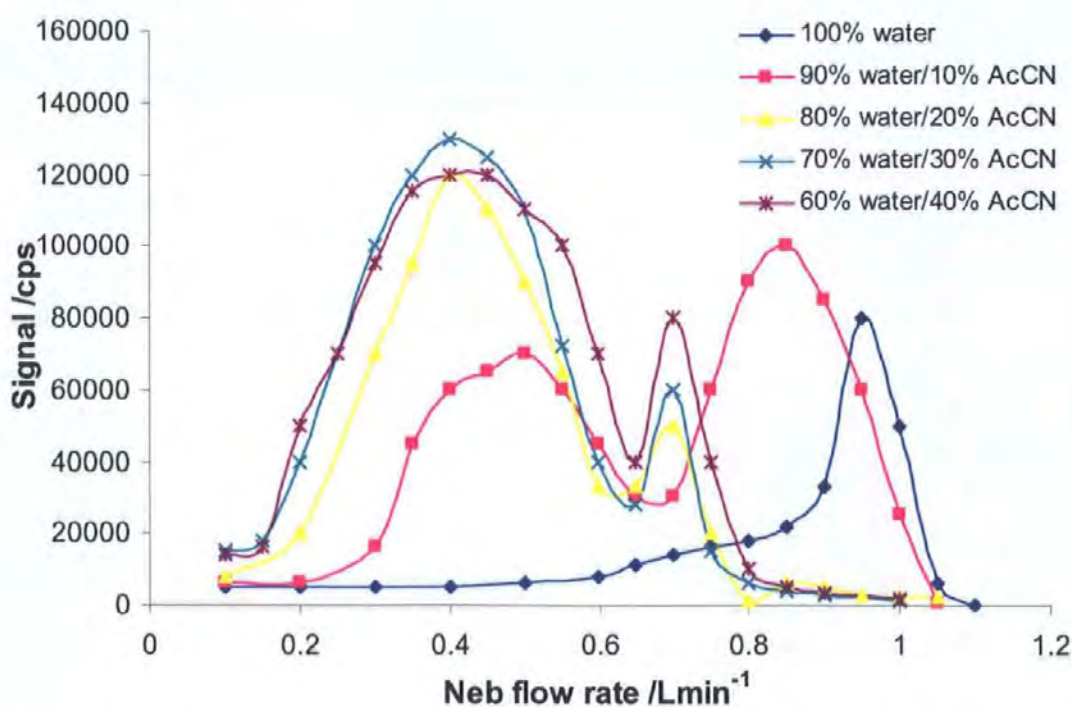
Figure 2.7 **The effect of nebuliser gas flow rate and acetonitrile content on** ^{115}In$^+$ **signal for a Micromist nebuliser (50 µL min^{-1}) with self aspiration**

Experiments with the 50 µL min⁻¹ nebuliser were repeated, but this time the spray chamber was cooled to -5 °C instead of 5 °C. The intention being that, at the lower spray chamber temperature, more of the nebulised solution would condense on the spray chamber walls, with a resultant decrease in solvent vapour reaching the plasma. Results are shown in Figure 2.8, with very similar trends to those with the spray chamber cooled to 5 °C being observed. In this case, the greatest signal was obtained for 10 % acetonitrile with a nebuliser argon gas flow of 0.85 L min⁻¹. Also, at this spray chamber temperature, some of the nebulised liquid froze on the spray chamber wall, thereby limiting the function of the spray chamber.
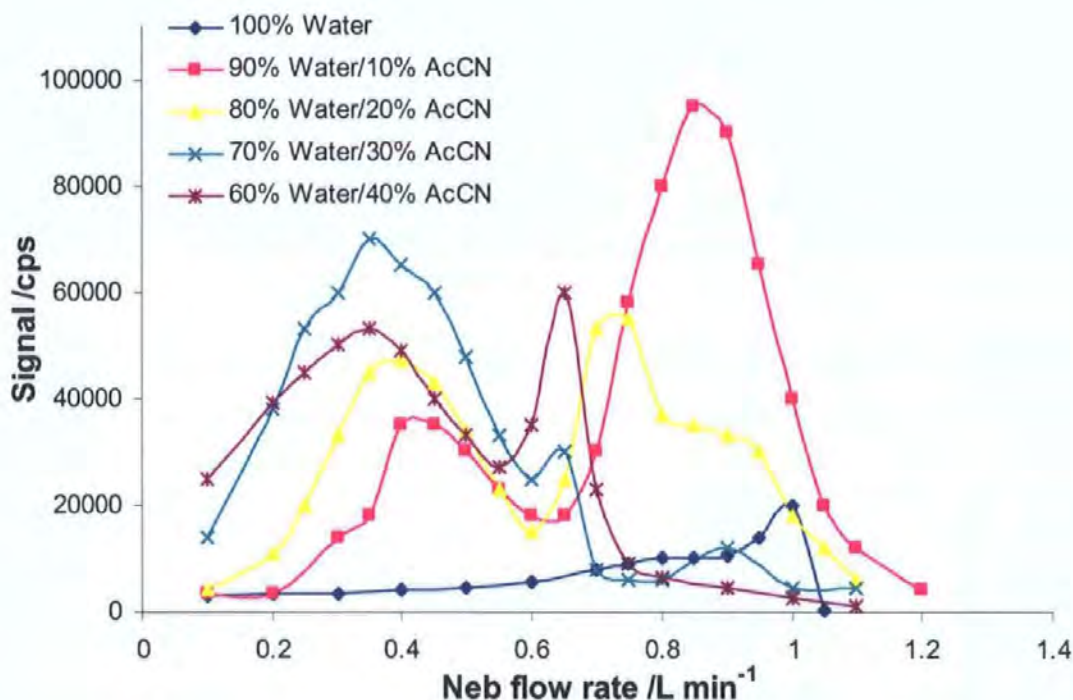
Figure 2.8   The effect of nebuliser gas flow rate and acetonitrile content on $^{115}In^+$ signal for a Micromist nebuliser (50 µL min⁻¹) self aspirating with the spray chamber cooled to -5 °C

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To asses whether the differences in signal intensity were due to the total amount of solution being nebulised, the experiments were repeated at a constant uptake rate. The 50 µL min^{-1} nebuliser was used but the self-aspirating 'T'-piece was removed and the nebuliser pumped at a constant flow rate of 0.1 mL min^{-1} with the spray chamber cooled to 5 °C. Results are shown in Figure 2.9. As can be seen, the largest signal was obtained with 100 % water. Results are shown for solutions containing only up to 30% acetonitrile, because at 40 % the reflected power increased above the threshold and the plasma extinguished.
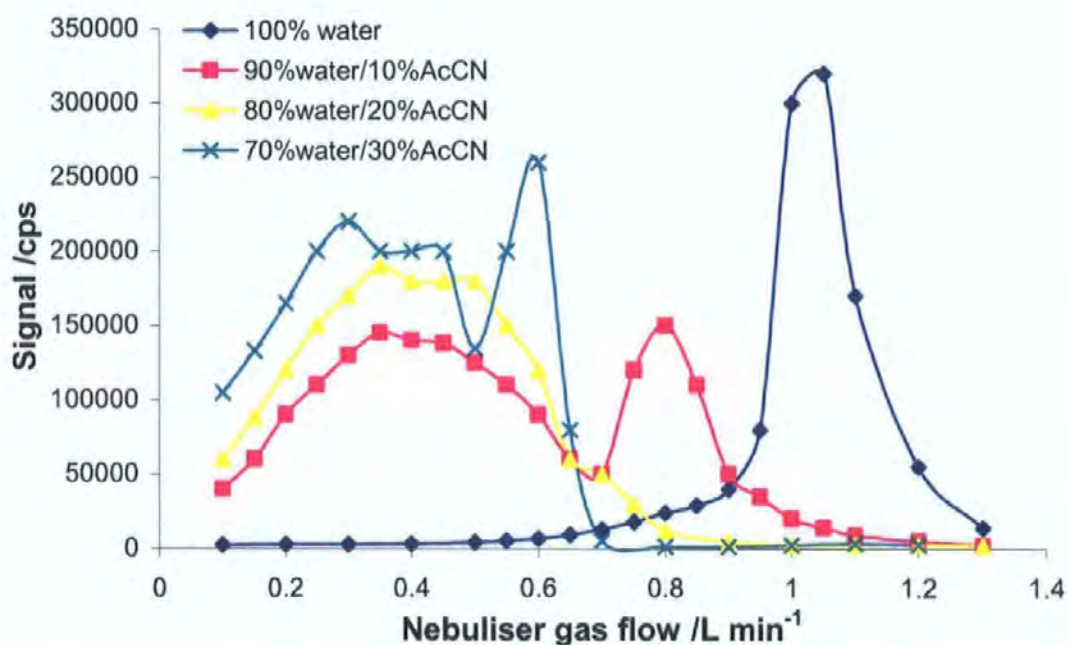
Figure 2.9 The effect of gas nebuliser flow rate and acetonitrile content on ^{115}In$^+$ signal for a Micromist nebuliser (50 µL min^{-1}) pumped at 0.1 mL min^{-1}

As can be seen (Figure 2.9) both the nebuliser gas flow and acetonitrile had a considerable effect on the signal obtained. At an acetonitrile volume fraction greater than 30%, the carbon build up on the cones became extensive and the increase in reflected power caused the plasma to extinguish. If this setup was to be used for chromatographic separations, then the organic content of the mobile phase at the elution time of the peak of interest would have to be known. Using the information in the graphs above, the optimum nebuliser gas flow rate can be determined and used to achieve the maximum sensitivity.

2.3.3 Effect of membrane desolvation

The Cetac Aridus[TM] and Vestec membrane desolvators were evaluated and compared to determine whether any decrease in solvent reaching the plasma could be achieved. When the desolvators were used, varying volume fractions of organic solvent (methanol and/or acetonitrile) in the mobile phase had no effect on the reflected power of the ICP-MS, even up to concentrations of 100%. The plasma was very stable, and did not extinguish throughout the experiments. Even after continued use for seven hours very little carbon build-up was observed on the cones, thus enabling many chromatographic runs to be performed without the need to switch off the instrument and clean the cones. For both systems, the signal was highly dependent on the flow of argon sweep gas. However, once optimised (usually with 100% aqueous) this optimised setting was constant throughout the chromatographic run. Typical count rates obtained for DDW blank samples run with the two different membrane desolvators are shown in Table 2.4. As these results show, the Vestec membrane desolvator generally gave lower background levels than the Cetac Aridus[TM], except in the case of sulphur where a higher background level was achieved with the Vestec unit. Both membrane

desolvators yielded the same sensitivity for 1 ng mL^{-1} ^{115}In, with a signal of 800,000 counts per second. One distinct disadvantage of the Aridus^{TM} membrane is that it utilises a low flow nebuliser which frequently blocked. For these reasons, it was decided that the Vestec membrane would be more suitable for our applications.

Table 2.4   Blank count rates for a DDW sample with the two membrane desolvation units using SF-ICP-MS

Element	Scanning Resolution	Counts per second achieved with Vestec	Counts per second achieved with Aridus^{TM}
^{32}S	3000	530,000	150,000
^{31}P	3000	500	3,500
^{35}Cl	3000	3,250	11,000
^{79}Br	420	275,000	1,000,000
^{81}Br	420	290,000	1,000,000
^{127}I	420	14,000	125,000
^{28}Si	420	50,000	2,500,000
^{19}F	3000	0	0
^{115}In	400	400	500

These membrane desolvators enabled mobile phases containing up to 100% organic solvent to be used and also removes many polyatomic interferences caused by the solvent. The most abundant polyatomic ions which have the same nominal mass as $^{31}P^+$ are shown in Table 2.5. These polyatomic ions contain oxygen and hydrogen, which are formed mainly from the sample introduction solvent. However, because the membrane desolvator removes almost all of the solvent, these interferences are reduced to a very low level. For illustrative purposes, the mass spectrum obtained in the region of m/z 31, at a resolution setting of 3000, is shown without (Figure 2.10) and with (Figure 2.11) desolvation. As can be seen $^{14}N^{16}OH^+$ was reduced to a level which enabled a quadrupole instrument to be used for phosphorus detection with a detection limit of 10 ng mL^{-1} (see chapter six).

Table 2.5   Accurate masses of $^{31}P^+$ and of the most abundant polyatomic ions at m/z 31

Ion	Accurate mass
$^{31}P^+$	30.9738
$^{15}N^{16}O^+$	30.9950
$^{14}N^{16}O^1H^+$	31.0058
$^{12}C^1H_3^{16}O^+$	31.0184
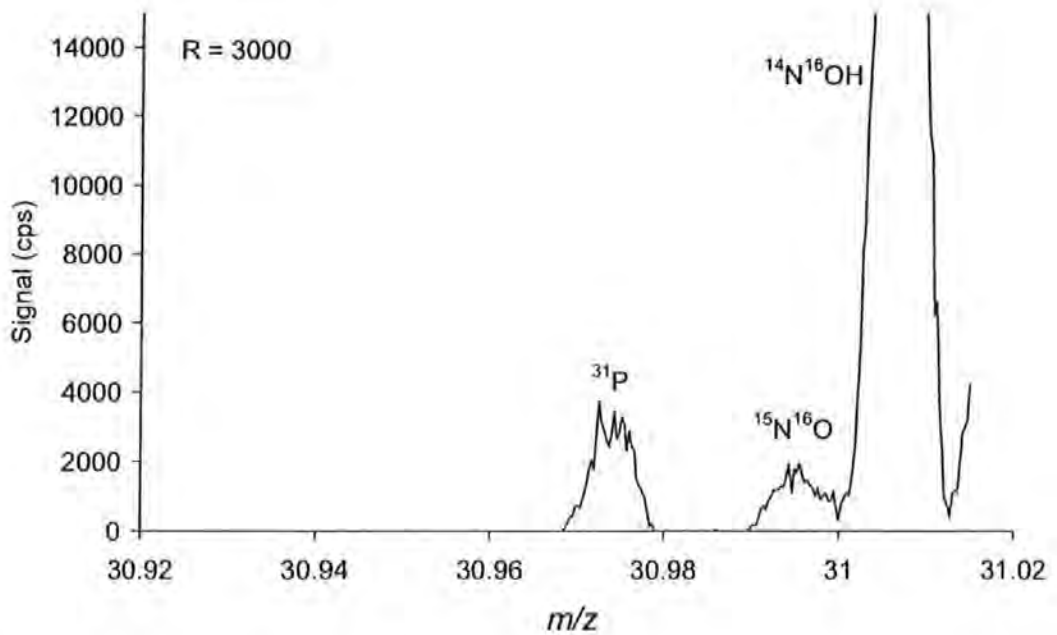
Figure 2.10 Background signal of phosphorus and the two major interferences without desolvation at a resolution setting of 3000
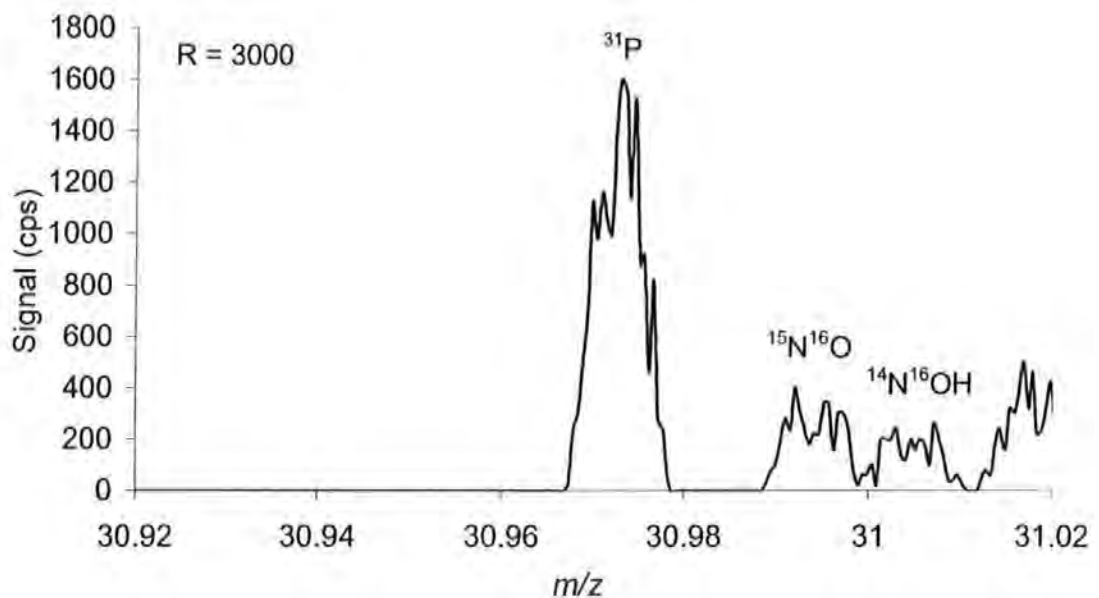
Figure 2.11 Background signal of phosphorus and the two major interferences after desolvation at a resolution setting of 3000

2.3.4 Lamotrigine Analysis

Lamotrigine is an anti-epileptic drug produced by GlaxoSmithKline. This drug contains two chlorine atoms within the molecule, so is ideally suited for chlorine selective detection using ICP-MS. In order to detect low-level chlorine containing impurities in the drug substance/product a sensitive technique (e.g. ICP-MS) is required. Impurities detected using HPLC-ICP-MS can be further characterised using HPLC-ESI-MS. Two probable impurities are 2,4-dichlorobenzoic acid and 2,4-dichlorobenzaldehyde, because both are involved in the production process of Lamotrigine (see Figure 2.12). ICP-MS has the capability to become a selective detector for all lamotrigine related impurities which contain at least one chlorine atom. LC-ESI-MS is not well suited since some impurities, e.g. dichlorobenzoic acid, only respond in negative ion mode, whereas lamotrigine itself and other related impurities respond in positive ion ionisation mode. Other impurities including dichlorobenzaldehyde do not ionise at all in ESI ionisation and would therefore need a different ionisation source. The aim of this experiment was to determine Lamotrigine, 2,4-dichlorobenzoic acid and 2,4-dichlorobenzaldehyde using HPLC-UV-ICP-MS with a view to determine the feasibility of this arrangement for $^{35}Cl^+$ selective detection for impurity profiling.
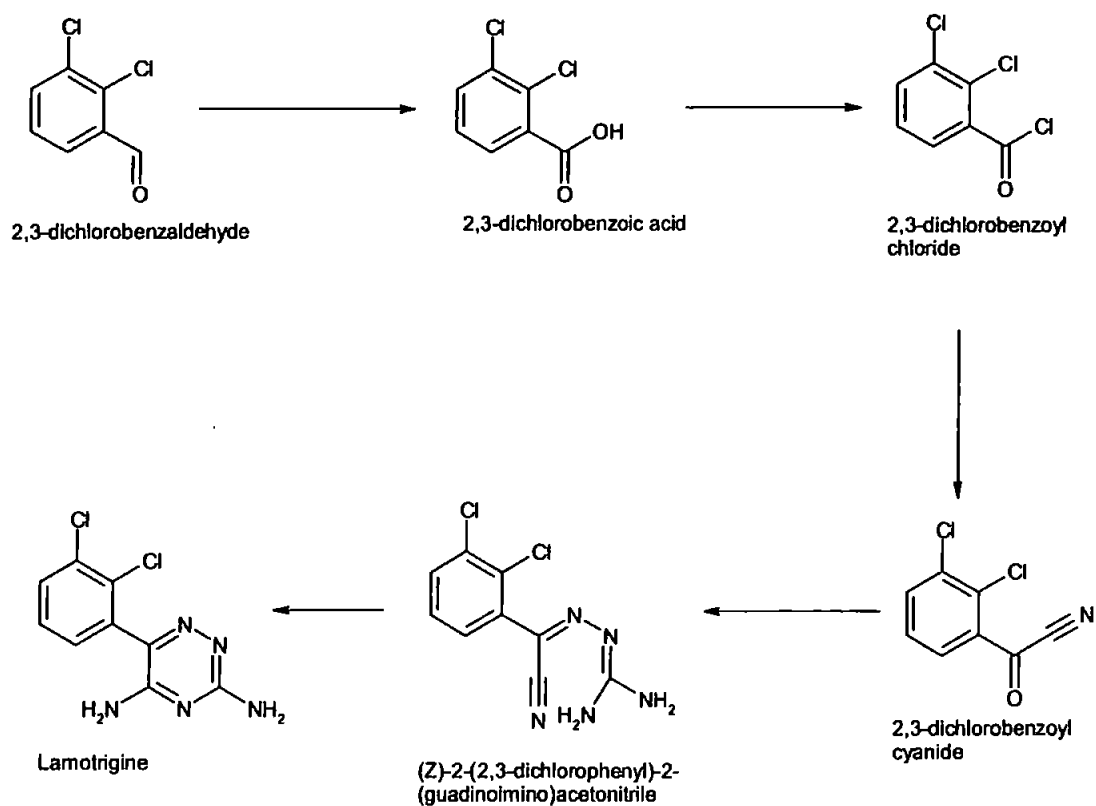
Figure 2.12 Reaction scheme showing the synthesis of Lamotrigine

The UV (at 306 nm) and $^{35}Cl^+$ selective ICP-MS chromatograms for Lamotrigine are shown in Figure 2.13. A large single peak for Lamotrigine was observed in both cases as expected. The variation in retention times for the UV and ICP-MS response was due to the time taken to transfer from the UV detector through the nebuliser (void volume of the system), spray chamber and desolvator to the ICP-MS. The response is normalised for each detection technique.
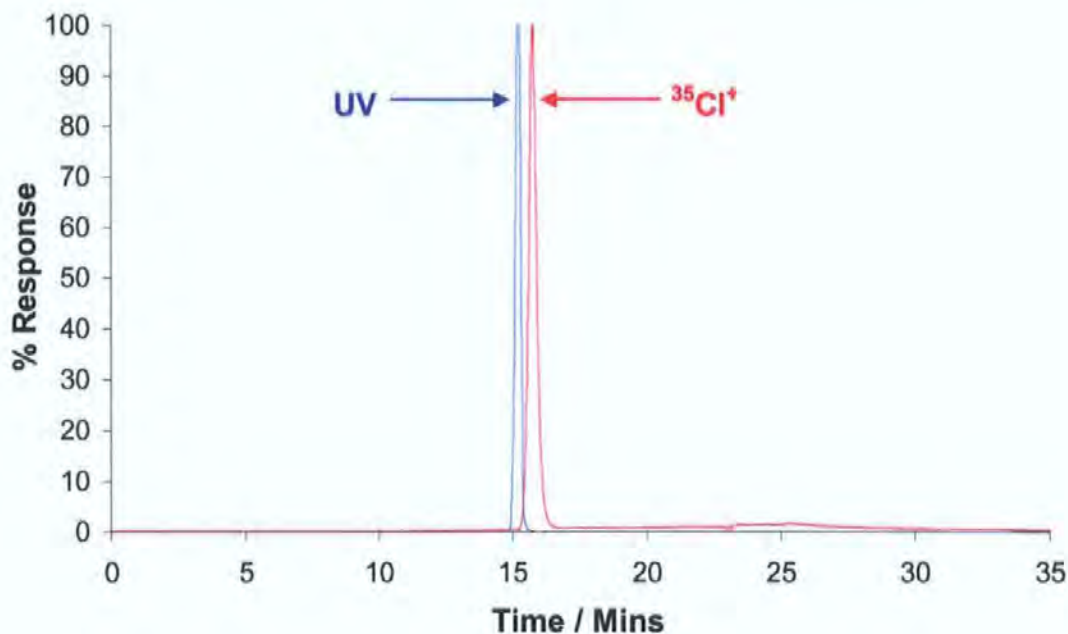
Figure 2.13 ^{35}Cl$^+$ **selective ICP-MS and UV at 306 nm chromatograms for Lamotrigine**

Figure 2.14 and Figure 2.15 show the UV and ICP-MS responses for 2,4-dichlorobenzoic acid and 2,4-dichlorobenzaldehyde respectively. The UV chromatograms for 2,4-dichlorobenzoic acid 2,4-dichlorobenzaldehyde were obtained at 278 nm and 256 nm respectively, because both these compounds have poor UV chromophores at 306 nm. Analytical peaks were observed in the UV, but no peaks were observed when ICP-MS was used for detection. The ICP-MS response appears to just show baseline noise and drift obtained when the gradient elution occurs. After consultation with the literature,[66, 67] it was postulated that the lack of ICP-MS response was caused by the membrane desolvator. Membrane desolvators are designed to allow small polar solvent molecules from the sample stream to pass through the membrane. Both 2,4-dichlorobenzoic acid and 2,4-dichlorobenzaldehyde are more polar and smaller than Lamotrigine so were probably removed by the desolvator, thus a desolvation membrane is not suitable for this type of analysis. This can be a major drawback when using a

membrane desolvator and it is worth noting that, when unknowns are analysed, other analytes may be present in the sample which do not reach the ICP-MS and are therefore not detected.
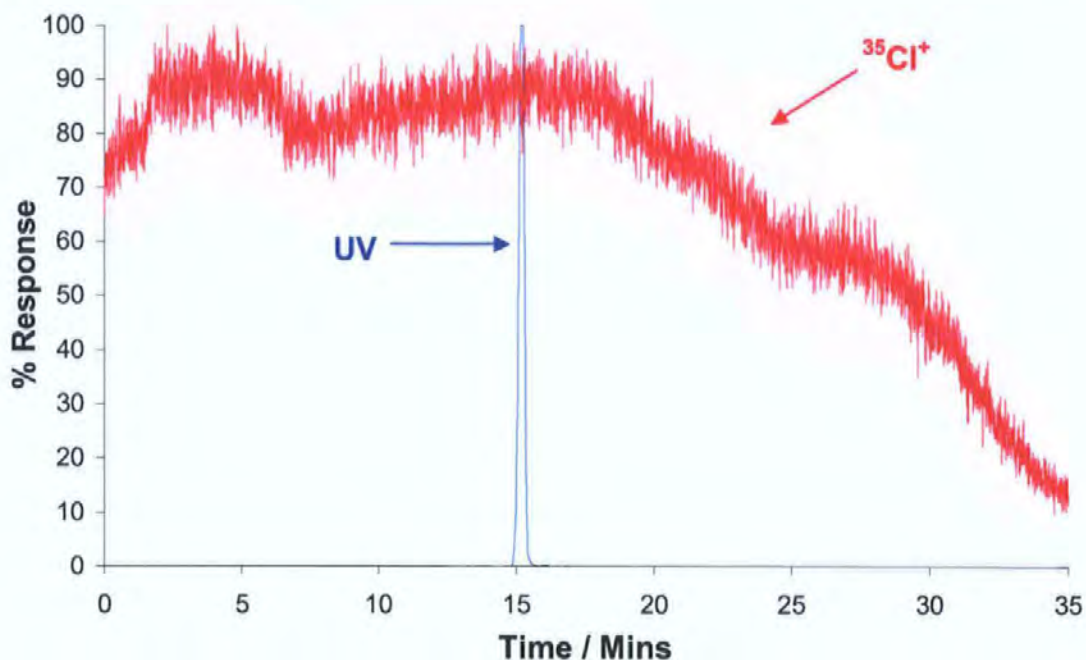
Figure 2.14 $^{35}Cl^+$ selective ICP-MS and UV at 278 nm chromatograms for 2,4-dichlorobenzoic acid
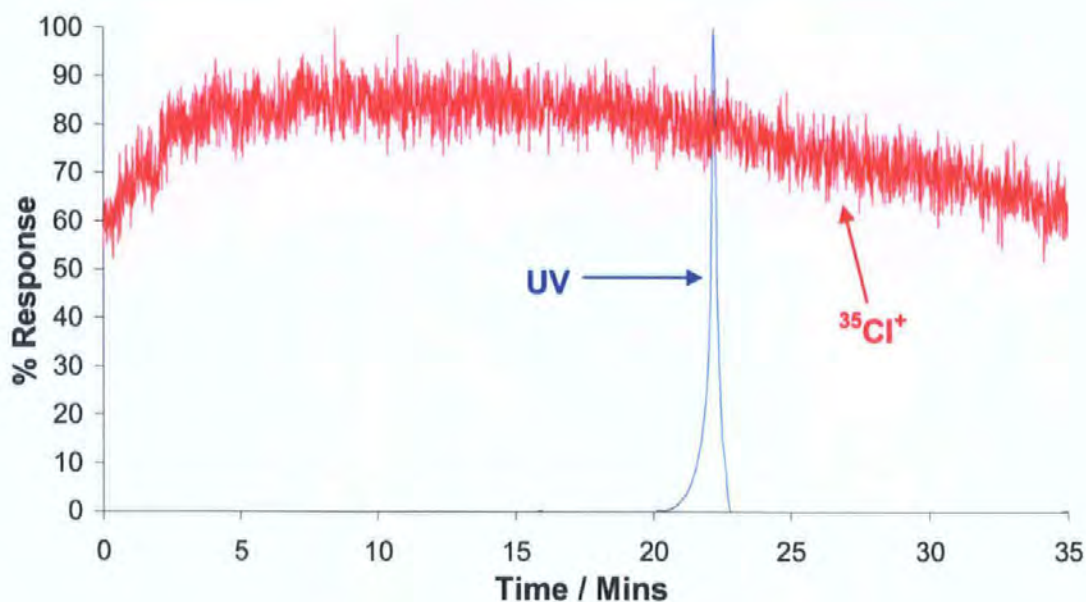
Figure 2.15 $^{35}Cl^+$ selective ICP-MS and UV at 256 nm chromatograms for 2,4-dichlorobenzaldehyde

2.4 Conclusions

Two different methods were investigated for interfacing HPLC with ICP-MS detection to enable organic solvents to be used for the chromatographic separation. The use of a low-flow nebuliser, with the nebuliser aspirating at its optimum rate, resulted in a much reduced solvent load on the plasma. However, as the concentration of the organic phase changed during a gradient elution, the uptake of the nebuliser, and hence the analyte signal, also changed. The optimal nebuliser gas flow rate for 100 % aqueous was totally different to the optimum gas flow rate for a 10 % volume fraction acetonitrile eluent. This would make it extremely difficult to perform quantitative analysis without making a correction for signal drift. Also, even with flow rates as low as 100 μL min^{-1}, too much solvent reached the plasma when the acetonitrile volume fraction reached 30%, causing the plasma to extinguish.

The use of a membrane desolvator prior to the aerosol entering the plasma did enable up to 100 % acetonitrile solutions to be nebulised with little detrimental effect on the plasma. Gradient elution was also possible, with the nebuliser gas flow rate being optimal throughout the chromatographic run. A desolvator also has the advantage of removing many polyatomic ion interferences thereby enabling phosphorus selective detection using low resolution quadrupole ICP-MS. The major problem encountered when using a desolvator membrane, was that some small polar analytes were removed along with the solvent. It is therefore limited to analyses where the analytes are large and not too polar.

Chapter Three

Derivatisation of Carboxylic Acids with Tris(2,4,6-trimethoxyphenyl) phosphonium propylamine bromide and Analysis by Liquid Chromatography - Electrospray Ionization Mass Spectrometry (LC-ESI-MS)

3.1   Introduction

3.1.1   Derivatisation of Carboxylic acids

Chemical derivatisation is an established process of modifying the properties of an analyte molecule to facilitate detection using a given analytical technique[68]. Derivatisation has been used to enhance detection in mass spectrometry[69], ultraviolet/visible (UV/Vis) spectroscopy[70] and fluorescence spectroscopy[71] and also to improve chromatographic properties in techniques such as LC-MS and GC-MS. The chemically added species, i.e. the derivatising reagent, can be chosen according to the properties it possesses and the application of these properties to the detector in use.

Many pharmaceutical drug substances contain carboxylic acids either as a main constituent or as impurities at low levels. Mass spectrometry is ideally suited to detection due to its high sensitivity and selectivity. However, carboxylic acids tend to be poorly ionised by ionisation sources used in mass spectrometry (in atmospheric ionisation sources like ESI, carboxylic acids ionise in negative ion

mode, which generally is less sensitive than positive ion mode), and hence the derivatisation of carboxylic acids to enhance their detection for chromatographic analysis has been a subject of some investigation[68, 72-76]. By exploring the nature and chemical properties of a molecule, a method for modification and subsequent analysis can be devised. Published methods for derivatising carboxylic acids generally involve the incorporation of fluorescent tags[71] or electrochemical detection methods[77], with very little published work on analysis by liquid chromatography-mass spectrometry (LC-MS).

The choice of derivatisation reagent is an important consideration when considering ESI-MS detection. Ideally, the reagent should be pre-charged to remove the dependence on the electrospray ionisation source to initiate ionisation. As ESI-MS is a soft ionisation source, some molecules are poorly ionised by the electrospray process, thus if the molecule is pre-charged this electrospray process becomes less important. The derivatising reagent must possess a suitable reactive group to react with the functional group of the analyte molecule and must be stable and remain intact throughout the reaction process in order to ensure that the maximum signal is achieved with the derivatised product. The molar mass and structure of the derivatisation reagent is also a consideration; smaller reagents have an advantage in that steric hindrance is not an important factor in the reaction process, but larger reagents have the advantage that their derivatised products have a high mass, and thus will enable a signal to be observed in the less noisy region of the mass spectrum. In addition, the reaction should be a rapid, single step reaction with a high yield, using inexpensive and commercially available (or easily synthesised) reagents. Also, the derivatisation product, when not pre-charged, is often less polar than the carboxylic acid, thus increasing the retention time on a conventional C18 column and improving ionisation in

electrospray (ESI) due to a more stable spray being generated with more organic solvent present.

There are some disadvantages in using a derivatisation reaction. The reaction procedure adds extra time to the analysis, with extra materials and glassware needed for the reaction. The reaction may also produce other compounds within the reaction, including partially derivatised products, unwanted side products, and the reaction of the carboxylic acid with any impurities present in the derivatising reagent. The use of chromatography prior to mass spectroscopy detection can overcome some of these problems by separating the derivatised analyte from any interfering by-products of the derivatisation reaction.

In this chapter tris(2,4,6-trimethoxyphenyl)phosphonium propylamine bromide (see Structure 3.1) is used as the derivatising reagent. This is a large positively-charged phosphonium reagent thus enabling detection by ESI-MS in the +ve ion mode. This reagent has an amine functional group, which reacts with a carboxylic acid to form an amide. However, amides are difficult to prepare by direct reaction of amines and carboxylic acids since the basicity of the amine converts the acidic carbonyl groups into their carboxylate anions and removes their reactivity. This derivatisation incorporates a coupling reaction with 1-chloro-4-methylpyridinium iodide and triethylamine to activate the carbonyl group on the carboxylic acid prior to nucleophilic attack by the amine group on the coupling reagent. The resulting derivatised carboxylic acid contains a stable amide bond linking the derivatising reagent with the carboxylic acid. The overall reaction is superficially similar to the kind of nucleophilic substitution that occurs during S_N2 reactions, but the mechanisms are different. Whereas an S_N2 reaction occurs in a single back-side displacement step of the leaving group, nucleophilic acyl substitutions take place

in two steps and involves a tetrahedral intermediate as detailed in Scheme 3.1. In this way, carboxylic acids can react with alcohols to form esters, amines to form amides or even other carboxylic acids to form acid anhydrides.

In this chapter, detection of underivatised maleic, fumaric, salicylic and sorbic acids by LC-ESI-MS-MS, operated in negative ion mode, has been compared with detection of the TMPP-derivatised carboxylic acids in positive ion mode.
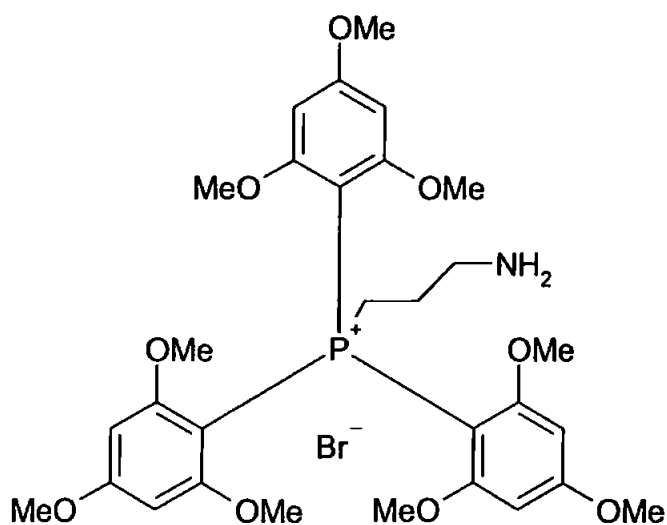
Molecular Formula =C30H41NO9P . Br

Structure 3.1 Tris(2,4,6-trimethoxyphenyl)phosphonium propylamine bromide (TMPP)
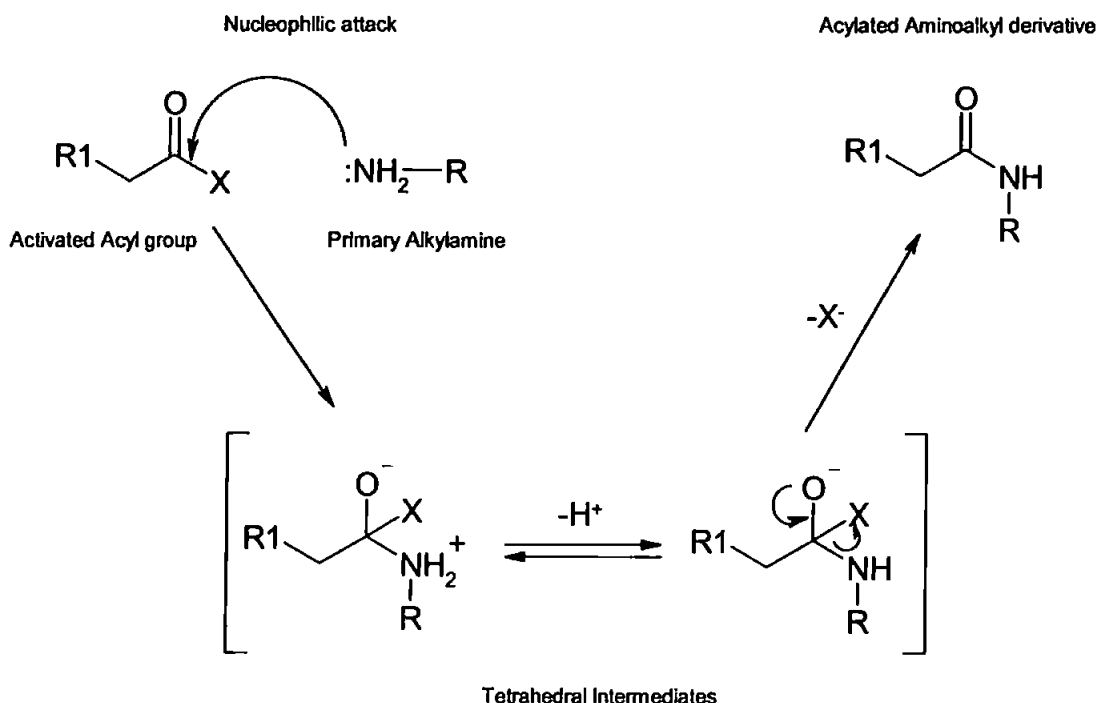
Scheme 3.1 The mechanism of acylation through nucleophilic attack

3.2 Experimental

3.2.1 Chemicals and reagents

Carboxylic acids, 2-chloro-1-methylpyridinium iodide (CMPI), triethylamine (TEA) and formic acid were obtained from Sigma-Aldrich (Poole, Dorset, UK). Acetonitrile (HPLC grade) was obtained from Fisher Scientific UK Ltd. (Loughborough, Leicestershire, UK). High purity water (18.2 MΩ) was obtained using an Elga Maxima water purifying system.

3.2.2 Synthesis of tris(2,4,6-trimethoxyphenyl)phosphonium propylamine bromide (TMPP) (Scheme 3.2)

TMPP was synthesised in-house using an adaptation of the procedure detailed by Leavens et al.[78] To a solution of 3-bromopropylamine hydrobromide (4.98 g, 22.74 mmol,) in water (20 mL) was added potassium carbonate (2.24 g) and toluene (20

mL) with stirring. The resultant toluene phase was isolated, clarified with saturated brine (20 mL), and dried over magnesium sulphate. This dry toluene phase was filtered directly into a pre-refluxing solution of tris(2,4,6-trimethoxyphenyl) phosphine (2.12 g, 3.98 mmol) in toluene (40 mL). The mixture was refluxed for a further 30 min and the resulting white precipitate was isolated by filtration, washed with toluene (2 x 5 mL) and diethyl ether (25 mL) and dried overnight at 30 °C to give the title compound.

Scheme 3.2 Synthesis of tris(2,4,6-trimethoxyphenyl)phosphonium propylamine bromide (TMPP)

3.2.3 Coupling of TMPP propylamine with carboxylic acids

A solution of CMPI was prepared by dissolving 37.98 mg of CMPI in approximately 20 mL of acetonitrile in a 25 mL volumetric flask. Triethylamine (41.4 µL) was added, and the solution made up to volume with acetonitrile. To 100 µL of 100 µM carboxylic acid in 90:10 % volume fraction water:acetonitrile were added 100 µL of CMPI/TEA coupling reagent (prepared as above). After thorough mixing for five minutes at room temperature, 100 µL of a 1 mM TMPP propylamine solution in acetonitrile was added. The solution was left to react for 30 minutes in an ultrasonic bath at room temperature. A schematic of the reaction is shown in Scheme 3.3.

Scheme 3.3 Activation of carboxylic acids with 2-chloro-1-methyl pyridinium iodide (CMPI) and reaction with TMPP propylamine.

3.2.4 Preparation of Panadol™ sample and subsequent derivatisation with TMPP propylamine

A commercially available Panadol™ tablet (GlaxoSmithKline), an analgesic containing paracetamol as the active pharmaceutical ingredient and potassium sorbate as preservative, was crushed with a pestle and mortar until a fine powder was achieved. A small amount of this powder (18.1 mg) was added to a centrifuge tube along with 1 mL of 90:10 % volume fraction water:acetonitrile and centrifuged at 13,000 rpm for 8 minutes. To 100 µL of this supernatant were added 100 µL of CMPI/TEA coupling reagent (prepared as above). After thorough mixing for five minutes at room temperature, 100 µL of a 1 mM TMPP propylamine solution in acetonitrile was added. The solution was left to react for 30 minutes in an ultrasonic bath at room temperature prior to analysis.

3.2.5 Chromatography

For the HPLC-ESI-MS-MS analyses, an Agilent 1100 chromatography system (Agilent Technologies, Stockport, U.K.) was used. All separations were performed using a Phenomenex Luna C18 (2) reversed phase column (100 x 4.6 mm, 3 µm particle size) at 40 °C. The mobile phases contained the following: (A) water / acetonitrile (90:10 v/v) containing 0.05% (v/v) formic acid and (B) water / acetonitrile (10:90 v/v) containing 0.05% (v/v) formic acid. For negative ion mode ESI-MS-MS a stepped gradient elution was used: 0-5 min, 100 % A; 5-5.5 min, 100-70 % A; 5.5-15 min 70 % A; 15-15.5 min, 70-100 % A; 15.5-20 min 100 % A. Similarly, for positive ion mode ESI-MS-MS an isocratic stepped gradient elution was used: 0-10 min, 70 % A; 10-10.1 min, 70-50 % A; 10.1-13 min 50 % A; 13-13.1 min, 50-70 % A; 13.1-20 min 70 % A. The flow rate was 1 mL min^{-1} with an

analyte injection volume of 5 µL. These step gradients were chosen to enhance quantitation using ESI-MS.

3.2.6 Electrospray mass spectrometry

For analysis of the underivatised carboxylic acids, negative ion mass spectra were acquired, whereas for derivatised carboxylic acids, positive ion mode was used to detect them. Spectra were acquired on an Applied Biosystems MDS Sciex API 4000 (Applied Biosystems Ltd, Foster City, California, USA) equipped with a TurboV™ Ion Spray ionisation source. The detection limits were determined using MRM (multiple reaction monitoring) which is the most sensitive technique available on a triple quadrupole instrument. The other parameters used are shown in Table 3.1 and Table 3.2. Data processing was performed using Analyst 1.3 software. The dwell time for each MRM transition was 250 ms and both quadrupoles were operated at unit mass resolution. For all the gases, the settings are arbitrary units. For characterisation of the synthesised TMPP, the same system set-up was used, except no HPLC column was adopted. The spectrum was recorded in the positive ion mode within 190-2000 m/z.

Table 3.1 ESI-MS/MS mass spectrometer operating conditions for analysis of underivatised acids

	Acid			
	Maleic	Fumaric	Salicylic	Sorbic
Quadrupole 1 (m/z)	115	115	137	111
Quadrupole 3 (m/z)	71	71	93	67
Curtain gas (arbitrary units)	10	10	10	10
Collision gas (arbitrary units)	6	6	6	6
Ionspray potential (arbitrary units)	-4200	-4200	-4200	-4200
Temperature (°C)	500	500	500	500
Nebuliser gas (arbitrary units)	20	20	20	20
Auxiliary gas (arbitrary units)	0	0	0	0
Interface heater	ON	ON	ON	ON
Declustering potential (V)	-25	-30	-30	-40
Entrance potential (V)	-10	-10	-10	-10
Collision energy (eV)	-14	-12	-24	-14
Collision cell exit potential (V)	-5	-5	-5	-5

Table 3.2 ESI-MS/MS mass spectrometer operating conditions for analysis of TMPP derivatised carboxylic acids

	Acid			
	Maleic	Fumaric	Salicylic	Sorbic
Quadrupole 1 (*m/z*)	688	688	710	684
Quadrupole 3 (*m/z*)	590	642	590	181
Curtain gas (arbitrary units)	10	10	10	10
Collision gas (arbitrary units)	4.00	4.00	4.00	4.00
Ionspray potential (arbitrary units)	5500	5500	5500	5500
Temperature (°C)	650	650	650	650
Nebuliser gas (arbitrary units)	20	20	20	20
Auxiliary gas (arbitrary units)	0	0	0	0
Interface heater	ON	ON	ON	ON
Declustering potential (V)	95	95	100	100
Entrance potential (V)	10	10	10	10
Collision energy (eV)	55	75	50	70
Collision cell exit potential (V)	8	8	8	8

3.2.7 Nuclear Magnetic Resonance

The ^{1}H NMR spectra were acquired at 270 MHz using a Jeol EX270 Fourier Transform NMR spectrometer. Deuterated acetonitrile (CD$_3$CN, Goss Scientific, Great Baddow, UK) was chosen as the solvent as the residual protio methyl (CD$_2$HCN) group, δ 1.9 ppm, which was used to reference the ^{1}H spectra does not interfere with the expected chemical shifts for the synthesised TMPP. ^{31}P NMR spectra were acquired using the same instrument at 109 MHz, but in this case the centre of the spectral window acquired, 700 ppm, was set to δ 0 ppm and used to reference the spectra because the solvent used did not contain any phosphorus to reference the signal to.

3.3   Results and Discussion

3.3.1 Synthesis of tris(2,4,6-trimethoxyphenyl)phosphonium propylamine bromide (TMPP) (Scheme 3.2)

Initial attempts to synthesise the TMPP derivatising reagent using the reference method[78] were unsuccessful, with the propylamine side chain appearing not to have attached to the tertiary phosphonium. This may be because, unlike the hydrobromide salt, the 3-bromopropylamine free-base was unstable and tended to condense with itself by nucleophilic displacement of bromide by the primary amine. The reference paper did not indicate the length of time necessary to liberate the free amine from the salt with K$_2$CO$_3$, nor the subsequent clarification with brine/drying over magnesium sulphate, so it is probably important to perform this part of the process as quickly as possible. Hence, the literature method was modified. The tris(2,4,6-trimethoxyphenyl) phosphine was weighed and dissolved

in enough toluene to ensure complete dissolution and refluxed for 20 minutes. The formation of 3-bromopropylamine free-base was prepared as quickly as possible, and the filtration of the dry toluene phase was directed straight into the pre-refluxing tris(2,4,6-trimethoxyphenyl) phosphine. This yielded a white crystalline precipitate, which was isolated with a yield of 0.74 g, 27.9%. Confirmation that TMPP had been synthesised was achieved using ^{1}H and ^{31}P NMR, and flow injection ESI-MS. The mass spectrum of the synthesised material yielded a large peak with a mass of 590 m/z in positive ion mode (Figure 3.1). This is the characteristic mass of TMPP and is the same as the mass observed by Leavens et al.[78] Moreover, two in-source formed fragment ions support this structure. The fragment ion at m/z 547 corresponds to the loss of CH_2=CH-NH_2 from the quaternary phosphonium ion and the ion at m/z 422 corresponds to the loss of one of the trimethoxyphenyl groups. The ^{1}H NMR spectrum obtained for the synthesised TMPP dissolved in deuterated acetonitrile is shown in Figure 3.2 along with the spectral data given in Table 3.3. For comparison, the ^{1}H NMR spectrum obtained for the tris (2,4,6-trimethoxyphenyl) phosphine starting material is shown in Figure 3.3 along with the spectral data shown in Table 3.4. By comparing these spectra, the synthesised TMPP showed peaks for the propylamine side chain (at 3.04, 2.62 and 1.34 δppm), which are not observed in the spectrum of the phosphine starting material. This data, along with the mass spectral data, confirmed that TMPP was synthesised successfully. This is confirmed by comparison with Leavens et al.[78] which quoted peak shifts and integrals identical to those obtained in these experiments. Further evidence to support the synthesis was provided by ^{31}P NMR experiments. These were performed for the tris(2,4,6-trimethoxyphenyl) phosphine starting material and for the synthesised TMPP. For the starting material, a chemical shift of -66.67 ppm was observed (Figure 3.4), whereas for the synthesised TMPP a chemical shift of

6.65 ppm was observed (Figure 3.5). This large difference in chemical shift gives more confidence in the successful synthesis of a quarternarised phosphorus.
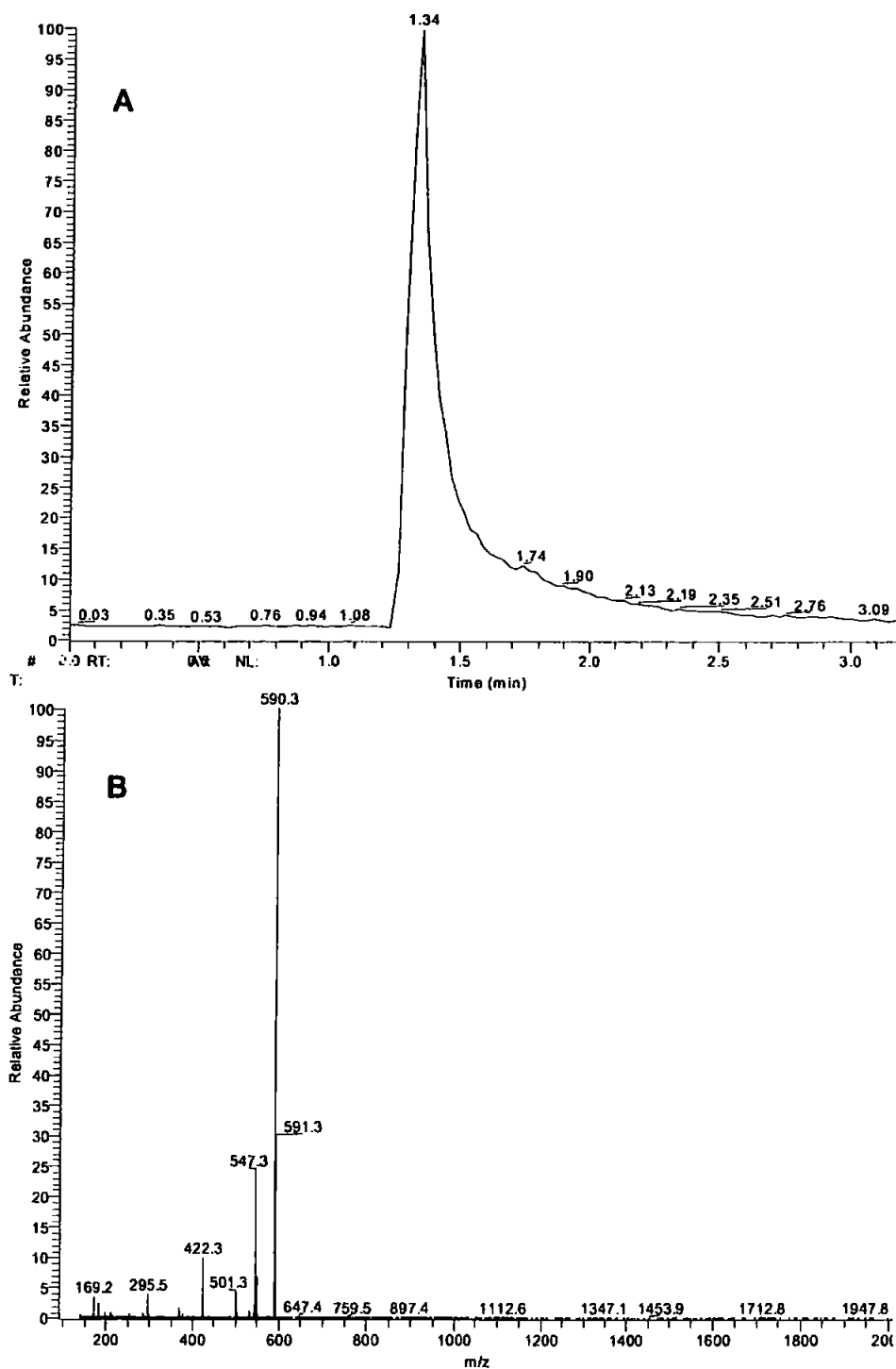
Figure 3.1   Positive ion ESI-MS of the synthesised TMPP: A, total ion current of the flow injected compound; and B, mass spectrum of the peak at 1.34 mins
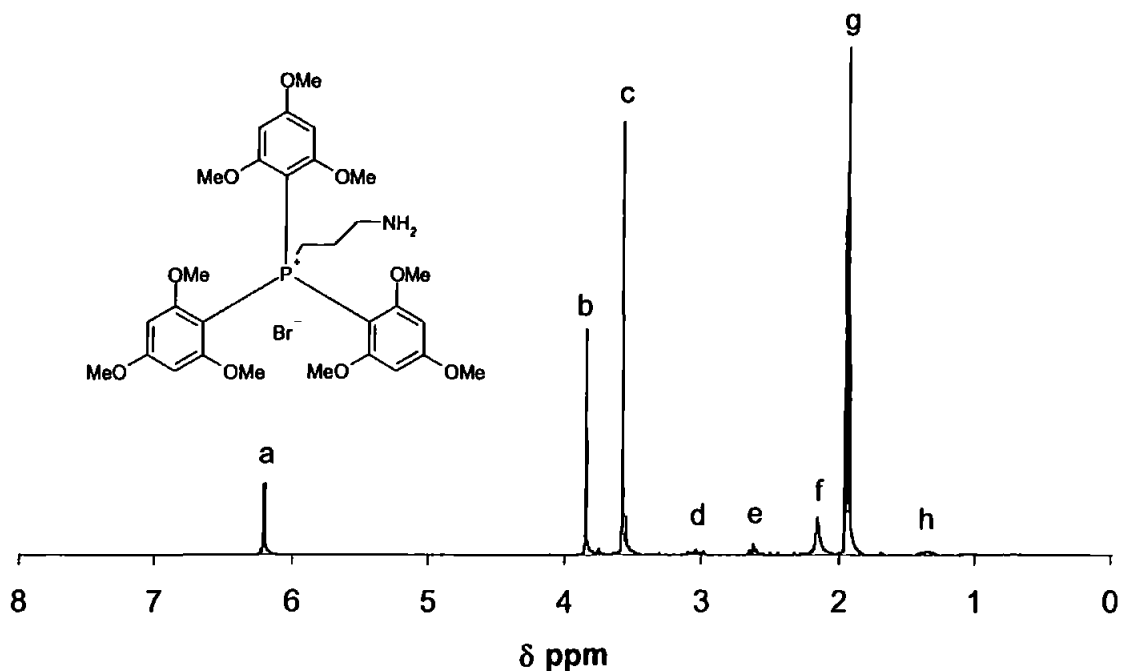
Figure 3.2 ^{1}H NMR spectrum for synthesised TMPP, the data for each signal (lettered) is shown in Table 3.3

Table 3.3 ^{1}H NMR spectral data and peak assignment for the synthesised TMPP

Peak I.D.	Chemical Shift (ppm)	Integral	Coupling pattern	Assignment
a	6.21	6	Doublet	3- and 5-H aromatic protons
b	3.85	9	Singlet	4- OC$\underline{H}_3$
c	3.58	18	Singlet	2- and 6- OC$\underline{H}_3$
d	3.04	2	Multiplet	C$\underline{H}_2$CH$_2$CH$_2$NH$_2$
e	2.62	2	Triplet	CH$_2$CH$_2$C$\underline{H}_2$NH$_2$
f	2.15			From Solvent
g	1.92			From Solvent
h	1.34	2	Multiplet	CH$_2$C$\underline{H}_2$CH$_2$NH$_2$
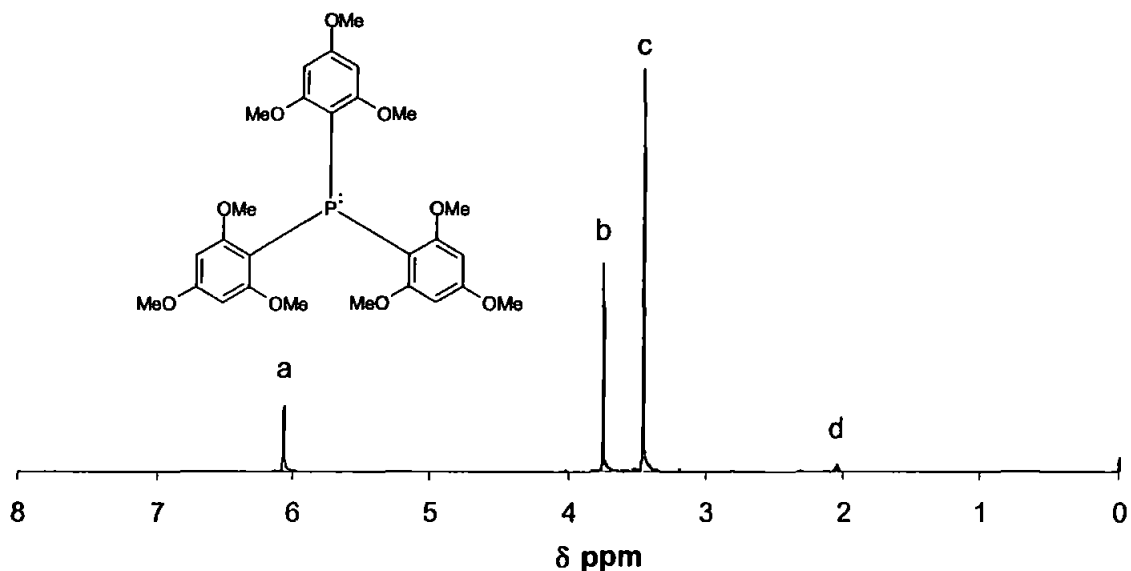
Figure 3.3 ^{1}H NMR spectrum for tris (2,4,6-trimethoxyphenyl) phosphine starting material, the data for each signal (lettered) is shown in Table 3.4

Table 3.4 ^{1}H NMR spectral data and peak assignment for the tris (2,4,6-trimethoxyphenyl) phosphine starting material

Peak I.D.	Chemical Shift (ppm)	Integral	Coupling pattern	Assignment
a	6.07	6	Doublet	3- and 5-H aromatic protons
b	3.75	9	Singlet	4- OCH$_3$
c	3.46	18	Singlet	2- and 6- OCH$_3$
d	2.04			From Solvent
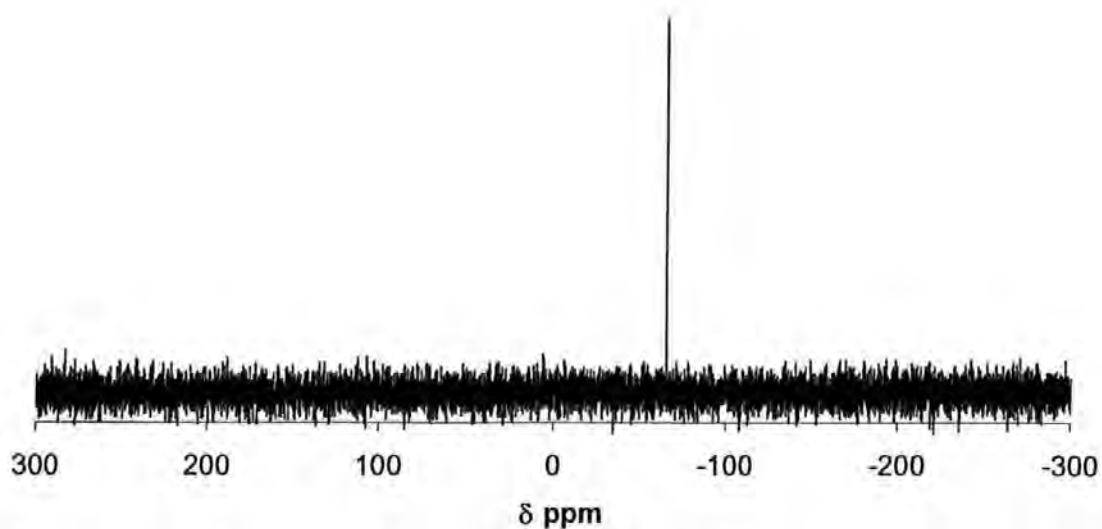
Figure 3.4 ^{31}P NMR spectrum for tris (2,4,6-trimethoxyphenyl) phosphine starting material
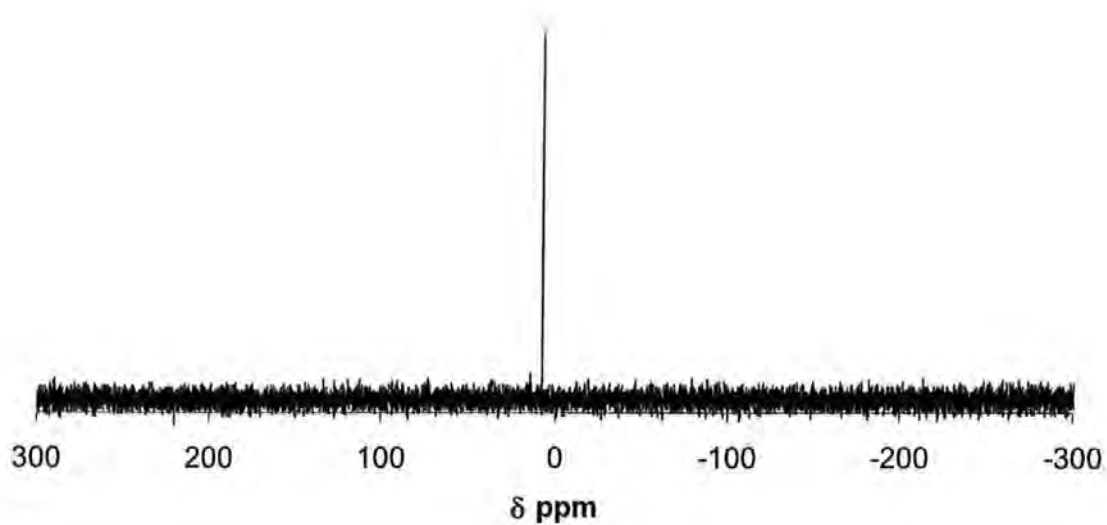
Figure 3.5 ^{31}P NMR spectrum for synthesised TMPP

3.3.2 Derivatisation of carboxylic acids

The determination of fumaric, sorbic, maleic and salicylic acid was investigated. These acids were chosen because they are commonly found in many pharmaceutical products as additives or impurities, and are difficult to determine using conventional ESI-MS because they do not form positive ions and have low molecular weights. Negative ion ionisation is possible, but due to their low molecular weight, there is a lot of chemical noise in the mass spectra, e.g. from common LC additives such as acetic acid, formic acid and especially trifluoroacetic acid (m/z = 113 and its cluster ion at m/z = 227). In addition, since they are poorly retained on conventional reversed phase columns which are typically used for pharmaceutical analysis, they elute in a highly aqueous matrix which is not ideal for electrospray ionisation. It leads to an unstable spray if the organic content of the eluent is too low. It would be advantageous to be able to determine these compounds using the standard approach of reversed phase LC, coupled with ESI-MS operated in positive ion mode, to enable co-determination of all analytes.

In order to evaluate any possible benefits of derivatisation of the acids with TMPP the analysis was performed using LC-ESI-MS-MS in negative ion mode without derivatisation and in positive ion mode with derivatisation. This represented the two best case scenarios for the analysis of the acids with and without derivatisation. Chromatograms showing the separation and detection of the four acids using both modes of detection are shown in Figure 3.6. Detection was performed by monitoring the appropriate precursor ion in quadrupole 1 and the most abundant product ion in quadrupole 3 (Table 3.5 and Table 3.6). Quadrupole 2 uses RF only and acts as a collision cell, where a collision gas is introduced at a pressure such that an ion entering the quadrupole undergoes one or several

collisions[9]. The ion then fragments and the product/fragment ions are analysed in quadrupole 3. In multiple reaction mode (MRM), the most abundant precursor ion, along with the most abundant product ion, combine to improve the sensitivity of the instrument, as two specific masses are being analysed. Thus, in theory, if all ions are fragmented each ion is detected twice. Also, because only ions transmitted by quadrupole 1 are detected at the m/z determined by quadrupole 3, a very low background signal is observed, resulting in an enhanced signal-to-noise ratio, hence lowering the limits of detection.

For the underivatised acids, the most abundant fragment ion corresponded to the loss of CO_2 (a loss of 44 m/z) from the precursor ion, whereas for the derivatised acids this fragmentation is variable, hence the most abundant fragment was that used in the analyses. In order to improve the duty cycle not all product ions of the compounds to be analysed were monitored throughout the chromatographic run, but instead the mass spectrometer switched to the most appropriate mass during elution of each individual carboxylic acid, hence the changing baseline observed in Figure 3.6 A.

A three point calibration was performed for each of the acids and detection limits were calculated based on 3σ of the baseline noise. This was calculated by taking the standard deviation of ten randomly picked points on the baseline, and calculating the standard deviation of these points. Using three times this standard deviation and correlating this to the linear response equation for each acid, the detection limits were calculated. Detection limits for the underivatised and derivatised carboxylic acids are given in Table 3.5 and Table 3.6 respectively. As can be seen, detection limits for fumaric, sorbic and maleic acids were approximately one order of magnitude lower after derivatisation and +ve ion

detection, thereby confirming the utility of this method for the enhanced detection of these acids. In contrast, a fivefold decrease for the detection limit was observed for salicylic acid. This is probably due to strong internal hydrogen bonding making salicylic acid a poor nucleophile leading to an inefficient reaction with the CMPI activating reagent

Table 3.5   **Detection limits obtained for the underivatised carboxylic acids obtained using LC-ESI-MS-MS in the –ve ion mode**

Acid	Q_1 m/z (precursor)	Q_3 m/z (product)	Detection limit / fmol
Fumaric	115	71	24
Sorbic	111	67	51
Maleic	115	71	2
Salicylic	137	93	117

Table 3.6   **Calculated detection limits for the derivatised carboxylic acids obtained using LC-ESI-MS-MS in the +ve ion mode**

Acid	Q_1 m/z (precursor)	Q_3 m/z (product)	Detection limit / fmol
Fumaric	688	642	2
Sorbic	684	181	4
Maleic	688	590	0.4
Salicylic	710	590	540

Figure 3.6   Chromatograms for a mixture of carboxylic acids (2 µmol L^{-1}) with detection by multiple reaction monitoring electrospray mass spectrometry: A, underivatised carboxylic acids and negative ion mode ESI-MS/MS; B, derivatised carboxylic acids and positive ion mode ESI-MS/MS

3.3.3 Analysis of Panadol™ tablets

Panadol Extra™ is the proprietary name of an oral analgesic medicine manufactured by GlaxoSmithKline, which would normally contain paracetamol, caffeine and potassium sorbate (used as preservative) together with excipients. Hence, the absence of sorbate in any so-called Panadol Extra™ product would indicate that the product was counterfeit. Analysis was performed using LC-ESI-MS/MS in both negative and positive ion mode after derivatisation with TMPP propylamine; the resulting chromatograms are shown in Figure 3.7. Derivatisation of the sorbic acid (Figure 3.7 B) resulted in an improvement in chromatographic resolution and signal-to-noise compared to the underivatised sample (Figure 3.7 A). An added advantage of the derivatisation is that analysis can be performed using positive ion mode ESI-MS, so both paracetamol and caffeine can also be determined simultaneously if necessary.
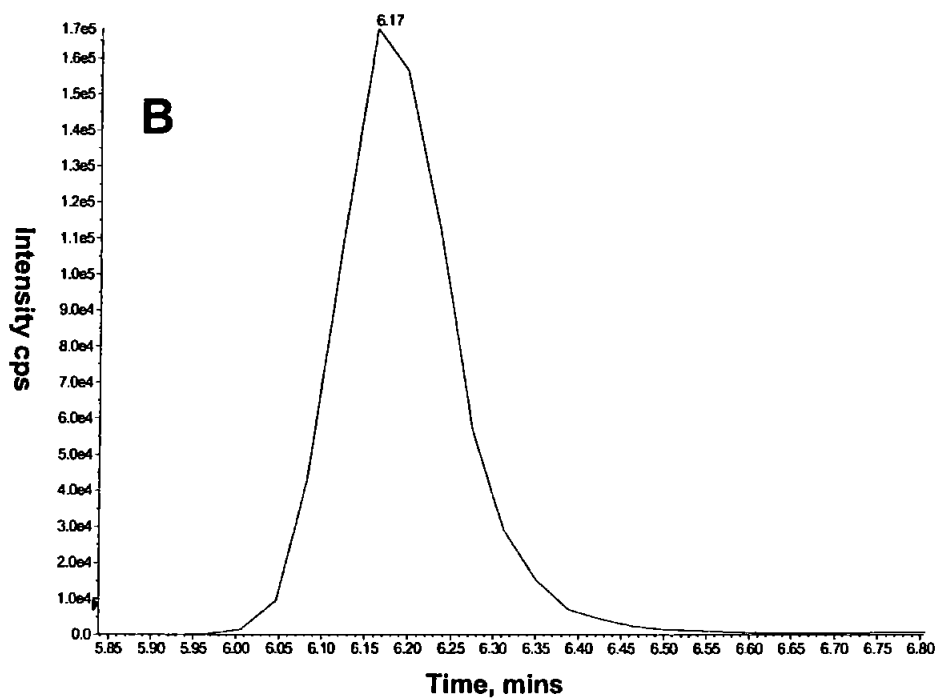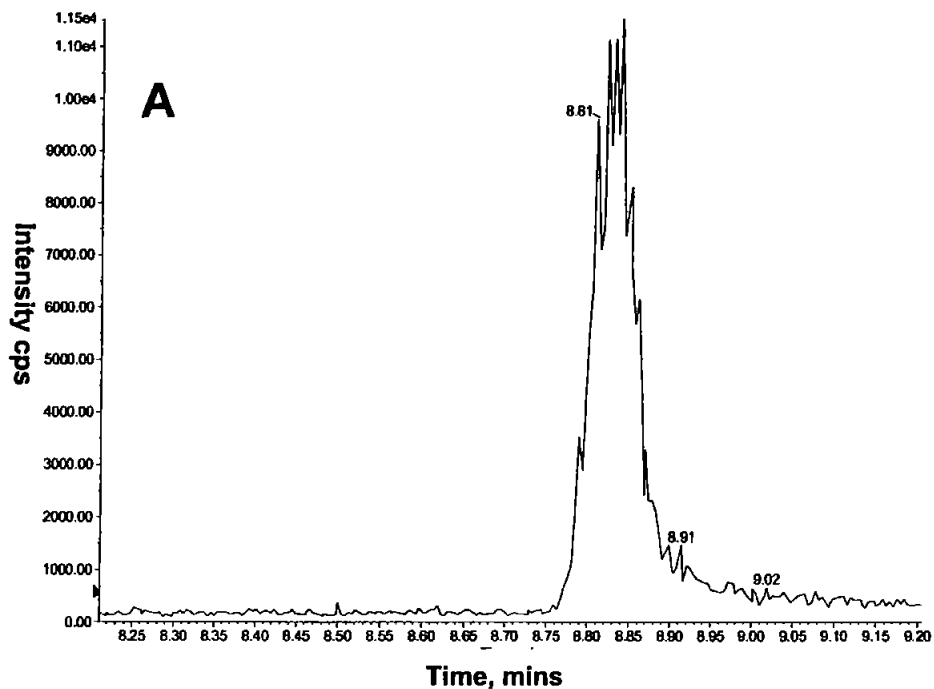
Figure 3.7   Selected ion chromatograms for Panadol™ samples, showing
the presence of sorbate, obtained in: A, negative ion mode
without derivatisation; B, positive ion mode after derivatisation
with TMPP

3.4 Conclusions

Enhanced detection of maleic, fumaric and sorbic acids has been achieved by derivatisation with TMPP and detection using positive ion LC-ESI-MS/MS in multiple reaction mode. The method was successfully used for the derivatisation of sorbic acid in a sample of Panadol™. The derivatisation of salicylic acid was not as successful, probably due to poor reaction efficiency.

Chapter Four

Detection of phosphorus tagged carboxylic acids using HPLC-SF-ICP-MS

4.1 Introduction

4.1.1 ICP-MS detection of carboxylic acids

The vast majority of organic compounds are essentially not detectable by inductively coupled plasma mass spectrometry (ICP-MS) because they only contain H, C, O and N. These elements have extremely high background signals because they are either present in the solvents used during sample introduction or present in atmospheric air entrained into the plasma, hence, trace analysis is impossible. The exceptions to this are the determination of C at minor and trace levels[79-81] and element selective detection of organic compounds which contain a hetero-atom such as P, S, Si, Cl, Br, I, a metal or metalloid. For example, detection of phosphorus containing compounds has previously been performed for the determination of phosphorylated peptides[82-84] and for sulphur-containing compounds in pharmaceuticals[56]. However, if a means of making all organic compounds detectable by ICP-MS could be achieved, many new applications would be realised. For example, high accuracy quantitative analysis of organic compounds could be achieved using external calibration; and many biological molecules could be determined in metabolic studies[85]. An alternative has recently been proposed by Smith et al.[86] which uses aqueous eluents only (at temperatures of 60 and 160 °C) or isotopically enriched solvents to detect carbon in organic compounds by LC-ICP-MS.

Leavens et al.[78] have previously reported on the synthesis of a large molecular weight phosphorus reagent, tris(2,4,6-trimethoxyphenyl) phosphonium propylamine bromide (TMPP), which was used to derivatise organic compounds to make them amenable to detection by +ve ion electrospray ionization mass spectrometry (ESI-MS), and it has been shown how this can be used for the determination of low molecular weight carboxylic acids in pharmaceutical samples[87]. As the majority of carboxylic acids do not contain a heteroatom so cannot be detected using ICP-MS, by derivatising these acids with TMPP, they have effectively been tagged with phosphorus, so $^{31}P^+$ selective detection using ICP-MS can be performed. This has useful applications in pre-screening for quality control and investigations involving intellectual property rights[56]. In this chapter some pharmaceutically important carboxylic acids (maleic, fumaric, salicylic and sorbic) were derivatised by TMPP and detected by phosphorus selective LC-ICP-MS. The derivatisation reaction was also examined to further enhance the derivatisation procedure to lead to more sensitive detection, and hence lower detection limits.

4.2 Experimental

4.2.1 Chemicals and reagents

Carboxylic acids, 2-chloro-1-methylpyridinium iodide (CMPI) and triethylamine (TEA) were obtained from Sigma-Aldrich (Poole, Dorset, UK). HPLC grade acetonitrile was obtained from Fisher Scientific UK Ltd. (Loughborough, Leicestershire, UK). High purity water (18.2 MΩ) was obtained using an Elga Maxima water purifying system. TMPP was synthesised as detailed in Section 3.2.2.

4.2.2 Preparation of coupling reagent

Solutions of CMPI were prepared by dissolving an appropriate amount (typically 38 mg) of CMPI in approximately 20 mL of acetonitrile in a 25 mL volumetric flask. The corresponding amount of triethylamine was added, and the solution made up to volume with acetonitrile.

4.2.3 Coupling of TMPP propylamine with carboxylic acids

To 500 μL of carboxylic acid in 90:10 % (v/v) water:acetonitrile were added 500 μL of CMPI/TEA coupling reagent (prepared as above). After thorough mixing for five minutes at room temperature, 500 μL of a TMPP propylamine solution in acetonitrile was added. The solution was left to react for 30 minutes in an ultrasonic bath at room temperature.

4.2.4 HPLC-SF-ICP-MS analyses

For HPLC-SF-ICP-MS analyses, a modular chromatography system (HP1050, Agilent Technologies, Stockport, U.K.) equipped with a C18(2) reversed phase column (Phenomenex Luna, 100 x 4.6 mm, 3 μm particle size) was used. The mobile phase comprised of a binary system of: eluent A, water/acetonitrile (90:10 % v/v) containing 0.05% formic acid (v/v); and eluent B, water/acetonitrile (10:90 % v/v) containing 0.05% (v/v) formic acid. The linear gradient employed started at 100% A, changing to 40% A and 60% B over 20 minutes. The flow rate was 1 mL min^{-1} with an injection volume of 5 μL. All experiments were performed using a sector-field inductively coupled plasma mass spectrometer (SF-ICP-MS, Thermo Elemental Axiom, Winsford, U.K.), using a mass resolution setting of 3000. Operating conditions are shown in Table 4.1.

Table 4.1   Operating conditions for SF-ICP-MS

ICP	
Nebuliser gas flow/Lmin^{-1}	1.10
Auxiliary gas flow/Lmin^{-1}	0.85
Coolant gas flow/Lmin^{-1}	14.0
Nebuliser	Micromist (Glass Expansion, Switzerland)
Spray Chamber	Jacketed quartz cyclonic, cooled to 5 °C
Torch	Quartz Fassel-type with quartz bonnet, without shield
Interface	
Sampling cone	Nickel 1 mm i.d.
Skimmer cone	Nickel, 0.7 mm i.d.
Single ion monitoring	
Resolution	3000
Slit settings	Source, 330; collector, 220
Masses monitored	Single ion monitoring for $^{31}P^+$ at m/z 30.974
Dwell time/ms	500
Scanning	
Resolution	3000
Slit setting	Source, 330; collector, 220
Masses monitored	Scanning between m/z 30.838 and 31.109
Dwell time/ms	50
Points	20

It is common, in large pharmaceutical companies, to adopt a standard HPLC elution method which can be used in the majority of cases. The method is often standardised to a simple linear elution of acetonitrile/water which can run up to 100 % organic phase (v/v). Such a high volume fraction of acetonitrile is incompatible with the normal operation of ICP-MS unless an extremely low flow nebuliser is used. One solution to this, which was adopted in this work, is to use a membrane desolvation system, which removes the vast majority of solvent and allows routine operation of gradient elution up to 100 % volume fraction acetonitrile at standard 1 mL min^{-1} flow rates (see chapter two). However, it should be noted that low molecular weight polar analytes may also be removed by the desolvation systems, so caution should be used when adopting this approach. In this work the HPLC column was coupled with the SF-ICP-MS simply by inserting the end of the tubing from the column into the sample uptake inlet of the nebuliser, and the output from the spray chamber was introduced into a Universal Interface Model B

desolvation device (Vestec Corporation, Houston, USA). The sweep gas was optimised daily at approximately 2.0 L min^{-1} to ensure the maximum signal for $^{31}P^{+}$ was achieved. The dry aerosol exiting the desolvator was then transferred to the ICP torch via 1 m of 0.25 in. i.d. Tygon tubing.

4.2.5 HPLC-Electrospray ionisation mass spectrometry

HPLC was performed using a binary pump (P580A, Dionex-Softron GmbH, Germering, Germany) coupled with a C18(2) reversed phase column (Phenomenex Luna, 100 x 4.6 mm, 3 μm particle size) with the same gradient elution used for HPLC-SF-ICP-MS. The flow rate (1 mL min^{-1}) was split post column (high pressure micro-splitter valve; Upchurch Scientific Ltd, Oak Harbor, WA, USA), and a portion (~200 μL min^{-1}) was diverted to the mass spectrometer and the residue to waste. Sample injections (5 μL) were made manually with a metal-free injection valve (Rheodyne 9125, CA, USA). Mass spectrometry analysis was performed using an ion trap mass spectrometer fitted with an electrospray interface (ThermoQuest Finnigan Mat LCQ, San Jose, CA). Data were acquired and processed with Xcalibur 1.0 software. Instrument optimization was performed by infusing a 100 ng mL^{-1} solution of TMPP at 3 μL min^{-1} monitoring for the characteristic positive ion at 590 m/z. The following instrument parameters were used: source voltage, +4.50 kV; capillary voltage, +20 V; tube lens offset, -10.00V; capillary temperature, 220 °C; nitrogen sheath gas flow rate, 60 (arbitrary units), and nitrogen auxiliary gas flow rate 20 (arbitrary units). Mass spectra were recorded in the positive ion mode within m/z 190-1000.

4.3 Results and Discussion

4.3.1 Phosphorus selective detection

Chromatograms of maleic acid (MA), after derivatisation with TMPP, are shown in Figure 4.1. Detection was performed by using SF-ICP-MS with $^{31}P^+$ selective detection at 30.974 m/z (Figure 4.1 A and B), and ESI-MS (Figure 4.1 C and D). As can be seen, the TMPP derivatising reagent contained many phosphorus containing impurities (Figure 4.1 A), so it was necessary to optimise the chromatography such that the derivatised maleic acid (MA) was separated from these peaks (Figure 4.1 B), and particularly the large peak for unreacted TMPP. For comparison, and for confirmatory purposes, detection was also performed using ESI-MS and the major peak at 8.43 min was found to have a mass of 590 m/z, which corresponds to the mass of TMPP. Likewise, the peak at 12.65 min was found to have a molecular ion at 688 m/z, indicating it to be MA derivatised with TMPP (Figure 4.1 D). A comparison of the chromatogram obtained using ICP-MS (Figure 4.1 B) with the total ion chromatogram obtained using ESI-MS detection (Figure 4.1 C) indicates that the former method yielded a slightly improved signal-to-noise ratio, which should result in better detection limits. However, in this case, ESI-MS showed better signal-to-noise when single ion monitoring at the base peak of 688 m/z was performed. This highlights both the advantages and disadvantages of the two techniques, namely that ICP-MS is useful for high sensitivity screening purposes when confirmation that a compound contains phosphorus (or any other element for that matter) is required. Hence, once these peaks in the chromatogram have been identified, then ESI-MS can be used in single ion monitoring mode to perform further quantitative analysis. In this case the TMPP was synthesised in-house so was not particularly pure, however, it

is envisaged that a purer form of the reagent would alleviate some of the problems

caused by interfering peaks in the ICP-MS chromatogram.
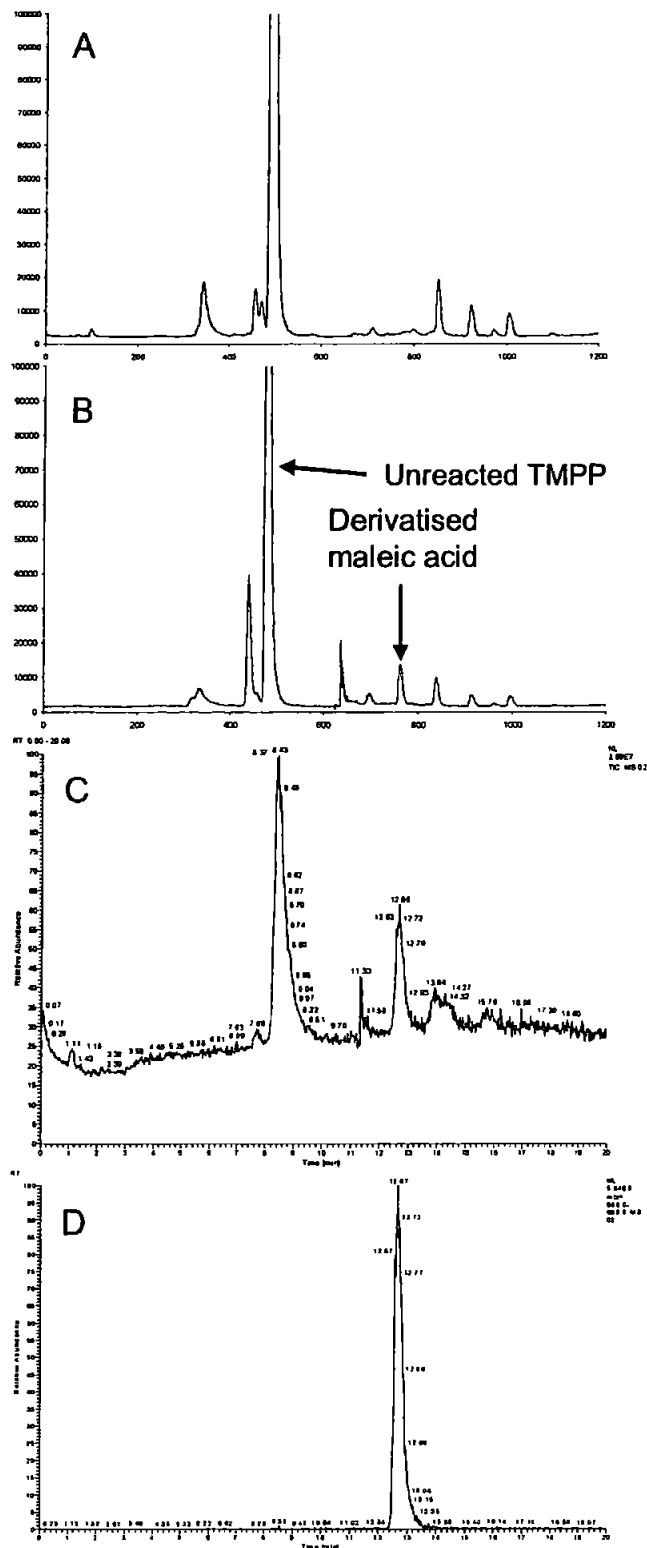
Figure 4.1 Chromatograms of derivatised maleic acid. A, blank injection of TMPP and $^{31}P^+$ selective detection using SF-ICP-MS; B, derivatised maleic acid and $^{31}P^+$ selective detection using SF-ICP-MS; C, derivatised maleic acid and total ion current using +ve ion ESI-MS; D, derivatised maleic acid and selective ion monitoring at 688 m/z using +ve ion ESI-MS.

4.3.2 Optimisation of derivatisation reaction

In order to achieve optimal sensitivity the concentrations of the derivatisation reagents were optimised using a 2-factor, 3-level factorial design (Table 4.2). Concentrations of TMPP and CMPI were set at 0.1 mM, 1 mM and 10 mM for the derivatisation of 0.1 mM of MA. Triethylamine (TEA) was also present as the base for the reaction so its concentration was adjusted to equal the sum of the concentrations of TMPP and MA, in order to maintain the stoichiometry of the reagents. At higher concentrations of TMPP, many impurities were observed, thus making it difficult to identify the peak due to derivatised MA (see Figure 4.2 B). It is desirable to achieve a situation where the derivatisation of MA is maximum for the lowest concentration of reagents, but that the reagents are always in sufficient excess to ensure constant reaction efficiency for varying concentrations of acid. In this case, maximum signal for derivatised MA (at 0.1 mM), and hence maximum reaction efficiency, was achieved at concentrations of 1 mM (TMPP) and 10 mM (CMPI), with the concentration of TEA at 11 mM. The effect of TMPP on the derivatisation of MA is shown in Figure 4.2. At a TMPP:MA ratio of 10:1 (1.0 mM TMPP, 0.1 mM MA) a net peak height signal of approximately 1,500 for derivatised MA was observed (Figure 4.2 A). At a TMPP:MA ratio of 100:1 (10 mM TMPP, 0.1 mM MA) the peak height signal was similar at approximately 1,700; however, impurities in the TMPP eluted close to the analyte peak (Figure 4.2 B) which made identification of the derivatised MA more difficult. A rough estimate of the efficiency of the reaction was obtained by ratioing the peak height of the derivatised MA to the sum of the derivatised acid plus the residual TMPP reagent peak, then expressing this as a percentage of the expected theoretical ratio calculated from the known concentrations. This assumes equal instrumental response for the two compounds, which should hold roughly true in this case given that a desolvator

was used and the nebuliser efficiency was similar over the duration of the gradient in which the peaks eluted. This is illustrated in Figure 4.2 A, where it is evident that the baseline signal, resulting from phosphorus impurities in the eluent, increased in a linear manner by only a factor of 2 over the course of the chromatographic run, and only by a factor of approximately 1.5 between the elution of TMPP and MA. Hence, for equimolar concentrations of MA and TMPP (1 mM) the reaction efficiency was 22%, and when TMPP was in 10-fold excess the reaction efficiency was 16%. Hence, it is likely that only between 10-20% of MA was derivatised under the conditions used and detection limits could be improved further by improving the efficiency of the reaction.

Table 4.2   Two factor, three level full factorial design experiment to determine the optimal conditions for derivatisation of 0.1 mM maleic acid

Solution Number	CMPI Concentration (mM)	TMPP Concentration (mM)	TEA Concentration (mM)
1	1.0	1.0	1.1
2	0.1	1.0	1.1
3	1.0	0.1	0.2
4	1.0	10.0	10.1
5	10.0	0.1	0.2
6	0.1	10.0	10.1
7	10.0	10.0	10.1
8	0.1	0.1	0.2
9	10.0	1.0	1.1

Figure 4.2 HPLC-ICP-MS chromatograms measuring $^{31}P^{+}$ at 30.974 m/z for the derivatisation of 0.1 mM maleic acid with: (A) 10 mM CMPI and 1.0 mM TMPP and (B) 10 mM CMPI and 10 mM TMPP

An approximate limit of detection for derivatised MA, using $^{31}P^+$ selective detection by ICP-MS, was calculated as the concentration of MA which resulted in a peak height of 3x baseline noise. This resulted in a detection limit of 0.05 mM for a 5 μL injection. This is confirmed by Figure 4.2 A, where a 0.1 mM injection can clearly be seen to be close to the detection limit. For comparison, detection limits for TMPP obtained in this work, and for malathion from previous work on the same instrument, are shown in Table 4.3. As can be seen, the compound specific detection limit for TMPP was approximately 5-6 times lower than that for derivatised MA (in molar terms) reflecting the 10-20% reaction efficiency of the latter. The $^{31}P^+$ specific detection limit for malathion achieved previously[88] was approximately 10-times lower than the detection limit for TMPP determined in this work. The probable explanation for this is that a plasma shield was not used on this occasion which resulted in a 10-fold reduction in sensitivity, with a consequent increase in the detection limit.

Table 4.3   Approximate absolute limits of detection for a 5 μL injection

	Compound specific		$^{31}P^+$ specific	
	(nmol)	(ng)	(ng)	Ref.
TMPP (bromide salt)	0.046	30	1.4	this work
MA (derivatised)	0.25	29	7.8	this work
Malathion[a]			0.16	88

[a] 100 μL injection

4.3.3 Analysis of derivatised carboxylic acids

The usefulness of the derivatisation reaction was tested for the detection of several other carboxylic acids, namely, fumaric, sorbic and salicylic acids. Detection using both HPLC-SF-ICP-MS ($^{31}P^+$ selective) and LC-ESI-MS (total ion and extracted ion ranges) was performed to verify which of the phosphorus containing peaks was due to the derivatised acids (Figure 4.3). Derivatised MA was observed at 12.6 minutes (Figure 4.2), sorbic acid at 14.4 minutes and fumaric acid at 12.0 minutes (Figure 4.3). No peak was observed with either $^{31}P^+$ selective ICP-MS or ESI-MS detection for derivatised salicylic acid. This is probably due to strong internal hydrogen bonding making salicylic acid a poor nucleophile, leading to an inefficient reaction with the CMPI activating reagent. It should be possible to derivatise a range of carboxylic acids using this method, as described by Leavens et al.[78] They did not give any indication as to the efficiency of the reaction or detection limits. It may be possible to improve the reaction efficiency by optimising the derivatisation chemistry, and utilising a solid phase analytical approach as described by Pilus et al.[89]

4.4 Conclusions

HPLC-SF-ICP-MS has been used for the detection of maleic, sorbic and fumaric acids after derivatisation with the phosphorus containing reagent tris(2,4,6-trimethoxyphenyl) phosphonium propylamine. This allowed $^{31}P^+$ selective detection to be performed on organic compounds, which are normally un-detectable by ICP-MS, at low concentrations. The derivatisation reaction was partially optimised for maleic acid; however, there is scope for further improving the reaction efficiency to achieve lower detection limits and improved quantitative analysis. Further work on the preparation of a multiply brominated TMPP reagent (described in Chapter 6)

should allow simultaneous bromine and phosphorus selective detection. Multiple bromination should improve detection limits and make possible the use of this reagent to determine the degree of phosphorylation of peptides. The scope of application for reagents such as these is great, particularly in proteomics and genomics where ever more selective and sensitive methods of analysis are required.
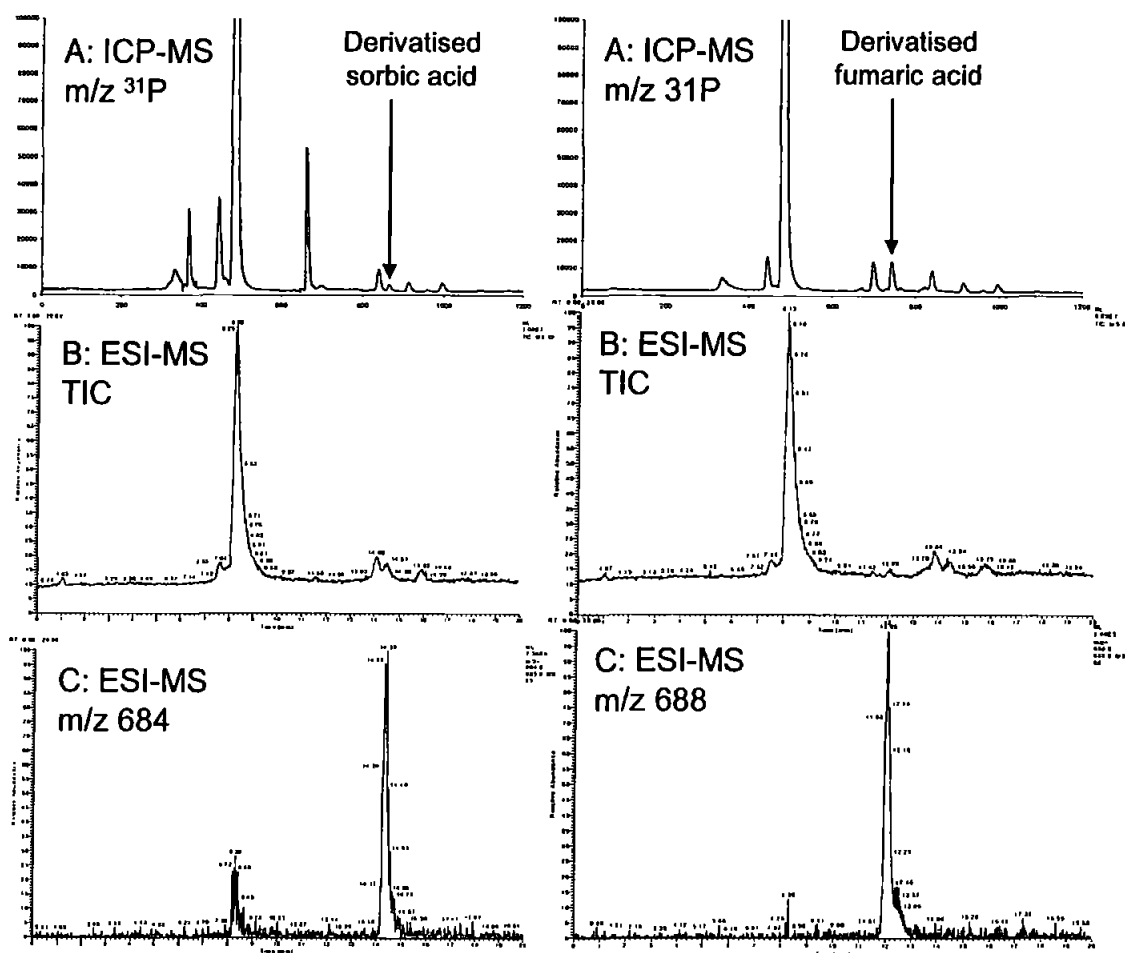
Figure 4.3 Chromatograms of derivatised sorbic (left) and fumaric (right) acids. A, $^{31}P^{+}$ selective detection using SF-ICP-MS; B total ion current using +ve ion ESI-MS; C, selective ion monitoring using +ve ESI-MS.

Chapter Five

Synthesis of Tris(3,5-dibromo-2,4,6-trimethoxyphenyl) phosphonium propylamine bromide (BrTMPP) and its use as a derivatising reagent for carboxylic acids

5.1 Introduction

The derivatisation of carboxylic acids with TMPP has enhanced detection using ESI-MS and made possible phosphorus selective ICP-MS detection of these acids. However, the TMPP derivatising reagent has a mass of 590 m/z but only a single phosphorus atom of 31 m/z per molecule is detected by ICP-MS. However, if the carboxylic acids were derivatised with a molecule containing multiple heteroatoms, then enhanced detection using ICP-MS may be possible. This could be achieved in a number of ways, either by developing an alternative derivatising reagent with the attendant problems associated with this, or by modifying the existing TMPP reagent to facilitate enhanced detection. This could be achieved by incorporating more heteroatoms onto the molecule using a commercially available halogen containing reagent for preparing the related derivatising reagent. One such reagent is tris(4-chlorophenyl) phosphine which could be used instead of tris(2,4,6-trimethoxyphenyl) phosphine in the synthesis of tris(4-chlorophenyl) phosphonium propylamine bromide according to Scheme 5.1. This synthesis would utilise the reaction scheme as detailed in section 4.2.2. Derivatisation with this reagent would yield three chlorine atoms to each phosphorus atom, leading to a potential increase in detection sensitivity for the acids.
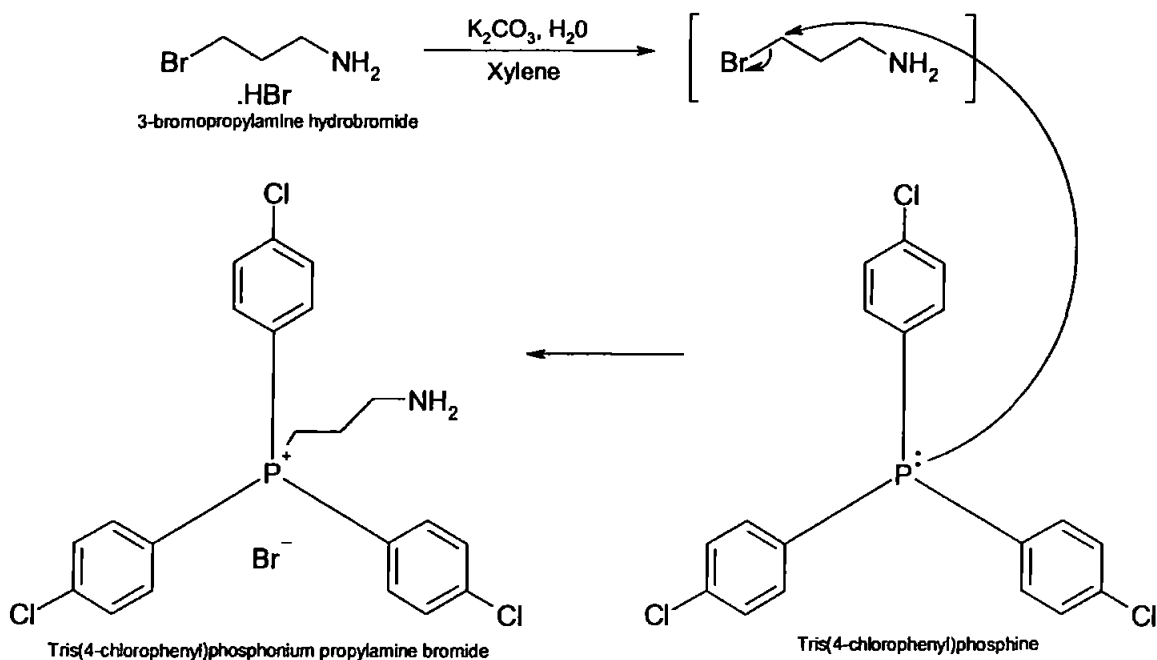
Scheme 5.1 Synthesis of tris(4-chlorophenyl)phosphonium propylamine bromide from tris(4-chlorophenyl) phosphine

A simpler alternative is to halogentate the existing TMPP molecule at the un-substituted carbon atoms on the aromatic rings. This halogenation of aromatics involves the following three steps.

1) Generation of a positive halogen (electrophile)

2) Attachment of the positive halogen to the aromatic ring (slow step)

3) Removal of a proton to yield a neutral halogenated aromatic species (fast step)

An example of this electrophilic aromatic substitution is the single chlorination of benzene[90] shown in Scheme 5.2. The key step (2) is the attachment of the positive chlorine electrophile to the aromatic ring. As free Cl^+ is not involved, ferric chloride is needed to form the complex in (1), from which chlorine is transferred to the ring, thus the generation of this electrophile is important. Other reagents that have been used to yield positive halogens for electrophilic aromatic substitution include

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trifluoroacetyl hypohalites[91], hypobromous acid[92, 93] hypochlorous acid[93, 94],

bromine[93], chlorine[93] and bromine chloride[93].

1) Cl_2 + $FeCl_3$ $\rightleftharpoons$ $\overset{\ominus}{Cl_3Fe}$—$\overset{\oplus}{Cl}$—$Cl$

2) $\overset{\ominus}{Cl_3Fe}$—$\overset{\oplus}{Cl}$—$Cl$ + C_6H_6 $\longrightarrow$ $C_6\overset{\oplus}{H_5}\overset{H}{\diagup}_{\diagdown Cl}$ + $FeCl_4^-$ *(Slow)*

3) $C_6\overset{\oplus}{H_5}\overset{H}{\diagup}_{\diagdown Cl}$ + $FeCl_4^-$ $\longrightarrow$ C_6H_5Cl + HCl + $FeCl_3$ *(Fast)*
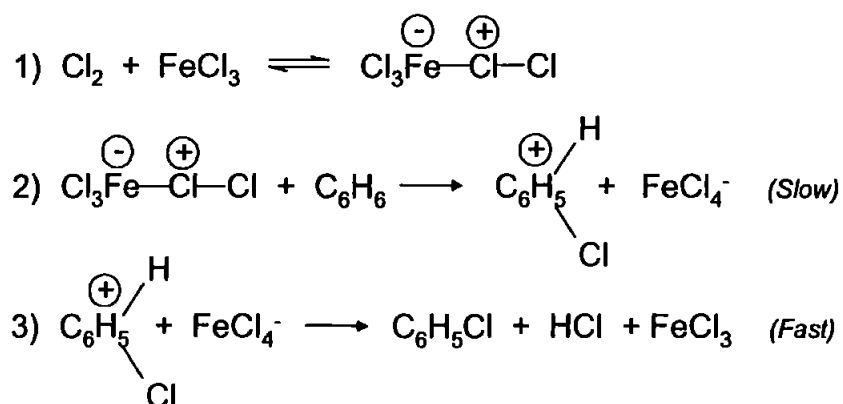
Scheme 5.2 Mechanism for the chlorination of benzene

The TMPP molecule contains three aromatic rings each of which is tri-substituted with methoxy functional groups. These methoxy groups are electron donating, thus yielding a more negative charge on the ring, leaving it more reactive to an electrophile. This enables even weaker electrophiles to be utilised to halogenate at the non-substituted 3 and 5-carbons on the ring. If all unsubstituted carbons are successfully halogenated, six halogen atoms will be incorporated into the molecule. This would enable both phosphorus and halogen detection to be utilised for the derivatised carboxylic acids, with the added benefit of detecting six halogens compared to the single phosphorus atom as described in chapter 4. In this chapter, the synthesis of tris(3,5-dibromo-2,4,6-trimethoxyphenyl) phosphonium propylamine bromide (BrTMPP) derivatising reagent and it's subsequent characterisation is described. The use of this reagent to derivatise carboxylic acids and detect them using bromine and phosphorus selective ICP-MS detection is also described.

5.2 Experimental

5.2.1 Chemicals and reagents

Maleic acid, 2-chloro-1-methylpyridinium iodide (CMPI), triethylamine (TEA), sodium bromate, sulphuric acid, perchloric acid and formic acid were obtained from Sigma-Aldrich (Poole, Dorset, UK). Acetonitrile (HPLC grade) was obtained from Fisher Scientific UK Ltd. (Loughborough, Leicestershire, UK). High purity water (18.2 MΩ) was obtained using an Elga Maxima water purifying system.

5.2.2 Preparation of hypobromous acid

Hypobromous acid was prepared from sodium bromate in strong acidic medium using an adaptation of the method detailed by Noszticzius et al.[95] Sodium bromate (2 mL of a 1 M aqueous solution) was added drop wise to a stirred mixture of 3 mL of water and 5 mL of concentrated sulphuric acid at 0 °C. The mixture was slowly warmed to 40 °C using a water bath. When the vigorous evolution of oxygen ceased, the mixture was stored in a refrigerator until needed. In the strong acidic medium, the bromate decomposes to hypobromous acid according to the following equation:

$$H^+ + BrO_3^- \longrightarrow HOBr + O_2$$
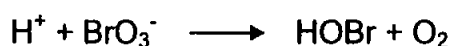
In this acidic medium, an equilibration reaction occurs yielding a bromine electophile according to the following equation:

$$HOBr + H^+ \rightleftharpoons H_2OBr^+ \rightleftharpoons Br^+ + H_2O$$
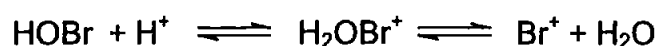
The hypobromous acid solution must be freshly prepared as the hypobromous acid further decomposes to bromine according to the following equation:

$$4 \, HOBr \longrightarrow 2Br_2 + 2H_2O + O_2$$

5.2.3 Synthesis of Tris(3,5-dibromo-2,4,6-trimethoxyphenyl) phosphonium propylamine bromide (BrTMPP)

TMPP was synthesised in-house using the procedure detailed in section 4.2.2. TMPP (90.3 mg of the previously synthesised material) was dissolved in 25 mL of ethanol. 1 mL of perchloric acid and all the hypobromous acid prepared as detailed in 5.2.2 were added. The solutions were left stirring on a magnetic stirrer for 1.5 hours. The solution was left overnight for the ethanol to evaporate off. A yellow precipitate was formed, which was isolated by filtration and dried overnight at 30 °C to give the title compound shown in Scheme 5.3.

TMPP (m/z 590)   **Excess Br$^+$**   **BrTMPP (m/z 1064)**
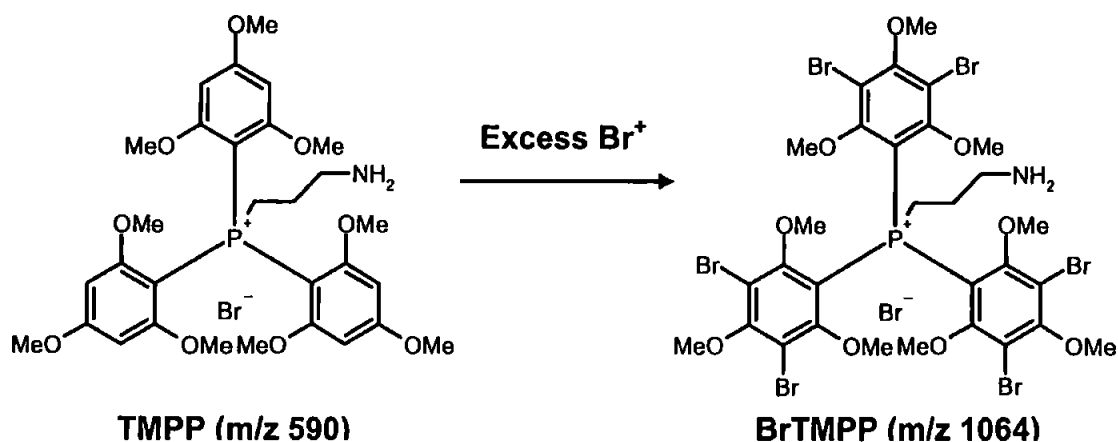
Scheme 5.3 Synthesis of Tris(3,5-dibromo-2,4,6-trimethoxyphenyl) phosphonium propylamine bromide (BrTMPP)

5.2.4 Preparation of coupling reagent

The optimum concentrations of 10 mM CMPI and 11 mM TEA for derivatisation were determined in Section 4.3.3. Thus, 68.3 mg of CMPI was dissolved in approximately 20 mL of acetonitrile in a 25 mL volumetric flask. Triethylamine (39.8 µL) was added, and the solution made up to volume with acetonitrile.

5.2.5 Coupling of BrTMPP propylamine with maleic acid

To maleic acid (500 µL) in 90:10 % (v/v) water:acetonitrile were added 500 µL of CMPI/TEA coupling reagent (prepared as above). After thorough mixing for five minutes at room temperature, 500 µL of 1 mM BrTMPP propylamine solution in acetonitrile was added. The solution was left to react for 30 minutes in an ultrasonic bath at room temperature. The reaction scheme is identical to that detailed in Scheme 4.2, except this time it is with brominated TMPP.

5.2.6 HPLC-ICP-MS analyses

For HPLC-ICP-MS analyses, a HP1090 chromatography system incorporating a UV/vis detector (Agilent Technologies, Stockport, U.K.) equipped with a C18(2) reversed phase column (Phenomenex Luna, 100 x 4.6 mm, 3 µm particle size) was used. The mobile phase comprised a binary system of: eluent A, water:acetonitrile (90:10 % v/v) containing 0.05% formic acid (v/v); and eluent B, water/acetonitrile (10:90 % v/v) containing 0.05% (v/v) formic acid. The linear gradient employed started at 100% volume fraction A, changing to 10% volume fraction A and 90% volume fraction B over 30 minutes. The flow rate was 1 mL min^{-1} with an injection volume of 5 µL. All experiments were performed using a

quadrupole inductively coupled plasma mass spectrometer (Thermo Elemental PQ3, Winsford, U.K.). Operating conditions are shown in Table 5.1.

Table 5.1   Operating conditions for quadrupole-ICP-MS

ICP	
Nebuliser gas flow/Lmin^{-1}	1.10
Auxiliary gas flow/Lmin^{-1}	0.85
Coolant gas flow/Lmin^{-1}	14.0
RF Forward Power/W	1450
Nebuliser	Glass Concentric (Glass Expansion, Switzerland)
Spray Chamber	Jacketed quartz cyclonic, cooled to 5 °C
Torch	Quartz Fassel-type without shield
Interface	
Sampling cone	Nickel 1 mm i.d.
Skimmer cone	Nickel, 0.7 mm i.d.
Time Resolved Analysis	
Masses monitored	Monitoring for $^{31}P^+$ at 31 m/z and $^{79}Br^+$ at 79 m/z
Dwell time/ms	500

The HPLC column was coupled with the Q-ICP-MS simply by inserting the end of the tubing from the column into the sample uptake inlet of the nebuliser, and the output from the spray chamber was introduced into a Universal Interface Model B desolvation device (Vestec Corporation, Houston, USA). The sweep gas was optimised daily at approximately 2.0 L min^{-1} to ensure the maximum signal for $^{31}P^+$ was achieved. The dry aerosol exiting the desolvator was then transferred to the ICP torch via 1 m of 0.25 in. i.d. Tygon tubing.

5.2.7 HPLC-Electrospray ionization mass spectrometry

HPLC was performed using a binary pump (P580A, Dionex-Softron GmbH, Germering, Germany) coupled with a C18(2) reversed phase column (Phenomenex Luna, 100 x 4.6 mm, 3 μm particle size) with the same gradient elution used for HPLC-ICP-MS. The 1 mL min^{-1} flow rate was split post column (high pressure micro-splitter valve; Upchurch Scientific Ltd, Oak Harbor, WA,

USA), and a portion of the stream (~200 μL min^{-1}) was diverted to the mass spectrometer and the residual to waste. Sample injections (5 μL) were made manually with a metal-free injection valve (Rheodyne number 9125, CA, USA). Mass spectrometric analysis was performed using an ion trap mass spectrometer fitted with an electrospray interface (ThermoQuest Finnigan Mat LCQ, San Jose, CA). Data were acquired and processed with Xcalibur 1.0 software. Instrument optimization was performed by infusing a 100 ng mL^{-1} solution of TMPP at 3 μL min^{-1} monitoring the characteristic positive ion at 590 m/z. The following instrument parameters were used: source voltage, +4.50 kV; capillary voltage, +20 V; tube lens offset, -10.00V; capillary temperature, 220 °C; nitrogen sheath gas flow rate, 60 (arbitrary units), and nitrogen auxiliary gas flow rate 20 (arbitrary units). Mass spectra were recorded in the positive ion mode within m/z 150-1500. For characterisation of the synthesised BrTMPP, the same system set-up was used, except no HPLC column was adopted. The mass spectrum was recorded in the positive ion mode within 190-2000 m/z.

5.2.8 Nuclear Magnetic Resonance

All ^{1}H NMR spectra were acquired at 270 MHz using a Jeol EX270 Fourier Transform NMR spectrometer in deuterated methanol (CD$_3$OD, Goss Scientific, Great Baddow, UK). ^{31}P NMR spectra were acquired using the same instrument at 109 MHz, but in this case the centre of the spectral window acquired, 700 ppm, was set to δ 0 ppm and used to reference the spectra as the solvent used does not contain any phosphorus to reference the signal to.

5.3   Results and Discussion

5.3.1   Synthesis of Tris(3,5-dibromo-2,4,6-trimethoxyphenyl) phosphonium propylamine bromide (BrTMPP)

Initial attempts to synthesise a halogenated phosphonium derivatising reagent focussed on the synthesis of a tris (4-chlorophenyl) phosphonium propylamine bromide, using the commercially available tris (4-chlorophenyl) phosphine. This utilised the synthetic procedure detailed in Scheme 5.1, starting with the commercially available tris (4-chlorophenyl) phosphine. Despite many attempts at this synthesis, it proved unsuccessful, with no reaction appearing to take place. This is probably due to the inductive effect of chlorine as opposed to the high activating effect of the methoxy groups on the phenyl rings for nucleophilic attack. The electron withdrawing effect of three chlorine atoms may result in some de-activation of the phosphorus lone pair, making it a poor nucleophile. This synthesis was repeated, but at a higher temperature to attempt to increase the rate of reaction. Xylene (boiling point 138 °C) was used as the solvent instead of toluene (boiling point 110 °C), but again this proved unsuccessful.

The second approach was to use the already synthesised TMPP reagent, and halogenate it at the non-substituted atoms of the phenyl rings. To do this a bromine electrophile would need to be prepared. Two initial ideas were to use bromine or hypobromous acid to produce the bromine electrophile. Although bromine generally needs a Lewis acid catalyst to react with aromatic rings, more highly reactive aromatic rings (i.e. those whose π-electrons are more available) do react with halogens in the absence of any Lewis acid[90]. As the TMPP molecule contains methoxy substituents, then a more reactive ring is formed, thus bromination using bromine liquid was a possibility. However, when this was

attempted, no bromination occurred, with only the original TMPP starting material being produced after the synthesis. This is likely to be due to the fact that bromine on its own yields only a very weak electrophile, produced by a dipole-dipole bond. It appears that a much stronger electrophile is needed for this electrophilic aromatic substitution.

A stronger bromine electrophile is produced by hypobromous acid. These are still relatively weak electrophiles[93], which only react with aromatic substrates with activated rings. As the TMPP molecule contains three methoxy substituents, which activate the ring, they are susceptible to electrophilic attack by relatively weak electrophiles. When the hypobromous acid was prepared fresh as detailed in Section 5.2.2, and was added to the TMPP, a yellow solution was produced. After the solvent was removed, a yellow precipitate was isolated with a yield of 0.80 g, 50.3%. To characterise this material, ^{1}H and ^{31}P NMR, and infusion ESI-MS were used. The mass spectrum of the synthesised material yielded a large peak with a mass of 1064 m/z (most abundant ion of isotope cluster) in positive ion mode (Figure 5.1), which is the characteristic mass of the most abundant monoisotopic peak of the already positively charged BrTMPP molecule. Further evidence that this material was BrTMPP was collected by examining the isotope pattern produced. The BrTMPP molecule contains six bromine atoms so a characteristic bromine isotope pattern would be expected. The BrTMPP molecule elemental composition was entered into an isotope distribution program software package (Anton Erasmuson, Upper Hutt, New Zealand) and its distinctive isotope pattern was calculated (Figure 5.2). The predicted/calculated isotope pattern was almost identical to that obtained experimentally from the synthesised material.
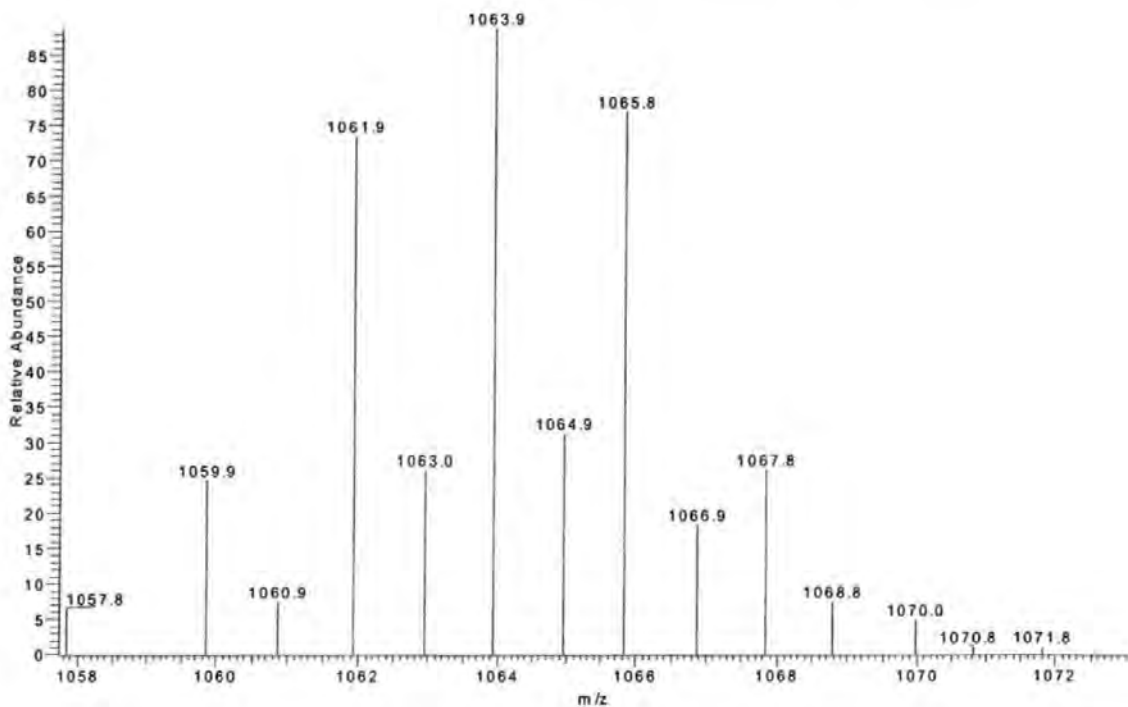
Figure 5.1   Positive ion ESI mass spectrum of the synthesised BrTMPP
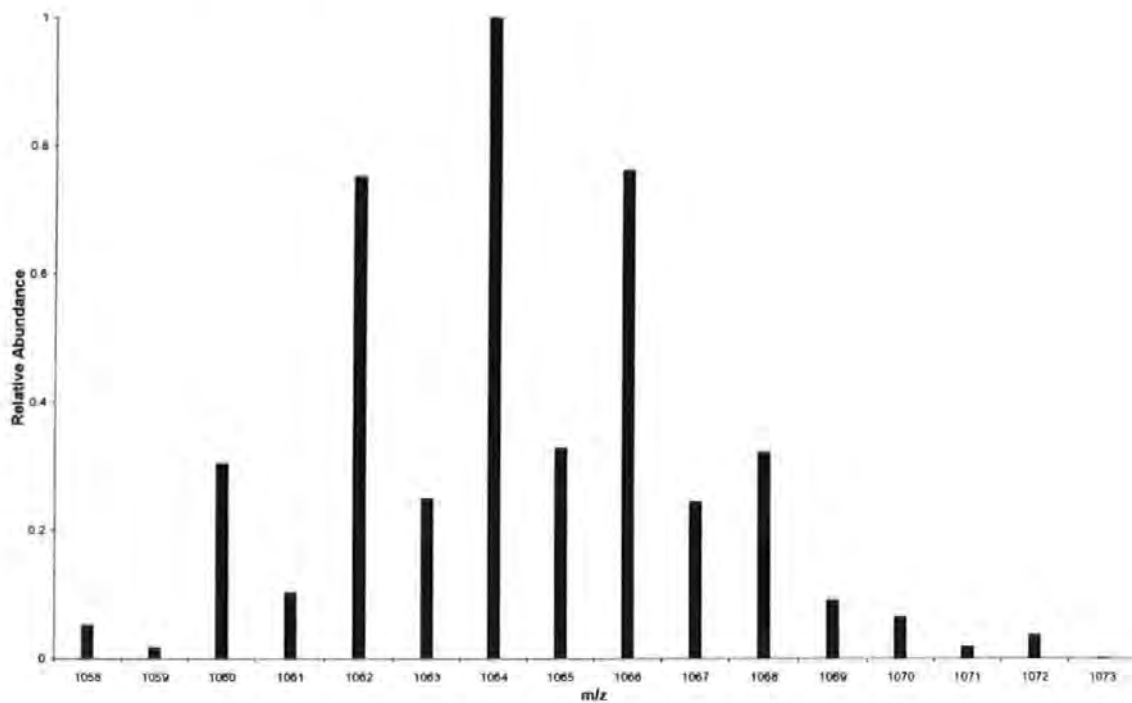
Figure 5.2   Calculated isotope pattern for BrTMPP

To further confirm that this material was BrTMPP, ^{1}H NMR and ^{31}P NMR spectra were obtained. The ^{1}H NMR spectrum obtained for this material dissolved in deuterated methanol is shown in Figure 5.3 along with the spectral data in Table 5.2. The ^{1}H spectrum showed no peaks at a chemical shift of 6-9 ppm, thereby confirming the absence of aromatic protons of the original TMPP (chemical shift of 6.21 ppm, see Figure 3.2). This suggested that electrophilic aromatic substitution of the hydrogens on the phenyl ring had taken place. However, when assigning the spectrum, it appeared that it did not yield the correct integral values for the propylamine side chain. This was probably due to the low purity of the synthesised product, and the weak signal of these protons. The spectrum also showed a large unidentified peak at 3.6 ppm (d) which was probably due to the solvent. Further evidence that the synthesised product was obtained is shown in the ^{31}P NMR spectrum which showed one major peak at a chemical of shift of 11.18 ppm (Figure 5.4). This was different to that obtained for TMPP which produced a chemical shift of 6.65 ppm (see Figure 3.5). The ^{1}H and ^{31}P NMR data obtained, along with the mass spectral data confirm that the BrTMPP was synthesised successfully.
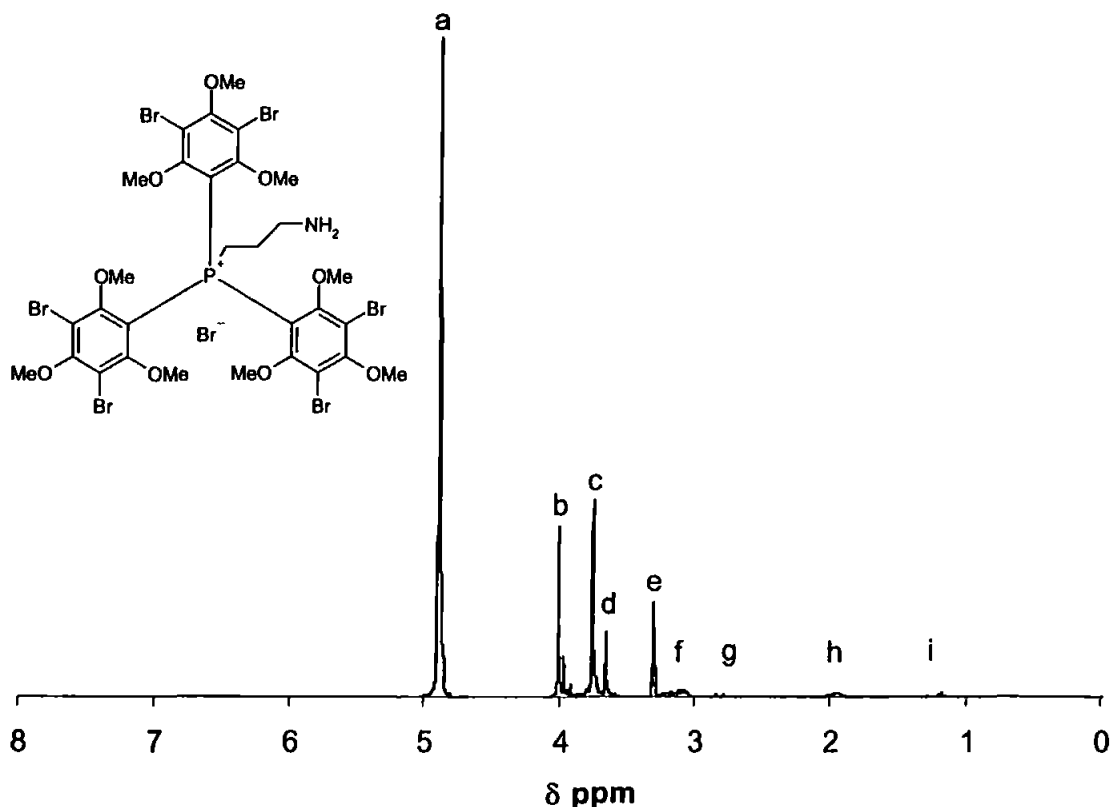
Figure 5.3 ^{1}H NMR spectrum for synthesised BrTMPP, the data for each signal (lettered) is shown in Table 5.2

Table 5.2 ^{1}H NMR spectral data and peak assignment for the synthesised BrTMPP

Peak I.D.	Chemical Shift (ppm)	Integral	Coupling pattern	Assignment
a	4.90			Residual water in solvent
b	4.01	9	Singlet	4- OC$\underline{H}_3$
c	3.76	18	Singlet	2- and 6- OC$\underline{H}_3$
d	3.66	5	Singlet	Unidentified
e	3.31			From Solvent
f	3.20	2.3	Multiplet	C$\underline{H}_2$CH$_2$CH$_2$NH$_2$
g	2.79	0.5	Triplet	CH$_2$CH$_2$C$\underline{H}_2$NH$_2$
h	1.94			From Solvent
i	1.34	0.4	Multiplet	CH$_2$C$\underline{H}_2$CH$_2$NH$_2$

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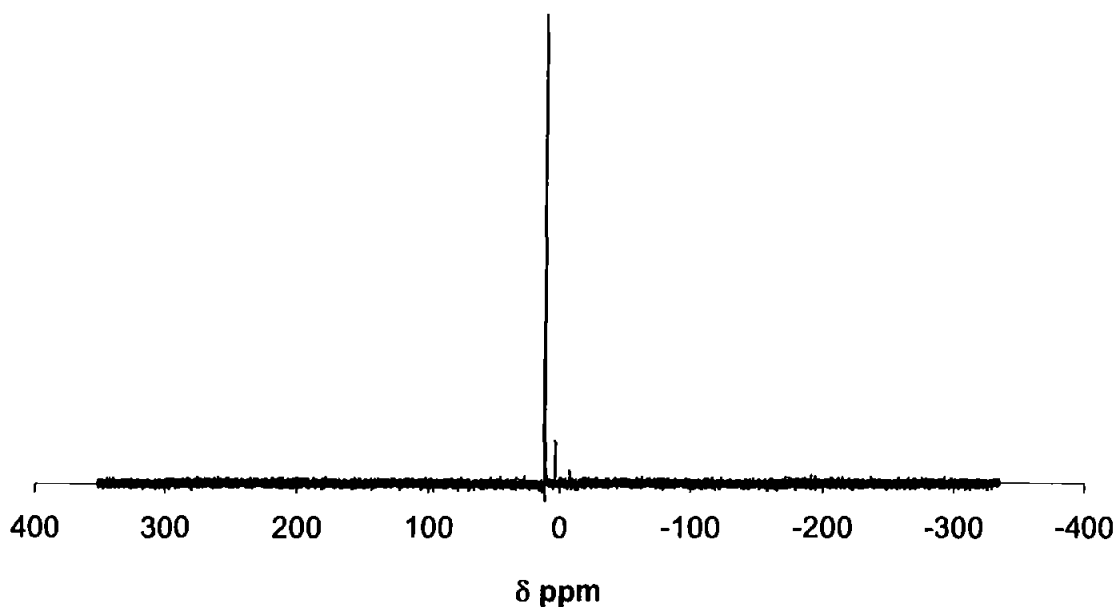
Figure 5.4 ^{31}P NMR spectrum for BrTMPP in deuterated methanol

5.3.2 Phosphorus and bromine selective detection

Chromatograms of maleic acid (MA), after derivatisation with BrTMPP, are shown in Figure 5.5 and Figure 5.6. Detection was performed using ICP-MS with ^{31}P$^+$ and ^{79}Br$^+$ selective detection at 31 m/z and 79 m/z respectively (Figure 5.5), and ESI-MS (Figure 5.6). As can be seen, the BrTMPP derivatising reagent contained many phosphorus and bromine containing impurities (Figure 5.5), so it was necessary to optimise the chromatography such that the derivatised maleic acid (MA) was separated from these peaks and particularly the large peak for unreacted BrTMPP at 11 minutes. For comparison, and confirmatory purposes, detection was also performed using ESI-MS (Figure 5.6) and the peak at 10.61 minutes was found to have a mass of 1064 m/z (most abundant monoisotpic peak of isotope cluster), which corresponded to the mass of the most abundant isotope of BrTMPP. Likewise, the peak at 16.55 min. was found to have a mass of 1162 m/z (most abundant monoisotopic peak of isotope cluster), indicating it to be MA

derivatised with BrTMPP (Figure 5.6 B). This was further confirmed by checking the bromine isotope ratio pattern, which was identical to that shown in Figure 5.1. There was a slight difference in retention time between ICP-MS and ESI-MS peaks, which was the result of the extra tubing required to connect the LC system to the ICP-MS and also the extra time required to transport the aerosol through the desolvator to the plasma. A comparison of the chromatograms obtained using ICP-MS indicates that by monitoring bromine rather than phosphorus a larger signal was achieved, as expected. After examining the chromatograms, a nine-fold increase in signal was achieved. The ICP-MS chromatogram contained many impurity peaks, so ESI-MS was essential in identifying and confirming which peak was the derivatised MA. In this case both the TMPP and the BrTMPP derivatising reagents synthesised in-house were not particularly pure. If the initial step of synthesising the TMPP and the subsequent BrTMPP synthesis were purified this may alleviate some of the problems caused by interfering peaks in the ICP-MS chromatograms.
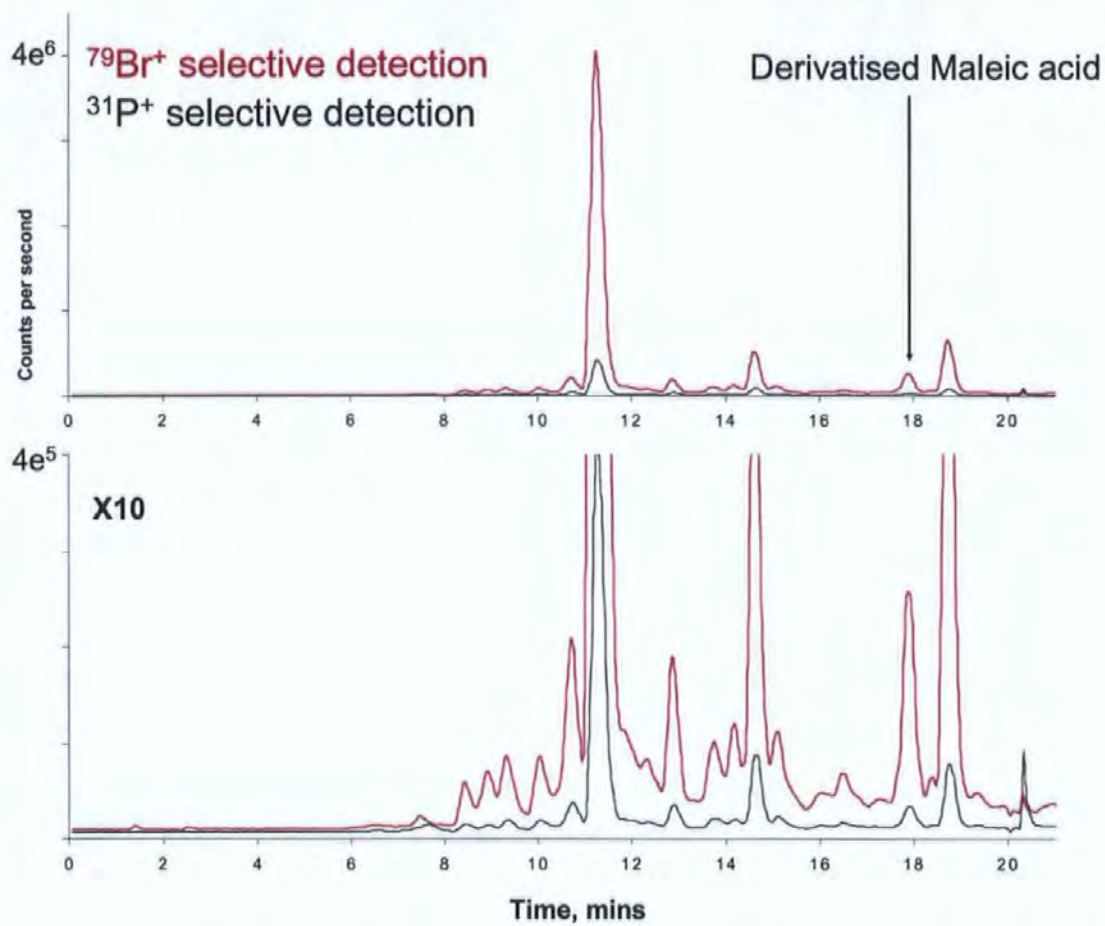
Figure 5.5   LC-ICP-MS chromatograms for derivatised maleic acid detecting $^{79}Br^+$ and $^{31}P^+$ at a concentration of 330 µM
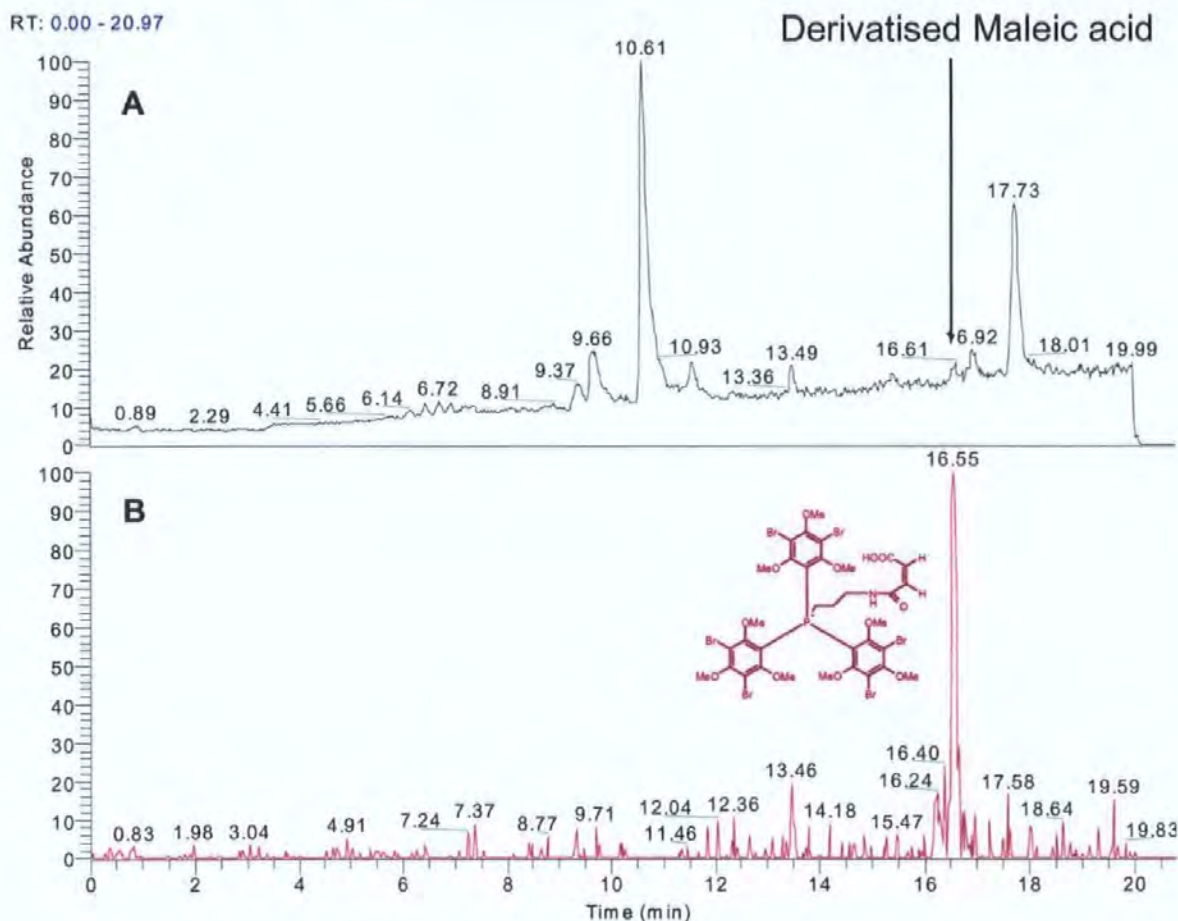
Figure 5.6 LC-ESI-MS chromatograms for derivatised maleic acid at a concentration of 30 µM. A, derivatised maleic acid and total ion current using +ve ion ESI-MS; B, derivatised maleic acid and extracted ion chromatogram for 1162 m/z using +ve ion ESI-MS

5.4 Conclusions

Tris(3,5-dibromo-2,4,6-trimethoxyphenyl) phosphonium propylamine bromide (BrTMPP) has been successfully synthesised using hypobromous acid to generate the bromine electrophile. The synthesised product was characterised by ^{1}H NMR and electrospray mass spectrometry. HPLC-SF-ICP-MS has been used for the determination of maleic acid, after derivatisation with BrTMPP. This allowed $^{31}P^+$ and $^{79}Br^+$ selective detection to be performed on organic compounds which are normally invisible to detection by ICP-MS at low concentrations. By derivatising

maleic acid with BrTMPP, an increase in signal of approximately nine times was achieved by monitoring bromine as apposed to phosphorus. This successful multiple brominated derivatising reagent has not only improved detection but also allows other applications such as the determination of degree of phosphorylation of peptides (see chapter 6).

Liquid Chromatography Coupled to Inductively Coupled Plasma Mass Spectrometry for the Determination of Phosphorylated Peptides

6.1 Introduction

6.1.1 Protein phosphorylation

Dynamic post-translational modification is a general mechanism for maintaining and regulating protein structure and function[96]. Among the many post-translational modifications that have been characterised, protein phosphorylation plays a very important role. It was over forty years ago since it was first recognised that the enzymatic phosphorylation and dephosphorylation of proteins and peptides, often referred to as 'reversible phosphorylation', was a dynamic process involved in the regulation of cellular functions. Prior to this, these proteins were generally thought to have nutritional functions, for example, providing a source of phosphorus for growing organisms. Up until the studies by Earl Sutherland and his colleagues[97-100] and by Ed Fischer and Edwin Krebs[101-103] there was no idea that rapid turnover of protein-bound phosphate might occur, and there was no knowledge of the mechanisms involved in the formation of phosphorylated proteins. These studies recognised the process involved for reversibly altering the function and structure of proteins.

The direct involvement of protein phosphorylation in a metabolic pathway of a eukaryotic system was first demonstrated by Krebs and Fischer in 1956[101, 102]. They showed that the activity of skeletal muscle glycogen phosphorylase, an enzyme involved in glycogenolysis, is regulated by reversible modification. The chemical reactions involved with this system were subsequently reported by the same group in 1958[103], which together with further work in this area won them the joint 1992 Nobel Prize in Physiology or Medicine[‡]. Almost at the same time, pioneering work by Burnett and Kennedy[104] on the phosphorylation of casein dispelled the theories that this reversible phosphorylation was utilised only by enzymes involved in glycogen metabolism. More recently, increasing numbers of phosphoenzymes and phosphoproteins have been characterised in a wide range of eukaryotic systems from fungi to mammals[105]. During this rapid spread of interest in protein phosphorylation, it has been apparent that this applies to many areas of research. These include, for example, researchers involved in protein synthesis, muscle contraction, oocyte maturation and the cell cycle, virology, transcriptional regulation, lymphocyte activation, secretion, and ion channels[106]. Protein phosphorylation is essentially incorporated in almost all areas of biological research[106].

Reversible phosphorylation of proteins at serine, threonine, and tyrosine residues is probably the most important regulatory mechanism in gene expression and protein synthesis[107, 108]. Cell signalling, controlled by protein phosphorylation, regulates many cellular functions at receptors, ion channels, transcription factors, kinases and contractile proteins[108]. This regulation can occur via modulation of co-operative binding of contractile proteins,[109] protein-protein interactions, or by

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[‡] The prize was awarded jointly to Edmond H. Fischer and Edwin G. Krebs "for their discoveries concerning reversible protein phosphorylation as a biological regulatory mechanism"

autoinhibition of protein kinases[110]. All these interactions involve the alteration of the charge of the newly formed phosphorylated amino acid, which instigates local or global changes[108]. Therefore, the alteration of a single residue via reversible phosphorylation is capable of initiating complex cellular signals involved in almost all-physiological processes: metabolism, differentiation, and contraction, etc[110-112]. This demonstrates the need to understand reversible phosphorylation qualitatively (i.e., the site where the modification is taking place) and quantitatively (to what degree the site is modified).

6.1.2 Protein phosphorylation mechanism

Protein kinases are enzymes which transfer phosphate groups from a nucleoside-triphosphate onto an acceptor amino acid in a substrate protein[105]. The protein kinase enzymes catalyse the transfer of a phosphate group from adenosine triphosphate (ATP) to the target protein. This can yield an activated or deactivated protein, depending on its function and its phosphorylated state. Protein phosphatase enzymes do the opposite; they are responsible for removing the phosphorylated group from the protein, yielding the unphosphorylated form again. For this reason, the process is described as reversible (Figure 6.1). Changes in the level, subcellular location and activity of these kinases and phosphatases have consequences on normal cell function and maintenance of cellular homeostasis. These protein kinases and phosphatases play an essential role in many signalling pathways, and therefore have the potential to contribute to diseases ranging from cancer and inflammation to diabetes and cardiovascular disorders, not to mention cell growth, survival and many other biological functions. Recent investigations reveal that there are over 500 human kinases, making them one of the most populated classes of druggable targets.
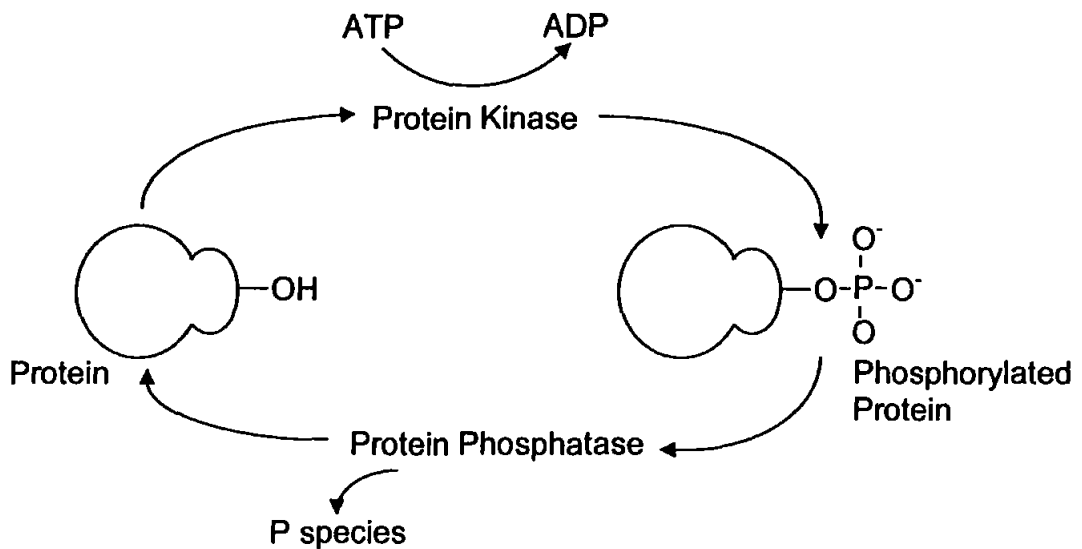
Figure 6.1   Simplified schematic diagram of reversible phosphorylation

6.1.3 Methods to determine protein phosphorylation

Many methods have been used to examine protein phosphorylation. The usual procedure is based on labelling the phosphate fraction with ^{32}P, purification and enzymatic digestion followed by peptide separation. The separated peptides are then subjected to Edman sequencing whereby the sites of phosphorylation are determined by the radioactive label during the Edman cycles[113]. In Edman sequencing, each amino acid is cleaved in sequence from the N-terminus of the protein/peptide, and detected. By piecing together this amino acid sequence, the structure of the peptide/protein can be deduced. This method does give a high degree of sensitivity and robustness, but there are some important disadvantages. The radiation emitted by the labelled ^{32}P can stress biological systems, and therefore can interfere with any in-vivo incorporation of ^{32}P-labelled phosphate, hampering the cell cycle studies. In vitro incorporation of ^{32}P can give access to attached phosphate groups in addition to those incorporated in the protein, and therefore provides no truly reliable insights into the sites of phosphorylation. Generally, ^{32}P labelling alone gives only poor information on the specific site of its

incorporation. Finally, as ^{32}P is radioactive, the extra safety precautions needed to be adopted for its use may be forbidden for some laboratories.

This has lead to a number of attempts to develop non-radioactive methods for analysing protein phosphorylation, primarily based on the combination of enzymatic digestion and mass spectrometry. The advantages of these techniques include the relative speed, sensitivity and adaptability of MS, with a +80 Da mass increase being indicative of the attachment of a phosphate group. This method was successfully adopted for the determination of phosphorylation sites in the internal repeat of rat profilaggrin[114]. In the work by Resing *et al.*, tryptic peptides of filaggrin and profilaggrin were fractionated by reversed-phase HPLC and analysed by electrospray ionisation mass spectrometry. Nine phosphopeptides were identified as those with masses of 80 Da (or multiples of 80 Da) greater than the unphosphorylated peptides. In order to determine the exact site of phosphorylation, peptide sequencing with collisionally induced dissociation (CID) is needed. Problems did occur in that several multiply-phosphorylated peptides underwent neutral loss of H_3PO_4 during collisional activation, leading to complications when interpreting the MS/MS spectra.

A more specific method for phosphopeptide identification uses the specificity of phosphatase enzymes[115]. Peptide mapping using MALDI before and after phosphatase treatment detects phosphatase by the shift of 80 Da due to the removal of the phosphate moiety. The 80-Da difference and the difference in retention times between the enzyme-treated and untreated sample identifies the phosphopeptides.

Protein phosphorylation sites have also been determined using in-gel digestion and nanoelectrospray tandem mass spectrometry[116]. This method detected the phosphopeptides of β-casein after in gel digestion at the level of 250 fmol of protein applied to the gel. This method is not very rapid so, if specificity about the site is required, then this method becomes very laborious.

A further problem with the use of mass spectrometry for phosphopeptide detection is that the ionisation efficiency of both MALDI and electrospray ionisation mass spectrometry is compound dependent, which leads to reduced ionisation efficiency for phosphopeptides compared to unmodified peptides. Thus, these methods tend to only yield qualitative information about the phosphorylation status of a protein. Large phosphopeptides (mol wt > 2500) may also not be detected due to their poor fragmentation characteristics. A recent paper published by Ruse et al.[108] compensated for this by using an internal standard that was present in the digest from the protein studied, but unmodified by the modification reaction. Liquid chromatography coupled to mass spectrometry was used for the analysis of the peptides produced by in-gel digestion and separated by SDS-PAGE. This method is ideally suited to measure a phosphorylation reaction, but is not necessarily ideally suited for a screening exercise.

Much of the recent work on protein phosphorylation analysis is based on element specific mass spectrometry with phosphorus selective detection. This approach should overcome some of the limitations of the current radioactive and mass spectrometric methods. This quantitative technique of element selective analysis has seen increasing biological applications in recent years, especially when coupled to chromatography.

Much of the phosphoprotein analysis using inductively coupled plasma mass spectrometry has been performed by Mathias Winds' group at the Cancer Research Centre in Germany. In the first paper, published in 2001[83], his group used capillary liquid chromatography with element specific $^{31}P^+$ detection using ICP-MS and electrospray ionisation mass spectrometry for confirmation of the phosphoproteins. This technique was successfully adopted for the analysis of digests of three phosphoproteins, β-casein, activated human MAP kinase ERK1, and protein kinase A catalytic subunit. The chromatography used for their separation did, however, only identify a single monophosphorylated fragment for β-casein, when in fact there are two phosphopeptides produced when β-casein is digested with trypsin. The other highly phosphorylated fragment was retained on the column, and hence was not detected by either ICP-MS or MS[83].

In a separate paper[82], Wind's group determined the degree of phosphorylation using the stoichiometric phosphorus to sulphur ratio (^{31}P to ^{32}S) determined in phosphopeptides containing cysteine and/or methionine residues. This was then converted into the degree of phosphorylation using protein/peptide sequence information. The major drawback with this approach is that the number of cysteine and/or methionine residues must be known.

Another separation technique often used in protein/peptide separations is gel electrophoresis. Marshall et al.[117] used this separation technique with laser ablation (LA) ICP-MS to determine protein phosphorylation on electrophoresis gel blots. The detection limit on the gel blots for phosphorus using ICP-MS were found to be 16 pmol. This does have considerable potential for protein phosphorylation and also for the determination of metals. This paper also detailed some of the

problems of using LA-ICP-MS for $^{31}P^+$ selective detection of electrophoresis gels due to the large background obtained from the gels themselves.

6.1.4 Aims of this study

The work described in this chapter utilises phosphorus selective detection by ICP-MS to detect phosphorylated peptides. Reversed phase chromatography was used, so a membrane desolvation device attached prior to the aerosol entering the plasma was necessary to enable gradient elution with up to 90% acetonitrile. By comparing UV and $^{31}P^+$ selective detection, phosphorylated peptides should be easily distinguishable from un-phosphorylated peptides. Detection of phosphorylated and un-phosphorylated peptides is compared, along with detection of an in-solution tryptic digest of bovine β-casein.

This only yields qualitative data of where the peptide is phosphorylated. To determine the actual extent of phosphorylation, the technology developed for the derivatisation of carboxylic acids with BrTMPP can be adopted. The BrTMPP molecule contains one phosphorus atom and six bromine atoms. Hence, it can be used to derivatise a peptide at the C-terminus and by monitoring the Br/P ratio using LC-ICP-MS the extent of phosphorylation can be determined. For example, if a singly phosphorylated peptide was derivatised with BrTMPP the Br/P molar ratio would be 9:2, whereas if a doubly phosphorylated peptide was derivatised, a ratio of 9:3 would be achieved, and so on. Problems do arise if the peptide contains carboxylic acid side chains. If the BrTMPP reagent successfully derivatises the side chain which, if derivatised along with the C-terminus, would yield a different Br/P ratio. Table 6.1 shows the calculated theoretical ratios that would be obtained depending on the extent of phosphorylation and derivatisation

of the phosphorylated peptides. The theory generally works but if a ratio of 9:2 or 9:3 was obtained, this could be interpreted in several ways. One solution would be to use LC-ESI-MS in parallel. The examination of the mass obtained for the peak of interest in LC-ESI-MS, and its bromine isotope ratio pattern, would show the number of BrTMPP molecules attached to the peptide. Hence, with this information the degree of phosphorylation could be deduced.

Table 6.1 **Bromine to phosphorus ratios theoretically obtained for BrTMPP derivatised phosphorylated peptides**

Number of BrTMPP molecules added	Number of Phosphorylation sites			
	0	1	2	3
1	9:1	9:2	9:3	9:4
2	18:2	18:3	18:4	18:5
3	27:3	27:4	27:5	27:6

A further problem with derivatising peptides comes with the chemistry involved in the derivatisation reaction. The reaction utilises the intermediate activation of the carboxylic acid, prior to a condensation reaction with an amine (from the derivatising reagent) to form a stable amide bond. However, both these reactive groups are present in peptides (which is the main reason CMPI reagents were developed) so the peptides can preferentially react with themselves to make polymers of the same peptide sequence. To overcome this preferential self reactions of the peptides, N-terminus protected peptides need to be used for this purpose.

6.2 Experimental

6.2.1 Chemicals and reagents

High purity water (DDW) (18.2 MΩ) was purified in house using an Elga Maxima water purifying system. Acetonitrile was of HPLC grade quality and was purchased from Fisher Scientific UK Ltd. (Loughborough, Leicestershire, UK). Trypsin, β-casein, HPLC peptide standard mix (H2016), ammonium acetate, sodium polyphosphate, bovine β-casein, trypsin, ammonium bicarbonate, 3-(methylphosphinico)propionic acid, potassium P,P-dimethylphosphonoacetate, diethylphosphonoacetic acid , 2-chloro-1-methylpyridinium iodide (CMPI), and triethylamine (TEA) were all obtained from Sigma-Aldrich (Poole, Dorset, UK). The phosphorylated peptide (pp60 c-src) was obtained from Bachem, (Weil am Rhein, Germany) and the other two synthesised phosphorylated peptides (PRN5220 and PRN5221) were obtained from Biomol International (Exeter, Devon, UK).

6.2.2 Preparation of samples for qualitative determination of phosphorylation

The peptide standard mix and the phosphorylated peptide (pp60 c-src) samples were prepared to contain 1 mg mL^{-1} of the compound dissolved in DDW. The sample was a 50:50 %(v/v) mixture of these two samples.

6.2.3 Tryptic Digest of bovine β-casein

Many different protocols exist in the literature for performing tryptic digests, but most of these are utilised for in-gel digestions. The following protocol was used due to its ease of use and it's applicability to in-solution digests. A 10:1 mass ratio of β-casein (0.3 g) to trypsin (0.03 g) was added into 25 mL of a solution

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containing 25 mM ammonium bicarbonate in DDW. The mixture was then incubated at 37 °C with shaking overnight, prior to analysis.

6.2.4 Preparation of coupling reagent

The optimum concentrations of CMPI (10 mM) and TEA (11 mM) for derivatisation were determined in Section 4.3.2. Thus, 68.3 mg of CMPI was dissolved in approximately 20 mL of acetonitrile in a 25 mL volumetric flask. Triethylamine (39.8 μL) was added, and the solution made up to volume with acetonitrile.

6.2.5 Coupling of BrTMPP propylamine with carboxylic acids and phosphorylated peptides

To 500 μL of 1 mM carboxylic acid (3-(methylphosphinico)propionic acid, potassium P,P-dimethylphosphonoacetate or diethylphosphonoacetic acid) in 90:10 % (v/v) water:acetonitrile or 10 mM phosphorylated peptide (see Section 6.4.4) in water were added 500 μL of CMPI/TEA coupling reagent (prepared as above). After thorough mixing for five minutes at room temperature, 500 μL of 1 mM BrTMPP propylamine solution in acetonitrile was added. The solution was left to react for 30 minutes in an ultrasonic bath at room temperature.

6.2.6 HPLC-ICP-MS analyses for qualitative phosphorylated peptide analysis

For HPLC-ICP-MS analyses, a HP1090 chromatography system incorporating a UV/vis detector (Agilent Technologies, Stockport, U.K.) equipped with a C18 250 x 4.6 mm analytical column (Hypersil-Keystone PEP 300Å, Thermo Electron

Corporation, Runcorn, UK) was used. The mobile phase comprised a binary system of: eluent A, 0.05 M ammonium acetate in water and eluent B, acetonitrile. For the determination of detection limits using sodium polyphosphate ($Na_5P_3O_{10}$) a simple isocratic elution with 99 %(v/v) eluent A, 1 %(v/v) eluent B was used. For the peptide HPLC test mix (a standard mixture of five peptides obtained from Sigma-Aldrich, Poole, Dorset) and/or phosphorylated peptide standard (pp60 c-src), a gradient of 0-30 min, 0-30% solvent B was used. For the β-casein analysis, the gradient was increased to 0-60 min, 0-50% solvent B to ensure all components were eluted from the HPLC column. All experiments used a 20 µL injection volume. All experiments were performed using a quadrupole inductively coupled plasma mass spectrometer (Thermo Elemental PQ3, Winsford, U.K.). Operating conditions are shown in Table 6.2. The HPLC column was coupled with the ICP-MS simply by inserting the end of the tubing from the column into the sample uptake inlet of the nebuliser, and the output from the spray chamber was introduced into a Universal Interface Model B desolvation device (Vestec Corporation, Houston, USA). The sweep gas was optimised daily at approximately 2.0 L min^{-1} to ensure the maximum signal for $^{31}P^+$ was achieved. The dry aerosol exiting the desolvator was then transferred to the ICP torch via 1 m of 0.25 in. i.d. Tygon tubing.

Table 6.2   Operating conditions for quadrupole-ICP-MS

ICP	
Nebuliser gas flow/Lmin^{-1}	1.10
Auxiliary gas flow/Lmin^{-1}	0.85
Coolant gas flow/Lmin^{-1}	14.0
RF Forward Power/W	1450
Nebuliser	Glass Concentric (Glass Expansion, Switzerland)
Spray Chamber	Jacketed quartz cyclonic, cooled to 5 °C
Torch	Quartz Fassel-type without shield
Interface	
Sampling cone	Nickel 1 mm i.d.
Skimmer cone	Nickel, 0.7 mm i.d.
Single Ion Monitoring	
Masses monitored	Monitoring for $^{31}P^+$ at 31 m/z and $^{79}Br^+$ at 79 m/z
Dwell time/ms	500

6.2.7 HPLC-ICP-MS analyses of derivatised carboxylic acids and phosphorylated peptides

For HPLC-ICP-MS analyses, a HP1090 chromatography system incorporating a UV/vis detector (Agilent Technologies, Stockport, U.K.) equipped with a C18(2) reversed phase column (Phenomenex Luna, 100 x 4.6 mm, 3 μm particle size) was used. The mobile phase comprised a binary system of: eluent A, water:acetonitrile (90:10 % v/v) containing 0.05% formic acid (v/v); and eluent B, water/acetonotrile (10:90 % v/v) containing 0.05% (v/v) formic acid. The linear gradient employed started at 100% A, changing to 10% A and 90% B over 30 minutes. The flow rate was 1 mL min^{-1} with an injection volume of 5 μL. All experiments were performed using a Q-ICP-MS (Thermo Elemental PQ3, Winsford, U.K.). Operating conditions are shown in Table 6.2.

6.2.8 HPLC-Electrospray ionisation mass spectrometry of derivatised carboxylic acids and phosphorylated peptides

HPLC was performed using a binary pump (P580A, Dionex-Softron GmbH, Germering, Germany) coupled with a C18(2) reversed phase column (Phenomenex Luna, 100 x 4.6 mm, 3 μm particle size) with the same gradient elution used for HPLC-ICP-MS. The 1 mL min^{-1} flow rate was split post column (high pressure micro-splitter valve; Upchurch Scientific Ltd, Oak Harbor, WA, USA), with a portion of the stream (~200 μL min^{-1}) diverted to the mass spectrometer and the residual to waste. Sample injections (5 μL) were made manually with a metal-free injection valve (Rheodyne model number 9125, CA, USA). Mass spectrometric analysis was performed using an ion trap mass spectrometer fitted with an electrospray interface (ThermoQuest Finnigan Mat LCQ, San Jose, CA). Data were acquired and processed with Xcalibur 1.0 software. Instrument optimization was performed by infusing a 100 ng mL^{-1} solution of TMPP at 3 μL min^{-1} and monitoring the characteristic positive ion at 590 m/z. The following instrument parameters were used: source voltage, +4.50 kV; capillary voltage, +20 V; tube lens offset, -10.00V; capillary temperature, 220 °C; nitrogen sheath gas flow rate, 60 (arbitrary units), and nitrogen auxiliary gas flow rate 20 (arbitrary units). Mass spectra were recorded in the positive ion mode within m/z 150-1500. For characterisation of the synthesised BrTMPP, the same system set-up was used, except no HPLC column was adopted. The spectrum was recorded in the positive ion mode within 190-2000 m/z for the derivatised carboxylic acids, and 190-3000 m/z for the derivatised phosphorylated peptides.

6.3 Results and Discussion

6.3.1 Phosphorus selective detection

For this work, a membrane desolvation device was used. As this removes the majority of the solvent, and thus the polyatomic interferences associated with ^{31}P detection, a low resolution ICP-MS instrument could be utilised. Using a standard quadrupole instrument with the desolvator, the background phosphorus signal was reduced to approximately 3000 counts per second, enabling low phosphorus levels to be detected even when using chromatography. The membrane desolvator also ensured the chromatography used (up to 90% volume fraction acetonitrile in this case) had little detrimental effect on the plasma. For the determination of the detection limit, samples containing between 1 ng mL^{-1} and 1 µg mL^{-1} of phosphorus from sodium polyphosphate were analysed by HPLC-ICP-MS. The chromatograms obtained were exported into MassLynx software (Waters, Massachusetts, USA). This software enabled the peaks to be integrated, yielding peak areas. Using the peak areas, a linear graph was obtained for peak area against phosphorus concentration based on a 20 µL injection. The limit of detection, calculated as the concentration of $^{31}P^{+}$ which resulted in a peak height of 3 times the baseline noise, was calculated to be 10 ng mL^{-1} of $^{31}P^{+}$.

6.3.2 Detection of phosphorylated peptides

To determine the feasibility of HPLC-UV-ICP-MS with membrane desolvation for determining phosphorylated peptides, a HPLC peptide standard mix was injected onto the HPLC-UV-ICP-MS system. The mix is a commercially available standard, containing five un-phosphorylated peptides. The chromatograms produced are shown in Figure 6.2. The UV chromatogram clearly shows the five peptides,

although two are not very well resolved. No peaks were observed on the $^{31}P^+$ selective ICP-MS chromatogram. This is exactly as expected because the HPLC standard did not contain any phosphorylated peptides. There is a small peak at approximately 27 minutes, but this is just about at the level of detection of the instrument, and was probably due to a small phosphorus containing impurity in the mixture.
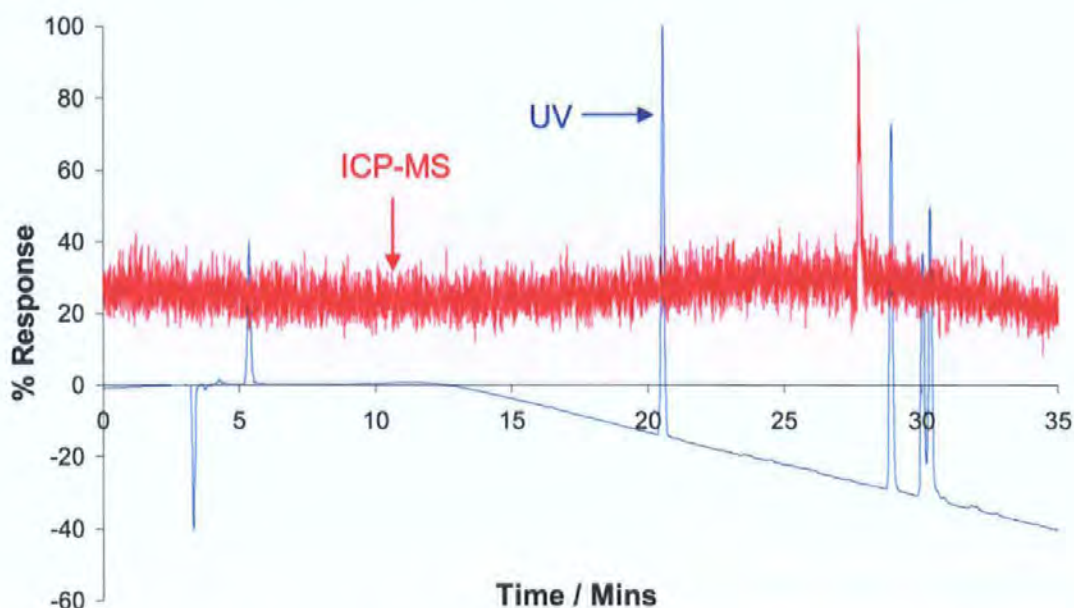
Figure 6.2   UV at 220 nm and $^{31}P^+$ ICP-MS chromatograms for the HPLC peptide test mix

The chromatogram obtained for a singly phosphorylated peptide (pp60 c-src) is shown in Figure 6.3, which produced only one peak in the UV chromatogram and one main peak using phosphorus selective ICP-MS detection, as expected. Several other small peaks were observed on the UV and ICP-MS chromatograms, but these were probably due to impurities in the sample, which was stated to only contain 86.3% of the peptide. It is also worth noting the slight difference in retention time between the UV and ICP-MS responses. This was due to the time delay between the UV and ICP-MS detectors.
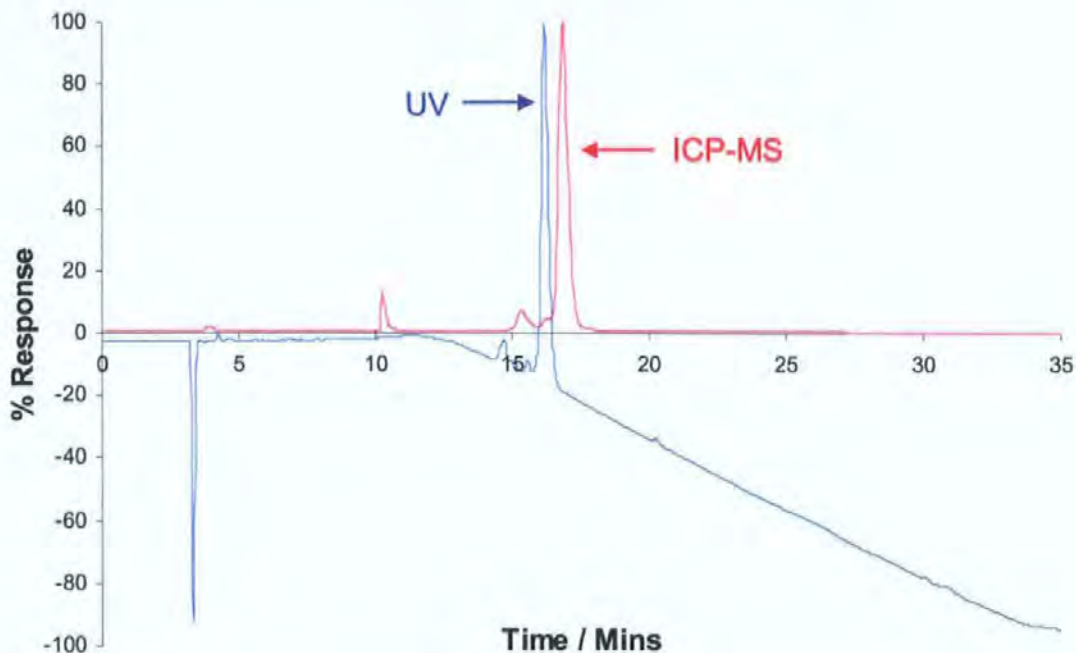
Figure 6.3   UV at 220 nm and $^{31}P^+$ ICP-MS chromatograms for the singularly phosphorylated peptide (pp60 c-src)

In order to demonstrate that the HPLC-UV-ICP-MS system could be used to distinguish between phosphorylated and un-phosphorylated peptides, a mixture of the peptide test mix and the phosphorylated peptide was prepared and injected. The UV and ICP-MS chromatograms are shown in Figure 6.4. As expected, only one major peak was observed using $^{31}P^+$ selective detection, but five peaks were obtained using UV detection. The last two peptides from the standard test mix appear to have co-eluted. However, by using both UV and ICP-MS together, this simple system enables phosphorylated peptides to be distinguished easily from un-phosphorylated peptides.
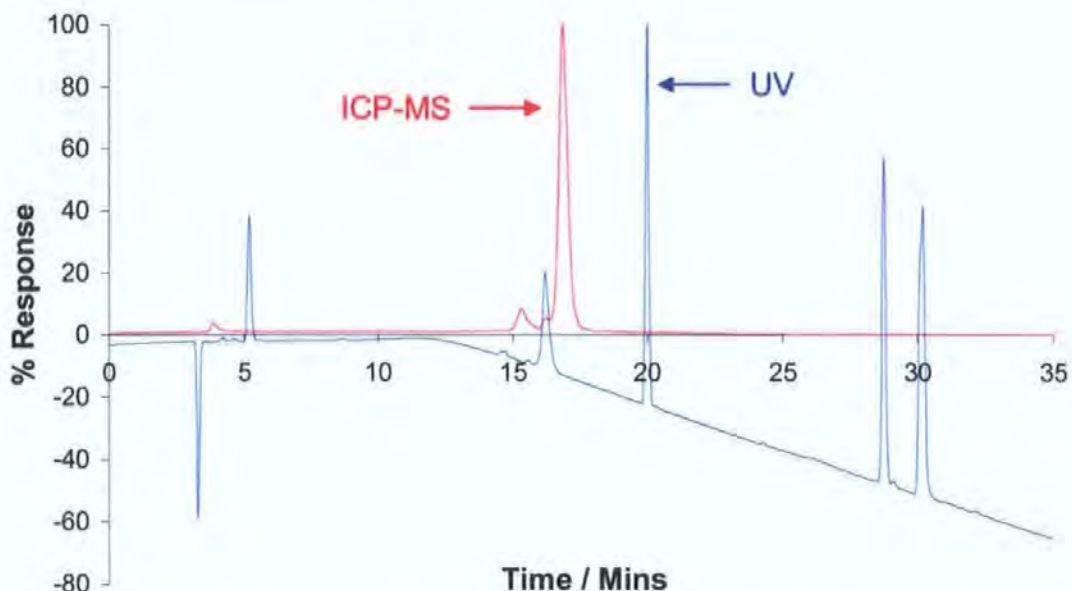
Figure 6.4   **UV at 220 nm and $^{31}P^+$ ICP-MS chromatograms for the peptide test mix spiked with the singularly phosphorylated peptide**

This approach has been shown to be suitable for model peptides, but in "real" life protein samples, there may be matrix effects and other interferences. To ensure that this system was suitable for "real" protein samples, a bovine β-casein sample was digested with trypsin overnight. Bovine β-casein was chosen as this is commercially available and is known to contain phosphorylated peptides, so is often used as a reference compound to demonstrate specific detection of phosphopeptides in tryptic digests[83]. Using the initial 30 minute gradient elution profile utilised for the peptide mix and phosphorylated peptide, it appeared that some of the digested compounds had not eluted from the column. Thus, the slower and longer 60-minute gradient elution was used. Figure 6.5 shows the UV and ICP-MS chromatograms obtained for this digest. Two peaks were observed in the ICP-MS response for the digest, which is as expected because a tryptic digest of β-casein produces two phosphorylated peptides[83]. One of these is a singly phosphorylated peptide, presumably the smaller peak, and the other which contains four phosphorylated amino acids, probably the larger peak. The second

larger peak is the phosphorylated peptide that was retained on the column and hence wasn't detected in the work by Wind *et al.*[83] The known difference in retention time observed with the phosphorylated peptide (pp60 c-src) can be calculated and used to determine which ICP-MS peak correlates to which UV peak. If electrospray ionisation mass spectrometry was utilised in parallel with ICP-MS detection, with the difference in retention times correlated, then both detection techniques could be used to provide ease of identification of the phosphorylated peptides along with molecular mass spectral data. If ESI-MS or UV alone was used, then it would be extremely difficult to find which peak was due to the phosphorylated peptides due to the large amounts of peaks in the spectrum (approximately 50). Hence, the use of ICP-MS simultaneously with ESI-MS permits the targeted analysis and characterisation of phosphorylated peptides within a single chromatographic run.
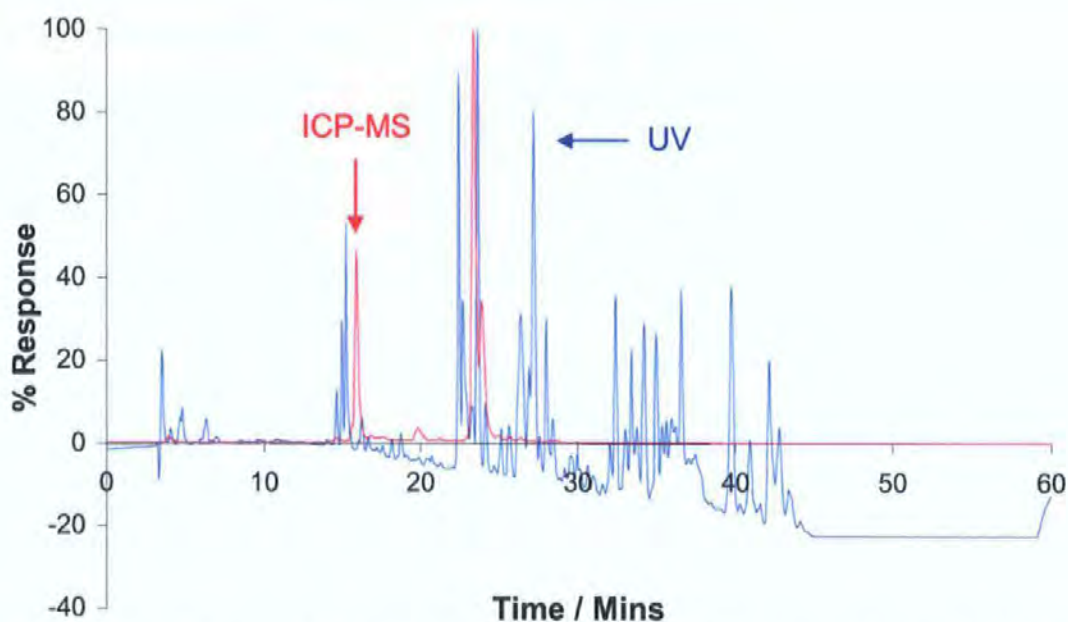
Figure 6.5   UV at 220 nm and $^{31}P^+$ ICP-MS chromatograms for an in-solution tryptic digest of bovine β-casein

6.3.3 Analysis of phosphorus containing acids

The BrTMPP reagent has already been successfully used for the derivatisation of maleic acid, with a consequent improvement in detection using Br selective detection (see Chapter 5). However, the Br/P ratio can also be used to determine the number of phosphorus atoms on the derivatised molecule. In order to test this theory, three phosphorus containing carboxylic acids were derivatised with BrTMPP. These were 3-(methylphosphinico)propionic acid, potassium P,P-dimethylphosphonoacetate and diethylphosphonoacetic acid (see Figure 6.6 for the structures of these acids), chosen because they are all commercially available in a high purity.
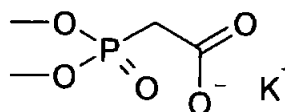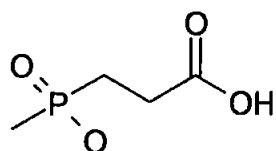
3-(methylphosphinico)propionic acid   Potassium P,P-dimethylphosphonoacetate
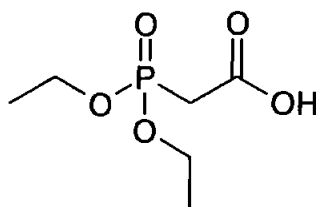
Diethylphosphonoacetic acid

Figure 6.6   Structure of the phosphorus containing carboxylic acids

LC-ESI-MS chromatograms of potassium P,P-dimethylphosphonoacetate, after derivatisation with BrTMPP are shown in Figure 6.7. When the total ion current chromatogram (Figure 6.7 A) was compared to a BrTMPP blank, a new peak at 15.80 minutes was observed. This was confirmed to be the derivatisation product of potassium P,P-dimethylphosphonoacetate when the theoretical mass of the product was extracted, and a single peak was observed in the single ion monitoring trace (Figure 6.7 B). This then enabled element selective detection of $^{31}P^+$ and $^{79}Br^+$ to be used. By examining the ICP-MS chromatograms at the elution time of the derivatised acid, a peak was observed at 1020 seconds (17.0 minutes) for both the bromine and phosphorus signals (Figure 6.8). The difference between this retention time, and that obtained by LC-ESI-MS was the difference in time for the eluent to get to the respective detectors. To prove the concept that this method could be used to determine the amount of phosphorus in the molecule, the Br:P ratios were calculated for the peak of interest and the excess BrTMPP peak at 780 seconds (13.0 minutes). For the underivatised BrTMPP, a Br:P ratio of 9.02 was obtained, however, for the derivatised acid, a ratio of 4.46 was obtained. This is almost exactly half the ratio obtained for underivatised BrTMPP, thus confirming that the acid has one phosphorus atom in the molecule. When the LC-ESI-MS chromatograms for the derivatised 3-(methylphosphinico)propionic acid and diethylphosphonoacetic acid were examined, and their selected masses extracted, no peaks were observed. This indicates that these acids may not be derivatised very efficiently with this BrTMPP reagent. The LC-ICP-MS chromatograms did not yield conclusive evidence for the derivatisation either, and thus the Br/P ratios could not be determined.
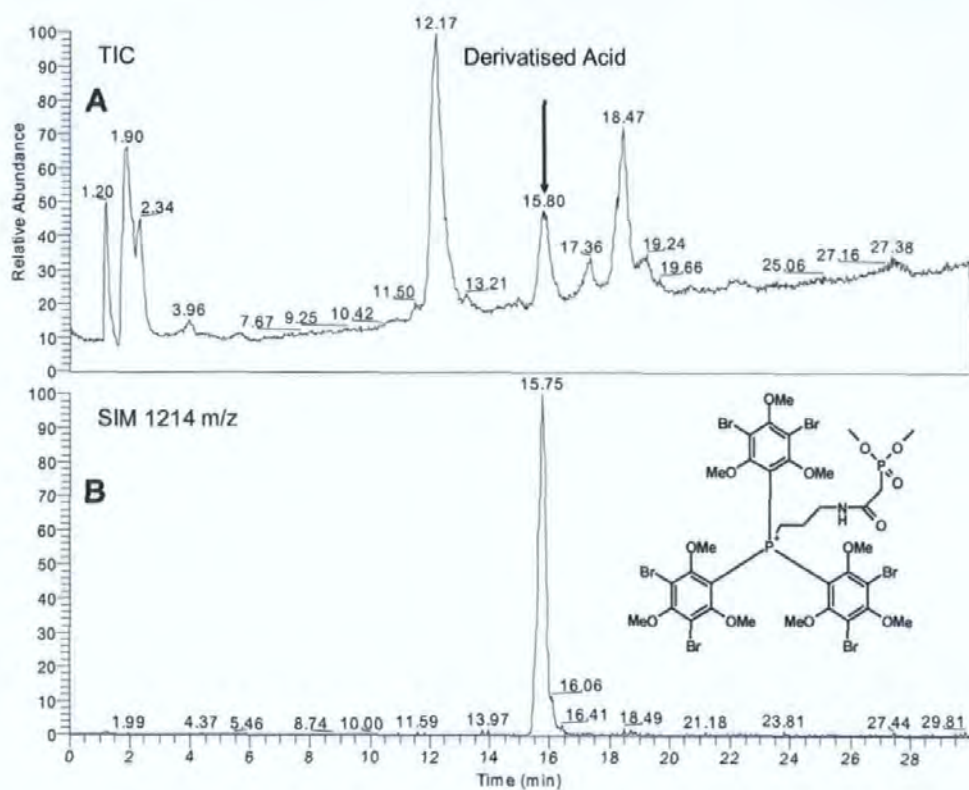
Figure 6.7   LC-ESI-MS chromatograms for BrTMPP derivatised potassium

P,P-dimethylphosphonoacetate
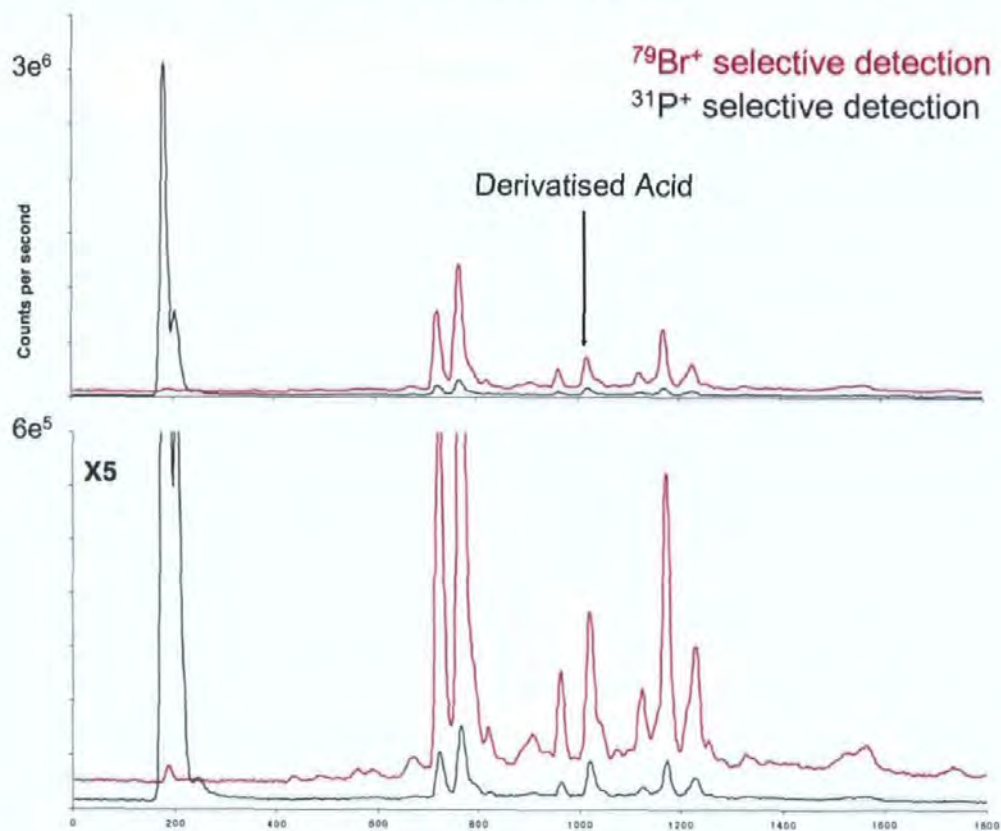
Figure 6.8   LC-ICP-MS chromatograms for BrTMPP derivatised potassium

P,P-dimethylphosphonoacetate

6.4.4 Derivatisation and analysis of phosphorylated peptides

For successful derivatisation using the method described here the phosphorylated peptides must be N-terminus end-capped, hence two peptides were specifically synthesised for this purpose by Biomol International (Exeter, Devon, UK). Both peptides were end-capped with an acetyl group to protect them from self polymerising during the derivatisation reaction. The amino acid sequences for the peptides obtained are shown below:

PRN5220 AC-Thr-Ser-Glu-Pro-Gln-Tyr(PO_3H_2)-Gln-Pro-Gly-Glu-Asn-Leu-OH

PRN5221 AC-Thr-Ser-Tyr(PO_3H_2)-Gln-Tyr(PO_3H_2)-Gly-Leu-OH

PRN5220 was chosen because this sequence is identical to the phosphorylated peptide used for the qualitative analysis in Section 6.3.1, and PRN5221 was chosen, because it contains two phosphorylated tyrosine amino acids, thus a different Br:P ratio would be expected.

The peptides were derivatised with 1 mM BrTMPP, and their LC-ESI-MS chromatograms are shown in Figure 6.9. When the theoretical masses of the derivatised peptides were calculated, and their masses extracted from these chromatograms, no peaks were observed. However, large peaks were obtained for the underivatised peptides at 7.49 minutes for PRN5220 (Figure 6.9 A) and 6.90 minutes for PRN5221 (Figure 6.9 B). Again this suggests that these peptides were not successfully derivatised by the BrTMPP derivatising reagent. This may be due to steric hindrance due to the bulky derivatisation group and the size of the peptides. Also these peptides may fold to yield a confirmation which does not leave the C-terminus available for reactions. The derivatisation efficiency of the reaction may also be too low for successful detection.
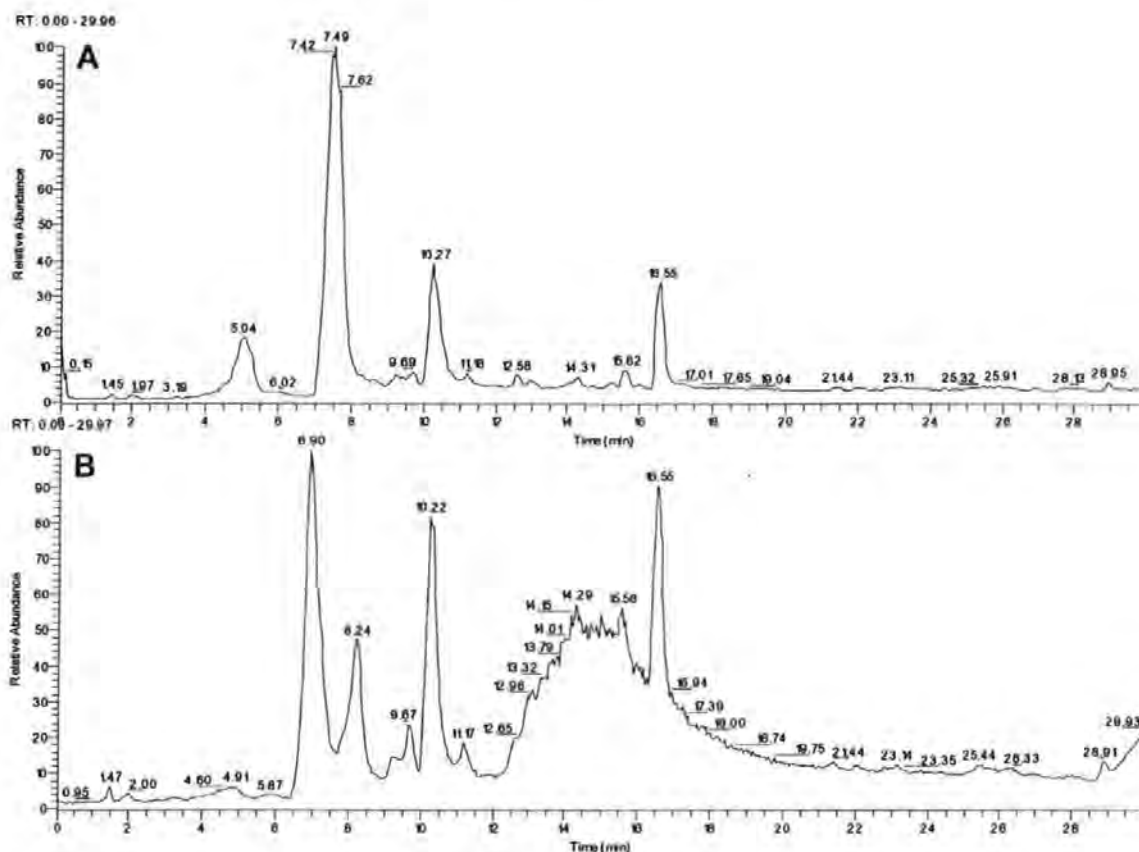
Figure 6.9   LC-ESI-MS chromatograms for the BrTMPP derivatised phosphorylated peptides; A PRN5220; B PRN5221

6.4   Conclusions

HPLC-UV-ICP-MS has been used to distinguish between phosphorylated and un-phosphorylated peptides. A detection limit of 10 ng mL^{-1} for phosphorus with $^{31}P^+$ selective detection was obtained based on a 20 µL injection volume. This system has been successfully utilised to distinguish the phosphorylated fragments obtained when bovine β-casein was digested in solution with trypsin. The applicability of using this system is wide ranging. It has been demonstrated that this, used in conjunction with electrospray ionisation mass spectrometry detection, would make it easier to identify phosphorylated peptides and gather structural information. This system is also applicable to chromatographic separations utilising strong organic modifiers needed in the elution.

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The methodology developed using BrTMPP to derivatise carboxylic acids has been shown to be successful in derivatising potassium P,P-dimethylphosphonoacetate and by determining the Br:P ratio the number of phosphorus atoms on the molecule was determined. Unfortunately, this BrTMPP molecule did not successfully derivatise 3-(methylphosphinico)propionic acid, diethylphosphonoacetic acid or the phosphorylated peptides PRN5220 and PRN5221. If the efficiency of this reaction could be improved, and made non-selective, the method has the potential to determine the extent of phosphorylation. To further enhance the derivatisation efficiency, solid-phase derivatisation may help to alleviate some of these problems.

Conclusions and suggestions for future work

7.1   Conclusions

A membrane desolvator has been evaluated for coupling HPLC with ICP-MS. This interface has enabled the introduction of a range of organic solvents including acetonitrile and methanol into an ICP. In addition, it facilitated the use of solvent gradients without the need to compensate for the solvent loading on the plasma, enabling up to 100 % organic eluents to be utilised for chromatographic separations. The membrane desolvation device removed the majority of the chromatographic eluent, thereby reducing or eliminating many polyatomic interferences such as NOH^+ on $^{31}P^+$, obviating the need for high resolution mass spectrometry. However, one major drawback was the propensity for small polar analytes to pass through the membrane. This was highlighted during the analysis of Lamotrigine where dichlorobenzaldehyde and dichlorobenzoic acid were not detected by $^{35}Cl^+$ selective ICP-MS, presumably because they were removed along with the solvent.

HPLC-ICP-MS has been used for the determination of some pharmaceutically important carboxylic acids after derivatisation with the phosphorus containing reagent TMPP. This allowed $^{31}P^+$ selective detection to be performed on organic compounds which are normally un-detectable by ICP-MS. The derivatisation also enabled the enhanced detection of maleic, fumaric and sorbic acids after

derivatisation with TMPP by HPLC-ESI-MS/MS. Detection was compared pre- and post-derivatisation, with up to a twelve-fold increase being observed for these three acids. The method was also successfully used for the determination of sorbic acid in a sample of Panadol™. The derivatisation of salicylic acid was not as successful resulting in an increased limit of detection observed with ESI-MS-MS detection. This was also highlighted with phosphorus selective ICP-MS detection, where derivatised salicylic acid was not detected.

TMPP was successfully brominated to yield Tris(3,5-dibromo-2,4,6-trimethoxyphenyl) phosphonium propylamine bromide (BrTMPP) using hypobromous acid to generate the bromine electrophile. ^{1}H NMR spectroscopy and infusion ESI-MS were used to verify that bromination had occurred. This BrTMPP derivatising reagent was used to successfully derivatise maleic acid, and was detected by HPLC-SF-ICP-MS. This enabled both $^{31}P^+$ and $^{79}Br^+$ selective detection to be performed on this organic acid which is normally invisible to detection by ICP-MS. By derivatising maleic acid with BrTMPP, an increase in signal of approximately nine times was achieved by monitoring bromine as opposed to phosphorus.

HPLC-UV-ICP-MS with a membrane desolvator interface has also been used to distinguish between phosphorylated and un-phosphorylated peptides. A detection limit of 10 ng mL^{-1} for phosphorus with $^{31}P^+$ selective detection was obtained using chromatography with a 20 μL injection volume and a quadrupole ICP-MS. This system was successfully utilised to distinguish the phosphorylated fragments obtained when bovine β-casein was digested in solution with trypsin. The applicability of using this system is wide ranging. It has been demonstrated that this, used in conjunction with electrospray ionisation mass spectrometry detection,

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would make it easier to identify phosphorylated peptides and gather structural information.

The methodology developed using BrTMPP to derivatise carboxylic acids was shown to be successful in derivatising a phosphorus containing carboxylic acid, potassium P,P-dimethylphosphonoacetate. By determining the Br:P ratio using ICP-MS, the number of phosphorus atoms on the molecule was determined. Unfortunately, this BrTMPP molecule did not successfully derivatise 3-(methylphosphinico)propionic acid, diethylphosphonoacetic acid or the phosphorylated peptides PRN5220 and PRN5221. If this reaction could be improved, and made non-selective, the method has the potential to determine the extent of phosphorylation.

7.2   Suggestions for future work

During the course of this work a number of areas have been identified for further study. Although the use of membrane desolvators has enabled highly organic reversed phase eluents to be utilised with ICP-MS analysis, some analytes of interest may be removed by the membrane. If a non-selective membrane could be sourced and evaluated so it did not remove analytes of interest, but successfully removed the solvents, this would yield higher confidence in detection of unknowns.

The derivatisation of carboxylic acids resulted in improved ESI-MS detection (in most acids) and also enabled these derivatised acids to be detected with ICP-MS. However, if the reaction efficiency of the derivatisation reaction could be improved, lower detection limits should be possible. This improvement in reaction efficiency may also be possible if other coupling reagents were used to aid the reaction.

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N,N'-carbonyldiimidazole[118-121] is one such reagent which was examined during this study, but this had an adverse effect on derivatisation efficiency with these compounds. Other such reagents could be sourced to test whether any enhancements may be achieved.

Other possible ways to enhance the derivatisation efficiency is to use solid phase analytical derivatisation[122-124]. Here, the reagents, derivatives and side products are retained on a solid phase during sorption and derivatisation and in principle, can be selectively eluted after the derivative has formed. Thus, derivatisation can occur at the same time with isolation from the matrix or can be carried out after sorption on the solid phase[122]. This can yield two possible improvements when compared with in-solution derivatisation. Not only can the derivatisation be improved by continually adding more reagents, but when the derivatised product is washed from the sorbent, theoretically some of the other compounds present (e.g. underivatised reagents, excess derivatising products, unwanted by-products) can be removed to yield a purer sample for further analysis.

The preparation of TMPP and subsequent synthesis of BrTMPP involved a multi-step reaction. If cleaning steps, including some re-crystallisation, were incorporated into the reaction scheme, then a purer reagent may be produced yielding cleaner chromatograms. This would enable easier identification of the peaks which correspond to those of the derivatised product and possibly lower the limits of detection.

Other derivatisations could be examined. Leavens et al.[78, 125] have already reported the use of other compounds based on tris(2,4,6-trimethoxyphenyl) phosphine, which have been used to derivatise alcohols, aldehydes, ketones and

amines. These derivatisations could be examined with ICP-MS detection, for compounds which have pharmaceutical relevance. The derivatisation of amines with tris(2,4,6-trimethoxyphenyl)phosphonium ethanoic acid bromide is especially interesting. This molecule could be examined with the same reaction scheme as that used for derivatising with TMPP. If this molecule could be successfully brominated, in a similar fashion as TMPP was, then this molecule could be reacted at the N-terminus of peptides. This may offer an alternative method for the determination of the degree of phosphorylation.

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Appendix

Papers Published

Detection of phosphorus tagged carboxylic acids using HPLC-SF-ICP-MS

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High performance liquid chromatography (HPLC) has been coupled with sector field inductively coupled plasma mass spectrometry (SF-ICP-MS) for the determination of maleic, sorbic and fumaric acids after derivatisation with the phosphorus containing reagent tris(2,4,6-trimethoxyphenyl)phosphonium propylamine (TMPP). This allowed $^{31}P^+$ selective detection to be performed for these compounds, which are normally invisible to detection by ICP-MS at low concentrations. Optimal reagent conditions for the derivatisation of 0.1 mM maleic acid were: 1 mM TMPP; 10 mM 2-chloro-1-methylpyridinium iodide (CMPI); 11 mM triethylamine. The efficiency of the derivatisation reaction was estimated to be between 10–20% and detection limits, estimated as 3 times baseline noise, were 0.046 nmol for TMPP and 0.25 nmol for derivatised maleic acid, for a 5 μl injection.

Introduction

The vast majority of organic compounds are essentially invisible to detection by inductively coupled plasma mass spectrometry (ICP-MS) because they only contain H, C, O and N. These elements have extremely high background signals because they are either present in the solvents used during sample introduction or present in atmospheric air entrained into the plasma, hence trace analysis is impossible. The exceptions to this are the determination of C at minor and trace levels[1–3] and the element selective detection of organic compounds which contain a hetero-atom such as P, S, Si, Cl, Br, I, a metal or metalloid. For example, detection of phosphorus containing compounds has previously been performed for the determination of phosphorylated peptides[4–6] and sulfur-containing pharmaceuticals.[7] An alternative has recently been proposed by Smith et al.[8] which uses aqueous eluents only (at temperatures of 60 °C and 160 °C) or isotopically enriched solvents to detect carbon in organic compounds by LC-ICP-MS. If a means of making all organic compounds detectable by ICP-MS could be achieved, many new applications would be realised. For example, high accuracy quantitative analysis of organic compounds could be achieved using external calibration; and many biological molecules could be determined in metabolic studies.[9]

Leavens et al.[10] have previously reported on the synthesis of a large molecular weight phosphorus reagent, tris(2,4,6-trimethoxyphenyl)phosphonium propylamine bromide (TMPP), which was used to derivatise organic compounds to make them amenable to detection by positive ion electrospray ionization mass spectrometry (ESI-MS). We have used the TMPP reagent for the determination of low molecular weight carboxylic acids in pharmaceutical samples, and have determined the limits of detection using ESI-MS-MS.[11] This reaction incorporates a coupling reaction with 1-chloro-4-methylpyridinium iodide and triethylamine to activate the carbonyl group on the carboxylic acid prior to nucleophilic attack by the amine group on the coupling reagent. The resulting derivatised carboxylic

acid contains a stable amide bond linking the derivatising reagent with the carboxylic acid. The majority of carboxylic acids do not contain a heteroatom so cannot be detected using ICP-MS. However, by derivatising these acids with TMPP they have effectively been tagged with phosphorus, so $^{31}P^+$ selective detection using ICP-MS can be performed. This has useful applications in pre-screening for quality control and investigations involving intellectual property rights.[7] In this paper, detection of some pharmaceutically important carboxylic acids (maleic, fumaric, salicylic and sorbic) by LC-ICP-MS, after derivatisation, is described. The derivatisation reaction was also examined to further enhance the derivatisation procedure to lead to more sensitive detection, and hence lower detection limits.

Experimental

Chemicals and reagents

Carboxylic acids, 2-chloro-1-methylpyridinium iodide (CMPI), triethylamine (TEA) and formic acid were obtained from Sigma–Aldrich (Poole, Dorset, UK). HPLC grade acetonitrile was obtained from Fisher Scientific UK Ltd. (Loughborough, Leicestershire, UK). Distilled deionised water (18.2 MΩ) was obtained using an Elga Maxima water purifying system.

Synthesis of tris(2,4,6-trimethoxyphenyl)phosphonium propylamine bromide (TMPP) (Scheme 1)

TMPP was synthesised in-house using an adaptation of the procedure detailed by Leavens et al.[10] Initial attempts to synthesize this derivatising reagent using the literature method were unsuccessful, possibly due to the propylamine side chain not attaching to the tertiary phosphonium. This may be because the 3-bromopropylamine free base was unstable and tended to condense with itself by nucleophilic displacement of bromide by the primary amine. Hence, it was decided to perform the liberation of the free amine from the salt, and
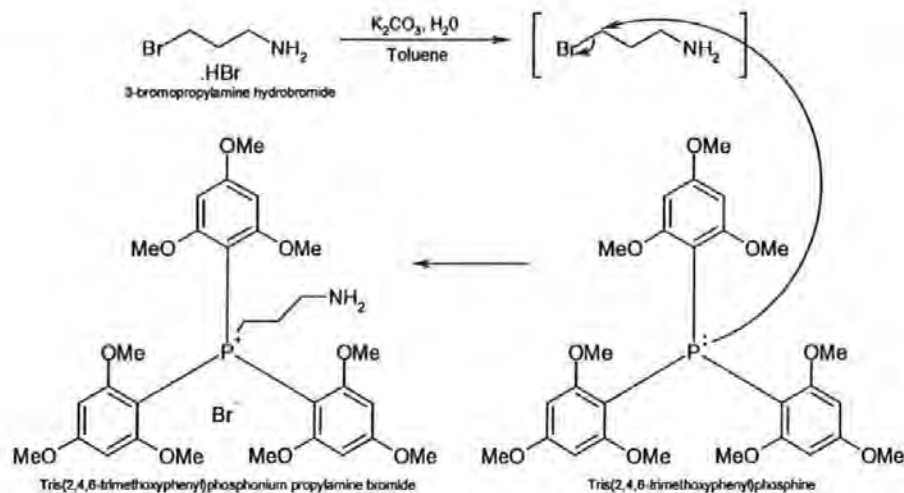
Scheme 1 Synthesis of tris(2,4,6-trimethoxyphenyl)phosphonium propylamine bromide (TMPP).

the subsequent clarification, as quickly as possible. Thus, the following method was developed successfully: To a solution of 3-bromopropylamine hydrobromide (4.98 g, 22.74 mmol, now in large excess) in water (20 ml) was added potassium carbonate (2.24 g) and toluene (20 ml) with stirring. The resultant toluene phase was isolated, clarified with saturated brine (20 ml), and dried over magnesium sulfate. This dry toluene phase was filtered directly into a pre-refluxing solution of tris(2,4,6-trimethoxyphenyl)phosphine (2.12 g, 3.98 mmol) in toluene (40 ml) to minimise the time for the 3-bromopropyla-mine free base to condense with itself. The mixture was refluxed for a further 30 min and the resulting white precipitate was isolated by filtration, washed with toluene (2 × 5 ml) and diethyl ether (25 ml) and dried overnight at 30 °C to give the title compound. A schematic of the reaction is shown in Scheme 1.

Preparation of coupling reagent

Solutions of CMPI were prepared by dissolving the amount of CMPI in approximately 20 ml of acetonitrile in a 25 ml volumetric flask. The corresponding amount of triethylamine was added, and the solution made up to volume with acetonitrile.

### Coupling of TMPP propylamine with carboxylic acids

To 500 µl of carboxylic acid in 90 : 10% (v/v) water : acetonitrile were added 500 µl of CMPI/TEA coupling reagent (prepared as above). After thorough mixing for 5 min at room

temperature, 500 µl of a TMPP propylamine solution in acetonitrile was added. The solution was left to react for 30 min in an ultrasonic bath at room temperature. A schematic of the reaction is shown in Scheme 2.

HPLC-SF-ICP-MS analyses

For HPLC-SF-ICP-MS analyses, a HP1050 modular chromatography system (Agilent Technologies, Stockport, UK) equipped with a Phenomenex Luna C18(2) reversed phase column (100 × 4.6 mm, 3 µm particle size) was used. The mobile phase comprised a binary system of: eluent A, water : acetonitrile (90 : 10% v/v) containing 0.05% formic acid (v/v); and eluent B, water–acetonitrile (10 : 90% v/v) containing 0.05% (v/v) formic acid. The linear gradient employed started at 100% A, changing to 40% A and 60% B over 20 min. The flow rate was 1 ml min^{-1} with an injection volume of 5 µl. All experiments were performed using a sector-field inductively coupled plasma mass spectrometer (SF-ICP-MS, Thermo Elemental Axiom, Winsford, UK), using a mass resolution setting of 3000. Operating conditions are shown in Table 1.

It is common, in large pharmaceutical companies, to adopt a standard HPLC elution method which can be used in the majority of cases. The method is often standardised to a simple linear elution of acetonitrile–water which can run up to 100% (v/v). Such a high concentration of acetonitrile is incompatible with the normal operation of an ICP-MS unless an extremely low flow nebuliser is used. One solution to this, which was
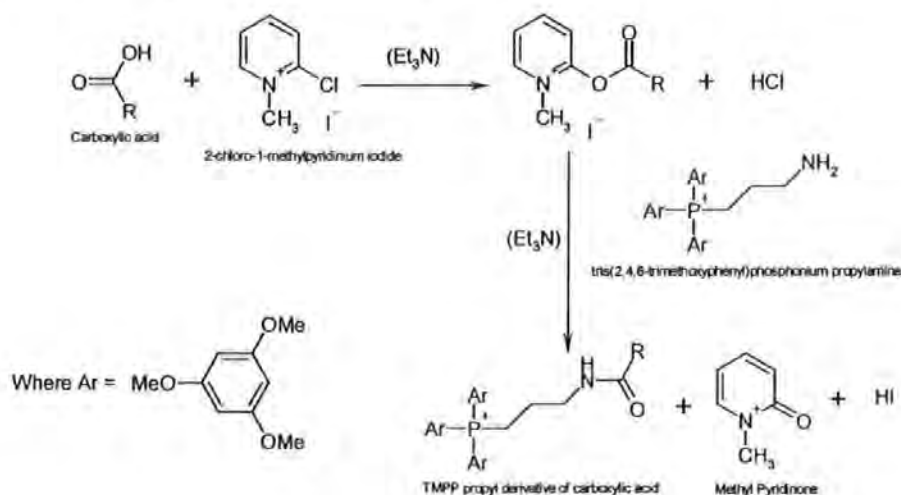
Scheme 2 Activation of carboxylic acids with 2-chloro-1-methylpyridinium iodide (CMPI) and reaction with TMPP propylamine.

Table 1 Operating conditions for SF-ICP-MS

ICP	
Nebuliser gas flow/l min^{-1}	1.10
Auxiliary gas flow/l min^{-1}	0.85
Coolant gas flow/l min^{-1}	14.0
Nebuliser	Micromist (Glass Expansion, Switzerland)
Spray chamber	Jacketed quartz cyclonic, cooled to 5 °C
Torch	Quartz Fassel-type with quartz bonnet, without shield
Interface	
Sampling cone	Nickel 1 mm id
Skimmer cone	Nickel, 0.7 mm id
Single ion monitoring	
Resolution	3000
Slit settings	Source, 330; collector, 220
Masses monitored	Single ion monitoring for $^{31}P^+$ at 30.974 m/z
Dwell time/ms	500
Scanning	
Resolution	3000
Slit setting	Source, 330; collector, 220
Masses monitored	Scanning between 30.838 and 31.109 m/z
Dwell time/ms	50
Points	20

adopted in this work, is to use a membrane desolvation system, which removes the vast majority of solvent and allows routine operation of gradient elution up to 100% acetonitrile at a standard 1 ml min^{-1} flow rate. However, it should be noted that low molecular weight polar analytes may also be removed by the desolvation system, so caution should be used when adopting this approach. In this work the HPLC column was coupled with the SF-ICP-MS simply by inserting the end of the tubing from the column into the sample uptake inlet of the nebuliser, and the output from the spray chamber was introduced into a Universal Interface Model B desolvation device (Vestec Corporation, Houston, USA). The sweep gas was optimised daily at approximately 2.0 l min^{-1} to ensure that the maximum signal for $^{31}P^+$ was achieved. The dry aerosol exiting the desolvator was then transferred to the ICP torch *via* 1 m of 0.25 in. id Tygon tubing.

HPLC–electrospray ionization mass spectrometry

HPLC was performed using a P580A binary pump (Dionex–Softron GmbH, Germering, Germany) coupled with a Phenomenex Luna C18(2) reversed phase column (100 × 4.6 mm, 3 μm particle size) with the same gradient elution as is used for HPLC-SF-ICP-MS. The 1 ml min^{-1} flow rate was split post-column (high pressure micro-splitter valve; Upchurch Scientific Ltd, Oak Harbor, WA, USA), and ~200 μl min^{-1} was diverted to the mass spectrometer and the residue to waste. Sample injections (5 μl) were made manually with a metal-free Rheodyne injector (model number 9125, CA, USA). Mass spectrometry analysis was performed using an ion trap mass spectrometer fitted with an electrospray interface (Thermo-Quest Finnigan Mat LCQ, San Jose, CA). Data were acquired and processed with Xcalibur 1.0 software. Instrument optimization was performed by infusing a 100 ng ml^{-1} solution of TMPP at 3 μl min^{-1}, monitoring for the characteristic positive ion at 590 m/z. The following instrument parameters were used: source voltage, +4.50 kV; capillary voltage, +20 V; tube lens offset, −10.00 V; capillary temperature, 220 °C; nitrogen sheath gas flow rate, 60 (arbitrary units) and nitrogen auxiliary gas flow rate 20 (arbitrary units). Mass spectra were recorded in the positive ion mode within m/z 190–1000.

Results and discussion

Phosphorus selective detection

Chromatograms of maleic acid (MA), after derivatisation with TMPP, are shown in Fig. 1. Detection was performed using SF-ICP-MS with $^{31}P^+$ selective detection at 30.974 m/z (Fig. 1A and 1B), and ESI-MS (Fig. 1C and 1D). As can be seen, the TMPP derivatising reagent contained many phosphorus containing impurities (Fig. 1A), so it was necessary to
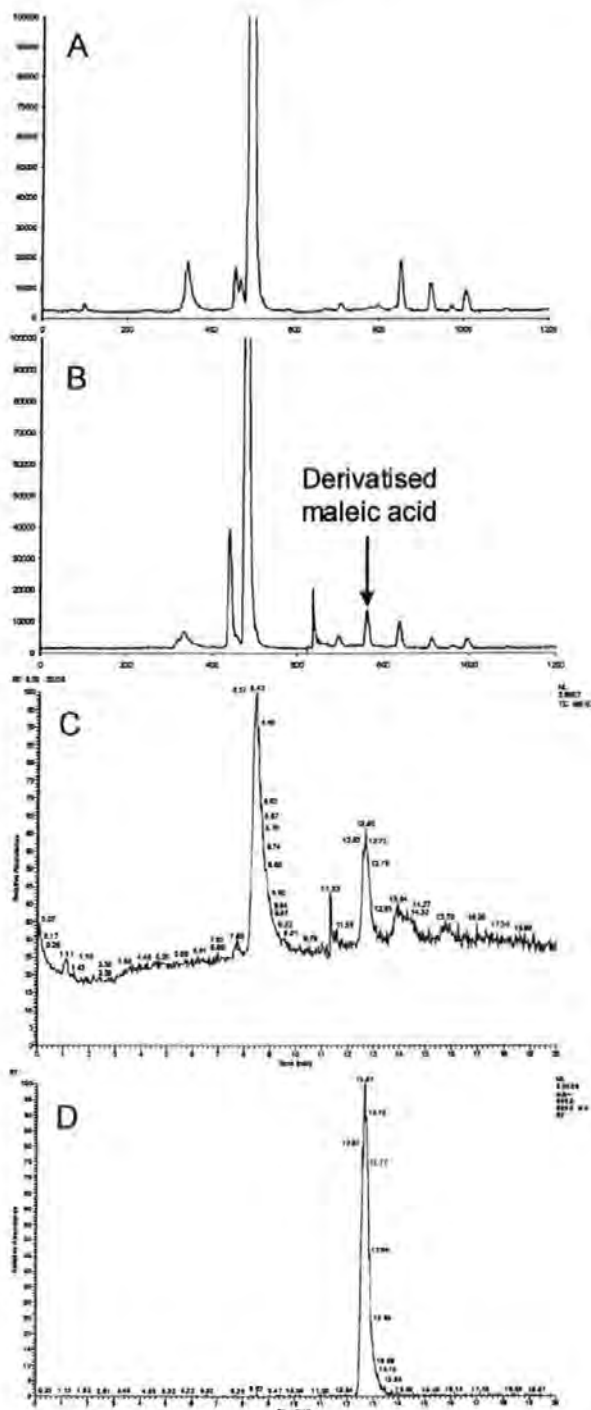

Fig. 1 Chromatograms of derivatised maleic acid: A, blank injection of TMPP and $^{31}P^+$ selective detection using SF-ICP-MS; B, derivatised maleic acid and $^{31}P^+$ selective detection using SF-ICP-MS; C, derivatised maleic acid and total ion current using positive ion ESI-MS; D, derivatised maleic acid and selective ion monitoring at 688 m/z using positive ion ESI-MS.

optimise the chromatography such that the derivatised MA was separated from these peaks (Fig. 1B), and particularly the large peak for unreacted TMPP. For comparison, and confirmatory purposes, detection was also performed using ESI-MS and the major peak at 8.43 min was found to have a mass of 590 m/z, which corresponds to the mass of TMPP. Likewise, the peak at 12.65 min was found to have a mass of 688 m/z, indicating it to be MA derivatised with TMPP (Fig. 1D). A comparison of the chromatogram obtained using ICP-MS (Fig. 1B) with the total ion chromatogram obtained using ESI-MS detection (Fig. 1C) indicates that the former method yielded a slightly improved signal-to-noise ratio, which should result in better detection limits. However, in this case, ESI-MS showed better signal-to-noise when single ion monitoring at the base peak of 688 m/z was performed. This highlights both the advantages and disadvantages of the two techniques, namely that ICP-MS is useful for high sensitivity screening purposes, when confirmation that a compound contains phosphorus (or any other element for that matter) is required. Hence, once these peaks in the chromatogram have been identified, then ESI-MS can be used in single ion mode to perform further qualitative analysis. In this case the TMPP was synthesised in-house so was not particularly pure: however, it is envisaged that a purer form of the reagent would alleviate some of the problems caused by interfering peaks in the ICP-MS chromatogram.

Optimisation of derivatisation reaction

In order to achieve optimal sensitivity the concentrations of the derivatisation reagents were optimised using a 2-factor, 3-level factorial design (Table 2). Concentrations of TMPP and CMPI were set at 0.1 mM, 1 mM and 10 mM for the derivatisation of 0.1 mM of MA. Triethylamine (TEA) was also present as the base for the reaction so its concentration was adjusted to equal the sum of the concentrations of TMPP and MA, in order to maintain the stoichiometry of the reagents. At higher concentrations of TMPP, many impurities were observed, thus making it difficult to identify the peak due to derivatised MA (see Fig. 2B). It is desirable to achieve a situation where the derivatisation of MA is at a maximum for the lowest concentration of reagents, but the reagents are always in sufficient excess to ensure constant reaction efficiency for varying concentrations of acid. In this case, maximum signal for derivatised MA (at 0.1 mM), and hence maximum reaction efficiency, was achieved at concentrations 1 mM (TMPP) and 10 mM (CMPI), with the concentration of TEA at 11 mM. The effect of TMPP on the derivatisation of MA is shown in Fig. 2. At a TMPP : MA ratio of 10 : 1 (1.0 mM TMPP, 0.1 mM MA) a net peak height signal of approximately 1500 for derivatised MA was observed (Fig. 2A). At a TMPP : MA ratio of 100 : 1 (10 mM TMPP, 0.1 mM MA) the peak height signal was
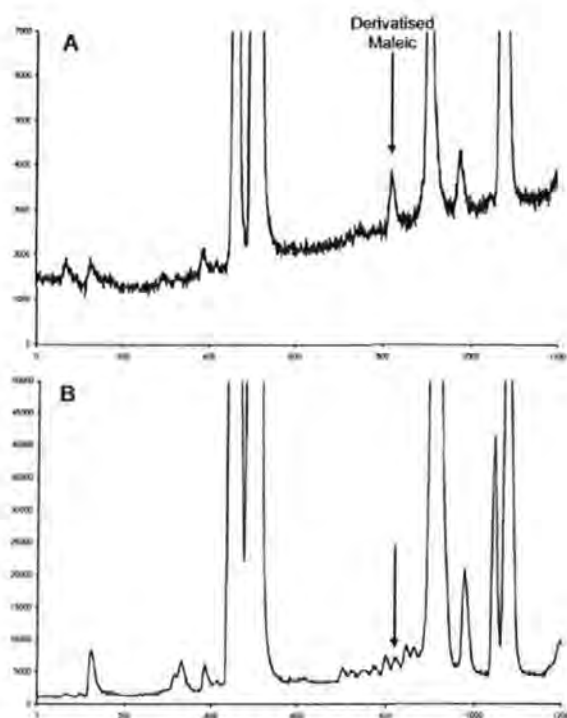

Fig. 2 HPLC-ICP-MS chromatograms measuring $^{31}P^+$ at 30.974 m/z for the derivatisation of 0.1 mM maleic acid with: (A) 10 mM CMPI and 1.0 mM TMPP; and (B) 10 mM CMPI and 10 mM TMPP.

similar at approximately 1700; however, impurities in the TMPP eluted close to the analyte peak (Fig. 2B), which made identification of the derivatised MA more difficult. A rough estimate of the efficiency of the reaction was obtained by ratioing the peak height of the derivatised MA to the sum of the derivatised acid plus the residual TMPP reagent peak, then expressing this as a percentage of the expected theoretical ratio calculated from the known concentrations. This assumes equal instrumental response for the two compounds, which should hold roughly true in this case given that a desolvator was used and the nebuliser efficiency was similar over the duration of the gradient in which the peaks eluted. This is illustrated in Fig. 2A, where it is evident that the baseline signal, resulting from phosphorus impurities in the eluent, increased in a linear manner by only a factor of 2 over the course of the chromatographic run, and only by a factor of approximately 1.5 between the elution of TMPP and MA. Hence, for equimolar concentrations of MA and TMPP (1 mM) the reaction efficiency was 22%, and when TMPP was in 10-fold excess the reaction efficiency was 16%. Hence, it is likely that only between 10–20% of MA was derivatised under the conditions used and detection limits could be improved further by improving the efficiency of the reaction.

An approximate limit of detection for derivatised MA, using $^{31}P^+$ selective detection by ICP-MS, was calculated as the concentration of MA which resulted in a peak height of 3× baseline noise. This resulted in a detection limit of 0.05 mM for

Table 2 Two factor, three level full factorial design experiment to determine the optimal conditions for derivatisation of 0.1 mM maleic acid

Solution number	CMPI concentration/ mM	TMPP concentration/ mM	TEA concentration/ mM
1	1.0	1.0	1.1
2	0.1	1.0	1.1
3	1.0	0.1	0.2
4	1.0	10.0	10.1
5	10.0	0.1	0.2
6	0.1	10.0	10.1
7	10.0	10.0	10.1
8	0.1	0.1	0.2
9	10.0	1.0	1.1

Table 3 Approximate absolute limits of detection for a 5 μl injection

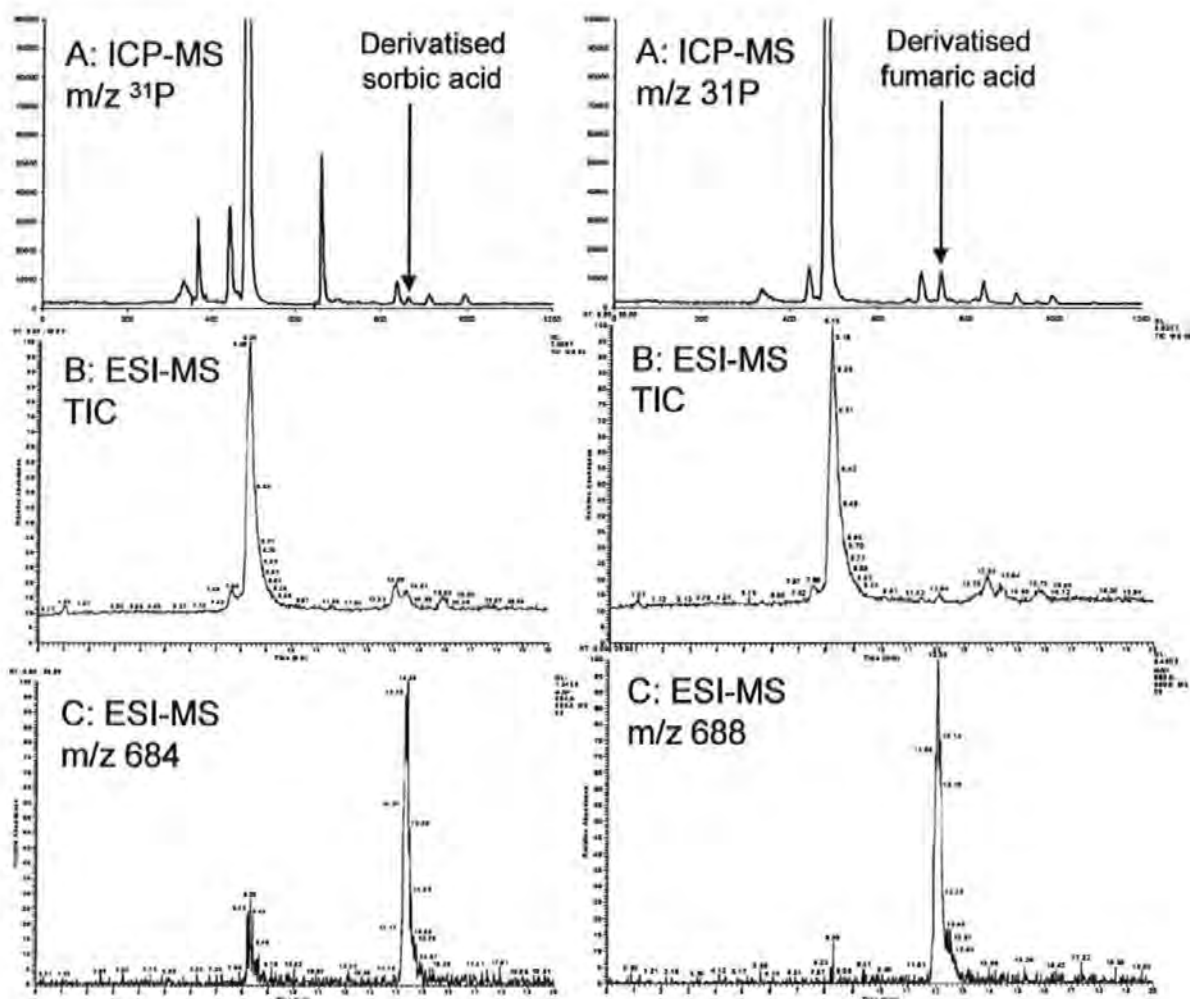|	Compound specific		^{31}P specific	
	/nmol	/ng	/ng	Ref.
TMPP (bromide salt)	0.046	30	1.4	This work
MA (derivatised)	0.25	29	7.8	This work
Malathion[a]			0.16	12

[a] 100 μl injection.

Fig. 3 Chromatograms of derivatised sorbic (left) and fumaric (right) acids: A, $^{31}P^+$ selective detection using SF-ICP-MS; B total ion current using positive ion ESI-MS; C, selective ion monitoring using positive ESI-MS.

a 5 μl injection. This is confirmed by Fig. 2A, where a 0.1 mM injection can clearly be seen to be close to the detection limit. For comparison, detection limits for TMPP obtained in this work, and for malathion from previous work on the same instrument, are shown in Table 3. As can be seen, the compound specific detection limit for TMPP was approximately 5–6 times lower than that for derivatised MA (in molar terms), reflecting the 10–20% reaction efficiency of the latter. The $^{31}P^+$ specific detection limit for malathion achieved previously[12] was approximately 10-times lower than the detection limit for TMPP determined in this work. The probable explanation for this is that a plasma shield was not used on this occasion, which resulted in a 10-fold reduction in sensitivity, with a consequent increase in the detection limit.

Analysis of derivatised carboxylic acids

The usefulness of the derivatisation reaction was tested for the detection of several other carboxylic acids, namely, fumaric, sorbic and salicylic acids. Detection using both HPLC-SF-ICP-MS ($^{31}P^+$ selective) and LC-ESI-MS (total ion and extracted ion ranges) was performed to verify which of the phosphorus containing peaks was due to the derivatised acids (Fig. 3). Derivatised MA was observed at 12.6 min (Fig. 2), sorbic acid at 14.4 min and fumaric acid at 12.0 min (Fig. 3). No peak was observed with either $^{31}P^+$ selective ICP-MS or ESI-MS detection for derivatised salicylic acid. This is probably due to strong internal hydrogen bonding making salicylic acid a poor

nucleophile, leading to an inefficient reaction with the CMPI activating reagent. It should be possible to derivatise a range of carboxylic acids using this method, as described by Leavens et al.[10] They did not give any indication as to the efficiency of the reaction or detection limits: however, this has been addressed by us in another paper.[11] It should be possible to improve reaction efficiency by optimising the derivatisation chemistry, and utilising a solid phase analytical derivatisation approach as described by Pilus et al.[13]

Conclusions

HPLC-SF-ICP-MS has been used for the determination of maleic, sorbic and fumaric acids after derivatisation with the phosphorus containing reagent tris(2,4,6-trimethoxyphenyl)-phosphonium propylamine. This allowed $^{31}P^+$ selective detection to be performed on organic compounds which are normally not amenable to detection by ICP-MS at low concentrations. The derivatisation reaction was partially optimised for MA: however, there is scope for further improving the reaction efficiency to achieve lower detection limits and a more quantitative analysis. Work is currently underway, in our laboratory, on the preparation of a multiply brominated TMPP reagent, which will allow simultaneous bromine and phosphorus selective detection. Multiple bromination should improve detection limits and make possible the use of this reagent to determine the degree of phosphorylation of peptides. The scope of application for reagents such as these is

great, particulalrly in proteomics and genomics where ever more selective and sensitive methods of analysis are required. The application of isotopically tagged reagents is a particular focus of our work and is now close to publication.

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Derivatisation of carboxylic acid groups in pharmaceuticals for enhanced detection using liquid chromatography with electrospray ionisation tandem mass spectrometry

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Tris(2,4,6-trimethoxyphenyl)phosphonium propylamine bromide (TMPP) has been used for the derivatisation of maleic, fumaric, sorbic and salicylic acids to facilitate determination using liquid chromatography/electrospray ionisation tandem mass spectrometry (LC/ESI-MS/MS) in positive ion mode. Detection limits, achieved using multiple reaction monitoring mode, were 2, 4, 0.4 and 540 fmol (5 μL injection) for derivatised fumaric, sorbic, maleic and salicylic acids, respectively. In comparison, detection limits achieved in negative ion mode for the underivatised acids were 24, 51, 2, and 117 fmol, respectively. The method was successfully used for the determination of sorbic acid in a sample of Panadol™. The derivatisation of salicylic acid was not as successful, probably due to poor reaction efficiency. Copyright © 2005 John Wiley & Sons, Ltd.

Many pharmaceutical drug substances contain carboxylic acids as either a main constituent or as impurities at low levels. Mass spectrometry is ideally suited to detection due to its high sensitivity and selectivity. However, carboxylic acids tend to be poorly ionised by ionisation sources used in mass spectrometry, and hence the derivatisation of carboxylic acids to enhance their detection for chromatographic analysis has been a subject of some investigation.[1–6] By exploring the nature and chemical properties of a molecule, a method for modification and subsequent analysis can be devised. Published methods for derivatising carboxylic acids generally involve the incorporation of fluorescent tags[7] or of functional groups suitable for electrochemical detection methods,[8] with very little concentration on analysis by liquid chromatography/mass spectrometry (LC/MS).

The choice of derivatisation reagent is an important consideration. Ideally, the reagent should be pre-charged to remove the dependence on the ionisation source to initiate ionisation, and must possess a suitable reactive group to react with the functional group of the analyte molecule. The derivatisation reagent must also be stable and remain intact throughout the reaction process to ensure that maximum signal is achieved with the derivatised product. The molar

mass and structure of the derivatisation reagent are also important considerations. Smaller reagents have an advantage in that steric hindrance is not an important factor in the reaction process, but larger reagents have the advantage that their derivatised products have a high mass and thus will enable a signal to be observed in the less noisy region of the mass spectrum. In addition, the reaction should be a rapid, single-step reaction with a high yield, using inexpensive and commercially available (or easily synthesised) reagents. Also, the derivatisation product, when not pre-charged, is often less polar than the carboxylic acid, thus increasing the retention time on a conventional C18 column and improving ionisation in electrospray (ESI) due to generation of a more stable spray with more organic solvent present.

There are some disadvantages in using a derivatisation reaction. The reaction procedure adds extra time to the analysis, with extra materials and glassware needed for the reaction. The reaction may also produce other compounds within the reaction, including partially derivatised products, unwanted side products, and the reaction of the carboxylic acid with any impurities present in the derivatising reagent. The use of chromatography prior to MS detection can overcome some of these problems.

In this paper tris(2,4,6-trimethoxyphenyl)phosphonium propylamine bromide is used as the derivatising reagent. This is a large positively charged phosphonium reagent that enables detection by ESI-MS in the positive ion mode. The synthesis of this reagent is described elsewhere by Leavens *et al.*,[9] and we have previously reported on its use as a derivatising agent for element-selective detection of organic

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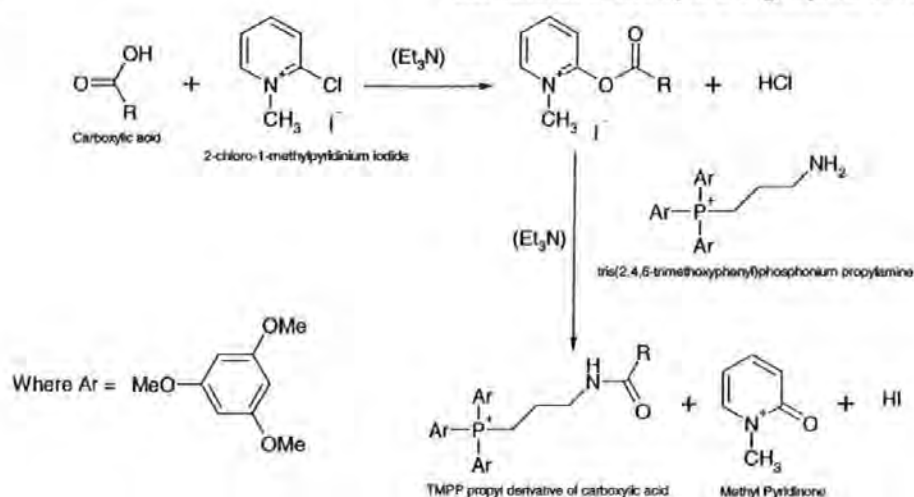
Scheme 1. Activation of carboxylic acids with 2-chloro-1-methylpyridinium iodide (CMPI) and reaction with TMPP propylamine.

compounds by LC with inductively coupled plasma mass spectrometry (ICP-MS).[10] The derivatisation incorporates a coupling reaction with 1-chloro-4-methylpyridinium iodide and triethylamine to activate the carbonyl group on the carboxylic acid prior to nucleophilic attack by the amine group on the coupling reagent. The resulting derivatised carboxylic acid contains a stable amide bond linking the derivatising reagent with the carboxylic acid.

In this work, detection of underivatised maleic, fumaric, salicylic and sorbic acids by LC/ESI-MS/MS, operated in negative ion mode, has been compared with detection of the TMPP-derivatised carboxylic acids in positive mode.

EXPERIMENTAL

Chemicals and reagents

The carboxylic acids, 2-chloro-1-methylpyridinium iodide (CMPI), triethylamine (TEA) and formic acid were obtained from Sigma-Aldrich (Poole, Dorset, UK). HPLC-grade acetonitrile was obtained from Fisher Scientific UK Ltd. (Loughborough, Leicestershire, UK). Tris(2,4,6-trimethoxyphenyl) phosphonium propylamine bromide (TMPP) was synthesised at GlaxoSmithKline using the procedure detailed by Leavens et al.[9] Distilled deionised water ($18.2\,M\Omega$) was obtained using an Elga Maxima water-purifying system.

Coupling of TMPP propylamine with carboxylic acids

A solution of CMPI was prepared by dissolving 37.98 mg of CMPI in approximately 20 mL of acetonitrile in a 25 mL volumetric flask. Triethylamine ($41.4\,\mu L$) was added, and the solution made up to volume with acetonitrile. To $100\,\mu L$ of $100\,\mu M$ carboxylic acid in 90:10% (v/v) water/acetonitrile were added $100\,\mu L$ of CMPI/TEA coupling reagent (prepared as above). After thorough mixing for 5 min at room temperature, $100\,\mu L$ of a 1 mM TMPP propylamine solution in acetonitrile were added. The solution was left to react for 30 min in an ultrasonic bath at room temperature. A schematic of the reaction is shown in Scheme 1.

Preparation of Panadol™ sample and subsequent derivatisation with TMPP propylamine

A commercially available Panadol™ (GlaxoSmithKline) tablet was crushed with a pestle and mortar until a fine powder was obtained. A small amount of this powder (18.1 mg) was added to a centrifuge tube along with 1 mL of 90:10% (v/v) water/acetonitrile, and centrifuged at 13000 rpm for 8 min. To $100\,\mu L$ of this supernatant were added $100\,\mu L$ of CMPI/TEA coupling reagent (prepared as described above). After thorough mixing for 5 min at room temperature, $100\,\mu L$ of a 1 mM TMPP propylamine solution in acetonitrile were

Table 1. ESI-MS/MS operating conditions for analysis of underivatised acids

	Acid			
	Maleic	Fumaric	Salicylic	Sorbic
Quadrupole 1 (m/z)	115	115	137	111
Quadrupole 3 (m/z)	71	71	93	67
Curtain gas (arbitrary units)	10	10	10	10
Collision gas (CAD) (arbitrary units)	6	6	6	6
Ionspray potential (IS) (V)	−4200	−4200	−4200	−4200
Temperature (°C)	500	500	500	500
Nebuliser gas (GS1) (arbitrary units)	20	20	20	20
Auxiliary gas (GS2) (arbitrary units)	0	0	0	0
Declustering potential (V)	−25	−30	−30	−40
Collision energy (eV)	−14	−12	−24	−14

Table 2. ESI-MS/MS operating conditions for analysis of derivatised acids

	Acid			
	Maleic	Fumaric	Salicylic	Sorbic
Quadrupole 1 (*m/z*)	688	688	710	684
Quadrupole 3 (*m/z*)	590	642	590	181
Curtain gas (arbitrary units)	10	10	10	10
Collision gas (CAD) (arbitrary units)	4.00	4.00	4.00	4.00
Ionspray potential (IS) (V)	5500	5500	5500	5500
Temperature (°C)	650	650	650	650
Nebuliser gas (GS1) (arbitrary units)	20	20	20	20
Auxiliary gas (GS2) (arbitrary units)	0	0	0	0
Declustering potential (V)	95	95	100	100
Collision energy (eV)	55	75	50	70

added. The solution was left to react for 30 min in an ultrasonic bath at room temperature prior to analysis.

Chromatography

For the LC/ESI-MS/MS analyses, a HP1100 chromatography system (Agilent Technologies, Stockport, UK) was used. All separations were performed using a Phenomenex Luna C18(2) reversed-phase column (100×4.6 mm, 3 µm particle size) at 40°C. The mobile phases contained the following: (A) water/acetonitrile (90:10% v/v) containing 0.05% (v/v) formic acid and (B) water/acetonitrile (10:90% v/v) containing 0.05% (v/v) formic acid. For negative ion LC/ESI-MS/MS a stepped gradient elution was used: 0–5 min, 100% A; 5–5.5 min, 100–70% A; 5.5–15 min 70% A; 15–15.5 min, 70–100% A; 15.5–20 min 100% A. Similarly, for positive ion ESI-MS/MS, an isocratic stepped gradient elution was used: 0–10 min, 70% A; 10–10.1 min, 70–50% A; 10.1–13 min 50% A; 13–13.1 min, 50–70% A; 13.1–20 min 70% A. The flow rate was 1 mL min^{-1} with an analyte injection volume of 5 µL. These step gradients were chosen to enhance quantitation using ESI-MS.

Electrospray mass spectrometry

For analysis of the underivatised carboxylic acids, negative ion mass spectra were acquired, whereas, for derivatised carboxylic acids, positive ion mode was used. The mass spectrometer was an Applied Biosystems MDS Sciex API 4000 (Applied Biosystems Ltd., Foster City, CA, USA) triple quadrupole equipped with a Turbo-V Ionspray ionisation source. The detection limits were determined using MRM (multiple reaction monitoring) which is the most sensitive technique available with a triple-quadrupole instrument. The other
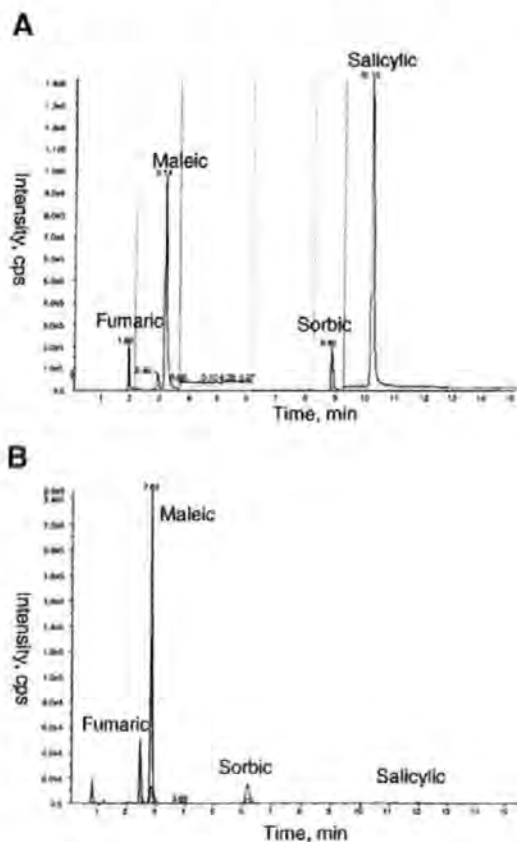

Figure 1. Chromatograms for a mixture of carboxylic acids (2 µmol L^{-1}) with detection by multiple reaction monitoring with electrospray mass spectrometry: (A) underivatised carboxylic acids and negative ion mode ESI-MS/MS and (B) derivatised carboxylic acids and positive ion mode ESI-MS/MS.

Table 3. Calculated detection limits obtained for the underivatised carboxylic acids obtained using LC/ESI-MS/MS in negative ion mode

Acid	Q_1 *m/z* (precursor)	Q_3 *m/z* (product)	Detection limit (fmol)
Fumaric	115	71	24
Sorbic	111	67	51
Maleic	115	71	2
Salicylic	137	93	117

Table 4. Calculated detection limits for the derivatised carboxylic acids obtained using LC/ESI-MS/MS in positive ion mode

Acid	Q_1 *m/z* (precursor)	Q_3 *m/z* (product)	Detection limit (fmol)
Fumaric	688	642	2
Sorbic	684	181	4
Maleic	688	590	0.4
Salicylic	710	590	540

A

TIC of -MRM (1 pair): Period 5, from Sample 1 (Panadol Sample) of R9123_030_06.wiff   Max. 1.2e4 cps.

B

XIC of +MRM (9 pairs): 684.0/181.0 amu from Sample 3 (Derivatised panadol) of R9123_031_05....   Max. 1.7e5 cps.
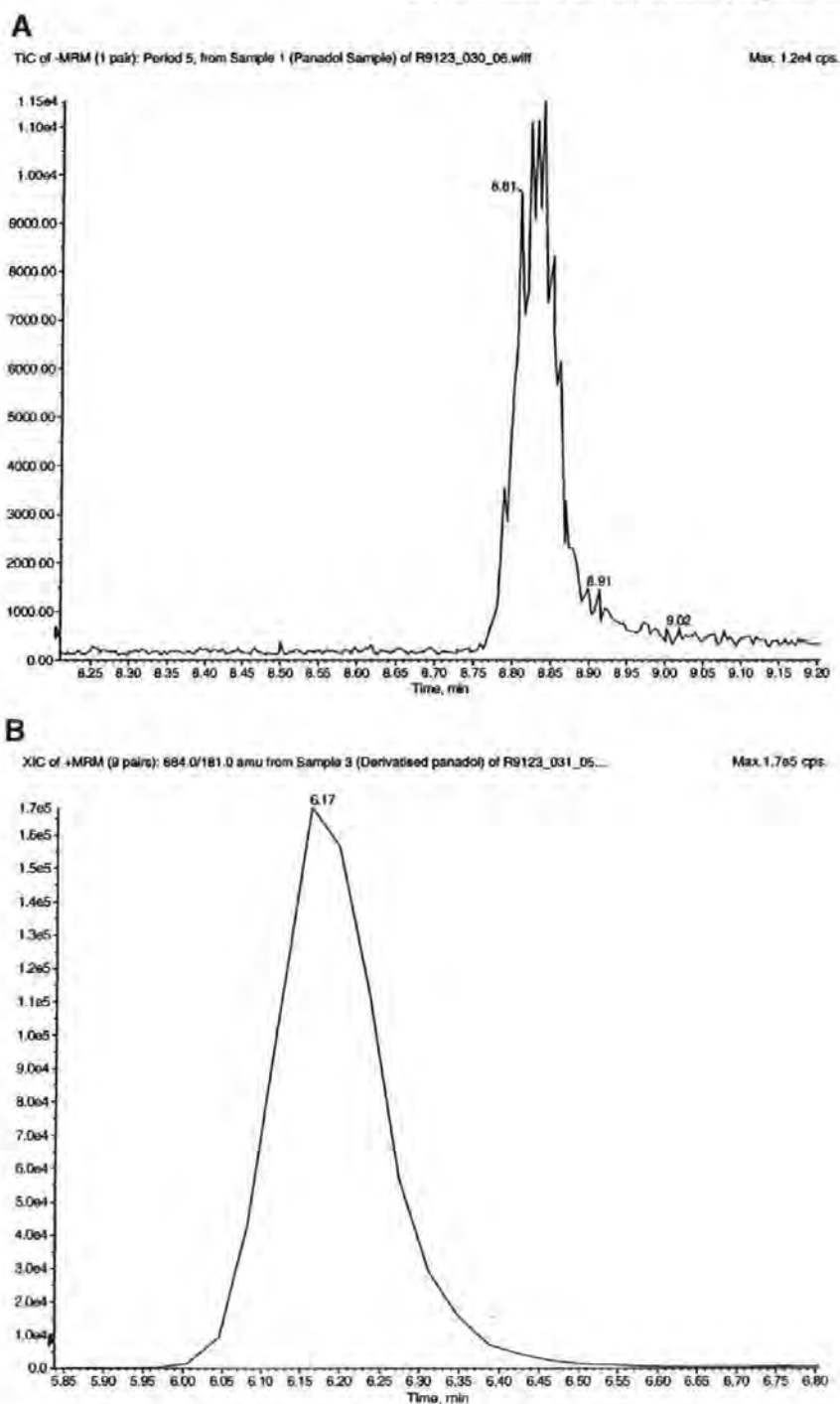

Figure 2. Selected ion chromatograms for Panadol™ samples, showing the presence of sorbate, obtained in (A) negative ion mode without derivatisation and (B) positive ion mode after derivatisation with TMPP.

parameters used are shown in Tables 1 and 2. Data processing was performed using Analyst 1.3 software.

RESULTS AND DISCUSSION

Derivatisation of carboxylic acids

The determination of fumaric, sorbic, maleic and salicylic acids was investigated. These acids were chosen because

they are commonly found in many pharmaceutical products as additives or impurities, and are difficult to determine using conventional ESI-MS because they do not form positive ions and have low molecular weights. Negative ion ionisation is possible but, due to the low molecular weights, there is a lot of chemical noise in the mass spectra, e.g. from common LC additives such as acetic acid, formic acid, and especially trifluoroacetic acid (m/z 113 and its proton-bound dimmer ion at

m/z 227). In addition, since these organic acids are poorly retained on conventional reversed-phase columns that are typically used for pharmaceutical analyses, they elute in a highly aqueous matrix which is not ideal for ESI (an unstable spray if the organic content of the eluent is too low). It would be advantageous to be able to determine these compounds using the standard approach of reversed-phase LC coupled with ESI-MS operated in positive ion mode, to enable co-determination of all analytes.

In order to evaluate any possible benefits of derivatisation of the acids with TMPP, the analysis was performed using both LC/ESI-MS/MS in negative ion mode without derivatisation and in positive ion mode with derivatisation. Chromatograms showing the separation and detection of the four acids using both modes of detection are shown in Fig. 1. Detection was performed by monitoring the appropriate precursor ion in quadrupole 1 and the most abundant fragment ion in quadrupole 3 (Tables 1 and 2). For the underivatised acids, the most abundant fragment ion corresponded to the loss of 44 Da (assigned as CO_2) from the $[M-H]^-$ precursor ion; for the derivatised acids the most efficient fragmentation was variable, so the most abundant fragment for each test analyte was used in the analyses. In order to improve the duty cycle all product ions were not monitored throughout the entire chromatographic run, but instead the mass spectrometer was switched to the most appropriate *m/z* values during elution of each individual carboxylic acid; this led to the changes in baseline observed in Fig. 1(A).

A three-point calibration was performed for each of the acids, and detection limits were calculated based on 3σ of the baseline noise. Detection limits for the underivatised and derivatised carboxylic acids are given in Tables 3 and 4, respectively. Detection limits for fumaric, sorbic and maleic acids were approximately one order of magnitude lower in positive mode after derivatisation, thereby confirming the utility of this method for the enhanced detection of these acids. In contrast, a fivefold increase in the detection limit was observed for salicylic acid; this is probably due to strong internal hydrogen bonding that makes salicylic acid a poor nucleophile, leading to an inefficient reaction with the CMPI activating reagent.

Analysis of Panadol™ tablets

Panadol Extra™ is the proprietary name of an oral analgesic medicine manufactured by GlaxoSmithKline, which would normally contain paracetamol, caffeine and potassium sorbate (used as preservative), together with excipients. Hence, the absence of sorbate in any so-called Panadol Extra™ product would indicate that the product was counterfeit. Analysis was performed using LC/ESI-MS/MS in both negative

and positive ion mode after derivatisation with TMPP propylamine; the resulting chromatograms are shown in Fig. 2. Derivatisation of the sorbic acid (Fig. 2(B)) resulted in an improvement in chromatographic resolution and signal-to-noise compared to the underivatised sample (Fig. 2(A)). An added advantage of the derivatisation is that the analysis can be performed using positive ion mode, so both paracetamol and caffeine can also be determined simultaneously if necessary. Using this method it should be possible to quantify the concentration of sorbate in Panadol Extra™ provided that appropriate validation is performed; however, the method was used in a purely qualitative fashion in this work.

CONCLUSIONS

Enhanced detection of maleic, fumaric and sorbic acids has been achieved by derivatisation with TMPP and detection using positive ion LC/ESI-MS in multiple reaction mode. The method was successfully used for the determination of sorbic acid in a sample of Panadol™. The derivatisation of salicylic acid was not as successful, probably due to poor reaction efficiency. So far, the reagents have only been tested under ideal conditions, and work is currently underway to derivatise peptides and proteins in samples of biological fluids. The use of these reagents in conjunction with complementary detection by ESI-MS and ICP-MS offers considerable potential in proteomics and genomics whenever more selective and sensitive methods of analysis are required.

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