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Development of Novel Methodologies for using ICP-MS in Bioanalysis and Drug Metabolism

Christopher John Smith

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Abbreviations

ICP	Inductively coupled plasma
MS	Mass spectrometry
HPLC	High performance liquid chromatography
MSMS	Triple quadrupole mass spectrometry
DMPK	Drug metabolism and pharmacokinetics
i.v.	Intravenous
AES	Atomic emission spectroscopy
ESI	Electospray ionisation
APCI	Atmospheric pressure ionisation
DRC	Dynamic reaction cell
RF	Radio frequency
UV	Ultraviolet
QC	Quality control
SN	Signal to noise
amu	Atomic mass unit
AUC	Area under the curve
$t_{½}$	Half life
SD	Standard deviation
Rt	Retention time
TCPP	Trichloro(2-picoline)platinate
TCAP	Trichloro amide platinate
HPMC	Hydroxypropylmethyl Cellulose
CLND	Chemiluminescent nitrogen detector
ELSD	Evaporative light scattering detector
SIR	Single ion recording
i.p.	Intra peritoneal
LOD	Limit of detection
LOQ	Limit of quantification
TIC	Total ion chromatogram

Abstract

Inductively coupled plasma mass spectrometry (ICP-MS) has been widely used for environmental and trace analysis since its introduction in the early 1980s. This thesis describes an exploration of the potential of HPLC-ICP-MS within the pharmaceutical industry.

Determination of platinum in the anticancer drug ZD0473 was performed in comparison with conventional HPLC-MSMS, investigating limit of detection, linearity and reproducibility on spiked samples. Both methods were capable of providing accurate and precise results with samples from rats dosed intravenously 0.5 mg/kg and orally at 6 mg/kg, but the HPLC-ICP-MS Pt method had extended linear range and superior sensitivity, providing a limit of quantification of 0.1 ng/mL compared to 5 ng/mL by HPLC-MSMS. Impurity and metabolite profiles for ZD0473, using platinum as a marker with HPLC-ICP-MS, were compared to profiles from [^{14}C] labelled compound with radioactivity detection, showing that the compound was converted to 2-picoline.

Since the number of compounds containing platinum found in the pharmaceutical industry is limited ICP-MS was then evaluated for other elements.

The detection of carbon was investigated, since this is present in all organic molecules, and a limit of detection of 0.47 µmol of carbon was achieved for sulphanilamide using superheated water as the mobile phase. Isotopically enriched solvents ([^{12}C]-methanol 99.95 atom %) were used as organic modifier to aid chromatography. Detection limits of 86 µmol for ^{13}C-triple-labelled caffeine and 79 µmol ^{13}C-double-labelled phenacetin.

Halogen (Br, I and Cl) detection was investigated. Metabolite profiling and excretion balance studies were carried out using these elements after dosing suitable model compounds (substituted anilines and benzoic acids) to rats. Limits of detection for Br and I were measured down to 0.1 mM. Profiling for 2-, 3-, 4-bromobenzoic acids showed glycine and glucuronide metabolites, in different proportions dependant on the position of the Br. This was also seen for the 2-, 3-, 4-iodobenzoic acids.

Sulphur and phosphorous containing drugs were analysed using the reaction cell of the ICP-MS to chemically enhance the signal by reacting the element with oxygen (e.g. to give SO^+), moving the detection away from a region of isobaric interference. Metabolite profiling of omeprazole was performed, with limits of detection of 800 pg of sulphur on column (an improvement of 100 fold in sensitivity from detection without oxygen). Similar studies with phosphorus containing drugs also showed a significant increase in sensitivity following reaction with oxygen compared to conventional analysis by ICP-MS.

The studies undertaken here demonstrate the significant potential of HPLC-ICP-MS as a contributor to the analysis of drugs and metabolites in the pharmaceutical industry.

Chapter 1

An introduction to DMPK and inductively coupled plasma mass spectrometry

1.0: Background

The pharmaceutical industry is required to conduct studies of the metabolic fate of potential drugs. The compound of interest is dosed into animals and subsequently blood, urine and bile samples are taken for analysis of parent and potential metabolites in order to establish the breakdown pathways and identify any toxic intermediates.

To do this, an easy traceable label must be attached to the parent drug so that its progress though the metabolic system can be determined. Traditionally, radiolabels (eg. ^{14}C) have been used for this, but the use of radiolabels is costly, ethically problematic and slows time to market.

Great advantage would be gained if the atoms within the drug molecule could be uniquely detected, preferably without labelling, but at least without the use of radioactivity. High performance liquid chromatography (HPLC) is the method of choice for separating metabolites in biological samples, while inductively coupled plasma spectrometry (ICP-MS) offers the potential for compound independent quantification. This project was therefore established to investigate the usefulness of HPLC-ICP-MS in the study of drug metabolism. ICP-MS is an instrumental analytical technique based on the use of a high temperature ionisation source (ICP) coupled to a mass spectrometer. Papers on ICP-MS started to appear in the 1970's; however commercial products did not appear until 1983 with the introduction

of the Sciex ELAN 250 and the VG PlasmaQuad [1]. Since then many instruments have been developed and marketed by numerous manufacturers utilising different mass spectrometer (MS) types such as time of flight and quadrupole instruments. The technique has quickly developed in to the instrument of choice for routine ultra trace-element analysis, mainly due to the advantages of combining an ICP with a MS [2]. The ICP gives qualities such as multi-elemental capability, good precision, a wide linear dynamic range and is relatively free from chemical interference, which plagued techniques such as atomic absorption spectrometry [3]. The benefits of the MS as detector are simple spectra and low backgrounds, resulting in low detection limits. The ICP has one additional feature, which should ensure very simple spectra indeed: it firsts atomises and then ionises the sample. Ions extracted from the plasma into the MS are therefore expected to produce a spectrum, which is elemental, consisting of as many peaks as there are stable isotopes for each element present. This means that a sample containing all the elements from the periodic table would result in a total of a few hundred peaks, which is quite a contrast to a technique such as ICP-AES where a single element such as Fe and U can emit thousands of peaks.

The present project was designed to gauge how the ICP-MS could be utilised within a drug metabolism and pharmacokinetics department (DMPK). Commonly the mass spectrometers found within the laboratory of a DMPK department are quadrupole or time of flight instruments with electrospray ionisation (ESI) or atmospheric pressure ionisation (APCI) sources used for detection of molecular ions or large fragments. For quantitative analysis in pharmacokinetic studies within DMPK, triple quadrupole instruments have mostly been used. These instruments revolutionised the industry in the early 1990s, enabling analysts to obtain shorter run times, better selectivity and sensitivity than with more conventional methods of detection such as UV absorbance. The gains in sensitivity made the instruments the industry

standard for quantitation. The quandrupole time of flight or triple quadrupole MS were the usual system of choice for qualitative metabolite identification studies within DMPK. The ability of the instrument to provide accurate mass information made it a useful tool in metabolite structure determination when used with identification software.

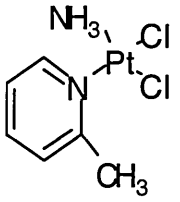


Figure 1.1 The structure of the platinum-containing anti cancer drug.

ICP-MS became an interest within the DMPK department when a problem in the analysis of an anti-cancer drug, similar to cis-platin, was encountered (Figure 1.1). The normal mode of detection, triple quadrupole mass spectrometry, in this case proved not to be the best mode of detection since the molecular ion of the compound could not be isolated [4]. With advances in the coupling of the ICP-MS to high performance liquid chromatography systems the instrument became capable of detecting low amounts of platinum eluting from a chromatographic system and therefore the combination became of interest within DMPK. Other characteristics of the instrument, such as its wide linear range made it a real alternative for the detection of platinum [5]. Because platinum anti-cancer drugs were relatively uncommon within the department, the instrument was also evaluated for the detection of elements more frequently found in drugs such as the halogens, sulphur, phosphorous and carbon [6-11].

The unique capability of ICP-MS in giving an accurate quantitative value for a particular element when related to a standard independent of structure is of great interest for the detection and quantification of drug metabolites. Once the metabolites have been separated chromatographically then they can be quantified against an external standard, to gain a metabolite profile, due to the efficient atomisation and ionisation of the ICP source. Normally such work is carried out using a radio-labelled compound which has been produced and dosed to follow the compound though the animal model. The use of a stable isotope within the structure of the compound enables the type of work to be carried out earlier in the discovery process before the radio-labelled compound becomes available. Clinical studies, where the radiolabel would normally be dosed to human volunteers, also became easier because of the omission of radioactivity [12].

1.1: Why ICP-MS ?

ICP-MS is of interest as a mode of detection for the analysis of drugs and metabolites within DMPK departments predominately due to the instrument's ability to give quantitative information based on an element contained within its structure, but independent of the structure. ICP-AES would also give this information, but was not considered because of the complicated spectrum gained, the generally lower sensitivity, and the inability to distinguish between isotopes. Given the complex mixtures in biological samples, the separation method is important. Gas chromatography (GC) can give high resolution separations, but most metabolites need derivatisation for GC because of their low volatility. The compounds under investigation are compatible with liquid chromatographic techniques without derivatisation.

Liquid chromatographic separation processes are the preferred choice in metabolite profile studies as well as in quantitative analysis. The modes of detection associated with LC are numerous such as MS, UV, and fluorescence. However, the technique of ICP-MS can obtain quantitative data without identical standards being required. Radio-flow counters do show this quality, but the synthesis required is expensive. The advantage of not having to change the molecule and use an element within the structure is not only financial but also practical because of the regulatory constraints on radio-labelling and the safety and disposal problems associated with radioactive compounds.

1.2: The inductively coupled plasma as an ion source

The ICP torch was first developed in the early 1960s S. Greenfield and V. A. Fassel [13]. The flame like discharges, which can reach up to temperatures of 10,000 K, are formed in a stream of argon flowing though a strong radio frequency electromagnetic field [14].

The ICP gas flows though three concentric tubes, which are assembled together in what is commonly referred to as the plasma torch (Figure 1.2). The flowing gas in the outer tube (commonly in the range of 13-17 L/min) is referred to as the cooling gas, with its role to ensure that the high temperature of the plasma does not melt the torch. It also has a secondary role in that it gives the distinctive shape to the plasma. The gas flowing in the middle concentric tube (commonly in the range of 1 L/min) is known as the plasma gas. It is this flow of gas, which is ionised to form the plasma. The inner tube gas flow is known as the carrier gas and is used to punch the plasma whilst carrying an aerosol or particulate form of the sample to be analysed.

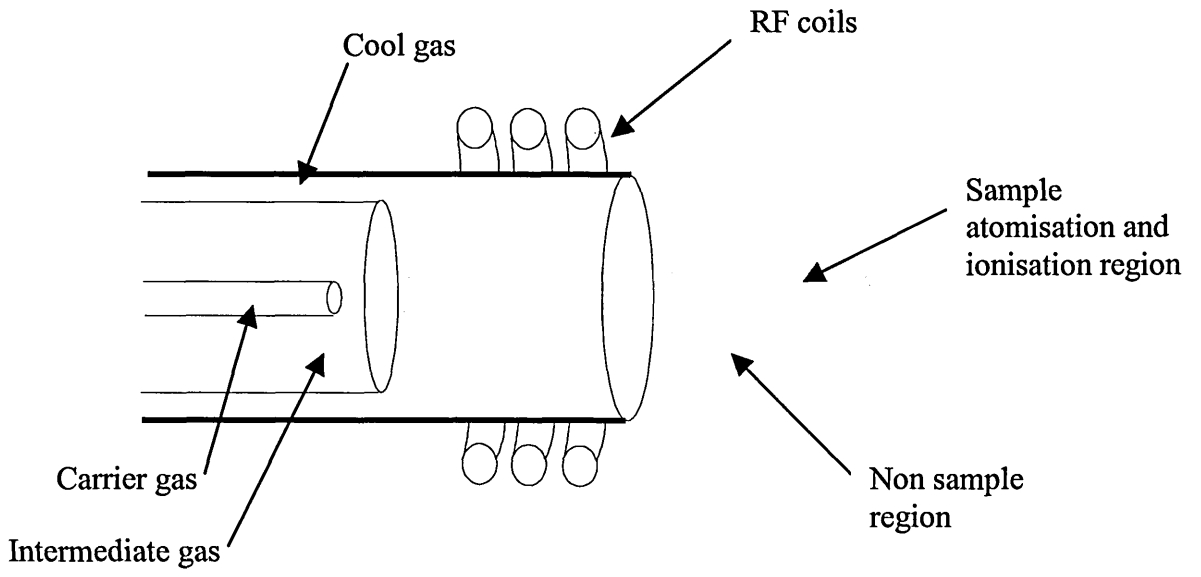


Figure 1.2 Schematic drawing of the ICP torch. Diagram used with the permission of GV Instruments [80].

A coil with a radio-frequency current flow encircles the torch to create an electromagnetic field in which the argon flows. A high voltage discharge spark is passed though the argon in the electromagnetic field to initiate the plasma. Electrons from the discharge gain energy which they transfer though collisions to argon atoms in the gas, thereby ionising them. This creates sets of ion-electron pairs that are in turn energised by the electromagnetic field and which take part in a cascading process of energy transfer from the coil to the gas. The result of this is a steady state plasma that is maintained as long as the radio-frequency current is maintained at a sufficient intensity and the gas flow is uninterrupted. The fire-ball like argon plasma is characterised by a bluish white discharge, which is a combination of emissions from the line spectrum of atomic argon and the continuous spectrum from ion-electron re-combinations taking place within the plasma.

When the plasma is formed the shape is that of a prolate spheroid, however the rapid expansion and acceleration of argon gas inside makes the introduction of sample difficult. This is achieved by punching a central channel inside the plasma thereby changing the shape of the plasma to the form of a doughnut with the outer plasma gas shielding the inner carrier gas so that virtually no mixing taking place between the two. As a result sample can be introduced in a gaseous or aerosol state along this central channel without disturbing the plasma or changing its composition. Whilst in the channel the sample will be desolvated, atomised, excited and ionised. Chemical species injected into the plasma will be broken down into their constituent elements, which are then ionised independently of their original form with efficiencies depending mainly on their ionisation energies. This can be predicted by the Saha equation [2].

Saha Equation

$$\frac{[M^+]}{[M]} = \frac{2}{n_e}\left(\frac{2\pi m_e kT_e}{h^2}\right)^{3/2}\frac{Q^+}{Q^0}\exp\left(-\frac{IP}{kT_{ion}}\right)$$

where:

$[M^+]$	=	the population of positively charged ions of the element M
$[M]$	=	the population of atoms of the element M
n_e	=	the electron number density in the plasma
m_e	=	the mass of the electron
h	=	Planck's constant
T_e	=	the free electron temperature
Q^+	=	the electronic partition function of the ion
Q^0	=	the electronic partition function of the atom
IP	=	the ionisation potential of the element
T_{ion}	=	the ionisation temperature

Most of the elements within the periodic table will be efficiently converted into singly charged ions. Figure 1.3 shows that the efficiency of ionisation decays as the ionisation energy of the element approaches that of argon (15.8 eV). This means that elements such as F, Ne and He are poorly ionised and the technique cannot be used for the analysis of these elements.

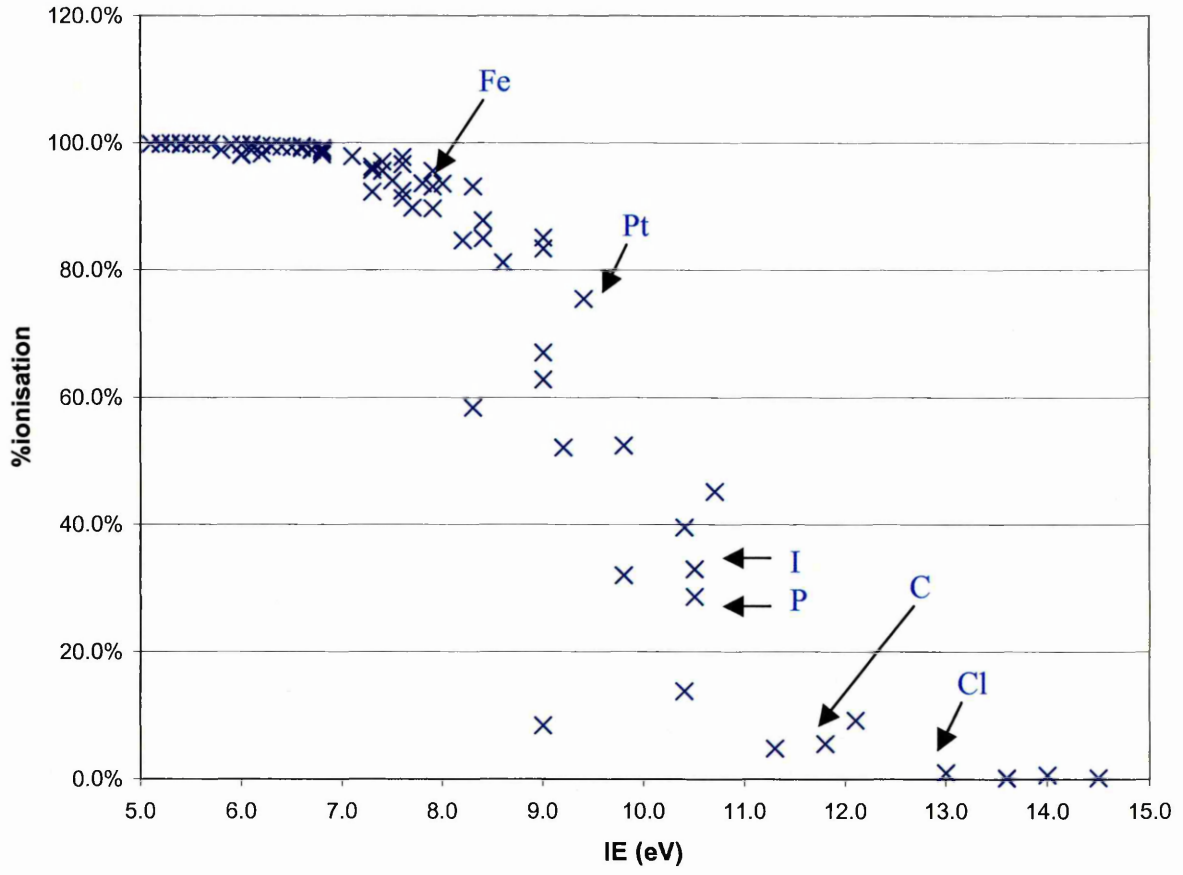


Figure 1.3 Efficiency of ionisation of elements in an ICP torch, with the elements in the study marked as a function of their ionisation energy [2].

1.3: Interfacing the Plasma Torch to the vacuum system

Interfacing the ICP torch to the MS requires transition from atmospheric pressure to a low operating pressure in order to avoid high voltage discharges and to ensure that the mean free path of ions is long enough to avoid collisions with the background atmosphere in the system [15]. The ICP-MS plasma is interfaced to the MS via a series of pumped chambers linked by extraction cones with small apertures. The design, shape and size of the apertures is important in the definition of the analytical performance for the instrument. Common to all ICP-MS systems is the presence of sampler and skimmer cones. The sampler is the interface between the plasma and the first vacuum chamber and therefore is subject to high temperatures since the plasma impinges directly on its surface. The sampler requires efficient cooling and therefore is made of material that has both good heat resistance and is an efficient thermal conductor. The most common material used is nickel, although depending on the application of interest Al, Cu and Pt have been used for this purpose. When the plasma makes contact with the surface of the sampler cone it cools down rapidly, forming a boundary layer. In this area ions recombine forming molecular species, such as $ArCl^+$ and ArO^+ that are not normally observed in bench chemistry. These types of molecular ions cause interferences and high backgrounds if they enter the mass analyser, so it is important that the opening aperture is designed to take ions from the central region only.

After entering the first vacuum chamber the sampled gas, which at this stage consists of ions, atoms and electrons, expands rapidly. The role of the skimmer cone is to ensure that a representative population of mixture is extracted into the next stage. To achieve this the skimmer cones are designed to have sharper edges and smaller apertures than the sampler cone (Figure 1.4).

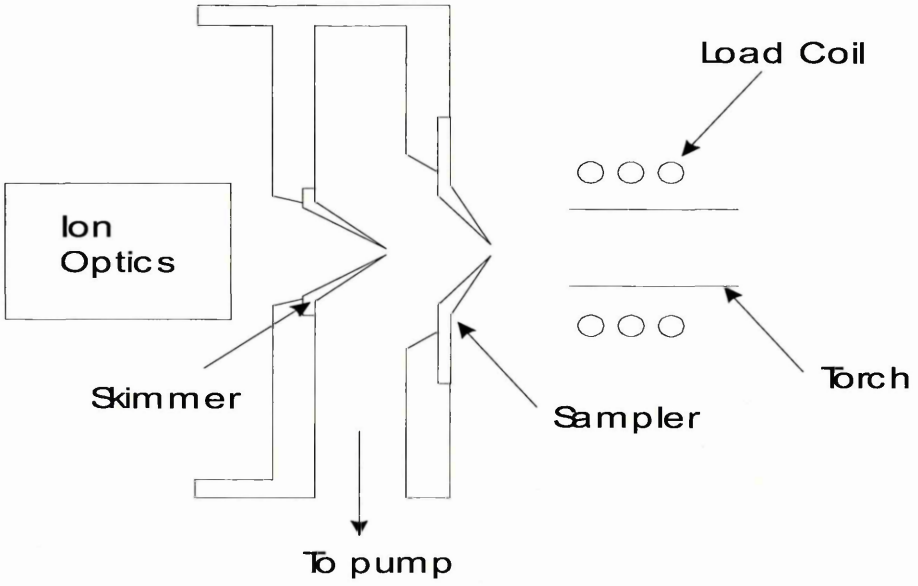


Figure 1.4 Schematic of an ICP-MS interface. The sampled region of plasma expands behind the sampler whilst a representative portion flows through the skimmer into the ion optic region. Diagram used with the permission of GV Instruments [80].

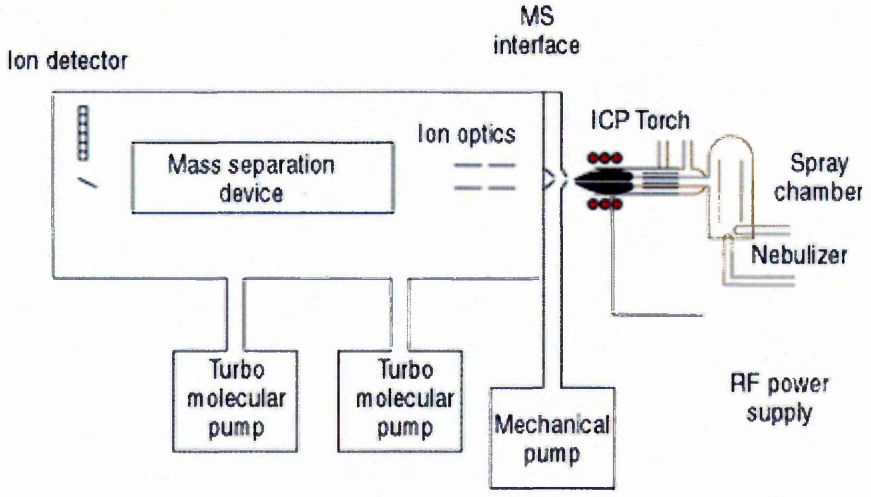


Figure 1.5 The building blocks of the ICP-MS instrument. Diagram used with the permission of GV Instruments [80].

1.4: Sample introduction into ICP-MS

The first block of an integrated ICP-MS instrument is the sample introduction device. This is a well researched component and is often considered the problem area of the technique. It is crucial that the liquid delivery device delivers a representative portion of the sample. The common unit for this process consists of a pump, a nebuliser and a spray chamber. The pump ensures a consistent flow of the sample to the nebuliser, with another pump removing waste from the spray chamber. The nebuliser is used to convert the stream of liquid sample into a fine aerosol and the spray chamber acts as a trap that filters out the larger droplets. This process is not particularly efficient, with only about 2 % of the sample reaching the plasma due to the varied size of the droplets. This efficiency is dependent on the type of nebuliser, however, and much work in this area is on going [16-17]. If 100 % of the sample is required to reach the plasma then inlet sources such as ultrasonic transducers can be utilised [18]. However, if a large amount of solvent is introduced to the plasma, the plasma may quench or change characteristics. This problem has been overcome by introducing a desolvating stage prior to the aerosol reaching the plasma, or alternatively a low flow rate to deliver the sample. High efficiency low flow rate nebulisers have been used widely in the past few years. The efficiency of these nebulisers in producing an aerosol with small size droplets is extremely high, however, since the sample consumption rate is low, there is little overloading of the plasma or gain in sensitivity.

The stability of the sample introduction source is also an important consideration. To gain good quality data the instrument must be stable over the short term as well as the long term. Short term instability is usually caused by the pulsating nature of the nebuliser source. This gives the spray chamber a secondary role in smoothing this effect out. Long-term stability

issues can be caused by partial blockages of the nebuliser and by physical parameters that affect the performance of the spray chamber such as temperature and surface wetting. Making the right choice of nebuliser for the application can reduce blocking. When dealing with clean samples and solvents the standard concentric nebuliser would be the correct choice since it is possible to match these to the flow rate used. With samples, and solvents, containing high levels of salts cross flow nebulisers are recommended since they are more tolerant of these systems. For samples with suspended particulates a V-groove type of nebuliser is recommended, but when these are used a reduction in sensitivity is observed [2].

The use of thermally stable spray chambers is necessary to avoid long-term drift. A thermoelectric Peltier cooling device can be used, or a jacket around the spray chamber with a continuous flow of cooling fluid from a chiller unit. In addition, cooling units are used to minimise drift associated with selectively reduced plasma loading from volatile solvents such as alcohol by maintaining the spray chamber temperature between –5°C and –10°C to condense the solvent.

1.5: Passage of Ions though the ICP-MS

As described earlier, the aerosol passing though the spray chamber is ionised in the ICP and introduced into the vacuum through an interface. After the interface the ions are guided into the mass spectrometer by a series of focusing lenses. These are electrostatic lenses and are designed to steer the ions without changing the composition of the ion beam exiting the skimmer. This process is difficult since the beam is positively charged and therefore the ions have a tendency to repel each other, which is known as the coulombic effect. This process is combated by accelerating the ions or by making use of a number of focusing lenses.

An additional use for the ion optics is to stop neutral species and protons reaching the detector, however, recently this has been prevented more efficiently by using reaction / collision cells.

The collision cell has an integral role in limiting spectral interferences. These interferences are predominantly ions generated from the argon gas, the solvent or the sample matrix. Figure 1.6 shows examples of the ions produced, indicating that for the determination of elements such as Fe the spectral interference $^{40}Ar^{16}O$ would have an effect on the sensitivity. The cold / cool plasma approach, which uses lower temperatures to reduce the formation of the interference, is one way around the problem. The low power results in less interference products forming [2]. However, this approach is time consuming for optimisation and does not take into account many of the elements of interest. Collision / reaction cells were developed in the late 1990's to deal with this problem [19]. Originally these were designed for mass spectrometers to generate product ion species to aid identification of precursor ions. When the technology was used in ICP-MS the collision cell was found to limit the formation of argon-based spectral interferences [20-22]. The ions enter the interface in the normal manner, from where they are extracted into an off-axis collision cell under vacuum. A gas such as hydrogen or helium is introduced into the collision cell, which is normally a hexapole or octapole, operated in rf-only mode. The rf-only mode acts as an ion focusing device, allowing collision and reaction with interferences, by a number of different mechanisms. These are predominantly ion-molecule reactions, with polyatomic interfering ions like $^{40}Ar^{16}O^{+}$, and $^{38}ArH^{+}$ converted to harmless non-interfering species. The analyte ions, free from interferences, then emerge from the collision cell into a quadrupole analyser for mass

separation (Figure 1.7). This technique removes most of the polyatomic interferences found in the system and shows advantages over the cool flame technique [23-24].

Another device for removing interferences is the dynamic reaction cell (DRC) [25-26], which works in a similar manner to the collision cell. The main difference is that quadrupoles are used instead of hexapoles because of the greater stability gained, and reactive gases such as ammonia, methane or oxygen are bled in, with the cell acting as a catalyst for ion-molecule chemistry to take place. The mechanisms taking place are predominantly reaction based, rather than collisions, and the interfering molecules are converted into an ion dissimilar to the analyte mass, or to a harmless neutral species (Figure 1.8). An example of the reactions that can occur is $NO^+ + O_2 \rightarrow NO_2^+ + O$. The analyte is then separated in a similar manner as before by an analyser quadrupole.

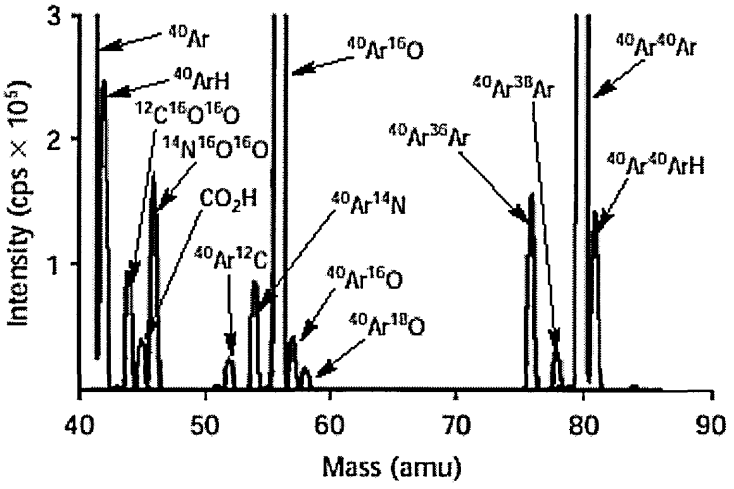


Figure 1.6 Mass spectrum from deionized water showing mass 40 to 85. The ions present represent interferences, which will reduce sensitivity of the species under investigation at the same mass. Taken from ref [20].

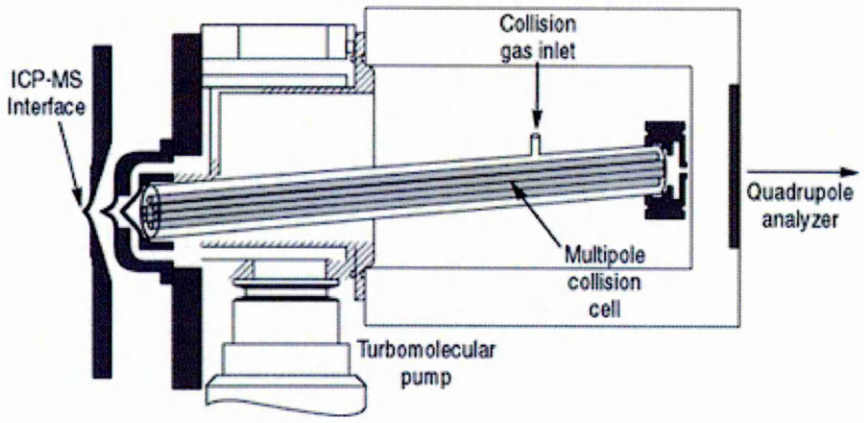


Figure 1.7 Multipole collision / reaction cell as used in GV instrument technology. Diagram used with the permission of GV Instruments [80].

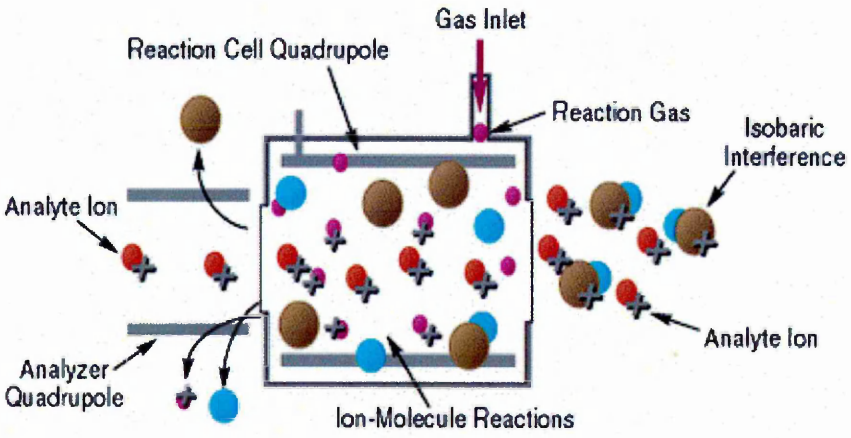


Figure 1.8 Principles of operation of a dynamic reaction cell (DRC). Diagram used with the permission of Perkin Elmer [25].

1.6: Coupling a HPLC system to an ICP-MS

The coupling of an HPLC system to the ICP-MS detector was an integral part of the project. The actual coupling of the HPLC system to the ICP-MS is not difficult, since the flow rates by the HPLC system (1 μL/min to 2-3 mL/min), are directly compatible with the sample introduction rates of the ICP infusion system. The first instance of this type of work was in the early 1980's when HPLC-ICP-MS was to identify elemental species [27].

The process of coupling the HLPC to the ICP-MS may be easy in principle, but the reality is somewhat different. There are a number of published reviews considering the problem of coupling chromatography to ICP-MS [28-30]. The main problem with such a process is associated with the mobile phase. The high levels of salts used in most ion chromatography mobile phases cause blockages at the nebuliser tip, cone apertures and other nebuliser source such as the ultrasonic nebuliser. Mobile phases containing high levels of organic solvent (such as those used in reversed phase HPLC) cause rapid blockages of the cones due to the deposition of carbon. In order to address these issues various techniques have to be employed to overcome the problems. Salt problems can be overcome by making use of on line dilution step or an exchange membrane, whereby Na, K and Ca ions are exchanged with hydrogen ions. The main problem associated with this work, however, is how to handle the organic part of a reversed phase chromatographic system. Since most separations of metabolites are carried out with an organic modifier mixed with an aqueous component it is important to understand the problems. The organic part causes two problems. Carbon is produced in the plasma from the mobile phase due to its relatively high ionisation energy (11.3 eV) [31]. As a result, an lower ionisation energy compared to argon is found within the plasma flame, which results in large volume of carbon atoms in the plasma. These atoms tend to deposit on the

apertures of the sampler and skimmer cones, thereby causing blockages. The problem is overcome by the addition of oxygen, which is mixed with the argon carrier gas. The oxygen reacts with the carbon atoms to form volatile products. However, the addition of oxygen to the system has the effect of changing the electrical impedance of the ICP. Consequently this process can only be used if the instrument has computer controlled RF circuitry, which can adjust to the impedance shift. The second problem found was the cooling effect of the organic phase on the plasma. With high levels of organic solvent the electrical impedance can be affected and without the RF adjustment ICP operation is very difficult. To counter this problem the mobile phase flow rates have to be considered in the method development, and the amount of organic must be kept as low as possible, in order to achieve optimum conditions for the instrument. When operating with organic components the use of a sub-zero spray chamber (below -5°C) is necessary to desolvate the aerosol, thus limiting the amount of organic reaching the plasma. To aid desolvation, specialist nebulisers have been developed [32]. These nebulisers generate an aerosol which is then heated, passed though a chamber kept at sub zero temperatures, and finally passed past a desolvating semi-porous membrane with a counter flow of argon on the outside. Although these nebulisers provide an ideal way for linking HPLC to ICP-MS, volatile species can be lost through the membrane.

Although these difficulties exist in running the ICP-MS with a chromatographic system, advances in technology, as well as an understanding of ICP-MS processes have made this technique feasible. HPLC-ICP-MS was therefore looked at in detail to determine its usefulness to a DMPK department.

Chapter 2

A comparison of quantitative methods for analysis of the platinum- containing anticancer drug {Cis-[amminedichloro(2-methylpyridine)] platinum(II)}(ZD0473) by HPLC coupled to either a triple quadrupole mass spectrometer or an inductively coupled plasma mass spectrometer.

2.1: Summary

The use of high performance liquid chromatography coupled with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) as a means for the quantitative determination of ZD0473, a platinum anticancer drug, and its related biologically active "aqua" compounds in biofluid samples, is described. The performance of the resulting HPLC-ICP-MS method was compared with that of a conventional HPLC-triple quadrupole mass spectrometer based (HPLC-MSMS) system for properties such as limit of detection, linearity and reproducibility using spiked samples. The methods were then applied to the determination of plasma ultrafitrate concentrations of ZD0473 in dog plasma samples obtained following intravenous and oral administration at 0.5 mg/kg and 6 mg/kg, respectively. These experiments showed that both methods were capable of providing accurate and precise results but that the HPLC-ICP-MS method had advantages of extended linear range and superior sensitivity, providing a limit of quantification of 0.1 ng/mL for ZD0473 compared to 5 ng/mL using the current HPLC-MSMS method. In addition, by using a single

combined HPLC-ICP-MS/MSMS system it was possible to determine the relative MSMS response of the aqua compounds for the first time.

2.2: Introduction

ZD0473 {cis[amminedichloro(2-methylpyridine)]platinum (II)} is a new generation platinum compound designed to deliver an extended spectrum of antitumour activity and overcome platinum resistance [33-34]. The structures of ZD0473 and the related biologically active "aqua" compounds are shown in Figure 2.1. Traditionally the analysis of platinum drugs has relied upon atomic absorption spectrophotometry, ICP-MS or electrochemical detection [35-37]. However, in the absence of a separation these techniques do not provide an adequate process for a quantitative assay, due to the failure to distinguish between the compound, the active aqua species and inactive metabolites (including amino acid adducts) / degradation products. If such information is required then a chromatographic separation coupled to fraction collection and subsequent off-line determination of the platinum content can be performed, but this is labour intensive, time consuming and prone to error. Because of these practical difficulties the initial method developed for the determination of ZD0473 in biological samples was based on the HPLC-MSMS system developed by Oe et al [4], which enabled the compound to be quantified down to concentrations of 10 ng/mL in biofluid samples such as plasma ultrafiltrate and urine. However, the advent of robust HPLC-ICP-MS systems offers great potential for the analysis of metal-containing compounds in biological matrices [38-41] as illustrated by a recent example for a platinum-containing compound in human plasma [5]. An HPLC-ICP-MS based method for the analysis of ZD0473 and related materials was therefore

developed. This method has been compared to an in-house assay, based on the published HPLC-MSMS procedure [4], via the analysis of both spiked samples and samples derived from a dog study following the intravenous and oral administration of ZD0473 at 0.5 mg/kg and 6 mg/kg, respectively.

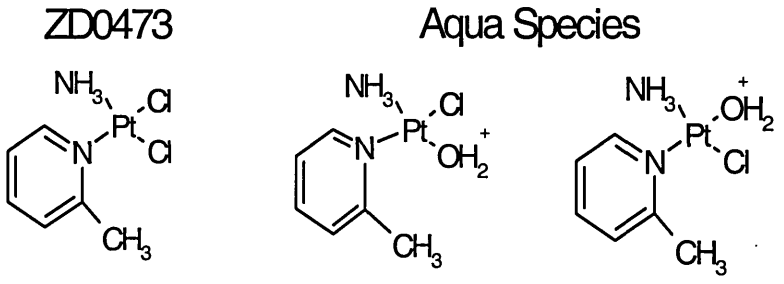


Figure 2.1 The structure of ZD0473 and the associated aqua species.

2.3: Experimental

2.3.1: Reagents

Chromatographic solvents together with formic and hydrochloric acids were purchased from Fisher Scientific UK Ltd (Loughborough, UK) and were of analytical or HPLC grade. Ammonium acetate and sodium chloride were obtained from BDH Ltd (Poole, UK) and were of analytical grade. ZD0473 was formulated as a sterile solution in 0.9 % sterile sodium chloride for intravenous administration, and a 50:50 w/w with lactose for oral administration, which was obtained from the Media Preparation Laboratory at AstraZenenca Pharmaceuticals (Alderley Park,

Macclesfield, UK). ZD0473 was supplied by AstraZeneca Pharmaceuticals (Alderley Park) and the deuterated internal standard, [$^2H_7$] ZD0473 (deuterated in the aromatic ring and methyl), was synthesised in the Isotope Chemistry Laboratory in the Dept. of Drug Metabolism and Pharmacokinetics at AstraZeneca Pharmaceuticals (Alderley Park).

2.3.2: Standard Solution Preparation

ZD0473 is light sensitive and care was taken when making up standard solutions for the two assays to avoid photo-degradation. Typically, 5 mg of ZD0473 was weighed out into an amber vial and 10 mL of 0.15 M sodium chloride was then added to achieve a concentration of 0.5 mg/mL. The presence of chloride ions stabilises ZD0473 by preventing the formation of the aqua species. Dissolution was obtained by stirring the sample for two hours at room temperature. The spiking standards were prepared by making the appropriate dilutions with sodium chloride (0.15 M) to provide standards of concentrations 500, 10, 1, 0.2 and 0.02 μg/mL. The initial solution was checked by UV spectroscopy at a concentration of 100 μg/mL and the data compared to previously generated data at this concentration to determine integrity of the preparation. The standard solution of the deuterated internal standard ([$^2H_7$]-ZD0473) employed in the HPLC-MSMS assay was prepared in the same manner and diluted with sodium chloride to give a final concentration of 5 μg/mL.

2.3.3: Intravenous and oral formulation of ZD0473 and sample collection

ZD0473 was formulated in physiological saline to a concentration of 0.5 mg/mL. ZD0473 was dissolved in 0.9% w/v sodium chloride by stirring overnight and then filtered through a 0.2 μm filter into a sterile vial. The formulation was then dosed intravenously as a bolus over 1 minute to a male beagle dog (12.8 kg) at a dose level of 0.5 mg/kg. Blood samples (4mL) were taken at 0.033, 0.083, 0.167, 0.25, 0.5, 0.75, 1, 2, 3, 6 and 12 hours post dose. The oral dose was formulated as a 50 % w/w solid mixture with lactose (90 mg) and dosed to a single male beagle dog (12.5 kg) at a level of 6 mg/kg. Blood samples were taken (4 mL) at 0.25, 0.5, 0.75, 1, 2, 3, 6 and 12 hours. On collection, blood samples were placed in lithium heparin tubes and centrifuged at 3000 rpm for 15 minutes to prepare plasma. Aliquots of these plasma samples were then taken (200 μL), placed into 6 Amicon Centrifree Filter devices (30,000 molecular weight cut-off, Millipore Corporation, Bedford, USA) and centrifuged at 3000 rpm for 30 minutes, at 4 °C to provide ultrafiltrates. The samples were then stored at -70 °C in glass vials wrapped in aluminium foil to protect the samples from photo-degradation until analysis (these conditions have been shown to provide stability for 5 months).

2.3.4: Preparation of standard curve and quality control samples.

The standards and quality control (QC) samples were prepared by spiking control dog plasma ultrafiltrate with standard solutions to produce the required concentrations. The standard curve prepared for the HPLC-MSMS assay contained samples spiked at 5, 10, 50, 200, 600, 1000, 2500, 5000, 7500 and 10000 ng/mL, respectively, whilst

that for the HPLC-ICP-MS method had additional standards at 0.1, 0.5 and 1 ng/mL. The QC samples for the HPLC-MSMS and HPLC-ICP-MS assays were prepared at 5, 100, 1000 ng/mL and 0.1, 0.5, 5, 1000, 10000 ng/mL, respectively and stored at –70°C in glass vials wrapped in aluminium foil until required.

2.3.5: Sample preparation.

Aliquots of the standards, QCs and samples (0.1 mL in each case) were placed into glass tubes (12 x 75mm). To these 25 µL of 0.15 M sodium chloride in 0.1 M hydrochloric acid was added, and the samples were then made up to 200 µL with 0.15 M sodium chloride. For the HLPC-MSMS method 25 µL of the internal standard solution (5 µg/mL) was added before the sample was made up to 200 µL. The mixtures were vortex mixed for 20 seconds and then transferred to HPLC vials ready for injection. The addition of hydrochloric acid was designed to ensure that the equilibrium favoured ZD0473 rather than the aqua species in the presence of the ultrafiltrate. The samples were transferred to autosampler vials and then placed in the autosampler and kept in the dark prior to injection. Blanks were prepared as above to check there were no interfering peaks in the chromatograms.

2.3.6: Chromatography

An isocratic reversed-phase HPLC method was used for both ICP-MS and the MSMS assays based on a mobile phase of methanol / water (20:80 V/V) containing 0.1 % formic acid and 0.15mM ammonium acetate (pH 3). The column used was a Phenomenex Synergi polar RP 150 x 4.6 mm (Phenomenex, Macclesfield, UK)

operated at ambient temperature with a flow rate of 1 mL/min, which give a run time of 7 minutes. An injection volume of 50 μL was used in the assays.

2.3.7: HPLC-ICP-MS

Chromatography for ICP-MS was performed using a Jasco PU-1580 HPLC system (Jasco Ltd, Great Dunmow, UK) with samples introduced via a PE 200 autosampler (PerkinElmer Ltd, Beaconsfield, UK). The injector was flushed using a methanol / water solution (1:1). ICP-MS was performed on a Platform ICP-MS, which uses a hexapole collision / reaction cell for simultaneous measurement of the platinum isotopes (Micromass, Wythenshawe, UK). The eluent from the column was introduced to the ICP-MS via either an ultrasonic nebuliser U-6000AT (Cetac Technologies, Omaha, Nebraska, USA) or a Meinhard concentric nebuliser coupled to a double pass spray chamber (Micromass, Wythenshawe, UK). MassLynx software (version 3.4) was used for instrument control, data acquisition and data handling. Table 2.1 shows operating and acquisition conditions of the ICP-MS including the flow of the nebuliser gases for sample introduction as well as an argon oxygen mix (95:5 V/V), which is used to reduce carbon build up from mobile phase combustion in the plasma.

Table 2.1 Instrument operating conditions for the Platform ICP-MS

Cooling gas	16.00 L/min	Plasma Power	1350 w
Plasma gas	0.65 L/min	Acquisition mode	SIR
Nebuliser gas	0.75 L/min	Dwell time	200 ms
Helium gas	5 mL/min	Masses monitored	195
Hydrogen gas	5 mL/min	Argon/Oxygen (95/5 %)	0.2 mL/min
Total analysis time	7 min		

2.3.8: HPLC-MSMS

Chromatography for HPLC-MSMS was performed using a PE 200 series pump (PerkinElmer Ltd, Beaconsfield, UK) with samples introduced via a CTC Analytics autosampler (Presearch, Hitchin, UK). The injector was flushed using a methanol / water solution (1:1). For quantification by MSMS an API-3000 mass spectrometer (Applied Biosystems, Warrington, UK) with a turbo ionspray inlet source was used for the multiple reaction monitoring of ZD0473 and its aqua species. Analyst software (version 1.2) was used for the instrument control, data acquisition and data handling. The operating conditions for the instrument are shown in Table 2.2.

Table 2.2 Instrument operating conditions for API-3000 MSMS

	Ion Transformation		
Component	Q1	Q3	Dwell time (mSec)
ZD0473	393.4	303.5	150
ZD0473D6	400.0	310.2	150
Aqua Species	358.0	303.5	150

2.3.9: HPLC-ICP-MS/MSMS

To evaluate the combination of HPLC-ICP-MS/MSMS, and enable the relative response for the aqua species by MSMS to be determined, the chromatographic

system used for HPLC-ICP-MS described above was taken and the effluent from the column split using a splitter valve (600; Jasco Ltd , Great Dunmow, UK), positioned directly after the outlet of the column; this directed half of the flow (0.5 mL/min) to the HPLC-MSMS and the remainder (0.5 mL/min) to the HPLC-ICP-MS. PEEK tubing (0.005" bore) was used to connect the instruments and the lengths adjusted to give similar retention times for ZD0473 in both systems.

2.4: Results and Discussion, Assay Development and Validation

2.4.1: Chromatography

ZD0473 is found in biological fluids together with a mixture of the pharmacologically active aqua species (structures in Figure 2.1). As the HPLC-ICP-MS method is based on the detection of platinum a chromatographic separation of ZD0473 from these aqua species was required to ensure specificity. Similarly, the use of MSMS for quantification necessitated the separation of the two aqua components as the same ion transformations are seen for both aqua species. The HPLC separation that was developed, based on reversed-phase chromatography with a methanol-ammonium acetate / formate (20:80) buffered eluent, provided this separation and was also compatible with both modes of MS detection [28, 42]. The formic acid and ammonium acetate components of the mobile phase were primarily there for the benefit of the HPLC-MSMS system with formic acid added to aid the ionisation of the compounds in the turbo ion spray source, whilst the ammonium acetate was present in order to produce the ammonium adducts required for MSMS detection (Figure 2.2). For HPLC-ICP-MS methanol-water 20:80 alone would be suitable for

chromatography, but a common mobile phase was used here to aid comparison of the two detection methods.

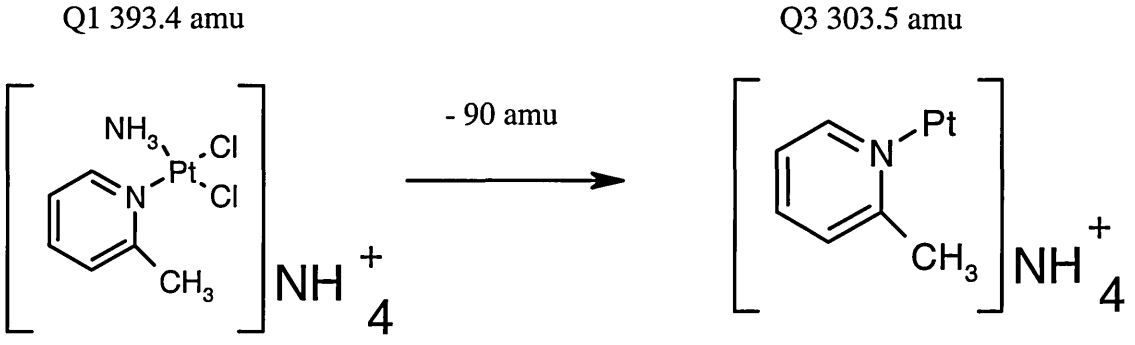


Figure 2.2 The multiple reaction monitoring transition for the HPLC-MSMS system with the mass 393.4 to 303.5 with a loss of 90 amu in the collision cell.

2.4.2: HPLC-ICP-MS

Having obtained a suitable chromatographic separation the development and validation of an HPLC-ICP-MS-based assay for ZD0473 was undertaken. For this, the various argon gas flows were optimised to produce the best sensitivity for platinum in the ICP-MS and the hydrogen and helium collision cell gases were set to produce the lowest background [20, 43] as detailed in Table 2.1. For successful HPLC-ICP-MS, efficient removal of the organic modifier prior to the introduction of the sample as an aerosol into the plasma is important. Various devices are available to achieve this [24] and in this study two types of nebuliser were investigated, namely

an ultrasonic nebuliser and a concentric nebuliser with a double pass spray chamber. The latter was water-cooled to -4°C. The ultrasonic nebuliser is far more efficient than the concentric nebuliser in producing an ultra fine aerosol, which is desolvated prior to its introduction into the ICP plasma. This increased efficiency leads to an increased sensitivity (5 pg on column limit of detection) and low backgrounds, but at the expense of linear range due to losses of the analyte at high concentration in the desolvating step. However, in use it has proved to be more time consuming to set up and was found to be less reliable than the concentric nebuliser / double pass spray chamber combination. The latter provides an extended linear range (but with higher background levels) in comparison with the ultrasonic nebuliser, and has also proved to be more robust. The higher backgrounds observed with the concentric nebuliser result from its poorer efficiency, compared to the ultrasonic nebuliser, in removing the organic solvent from the mobile phase. However, this combination still offered a limit of quantification of 0.1 ng/mL (10 pg on column) at a signal to noise (S/N) of 10, combined with a linear range extending over 8 orders of magnitude. Because of the extended linear range afforded by this method of sample introduction, further method development employed this nebuliser. If greater sensitivity was to be required and reduction in the calibration range was acceptable, then the increased set up time for the ultrasonic nebuliser would become worthwhile. Examples of the calibration curves for HPLC-ICP-MS using both nebuliser systems are given in Figure 2.3, with representative chromatograms obtained using the concentric nebuliser illustrated in Figure 2.4. The equation of the calibration line for the concentric nebuliser was $y = 653.5x + 335.7$ which was calculated by least squares linear regression for a range of 0.1 – 10000 ng/mL.

Based on these conditions using the concentric nebuliser the method was validated for spiked plasma samples over the range 0.1 to 10000 ng/mL. The results, shown in Table 2.3, clearly indicate that the method is suitable for use over the concentration range examined. The excellent accuracy and precision data obtained, in the absence of an internal standard, are noteworthy. No reduction in response throughout the course of the analysis was noted, as shown by QC sample sets at the start and finish of the individual runs. The limit of quantification of the method using these conditions was 0.1 ng/mL (10 pg on column), using arguments based on either a S/N of 10:1 or the precision being better than 20%. A limit of detection based on a criterion of the signal to noise ratio being 3:1 would allow the detection of ZD0473 at 0.05 ng/mL.

Table 2.3 Accuracy and precision data for HPLC-ICP-MS on spiked ultrafiltrate samples over the range 0.1 to 10000 ng/mL (n=6). For experimental conditions see section 2.3.

Quality control samples	mean (ng/mL)	accuracy (%)	coefficient of variation (%)
LLQ (0.1 ng/mL)	0.101	101	19.9
LQC (0.5 ng/mL)	0.47	95	9.3
MQC (5 ng/mL)	4.6	92	1.8
HQC (1000 ng/mL)	913	91	2.5
UQC (10000 ng/mL)	9949	99	3.2

LLQ Lower Limit of quantification

LQC Lower Quality of Control

MQC Middle Quality of Control

HQC Higher Quality of Control

UQC Upper Quality of Control

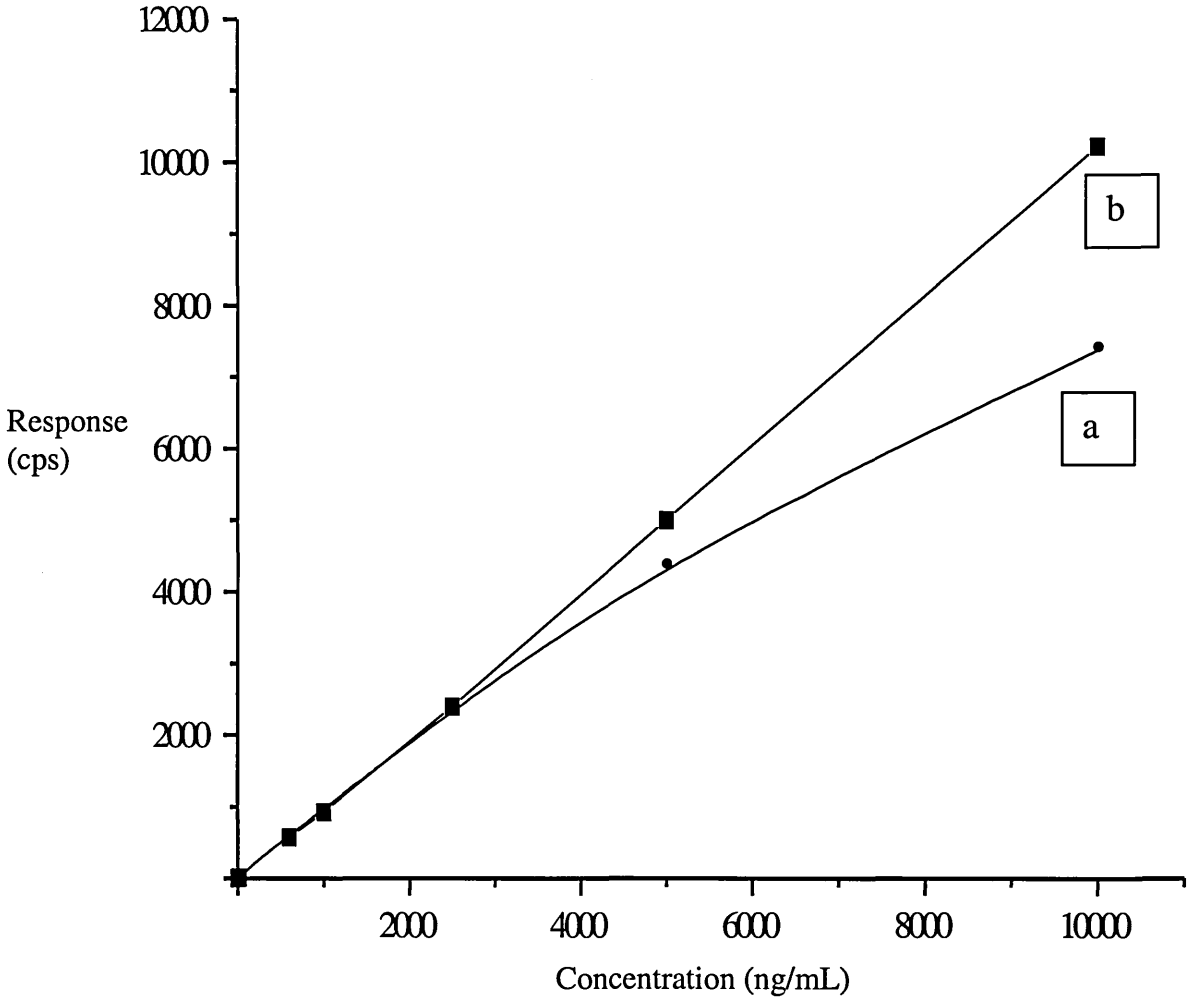


Figure 2.3 Examples of the calibration curves produced in the determination of platinum by ICP-MS using two types of nebulising sources a) Ultrasonic nebuliser showing saturation after 1000 ng/mL and b) Meinhard nebuliser with a double pass spray chamber showing extended linear range. For experimental conditions see section 2.3.

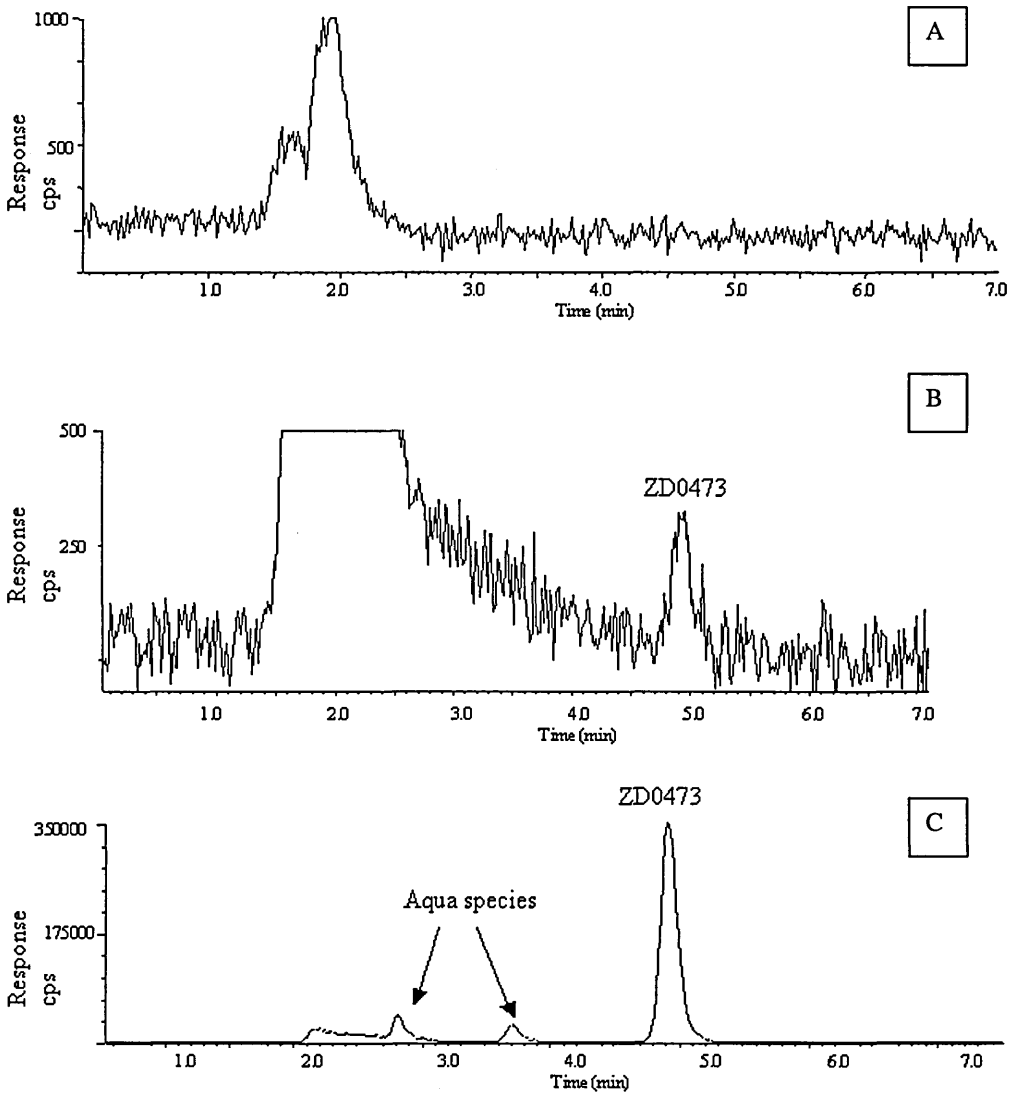


Figure 2.4 Example chromatograms for HPLC-ICP-MS monitored separation of platinum at *m/z* 195. Chromatogram A) represents a blank ultrafiltrate sample, B) represents the Limit of detection at 10 pg on column of ZD0473 and C) a intravenous sample taken at 0.5 hour after a dose of 0.5 mg/kg to a male dog. For experimental conditions see section 2.3.

2.4.3: HPLC-MSMS

ZD0473 is not a particularly good candidate for HPLC-MSMS because the compound could not be isolated in a Q1 scan due to decomposition in the source. The method developed by Oe et al [4] overcame this problem by making use of the presence of ammonium acetate in the mobile phase to produce an ammonium adduct, which was then fragmented to form product ions, one of which was then used in the multiple reaction monitoring transition (Figure 2.2).

For tuning purposes on the API-3000 mass spectrometer to achieve the required sensitivity, the resolution setting in the Q1 region was changed from unit resolution (peak width 0.7 ± 0.1 amu) to low resolution (peak width 0.8 ± 0.1 amu) with peak width determined at 50 % height of peak. This process allowed more ions through to the Q3 region, thereby increasing sensitivity, albeit at the expense of reducing the selectivity of the assay. The linear range of the assay was, however, limited to 5 – 1000 ng/mL with the equation of the calibration line given by $y = 0.00136x + 0.00101$ calculated by least squares linear regression. Above 1000 ng/mL the assay showed signs of saturation and the coefficients of variation were unacceptably large. Typical mass chromatograms are illustrated in Figure 2.5. These conditions were used to develop an HPLC-MSMS method for ZD0473 over the range 5 to 1000 ng/mL. The resulting data are summarised in Table 2.4. These results show the method to be suitable for the analysis of ZD0473 with a limit of quantification of 5 ng/mL, corresponding to an on column loading of 125 pg. This limit of quantification was calculated based on a signal to noise ratio of 10:1. Using a signal to noise ratio of 3:1 would result in a limit of detection of ca. 2 ng/mL.

The method, as developed, has proved to be robust in practice and has been used to examine numerous samples from animal studies.

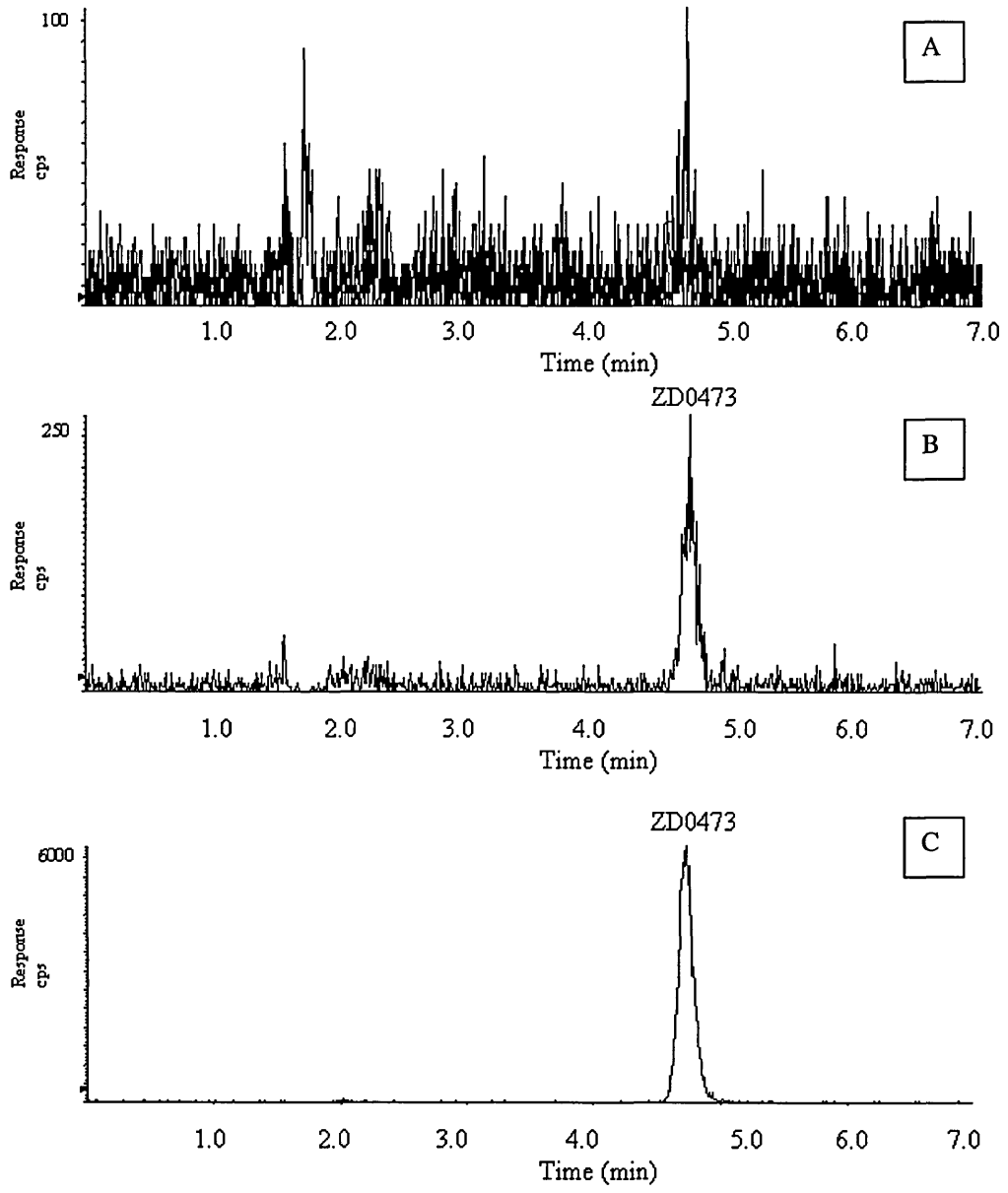


Figure 2.5 Example chromatograms obtained from the HPLC-MSMS assay of ZD0473 with MRM transition 393 to 303 amu. Chromatogram A) represents a blank ultrafiltrate sample, B) represents a 5 ng/mL standard of ZD0473 and C) a intravenous sample taken at 0.5 hour after a dose of 0.5 mg/kg to a male dog. For experimental conditions see section 2.3.

As indicated above, the HPLC-ICP-MS method was able to provide accurate and precise results in the absence of an internal standard. For comparison, the data from the HPLC-MSMS method was evaluated without taking the internal standard into account to see if a similar approach could be adopted. However, this analysis showed that there was a gradual decrease in the response of the MSMS during the course of the run of 150 samples, with a difference of ca. 24% for QC samples analysed at the start compared to those at the end of the batch. A build up of deposits on the source probably caused this effect, so reducing the response of the HPLC-MSMS. The use of a suitable internal standard is, therefore, mandatory for the assay of ZD0473 by HPLC-MSMS.

Table 2.4 Accuracy and precision data for LC-MSMS (n=6)

Quality control samples	mean (ng/mL)	accuracy (%)	coefficient of variation (%)
LLQ (5 ng/mL)	5.2	104	12.3
MQC (100 ng/mL)	109	109	5.3
HQC (1000 ng/mL)	1076	107	5.4

Abbreviations as described on page 30

2.4.4: Application of HPLC-MSMS and HPLC-ICP-MS for the determination of ZD0473 in dog plasma.

In order to enable a comparison of the two methods to be performed on study-derived rather than spiked samples, both the HPLC-MSMS and the HPLC-ICP-MS methods were used to analyse plasma ultrafiltrate obtained following intravenous

administration of ZD0473 at 0.5 mg/kg to a single dog. The data obtained using both methods are graphically represented in Figure 2.6 with the corresponding QC data in Table 2.5. Peak observed plasma concentrations were a little over 2000 ng/mL, falling rapidly to approximately 10 ng/mL by 3 hr post dose. Using HPLC-ICP-MS the concentrations of ZD0473 in the 6 and 12 hr post dose samples could be determined (2.0 and 1.4 ng/mL, respectively). HPLC-MSMS was, in contrast, unable to provide quantitative data for these samples. For those samples where both methods were able to provide results the correlation between the two sets of data was good, even though a small bias was observed. The HPLC-MSMS method generally shows a 10-20 % higher value compared to the HPLC-ICP-MS method. However, when the data was put though statistical analysis (see below), where QC (validation study) and intravenous study sample data were included, the methods were shown to be equivalent as discussed in section 2.4.5.

Table 2.5 Quality control data for both HPLC-MSMS and HPLC-ICP-MS obtained during the analysis of plasma samples from a single dog dosed intravenously at 0.5 mg/kg with ZD0473.

QC Levels (ng/mL)	5	10	400	2500
HPLC-MSMS QC set 1	4.5	9.0	419	2670
HPLC-MSMS QC set 2	4.3	8.8	393	2490
mean	4.4	8.9	406	2580
accuracy (%)	88	89	101	103
HPLC-ICP-MS QC set 3	5.6	9.1	321	2700
HPLC-ICP-MS QC set 4	5.6	9.3	320	2568
mean	5.6	9.2	320	2634
accuracy (%)	112	92	80	105

NB The HPLC-MSMS and HPLC-ICP-MS were carried out on different days.

The HPLC-ICP-MS and HPLC-MSMS-derived intravenous plasma profiles as illustrated in Figure 2.6 show the differences in the extent to which each technique can follow the plasma concentration of the drug leads to different plasma profiles being calculated. A two-compartment pharmacokinetic model consisting of a distribution phase and an elimination phase best fitted the HPLC-MSMS method, which followed the curve out to 3 hr when modelled. The $t_{½}$ for the terminal phase was 0.46 hr. When the HPLC-ICP-MS data were modelled the best fit was obtained using a three-compartment model. This consisted of a distribution phase followed by two apparent elimination phases. The first elimination phase gave a $t_{½}$ of 0.48 hr, which is comparable with the data obtained from the HPLC-MSMS system. The second apparent elimination phase gave a $t_{½}$ of 13.3 hr, however, more data points would be required to increase the degree of confidence in this value as it is based on data over a limited sampling period (12h). The AUC value for the HPLC-ICP-MS method was calculated to be 745 hr.ng/mL compared with 820 hr.ng/mL for the HPLC-MSMS method. The difference is primarily due to the slight bias observed in the methods with the HPLC-MSMS system having higher results; the longer terminal phase $t_{½}$ value for the HPLC-ICP-MS method has a much smaller impact.

Overall, the pharmacokinetic data obtained for the two systems were shown to be comparable, with additional information gained from the HPLC-ICP-MS method as a result of the increased sensitivity of the instrument.

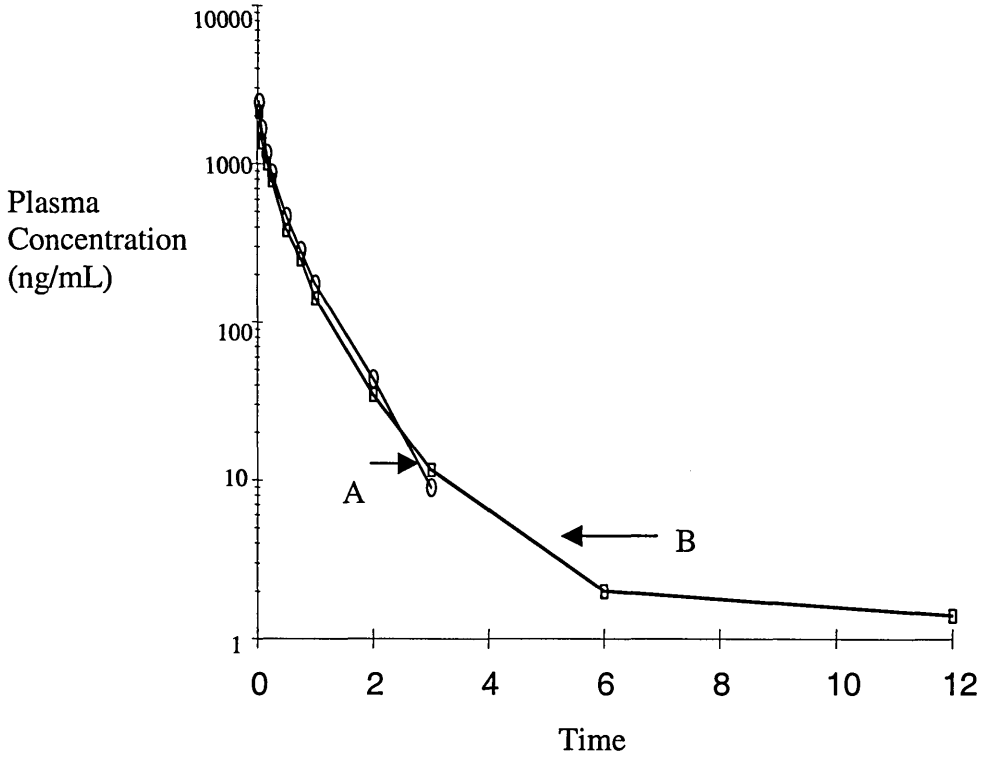


Figure 2.6 Intravenous profiles gained from a male dog dose with ZD0473 at 0.5 mg/kg. Two traces are represented with A) using plasma concentrations obtained from the more conventional technique of HPLC-MSMS. Trace B) shows plasma concentrations obtained using HPLC-ICP-MS demonstrating the extended range due to the lower detection ability.

2.4.5: Statistical Comparison of Methods

In order to compare results statistically from two different instruments it is necessary to look at random error and systematic error. The statistical method used to investigate these parameters was based on the methodology of Gilbert et al [44], which requires at least 30 data points made up of QC and real samples. The data used for statistical comparison was therefore made up of the QC samples, obtained from the validation experiments, together with the samples obtained from a single dog dosed intravenously. A total of 35 data pairs were included in this comparison.

Random error was compared by examining the differences between the two sets of results, normalized to the reference concentration. The standard deviation was calculated and then the range of the mean ± 2*s.d. If this range then includes the value of zero then there is no significant random error between the two sets of results. The data from the two systems showed +2 s.d. of 272 and –2 s.d. –131, therefore showing that there was no random error associated with the data.

If the ratio between each paired result is calculated, the standard deviation is then calculated from these ratios and then the range of the mean ± 2* s.d. If this range includes the value of 1.0 then there is no significant systematic error between the two sets of results. The data from the two systems showed + 2 s.d. of 1.4 and –2 s.d. 0.8. This data therefore confirms that the error is within acceptable limits.

Even with the apparent bias observed between the two analytical techniques when the single dose intravenous data were subjected to statistical analysis the QC and real

sample data sets were comparable within the limits of the test indicating that the methods are equivalent.

2.4.6: Aqua species comparison of HPLC-ICP-MS and HPLC-MSMS

Additional data were obtained from the HPLC-ICP-MS method, which enabled the determination of the aqua species in addition to ZD0473. Thus, the response for platinum remains constant no matter which compound contains the element and the concentrations of platinum-containing analytes such as the aqua species can be determined (Table 2.6), even in the absence of an authentic standard, in plasma samples. These data show that, even though acid was added to the samples to force the equilibrium in favour of ZD0473, the aqua species were still present to some extent (e.g. see Figure 2.4C). In the case of MSMS, where the relative ionisation efficiencies of the aqua species were not known, only qualitative data for these compounds could be obtained in the absence of response factor data. In order to determine the relative responses for these aqua compounds compared to ZD0473, a hyphenated HPLC-ICP-MS/MSMS system was constructed and samples from an oral dog study analysed. From the chromatograms illustrated for the combined system (Figure 2.7) it can be seen that the integration of the two spectrometers into a single system readily enables the determination of the MSMS responses for the aqua species by comparison with the values obtained simultaneously by ICP-MS. Thus, the relative responses for the aqua species, calculated by comparing the ratio of the responses for the ICP-MS based assay to the MSMS assay, were 1.1 ± 0.1 for the aqua species at Rt 2.3 min and 0.9 ± 0.2 for the aqua species at Rt 3.2 min ($N = 4$). In this instance therefore, the relative MSMS response for the aqua species was in fact

essentially the same as that of ZD0473. The results for the aqua species present in the oral samples provided by both HPLC-ICP-MS and HPLC-MSMS (calculated using the above response factors) are given in Table 2.6.

Table 2.6 Plasma concentrations obtained from HPLC-ICP-MS and HPLC-MSMS run in parallel after ultrafiltrate plasma samples were collected from a single male dog dosed orally at 6 mg/kg.

	HPLC-ICP-MS		
sample (hour)	ZD0473 (ng/mL)	Aqua species at 2.3 min (ng/mL)	Aqua species at 3.2 min (ng/mL)
0.25	200	14.1	9.4
0.5	326	25.9	21.8
0.75	230	17.9	12.0
1	196	20.8	14.2
	HPLC-MSMS *		
0.25	224	15.8	10.6
0.5	352	28.1	22.8
0.75	253	19.7	15.7
1	210	21.4	14.8

As shown elsewhere, such combined systems also provide a powerful technique in metabolite identification studies [40] where the ICP-MS data can be used to direct MSMS analysis to compound-related materials.

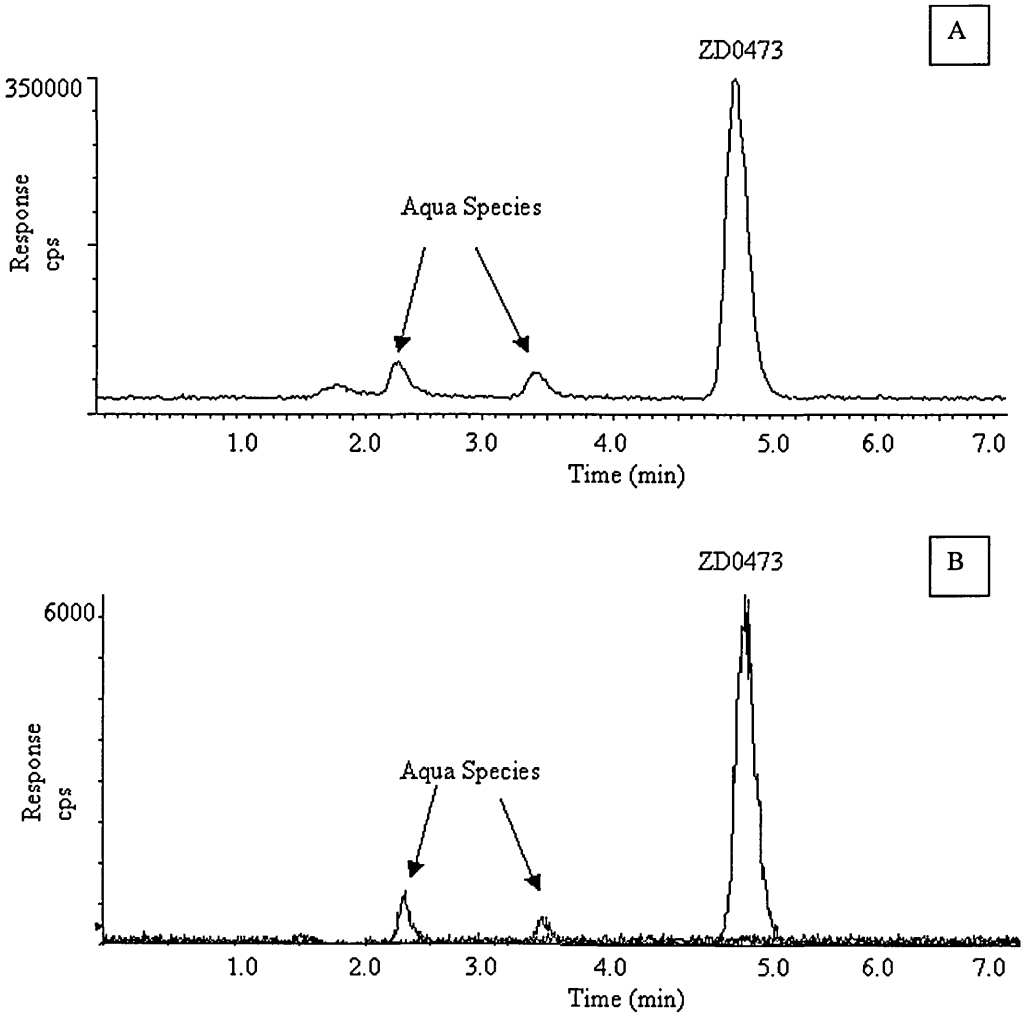


Figure 2.7 Chromatograms obtained from the HPLC-ICP-MS and HPLC-MSMS assays run in parallel on a sample taken at 0.5 hours from a single dog dosed orally at 6 mg/kg. Chromatogram A) represents HPLC-ICP-MS trace monitored at *m/z* 195 for platinum and chromatogram B) representing an HPLC-MSMS trace monitoring the mass transitions as described in Table 2.2. Both traces show the two aqua species and the parent component ZD0473. For experimental conditions see section 2.3.

2.5: Conclusion

Both HPLC-ICP-MS and HPLC-MSMS enabled the detection and quantification of ZD0473 in plasma samples and provided the basis for robust bioanalytical methods. However, the HPLC-ICP-MS system was found to have greater sensitivity, with a limit of quantification for ZD0473 of 0.1 ng/mL (0.05ng/mL of platinum) compared to 5 ng/mL for the HPLC-MSMS method. Because it is based on the quantification of platinum, the HPLC-ICP-MS method also enables the facile quantification of the platinum-containing aqua species, even in the absence of standards. HPLC-ICP-MS provided an assay with a much greater linear range than HPLC-MSMS, being linear over 8 orders of magnitude, compared to just over 3 orders of magnitude for HPLC-MSMS. These studies show that HPLC-ICP-MS provides the basis for a routine method for the sensitive determination of ZD0473 and the related aqua species in biofluid samples.

The results presented in this chapter were published in Analytical Chemistry **75**, 2003, 1463-1469. (appendix A).

Chapter 3

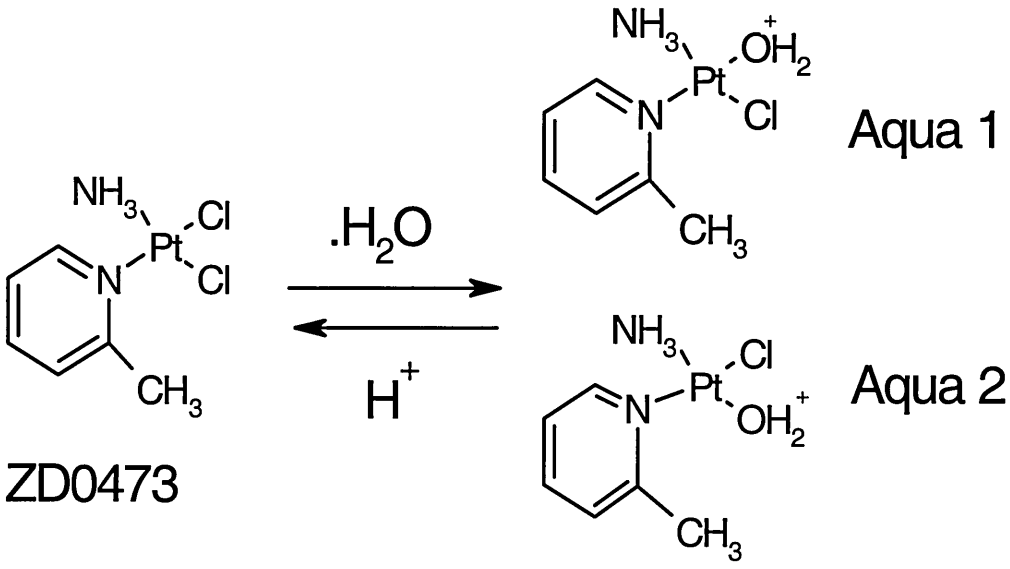
Analysis of a [^{14}C]-labelled platinum anticancer compound in dosing formulations and urine using a combination of HPLC-ICP-MS and flow scintillation counting.

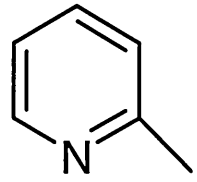
3.1: Summary

HPLC-ICP-MS was used in combination with on-line radioactivity detection for the determination of [^{14}C]-ZD0473, a platinum anticancer drug. Initially the system was set up to look at the purity of the formulations used in a dog study. This analysis showed that the oral formation had a purity of 93 %, with 91 % for the intravenous (*i.v.*) dose. The system was then adapted for use within the dog urine study following *i.v.* administration to investigate the metabolism of ZD0473. The main purpose of the study was to investigate the difference between the values gained from the [^{14}C]-labelled study compared to the data obtained from the total platinum results from an external laboratory. The results showed that the platinum levels were higher than that was seen for the [^{14}C] data and it was postulated that the differences were due to the compound converting to the 2-picoline moiety, which retained the [^{14}C]-label but lost the platinum element. The metabolism study in the dog showed 2-picoline to be present in urine samples. Samples from studies in rats were also successfully analysed.

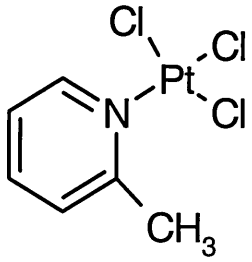
3.2: Introduction

As described in chapter 2, ZD0473 {cis-[amminedichloro(2-methylpyridine)] platinum(II)} is a new generation platinum compound designed to deliver an extended spectrum of antitumour activity and overcome platinum (Pt) resistance [45]. The structure of ZD0473 and related compounds, including the biologically active "aqua" compounds is shown in Figure 3.1. Having shown that HPLC-ICP-MS provided a suitable analysis for ZD0473 and related Pt-containing molecules in plasma, this methodology was used to provide a rapid, efficient and sensitive method of analysis for Pt in other samples containing ZD0473 and related materials. Here the use of HPLC-ICP-MS coupled with a radio flow scintillation counter and a UV detector is described to provide a method of analysis for [^{14}C]-labelled ZD0473. The techniques were used in combination to determine whether the profile for [^{14}C]-labelled compounds matched the profile obtained for Pt in chromatograms obtained for dose formulations and urine samples from a metabolism study.



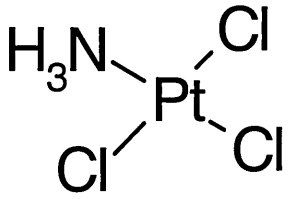


2-Picoline



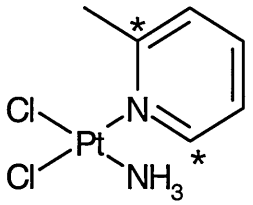
(TCPP)

Trichloro(2-picoline)platinate



(TCAP)

Trichloro amine platinate



ZD0473 with [^{14}C] label positions

Figure 3.1 The structure of ZD0473 with the associated aqua species and position of radiolabels, and known impurities.

3.3: Experimental

3.3.1: Reagents

Trifluoroacetic acid was purchased from Fisher (Fisher Scientific UK Ltd, Loughborough). Solvents for chromatography were of HPLC grade and were obtained from Fisher Scientific. The physiological saline used in the study for the dilution of samples and for the preparation of *i.v.* formulations, together with the hydroxypropylmethyl cellulose (HPMC) used in the preparation of the oral dose solution, was obtained from the Media Preparation Laboratory at AstraZeneca Pharmaceuticals. The scintillant used was Ultima-Flo M from Packard Ltd (Berkshire, UK).

ZD0473 was obtained from AstraZeneca Pharmaceuticals (Alderley Park, Macclesfield, UK) whilst [^{14}C]-labelled ZD0473 was synthesised in the Isotope Chemistry Laboratory at AstraZeneca Pharmaceuticals at a specific activity of 55 μCi/mg. The [^{14}C]-labelled isotope was placed in the aromatic ring of ZD0473.

3.3.2: Reference compound

ZD0473 was dissolved at a concentration of 0.005 mg/ml in 0.5M sodium chloride. In aqueous solution, ZD0473 forms equilibrium species (Figure 3.1). The concentrations of these species were dependent on the chloride ion. Radioactive label positions were shown in Figure 3.1.

3.4: Samples for Oral and *i.v.* Dose Formulations

3.4.1: Oral and *i.v.* Dose Formulations of ZD0473

ZD0473 was formulated in physiological saline or 0.5 % HPMC for *i.v.* and oral administration respectively at a concentration of 0.5 mg/mL and a specific activity of 55 μCi/mg. To ensure that ZD0473 was completely dissolved the formulations were made up and stirred for 3 hr prior to analysis.

3.4.2: Urine

Urine was collected for the period 6-12 hr following *i.v.* administration to a male beagle dog (Age 11 months, Weight 12.8 kg, Dose 0.5 mg/kg, 55 μCi/mg of [^{14}C]-ZD0473). At the end of the sample period the weight was recorded and the urine split into two 20 mL portions and stored at –70°C in a freezer. Thawed samples were pre-treated by centrifuging in an Eppendorf 5417C centrifuge (Eppendorf, Hamburg, D) for 5 minutes at 14000 rpm before injection (25 μL) onto the HLPC system.

3.4.3: Chromatography

Reversed-phase HPLC was performed using a Jasco gradient HPLC system PU-1580 (Jasco Ltd, Great Dunmow, UK) with samples introduced via a PE 200 autosampler (PerkinElmer Ltd, Beaconsfield, UK). Chromatography was performed on an Inertsil ODS-3 150 mm x 4.6 mm 5 μm column (Hichrom Ltd, Reading, UK) at a flow rate of

1.0 mL/min. The solvent system used for these separations was a gradient formed from 0.2% trifluoroacetic acid in water (solvent A) and methanol (solvent B). The gradient conditions are as shown in Table 3.1.

Table 3.1 Chromatographic gradient conditions

Time minutes	Pump programming	Mobile Phase A %	Mobile Phase B %
0 - 4	Isocratic	98	2
4 - 11	Linear gradient	98 to 80	2 to 20
11 -14	Isocratic	80	20
14 - 20	Linear Gradient	80 to 98	20 to 2

3.4.4: UV and [^{14}C]-Detection

The eluent from the column was directed via an splitter (LC Packings, Presearch, Hitchin, UK) to either a Micromass Platform ICP-MS (GV Instruments UK Ltd, Wythenshawe, UK) (250 µL/min) or to a Jasco UV-1575 intelligent vis / UV detector set at 265 nm (Jasco Ltd, Great Dunmow, UK) and a Flow One radio scintillation analyser (Packard, Berkshire, UK) (750 µL/min) connected in series. The Flow One detector was fitted with a low volume (50 µL) flow cell in order to preserve the resolution between peaks. For both UV and [^{14}C]-detection Masslynx software version 3.4 (Micromass UK, Wythenshawe, UK) was used for data analysis.

3.4.5: ICP-MS

The Platform ICP-MS instrument employed in these studies used a hexapole collision / reaction cell based ICP mass spectrometer [42] for the simultaneous measurement of ^{14}C and ^{195}Pt. The eluent from the column was introduced to the ICP-MS via a concentric nebuliser and a double pass spray chamber, which was cooled to –3 °C to allow an increase in the percentage of methanol used within the system. Masslynx software was used for the instrument control, data acquisition and data handling. The flow of the nebuliser gases and the operating and acquisition conditions of the ICP-MS are shown in Table 3.2.

The isotope envelope for Pt contains masses at 194, 195, 196 and 198 (at percentage abundance's of 32.9, 33.8, 25.3, 8.0 %). Although it is possible to run the instrument in such a way as to sum these ions (which would theoretically give rise to an increased signal) in practice the increase in the background caused the process not to be beneficial in that the signal to noise ratio was not increased. Therefore the ion 195 was monitored because it is the most abundant isotope of Pt.

Table 3.2 Instrument operating conditions for the ICP-MS

Cooling gas	16.00 L/min	Plasma Power	1350 w
Plasma gas	0.65 L/min	Acquisition mode	SIR
Nebuliser gas	0.75 L/min	Dwell time	200 ms
Helium gas	1 mL/min	Masses monitored	195, 14
Hydrogen gas	4.0 mL/min	Total analysis time	20 min

3.5: Results and Discussion

3.5.1: Assay Development

The chromatographic system designed for this assay had to achieve the objectives of separation of the components in Figure 3.1, whilst under the constraints of solvent composition imposed by the ICP-MS [24, 49]. This meant that the percentage of organic modifier had to be kept below approximately 40 % or the plasma would be compromised and / or extinguished. To overcome this limitation ion pair buffers (1-hexanesuluphonate, sodium salt) with a low percentage of methanol were investigated to obtain the required separation. This approach provided an adequate separation but was ultimately unsuccessful due to the buffer creating blockages within the ICP-MS system and poor chromatographic reproducibility between runs (Figure 3.2). The buffers were therefore, replaced with a water-methanol gradient system, which had a

final methanol composition of 30 %. The instrument was able to run at this percentage of methanol due to the low flow rate (250 μL/min) and the spray chamber being cooled to –3 °C. The system was further optimised and the final chromatographic conditions were as described in Table 3.1. A standard chromatogram is shown in Figure 3.3. The change of HPLC system resulted in improved equipment reliability and more robust chromatographic separation.

3.5.2: Oral and IV Dose Formulation Purity Determination

The dose solutions prepared for the *i.v.* and oral formulations were analysed to see if they were of the required purity before dosing was carried out. Typical results for the UV, ^{195}Pt, and [^{14}C]-profiles generated for the oral and *i.v.* formulations are shown in Figures 3.4 and 3.5 respectively. The dose purity can be calculated by summing the ZD0473 peak area with those for the aqua species to give the total percentage of active drug. In this instance this was found to be 91 % for the oral dose solution and 93% for the *i.v.* dose by ICP-MS. These results show that for the dose solutions there was a good degree of correspondence between the chromatograms obtained for the 3 modes of detection.

The method demonstrated that the ratio of aqua species was different for each sample analysed. The reason for the increased levels and different ratio of aqua species in the oral sample was probably due to the formulation having a higher salt content and therefore driving the equilibrium in the direction of the aqua species.

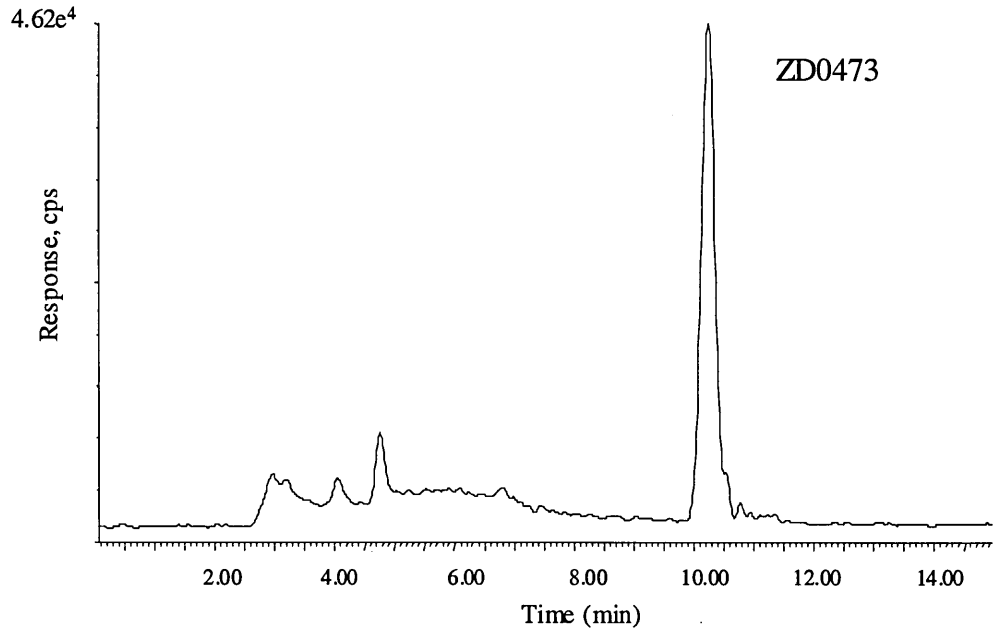


Figure 3.2 A typical example of a chromatogram obtained using an ion pair reagent with ICP-MS detection of Pt at *m/z* 195. A large peak for ZD0473 is shown at 10.0 min for a purity sample. However, the chromatography at the start of the run shows a loss in resolution due to poor chromatography caused by the sodium 1-hexanesulphonate ion pair reagent. The reagent also caused problems with blockages with the nebuliser making the system unreliable. For experimental conditions see section 3.3.

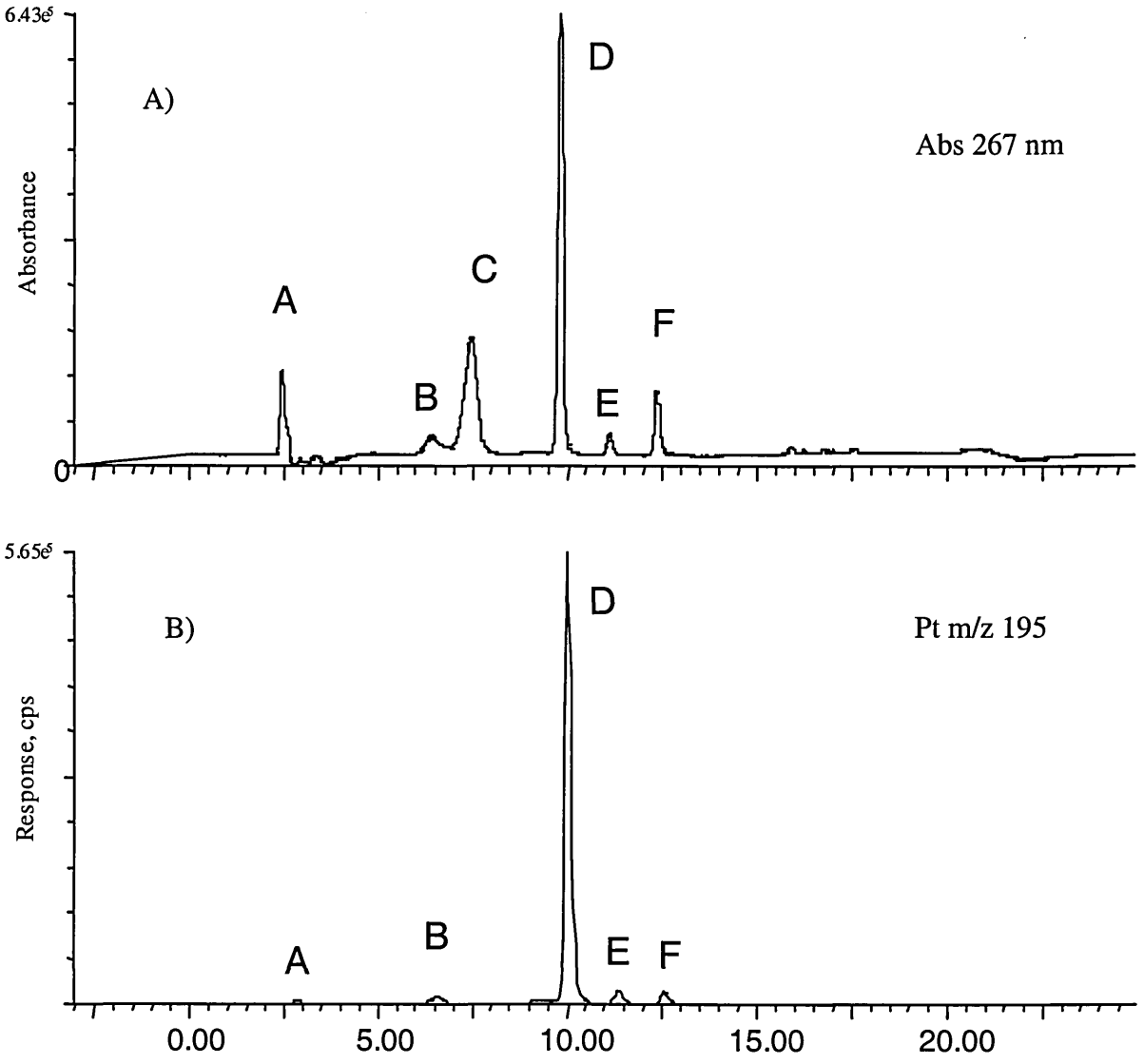


Figure 3.3 A typical chromatogram obtained from of the methanol-water gradient elution developed for the separation of the aqua species and impurity components. A: TCAP B: Aqua species 1 C: 2-Picoline D: ZD0473 E: Aqua species 2 F: TCPP.

a) UV absorbance trace at 267 nm, b) ICP-MS trace at *m/z* 195 for Pt. For experimental conditions see section 3.3.

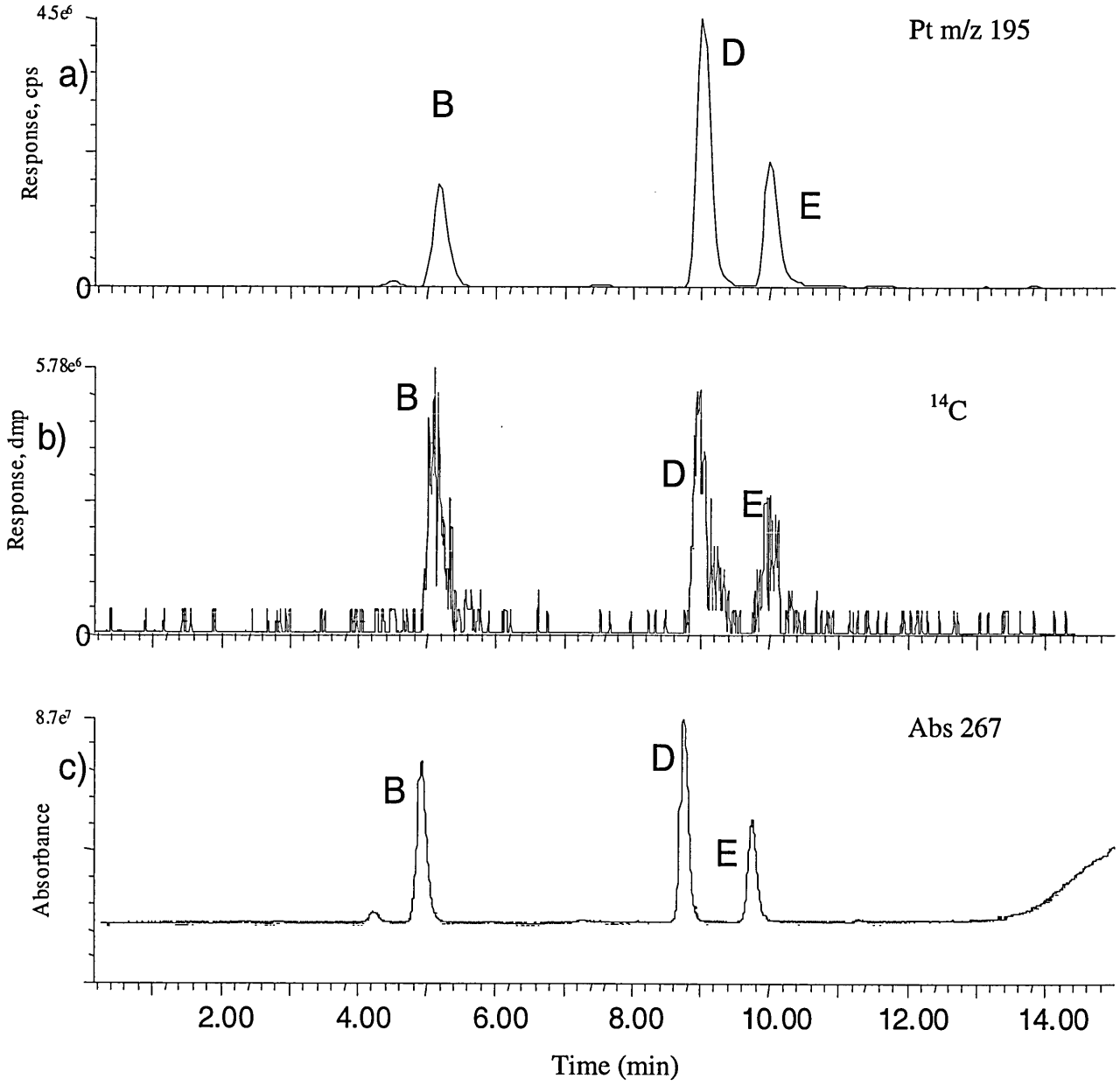


Figure 3.4 Typical a) ICP-MS platinum, b) radioactivity and c) UV absorbance traces obtained for the oral dose solution at concentration of 0.5 mg/mL. Peaks B, D and E represents aqua species 1, ZD0473 and aqua species 2 respectively. A purity value of 91 % was obtained when the peaks from the Pt trace were summed. For experimental conditions see section 3.3

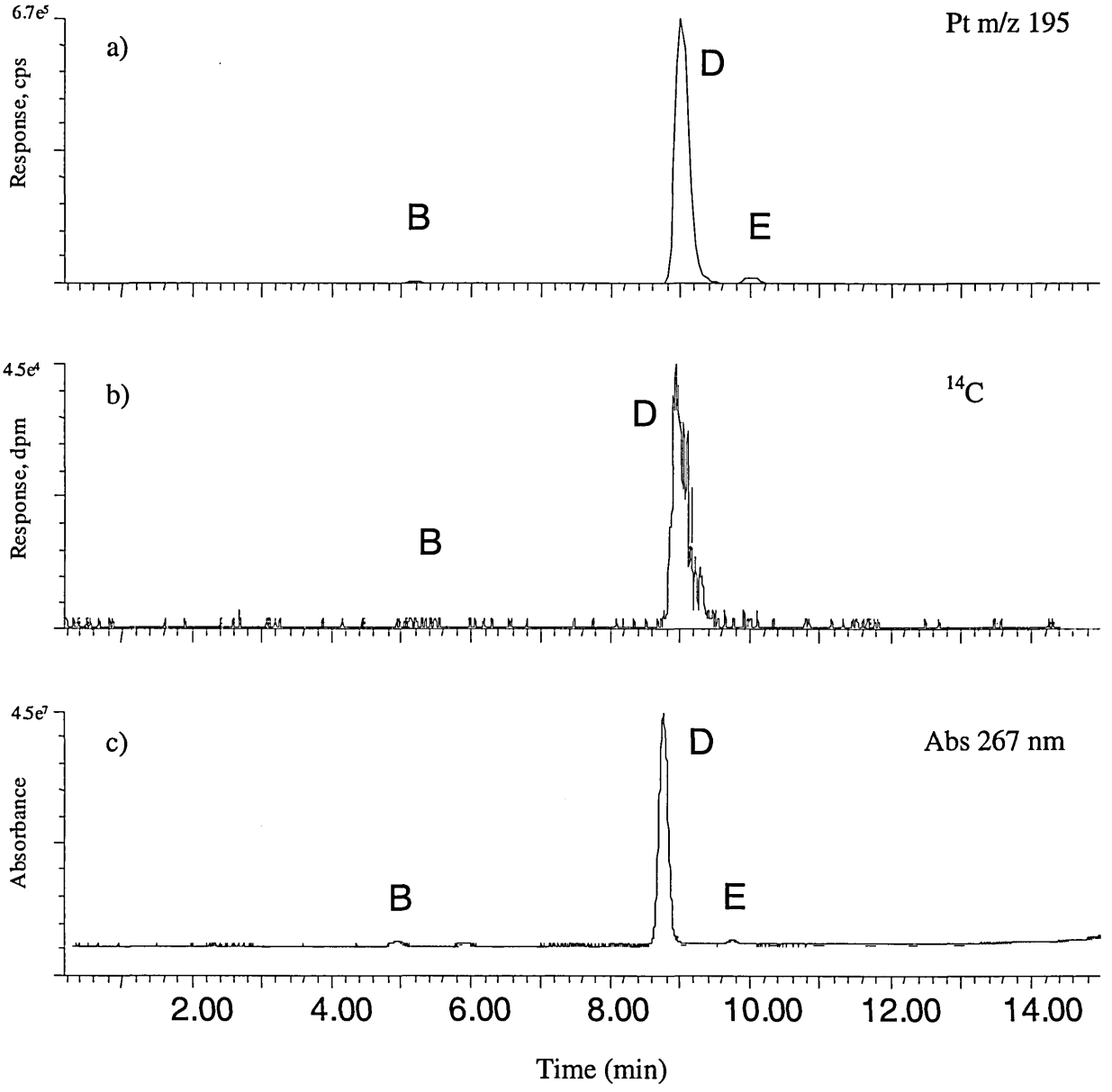


Figure 3.5 Typical a) ICP-MS platinum, b) radioactivity and c) UV absorbance traces obtained for the IV dose solution at concentration of 0.5 mg/mL. Peaks B, D and E represents aqua species 1, ZD0473 and aqua species 2 respectively. A purity value of 93 % was obtained when the peaks from the Pt trace were summed. For experimental conditions see section 3.3

3.5.3: Analysis of Urine samples obtained from *i.v.* dose.

The chromatographic system developed for the analysis of the formulation samples was also used for urines obtained from a radio-labelled excretion balance study carried out in the dog, following intravenous administration of [^{14}C]-ZD 0473. The UV absorbance (254 nm), ^{14}C radio detection and ICP-MS at *m/z* 195 traces obtained for a single dog (6-12hr post dose) are shown in Figure 3.6. From the ^{195}Pt trace unchanged ZD0473 accounted for 6.95% of the total based on the retention time. The remainder consisted of 8 Pt-containing peaks, eluting between 2 and 14 minutes, but none of these peaks corresponded to any of the degradation products represented in Figure 3.1. Comparison of the ^{195}Pt trace with the radioactivity profile revealed several noticeable differences. In particular a large radio-labelled peak was observed at 13.10 min, which was not present in the ^{195}Pt trace. This discrepancy in the profiles obtained for the ^{195}Pt and ^{14}C traces indicates that at some stage in the metabolism of the compound by the dog, the 2-picoline moiety, which contains the radio-label, has been detached from the platinum. The use of both detectors, therefore, provides valuable and more robust metabolic information that would not have been obtained had only one been used. The identity of these metabolites is considered in the following sections in relating to [^{14}C] and ICP-MS detection. No useful metabolic information could be gained from UV absorbance detection at 267 nm due to the interference from endogenous material.

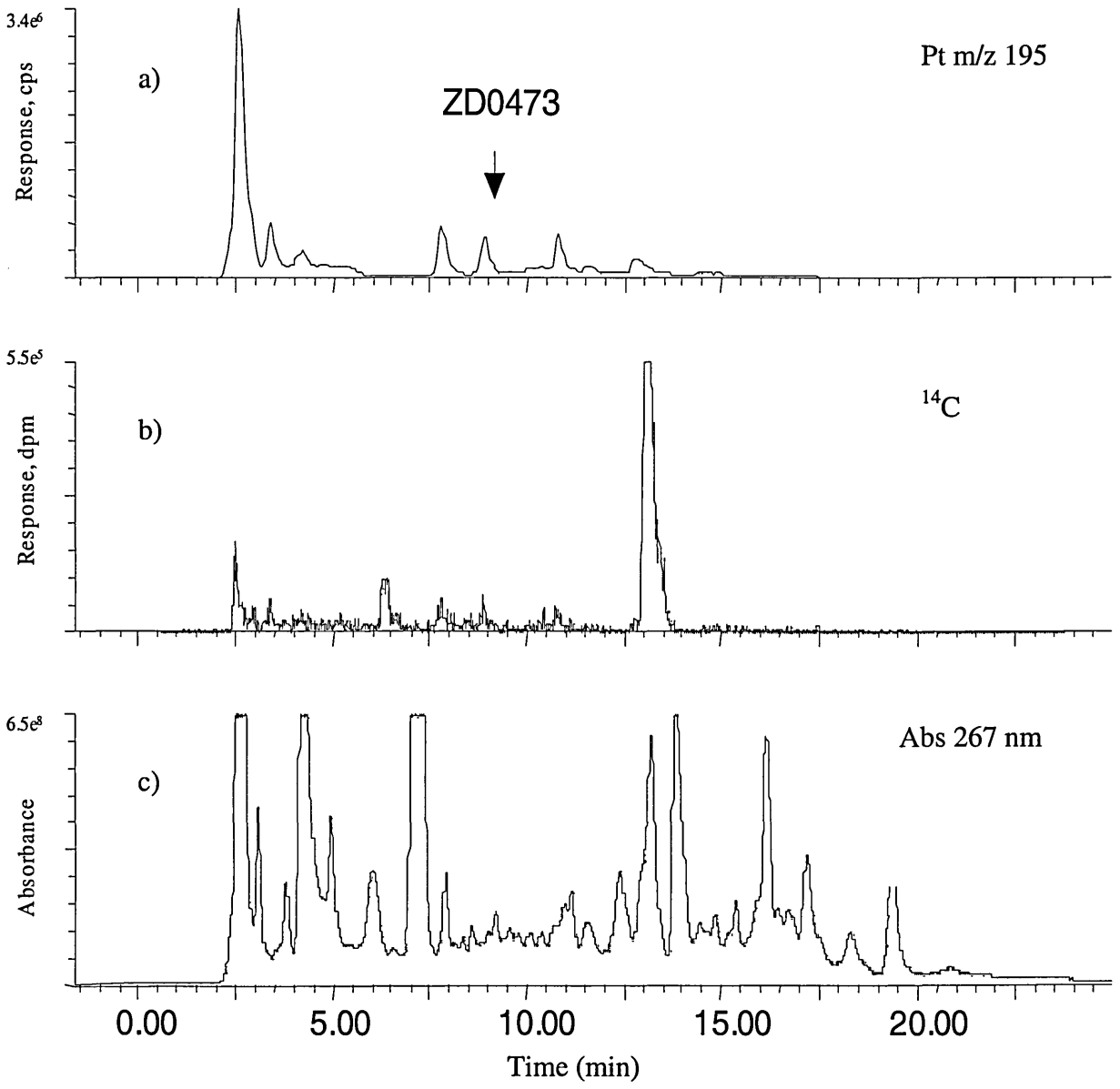


Figure 3.6 A typical set of traces for A) ICP-MS platinum, B) radioactivity and C) UV absorbance (267 nm) obtained for a dog urine sample. The ICP-MS trace shows that ZD0473 is present and can be quantified against an external standard. The [^{14}C]-trace shows the presences of metabolite peaks, which have lost the Pt element. The UV trace is not selective enough to provide useful information. For experimental conditions see section 3.3

3.6: Radioactivity and platinum profiling of urine samples from tissue distribution and excretion studies in the rat.

The previously described analytical system was set up to investigate the metabolism of ZD0473 in samples from rodents. The purpose of this study was to compare the chromatographic profiles of platinum and ^{14}C in urine, following single and multiple intravenous administration of [^{14}C]-ZD0473 to male rats. A mass spectrometer was also run in parallel to gain molecular information.

3.7: Radioactive samples for dosing

Three sets of dosings were carried out in the study.

3.7.1: Single dose

Three male rats each received a single intravenous dose of [^{14}C]-ZD0473 at 30 mg/kg with a target radioactive dose of 150 μCi/kg. Urine was collected at 0-6, 6-12, 12-24 hours and subsequently at daily intervals up to 7 days post dose. Pooled urine samples collected over the first 24 hours from one animal were analysed in this study. This represented more than 80% of the urinary excretion of total radioactivity from this animal.

3.7.2: Low multiple dose

Three male rats each received an intravenous multiple dose of [^{14}C]-ZD0473 at 5 mg/kg/day for 5 days with a target radioactivity dose of 175 μCi/kg. Urine was collected at 0-6, 6-12 and 12-24 hours post dose on days 1 to 5 and at daily intervals up to 7 days post the fifth dose. Pooled urine samples from one animal were analysed in this study. This represented greater than 90% of the urinary excretion of total radioactivity from this animal.

3.7.3: High multiple dose

Three male rats each received an intravenous multiple dose of [^{14}C]-ZD0473 at 7.5 mg/kg/day for 5 days with a target radioactivity dose 260 μCi/kg. Urine was collected at 0-6, 6-12 and 12-24 hours post dose on days 1 to 5 and at daily intervals up to 7 days post the fifth dose. Pooled urine samples from one animal were analysed in this study. This represented greater than 95% of urinary excretion of total radioactivity from this animal.

3.8: Analytical design and sample preparation

For the single 30 mg/kg and the multiple 7.5 mg/kg/day doses, pooled urine samples were generated by taking 40-50% of the sample generated over the time periods, 0-6, 6-12 and 12-24 post doses on days 1 and 5, respectively. Samples analysed were from one animal for each dosing regimen. For the 5 mg/kg/day dose, a single urine

sample from one animal, generated over the first 24 hours following the fifth dose, was analysed.

The samples were injected onto the HPLC system either undiluted or following dilution with an equal volume of distilled water, with no pre-treatment.

3.9: Analytical procedures and equipment

Injections were made using a Perkin Elmer series 200 autoinjector on to a reversed phase HPLC column, (Inertsil ODS-3 5µm, 150 x 4.6 mm id) in a column oven set at 35 °C.

The HPLC mobile phase was delivered using a Jasco PU-1580 pump delivering the gradient shown in Table 3.1 with mobile phase made up of 0.2% TFA in distilled water and methanol (eluents A and B, respectively). The HPLC flow was split twice, the initial split, using an accurate splitter, directing 250 µL/min to the radioactivity detector and 750 µL/min towards the mass spectrometer. The second split, of the 750 µL/min flow, was achieved using a model 600 (Jasco) splitter valve directing 375 µL/min to both the ICP-MS and the API 3000 mass spectrometer.

The UV detector was a model 1575 (Jasco) set at 267 nm. The radio-detector was a series 150TR instrument (Packard) fitted with a liquid flow cell.

A Sciex API 3000 triple quadrupole LC-MSMS mass spectrometer operated in positive mode, scanning between 50-150 and 300-500 amu, and a Micromass ICP-MS

were operated in parallel to the UV and radioactivity detectors. Figure 3.7 shows the system used in the study.

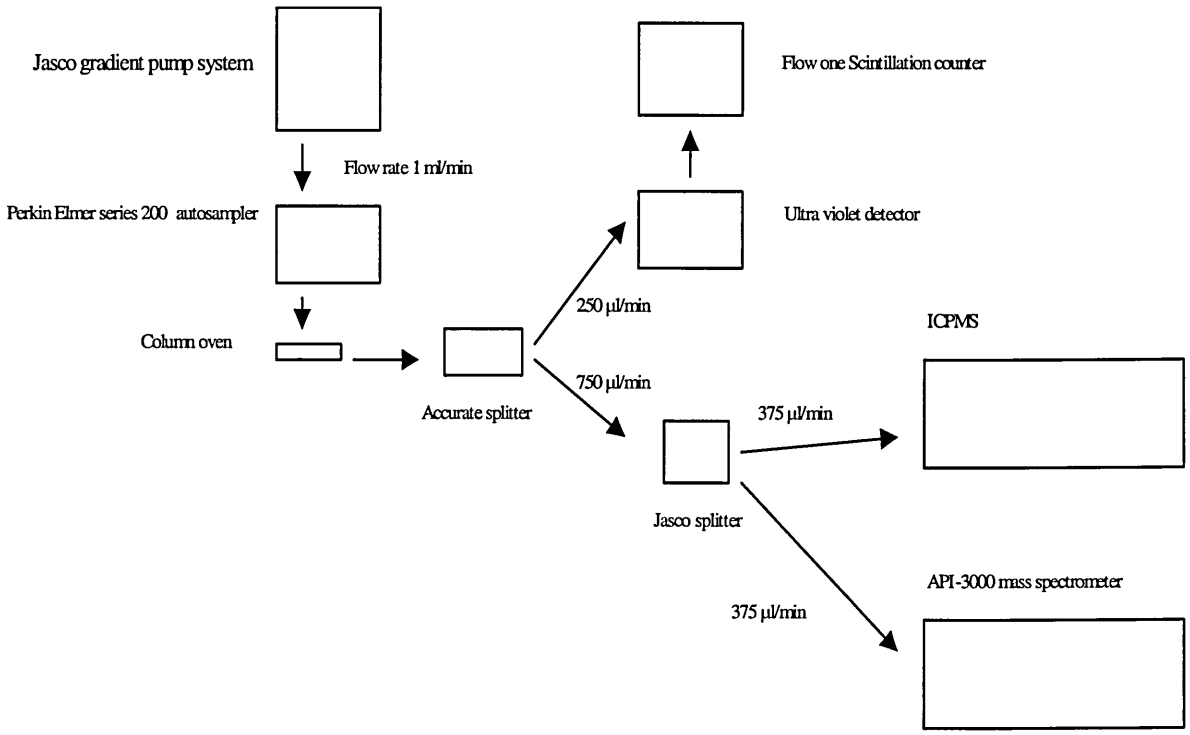


Figure 3.7 Schematic diagram of the system used for the analysis of ZD0473 and its associated metabolites and impurities with simultaneous measurement of ^{14}C, UV absorbance, organic mass spectra and Pt.

3.10: Results and discussion (rat urine profiling experiment)

A typical Pt trace for a rat urine sample is shown in Figure 3.8, and a radio HPLC chromatogram in Figure 3.9. The relative proportions of radio-labelled components detected, as a percentage of the traces, are shown in Table 3.3, with retention times in Table 3.4. HPLC-MSMS traces and fragmentation patterns for ZD0473 and 2-picoline standards are shown in Figure 3.10. HPLC-MS traces and fragmentation patterns for two unknown components are shown in Figure 3.11. Comparison of the components seen by the three detection methods is given in Table 3.5.

The radio-chromatograms of urine showed three major components in all samples accounting for a total of approximately 50 to 70 % on the trace (Figure 3.9 and Table 3.3). The remaining activity consisted of several components, which could not reliably be quantified above the background levels.

The proportion of ZD0473 in urine [^{14}C]-traces was in the range 4.5 to 24 %. Levels of ZD0473 were at the lower range for samples taken from animals following 5 daily intravenous administrations of ZD0473 as compared to those collected following single administration (Table 3.3).

The aqua species were not detectable in the radio-labelled traces, but were seen with platinum detection at approximate relative retention times of 0.63 and 1.1, corresponding with those of the aqua standards (Figure 3.3 and Table 3.6). The identity of the component at relative retention time of 0.63 was confirmed as being

one of the aqua species, due to the presence of the characteristic fragmentation pattern for ZD0473 shown in Figure 3.10.

The peak at relative retention time 0.72 in the [^{14}C]-trace had no corresponding platinum peak (Table 3.6) suggesting this to be a product derived from cleavage of the platinum - 2-picoline ring bond as suggested, and furthermore the relative retention time was similar to the 2-picoline standard, suggesting this to be the identity of this component. The identity of 2-picoline was confirmed from the MS data, since the product ion was characteristic for 2-picoline at 94 amu (Figure 3.11).

In the radioactivity and ICP-MS platinum chromatograms for all samples, a component eluted later than ZD0473, with relative retention time of approximately 1.4. The identity of this component could not be identified, but since it contains both platinum and [^{14}C]-labels, this suggests that the platinum to 2-picoline bond was intact. There was platinum isotope pattern data at around 400 amu suggesting this component to have molecular weight greater than ZD0473. This suggests that the component may be a conjugate or adduct, e.g. with an endogenous amino acid, however there was insufficient data to make an identification.

There may be additional, but minor, components present, which may be adducts or conjugates with higher molecular weights than those discussed above. However, MS analysis was only performed for mass units of up to *m/z* 500 in this study due to limitation in the spectrometer, in order to maintain sensitivity.

No data were obtained to aid the identification of the relatively minor components seen at a relative retention time of 0.34 to 0.38 in the platinum and [^{14}C]-traces.

Table 3.3 Components detected in the radio-chromatogram from 0-24 hour pooled urine samples following intraveneous doses of ZD0473 to male rats given either as single doses or as multiple once daily administration over 5 days.

		Relative retention time to ZD0473 =1 (min)					
Dose level	Sample	0.34	0.5	0.72	ZD0473	1.45	
		Percentage amounts for peaks in the radio trace					Total %
30 mg/kg	M15 Day 1	1.43	-	12.7	23.8	34.2	72.1
5 mg/kg	M10 Day 1	1.33	-	13.2	15.9	35	65.4
5 mg/kg	M10 Day 5	12.9	-	15.6	4.49	22.3	55.3
7.5 mg/kg	M19 Day 1	0.98	-	14.7	16.8	35.9	68.4
7.5 mg/kg	M19 Day 5	7.7	6.5	16.6	7.6	30.5	68.9

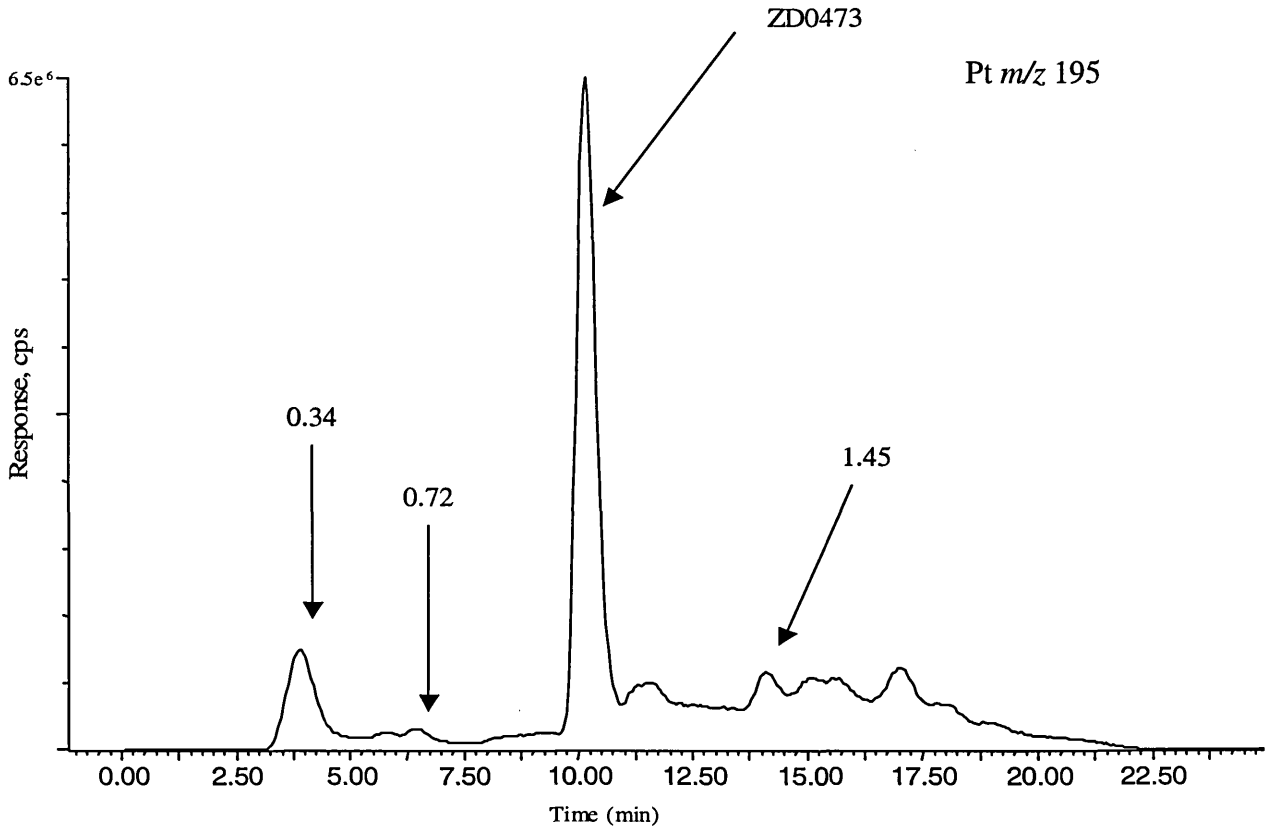


Figure 3.8 HPLC platinum trace of a 0-24 hour urine sample (animal 15M) following a single intravenous dose of [^{14}C]-ZD0473 at 30 mg/kg. The peak at relative retention time 0.34 is an unknown. No Pt peak was seen at 0.72, which corresponds to 2-picoline retention time. The peak at 1.45 has been postulated as a conjugate or adduct. For experimental conditions see section 3.3

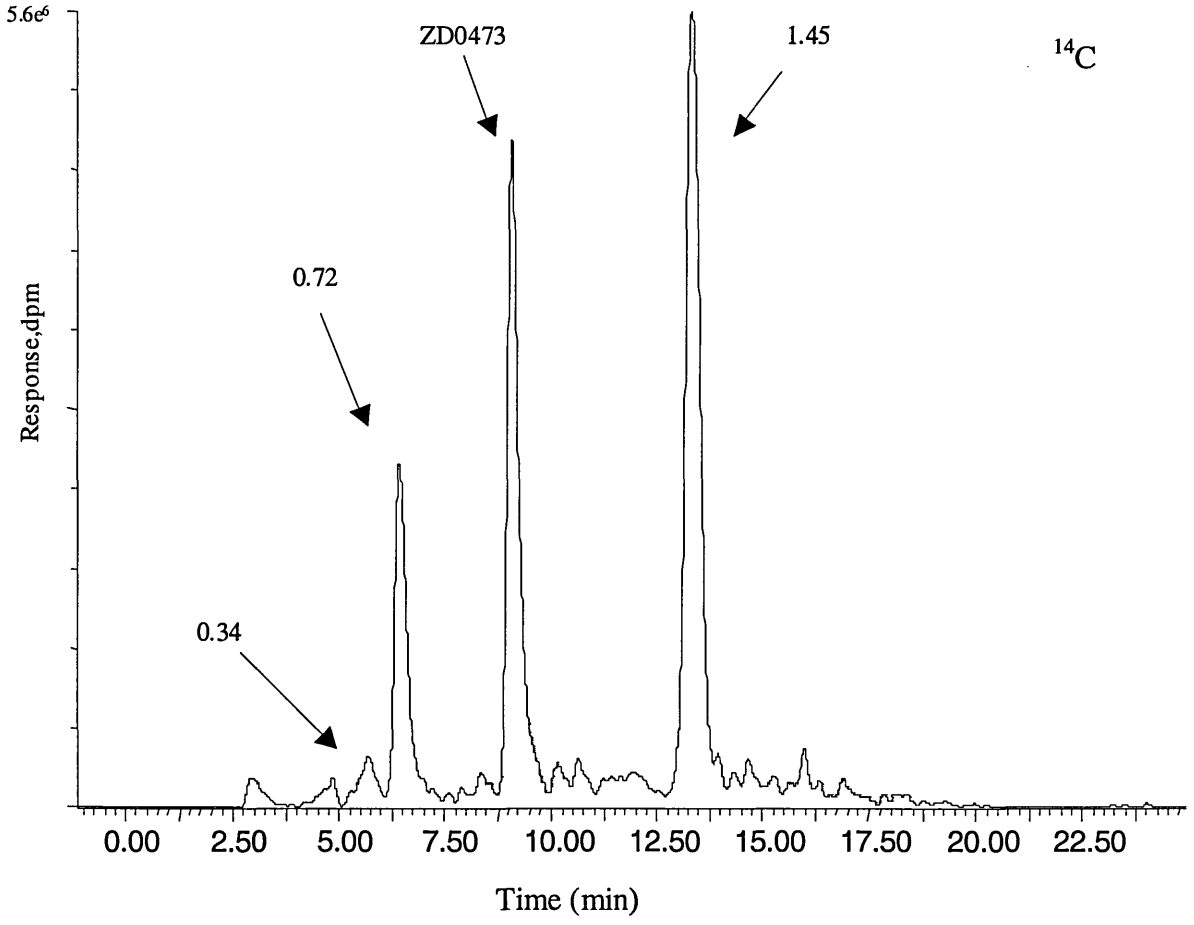


Figure 3.9 HPLC radiochromatogram of a 0-24 hour urine sample (animal 15M) following a single intravenous dose of [^{14}C]-ZD0473 at 30 mg/kg. The peak represented at relative retention time of 0.34 is an unknown. The peak at 0.72 having a [^{14}C] response represents 2-picoline moiety. The peak at 1.45 represents a metabolite suggesting a conjugate or adduct. For experimental conditions see section 3.3.

Table 3.4 Mean retention times relative to ZD0473 for HPLC-ICP-MS components detected in all 0-24 urine samples obtained following intravenous doses of ZD0473 to male rats given as single doses or as multiple once daily administrations over 5 days.

	Retention times (min) relative to ZD0473							
mean	0.38	0.62*	ZD0473 (1.00)	1.12	1.24**	1.39	1.52*	1.68*

* Not present in all samples
** Present only in day 5 samples

Table 3.5 Mean retention times relative to ZD0473 for HPLC-MS components detected in all 0-24 urine samples obtained following intravenous doses of ZD0473 to male rats given as single doses or as multiple once daily administrations over 5 days.

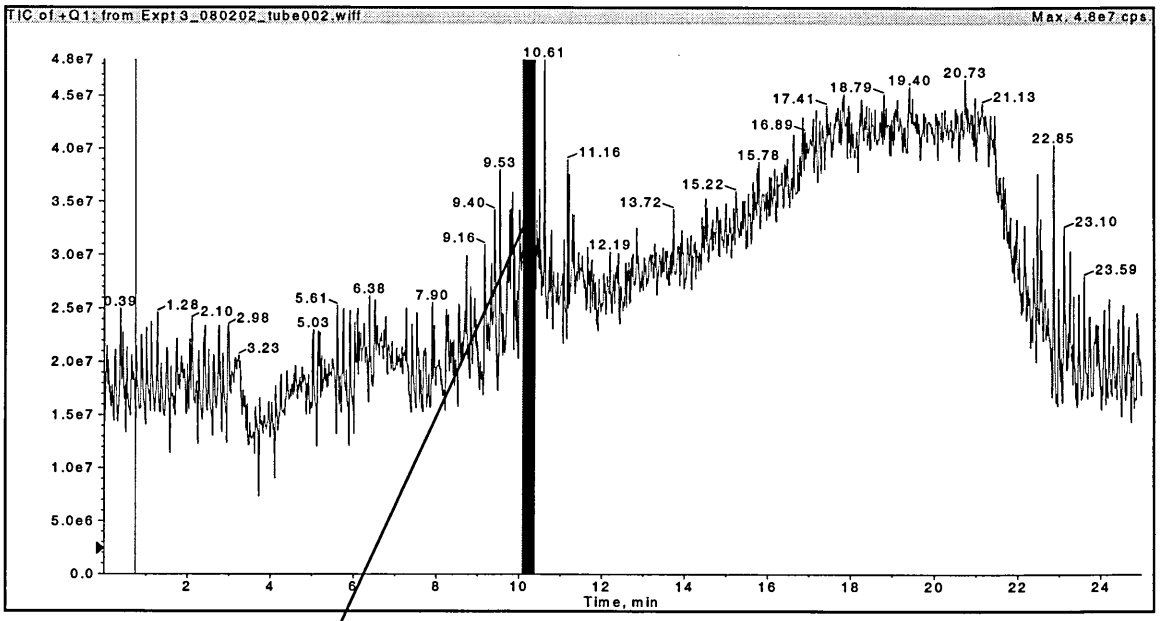
	Retention times relative to ZD0473					
mean	0.41	0.63	0.74	ZD0473 (1.00)	1.38	1.71

Table 3.6 Summary of the relative retention times (min) with all detection methods for all 0-24 hr urine samples analysed following intravenous doses of ZD0473 to male rats given as single doses or a multiple once daily administrations over 5 days.

[^{14}C]	Platinum data	MS data	Tentative component Identification
0.34	0.38	0.41	Unknown**
n.d.	0.62	0.63	Aqua
0.72	n.d.	0.74	2-picoline
ZD0473	ZD0473	ZD0473	ZD0473
n.d.	1.12	n.d.	Aqua
1.45	1.39	1.38 (Pt isotope pattern at 400 amu)	Unknown 1
n.d.	1.68	1.71	Unknown 2

** eluted at the solvent front and hence may be a mixture of components
n.d. Not detected

A



B

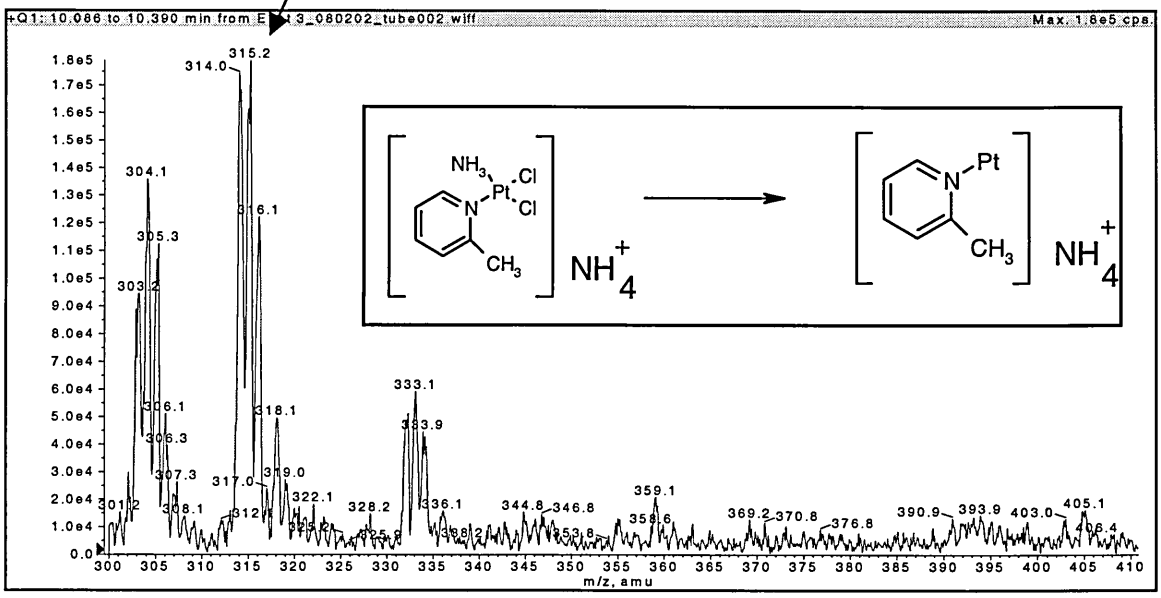


Figure 3.10 HPLC-MS reference compound trace of ZD0473 at a concentration of 1 µg/mL. A. Represents a Q1 scan of ZD00473 showing a high background due to the wide scan range. B. Mass spectrum showing the fragmentation product indicative of ZD0473. For experimental conditions see section 3.3.

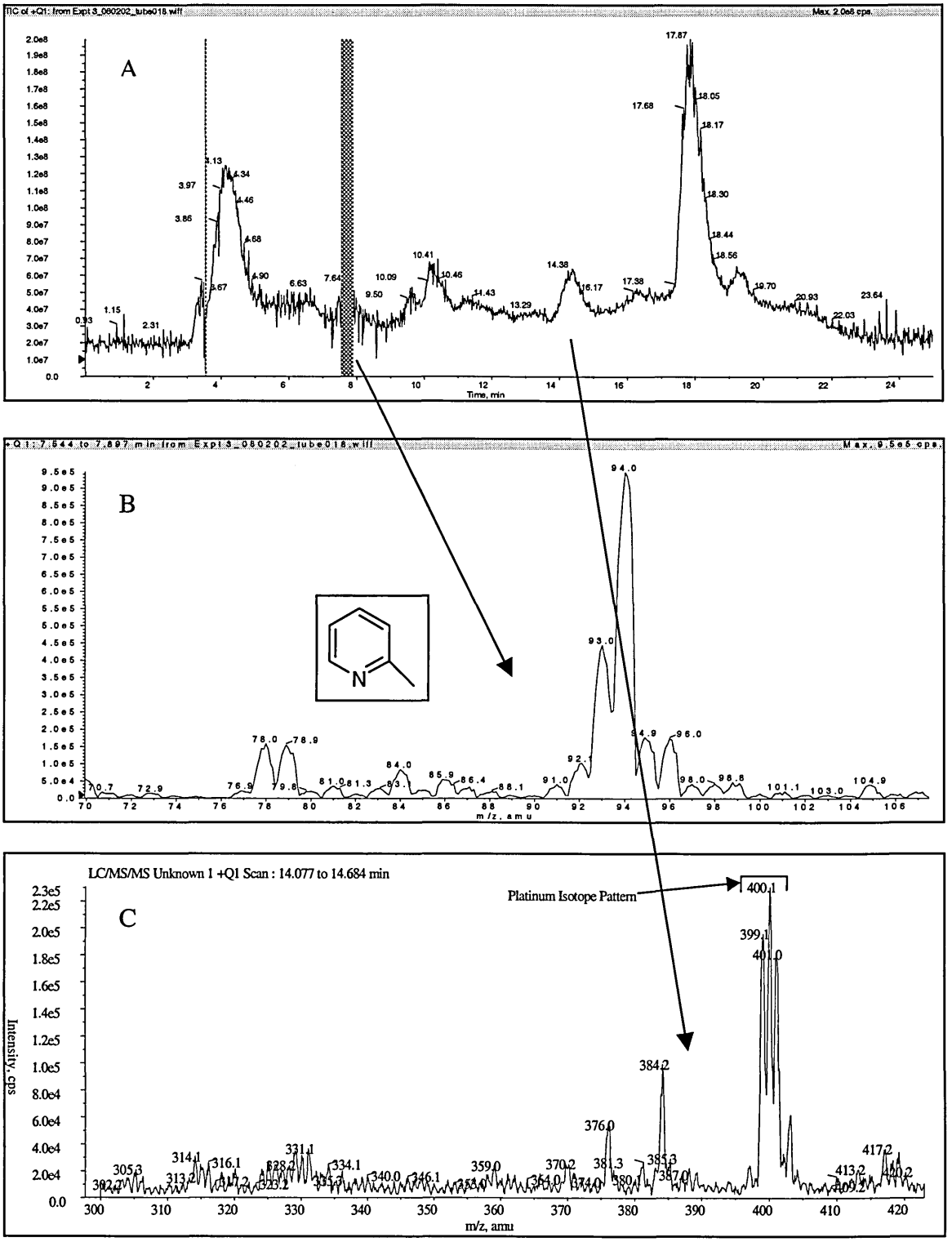


Figure 3.11 A. HPLC-MS TIC from a 0-24 hour pooled urine sample following a single dose of [^{14}C]-ZD0473 at 30 mg/kg to a male rat. B. Mass spectrum of 2-picoline. C. Mass spectrum of unknown showing Pt-isotope pattern near 400 amu. For experimental conditions see section 3.3

3.11: Study conclusions (rat urine profiling experiment)

The primary objective was to obtain HPLC profiles of platinum and radio-activity in rat urine samples taken from studies following administration of [^{14}C]-ZD0473 as single doses at 30 mg/kg and multiple, once daily intravenous doses at 5 and 7.5 mg/kg/day over 5 days. A second aim was to identify components where possible.

Samples were analysed by HPLC interfaced with radio-detection, ICP-MS for platinum detection and MS for tentative identification.

Following single and multiple dosing regimes, 3 major radio-labelled components and 2 major and at least 2 minor platinum containing components were detected in rat urine, as summarised in Table 3.6.

ZD0473 was detected in both [^{14}C] radio-activity and ICP-MS-Pt chromatograms.

One of the components in the [^{14}C] trace was absent from the platinum trace and was identified by LC-MS as 2-picoline suggesting cleavage of the platinum to 2-picoline bond in the ZD0473 molecule.

Two components which eluted later than ZD0473 were detected. The first of these components contained both platinum and [^{14}C] labels indicating the platinum to 2-picoline bond to be intact. HPLC-MS showed the molecular weight of this component to be greater than ZD0473 suggesting it to be a conjugate or adduct.

Further work is required to establish the metabolism of ZD0473 in the rat.

3.12: Conclusion

HPLC-ICP-MS provided a sensitive and specific method for the detection of the anticancer drug ZD0473 and its Pt-containing impurities and metabolites in formulations and biological fluids. The use of ICP-MS and a radio activity detector in parallel enabled the simultaneous detection of radioactivity and Pt providing a more complete and robust profile of components in samples than would have been possible using either technique on its own. Addition of an HPLC-MS into the system to provide molecular mass information aided identification of the peaks observed with the other techniques. This combination showed clear benefits in that components were observed which contained only Pt or [^{14}C] giving both quantification and identification.

Some of data presented within this chapter were published in Smith etal, Chromatographia **55**, 2002, 151-155. (appendix A).

Chapter 4

HPLC-ICP-MS and HPLC-ICP-MS-MSMS for the detection of carbon-containing compounds.

4.1: Summary

High performance liquid chromatography (HPLC) combined with inductively coupled plasma mass spectrometry (ICP-MS) has been studied as a means for the detection of carbon to provide a "universal" method for detecting organic compounds in chromatographic eluents. Initial interest was generated in the detection of carbon when analysis of a platinum anti cancer compound containing a [^{14}C]-label was carried out. The ICP-MS was set up to look for the [^{14}C]-element and a response was gained from a peak containing 25600 dpm from an initial loading of 1.37 x 10^6 dpm. An attempt was therefore made to try to detect the non-radioactive isotopes of carbon. Carbon is particularly difficult to ionise and the amount of carbon present in normal chromatographic systems leads to high backgrounds, making detection a challenge. Novel separation approaches were therefore employed, using either entirely aqueous eluents (at temperatures of 60 and 160°C, dependent on the column used) to eliminate the organic modifier completely, or isotopically enriched solvents. For the aqueous eluents, detection limits for sulphanilamide were found to be 1.13 μmol (0.47 μmol of carbon), injected on a conventional 4.6 mm i.d. column. The use of a narrow bore column with highly isotopically enriched [^{12}C]-methanol (99.95 atom%) as organic modifier for the mobile phase enabled the detection of 86 μmol for [^{13}C]-triple-labelled caffeine and 79 μmol for [^{13}C]-double-labelled phenacetin. The sensitive

detection of [^{12}C]-compounds with [^{13}C]-enriched methanol as organic modifier proved impractical due to a lower level of isotopic purity (99 % atom) of this solvent, with the residual [^{12}C]-methanol resulting in significant interference.

4.2: Introduction

The combination of ICP-MS with liquid chromatography has resulted in renewed interest in element specific detection in HPLC. In addition to the detection of metals such as platinum [5, 54], the technique has been found to be suitable for the detection of elements such as bromine [6, 41], chlorine [55], iodine [8, 56], phosphorus [16, 42] and sulphur [9, 57, 58] for both quantitative and qualitative analysis.

As part of an investigation on the use of HPLC-ICP-MS for drug analysis the ability of HPLC-ICP-MS to detect a [^{14}C]-labelled anti-cancer compound in a formulation [39], based on the presence of $^{14}C^+$ was investigated. The specific detection of $^{14}C^+$ by ICP-MS was possible because of the low natural abundance of this isotope. This meant that interference from endogenous carbon, for example from the HPLC mobile phase, was negligible. Despite the fact that the resolving power of quadrupole ICP-MS instruments is insufficient to separate the masses $^{14}N^+$ (14.0031) and $^{14}C^+$ (14.001), the addition of H_2 to the collision cell to remove $^{14}N^+$ allowed $^{14}C^+$ to be detected with good specificity. These results led us to consider the possible use of ICP-MS to provide a "universal" method for the detection of organic compounds based on the detection of the more abundant isotopes of carbon (as has been investigated to some extent in HPLC-AES [59]). Such a technique would potentially be useful in that it would enable the amounts of individual components in a mixture

(e.g. from combinatorial synthesis) to be determined in the absence of authentic standards or knowledge of e.g. molar UV extinction coefficients etc. Such information is currently sought using e.g. chemiluminescent nitrogen detectors (CLND) for N and S-containing compounds, or evaporative light scattering detectors (ELSD) [60], but similar results should be obtainable though HPLC-ICP-MS with carbon detection. Such information might be especially valuable when combined with some means of determining the molecular mass of analytes, such as mass spectrometry, to enable molar responses to be calculated. The problem with the detection of $^{12}C^+$ and $^{13}C^+$ is that they are omni-present, from the organic modifiers used for conventional chromatography. This leads to high background interferences, so that sensitive detection is not possible. However, analysis of the more common isotopes of carbon might be possible if such interferences could be removed from the system. One possible answer explored here is the use of entirely aqueous eluents, including superheated water [61], where the mobile phase consists entirely of water, with separation controlled by changing the column temperature. Other background interferences can be controlled by chemical reaction with collision gases in the quadrupole [23]. An alternative way of reducing background interferences to manageable levels, also investigated here, is the use of ^{12}C or ^{13}C enriched solvents for the detection of [^{13}C]-enriched and normal [^{12}C]-containing compounds respectively.

4.3: Detection of [^{14}C] by ICP-MS

In addition to the detection of ^{195}Pt as described in chapter 3, we also undertook an investigation of the potential of HPLC-ICP-MS to detect the $^{14}C^+$ isotope present in [^{14}C]-ZD0473. Detection of $^{14}C^+$ is subject to two major difficulties namely; 1) high background at mass 14 from the presence of $^{14}N^+$ ions, and 2) relatively low sensitivity due to the high ionisation energy of carbon.

The atomic mass of $^{14}N^+$ is 14.00307 amu whereas the atomic mass of $^{14}C^+$ is 14.00324 amu. Therefore, the resolution required to separate these two ions by mass spectrometry is $m/\Delta m = 82371$. Commercially available high-resolution ICP-MS systems do not offer much higher resolution than $m/\Delta m = 10000$. As such, it is impossible to separate $^{14}N^+$ from $^{14}C^+$ by high resolution ICP-MS. However, the ICP-MS instrument used in this study, albeit being a quadrupole based system with a nominal resolution of $m/\Delta m = 400$, is equipped with a hexapole collision / reaction cell. It was therefore decided to investigate the potential of using this cell to chemically separate the $^{14}C^+$ and $^{14}N^+$ ions. Literature data on ion / molecule reactions clearly show that C^+ ions do not react with H_2 whilst the reaction of N^+ and H_2 ($N^+ + H_2 \rightarrow NH^+ + H^+$) is very efficient [52]. In agreement with these data, we observed that the addition of 4 mL/min of H_2 in the cell resulted in a virtually complete conversion of the N^+ ions into NH^+ without significantly affecting the sensitivity for $^{14}C^+$.

Because the first ionisation energy of C is high (11.3 eV) the population of C^+ ions formed in the ICP is relatively low (c.a. 5%). Therefore, the sensitivity of ICP-MS

for $^{14}C^{+}$ is about 20 times lower than it is for elements with low ionisation energies (where the ionisation efficiency is nearly 100%). However, it is possible to optimise for maximum sensitivity for $^{14}C^{+}$ by adjusting the plasma operating conditions (though changes in the argon gas flows) and the ion extraction conditions (via changes the cone voltages within the Masslynx software).

As a result of tuning the instrument and using H_2 in the collision cell, it was possible to detect $^{14}C^{+}$ in the samples under investigation. The chromatogram shown in Figure 4.1 was obtained for radio-labelled ZD0473 in the IV formulation with initial loading of 1.36×10^{6} dpm. When this quantity was injected onto the column a measurement of 25,600 dpm was found. It is apparent from this chromatogram that minor peaks were not observed and that in its current state of development ICP-MS detection could not be used alone for the determination of both ^{195}Pt and $^{14}C^{+}$, nevertheless the detection of the radiolabel ^{14}C associated with the main peak of ZD0473 was promising.

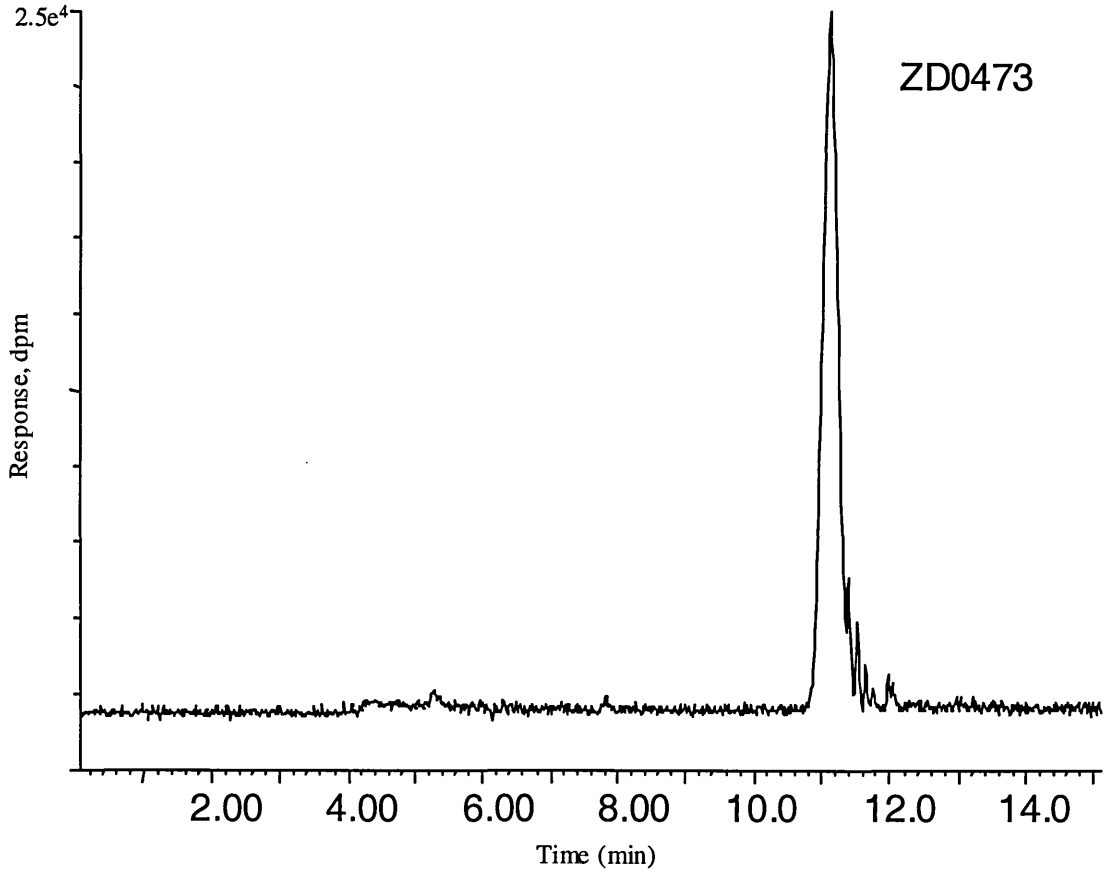


Figure 4.1 HPLC-ICP-MS chromatogram obtained for the monitoring of the radio label [^{14}C] at *m/z* 14 after 100 µL injection of 0.5 mg/mL (1.37 x 10^6 dpm) ZD0473 standard. 95 % of the molecule was labelled and a response of 25,600 dpm was achieved. For experimental conditions see section 3.3.

The work carried out on $^{14}C^{+}$ showed it was possible to detect carbon using ICP-MS. This was the first time the ICP-MS had been used in this manner and the data were published in Chromatographia [39]. The full potential of the HPLC-ICP-MS for this type of work was further investigated, utilising new modes of operation to allow the detection of $^{12}C^{+}$ and $^{13}C^{+}$.

4.4: Experimental

4.4.1: Reagents

Sulphanilamide, caffeine, antipyrine, and paracetamol (acetaminophen) used in this study were obtained from Sigma-Aldrich (Dorset, UK) at a purity of 99 %. The [^{13}C]-enriched caffeine (^{13}C-triple-labelled, 99 atom %), phenacetin (^{13}C-double-labelled, 99 atom %) and the [^{12}C]-enriched methanol (99.95 atom %) were also supplied by Sigma-Aldrich. The [^{13}C]-enriched methanol (99 atom %) was obtained from Cambridge Isotope Laboratories (Andover, USA).

4.4.2: Standard Solution Preparation

Caffeine, antipyrine and paracetamol stock solutions were prepared in water at a concentration of 5 mg/mL in 10 mL volumetric flasks with gentle heating to aid dissolution. Further dilutions were prepared in water to the concentrations of 1 and 0.1 mg/mL. [^{13}C]-enriched caffeine and phenacetin were dissolved in [^{12}C]-enriched methanol / water (1:1) mixture to a concentration of 1 mg/mL.

4.4.3: Preparation of standard curve and quality control samples.

A standard curve was constructed for caffeine with quality control data also obtained at each calibration point by injecting the sample six times. The caffeine standard curve was prepared at concentrations of 10, 5, 2, 1, 0.75, 0.5, 0.25, 0.1 and 0.05 mg/mL. A similar experiment was carried out for the [^{13}C]-labelled caffeine with concentrations of 1, 0.5, 0.3, 0.15 and 0.075 mg/mL used to construct the standard curve.

4.3.4: HPLC-ICP-MS and HPLC-ICP-MS-MSMS

The HPLC-ICP-MS was set up in three different configurations as described below.

Chromatography for the ICP-MS with entirely aqueous solvent systems was performed using a Jasco PU-1580 HPLC pump (Jasco Ltd, Great Dunmow, UK) with samples introduced via a PE 200 autosampler (PerkinElmer Ltd, Beaconsfield, UK). Column heating was carried out using an Eppendorf TC-50 column heater (Presearch, Hitchin, UK). ICP-MS was performed on a Platform ICP-MS, which used a hexapole collision / reaction cell for simultaneous measurement of the carbon isotopes (GV Instruments, Wythenshawe, UK). MassLynx software was used for instrument control, data acquisition and data handling. Flow rates of the nebuliser gases and operating conditions of the ICP-MS are shown in Table 4.1.

Table 4.1 Instrument operating conditions for the ICP-MS

Cooling gas	16.00 L/min	Plasma Power	1350 w
Plasma gas	0.65 L/min	Acquisition mode	SIR
Nebuliser gas	0.75 L/min	Dwell time	200 ms
Helium gas	1 mL/min	Masses monitored	12, 13
Hydrogen gas	4.0 mL/min	Total analysis time	7 min

For some experiments, see below, an API-365 MS (Applied Biosystems, Warrington UK) was connected in parallel via an in line splitter to a turbo ionspray inlet source, scanning over 100 to 400 *m/z*. Analyst software was used for the instrument control, data acquisition and data handling. The system was connected into the HPLC-ICP-MS system via PEEK tubing (0.13 mm) with the flow controlled by an accurate splitter (Presearch, Hitchin, UK).

4.4.4: Aqueous Separations at 60°C

The separation of caffeine, antipyrine and paracetamol with an aqueous mobile phase at 60°C was performed with 20 µL injection into a Discovery Zirconia-PDB 150 x 4.6 mm, 5 µm column (Supelco, Bellefonte USA) at a flow rate of 1 mL/min. The mobile phase was heated to 60°C and for this experiment the flow was split using an accurate splitter with 700 µL/min to an API-365 mass spectrometer, and 300 µL/min to the ICP-MS using a concentric nebuliser with a double pass spray chamber (GV Instruments, Wythenshawe, UK), for sample introduction. The flow into the mass

spectrometer was adjusted to allow 200 μL/min to enter the turbo ionspray source via a 1 to 5 splitter.

4.4.5: Aqueous Separations at 160°C

The separation of caffeine, paracetamol and phenacetin was also performed using superheated water at 160°C as the mobile phase on an XTerra C8 150 x 4.6 mm 5 μm column (Waters, Watford, UK) at a flow rate of 1 mL/min with an IS6000 ultrasonic nebuliser (Cetec Technologies, Omaha, Nebraska, USA) for sample introduction to the ICP-MS. The injector was flushed with 100 μL of water between samples with an injection volume of 20 μL.

4.4.6: Application to Caffeine Determination in Coffee

Instant coffee, 20 mg, (Carte Noire), purchased from a local supermarket, was dissolved in water, with gentle heating to aid dissolution. An aliquot (100 μL) of this sample was analysed by HPLC-ICP-MS using an XTerra column operated at 160°C and a flow rate of 1 mL/min as described above.

4.4.7: Separations with Isotopically Enriched Solvents

For both the [^{12}C] and [^{13}C]-istopically enriched mobile phases a microbore chromatographic system was used. Jasco PU-1580 pumps were used for solvent delivery at a flow rate of 0.1 mL/min. Separations were performed on a Polaris 50 x 1 mm i.d., 3μ column (Varian, Lake Forest, USA) with injection volumes of 10 μL

from a PerkinElmer 200 series autosampler. Mobile phases were made up of the enriched [^{12}C] or [^{13}C] solvent with water (20/80 V/V).

Gradient elution was also employed for the chromatography of phenacetin (from 95 % water 5% enriched methanol to 50 % water 50 % enriched methanol linearly over 10 minutes).

4.5: Results and Discussion

It was possible to detect $^{14}C^{+}$ in the eluent from an HPLC column using ICP-MS [39] because of the absence of significant interference from background levels of $^{14}C^{+}$ in the organic modifier used for the eluent. However, for sensitive detection of the naturally abundant isotopes of carbon, the ICP-MS clearly has to be operated in such away that as much of the external carbon interference as possible is eliminated to minimise background levels. One strategy to achieve this is the use of entirely aqueous eluents, as this removes organic modifier that would otherwise make carbon detection impractical. This is possible for a limited number of polar analytes and this range can be extended by performing chromatography at elevated temperatures (e.g. above 100°C with so-called superheated water [61]). Indeed, in many ways water provides the prefect chromatographic eluent to use with ICP-MS since there are no organic components present to quench the plasma.

4.5.1: HPLC-ICP-MS with Entirely Aqueous Mobile Phases.

Initial experiments with sulphanilamide, using superheated water chromatography at 160°C on the XTerra phase, confirmed the principle and indicated that a detection limit of ca 1.13 μmol of material could be achieved (470 nmol of carbon). Under these conditions sulphanilamide eluted rapidly from the column, with a retention time of just over 2 min. as shown in Figure 4.2a. These early investigations highlighted the importance of eliminating organic solvents from the system if possible. Thus, in one experiment, the compound was taken up in methanol : water (5:95 % V:V) and then diluted with water (1:10) for injection on to the system. As shown in Figure 4.2b, whilst the peak corresponding to analyte is still clearly visible it is superimposed on a much larger, and very broad, peak due to the residual methanol present in the injection solvent. It is noteworthy that the methanol peak required ca. 40 minutes of elution before it returned to baseline again (Figure 4.2 C). Thus the required solvent for both sample dissolution and chromatography is clearly water, though this does place constraints on the type of compounds for which this strategy would be suitable. We also noted that when the PEEK tubing was used at these high operating temperature it showed signs of degradation, which caused an elevated background level. Steel stainless tubing was therefore substituted to minimise this problem.

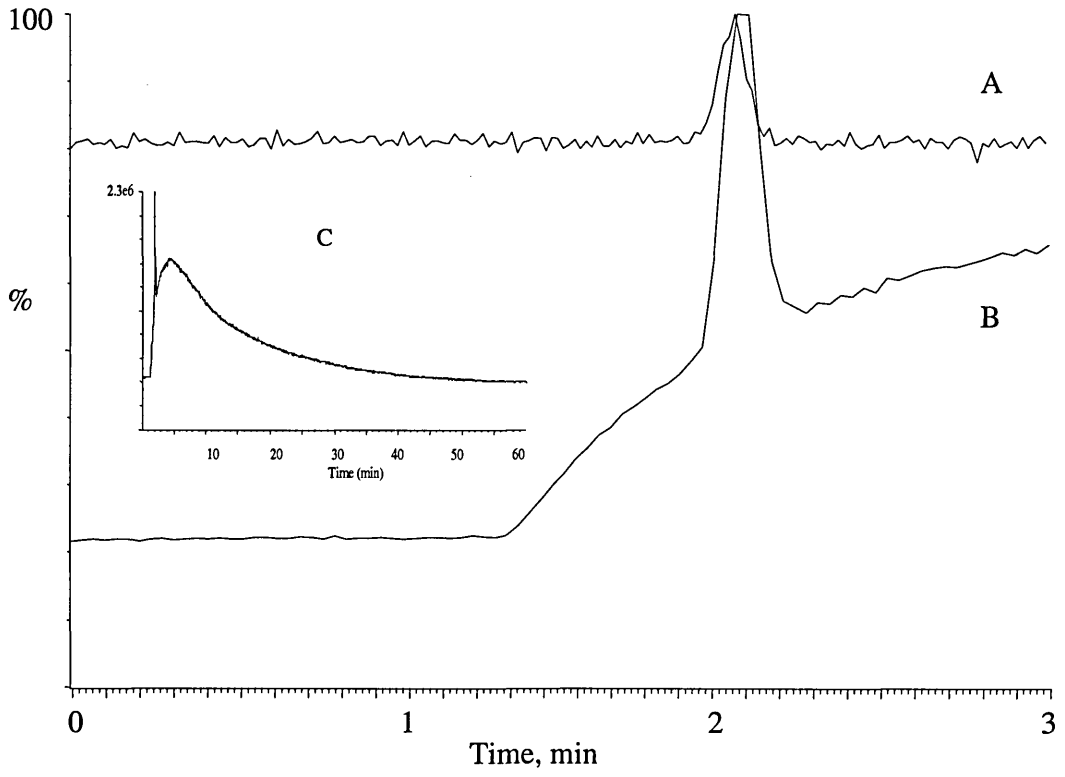


Figure 4.2. (A) ^{12}C HPLC-ICP-MS chromatography of 2.26 µg of sulphanilamide (1.13 µmol, 470 nmol of carbon) on an Xterra column at 160°C and 1 mL/min. (B) The same analysis for a sulphanilamide sample made up in 0.5 % methanol on the same time span for comparison. The insert, chromatogram (C), shows the baseline disturbance due to the methanol produced in chromatogram (B).

Chromatography was then performed on a mixture of three model compounds, caffeine, paracetamol and antipyrine, on Zirconia-based and XTerra stationary phases, with pure water as the mobile phase, and column oven temperatures of 60°C and 160°C respectively. Representative chromatograms are shown in Figures 4.3a and b for XTerra and Zirconica with the phases producing similar separation and peak shape.

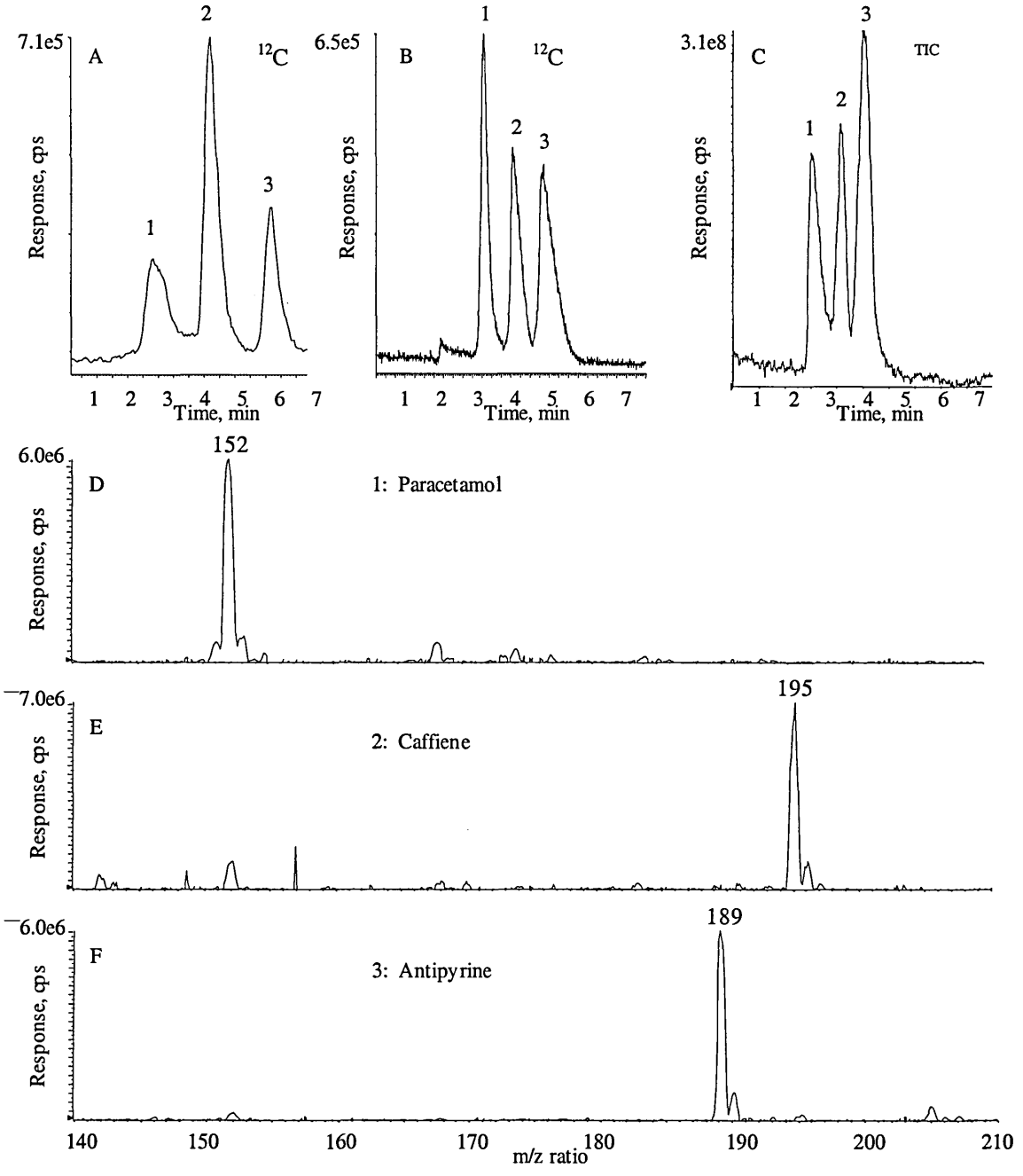


Figure 4.3. (A) ^{12}C HPLC-ICP-MS chromatogram showing the separation of paracetamol (1), caffeine (2) and antipyrine (3) on a Xterra column at 160°C. (B, C) Chromatograms showing the detection of the same compounds, separated on a Zirconica column at 60°C, detected by ICP-MS and MS, respectively. (D, E, F) Mass spectra obtained for the three compounds.

The response of the ICP-MS to $^{12}C^{+}$ for these compounds was found to be linear over the range examined (16 to 320 μmol) with detection limits down to 1.28 μmol (0.64 μmol of carbon) calculated for both columns and temperatures. Whilst not investigated exhaustively, reproducibility was examined by multiple injections of standards (six replicates) at a concentration of 16 μmol on column for the Zirconia column at 60 °C. The standard deviations obtained for this experiment were ca. 6.8, 7.8 and 6.5 % for paracetamol, caffeine and antipyrine respectively.

Clearly, as detection in the ICP-MS is proportional to the amount of carbon in each compound, the potential exists for the quantitative analysis of compounds in a mixture as, if the atomic composition of the compound is known, an absolute value can be obtained for the response, which relates to the amount of carbon present in the molecule. Table 4.2 shows an example of this with the area response obtained proportional to the amount of carbon atoms present divided by relative molecular mass (RRM) in each compound. This information would allow the amount of compound to be quantified relative to an external standard. With unknowns the molecular masses of the individual components could be obtained by dividing the effluent from the column between the ICP-MS and a conventional MS, as described by O'Corcoron etal [62]. To illustrate this, an API-365 triple quadrupole mass spectrometer was connected into the system by splitting the eluent from the column to the ICP-MS as described in the experimental section. This system was used to obtain simultaneous HPLC-ICP-MS and MS data from the mixture of paracetamol, caffeine and phenacetin, separated on the Zirconia-HBD phase. Figure 4.3c shows the total ion chromatogram obtained from a scan over the range 100 to 400 *m/z* representing

the three peaks of paracetamol, caffeine and antipyrine. The resulting mass spectra are shown in Figures 4.3 d, e and f giving the molecular masses of the components.

Table 4.2 Area response obtained proportional to the amount of carbon present in each compound.

Compound	No. of Carbons present	Mass With formulae	Carbon atoms / MW Ratio	Area Response
Paracetamol	8	152: $C_8H_9NO_2$	0.053	18278
Caffeine	8	194: $C_8H_{10}N_4O_2$	0.041	14885
Antipyrine	11	188: $C_{11}H_{12}N_2O$	0.058	21109

The XTerra column, operated at 160°C, was used to determine the amount of caffeine in a coffee sample. The chromatogram shown in Figure 4.4 shows a large endogenous peak, with the caffeine peak detected on the tailing edge, at a retention time of 5.5 minutes. By comparison to an external standard an amount of ca. 2 µg of caffeine on column was calculated for the response (100 µg/g of instant coffee). For a quantitative method further work would clearly be required to separate the caffeine peak from the endogenous interferences.

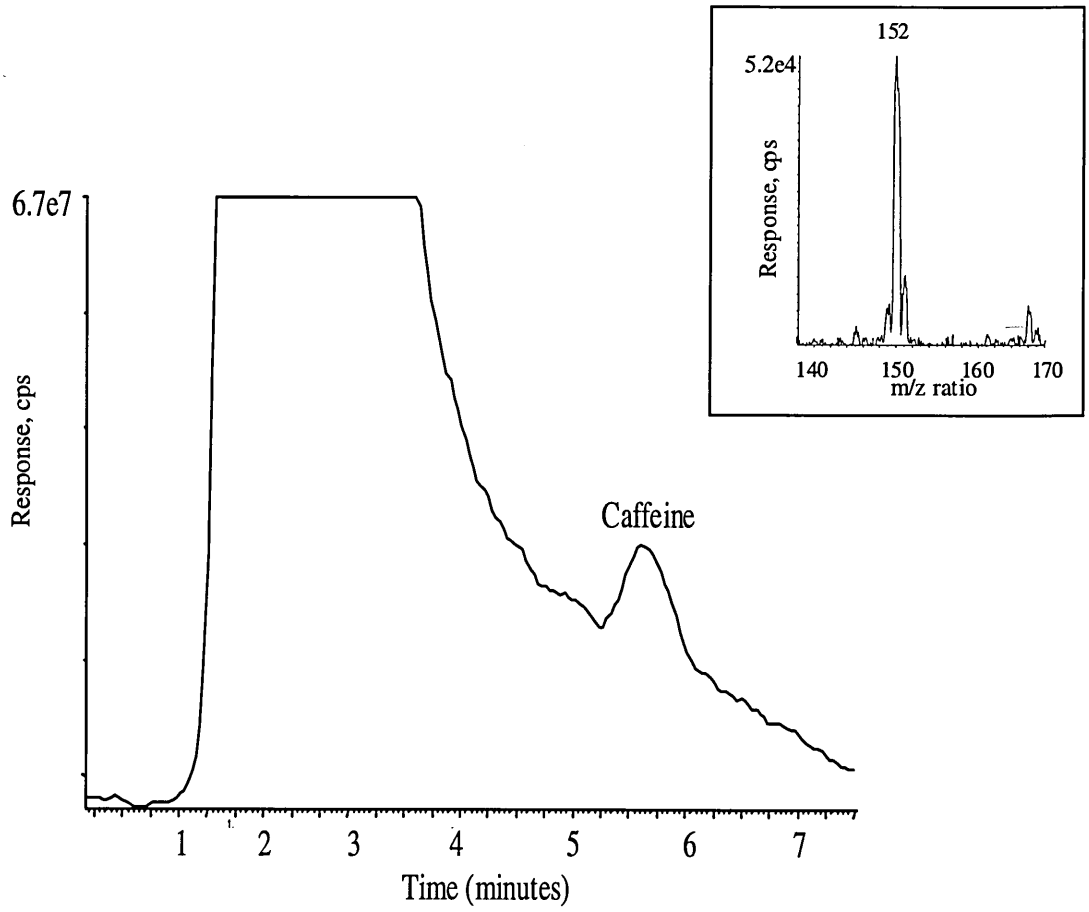


Figure 4.4. Instant coffee sample analysed on an XTerra column at 160°C and 1 mL/min using HPLC-ICP-MS, ca. 2 µg on column of caffeine (100 µg/g 'instant coffee'). The insert shows the $[M-H]^+$ ion for caffeine obtained by parallel MS.

4.5.2: HPLC-ICP-MS With Isotopically Enriched Eluents

Although the aqueous mobile phases described above have many advantages for ICP-MS there are clear limitations on the types of compound that can be separated in this way. With this in mind alternative chromatographic separations were investigated using isotopically enriched methanol as the organic modifier for chromatography. Both [^{12}C] and [^{13}C]-enriched methanol can be obtained commercially and we therefore investigated the detection of [^{13}C]-labelled analytes in a [^{12}C]-solvent as well as [^{12}C] detection of compounds in a [^{13}C]-mobile phase. As the cost of such enriched solvents is not insignificant chromatography was performed on a narrow bore column in order to minimise solvent consumption. This had the added benefit that the low flow rates employed reduced the amount of methanol in the plasma, thereby aiding detection.

Highly isotopically enriched [^{12}C]-methanol (99.95 atom %) was used as the organic modifier for the detection of [^{13}C]-triple-labelled caffeine and [^{13}C]-double-labelled phenacetin. The high degree of enrichment of the [^{12}C]-labelled methanol used to make up the organic modifier meant that the interference on the *m/z* 13 channel was negligible. An example of this is shown in Figures 4.5a and b where the peaks for the [^{13}C]-labelled caffeine (86 µmol of ^{13}C on column) and phenacetin (79 µmol of ^{13}C on column) are shown.

When gradient chromatography was investigated an increase in the background was noted as the proportion of organic modifier was increased, despite the high isotopic purity of the solvent. An example of the use of gradient HPLC-ICP-MS for the detection of [^{13}C]-labelled phenacetin is shown in Figure 4.5c.

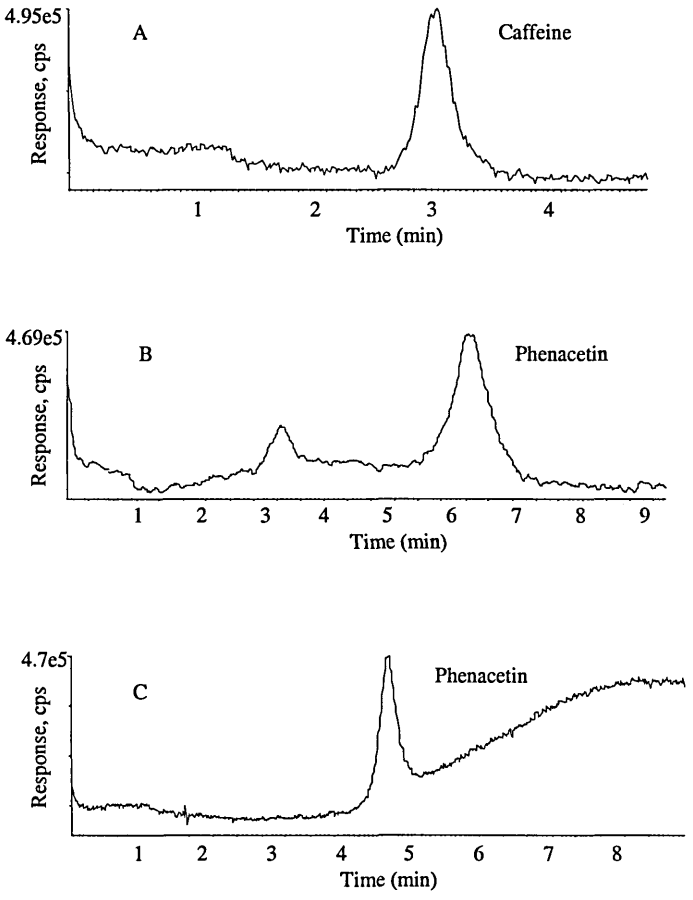


Figure 4.5. HPLC-ICP-MS traces obtained for (A) a $^{13}C^{+}$ caffeine peak 86 μmol for the three ^{13}C- labels, (B) a $^{13}C^{+}$ phenacetin peak 79 μmol for the two [^{13}C]-labels. (C) Shows the chromatography of phenacetin using gradient elution with the enriched solvent. The rising baseline reflects the changing solvent composition. For experimental conditions see section 4.4.

These preliminary experiments clearly demonstrate the potential for the detection of [^{13}C]-enriched analytes in reversed-phase eluents. However, when [^{13}C]-enriched methanol was used in an attempt to detect un-enriched $^{12}C^{+}$ carbon similar success was not achieved. This was due to the lower degree of isotopic enrichment in the [^{13}C]-methanol (99 atom %) compared to the [^{12}C]-methanol. Thus, the residual 1 % of [^{12}C]-methanol that remained in the [^{13}C]-enriched methanol ensured that there was a significant $^{12}C^{+}$ signal that precluded detection of the analytes at these concentrations. No examples of chromatograms are available because no peaks were observed.

4.6: Conclusion

The results presented here demonstrate that HPLC-ICP-MS can be used for the detection of carbon under a limited set of analytical conditions, and under these conditions HPLC-ICP-MS can provide a "universal detector" for organic compounds. In practice, given both the expense and lack of high enough levels of isotopic enrichment, only those separations based on entirely aqueous eluents are likely to be economically viable. The combination of carbon detection via HPLC-ICP-MS-MSMS, for molecular mass determination and structure elucidation, may prove attractive for the quantification and identification of unknowns.

The data presented in this chapter was published in Rapid Communications in Mass Spectrometry **18**, 2004, 1487-1492. (Appendix A).

Chapter 5

Halogen detection by HPLC-ICP-MS for metabolite profiling of Cl, Br and I containing compounds.

5.1: Summary

In order to determine the pharmacological and toxicological properties of xenobiotics in biological systems it is often necessary to determine the disposition and metabolic fate of the compound in experimental animals and man. In the initial phases of determining the metabolism of new compounds the first step is to develop analytical methods that enable the detection and quantification of metabolites in biofluids and excreta, followed by identification of any unknowns. This process usually takes the form of placing a radiolabel in the structure of the compound and carrying out profile experiments looking at the label and quantifying the response. As previously described, platinum within a compound could be used as the label, which can be utilised to follow the metabolism of a compound [39]. However the numbers of compounds used in the pharmaceutical industry, which have a metallic element, are few and therefore the use of ICP-MS for metal detection is of limited use. Halogens, however, are more often found in compounds, and would make the use of ICP-MS on attractive technique. Studies were set up to look at Br, Cl and I and their use within metabolism work with HPLC- ICP-MS detection in parallel with a mass spectrometer to aid identification of metabolites.

5.2: Introduction

The coupling of HPLC to ICP-MS has opened a new tool for metabolism studies. The ability to assign quantitative information to peaks found in the profile, without the need to produce a standard to quantify against or make a labelled compound for dosing, is extremely useful. Metallic elements such as platinum are relatively easy [39, 54], but application of the technique is then limited due to the small number of compounds containing such elements.

The evaluation of a halogen as the detection element was therefore considered because these elements are more prevalent in novel compounds. The main difficulty in using the halogens compared to metals is the lower sensitivity of detection with ICP-MS. The ionisation energy of iodine is 10.4 eV, resulting in an ionisation efficiency of between 14 and 33 %. For bromine and chlorine, ionisation efficiencies are only 18 and 5 % respectively, due to high first ionisation energies of 11.8 eV and 13.0 eV. Fluorine was not considered because the ionisation potential of the element is 17.4 eV, is greater than that of the carrier gas argon (15.8 eV), therefore rendering fluorine ionisation virtually impossible. Instruments available at the present do not allow for detection in negative mode therefore F^- cannot be examined.

Another complication that has to be taken in to account is that the halogens are affected by polyatomic interferences. Isobaric interferences are probably the largest class of interferences in ICP-MS and are caused by atomic or molecular ions that have

the same mass to charge ratio as the analytes of interest. The polyatomic interference that cause problems for the halogens are shown in Table 5.1 [63]

Table 5.1 Possible spectral interferences for halogen elements

Halogen Isotope	Polyatomic interferences		
^{79}Br	$^{40}Ar^{39}K^{+}$	$^{31}P^{16}O_3^{+}$	$^{38}Ar^{40}Ar^{1}H^{+}$
^{81}Br	$^{32}S^{16}O_3{}^{1}H^{+}$	$^{40}Ar^{40}Ar^{1}H^{+}$	$^{33}S^{16}O_3^{+}$
^{35}Cl	$^{16}O^{18}O^{1}H^{+}$	$^{34}SH^{+}$	
^{37}Cl	$^{36}Ar^{1}H^{+}$	$^{36}S^{1}H^{+}$	

Isobaric interferences are not such a problem when considering iodine since the region of *m/z* 127 is clear of such problems, therefore allowing lower limits of detection for compounds containing this element.

Bromine and chlorine do suffer from isobaric problems, which affect the efficiency of the assay to detect the elements. These problems can be controlled by the use of the instrument collision cell, which allows gases into the hexapole region of the ICP-MS [24]. These gases, such as hydrogen and helium, react with the polyatomic interferants and stop them from reaching the detector of the ICP-MS, allowing detection of the halogens.

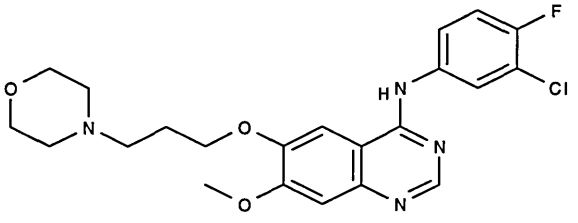
An additional manner of controlling interferences is to have knowledge of the type of polyatomic component causing the problem. Such areas as sample matrix, reagents

used in preparation, plasma gases and entrained atmospheric gases are responsible for introducing some isobaric problems. Therefore if the mechanism of production of these components is known then the assay can be designed to preclude or at least reduce the possibility of their formation.

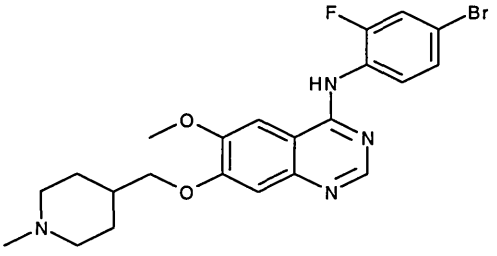
With this in mind an evaluation was undertaken of the usefulness of the halogens as elemental markers within the compound for balance and metabolism profiling studies.

5.3: Initial Evaluation of Bromine and Chlorine

Bromine and chlorine were considered initially because these elements are found more extensively in novel drug entities within the pharmaceutical industry. The compounds used are shown in Figure 5.1. They were chosen because they have similar structures and molecular weights, allowing for ease of comparison.



ZD1839
Molecular weight
483 amu



ZD6474
Molecular weight
479 amu

Figure 5.1 ZD1839 and ZD6126 development compounds from Astrazeneca used for the evaluation of bromine and chlorine detection.

5.4: Experimental

5.4.1: Standard Solution Preparation

The compounds were weighed and dissolved in methanol to a concentration of 1 mg/mL. These two solutions were then further diluted to a concentration of 10 μg/mL in water.

5.4.2: Instrumentation

Flow injections of 20 μL were made using by a PE 200 series autosampler (PekinElmer Ltd, Beaconsfield, UK) in a flow of water (1 mL/min) generated from a Jasco PU-1580 HPLC pump (Jasco Ltd, Great Dunmow, Uk). A meinhard concentric nebuliser used with a double pass spray chamber, which passed into a Platform ICP-MS (GV Instruments, Wythenshawe, UK). MassLynx software (version 3.4) was used to collect data and quantify the results (GV Instruments, Wythenshawe, Uk).

5.5: Discussion

The difficulty in studying halogens by ICP-MS is that the sensitivity is low because the ionisation potentials of bromine and chlorine are close to that of argon, as discussed in section 5.2. When coupled with the problem of isobaric interferences the usefulness of the approach may be called into question. Figure 5.2 represents the response of the two test compounds for chlorine and bromine. The trace shows that the response for bromine is significantly better than for chlorine, which is expected

since the ionisation energy for bromine is (11.8 eV) is lower than that for chlorine (13.0 eV), Figure 1.3 shows the percentage ionisation of elements in the plasma.

Sensitivity is also affected by the presence of isobaric polyatomic species and the collision cell was used to minimise the problem as described in section 5.2. However, the amount of the gas had to be monitored very closely, because an excess of gas added to the cell would not be a benefit. The gas may well react with the polyatomic interferences but it also inhibits the transition of the element of interest though to the multiplier for detection. A balance has to be found in the optimising step for all elements that require gas to aid detection.

Whilst the optimisation step was being carried out for chlorine, it was noted that the free radical of chlorine was able to react with hydrogen in the collision cell. This proved to be a beneficial since the chlorine collected two hydrogens, moving the detection masses from *m/z* 35, 37 to *m/z* 37, 39. This enabled the major isotope of chlorine (35) to be detected, but at *m/z* 37 where the interference was found to be less than at *m/z* 35. Table 5.2 shows the conditions used in the analysis of the halogens.

Table 5.2 Instrument operating conditions for chlorine and bromine analysis

Cool gas	16.00 L/min	Plasma power	1400 W
Intermediate gas	0.80 L/min	Acquisition mode	SIR
Nebuliser gas	0.60 L/min	Dwell time	0.5 s
Helium gas	2.0 mL/min	Masses monitored	37, 79
Hydrogen gas	2.0 mL/min	Total analysis time	4 min

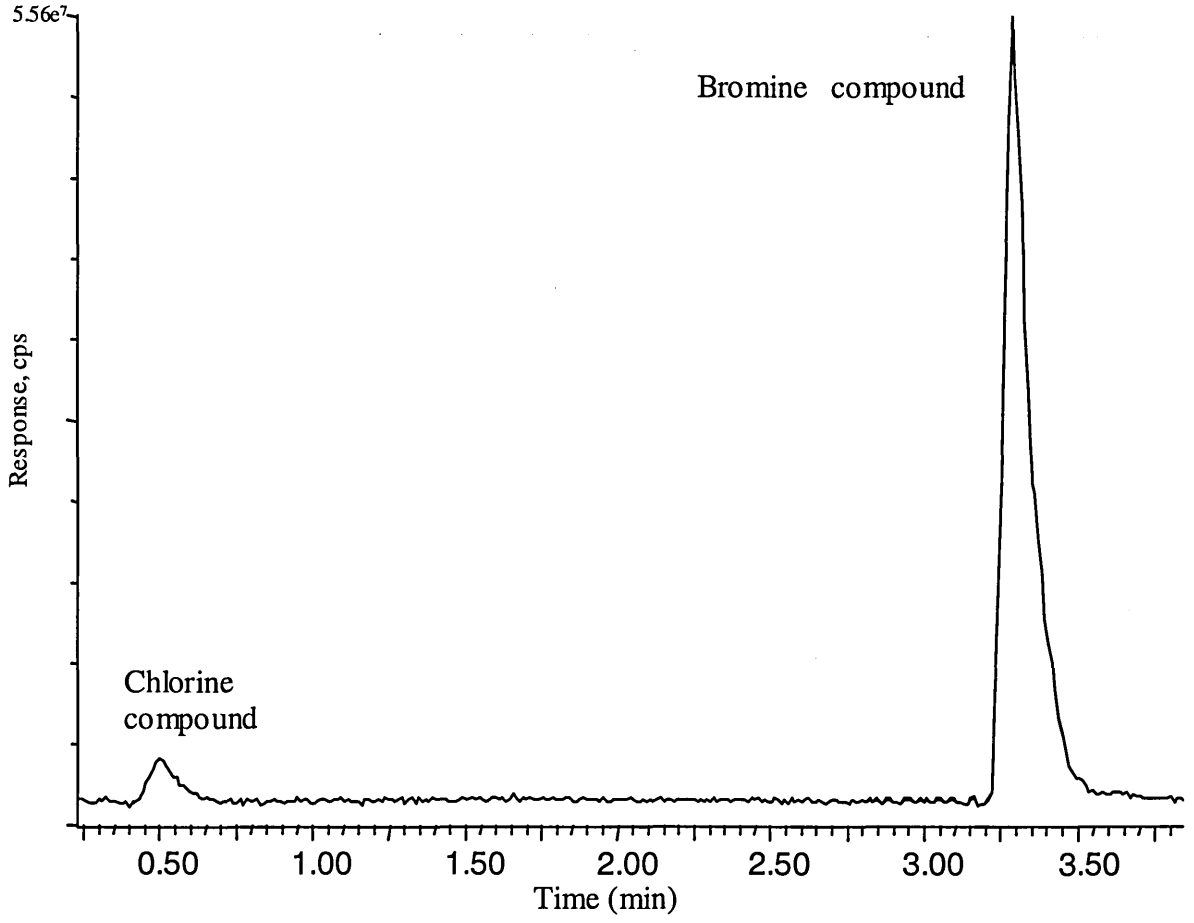


Figure 5.2 Flow injection ICP-MS responses for ZD1835 and ZD6474, with bromine monitored at *m/z* 79 and chlorine monitored at *m/z* 37 after reaction with hydrogen to allow the major isotope of chlorine to be monitored. Analysis carried out at a concentration of 10 μg/mL for both compounds. The signal to noise ratio for chlorine was 26 and for bromine was 686.

5.6: Conclusion

From this initial work it was decided that bromine would be a better candidate for ICP-MS detection and is therefore considered in detail first, since the data indicated that more sensitivity and value could be gained from the work. An excretion balance study in the rat was used as an example application.

5.7: Bromine excretion balance studies

5.8: Summary (4-Bromoaniline study)

In order to determine the disposition of dosed compounds, an "excretion balance" study is normally performed, whereby the amount of compound-related material is determined in the excreta. This provides quantitative information on the rate and routes of elimination of the drug or xenobiotic. For the metabolic profiling of novel drug candidates it is usually necessary for the drug to have a radiolabel placed in to its structure to enable accurate quantitative values for metabolite concentrations to be gained. The safety and toxicity issues associated with radiolabel production along with the expense means that if the same work could be done without a radiolabel then considerable benefit would ensue. The ICP-MS enables this process, in that instead of a [^{14}C]-label for profiling, an element within the structure of the compound may be used, in this case bromine.

5.9: Introduction (4-Bromoaniline study)

ICP-MS results in the complete atomisation of a biomolecule, and thus the detected signal is independent of the chemical properties of the parent molecule. For this reason, all of the element present in the sample should be detected and ICP-MS can be considered quantitative. 4-bromoaniline (Figure 5.3) was used as an evaluation compound to see if ICP-MS could offer an alternative for balance and metabolite profiling studies.

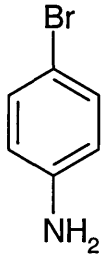


Figure 5.3 Structure of 4-Bromoaniline

5.10: Experimental (4-Bromoaniline study)

5.10.1: Reagents (4-Bromoaniline study)

4-Bromoaniline, purity 98 %, was obtained from Sigma-Aldrich Co Ltd (Dorset, UK). Methanol used for the chromatographic separation was high purity HPLC grade and, along with ammonium formate (purity 99 %) obtained from Fisher Scientific UK Ltd.

Water was obtained from an Elga water purification system (Elgastat Maxima, Elga, High Wycombe, UK).

5.10.2: Animal Dosing (4-Bromoaniline study)

Three male Wistar-derived rats (200-250 g) were fitted with biliary cannulae. Rats were permitted free access to food (R&M No. 1 Modified Irradiated Diet) and water throughout the study. The water was supplemented with glucose, NaCl and KCl for 24 hours prior to surgery until 48 hours after surgery, then with only NaCl and KCl until the end of the study. The rats were dosed with 4-bromoaniline, *i.p.*, at 50 mg/kg in ethanol-water (50:50), at a concentration of 50 mg/mL. Urine was collected over solid CO_2 into sterile containers 0-12, 12-24 and 24-48 hr post dosing. Bile was collected for the periods 0-6, 6-12, 12-24 and 24-48 hr post dosing. The samples were stored frozen at (-20°C) until analysis. At the end of the study, animals were killed by halothane inhalation, with death confirmed by cervical dislocation.

5.11: ICP-MS instrument settings (4-Bromoaniline study)

5.11.1: Instrumentation configuration (4-Bromoaniline study)

Ultra pure water was pumped though the system at 1 mL/min by a series 200 PerkinElmer pump and the sample was introduced by a series 200 PerkinElmer autosampler, with an injection volume of 50 µL. The sample was passed though a concentric nebuliser to a double pass spray chamber before entering the ICP-MS (GV instrument, Platform). The run time for the excretion balance samples was 1.5

minutes. The flow of the nebuliser gases and the operating and acquisition conditions of the ICP-MS are shown in Table 5.3. MassLynx software (version 3.4) was used for instrument control, data acquisition and handling.

Table 5.3 Instrument operating conditions for ICP-MS for excretion balance study

Cool gas	18.00 L/min	Plasma power	1600 W
Intermediate gas	0.80 L/min	Acquisition mode	SIR
Nebuliser gas	0.60 L/min	Dwell time	0.5 s
Helium gas	2.0 mL/min	Masses monitored	79, 81
Hydrogen gas	2.0 mL/min	Total analysis time	1.5 min
Hexapole auxiliary gas	0 mL/min		

5.11.2: Construction of Calibration Curves (4-Bromoaniline study)

Ten calibration standards were prepared of bromobenzene (Aldrich, purity ~ 99 %) in control (human) urine and in control (rat) bile. This compound was used because it was ready available and, as long as bromine is in the structure, any compound can be used for standard preparation. Concentrations ranged from 4 mg/mL of bromobenzene to 0.00004 mg/mL of bromobenzene (40 ng/mL) for both series of standards. This was carried out in duplicate for each standard and the average of the area under the peak (y axis) plotted against the bromine concentration (x axis) to give two straight-line graphs, one for bromine in urine, and one for bromine in bile (Figure 5.4 and 5.5).

5.11.3: Biofluid Analysis (4-Bromoaniline study)

Urine and bile samples from each timepoint from the one animal were centrifuged and either 25 μL or 50 μL injections made directly into the Platform ICP-MS, monitoring both ^{79}Br and ^{81}Br isotopes. The response was measured over 1.5 minutes. This was carried out in duplicate for each sample, and the equation from the relevant straight-line graph applied to half of the average of these two values (to take into account monitoring of both bromine isotopes [ratio 50.69:49.31] in the biofluid analysis, and monitoring of only one when analysing the calibration standards). Hence the bromine concentration could be calculated for 1 mL of urine / bile, which when multiplied by the total volume of biofluid excreted during that timepoint, gave the overall bromine excretion.

5.11.4: HPLC-ICP-MS (4-Bromoaniline study)

The HPLC system comprised an Alliance 2695 separation module (Waters Corporation), coupled to a Platform ICP-MS (GV Instruments, Wythenshawe, UK Ltd.) tuned to monitor ^{79}Br and ^{81}Br. In order to avoid excessive carbon build-up on the cones of the instrument, the nebuliser gas was mixed with 5 % v/v oxygen using an onboard mass flow controller. Additionally, oxygen was used as the hexapole auxiliary gas. The flow of the nebuliser gases and the operating and acquisition conditions of the ICP-MS are shown in Table 5.4. The chromatographic separation was carried out using a Hichrom H5BDS C_{18} column (250 x 4.6 mm) (Waters Corporation) with 5 μm particles using a linear reversed-phase gradient method based

on 0.01 M ammonium formate (solvent A) and methanol (solvent B) as follows; 0 to 10 min 95 % A, 10 to 35 min 5 to 60 % B, 35 to 40 min 60 to 80 % B, then from 40 to 50 min 80 to 5 % B. The flow rate was 1 mL/min, which was split to give 200 μL/min entering the MS. 20 μL injections of urine or bile samples from one animal were made. The samples were initially run using a fast gradient method with a run time of 15 minutes to ensure the plasma would be stable at high methanol concentrations and additionally for comparative purposes. MassLynx software was used for instrument control, data acquisition and handling.

Table 5.4 Instrument operating conditions for HPLC-ICP-MS of Br containing drugs

Cool gas	13.0 L/min	Plasma power	1450 W
Intermediate gas	0.7 L/min	Acquisition mode	SIR
Nebuliser gas	0.75 L/min	Dwell time	0.2 s
Helium gas	2.0 mL/min	Masses monitored	79, 81
Hydrogen gas	2.0 mL/min	Total analysis time	15.5 min
Hexapole auxiliary gas (O_2)	0.2 mL/min		

5.12: Results and Discussion (4-Bromoaniline study)

5.12.1: Excretion Balance

Figure 5.4 and 5.5 show the ICP-MS bromine response versus bromine concentration for the two series of standards in urine respectively. The calibrations were both linear

(for urine $R^2 = 0.998$, for bile $R^2 = 0.996$), demonstrating that no matrix suppression effects or background effects affected the calibrations. No extrapolations had to be carried out when analysing the biofluids since all bromine responses of the biofluid samples fell within the region of the calibrations standards analysed.

Tables 5.5 and 5.6 show that both the urine and the bile excretion data match the metabolite profiles when the areas of the different peaks are summed together. Figures 5.5 and 5.6 show the profile over the time course for both bile and urine. These data show the usefulness of the technique in quantitative mapping of the excretion pathways of drugs containing bromine.

5.13: Conclusion (4-Bromoaniline study)

The bromine excretion balance study described was undertaken in part at GV Instruments and showed that the use of bromine as a marker in the study was possible. The process was therefore repeated for 2-, 3- and 4-bromobenzoic acids.

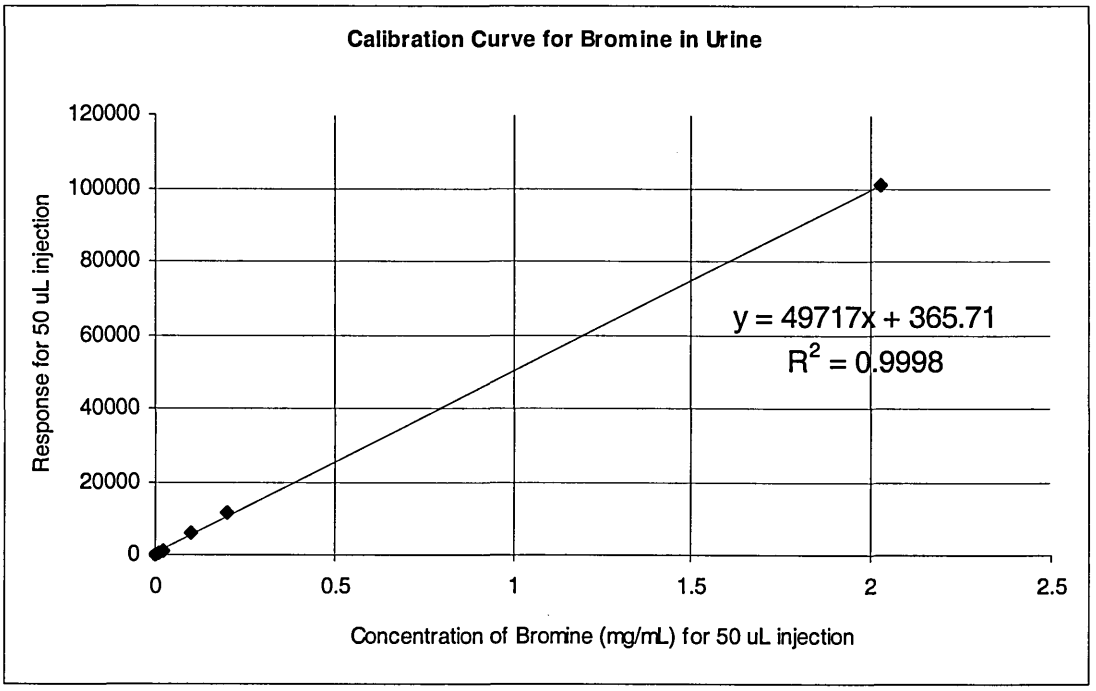


Figure 5.4: Bromine ICP-MS response versus concentration of bromobenzene in rat urine. For experimental conditions see section 5.10.

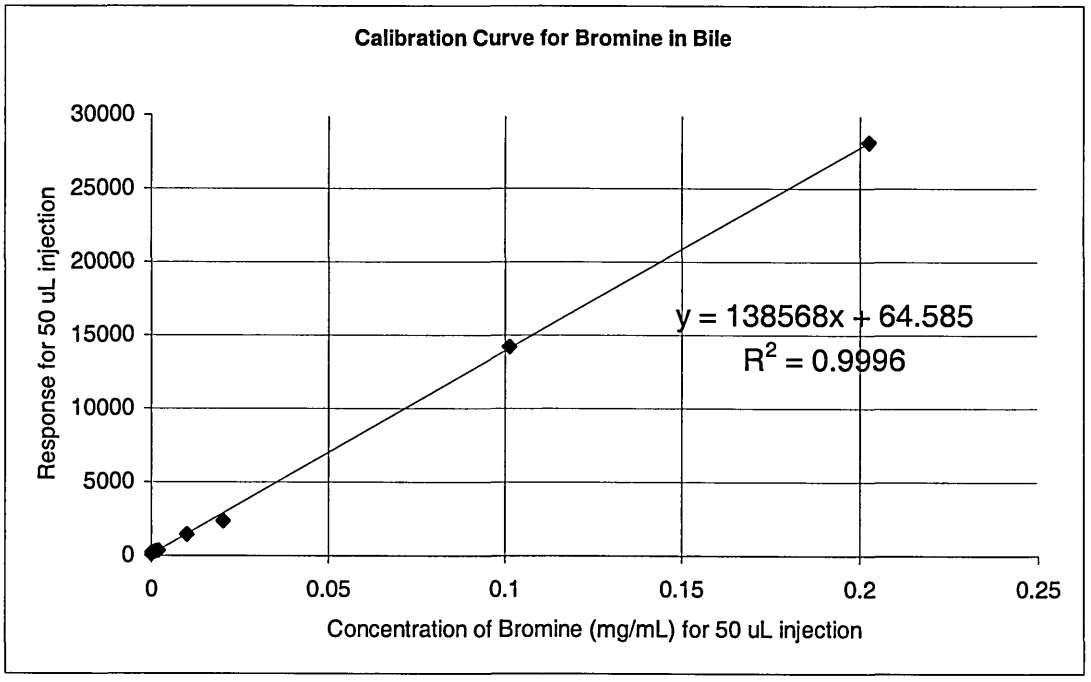


Figure 5.5: Bromine ICP-MS response versus concentration of bromobenzene in rat bile. For experimental conditions see section 5.10.

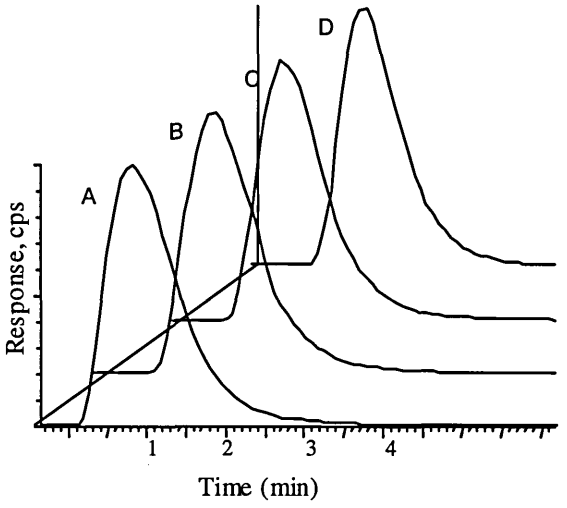


Figure 5.6A: Bromine ICP-MS response from flow injected bile samples from rat dosed at 50 mg/kg with 4-bromoaniline. The area underneath each peak represents the total amount of bromine in the sample.

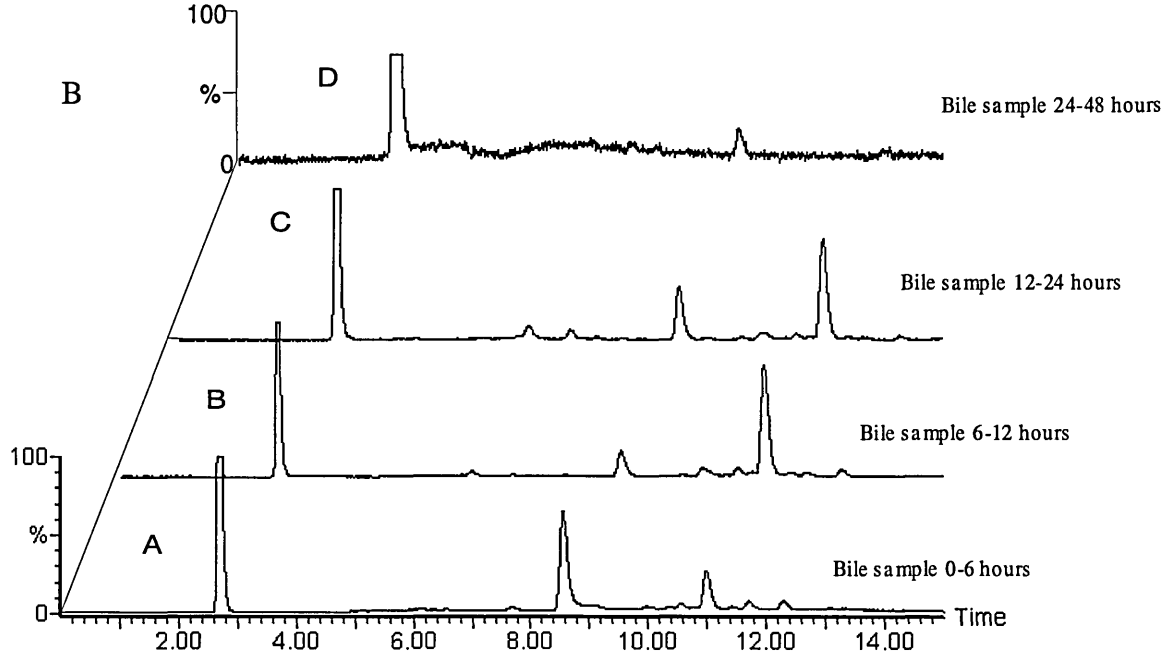


Figure 5.6B: Bromine HPLC-ICP-MS chromatograms from rat bile samples, showing differences in the metabolite profile over time. For experimental conditions see section 5.10.

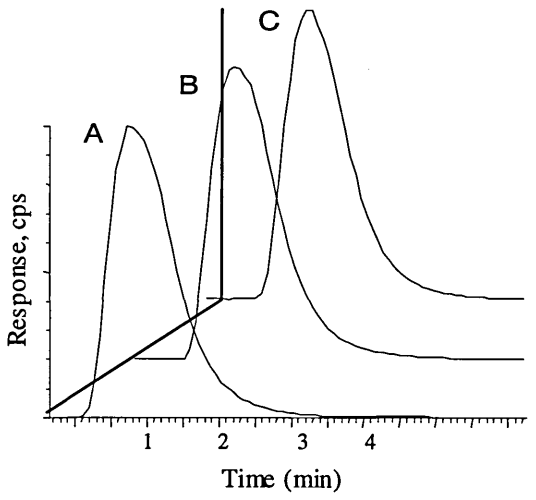


Figure 5.7A: Bromine ICP-MS response from flow injected urine samples from rat dosed at 50 mg/kg with 4-bromoaniline. The area underneath each peak represents the total amount of bromine in the sample.

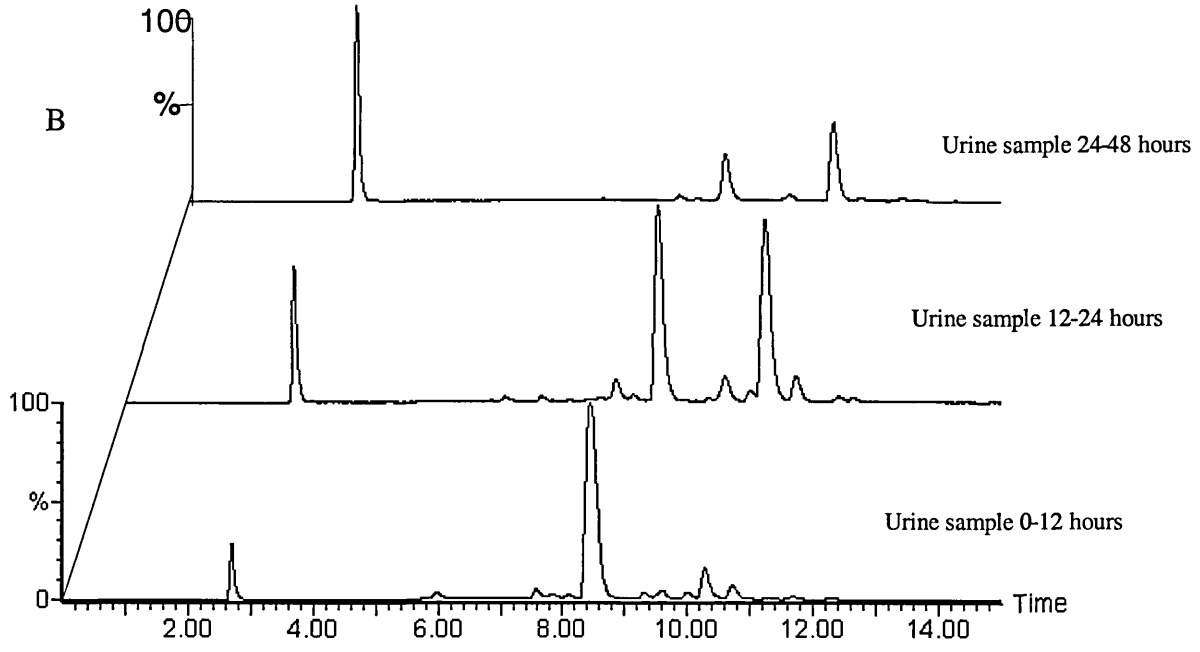


Figure 5.7B: Bromine HPLC-ICP-MS chromatograms from rat urine samples, showing differences in the metabolite profile over time. For experimental conditions see section 5.10.

Table 5.5: Bromine recovery from metabolite profiling of rat bile. Areas calculated in cps. For experimental conditions see section 5.10.

	Bile recoveries (cps)			
Time	(A) 0-6 hr	(B) 6-12 hr	(C) 12-24 hr	(D) 24-48 hr
Profile	22617	206198	135221	56539
Total	231048	222846	132844	55056
percentage recocovery %	95.6	90.9	101.5	102.5

Table 5.6: Bromine recovery from metabolite profiling of rat urine. Areas calculated in cps. For experimental conditions see section 5.10.

	Urine recoveries (cps)		
Time	(A) 0-12 hr	(B) 12-24 hr	(C) 24-48 hr
Profile	1996714	1292072	197534
Total	1861106	1193745	185959
percentage recocovery %	106.9	108.4	106.5

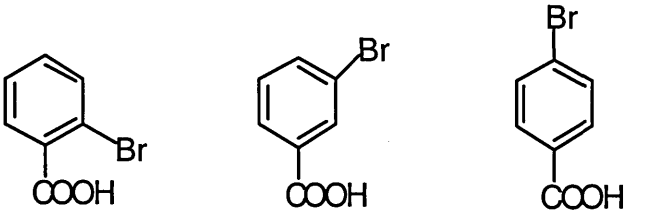
5.14: Excretion balance study on 2-, 3-, and 4-bromobenzoic acids

Work carried out in cooperation with Berit Packert Jensen from University of Pharmaceutical Sciences Copenhagen.

5.15: Experimental (Bromobenzoic acids)

5.15.1: Reagents (Bromobenzoic acids)

2-, 3- and 4-bromobenzoic acids (98% purity) were purchased from Sigma-Aldrich Co Ltd (Dorset, UK) Figure 5.8. Ethanol, formic acid, sodium hydroxide and hydrochloric acid (37%) were of analytical grade and purchased from Fisher Scientific UK Ltd (Loughborough, UK). Methanol was of HPLC grade (Fisher Scientific UK Ltd, Loughborough, UK). Water was obtained from an Elga water purification system (Elgastat Maxima, Elga, High Wycombe, UK).



2-bromobenzoic acid 3-bromobenzoic acid 4-bromobenzoic acid

Figure 5.8 Structures of the bromobenzoic acids used for metabolism studies by ICP-MS.

5.15.2: Animal dosing (Bromobenzoic acids)

Three male Wistar derived rats (230-260 g) were anaesthetised using halothane, bile duct-cannulated and acclimatised individually in glass metabolism cages 3 days prior to dosing. The animals were subjected to 12 h artificial light / dark cycles and were permitted free access to food and water throughout the study. Dosing solutions were made up by dissolving 2-, 3- and 4-bromobenzoic acid in ethanol-water (50:50), adjusting the pH to 6-8 using diluted NaOH, to reach a final concentration of 50 mg/mL for 2- and 3-bromobenzoic acid and 40 mg/mL for 4-brombenzoic acid. The rats were dosed i.p. one compound per rat at 50 mg/kg. The remainder of the dosing solutions were stored at -20°C until analysis. Urine and bile were collected prior to dosing and for the periods 0-6, 6-12, 12-24 and 24-48 h post dose and stored at -20°C until analysis. Cage wash was collected at 0-24 and 24-48 h post dose and stored at 4°C until analysis.

5.15.3: Instrumental (Bromobenzoic acids)

Jasco PU-1580 HPLC pumps (Jasco Ltd, Great Dunmow, UK) and a CTC-HTC Pal autosampler (Presearch, Hitchin, UK) were used to introduce samples to a GVI Platform ICP-MS instrument (GV Instruments Ltd, Manchester, UK). The ICP-MS instrument was equipped with a concentric nebuliser and a cooled double pass spray chamber. Helium gas was added to the collision / reaction cell of the ICP-MS to reduce argon-based interferences in the mass 80 region ($^{40}Ar^{40}Ar^{1}H^{+}$, $^{40}Ar^{38}Ar^{1}H^{+}$) [63] and both ^{79}Br and ^{81}Br could be measured. MassLynx software (GV Instruments Ltd) was used for instrument control, data acquisition and analysis. The operating conditions of the instrument are shown in Table 5.7.

Table 5.7 Instrument operating conditions for ICP-MS for 4-brombenzoic acid metabolism study.

Parameter	Excretion balance	Metabolite profiling
Cooling gas flow	15.0 L/min	18.0 L/min
Plasma gas flow	0.8 L/min	0.8 L/min
Nebuliser gas flow (argon)	0.8 L/min	0.8 L/min
Helium gas flow	1.0 mL/min	1.0 mL/min
Plasma power	1450 W	1600 W
Dwell time	300 ms	300 ms
Mass monitored	79	79,81
Spray chamber temperature	8°C	-7°C

For determining the total bromine content of urine and bile for the excretion balance study, no chromatography was performed and samples were introduced into the ICP-MS by flow injection into a carrier stream of water at a flow rate of 1.0 mL/min. For metabolite profiling, chromatographic separations were performed on a Polaris C18-A, 3 μ, 150 x 4.6 mm column with a matching guard column (Varian BV, Middelburg, the Netherlands) at a temperature of 40°C obtained with an Eppendorf TC-50 column heater (Presearch). The mobile phase consisted of 50 % methanol and 0.1% formic acid in water. The flow rate of 1 mL/min was split using an accurate splitter (Presearch) allowing 250 μL/min into the ICP-MS and the remainder into a Jasco UV-1575 detector set at 254 nm.

For the multiple hyphenation experiments, where both ICP-MS and MS techniques were used, the eluent (1 mL/min) was split letting 250 μL/min into the ICP-MS and

the remainder into the UV detector and then into an MS (Applied Biosystems, Warrington, UK). A turbo ionspray inlet source was used in the positive mode and a Q1 full scan over the range *m/z* 100-600 was acquired. Analyst software was used for instrument control, data acquisition and analysis.

5.15.4: Sample analysis (Bromobenzoic acids)

For the excretion balance study, a stock solution of 150 mM 3-bromobenzoic acid was prepared in 0.2 M NaOH. This was further diluted in control urine to prepare standard solutions of 0.1, 0.5, 1, 5, 10, 25 and 50 mM. A similar procedure was used to prepare quality control samples at final concentrations of 0.1, 10 and 50 mM. Standard solutions in bile were prepared by diluting the stock solution in control bile to concentrations of 0.1, 0.25, 0.5 and 1 mM with quality control samples at 0.5 and 1.25 mM. For analysis of cage wash and dosing solutions, aqueous standard solutions were prepared at concentrations of 0.05, 0.1, 5 and 10 mM and quality control samples of 0.1, 5 and 10 mM. The dosing solutions were diluted with water (1 in 49). The 0-6 hour urine samples and dosing solutions were analysed in triplicate, the remaining samples in singlicate. Samples were introduced by flow injection to the ICP-MS with an injection volume of 10 µL.

For metabolite profiling, 10 µL of neat urine and bile samples were injected on column. Selected urine and bile samples were further analysed by HPLC-MS. All samples were subjected to alkaline hydrolysis to obtain information on the identity of the metabolites and to estimate the distribution of metabolites. The alkaline hydrolysis was performed by incubating samples with 1 M NaOH (2 in 1) at 37°C for

24 hours and then restoring the pH by adding an equivalent amount of 1 M HCl. 20 µL aliquots of the hydrolysed samples were injected on column.

5.16: Results and discussion (Bromobenzoic acids)

5.16.1: Excretion balance study (Bromobenzoic acids)

Bile duct-cannulated animals were used to avoid the problems often associated with poor extraction recoveries from faeces. For sample analysis, a method based on total bromine content in the samples was developed and partially validated. Linear standard curves ($r^2 > 0.99$) were obtained in urine, bile and water in the concentration range 0.05-50 mM bromine, which covered the concentration ranges found in the samples, with concentrations below 0.05 mM considered irrelevant. Based on the quality control samples, values for accuracy were found in the range 93-108% with relative standard deviations below 7% as seen in Table 5.8. Similar values were obtained regardless of the matrix used, indicating that the performance of the method was not matrix dependent. The ICP-MS method thus appeared to be well suited to determining bromine content in urine, bile and aqueous samples.

Table 5.8 Accuracy and precision data for ICP-MS analysis of bromine at *m/z* 79

Matrix	Bromine conc. (mM)	n	Accuracy (%).	coefficient variation (%)
Urine	0.1	4	99.2	5.8
	10	4	93.7	3.4
	50	4	107.5	3.4
Bile	0.5	3	98.7	6.5
	1.25	2	98.3	0.9
Water	0.1	3	94.1	5.2
	5	2	93.0	4.7
	10	3	96.1	5.6

Following administration of 2-, 3- and 4-bromobenzoic acids at 50 mg/kg *i.p.* to bile duct-cannulated rats, urine, bile and aqueous cage wash were collected. The measured bromine concentrations in the dosing solutions, cage wash, urine and bile samples are shown in Table 5.9. It was noticed that the predose urine contained substantial amounts (0.4-0.5 mM) of bromine. HPLC-ICP-MS showed that for the predose samples, bromine was only detected in the solvent front (t_r =1.8 min) indicating inorganic bromine. This could be due to bromide originating from the halothane used to anaesthetise the rats 3 days prior to dosing, as bromide is a known halothane metabolite. No bromine patterns were found in the MS Q1 scan of the solvent front, but since the scan was performed over 100-600 amu in the positive mode, inorganic bromide at *m/z* 79 and 81 would not be detected. HPLC-ICP-MS analysis of all urine and bile samples revealed that the bromine peak in the solvent

front decreased in area the later the sample was taken after anaesthesia of the rats. This supports the assumption that the peak is due to bromide being excreted as a halothane metabolite. No studies on the metabolism of bromobenzoic acids have been found in the literature, but, for bromobenzene, bromine is retained in the metabolites [64]. It was therefore assumed that the bromide peak found in the samples was not related to bromobenzoic acid metabolism. Based on the HPLC-ICP-MS chromatograms, the area percent bromide of each sample was found and used to correct the concentrations found in the excretion balance study with results shown in Table 5.9. It was noticed that practically no bromine was found in the cage wash, and the highest bromine concentrations were found in the 0-6 hour urine samples.

Table 5.9 Bromine concentrations obtained in dosing solutions (n=3) and rat urine samples (0-6 hour urine: n=3, otherwise n=1) after *i.p.* dosing of 2-, 3- and 4-bromobenzoic acids (one compound per rat at 50 mg/kg). Concentrations corrected for inorganic bromide.

	Bromine conc. (mM)					
	2-bromobenzoic acid		3-bromobenzoic acid		4-bromobenzoic acid	
Sample	Measured	Corrected	Measured	Corrected	Measured	Corrected
Dosing solution	4.88	4.88	4.52	4.52	3.55	3.55
Urine predose	0.46	<0.1	0.48	<0.1	0.41	<0.1
Urine 0-6 h	24.2	23.9	9.91	9.71	8.19	8.03
Urine 6-12 h	4.10	4.05	0.88	0.69	0.70	0.54
Urine 12-24 h	0.62	0.46	0.42	0.24	0.62	0.53
Urine 24-48 h	0.61	<0.1	<0.1	<0.1	0.22	<0.1
Bile predose	<0.1	<0.1	<0.1	<0.1	0.15	<0.1
Bile 0-6 h	0.29	0.18	0.45	0.28	0.78	0.70
Bile 6-48 h	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Cage wash	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05

Based on the corrected bromine concentrations and the volumes of the samples collected from the rats, the recovery of 2-, 3- and 4-bromobenzoic acid could be calculated (Table 5.10). As seen in the table, most of the dose given was recovered in the urine and bile, with recoveries of 82, 85 and 98% for 2-, 3- and 4-bromobenzoic acid, respectively. The excretion of all three bromobenzoic acids was rapid with

approximately 70% of the dose recovered in the 0-6 hour urine. Only 2-7% of the dose was recovered in the bile.

Table 5.10 Percent recovery in urine, bile and cage wash of 2, 3- and 4-bromobenzoic acids dosed *i.p.* to one rat per compound at 50 mg/kg

	% dose recovered as total bromine		
Sample	2-bromobenzoic acid	3-bromobenzoic acid	4-bromobenzoic acid
Urine 0-6 h	66.7	70.5	79.7
Urine 6-12 h	8.6	5.4	4.1
Urine 12-24 h	2.7	5.8	5.3
Urine 24-48 h	1.4	0.0	1.8
Bile 0-6 h	2.3	2.9	7.1
Bile 6-48 h	0.0	0.0	0.0
Cage wash	0.0	0.0	0.0
Total	81.7	84.6	97.9

5.16.2: Metabolite profiling (Bromobenzoic acids)

As well as excretion balance data, additional information regarding the metabolic fate of test compounds is needed. The metabolism of 2-, 3- and 4-bromobenzoic acid was therefore investigated by profiling the urine and bile samples by HPLC-ICP-MS. Previous studies have demonstrated the use of reversed phase HPLC with ICP-MS as

a bromine detector for xenobiotics and their metabolites [50, 54]. Methanol was chosen as organic modifier rather than acetonitrile because of its lower carbon loading and less change in electrical impedance of the plasma. The chromatographic method developed, based on 50 % methanol and 0.1 % formic acid, separated the metabolites from parent compound as well as from inorganic bromide. The metabolite profiles of the 0-6 h urine and 0-6 hour bile from 2-, 3- and 4-bromobenzoic acid dosing, based on bromine detection, are shown in Figure 5.9 and 5.10. The pre-dose samples showed no bromine peaks except in the solvent front.

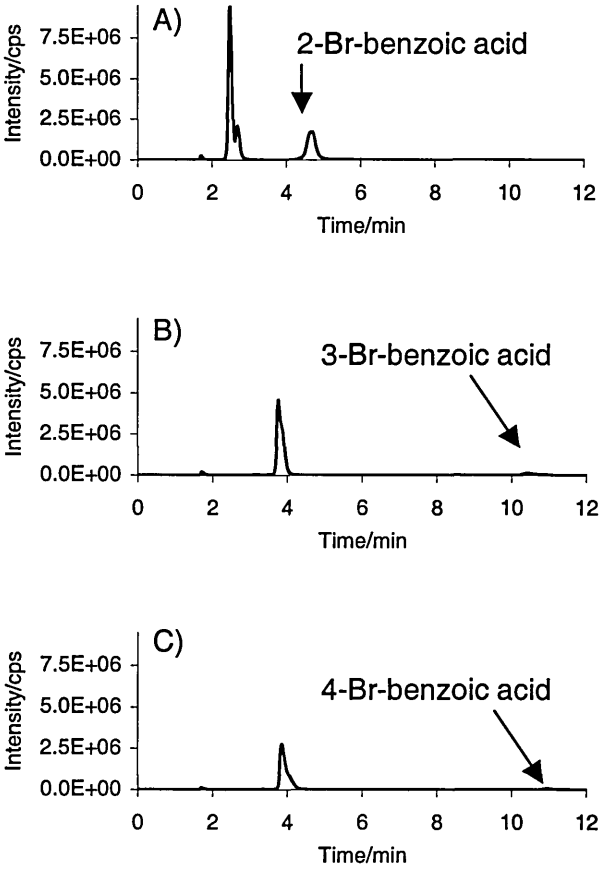


Figure 5.9 HPLC-ICP-MS bromine chromatograms of 0-6 hour urine profiles after dosing with A) 2-bromobenzoic acid B) 3-bromobenzoic acid and C) 4-bromobenzoic acid. 10µL neat urine was injected on a Polaris C18-A, 150 x 4.6 mm column, 40°C, eluted isocratically with 50 % methanol and 0.1% formic acid and detected with $^{79/81}$Br-ICP-MS.

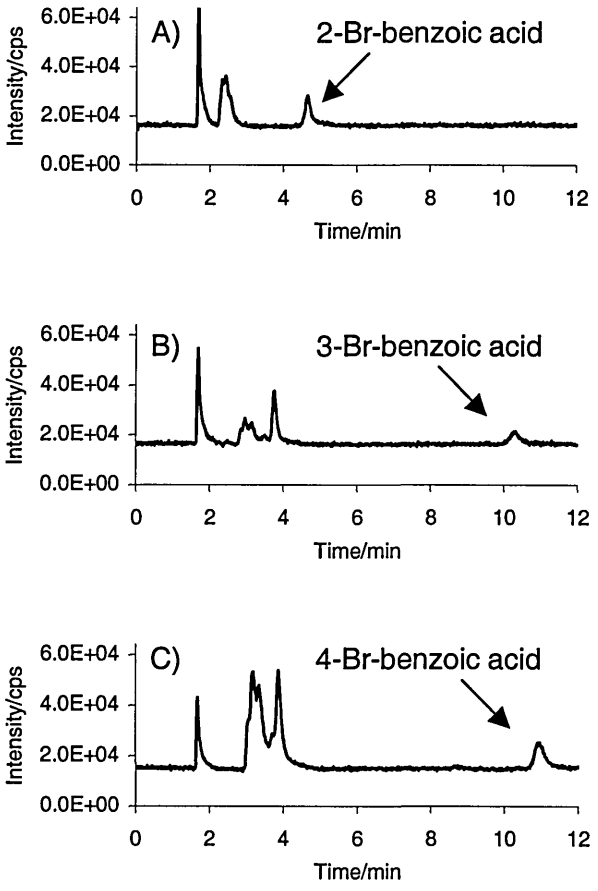


Figure 5.10 HPLC-ICP-MS bromine chromatograms of 0-6 hour bile profiles after dosing with A) 2-bromobenzoic acid B) 3-bromobenzoic acid and C) 4-bromobenzoic acid. 10μL neat bile was injected on a Polaris C18-A, 150 x 4.6 mm column, 40°C, eluted isocratically with 50 % methanol and 0.1% formic acid and detected with $^{79/81}$Br-ICP-MS.

The profiles reveal that 2-, 3- and 4-bromobenzoic acids are extensively metabolised as little parent compound is excreted. There appears to be one major metabolite in the urine profiles, however, the metabolites are not well separated from each other. Since ICP-MS is an element-detection technique, these profiles do not yield any structural information for the metabolites, and so other measures are required to elucidate the identity of the metabolites and purity of the peaks. For this purpose, MS was connected in parallel (as described elsewhere [6-8]) to the HPLC-ICP-MS instruments, along with a UV detector. As an example of the type of data acquired, typical total ion current full scan MS, UV and bromine detected ICP-MS chromatograms are shown in Figure 5.12a-c, respectively for urine from a rat dosed with 4-bromobenzoic acid. Both the UV and the MS chromatograms show peaks from compound-related material as well as endogenous compounds further highlighting, if it were needed, the selectivity of the bromine-detected ICP-MS for quantitative metabolite profiling.

In the mass spectrum, the bromine related peaks are easily identified by the bromine pattern of the two isotopes. For the urinary profile of 4-bromobenzoic acid (Figure 5.12), several bromine-containing ions were observed. A glycine conjugate is a likely metabolite for benzoic acids and this is supported by the observed *m/z* values of 258/260 (and 280/282 for the sodium adducts). The *m/z* values of 399/401 suggest sodium adducts of ester glucuronide conjugates. The *m/z* 183/185 is a likely fragment of both metabolites due to in-source fragmentation as indicated in Figure 5.11. In the bile, similar *m/z* values were found and the pattern was similar for 2- and 3-bromobenzoic acids. These findings are consistent with the literature, in that glycine and glucuronide conjugates have been found as the major metabolites of substituted

benzoic acids as well as unchanged parent [66]. Structures of the metabolites for benzoic acids are shown in Figure 5.11.

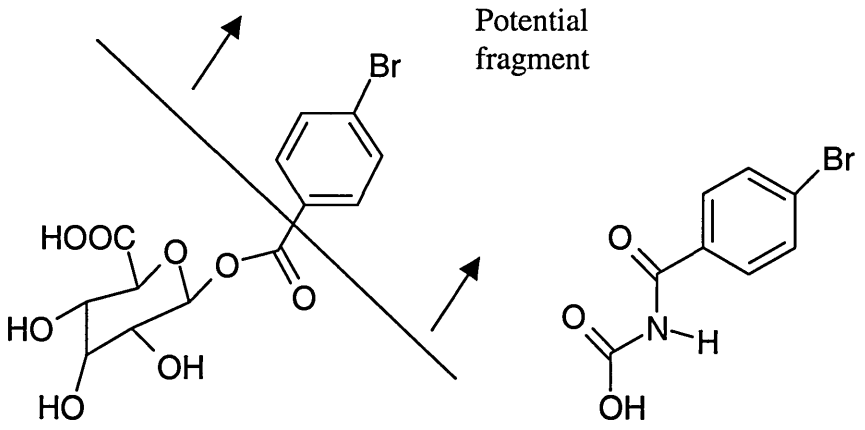


Figure 5.11 Structures of the glucuronide and glycine metabolites of 4-bromobenzoic acid with the potential site indicated for in source fragmentation occurring.

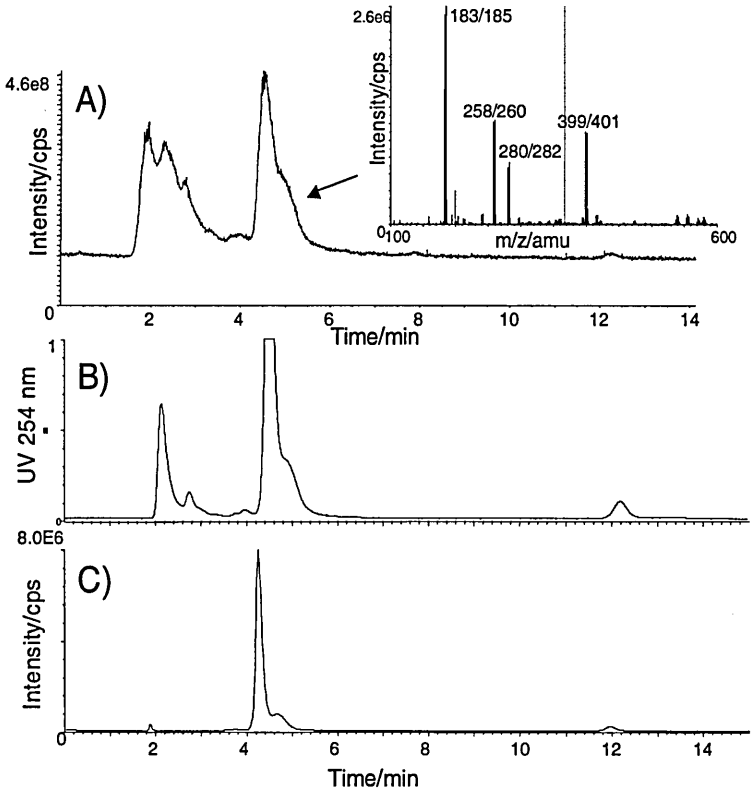


Figure 5.12 0-6 hour urine profile following dosing of 4-bromobenzoic acid. 10 µLof neat urine was injected on to a Polaris C18-A, 150 x 4.6 mm column at 40°C, eluted isocratically with 50 % methanol and 0.1 % formic acid, detected by: A) Total ion current full scan MS *m/z* 100-600, B) UV 254 nm and C) $^{79/81}$Br-ICP-MS. Insert in A shows mass spectrum recorded between 4 and 5 min.

Due to the overlap of metabolite peaks and to further investigate the identity of the peaks, the urine and bile samples were treated with alkali to hydrolyse ester glucuronides (glycine and ether glucuronide conjugates are stable under these conditions). As seen in the urine HPLC-ICP-MS profiles in Figure 5.13, the majority of the metabolites in urine are resistant to alkali treatment indicating that glycine conjugates are primarily formed. In the bile, the metabolites observed accounted for a very small proportion of the dose, but out of these, some of the metabolites disappeared on alkaline hydrolysis with a related increase in peak area of the parent compound. The alkaline hydrolysis thus supported the MS data in that both glycine and glucuronide conjugates were present.

To estimate the percentage of glycine and ester glucuronide conjugates respectively, the relative areas of the peaks in the chromatograms with and without alkali treatment were calculated. The amount of metabolite remaining after alkali treatment was assumed to be due to a glycine conjugate, with the proportion of glycine assumed constant regardless of treatment. From the chromatograms of the untreated samples, the percentage of parent was found, as well as total metabolite percentage. Knowing the proportion of glycine, the remaining proportion of metabolites originating from glucuronides could then be estimated. Finally, these percentages were related to the total amount of bromine as found in the excretion balance study. By using this approach, the total degree of glycine and glucuronide conjugate formation of the 2-, 3- and 4- bromobenzoic acids could be estimated (Table 5.11). The results show that for 2-, 3- and 4-bromobenzoic acid, glycine conjugation was the major metabolic route, most markedly seen for 4- and 3-bromobenzoic acid. 2-bromobenzoic acid was

metabolised to a lesser extent and a notable proportion of glucuronides was found as well.

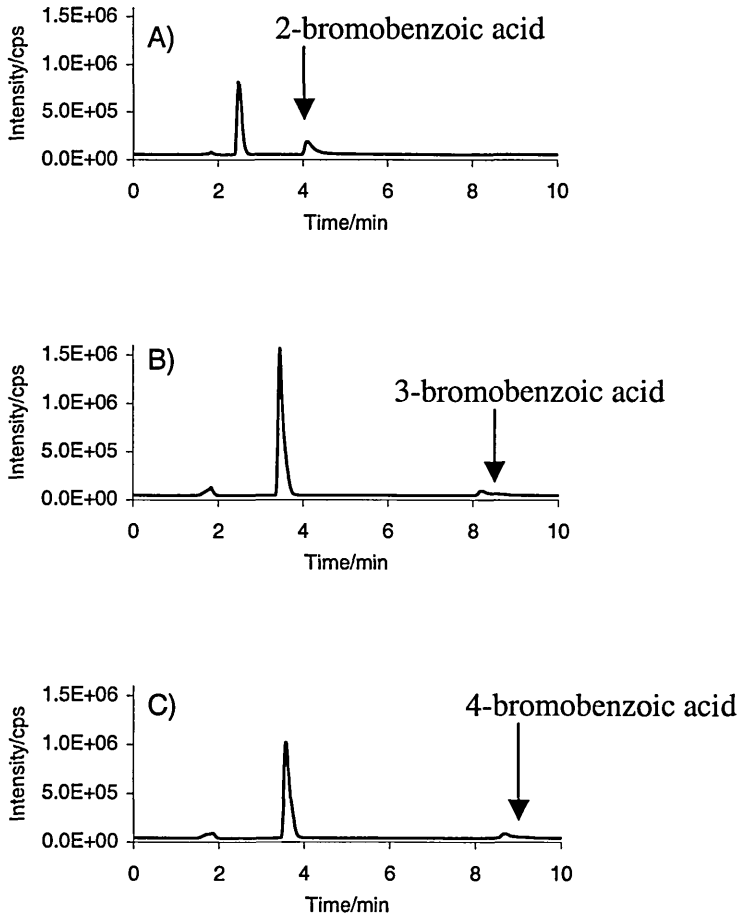


Figure 5.13 Profiles after alkaline hydrolysis of urine samples 0-6 hour from rat dosed with A) 2-bromobenzoic acid B) 3-bromobenzoic acid and C) 4-bromobenzoic acid. 20 µl neat urine was treated with 1 M NaOH and injected on a Polaris C18-A, 150 x 4.6 mm column, 40°C, eluted isocratically with 50 % methanol and 0.1% formic acid and detected with $^{79/81}$Br-ICP-MS.

Table 5.11 Percent formation of ester glucuronides and glycine conjugates in urine and bile after dosing of 2-, 3- and 4-bromobenzoic acid *i.p.* to rats at 50 mg/kg.

	% Formation based on bromine content								
	2-bromobenzoic acid			3-bromobenzoic acid			4-bromobenzoic acid		
	Urine	Bile	Total	Urine	Bile	Total	Urine	Bile	Total
Glycine conj.	48	0	48	76	1	77	84	3	87
Glucuronides	10	2	11	0	1	2	3	3	6
Parent	21	1	22	5	1	6	4	1	5
Total	79	2	82	82	3	85	91	7	98

5.17: Conclusion (Bromobenzoic acids)

This limited study of the application of ICP-MS to bromine-specific detection of the metabolites of 2-, 3- and 4-bromobenzoic acids in the urine and bile of rats clearly demonstrates the potential of using ICP-MS in metabolism studies, eliminating the need for radiolabelling. Quantitative excretion balances were performed as well as metabolite profiling using HPLC-ICP-MS. The study further shows the advantages of having MS detection in parallel with ICP-MS detection to gain structural information simultaneously.

5.18: Iodine excretion study

Work carried out in cooperation with Berit Packert Jensen from University of Pharmaceutical Sciences Copenhagen.

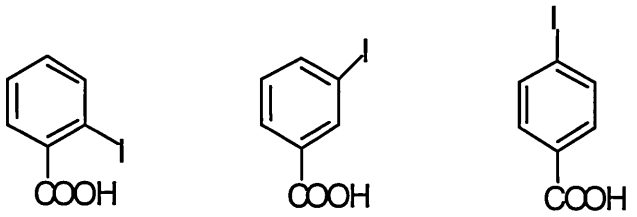
5.19: Introduction (Iodobenzoic acids study)

Iodine, when present in a molecule, also provides an interesting opportunity for sensitive and specific detection with quantification, as demonstrated in X-ray contrast media [42], thyroid hormones [70], enzyme digests of bovine thyroid gland in urine [71] and serum [72]. A recent study has also demonstrated this approach to obtain metabolite profiles following exposure of earthworms to 2-fluoro-4-iodoaniline [8]. In this work, an excretion balance study and metabolite profiling was carried out in rats dosed with 2-, 3-, and 4-iodobenzoic acids.

5.20: Experimental (Iodobenzoic acids study)

5.20.1: Reagents (Iodobenzoic acids study)

2-, 3- and 4-iodobenzoic acids (98% purity) were purchased from Sigma-Aldrich Co Ltd (Dorset, UK). Ethanol, formic acid, sodium hydroxide and hydrochloric acid (37%) were of analytical grade and purchased from Fisher Scientific UK Ltd (Loughborough, UK). Acetonitrile was of HPLC grade (Fisher Scientific UK Ltd, Loughborough, UK). Water was obtained from an Elga water purification system (Elgastat Maxima, Elga, High Wycombe, UK).



2-iodobenzoic acid 3-iodobenzoic acid 4-iodobenzoic acid

Figure 5.14 Structures of the compounds used for the iodine evaluation for ICP-MS.

5.20.2: Animal dosing (Iodobenzoic acids study)

Three male Wistar derived rats (250-300 g) were bile duct cannulated and acclimatised individually in glass metabolism cages 3 days prior to dosing. The animals were subjected to 12 h artificial light / dark cycles and were permitted free access to food and water throughout the study. Dosing solutions were made up by dissolving 2-, 3- and 4-iodobenzoic acid in 50 % ethanol, and adjusting pH to 6.5-8 using diluted NaOH, to reach a final concentration of 50 mg/mL for 2- and 3-iodobenzoic acid and 40 mg/mL for 4-iodobenzoic acid. The rats were dosed *i.p.* with one compound per rat at 50 mg/kg. The remainder of the dosing solutions were stored at -20°C until analysis. Urine and bile were collected prior to dosing and for the periods 0-6, 6-12, 12-24 and 24-48 hr post dose and stored at -20°C until analysis. Cage wash was collected at 0-24 and 24-48 hr post dose and stored at 4°C until analysis.

5.20.3: Instrumental (Iodobenzoic acids study)

As described in section 5.15.3 with specific operating conditions described in Table 5.12.

Table 5.12 Instrument operating conditions for ICP-MS for iodine excretion study

Parameter	Excretion balance	Metabolite profiling
Cooling gas flow	17.0 L/min	18.0 L/min
Plasma gas flow	0.8 L/min	0.8 L/min
Nebuliser gas flow (argon)	0.8 L/min	0.2 L/min
Nebuliser gas flow (argon/oxygen, 95/5% v/v)	0 L/min	0.5 L/min
Plasma power	1400 W	1650 W
Dwell time	300 ms	300 ms
Mass monitored	127	127
Spray chamber temperature	8°C	-7°C

For the excretion balance study chromatography was not performed and samples were introduced directly into the ICP-MS by flow injection into a carrier stream of water at a flow rate of 0.6 mL/min. For metabolite profiling the chromatographic separations were performed on a Polaris C18-A, 3 μ, 150 x 4.6 mm column with a matching guard column (Varian BV, Middelburg, the Netherlands) at a temperature of 40°C obtained with an Eppendorf TC-50 column heater (Presearch). The mobile phase consisted of 30 % acetonitrile and 0.1 % formic acid in water for analysis of 3- and 4-iodobenzoic acid metabolites or 20 % acetonitrile and 0.1 % formic acid in water for analysis of 2-iodobenzoic acid metabolites. The flow rate of 1 mL/min was split

using an accurate splitter (Presearch), allowing 50 μL/min into the ICP-MS and the remainder into a Jasco UV-1575 detector set at 254 nm. To avoid excess carbon build-up on the cones of the ICP-MS, the nebuliser gas was mixed with argon / oxygen (95/5 % v/v).

For the multiple hyphenation experiments, the eluent (1 mL/min) was split with 50 μL/min directed into the ICP-MS and 250μL/min into the UV detector and then to an API-365 MS (Applied Biosystems, Warrington, UK). A turbo ionspray inlet source was used in the positive mode and full scan over the range m/z 100-500 was acquired. Analyst software was used for instrument control, data acquisition and analysis.

5.20.4: Analysis (Iodobenzoic acids study)

For the excretion balance study, a stock solution of 150 mM 2-iodobenzoic acid was prepared in 0.2 M NaOH. This was further diluted in control urine to prepare standard solutions of 0.1, 0.5, 1, 5, 10, 15 and 20 mM I. A similar procedure was used to prepare quality control samples at final concentrations of 0.1, 5 and 20 mM I. Standard solutions in bile were prepared by diluting the stock solution in control bile to concentrations of 0.1, 0.5, 1 and 5 mM I with quality control samples at 0.15 and 5 mM I. For analysis of cage wash and dosing solutions, aqueous standard solutions were prepared at concentrations of 0.1, 5, 10, 20 mM I and quality control samples of 5 and 10 mM I. The dosing solutions were diluted with water (1 in 49) and analysed in triplicate. The urine, bile and cage wash samples were analysed in duplicate. Samples were introduced by flow injection to the ICP-MS with an injection volume of 10 μL.

For metabolite profiling, 20 µL of neat urine and bile samples were injected on column. Selected urine and bile samples were further analysed by MS and subjected to base hydrolysis to obtain information on the identity of the metabolites. Base hydrolysis was performed by incubating samples with 1 M NaOH (2 in 1) at 37°C for 24 hours and then restoring the pH by adding an equivalent amount of 1 M HCl. 40 µl of the hydrolysed samples were then injected on column.

5.21: Results and discussion (Iodobenzoic acids study)

5.21.1: Excretion balance study (Iodobenzoic acids study)

An experiment was set up similar to that described for the analysis of 2-, 3- and 4-bromobenzoic acid. Linear standard curves ($r^2 > 0.993$) were obtained in urine, bile and water over the concentration range 0.1-20 mM iodine, which covered the concentration ranges found in the samples, with concentrations below 0.1mM considered irrelevant. Based on the quality control samples, values for accuracy were found in the range 94-105% with relative standard deviations below 11% as seen in Table 5.13. Similar values were obtained regardless of the matrix used, indicating that the performance of the method was not matrix dependent. The ICP-MS method thus appeared to be well suited to determining iodine content in urine, bile and aqueous samples, in for example to obtain excretion balance data for iodobenzoic acid dosed to rats.

Table 5.13 Accuracy and precision data for ICP-MS analysis of iodine at *m/z* 127 in three different media to determine any differences from the matrix.

Matrix	Iodine conc. (mM)	n	accuracy (%)	coefficient of variation (%)
Urine	0.1	6	101.5	8.2
	5	6	94.2	6.0
	20	6	97.9	6.7
Bile	0.15	6	104.4	10.2
	5	6	100.4	10.2
Water	5	3	98.5	9.9
	10	3	100.2	3.7

Following administration of 2-, 3- and 4-iodobenzoic acids at 50 mg/kg *i.p.* to bile duct cannulated rats, urine, bile and aqueous cage wash were collected. The measured iodine concentrations in the dosing solutions, cage wash, urine and bile samples are shown in Table 5.14. These results show that practically no iodine was present in the predose samples or cage wash, and the highest iodine concentrations were found in the 0-12 hour post dose urine samples. As shown in Table 5.15, essentially a quantitative recovery of the test compounds was seen over the 24 hr time course of the study via urine and bile (102, 101 and 93% for 2-, 3- and 4-iodobenzoic acids respectively). The excretion of all three iodobenzoic acids was rapid with approximately 90 % of the dose recovered in the 0-24 hour urine and the remainder excreted in the bile within 6 hours.

These results clearly demonstrate that, as for the bromine compounds, there is potential for the ICP-MS to be used in quantitative excretion balance studies to be performed based on iodine-containing compounds without the need for radio-labelling.

Table 5.14 Average iodine concentration obtained in dosing solutions (n=3) and samples (n=2) after *i.p.* dosing of 2-, 3- and 4-iodobenzoic acids to one rat per compound at 50 mg/kg) I conc.<0.1mM.

	Iodine conc (mM)		
Sample	2-iodobenzoic acid	3-iodobenzoic acid	4-iodobenzoic acid
Dosing solution	3.52	3.08	2.62
Urine predose	-	-	-
Urine 0-6 h	12.3	9.36	10.1
Urine 6-12 h	3.84	4.18	18.1
Urine 12-24 h	0.33	0.19	0.53
Urine 24-48 h	-	-	-
Bile predose	-	-	-
Bile 0-6 h	0.52	0.43	0.96
Bile 6-48 h	-	-	-
Cage wash	-	-	-

Table 5.15 Percent recovery based on iodine ICP-MS data in urine, bile and cage wash of 2, 3- and 4-iodobenzoic acids dosed *i.p.* to one rat per compound at 50 mg/kg. The data indicates that the urine is the main route of excretion.

	% dose recovered		
Sample	**2-iodobenzoic acid**	**3-iodobenzoic acid**	**4-iodobenzoic acid**
Urine 0-6 h	70.1	76.4	22.5
Urine 6-12 h	16.0	6.1	32.4
Urine 12-24 h	4.8	10.9	26.4
Urine 24-48 h	0.0	0.0	0.0
Bile 0-6 h	11.1	8.2	12.1
Bile 6-48 h	0.0	0.0	0.0
Cage wash	0.0	0.0	0.0
Total	**102.0**	**101.6**	**93.4**

5.21.2: Metabolite profiling and identification (Iodobenzoic acids study)

As before, additional information regarding the metabolic fate of test compounds was gained by HPLC-ICP-MS. For HPLC-ICP-MS, methanol is generally preferred as the organic modifier over acetonitrile (5.16.2). However, acceptable resolution of the iodobenzoic acid metabolites was not achieved within a reasonable analysis time using an isocratic methanol-based solvent system. Chromatographic methods based on 20-30 % acetonitrile and 0.1 % formic acid were however able to achieve the

required separation. To minimise the organic loading to the plasma, the flow was split to direct only 50 μL/min to the ICP-MS. Oxygen was also added to the nebuliser gas to avoid excess carbon build-up on the torch and cones of the ICP-MS.

The metabolite profiles of the 0-6 hr urine and 0-6 hr bile samples obtained for 2-, 3- and 4-iodobenzoic acids, based on iodine detection, are shown in Figures 5.15 and 5.16. The pre-dose samples showed only trivial amounts of iodine indicating that all the iodine-containing peaks were iodobenzoic acid-related compounds. These profiles reveal that 2-, 3- and 4-iodobenzoic acids were extensively metabolised with little unchanged parent compound excreted. For all three iodobenzoic acids, one or two major peaks dominated the urinary metabolite profile. For bile a more complex profile was generally obtained, with no particular metabolite predominating.

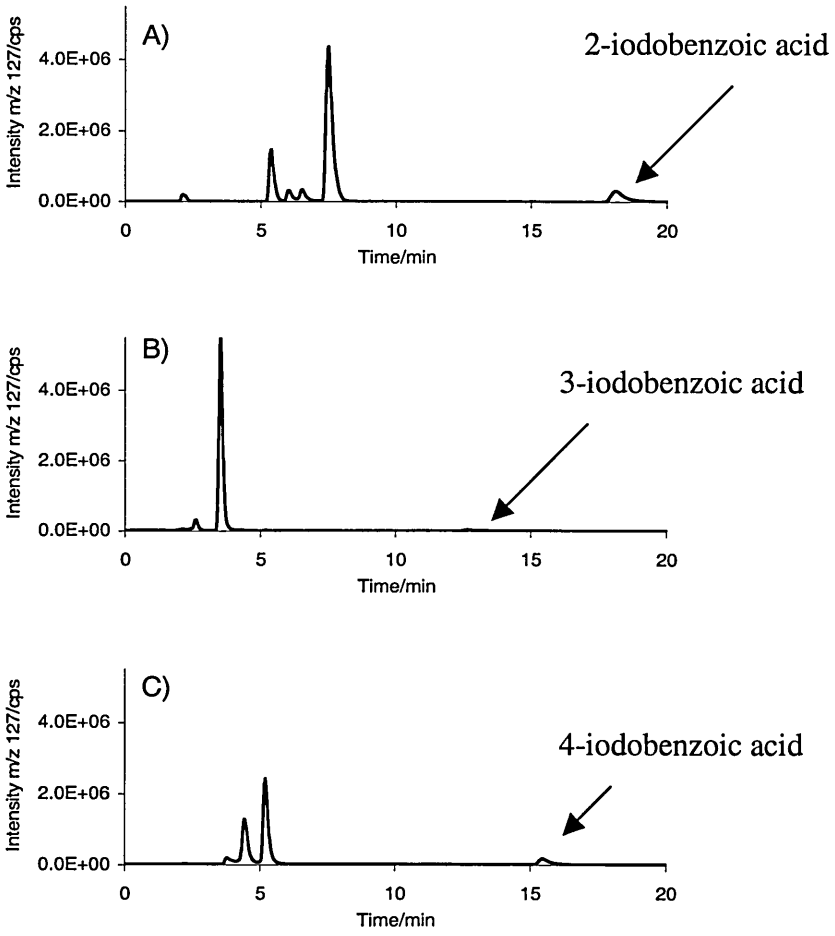


Figure 5.15 HPLC-ICP-MS iodine chromatogram of 0-6 hour urine profiles from rats dosed with A) 2-iodobenzoic acid B) 3-iodobenzoic acid and C) 4-iodobenzoic acid. 20µl neat urine was injected on a Polaris C18-A, 150 x 4.6 mm column, 40°C, eluted isocratically with 20 % (in A) or 30 % (in B and C) acetonitrile and 0.1 % formic acid. Detection with ICP-MS at *m/z* 127.

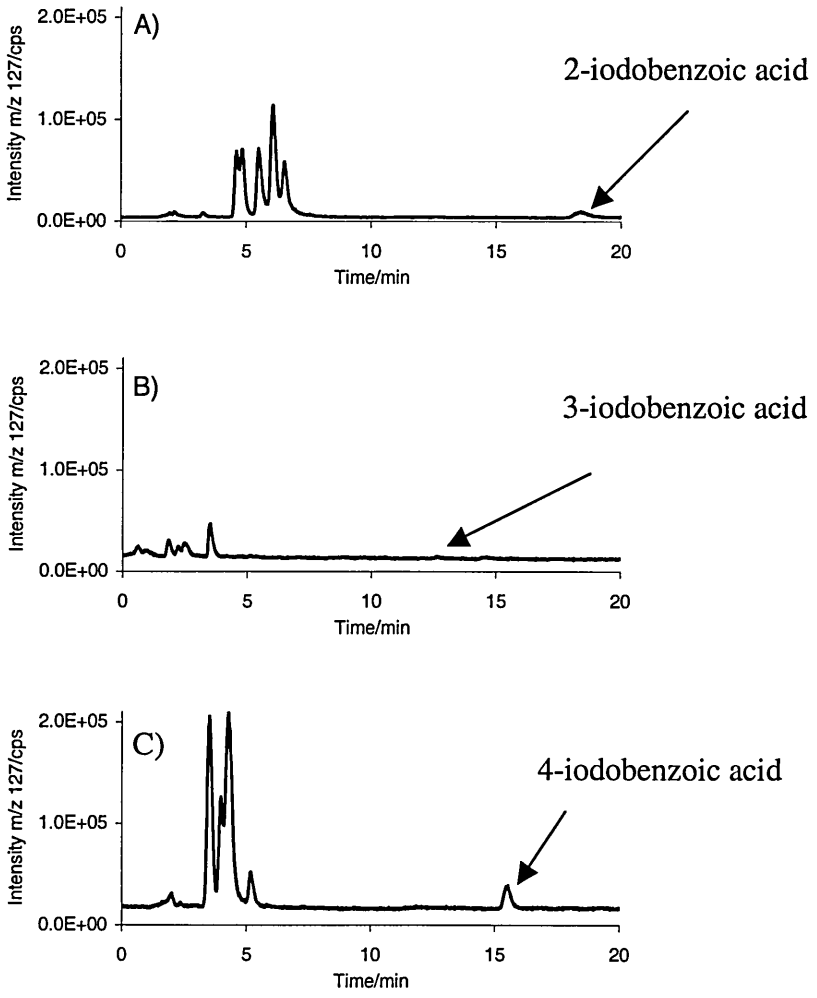


Figure 5.16 HPLC-ICP-MS iodine chromatogram of 0-6 hour bile profiles from rats dosed with A) 2-iodobenzoic acid B) 3-iodobenzoic acid and C) 4-iodobenzoic acid. 20µl neat bile was injected on a Polaris C18-A, 150 x 4.6 mm column, 40°C, eluted isocratically with 20 % (in A) or 30 % (in B and C) acetonitrile and 0.1 % formic acid. Detection with ICP-MS at *m/z* 127.

Coupling of a standard MS to the system was carried out to gain structural information. Typical total ion current full scan MS, UV and iodine detected ICP-MS chromatograms are shown in Figure 5.17a-c, respectively for 4-iodobenzoic acid. Both the UV and the MS chromatograms show peaks from compound-related material as well as endogenous compounds comparison of the traces further highlights the selectivity of the iodine-detection ICP-MS trace for quantitative metabolite profiling.

To identify the individual metabolites, iodine-detected peaks in the ICP-MS chromatogram were used as guides to pinpoint relevant peaks in the total ion current mass chromatogram from which mass spectra were extracted. For the urine profile of 4-iodobenzoic acid, shown in Figure 5.18, the major peak at 5.2 min had a *m/z* of 306 suggesting a glycine conjugate. An ion with *m/z* of 447 was found in the other major peak (eluting at 4.4 min) suggesting the presence of an ester glucuronide (a typical metabolite for a small aromatic acid such as this), seen as sodium adduct. A minor component with *m/z* of 399 at 3.8 min was also observed, but the identification of this metabolite was not pursued further as it accounted for a very small proportion of the dose. For the bile profile of 4-iodobenzoic acid the same metabolites were observed but in addition several further peaks with a *m/z* of 447 were present suggesting the presence of ester glucuronides. These findings are consistent with the literature, in that both glycine and ester glucuronide conjugates are widely found as benzoic acid metabolites [73]. In the urine profiles from 2- and 3-iodobenzoic acid, similar *m/z* values (306, 447) were found. However, in the case of 2-iodobenzoic acid the glycine conjugate was observed to elute before the ester glucuronides, reversing the order seen for both the 3- and 4-iodobenzoic acid metabolites. In the bile, several peaks at *m/z* 447 for putative transacylated ester glucuronides were observed in the profiles of

both 2- and 3-benzoic acids. However, the ion at *m/z* 306 for the glycine conjugate was only detected in the 3-iodobenzoic acid samples.

To further investigate the identity of the metabolites, the urine and bile samples were treated with alkali to hydrolyse ester glucuronides (glycine and ether glucuronide conjugates are stable under these conditions). As seen in the urine HPLC-ICP-MS profiles in Figure 5.19, the peaks postulated to be ester glucuronides all disappeared after alkaline hydrolysis with a related increase in peak area of the parent compound. In contrast, the glycine conjugates remained unaffected as did the minor unknown metabolite of 4-iodobenzoic acid. Similar effects were observed for the bile samples when alkaline hydrolysis was performed.

In summary 2-, 3- and 4-iodobenzoic acid were metabolised in the rat primarily to either glycine conjugates or ester glucuronides, but to varying degrees depending on the structure of the parent compound.

Quantification of the individual metabolites based on the HPLC-ICP-MS chromatogram was performed based on the peak areas of iodobenzoic acids standards, in a similar way to that performed for the excretion balance study. By using this approach, the total degree of glycine and ester glucuronide formation from the 2-, 3- and 4-iodobenzoic acids was determined (Table 5.16). The results show that 2-iodobenzic acid was mainly glucuronidated whereas 3-iodobenzoic acid primarily formed glycine conjugates. For 4-iodobenzoic acid, the balance between glycine conjugation and ester glucuronidation was similar but the former still accounted for

the majority of the dose. The unknown metabolite of 4-iodobenzoic acid accounted for less than 1 % and is not presented in the table.

Table 5.16 Percent formation of ester glucuronides and glycine conjugates in urine and bile after dosing of 2-, 3- and 4-iodobenzoic acid i.p. to rats at 50 mg/kg based on HPLC-ICP-MS iodine measurements.

	2-iodobenzoic acid			**3-iodobenzoic acid**			**4-iodobenzoic acid**		
	Urine Dose %	Bile Dose %	Total Dose %	Urine Dose %	Bile Dose %	Total Dose %	Urine Dose %	Bile Dose %	Total Dose %
Glucuronide	60.3	10.7	71.0	5.4	4.6	10.0	18.6	9.7	28.3
Glycine conj.	19.5	0.0	19.5	87.1	3.6	90.7	58.1	0.7	58.8
Parent	11.0	0.4	11.4	0.8	0.0	0.8	3.7	1.8	5.5
Total	90.8	11.1	101.9	93.3	8.2	101.5	80.4	12.2	92.6

5.22: Conclusion (Iodobenzoic acids study)

This limited study of the application of HPLC-ICP-MS to iodine-specific detection of the metabolites of 2-, 3- and 4-iodobenzoic acids in the urine and bile of rats clearly demonstrates that this element (iodine) can be used as an alternative to the use of radio-labelled compound allowing quantitative excretion balance studies can be performed as well as metabolite profiling.

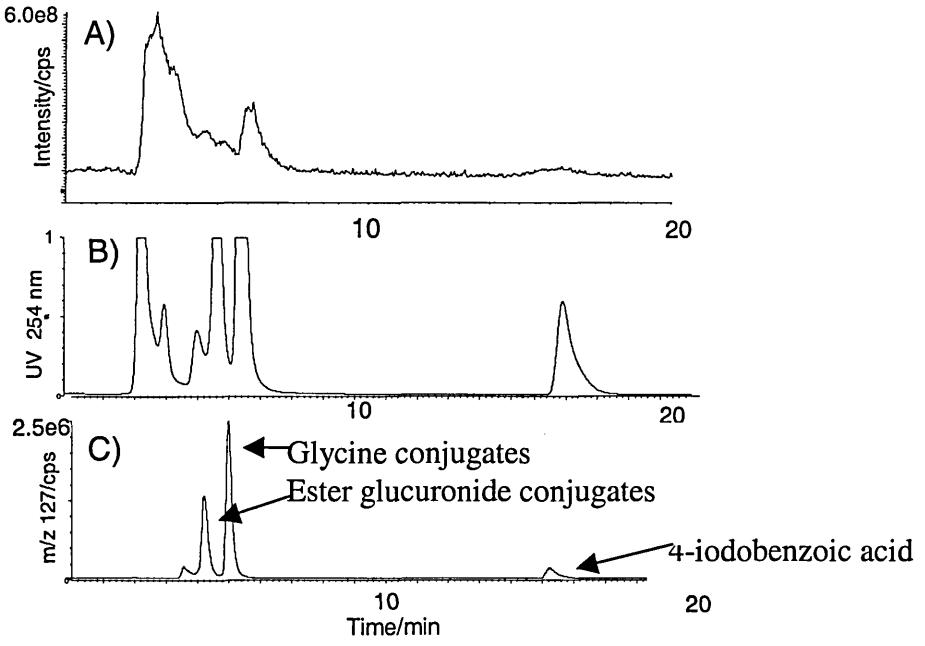


Figure 5.17 0-6 hour urine profile following dosing of rats with 4-iodobenzoic acid. Reversed phase isocratic HPLC with 3 modes of detection: A) Total ion current full scan MS *m/z* 100-500, B) UV 254 nm and C) Iodine detection by ICP-MS at *m/z* 127. For experimental conditions see section 5.20.

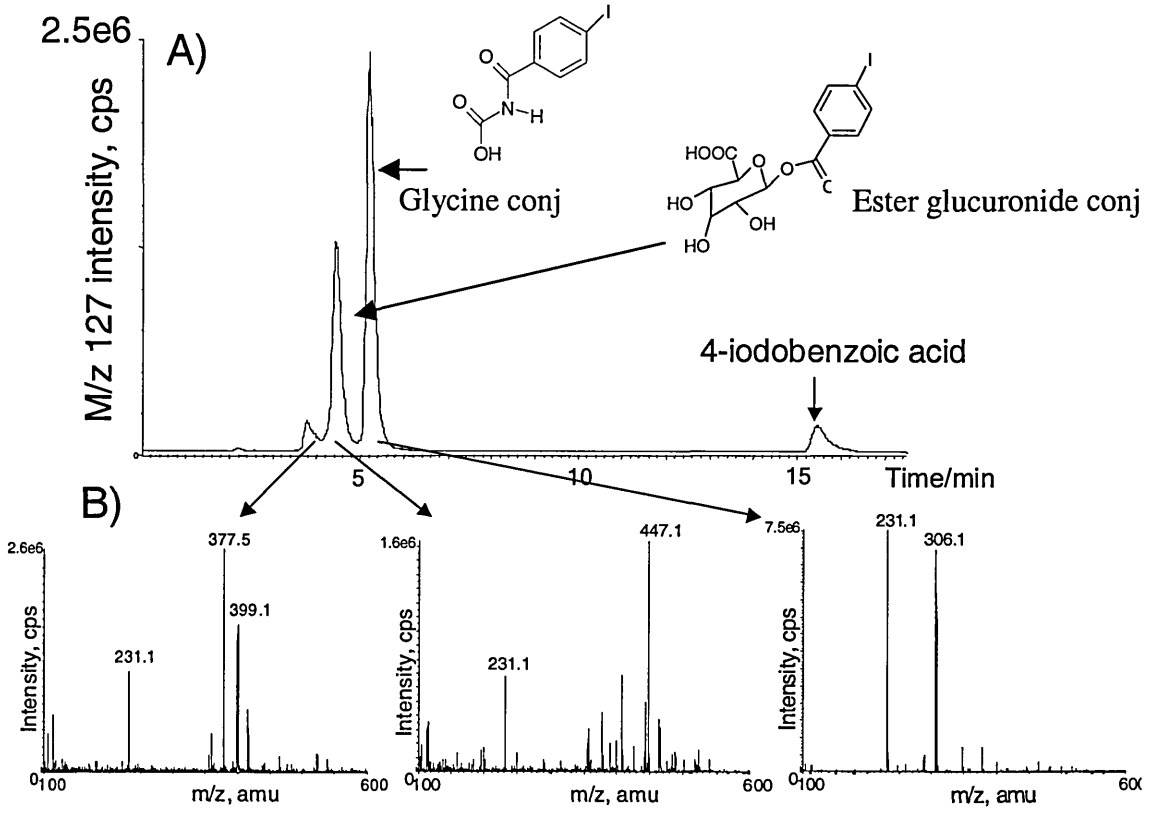


Figure 5.18 0-6 hour urine profile following dosing of rats with 4-iodobenzoic acid. Reversed phase isocratic HPLC with A) Iodine detection by ICP-MS at *m/z* 127 and B) MS spectra obtained from metabolite peaks at same times in conventional MS recording. For experimental conditions see section 5.20.

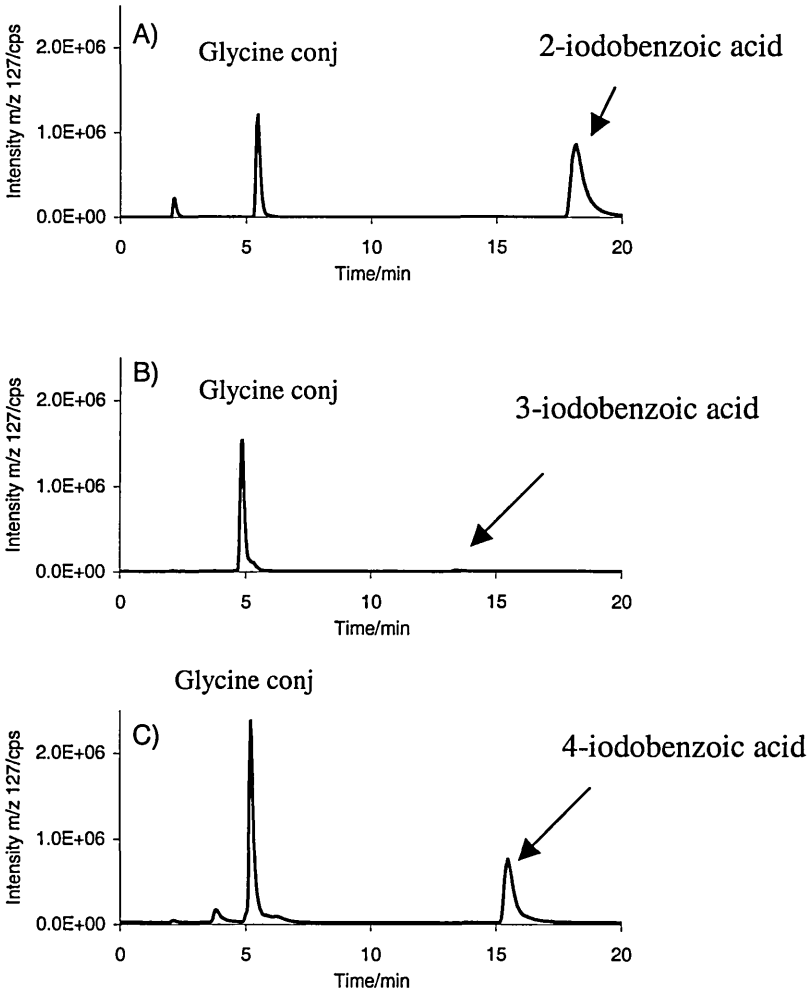


Figure 5.19 Profiles after basic hydrolysis of urine samples 0-6 hour from rats dosed with A) 2-iodobenzoic acid B) 3-iodobenzoic acid and C) 4-iodobenzoic acid. 20 µl neat urine was treated with 1 M NaOH and injected on a Polaris C18-A, 150 x 4.6 mm column, 40°C eluted isocratically with 20 % (in A) or 30% (in B and C) and 0.1% formic acid. Detection with ICP-MS at *m/z* 127 (100% = 2.5 x 10^6 cps).

5.23: Chlorine detection

The use of chlorine instead of Br or I was not possible. This is due to the amount of endogenous Cl found in biological matrices causing a high response in blank samples. Although chlorine may not be suitable for excretion studies it still has an important role in metabolite profiling [47].

The process is, however, more challenging than the two previous investigations with Br and I since the mass of Cl is in a region of isobaric interferences with polyatomic species such as $^{16}O^{18}O^{1}H^{+}$, $^{34}S^{1}H^{+}$, $^{35}Cl^{+}$ for the ^{35}Cl-isotope and $^{36}Ar^{1}H^{+}$, $^{36}S^{1}H^{+}$, $^{37}Cl^{+}$ for the ^{37}Cl-isotope, causing background levels to be elevated [63]. The sensitivity of Cl-element is not particularly high as shown in the initial work described in section 5.3 due to the small difference in ionisation potential between Cl and Ar. Therefore the problems of sensitivity and isobaric interferences complicate the use of Cl for the metabolite.

Work carried out by Duckett with Micromass [66] showed that data could be obtained for a profiling experiment as seen in Figure 5.20. The chromatograms show metabolites of urine collected over sequential time intervals after dosing a rat with 3-chloro-4-fluoroaniline (50 mg/kg).

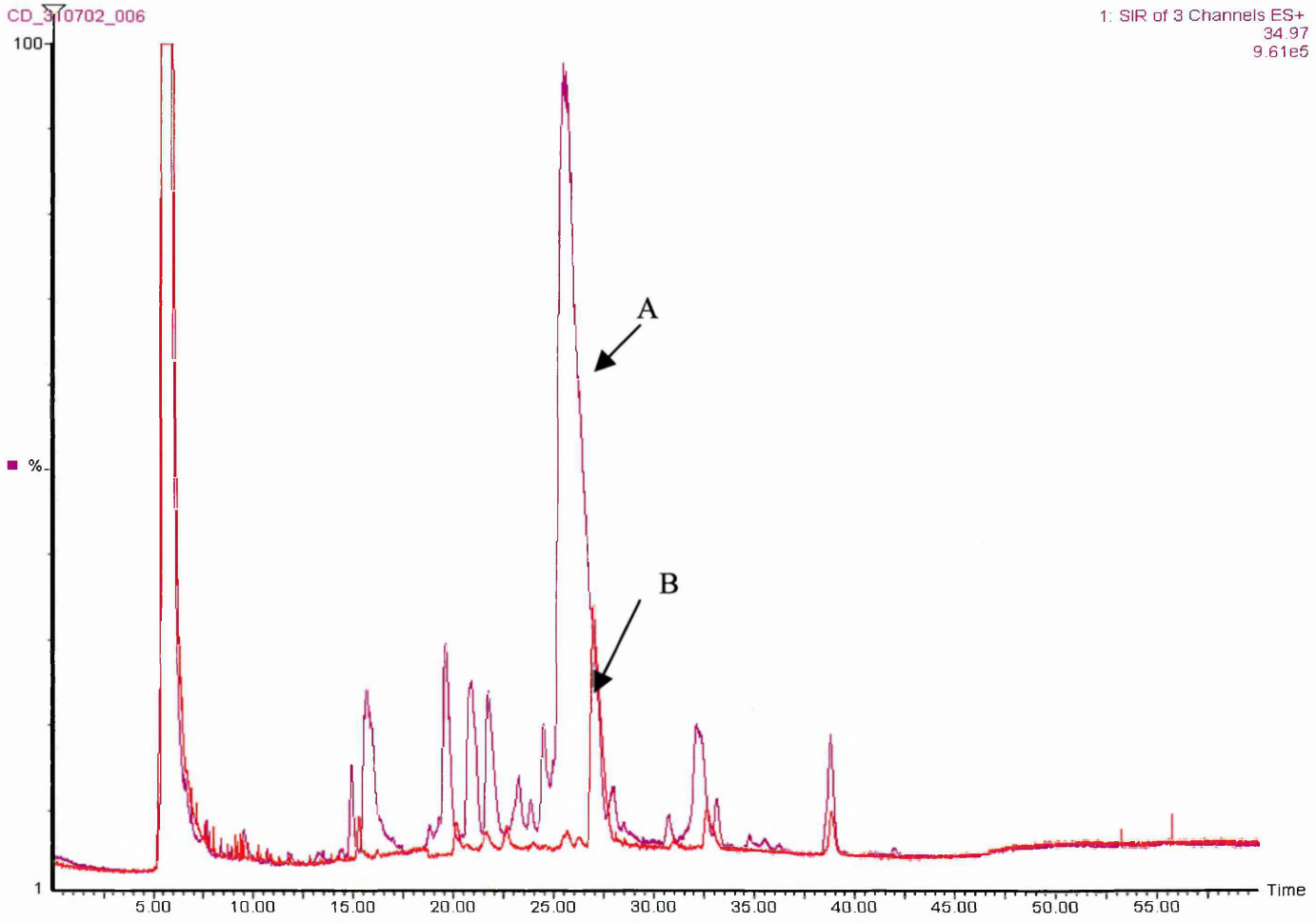


Figure 5.20 (A) HPLC-ICP-MS chromatogram of a metabolite profile using Cl-detection, sample taken at 0-6h after dosing 3-chloro-4-fluoroaniline. (B) HPLC-ICP-MS chromatogram of a sequential metabolite profile with the sample taken at 6-12h. The chromatograms show how the amount of the metabolite decreases with time.

Chromatogram used with permission from GV instruments obtained from reference [66].

This method used here was taken and transferred to the instrument in house with the same set-up, but the chromatograms could not be repeated and the background was too great to gain any results. The background isobaric inferences causing this problem can be controlled by the use of gasses in the collision cell. Hydrogen and helium are used with the Platform ICP-MS and react with the polyatomic atoms to remove them from the *m/z* value detected. When the same setting for the collision gases were used in house as at Micromass, the interferences appeared to get worse instead of better. The difference between the two set-ups was the source of hydrogen. The hydrogen was obtained from a generator in house while at Micromass high purity hydrogen was used from a cylinder. The generator, although advertised as giving the required level of purity, showed with standards not to give appropriate sensitivity changes, therefore indicating a problem. Due to regulations in the laboratory it was not possible to install a hydrogen cylinder to investigate the differences between the systems fully.

5.24: Conclusion

The use of halogens for excretion studies has been shown possible with little or no sample preparation, minimum analysis time per sample, low detection limits and avoiding the use of radiolabels. When used for profiling experiments then the technique is very useful within a Drug Metabolism and Kinetics Department for quantification of potential metabolites without expensive labelling.

Chapter 6

Halcogen detection by HPLC-ICP-MS for sulphur and phosphorus containing compounds.

6.1: Summary

The sensitive detection of sulphur and phosphorus-containing compounds using HPLC-ICP-MS is difficult due to the high background caused by polyatomic isobaric interferences. One potential solution to the problem of polyatomic interferences of this sort for S and P is to react the latter with oxygen in the collision hexapole to separate them from the interferences (O_2 on S and NOH on P). This has the effect of moving the detection mass from 31 and 32 to 47 and 48 *m/z* for P and S respectively. This region is clear from interferences thereby providing an increase in the sensitivity of detection for the analytes of approximately 50-100 fold. For omeprazole, a model S containing compound, a limit of detection (LOD) of 800 pg on column was achieved, an increase of ca. 100 fold in sensitivity. Similarly in the case of ZD6126, a phosphorus-containing pro-drug, an increase in sensitivity of 50 times was observed with an LOD of 1 ng on column. The technique of reacting sulphur with O_2 was then applied to the metabolite profiling of omeprazole, a sulphur containing selective inhibitor of gastric acid secretion.

6.2: Introduction

Inductively coupled plasma mass spectrometry (ICP-MS) has been used as an analytical technique since the 1980's [2, 14], but the use of the instrument in combination with high performance liquid chromatography (HPLC) is more recent. Whilst mainly used for the analysis of metals [5, 39, 54] (e.g. Pt) HPLC-ICP-MS has also found some applications in drug and xenobiotic metabolism studies of halogen-containing compounds (Cl, Br and I) [40, 41, 47, 51], as well as initial studies on S and P-containing compounds [9, 42]. However, in the case of S and P, detection sensitivity is compromised by the presence of a range of polyatomic interferences such as $^{14}N^{18}O^{+}$ and $^{15}N^{16}O^{1}H^{+}$which lead to isobaric interference [25]. To improve the specificity of detection for S and P, and thereby enhance sensitivity, chemical reaction of S and P with O_2 can be used to move them away from the region of interference [16]. The main difference between the analytes and the interferences is seen in the O-atom affinity. O-atom transfer from O_2 to P^{+} and S^{+} is thermodynamically favourable while for NO^{+}, NOH^{+}, and O_2^{+} it is endothermic and forbidden under thermal conditions. When oxygen is reacted with sulphur to produce a sulphoxide (SO^{+}) the detection *m/z* value changes from 32 to 48. Similarly for P, the production of PO^{+} results in an increase in *m/z* from 31 to 47. Table 6.1 shows the reaction enthalpy change and thermal reaction rate constant for oxygen with S, P and the interfering species.

The detection of some sulphur-containing metabolites can be important for understanding their toxicity. Certain toxic reactions arise as a result of the formation of reactive metabolites. These can be detected as they can react with the sulphur-

containing tripeptide glutathione within the cell and can are then found in the excreta as sulphur-containing mercapturates [81].

Table 6.1 Reaction thermodynamics and kinetics of oxygen with sulphur, phosphorus and relevant interfering ions.

Reaction	Reaction enthalpy change Kcal mol^{-1}	Thermal reaction rate constant K_r, $molecule^{-1}$ cm^3s^{-1}
$P^+ + O_2 \rightarrow PO^+ + O$	-71.4	5.3×10^{-10}
$S^+ + O_2 \rightarrow SO^+ + O$	-6.2	1.8×10^{-11}
$CO^+ + O_2 \rightarrow CO_2^+ + O$	-13.5	$<2 \times 10^{-14}$ (no reaction)
$HCO^+ + O_2 \rightarrow COOH^+ + O$	3.3	$<2 \times 10^{-14}$ (no reaction)
$NO^+ + O_2 \rightarrow NO_2^+ + O$	57.4	$<1 \times 10^{-11}$ (no reaction)
$NOH^+ + O_2 \rightarrow NO_2H^+ + O$	19.3	no data
$O_2^+ + O_2 \rightarrow O_3^+ + O$	102.1	no data

Table taken from ref [25]

In this section the utility of this approach for the quantitative analysis of sulphur compounds (omeprazole and sulphanilamide) and the phosphorus-containing compound ZD6126 (Figure 6.1) by HPLC-ICP-MS is described.

6.3: Experimental

6.3.1: Reagents

Chromatographic grade organic solvents were obtained from Riedel-de Haën (Sigma-Aldrich Ltd, UK) with formic acid purchased from Fisher (Fisher Scientific Ltd, UK),

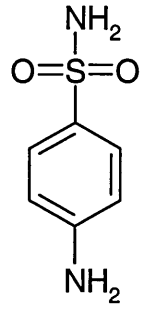
which were of analytical or HPLC grade. Sulphanilamide (purity 99 atom %) and omeprazole (purity 99 atom %) were purchased from Sigma (Sigma-Aldrich Ltd, UK) and the phosphorus-containing pro-drug was supplied by AstraZeneca Pharmaceuticals (Alderley Park, Macclesfield, UK).

6.3.2: Standard and Calibration Solution Preparation for Sulphur and Phosphorus.

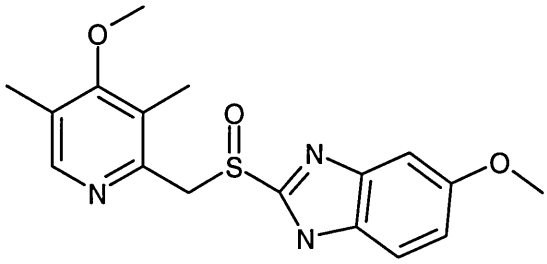
A standard solution of sulphanilamide was prepared at a concentration of 10 μg/mL by dissolution in water aided by sonication for 10 min in an ultrasonic water bath (Ultrawave Ltd, Cardiff UK).

Omeprazole stock solution was prepared in mobile phase at a concentration of 1 mg/mL in a 10 mL volumetric flask. The dissolution was aided by sonication for 10 min in an ultrasonic water bath. The stock solution was then diluted with mobile phase to give final concentrations of 1000, 700, 500, 400, 300, 100, 50 20 and 10 ng/mL.

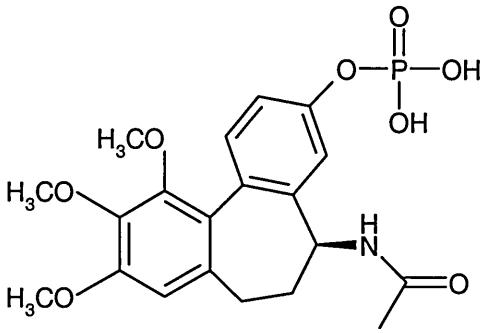
ZD6126 stock solution was prepared by dissolving the compound in mobile phase to a concentration of 1 mg/mL, and sonication for 10 min to aid dissolution. Dilutions were prepared in mobile phase to give final concentrations of 1000, 700, 500, 400, 300, 100, 50 and 10 ng/mL.



Sulphanilamide (Mwt = 172)



Omeprazole



ZD6126 Phosphate pro-drug

Figure 6.1 Structures of the compounds used for sulphur and phosphorus HPLC-ICP-MS studies.

6.3.3: Plasma Samples

200 μL samples of control plasma were spiked with either sulphanilamide or the phosphate pro-drug to a concentration of 100 ng/mL. These samples were then diluted with 100 μL of water, and 50 μL aliquots were injected on to the HPLC-ICP-MS system. Blanks were also prepared to check there were no interfering peaks present in the chromatograms.

6.4: Chromatography for Sulphur and Phosphorus Detection

An isocratic HPLC system was used for the analysis of sulphanilamide and the phosphate pro-drug with a Synergi Polar Rp column 150 x 4.6 mm (Phenomenex, Macclesfield, UK). The mobile phase consisted of acetoniltrile, water and formic acid (40:60 0.1 %), at a flow rate of 1 mL/min. An injection volume of 50 μL was used.

An isocratic HPLC system was used for the analysis of omeprazole with a C18 Xterra 150 x 2.1 mm, 3 μm column (Waters, Milford, USA). The mobile phase consisted of acetonitrile water (20:80) at a flow rate of 200 μL/min. The injection volume was 50 μL.

6.5: HPLC-ICP-MS

Initial work using this technique for sulphur-specific detection with sulphanilamide as the test analyte was carried out with an Elan DRC II ICP-MS at PerkinElmer Beaconsfield, equipped with a PE series 200 pump and a PE series 200 autosampler. The sample was introduced into the plasma via a concentric nebuliser. Interactive software was used for the instrument control and analysis (PerkinElmer, Beaconfield, UK). The flow of the nebuliser gases and the operating acquisition conditions of the ICP-MS are shown in Table 6.2.

Chromatography for the HPLC-ICP-MS of omeprazole and the phosphate-pro-drug was performed using a Jasco 1580 gradient HPLC system (Jasco Ltd, Great Dunmow, UK) with samples introduced via a PE 200 autosampler (PerkinElmer). The injector was flushed with mobile phase. ICP-MS was performed on a Platform ICP-MS (GV Instruments Ltd, Wythenshawe, UK) and the eluent was introduced to the ICP-MS via an Aridus nebuliser (Cetac Technologies, Omaha, Nebraska, USA). Masslynx software (GV Instruments, Wythenshawe, UK) was used for data analysis and instrument control. The flow of the nebuliser gases and the operating acquisition conditions of the ICP-MS are shown in Table 6.3.

Table 6.2 Instrument operating conditions for the Elan DRC II with Sulphanilamide.

Nebulizer Gas Flow	0.77 L/min	Analog Stage Voltage	-2000
Auxiliary Gas Flow	0.8 L/min	ICP RF Power	1500 w
Plasma Gas Flow	20.00 L/min	O_2 gas	0.5 mL/min
Lens voltage	6.5	Mass monitored	31, 32, 47, and 48

Table 6.3 Instrument operating conditions for the Platform ICP-MS with oeprazole and ZD6126.

Cooling gas	17.00 L/min	Plasma Power	1700 w
Plasma gas	0.65 L/min	Acquisition mode	SIR
Nebuliser gas	0.6 L/min	Dwell time	200 ms
Helium gas	0 mL/min	Masses monitored	31, 32, 34, 47 and 48
Hydrogen gas	0 mL/min	Argon/Oxygen (95/5 %)	0.2 mL/min
Oxygen gas	0.8 mL/min	Total analysis time	6 min

6.6: Results and Discussion

In order to perform "chemical resolution" a small amount of oxygen (Table 6.2 and 6.3) was introduced into the dynamic reaction cell of the Elan DRC II or the hexapole collision cell of the GV Platform through which the analytes are passed after the inductively coupled plasma. Prior to their exit from the reaction area into the MS detector chemical reaction occurred to produce the oxidised species for detection.

6.6.1: Sulphur Detection

Initial studies to investigate the potential of chemical resolution were carried out using sulphanilamide as a model compound on an Elan DRC II ICP-MS. The resulting increase in sensitivity obtained using such an approach is shown in Figure 6.2 A and B. Thus in Figure 6.2A, when the compound was monitored at *m/z* 32 only a relatively small signal was observed. In contrast, monitoring the signal for *m/z* 48 after introduction of oxygen in to the dynamic reaction cell resulted in a dramatic increase in signal to noise as shown in Figure 6.2B. The calibration curve was found to be linear over a range of 0.2 ng to 19 ng (coefficient regression 0.9998). In Figure 6.3 the chromatogram obtained following the injection of a spiked plasma sample (100 ng/mL, responding to 5 ng of analyte on-column) is shown.

Similar data were subsequently obtained for another sulphur-containing compound, omeprazole, on a Platform ICP-MS. Figure 6.4A-C shows the results obtained for 5 μg on column of omeprazole with detection either of ^{32}S, ^{34}S or $^{32}S^{16}O$. Although only a minor isotope of sulphur (4.2 % abundance) the detection of ^{34}S has been employed previously as a means of avoiding polyatomic interferences [67] and, as shown in Figure 6.4B there is a useful increase in sensitivity for this isotope compared to ^{32}S. However, comparison with the trace shown in Figure 6.4C with oxygen infused in to the hexapole reaction cell, clearly indicates the superiority of the latter approach. The improvement in response was found to be an increase of ca. a 100 times over the response found for ^{34}S isotope and a 1000 times for the ^{32}S isotope.

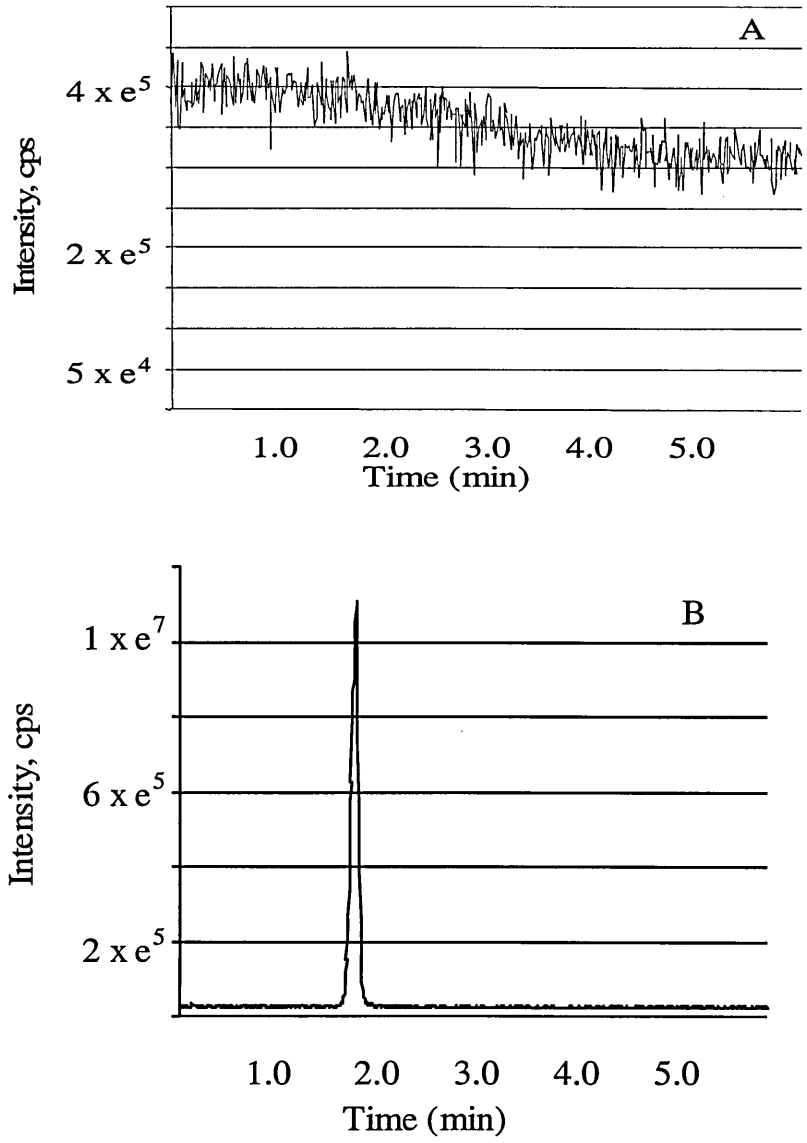


Figure 6.2 HPLC-ICP-MS chromatogram A shows the response for sulphanilamide (100 µL injected of 10 µg/mL standard solution) monitored at *m/z* 32 (1 µg on column). HPLC-ICP-MS chromatogram B shows the improvement in response, of the same sample, after addition of oxygen to the collision cell to produce an SO^+. ICP-MS data collected at *m/z* 48. For experimental conditions see section 6.3.

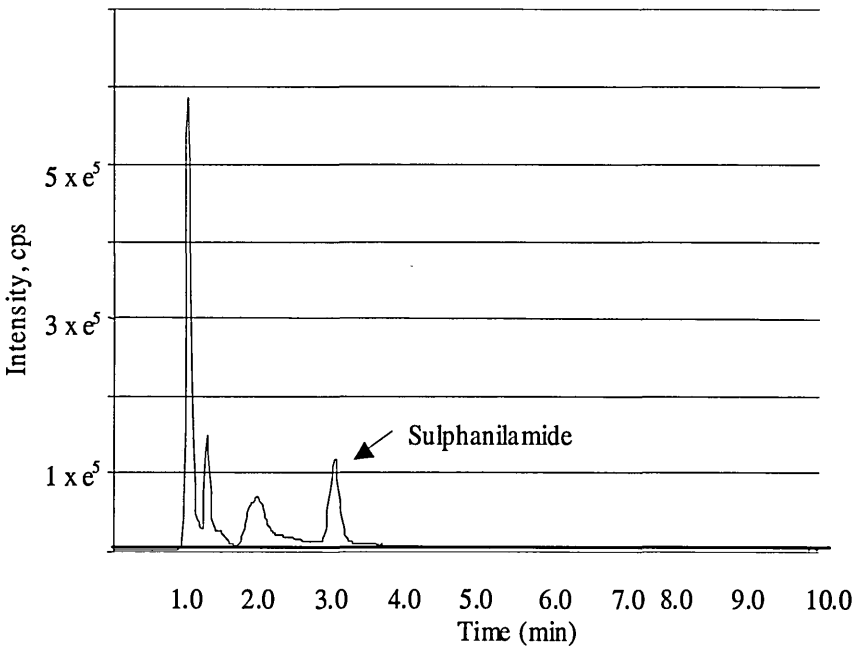


Figure 6.3 HPLC-ICP-MS chromatogram of a diluted plasma sample spiked with sulphanilamide, detected at *m/z* 48 after sulphur was reacted with oxygen (4 ng on column). The compound could be detected in a plasma matrix using chromatography to separate the endogenous material from the peak of interest. For experimental conditions see section 6.3.

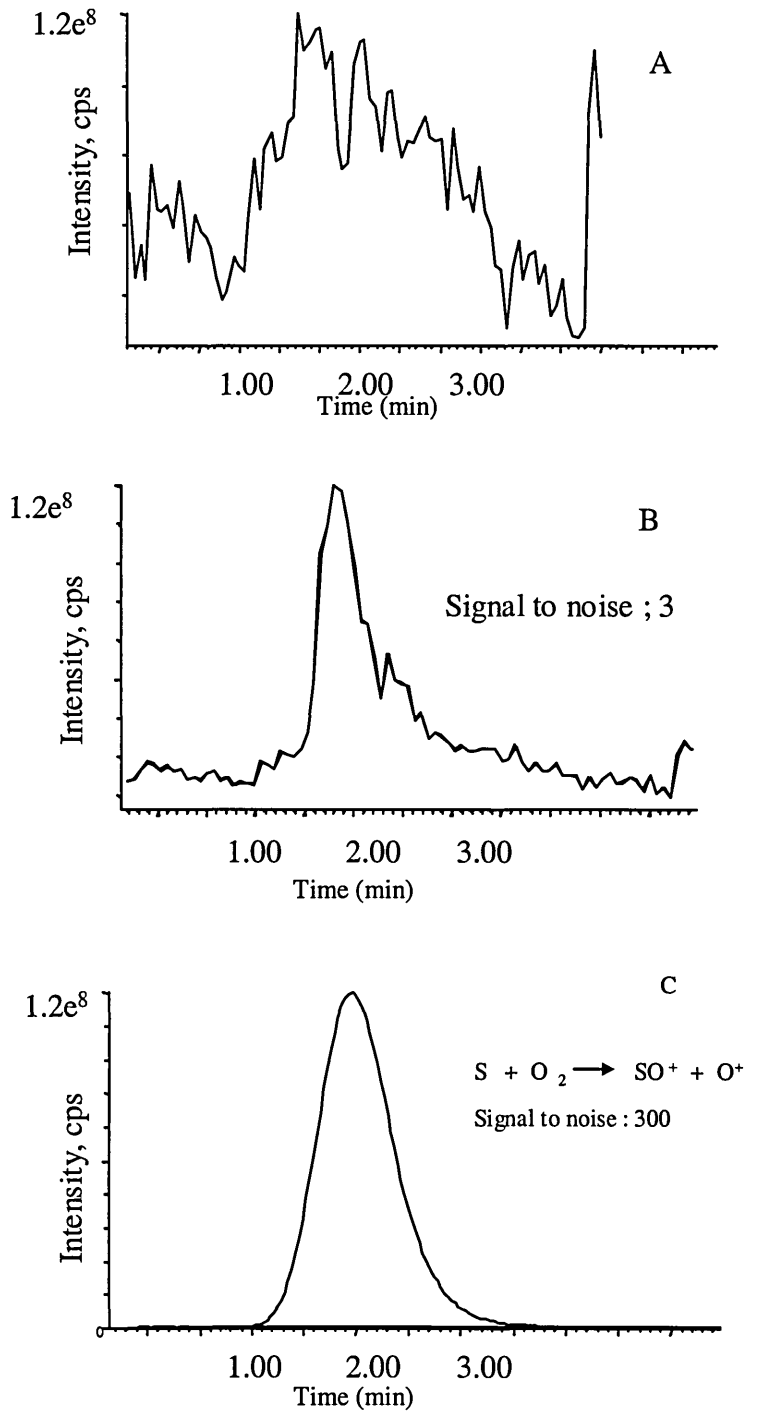


Figure 6.4 HPLC-ICP-MS chromatograms for omeprazole at 5 µg on column: (A) S monitored at *m/z* 32, (B) S monitored at *m/z* 34 and (C) SO^+ monitored at *m/z* 48. The figure demonstrates the reduction in the background when using SO^+ for detection.

The properties of the system with respect to sensitivity, accuracy, reproducibility and linear range were then investigated. Table 6.4 shows the accuracy and precision data collected using omeprazole with the chromatographic conditions described earlier, at three concentrations with six replicates at each concentration. This experiment was carried out with aqueous standards to show that the sulphur - oxygen reaction in the collision cell would not saturate rapidly and would maintain stability and sensitivity over a range of analyte concentrations. The system was linear over the range 800 pg to 400000 pg on column (regression coefficient 0.9998) with an accuracy at the lowest level (800 pg on column) of 98 % and coefficient of variation of 7.2 %. The data collected indicate that the use of oxygen in this manner was a reliable means of increasing sensitivity for S-containing compounds. Chromatographic traces obtained from the precision and accuracy experiment can be seen in Figure 6.5, where examples of the LOQ (800 pg compound on column) and a blank are represented. The amount of the sulphur detected corresponds to ca. 80 pg on column since there is only one S atom per molecule in the compound (Figure 6.1).

Table 6.4 Sulphur precision and accuracy data to determine the validity of using SO^+

Quality control samples (pg on column)	Observed mean (pg on column)	Accuracy (%)	Coefficient of variation (%) (n=6)
800	784	98	7.2
40000	35160	88	5.6
400000	438240	109	9.1

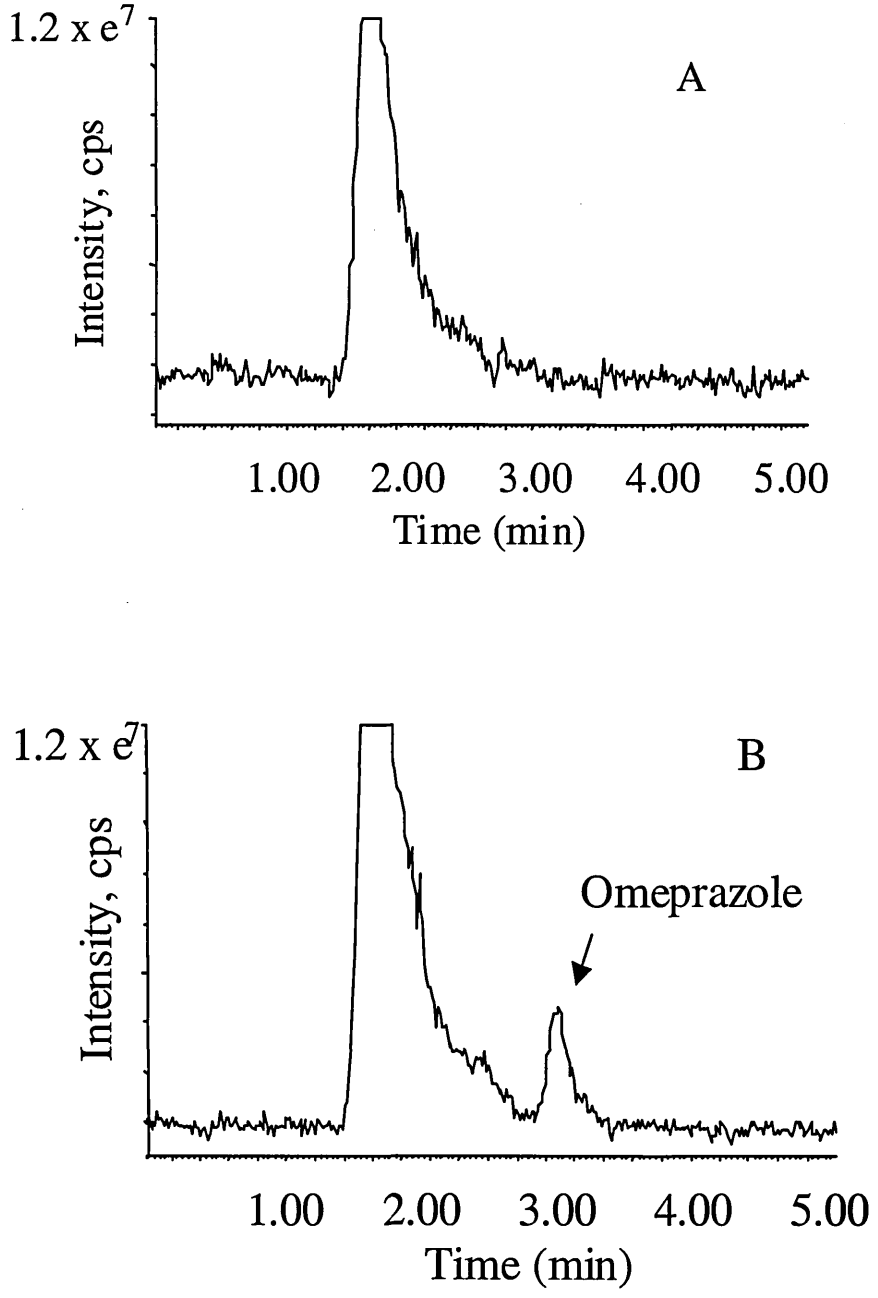


Figure 6.5 HPLC-ICP-MS chromatograms from omeprazole evaluation monitored at *m/z* 48 to avoid polyatomic interferences: (A) Blank showing endogenous sulphur from the matrix and (B) lower limit of quantification for omeprazole (800 pg on column). For experimental conditions see section 6.3.

6.7: Phosphorus-Detection

Evaluation of this methodology for the phosphate pro-drug ZD6126 was carried out on the Platform ICP-MS. Figure 6.6 shows that an increase in response of ca. 50 times was obtained when PO^+ instead of P^+ detection was used. In Table 6.5 accuracy and precision data are shown, indicating an accuracy of 109 % at the LOD (1000 pg on column corresponding to 100 pg of P), with a coefficient of variation of 9.7 %. The calibration was linear over the range tested (10 ng to 100 ng on column), with a coefficient of regression of 0.999. Although the linear range for this test was small, the characteristics of the ICP-MS should allow a much greater range to be used.

Table 6.5 Phosphorus precision and accuracy data.

Quality control samples (ng on column)	mean (ng on column)	accuracy (%)	coefficient of variation (%) (n=4)
10	10.9	109	9.7
50	49.9	99.8	9.0
100	102.1	102.1	3.0

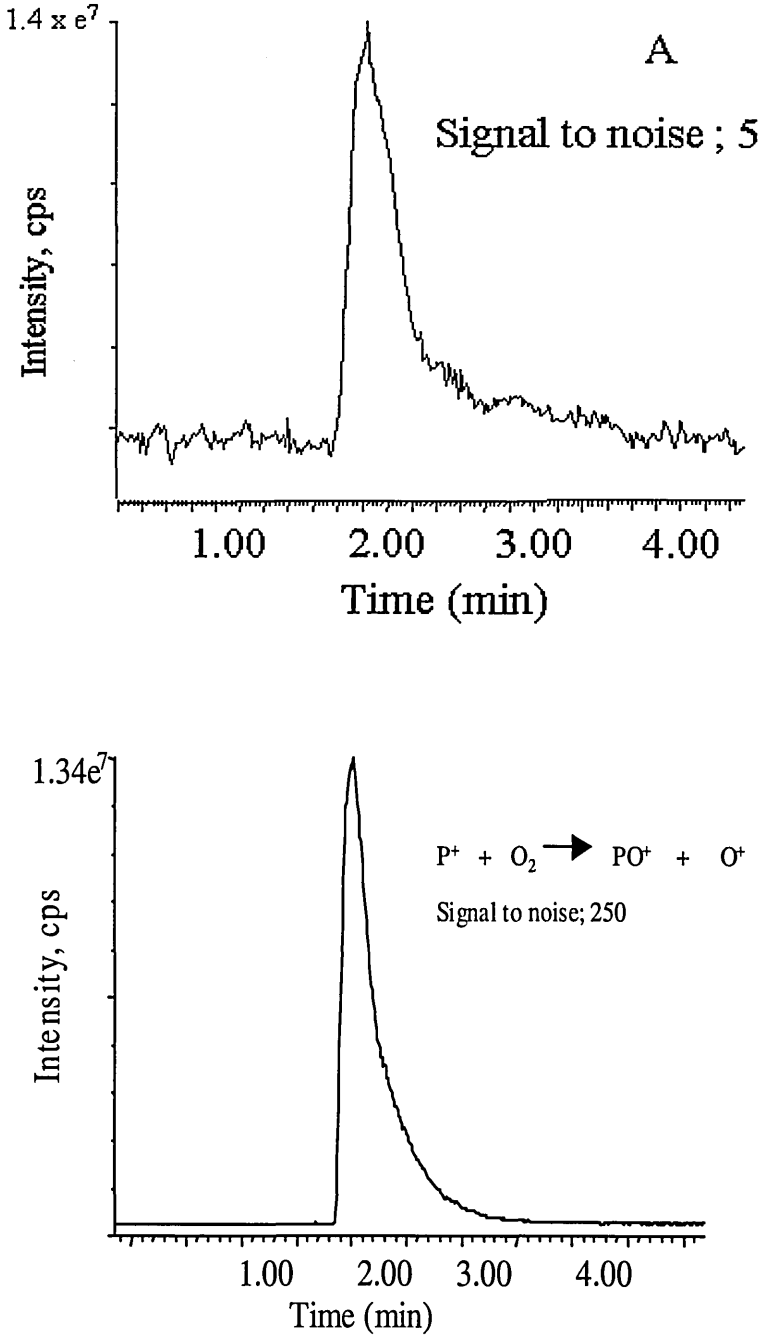


Figure 6.6 HPLC-ICP-MS chromatograms for the phosphorous containing pro-drug ZD6126 at 5 μg on column: (A) P monitored at *m/z* 31 and (B) P monitored at *m/z* 47. The figure shows the decrease in the background noise when the PO^+ reaction is used for monitoring. For experimental conditions see section 6.3.

Similar improvements in sensitivity to those seen for S were also obtained with this approach using the Elan DRC II for the detection of P. When P is reacted with oxygen, moving the detection from 31 to 47, polyatomic interference such as $^{15}N^{16}O^{+}$, $^{14}N^{16}O^{1}H$ and $^{12}C^{1}H_{3}{}^{16}O^{+}$ [25] are avoided. This is due to the reaction of P and O being exothermic and therefore going to completion, unlike the reaction of N and O, which is endothermic and therefore is unfavourable. The initial results for PO^{+} showed a calibration plot for the process to be linear over the range 100 ng to 1900 ng (regression coefficient of 0.999) for directly injected standards. A plasma sample analysed by HPLC-ICP-MS (Figure 6.7) to be showed the P-containing pro-drug (5 ng on columns) resolved from endogenous phosphate.

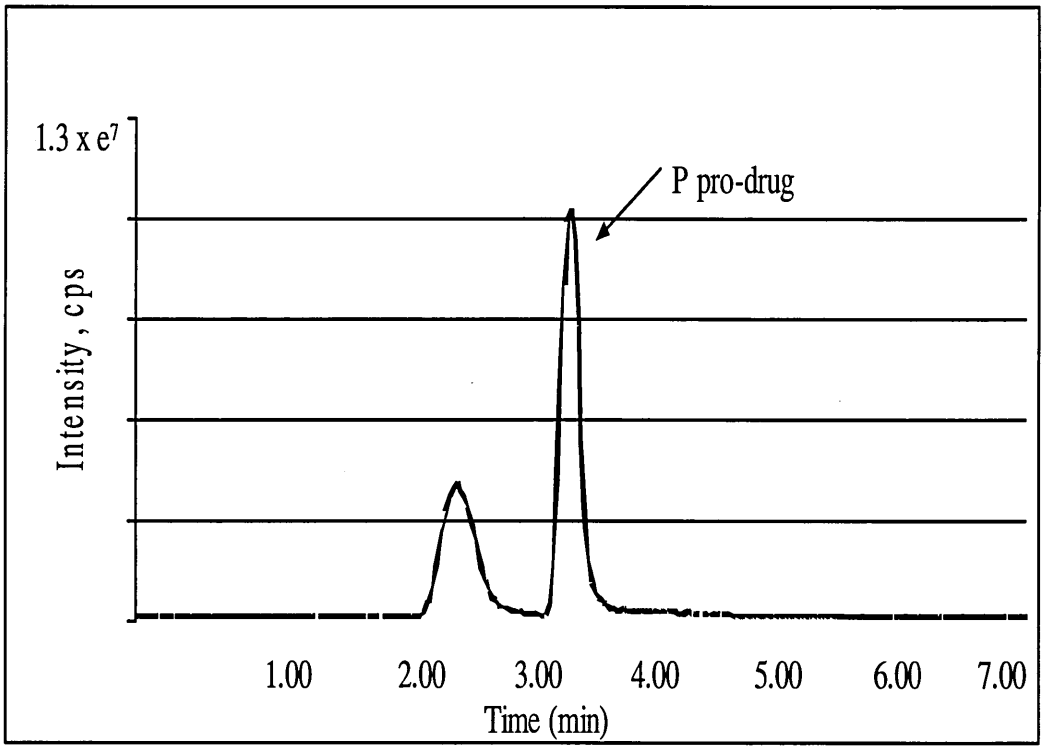


Figure 6.7 HPLC-ICP-MS chromatogram of a diluted plasma sample of ZD6126 (100 ng on column) monitored at *m/z* 47. The trace shows an endogenous peak separated from the phosphourus pro-drug. For experimental conditions see section 6.3.

6.8: Sulphur-specific detection of omeprazole metabolites in rat urine by ICP- MS

6.9: Introduction (Omeprazole metabolism study)

HPLC-ICP-MS with sulphur-specific detection was investigated as a method for obtaining metabolite profiles for the drug omeprazole administered as a 1:1 mixture of ^{32}S- and ^{34}S-labelled material. Analysis based on the monitoring of the chromatographic eluent at either *m/z* 32 or 34 was not successful due to insufficient sensitivity caused by interferences from polyatomic ions as discussed earlier. However, reaction of sulphur with oxygen in the hexapole collision cell, combined with monitoring at *m/z* 48 (for ^{32}S) or *m/z* 50 (for ^{34}S) provided a facile method for metabolite profiling. Detection of *m/z* 48 was superior in sensitivity to *m/z* 50.

6.10: Experimental (Omeprazole metabolism study)

6.10.1: Reagents

Omeprazole was purchased from Sigma (Sigma-Aldrich Co, St. Louis, MO, USA), [^{34}S] omeprazole was synthesized at AstraZeneca R&D (Mölndal, Sweden). Methanol (HPLC-grade) and ammonium acetate were purchased from Fisher Scientific UK Ltd (Lougborough, UK). Water was obtained from an Elga water purification system (Elgastat Maxima, Elga, UK).

6.10.2: Urine samples

Pooled urine samples (0-9 h post-dose), concentrated using C_{18}-solid phase extraction, were obtained from three male Sprague-Dawley rats dosed orally with 200 μmol/kg of a 1:1 mixture of [^{34}S]-labelled and unlabelled omeprazole. The samples were produced by Lars Weidolf of AstraZencea and preparation and extraction is described in [68, 69]. 100 μL was injected on column.

6.10.2: Instrumental

Chromatography was performed using a Jasco gradient HPLC system (Jasco Ltd, Great Dunmow, UK) with samples introduced via a PE 200 autosampler (PerkinElmer Ltd, Beaconsfield, UK). A C_{18} Hichrom column, HI-RPB, 5μm, 150x4.6 mm was used at a temperature of 40°C. The flow rate of 1 mL/min was split with 250 μL/min introduced to the ICP-MS. 0.13 mm PEEK tubing was used throughout. A gradient was employed based on A) methanol / H_2O (5/95), 2 mM ammonium acetate pH=7.0 and B) methanol / H_2O (95/5), 2 mM ammonium acetate pH=7.0 as follows: 0-5 min 95 % A, 5-20 min 95-50 % A, maintained from 20 to 25 min, 25-30 min 50-95 % A, remaining at 95 % A until 35 min.

ICP-MS was performed on a GVI Platform ICP-MS (GV Instruments Ltd, Wythenshawe, UK) equipped with a concentric nebulizer coupled to a cooled (-7°C) double pass spray chamber. Masslynx software (GV Instruments Ltd, Wythenshawe, UK) was used for data analysis and instrument control. The operating conditions of the instrument are shown in Table 6.6.

Table 6.6 Instrument operating conditions for ICP-MS for omeprazole metabolite profiling.

Parameter	Without oxygen reaction	With oxygen reaction
Cooling gas	17.0 L/min	15.0 L/min
Plasma gas	0.8 L/min	0.8 L/min
Nebuliser gas	0.8 L/min	0.8 L/min
Helium gas	9.8 mL/min	0 mL/min
Hydrogen gas	2.0 mL/min	0 mL/min
Oxygen gas	0 mL/min	0.60 mL/min
Plasma power	1700 W	1700 W
Acquisition mode	SIR	SIR
Dwell time	300 ms	300 ms
Masses monitored	32, 34	48, 50

6.11: Results and Discussion (Omeprazole metabolism study)

Previous studies have shown that, with suitably concentrated samples, sulphur-containing compounds can be monitored in chromatographic eluents without the need for special measures such as reaction with oxygen [57, 58]. For completeness therefore, the urine extract sample was initially analysed by ICP-MS for both ^{32}S and ^{34}S without using the oxygen reaction to move from the region of polyatomic interferences. However, as Figure 6.8 shows, adequate sensitivity was not obtained

for either isotope of sulphur. Thus, in the case of ^{32}S, the high background due to polyatomic interferences meant that no S-containing peaks for the compound or its metabolites could be detected by monitoring at *m/z* 32. At *m/z* 34 it was possible to detect a few peaks but it is clear, even with the use of an isotopically labelled compound, that sensitive detection would not be obtained and that minor metabolites present in this sample would not be detected. The addition of oxygen to the hexapole collision cell and monitoring at *m/z* 48 and 50 was then attempted. As shown in Figure 6.9, the result was a dramatic increase in signal to noise ratio and many sulphur-containing peaks appeared.

Thus, in the case of ^{32}S, where no peaks were detected with the conventional ICP-MS, more than 30 resolved sulphur - containing peaks were observed following addition of oxygen to the collision cell. In this study, omeprazole was dosed in a 1:1 ratio of ^{32}S and ^{34}S but, as seen in Figure 6.9, a higher sensitivity was obtained at *m/z* 48 (i.e. detection of ^{32}S) compared to *m/z* 50 (^{34}S). It is also noteworthy that, due to the lower sensitivity a generally higher background at 50 was observed. However, whilst these results show that there is no advantage in terms of sensitive detection to be obtained from isotopically labelling a compound with ^{34}S for HPLC-ICP-MS, a comparison of the two profiles is instructive. The use of the labelled compound enables the omeprazole-related peaks are distinguished from endogenous sulphur-containing substances, as the former requires peaks to be present in both the ^{32}S- and ^{34}S-chromatograms. From the results obtained here, the bulk of the endogenous sulphur-containing material seems to elute early in the gradient.

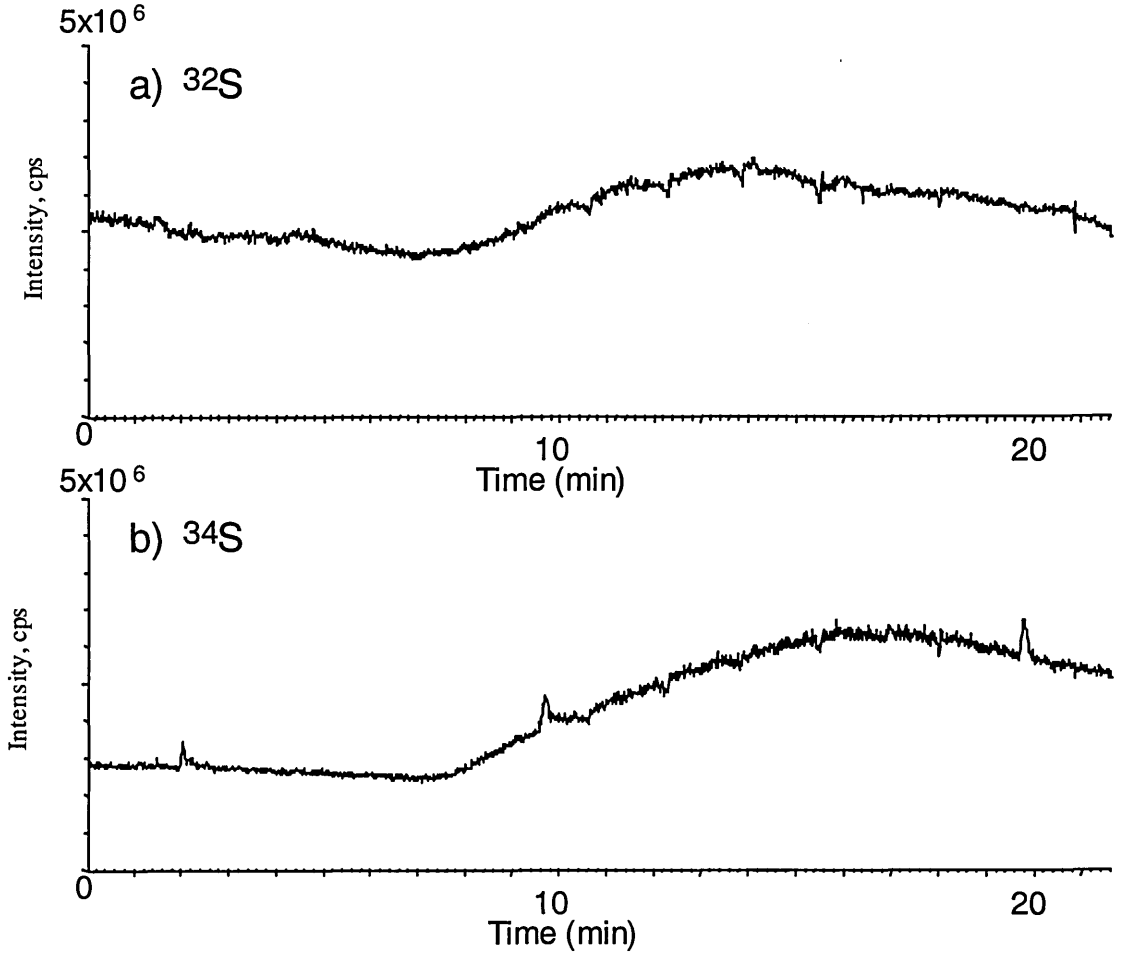


Figure 6.8 HPLC-ICP-MS chromatogram of omeprazole metabolites in rat urine extract detected at a) *m/z* 32 and b) *m/z* 34. The chromatograms show the poor response obtained without the enhancement shown with the addition of oxygen (Figure 6.9). 100 μL of sample was inject ed onto the column undiluted. For experimental conditions see section 6.10.

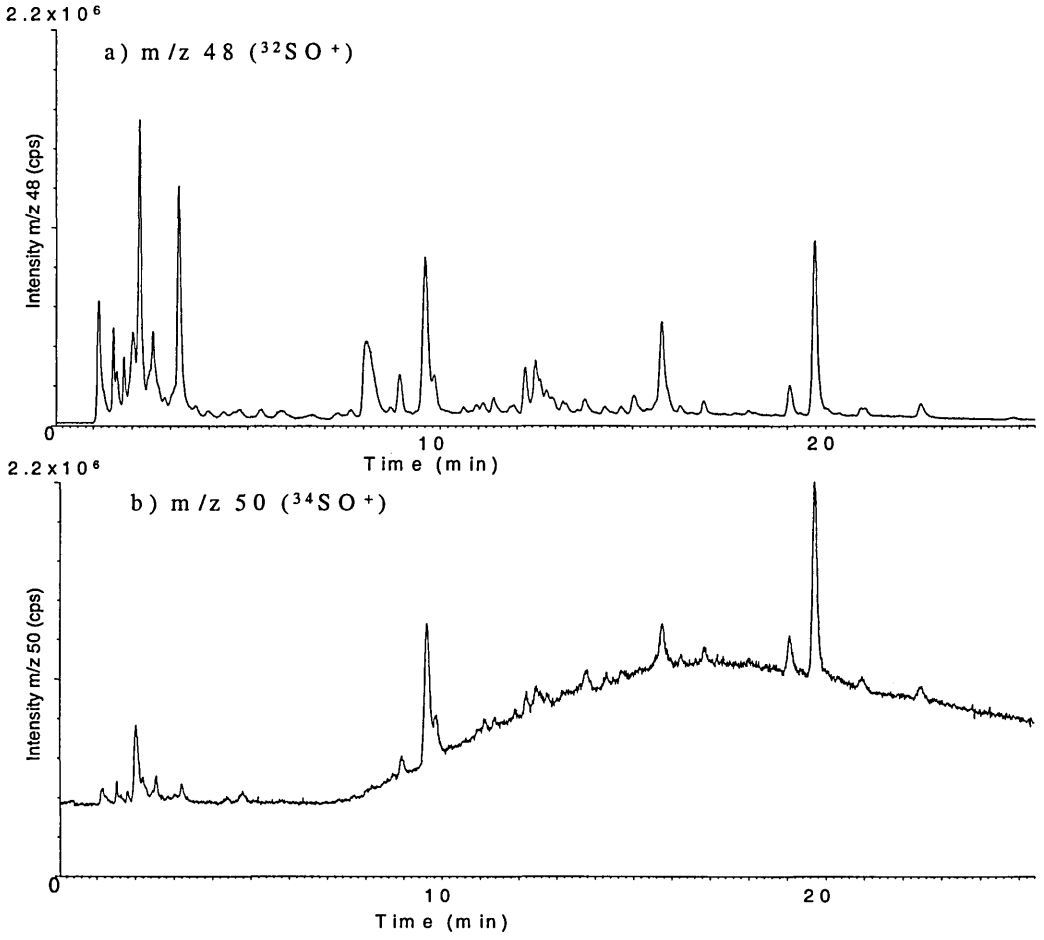


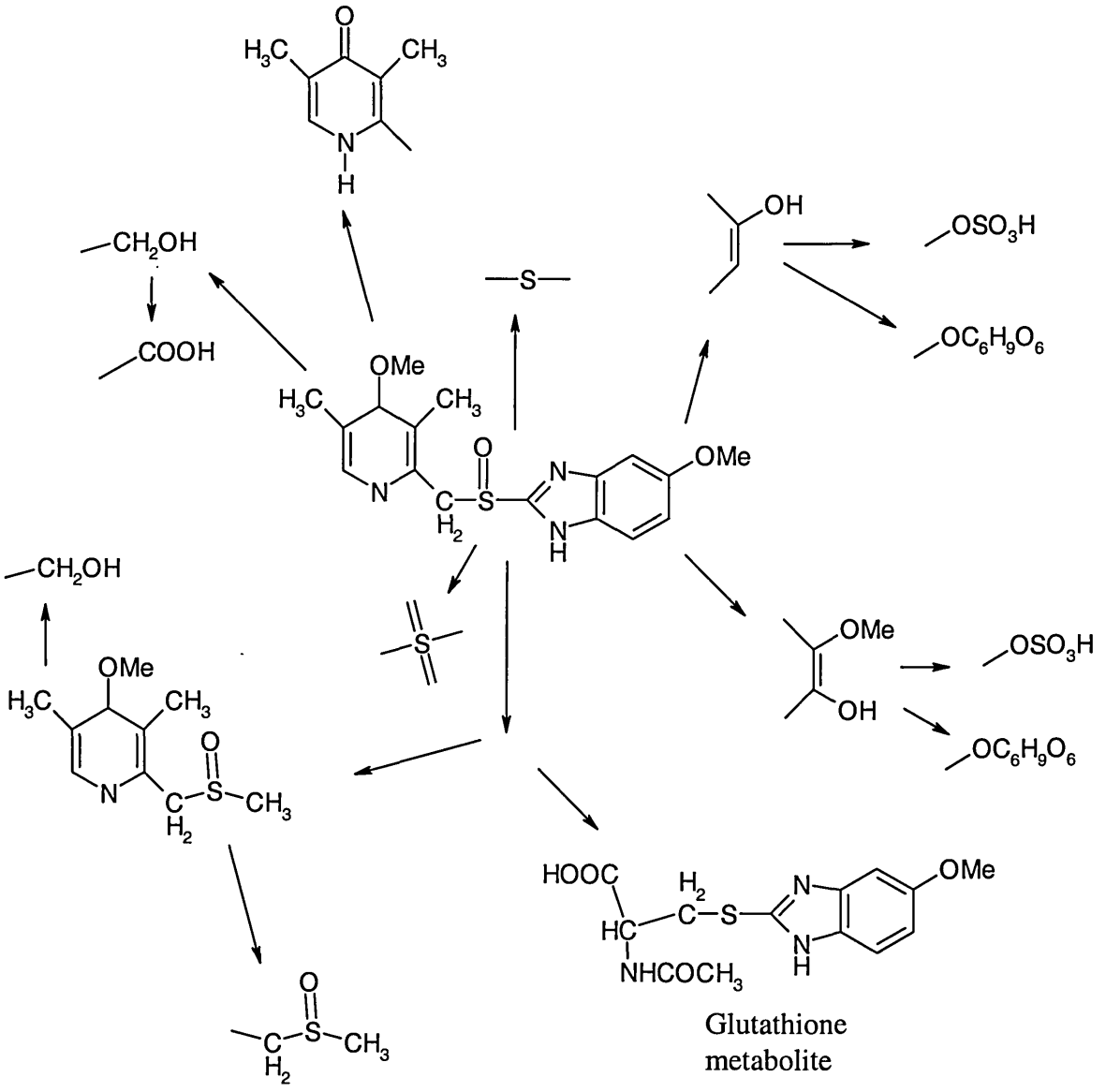
Figure 6.9 HPLC-ICP-MS chromatogram of omeprazole metabolites in a rat urine extract detected at a) *m/z* 48 ($^{32}SO^{+}$) and b) *m/z* 50 ($^{34}SO^{+}$) run on the same HPLC conditions as in Figure 6.8. Comparison of the traces shows the difference between endogenous sulphur and sulphur related to a metabolite. For experimental conditions see section 6.10.

ICP-MS provides no structural information so that the identity of the various peaks cannot be determined by this method. However, a peak corresponding to unchanged omeprazole was not detected (the drug would have eluted at 23.4 min in this system) clearly showing that the compound was extensively metabolised. Structural information was then obtained by simultaneously monitoring the chromatographic eluent by conventional mass spectrometry, as described for other elements in previous chapters.

6.12: Structural identification of omeprazole metabolites by HPLC-MS run in parallel with HPLC-ICP-MS.

6.12.1: Introduction (Omeprazole structural study)

The samples used in the experiment to determine the metabolic profile of omeprazole were then re-analysed in a hyphenated system where the ICP-MS was linked in parallel with an MS (Applied Biosystems, Warrington UK) to gain simultaneous data for the profile and mass information for the sulphur-containing metabolites. One potential metabolite route for omeprazole is the glutathione pathway (scheme 6.1).



Scheme 6.1 Previously identified metabolic pathway of omeprazole [68]

6.12.2: Experimental (Omeprazole structural study)

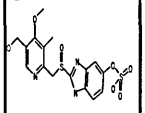
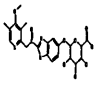
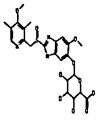
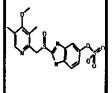
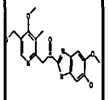
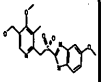
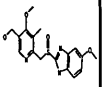
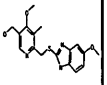
The same chromatographic conditions were used as in section 6.10.2 with a split to direct flow from the column to the ICP-MS (250 μL) and MS (750 μL). The mass spectrometer was set up in scan mode to capture data over the range 100 to 600 amu. The sample used in section 6.10.2 was injected (100 μL) on to the system.

6.13: Results and Discussion (Omeprazole structural study)

Parallel use of the ICP-MS with the MS was designed to maximise the amount of information gained from one sample committed to the analysis. The urine sample, from rats dosed with omeprazole as described in section 6.10.2, was profiled to gain quantitative data and related mass spectral data to enable tentative structural identification. From the ICP-MS trace of $^{32}S^{16}O$ an selection of peaks were chosen for quantification and these were compared to an external standard of omeprazole (10 μg/mL concentration). The results are shown in Table 6.7, where the retention time of the peaks of interest are given as well as the amount of sulphur present in the metabolite. These were compared to the total ion current (TIC) scan from the MS, which is shown in Figure 6.10. The TIC chromatogram peaks were labelled to relate them back to the ICP-MS chromatogram and are summarised in Table 6.7. With this information the amount of sulphur in each peak could then be used to estimate the concentration of each potential metabolite. The omeprazole external standard has a molecular weight of 346, in which sulphur represents 10 percent of the structure by mass. The masses for the related peaks were obtained from the parallel MS system, then these were used with the amount of sulphur found in each peak to give a total metabolite concentration. The MS was also used to gain structural information, and the data compared with previous results from Lars Weidolf et al. [68, 69]. Parent omeprazole was not observed in the chromatogram since the animal does not excrete unchanged parent, however the peak would have eluted at 19.5 minutes according to the standard. Due to the isotope cluster in the spectrum it is possible to identify the metabolites in the trace relatively easily. The ions observed at *m/z* 346/348 have the same mass as the parent but elute later. These components have been reduced to

sulphides after or prior to metabolic incorporation of an oxygen atom (Figure 6.19). Other metabolic transformations that involved oxidation are shown in Figures 6.16 and 6.17, with the major metabolite 5-hydroxyomeprazole shown in Figure 6.18 (concentration of 8.5 µg/mL). Sulphated conjugates appeared in the region *m/z* 396/398 to 442/444 including the compounds identified in Figures 6.11, 6.14 and 6.15. The final set of compounds found in the samples analysed were in the *m/z* region 492/494 to 538/540, where glucuronides are found. These are shown in Figures 6.12 and 6.13. The data obtained from the system are summarised in Table 6.7.

Table 6.7 Profile data obtained from ICP-MS trace of urine from omeprazole-dosed rats, monitored at $^{32}S^{16}O+$ against an external standard of omeprazole. The table shows the amount of sulphur present for each peak for the ICP-MS trace, which was related to the TIC of the MS trace and to allow the amount of each metabolite to be estimated.

Profile of omeprazole urine sample									
	A	B	C	D	E	F	G	H	I
Isotope masses	428/430	508/510	538/540	412/414	396/398	378/380	378/380	362/364	346/348
Structures									
Retention time (min) on ICPMS trace	9.3	9.9	12.1	12.8	16.1	17.2	19.2	20.1	22.8
amount of sulphur present in metabolite (µg/mL)	0.24	1.04	0.2	0.57	0.61	0.22	0.22	8.5	0.32
Quantity of metabolite present (µg/mL)	0.21	0.77	0.14	0.52	0.58	0.22	0.22	8.5	0.32

Full size diagrams of the structures are presented in Figures 6.11 though to 6.19.

6.14: Conclusion (Omeprazole metabolism study)

The data shows that a parallel system consisting of HPLC-ICP-MS and MS works well to give a significant amount of information from a single injection. The integrated system provided quantitative and structural information for metabolite peaks. The work demonstrated what could be achieved for one injection of a sample, however if more structural data was required then the triple quadrupole MS could be used to provide positive identification of metabolites.

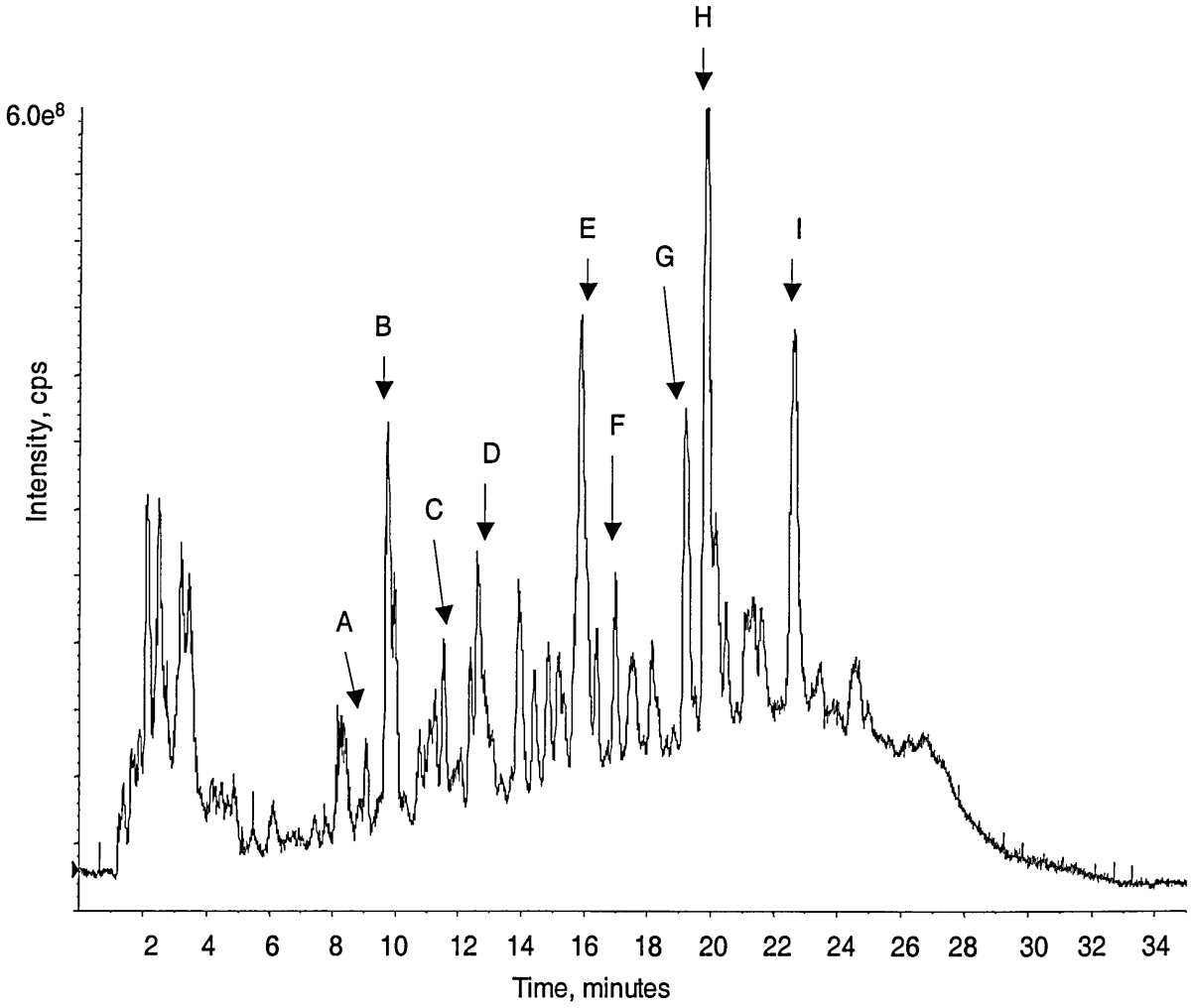


Figure 6.10 HPLC-MS total ion current chromatogram obtained for the omeprazole rat urine sample dosed orally with omeprazole at 200 μmol/kg (data gained over the range 100 to 600 amu). The labelled peaks are potential metabolites and were looked at in detail in the ICP-MS trace as well as in ion spectra. For experimental conditions see section 6.10.

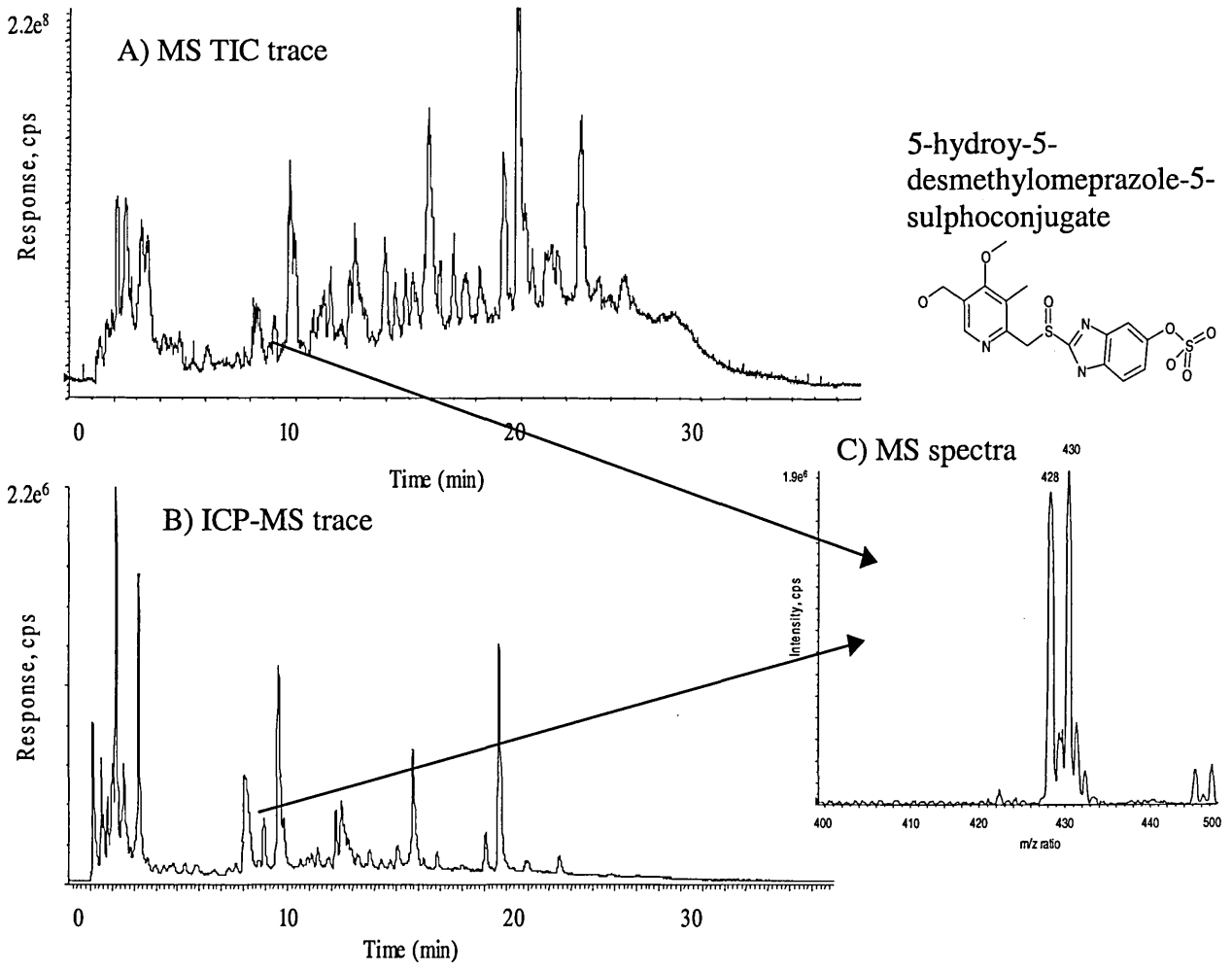


Figure 6.11 A) MS TIC trace showing peak A. B) ICP-MS trace gained from monitoring at *m/z* 48 showing peak A (concentration calculated against an external standard: 0.21 µg/mL, 10.5 ng on column) C) MS spectrum of peak A as shown in both chromatograms showing a *m/z* of 428/430 indicating 5-hydroy-5-desmethylomeprazole-5-sulphoconjugate [68, 69]. For experimental conditions see section 6.10.

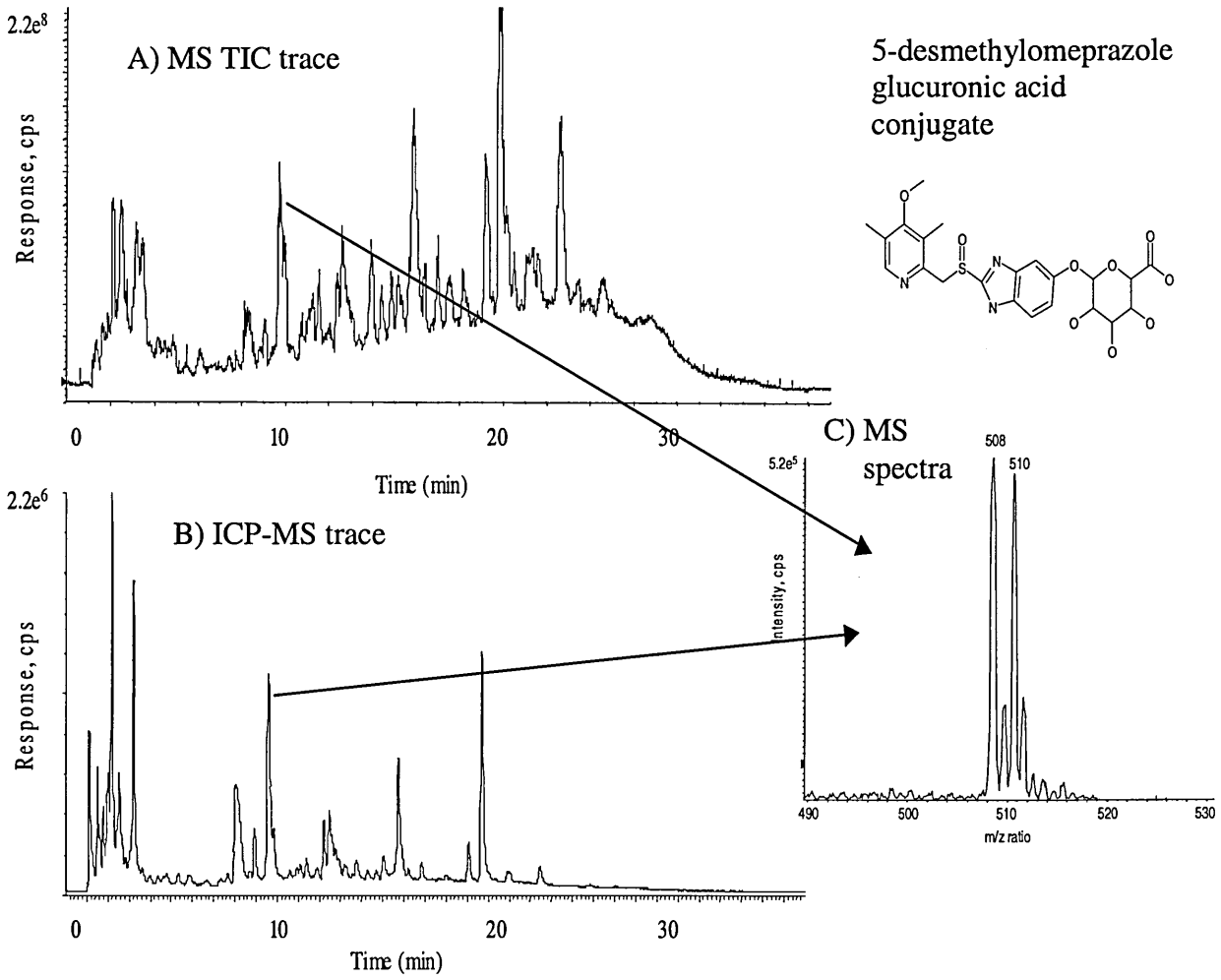


Figure 6.12 A) MS TIC trace showing peak B. B) ICP-MS trace gained from monitoring at *m/z* 48 showing peak B (concentration calculated against an external standard: 0.77 µg/mL, 38.5 ng on column) C) MS spectrum of peak B as shown in both chromatograms showing a *m/z* of 508/510 indicating 5-desmethylomeprazole glucuronic acid conjugate [68, 69]. For experimental conditions see section 6.10.

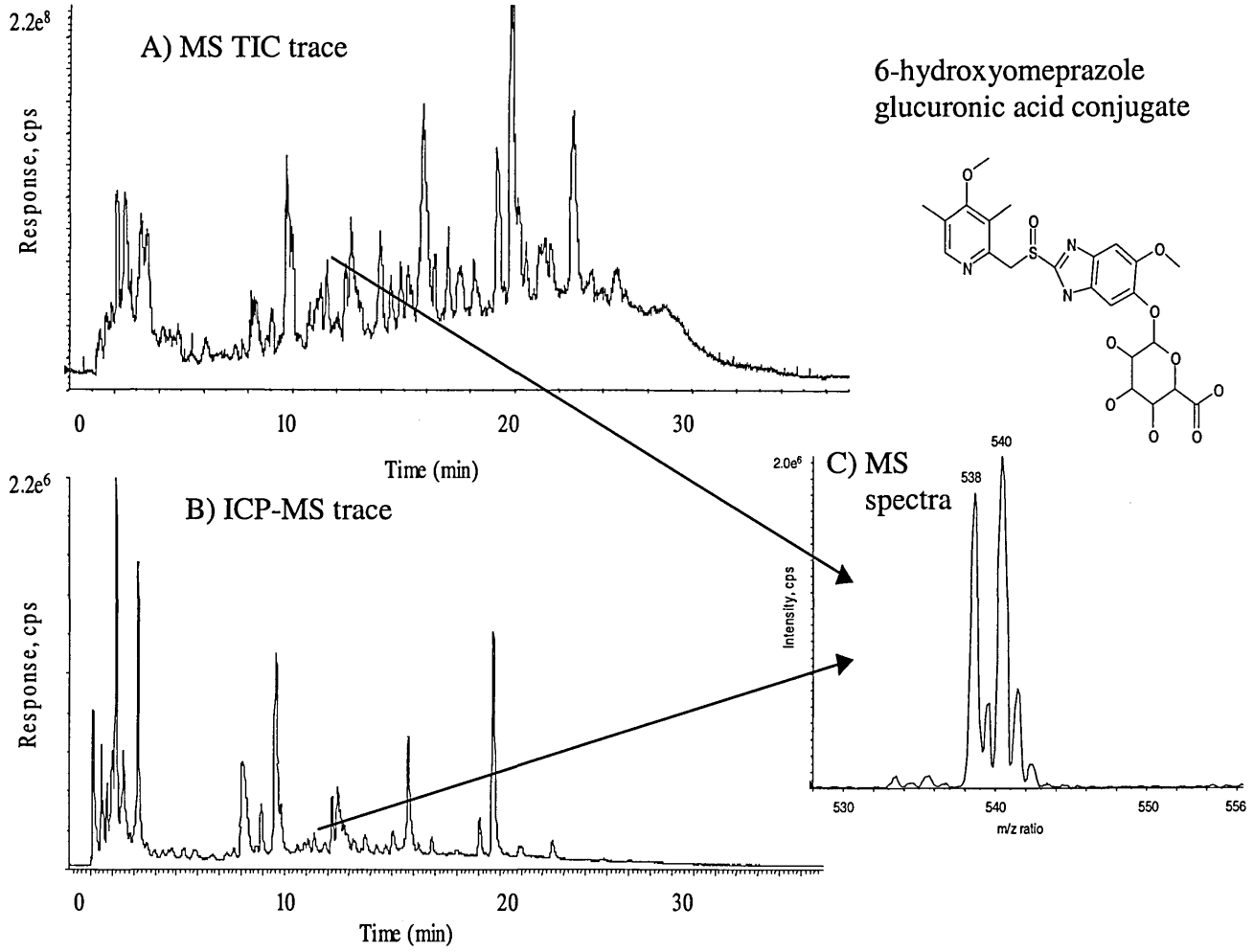


Figure 6.13 A) MS TIC trace showing peak C. B) ICP-MS trace gained from monitoring at *m/z* 48 showing peak C (concentration calculated against an external standard: 0.14 µg/mL, 7 ng on column) C) MS spectrum of peak C as shown in both chromatograms showing a *m/z* of 538/540 indicating 6-hydroxyomeprazole glucuronic acid conjugate [68, 69]. For experimental conditions see section 6.10.

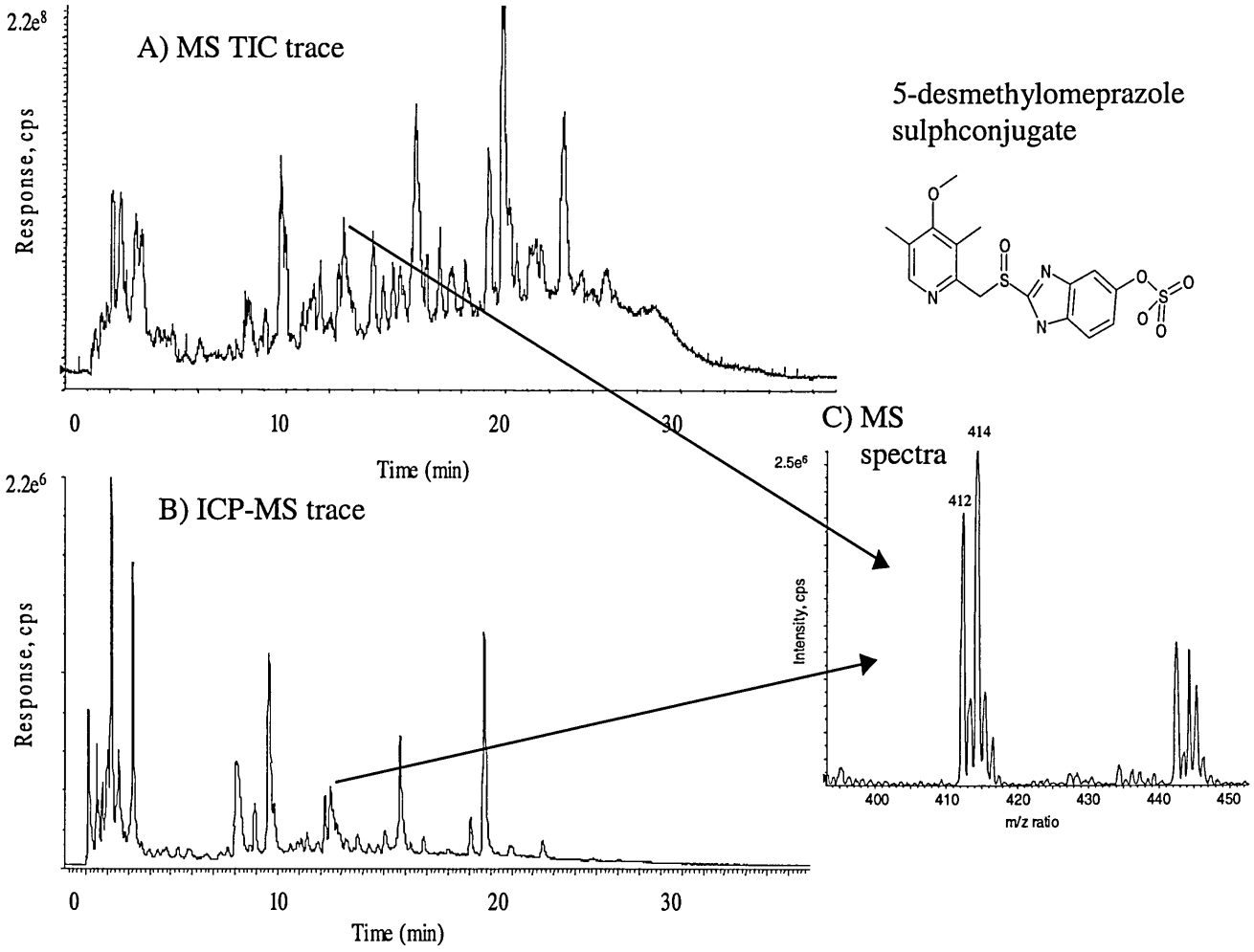


Figure 6.14 A) MS TIC trace showing peak D. B) ICP-MS trace gained from monitoring at *m/z* 48 showing peak D (concentration calculated against an external standard: 0.52 µg/mL, 26 ng on column) C) MS spectrum of peak D as shown in both chromatograms showing a *m/z* of 412/414 indicating 5-desmethylomeprazole sulphconjugate [68, 69]. For experimental conditions see section 6.10.

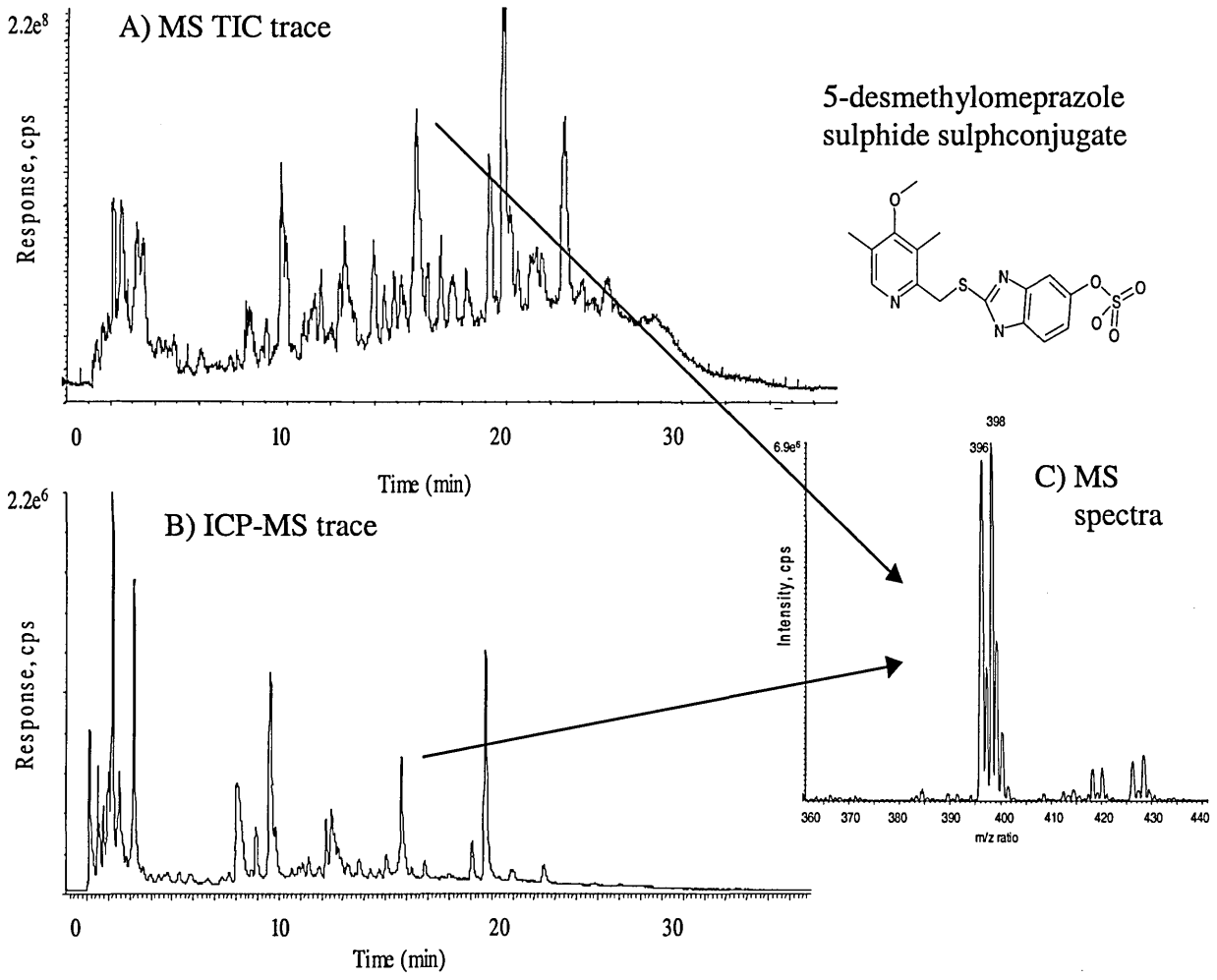


Figure 6.15 A) MS TIC trace showing peak E. B) ICP-MS trace gained from monitoring at *m/z* 48 showing peak E (concentration calculated against an external standard: 0.58 µg/mL, 29 ng on column) C) MS spectrum of peak D as shown in both chromatograms showing a *m/z* of 396/398 indicating 5-desmethylomeprazole sulphide sulphconjugate [68, 69]. For experimental conditions see section 6.10.

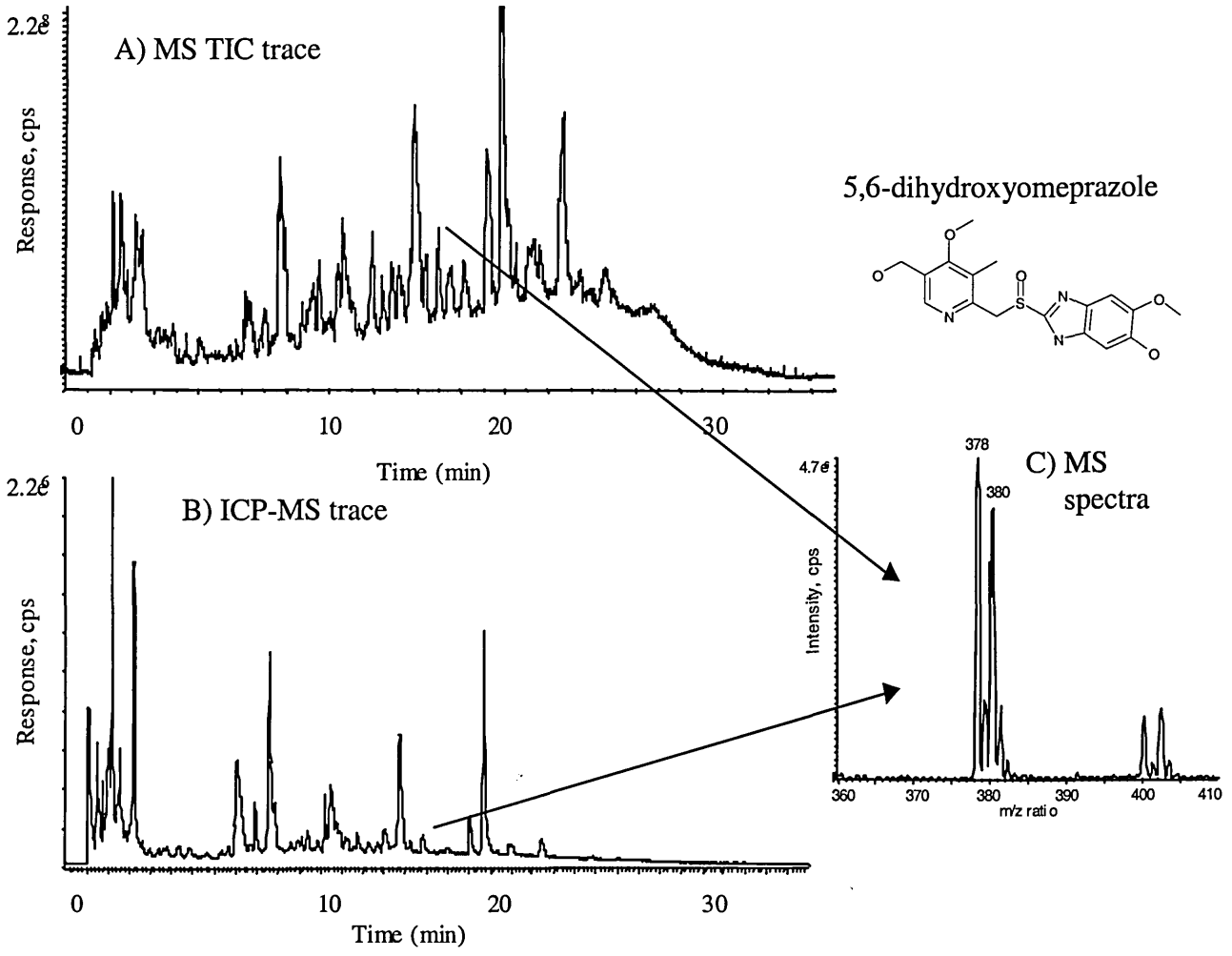


Figure 6.16 A) MS TIC trace showing peak F. B) ICP-MS trace gained from monitoring at *m/z* 48 showing peak F (concentration calculated against an external standard: 0.22 µg/mL, 11 ng on column) C) MS spectrum of peak F as shown in both chromatograms showing a *m/z* of 378/380 indicating 5,6-dihydroxyomeprazole [68, 69]. For experimental conditions see section 6.10.

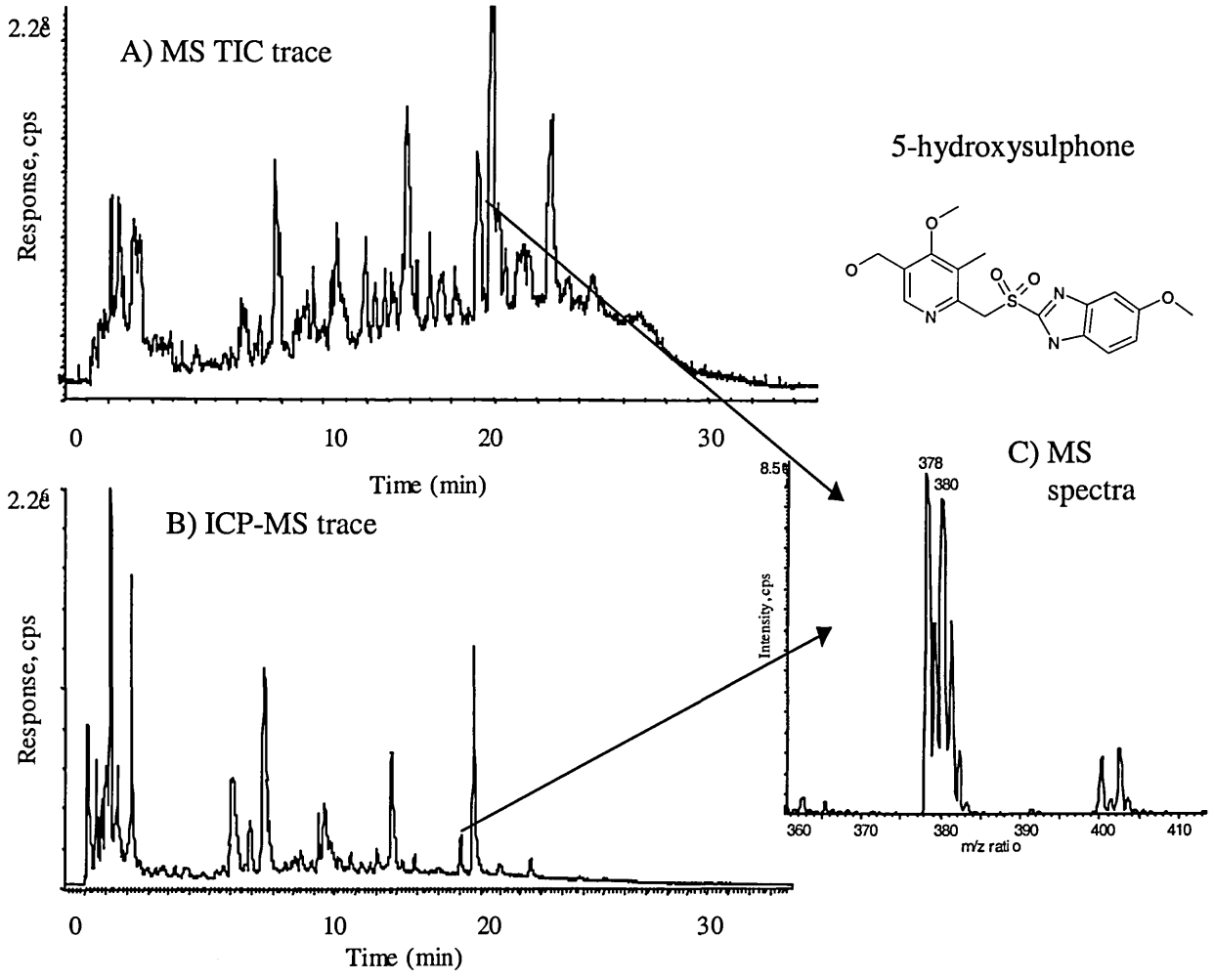


Figure 6.17 A) MS TIC trace showing peak G. B) ICP-MS trace gained from monitoring at *m/z* 48 showing peak G (concentration calculated against an external standard: 0.22 µg/mL, 11 ng on column) C) MS spectrum of peak G as shown in both chromatograms showing a *m/z* of 378/380 indicating 5-hydroxysulphone [68, 69]. For experimental conditions see section 6.10.

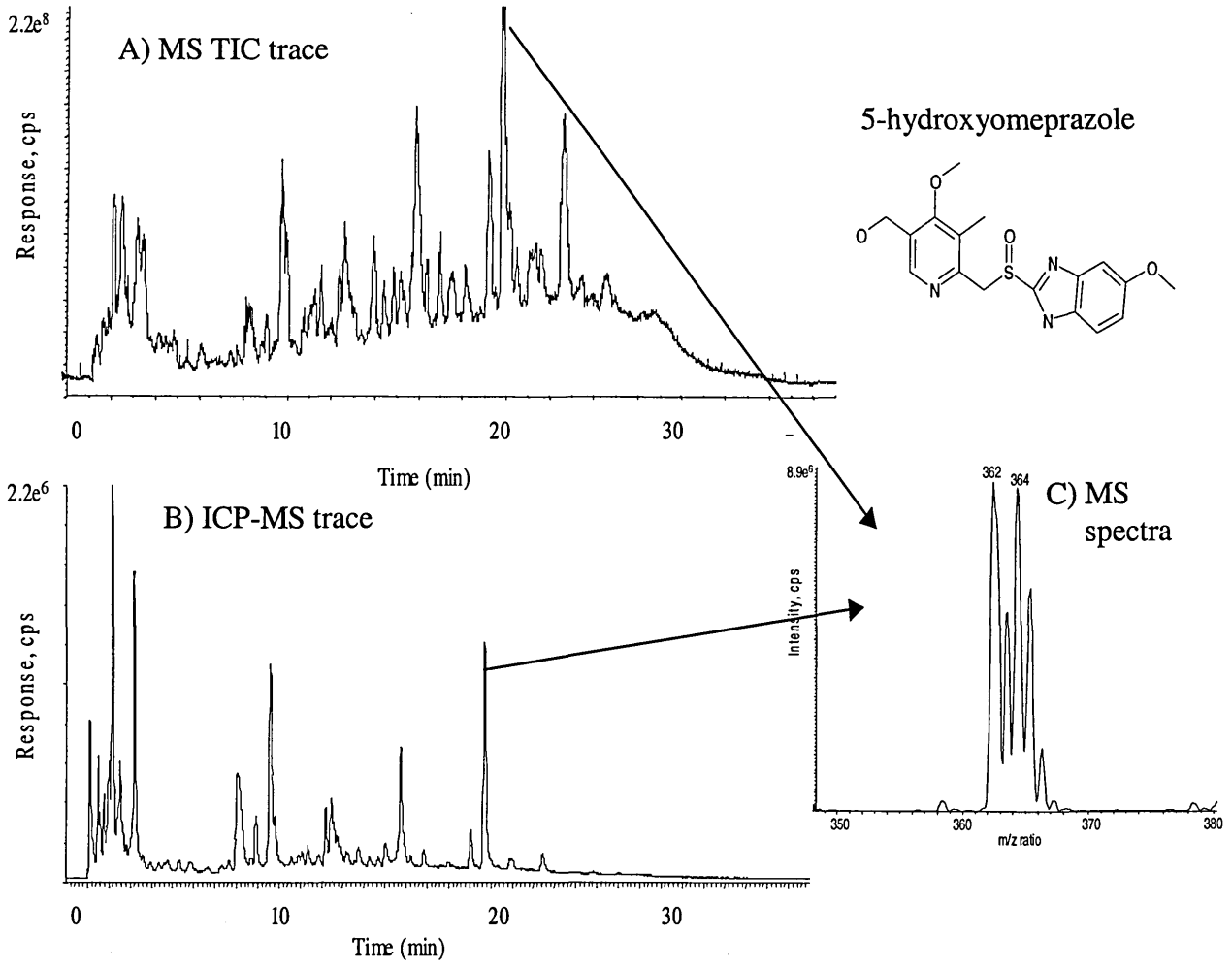


Figure 6.18 A) MS TIC trace showing peak H. B) ICP-MS trace gained from monitoring at *m/z* 48 showing peak H (concentration calculated against an external standard: 0.8.5 µg/mL, 42.5 ng on column) C) MS spectrum of peak H as shown in both chromatograms showing a *m/z* of 362/364 indicating 5-hydroxyomeprazole [68, 69]. For experimental conditions see section 6.10.

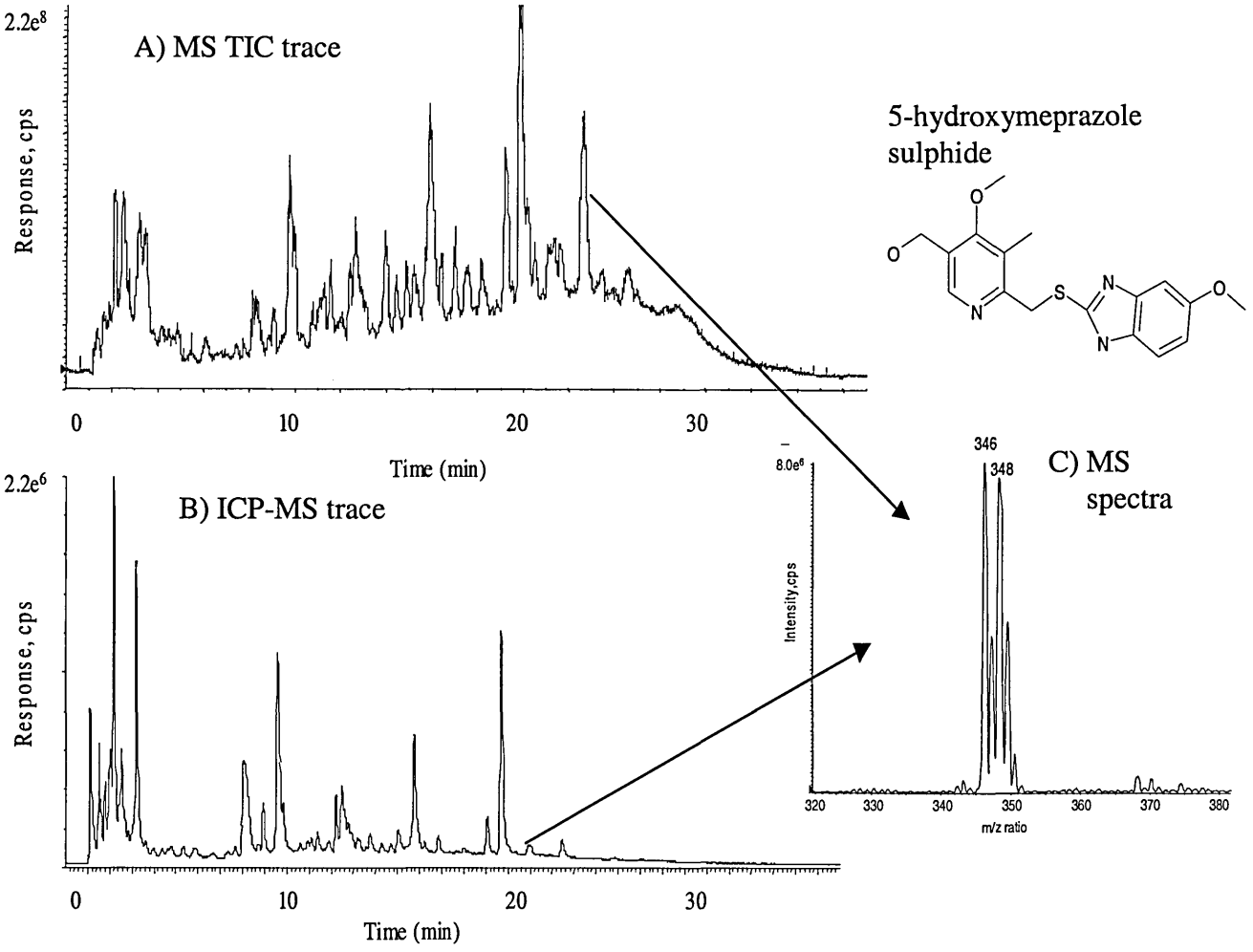


Figure 6.19 A) MS TIC trace showing peak I. B) ICP-MS trace gained from monitoring at *m/z* 48 showing peak I (concentration calculated against an external standard: 0.32 µg/mL, 16 ng on column) C) MS spectrum of peak I as shown in both chromatograms showing a *m/z* of 346/348 indicating 5-hydroxymeprazole sulphide [68, 69]. For experimental conditions see section 6.10.

6.15: Conclusion

The use of ICP-MS with the addition of oxygen to the collision cell to increase the sensitivity of methods for compounds containing S and P elements has been demonstrated. An increase in response for S of 100 times for the SO^+ compared to the ^{34}S isotope, and 1000 times compared to ^{32}S, was shown. Similar gains in sensitivity were achieved for P. This order of increase in sensitivity enabled the use of HPLC-ICP-MS for the analysis of compounds containing these elements at trace levels in complex matrices such as biological fluids.

The results presented in this chapter were published in, Chromatographia **59,** 2004, 165-170 and Rapid Communications in Mass Spectrometry **18,** 2004, , 181-183. [Appendix A].

Chapter 7

Conclusions and Future Work

7.1: Conclusions

ICP-MS has been shown to have a place within the quantitative and qualitative environment of a drug metabolism department. The complete atomisation of the biomolecule and the subsequent quantitative detection of a signal independent of chemical properties of the parent was the feature of interest in the technique. The elements contained in the sample are detected quantitatively, enabling the instrument sometimes to replace the use of [^{14}C] radio-labels in excretion balance studies and metabolite profile experiments.

Excretion balance studies using Br and I proved the concept that an element within the structure of the compound can be used to produce meaningful data. Once the excretion route was determined quantitative profiling could be used to determine the amounts of the individual metabolites present.

HPLC coupled to ICP-MS, separation of metabolite mixtures was possible and an element within the structure determined to give accurate quantification data for each metabolite as long as the element remained in the structure. Metabolite successfully were quantified against a non-related compound with the same element present, with due regard for the relative molar stereochemistry of the element within each compound. Profiling experiments were carried out with Br and I containing samples as well as for S and Pt containing compounds.

ICP-MS, however is an element detector so no structural information is gained from it. With this in mind, MS detection was run in parallel with the ICP-MS to gain maximum information from each sample. This was taken even further for Pt anti cancer drug profiling (ZD0473) where a UV absorbance detector and a scintillation counter were also placed in line for each sample injected. The added benefit of such a system was that the [^{14}C] radio-label was located in a different part of the molecule from the Pt and also more metabolites could be detected. Different metabolic pathways could be monitored with this extra information.

In most examples ICP-MS was run with MS only since the idea behind the system was to produce a technique that did not require [^{14}C] radio-labels. Detection of [^{13}C], either to produce a universal detector or to provide label-selective detection without radio isotopes, proved difficult because of the low ionisation efficiency for carbon, but proof of principle was established. Sulphur analysis was of particular interest since its presence in metabolites and drugs is not uncommon. The main problem found with this element was the sensitivity, because the interferences experienced with detection at *m/z* 32 showed the technique to be ineffectual. The problem was overcome by using a technique previously reported in environmental applications. By reacting oxygen with sulphur in the collision cell of the instrument to produce SO^+, the detection mass was increased to *m/z* 48, which had the effect of increasing the sensitivity 100 fold. It was not possible to use sulphur in excretion balance studies due to the amount of endogenous sulphur present in samples, but it was of use in metabolite profiling where individual S-containing compounds derived from the parent drug could be quantified in DMPK.

Excretion balance and metabolite profile experiments involving halogen were also investigated. Excretion balance study for Cl is not practical, since Cl is present in the plasma

making the background levels too great. Br also suffered from matrix Br and correction valves form the pre-dose samples were introduced to give an increased accuracy to the data. Iodine proved to be the most successful element in excretion balance experiment due to the lack of background interference from the matrix. Metabolite profiling in all cases was successful, using chromatography to separate the metabolite mixtures to quantifying metabolites against an none related standard.

Metabolism studies are only one area of interest; the quantification of drugs in kinetic studies is also of great importance. The sensitivity of ICP-MS to individual elements varies due to the ionisation energy of the element in relation to the ionisation energy of argon, the energy transfer gas. Pt was the only element, which showed sufficient sensitivity for kinetic studies to be carried out. For other elements the conventional method of HPLC-MSMS was superior. The Pt element used for the kinetic analysis of ZD0473 showed greater sensitivity compared with its HPLC-MSMS equivalent, 0.1 ng/mL compared to 5 ng/mL respectively. This increase in detection allowed the kinetic data to be followed to later time points.

Phosphorus was considered as possible element due to its importance within peptides as well as pro-drugs. The initial work using phosphorus used detection at m/z 31 which showed that detection limits were low. As for the sulphur, when phosphorus was reacted with oxygen an enhancement was seen which increased the sensitivity by a factor of 50. This work will by of great use in future projects into peptides and immunoassays.

Carbon was considered for a possible ICP-MS detection. The choice of carbon is a good choice since this element is present in all pharmaceutical drugs therefore goes to produce "universal detection system". Ionising carbon is a challenge in itself but was managed to a

degree and a proof of principle was achieved. If problems associated with mobile phase and poor sensitivity could be overcome the potential use for carbon detection within drug analysis is very high.

7.2: Future Work

7.2.1: Advancements in hardware

Throughout this project the equipment was being changed and enhanced to improve data and equipment reliability. An example of this was the addition of an automated RF box, so when the electrical impedance changes in the plasma occur due to introduction of organic modifier, the RF frequency can change to match the new conductivity. This stabilises the plasma and prevents it from extinguishing. Developments are continuing, and there are two main areas where work is still required.

Sensitivity is a major consideration for biological assays and for ICP-MS is governed by ionisation efficiency, background signal and nebulisation efficiency. The latter area is one where developments are occurring rapidly. Investments into new perfluoroalkoxy (PFA) nebulisers have produced more robust assays with slight increases in sensitivity with improvement in droplet size.

A particular interest would be the use of direct injection high efficiency nebulisation (DIHEN) [74]. This allows flow rates in the range of 1 to 100 μL /min to spray directly into the plasma. The advantage of this would be that the losses experienced in the spray chamber (which can be as high as 96 %) would be eliminated. An added advantage would be eradication of the

peak broadening seen in assays using a spray chamber. Sharper peaks would give increased sensitivity and better resolution. There is however a down side in that the spraying of organic modifier directly into the plasma may cause too great a change in electrical impedance compromising the plasma flame. Narrow bore chromatography, which is becoming more widely used in industry, may work with this technique [75, 76].

Chromatographic separation is an integral part of metabolite profiling, whether with the ICP-MS or any other technique. Improving the efficiency of chromatographic separation is therefore an important task. Ultra high pressure liquid chromatography (UPLC) is one possibility for improved chromatographic separations. This process makes use of narrow bore columns with very fine packing, which require very high pressure and run at low flow rates. This allows more flexibility in mobile phase composition, producing shorter run times and high resolution. Examples of the potential benefit are shown in Figure 7.1, which shows the results for a separation with UV detection. When the same sample injected into the ICP-MS, peak broadening reduced the resolution, even though a reduction in run time was achieved. Improvement in reduction of dead volume in the ICP-MS coupling should allow the benefits of fast, high resolution UPLC to be seen with element-selective detection.

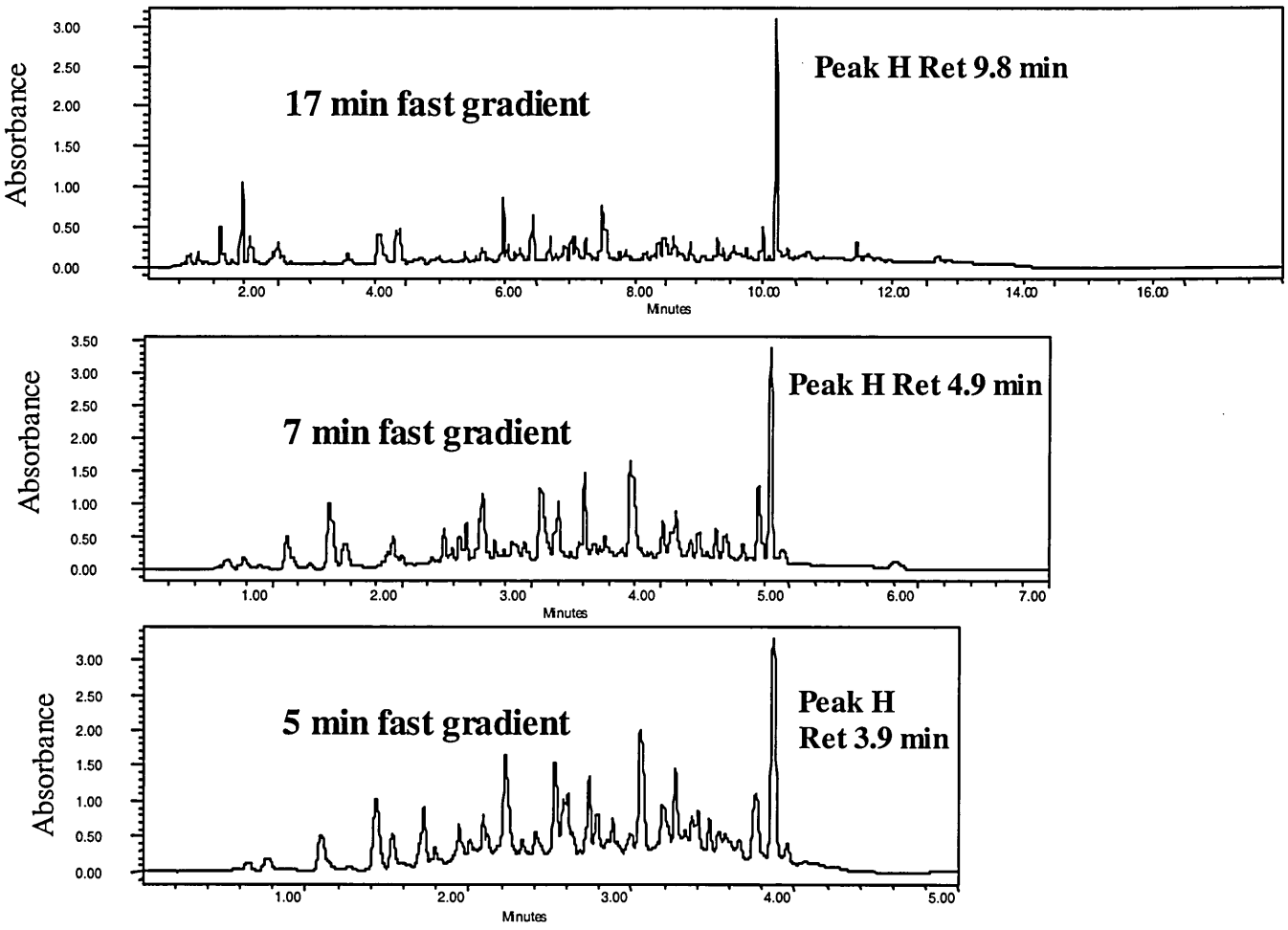


Figure 7.1 Example of the flexibility provided by UPLC, with an omeprazole metabolite sample detected by UV absorbance at 254 nm. A) A gradient run over 17 minutes which is faster than the original chromatography as shown in Figure 6.9. B) The same gradient mixture but the rate speeded up by a factor of two; resolution was maintained. C) The same gradient mixture but with the rate increased by a factor of seven resolution was still maintained. The column used in the experiment was a waters C18 1.7um 2.1 x 100 mm with mobile phase conditions as described in section 6.10.

7.2.2: Future applications

The use of ICP-MS in metabolite profiling is a continuing process, but needs improvement in the sensitivity and better separation techniques to maximise its usefulness. Technical developments may make the detection of halogens and halcogens easier. Improvement in phosphorus detection might be useful in the study of protein phosphorylation [77]. Reversible protein phosphorylation has been known for some time to control a wide range of biological functions and HPLC-ICP-MS for phosphorus may be of use in proteomics in parallel with MS to aid identification.

The element tagging work reported by Quinn etal and Baranov etal [78, 79] is of interest. This showed the advantages of tagging proteins with metals to improve the sensitivity of protein immunoassay. This work is particular interesting since it widens the application into an area where components not normally detectable can be detected due to the tag. Fluorescent tags and enzyme labels are widely used in immunoassay and nucleic acid research. Elemental tags will be smaller, which may benefit use in immunoassay in particular. The sensitivity of ICP-MS, and the number of metal's available, may make multi-tagging attractive in the biological field. DNA sequence analysis currently uses four fluorescent dyes, with some difficulty in distinguishing between then, while in security applications tags of as many as 20 different metals have been used with ICP-MS detection.

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Appendices

Appendix A

Paper published during project

A. C. J. Smith, I. D. Wilson, F. Abou-Shakra, R. Payne, T. C. Parry, P. Sinclair and D. W. Roberts: Anal. Chem. **75**, 2003, 1463-1469. (Chapter 2).

B. C. J. Smith, I. D. Wilson, F. Abou-Shakra, R. Payne, H. Grisedale, A. Long, D. Roberts and M. Malone: Chromatographia, **55**, 2002, 151-155. (Chapter 3)

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D. C. J. Smith, I. D. Wilson, L. Weidolf, F. Abou-Shakra, M. Thomsen: Chromatographia, **59**, 2004, 165-170. (Chapter 6).

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A Comparison of the Quantitative Methods for the Analysis of the Platinum-Containing Anticancer Drug {*cis*-[Amminedichloro(2-methylpyridine)]-platinum(II)} (ZD0473) by HPLC Coupled to Either a Triple Quadrupole Mass Spectrometer or an Inductively Coupled Plasma Mass Spectrometer

Christopher J. Smith,* Ian D. Wilson, Fadi Abou-Shakra,† Richard Payne, Tony C. Parry, Peta Sinclair, and David W. Roberts

Department of Drug Metabolism and Pharmacokinetics, AstraZeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, U.K., and Micromass UK Ltd, Floats Rd, Wythenshawe, Manchester M23 9LZ, U.K.

The use of high performance liquid chromatography coupled with inductively coupled plasma mass spectrometry (HPLC–ICPMS) as means for the quantitative determination of ZD0473, a platinum anticancer drug, and its related biologically active "aqua" compounds in biofluid samples is described. The performance of the resulting HPLC–ICPMS method was compared with that of a conventional HPLC–triple quadrupole mass spectrometer-based (HPLC–MS/MS) system for properties such as limit of detection, linearity, and reproducibility using spiked samples. The methods were then applied to the determination of plasma ultrafiltrate concentrations of ZD0473 in dog plasma samples obtained following intravenous and oral administration at 0.5 and 6 mg/kg, respectively. These experiments showed that both methods were capable of providing accurate and precise results but that the HPLC–ICPMS method had advantages of extended linear range and superior sensitivity, providing a limit of quantification of 0.1 ng/mL for ZD0473, as compared to 5 ng/mL using the current HPLC–MS/MS method. In addition, by using a single combined HPLC–ICPMS/MS/MS system, it was possible to determine the relative MS/MS response of the aqua compounds for the first time.

ZD0473 {cis[amminedichloro(2-methylpyridine)]platinum (II)} is a new generation platinum compound designed to deliver an extended spectrum of antitumor activity and overcome platinum resistance.[1-2] The structure of ZD0473 and the related biologically active "aqua" compounds are shown in Figure 1. Traditionally, the analysis of platinum drugs has relied upon atomic absorption spectrophotometry, ICPMS, or electrochemical detection.[3-5] However, in the absence of a separation, these techniques do not provide an adequate process for a quantitative assay because of the failure to distinguish between the compound, the active aqua species, and inactive metabolites (including amino acid adducts)/degradation products. If such information is required, a chromatographic separation coupled to fraction collection and subsequent off-line determination of the platinum content can be performed, but this is labor-intensive, time-consuming, and prone to error. Because of these practical difficulties, the initial method developed for the determination of ZD0473 in biological samples was based on the HPLC–MS/MS system developed by Oe et al.,[6] which enabled the compound to be quantified down to concentrations of 10 ng/mL in biofluid samples, such as plasma ultrafiltrate and urine. However, the advent of robust HPLC–ICPMS systems offers great potential for the analysis of metal-containing compounds in biological matrixes,[7-10] as illustrated by a recent

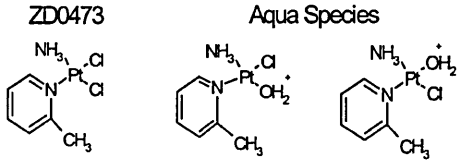

Figure 1. The structure of ZD0473 and the associated aqua species.

* To whom correspondence should be addressed. E-mail: christopher.smith1@astrazeneca.com.

† Micromass UK Ltd.

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example for a platinum-containing compound in human plasma.[11] We have, therefore, developed an HPLC–ICPMS-based method for the analysis of ZD0473 and related materials. This method has been compared to an in-house assay, based on the published HPLC–MS/MS procedure,[6] via the analysis of both spiked samples and samples derived from a dog study following the intravenous and oral administration of ZD0473 at 0.5 mg/kg and 6 mg/kg, respectively.

EXPERIMENTAL SECTION

Chemicals. Chromatographic solvents together with formic and hydrochloric acids were purchased from Fisher Scientific UK Ltd (Loughborough, U.K.) and were of analytical or HPLC grade. Ammonium acetate and sodium chloride were obtained from BDH Ltd (Poole, U.K.) and were of analytical grade. ZD0473 was formulated as a sterile solution in 0.9% sterile sodium chloride solution at a concentration of 0.5 mg/mL for intravenous administration, and at 50:50 w/w with lactose at a concentration of 90 mg for oral administration, which was obtained from the Media Preparation Laboratory at AstraZenenca Pharmaceuticals (Alderley Park, Macclesfield, U.K.). ZD0473 was supplied by AstraZeneca Pharmaceuticals (Alderley Park), and the deuterated internal standard, [$^2H_7$] ZD0473 (deuterated in the aromatic ring and methyl), was synthesized in the Isotope Chemistry Laboratory in the Department of Drug Metabolism and Pharmacokinetics at AstraZeneca Pharmaceuticals (Alderley Park).

Standard Solution Preparation. ZD0473 is light-sensitive, and care was taken when making up standard solutions for the two assays to avoid photodegradation. Typically, 5 mg of ZD0473 was weighed out into an amber vial, and 10 mL of 0.15 M sodium chloride was then added to achieve a concentration of 0.5 mg/mL. The presence of chloride ions stabilizes ZD0473 by preventing the formation of the aqua species. Dissolution was obtained by stirring the sample for 2 h at room temperature. The spiking standards were prepared by making the appropriate dilutions with sodium chloride (0.15 M) to provide standards of concentrations 500, 10, 1, 0.2 and 0.02 μg/mL. The initial solution was checked by UV spectroscopy at a concentration of 100 μg/mL, and the data were compared to previously generated data at this concentration to determine integrity of the preparation. The standard solution of the deuterated internal standard ([$^2H_7$]-ZD0473) employed in the HPLC–MS/MS assay was prepared in the same manner and diluted with sodium chloride to give a final concentration of 5 μg/mL.

Intravenous and Oral Formulation of ZD0473 and Sample Collection. ZD0473 was formulated in physiological saline at a concentration of 0.5 mg/mL. ZD0473 was dissolved in 0.9% w/v sodium chloride by stirring overnight and then filtering through a 0.2-μm filter into a sterile vial. The formulation was then dosed intravenously as a bolus over 1 min to a male beagle dog (12.8 kg) at a dose level of 0.5 mg/kg. Blood samples (4 mL) were taken at 0.033, 0.083, 0.167, 0.25, 0.5, 0.75, 1, 2, 3, 6, and 12 h post dose. The oral dose was formulated as a 50% w/w mixture with lactose (90 mg) and dosed to a single male beagle dog (12.5 kg) at a level of 6 mg/kg. Blood samples were taken (4 mL) at 0.25, 0.5, 0.75, 1, 2, 3, 6, and 12 h. On collection, blood samples were placed in lithium heparin tubes and centrifuged at 3000 rpm for 15 min to prepare plasma. Aliquots of these plasma samples were then taken (200 μL), placed into Amicon Centrifree Filter devices (30 000 molecular weight cutoff, Millipore Corporation, Bedford, MA), and centrifuged at 3000 rpm for 30 min at 4 °C to provide ultrafiltrates. The samples were then stored at −70 °C in glass vials wrapped in aluminum foil to protect the samples from photodegradation until analysis (these conditions have been shown to provide stability for 5 months, unpublished observations).

Preparation of Standard Curve and Quality Control Samples. The standards and quality control (QC) samples were prepared by spiking control dog plasma ultrafiltrate with standard solutions to produce the required concentrations. The standard curve prepared for the HPLC–MS/MS assay contained samples spiked at 5, 10, 50, 200, 600, 1000, 2500, 5000, 7500, and 10 000 ng/mL, respectively, whereas that for the HPLC–ICPMS method had additional standards at 0.1, 0.5, and 1 ng/mL. The QC samples for the HPLC–MS/MS and HPLC–ICPMS assays were prepared at 5, 100, and 1000 ng/mL and 0.1, 0.5, 5, 1000, and 10000 ng/mL, respectively, and stored at −70 °C in glass vials wrapped in aluminum foil until required.

Sample Preparation. Aliquots of the standards, QCs, and samples (0.1 mL in each case) were placed into glass tubes (12 × 75 mm). To these, 25 μL of 0.15 M sodium chloride in 0.1 M hydrochloric acid was added, and the samples were then made up to 200 μL with 0.15 M sodium chloride. For the HLPC–MS/MS method, 25 μL of the internal standard solution (5 μg/mL) was added before the sample was made up to 200 μL. The mixtures were vortex-mixed for 20 s and then transferred to HPLC vials ready for injection. The addition of hydrochloric acid was designed to ensure that the equilibrium favored ZD0473 rather than the aqua species in the presence of the ultrafiltrate. The samples were transferred to autosampler vials and then placed in the autosampler and kept in the dark prior to injection.

Chromatography. An isocratic reversed-phase HPLC method based on a mobile phase of methanol water (20:80 V/V) containing 0.1% formic acid and 0.15 mM ammonium acetate (pH 3) was used for both ICPMS and the MS/MS assays. The column used was a Phenomenex Synergi Polar RP 150 × 4.6 mm (Phenomenex, Macclesfield, U.K.) operated at ambient temperature with a flow rate of 1 mL/min, which gave a run time of 7 min. An injection volume of 50 μL was used in the assays.

HPLC–ICPMS. Chromatography for ICPMS was performed using a Jasco HPLC system (Jasco Ltd, Great Dunmow, U.K.) with samples introduced via a PE series 200 autosampler (Perkin-Elmer Ltd, Beaconsfield, U.K.). The injector was flushed using a methanol/water solution (1:1). ICPMS was performed on a Platform ICPMS, which uses a hexapole collision/reaction cell for simultaneous measurement of the platinum isotopes (Micromass, Wythenshawe, U.K.). The eluent from the column was introduced to the ICPMS via either an ultrasonic nebulizer U-6000AT (Cetac Technologies, Omaha, NB) or a Meinhard concentric nebulizer coupled to a double pass spray chamber (Micromass, Wythenshawe, U.K.). MassLynx software was used for instrument control, data acquisition, and data handling. Table 1 shows operating and acquisition conditions of the ICPMS, including the flow of the nebulizer gases for sample introduction

(11) Galettis, P.; Carr, J.; Paxton, J. W.; McKeage, M. J. *J. Anal. At. Spectrom.* **1999**, *14*, 953–956.

Table 1. Instrument Operating Conditions for the Platform ICPMS

cooling gas	16.00 L/min	plasma power	1350 w
plasma gas	0.65 L/min	acquisition mode	SIR
nebulizer gas	0.75 L/min	dwell time	200 ms
helium gas	5 mL/min	masses monitored	195
hydrogen gas	5 mL/min	argon/oxygen (95/5%) 0.2 mL/min	
total analysis time	7 min		

Table 2. Instrument Operating Conditions for API-3000 MS/MS

	ion transformation		
component	Q1	Q3	dwell time (ms)
ZD0473	393.4	303.5	150
ZD0473D6	400.0	310.2	150
aqua species	358.0	303.5	150

as well as an argon oxygen mix (95:5 v/v), which is used to reduce carbon buildup from mobile phase combustion in the plasma.

HPLC–MS/MS. Chromatography for HPLC–MS/MS was performed using a PE series 200 pump (PerkinElmer Ltd, Beaconsfield, U.K.) with samples introduced via a CTC HTC-PAL autosampler (Presearch, Hitchin, U.K.). The injector was flushed using a methanol/water solution (1:1). For quantification by MS/MS, an API-3000 mass spectrometer (Applied Biosystems, Warrington, U.K.) with a turbo ionspray inlet source was used for the multiple reaction monitoring of ZD0473 and its aqua species. Analyst software was used for the instrument control, data acquisition, and data handling. The operating conditions for the instrument are shown in Table 2.

HPLC–ICPMS/MS/MS. To evaluate the combination of HPLC–ICPMS/MS/MS, and enable the relative response for the aqua species by MS/MS to be determined, the chromatographic system used for HPLC–ICPMS described above was taken, and the effluent from the column was split using a splitter valve (Jasco Ltd, Great Dunmow, U.K.) positioned directly after the outlet of the column; this directed one-half of the flow (0.5 mL/min) to the HPLC–MS/MS and the remainder (0.5 mL/min) to the HPLC–ICPMS. PEEK tubing (0.005-in. bore) was used to connect the instruments, and the lengths were adjusted to give similar retention times for ZD0473 in both systems.

RESULTS AND DISCUSSION

Assay Development and Validation. *Chromatography.* ZD0473 was found in biological fluids together with a mixture of the pharmacologically active aqua species (structures in Figure 1). Because the HPLC–ICPMS method is based on the detection of platinum, a chromatographic separation of ZD0473 from these aqua species was required to ensure specificity. Similarly, the use of MS/MS for quantification necessitated the separation of the two aqua components, because the same ion transformations are seen for both aqua species. The HPLC separation that was developed, based on reversed-phase chromatography with a methanol–ammonium acetate/formate (20:80) buffered eluent, provided this separation and was also compatible with both modes of MS detection.[12,13] The formic acid and ammonium acetate components of the mobile phase were there primarily for the benefit of the HPLC–MS/MS system, with formic acid added to aid the ionization of the compounds in the turbo ionspray source, whereas the ammonium acetate was present in order to produce the ammonium adducts required for MS/MS detection (Figure 2). For HPLC–ICPMS, methanol–water 20:80 alone would be suitable for chromatography, but a common mobile phase was used here to aid comparison of the two detection methods.

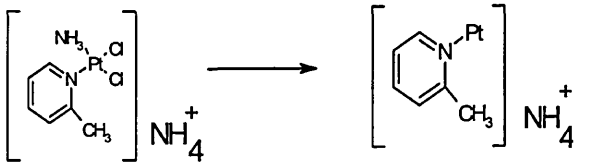

Figure 2. The multiple reaction monitoring transition for the HPLC–MS/MS system.

HPLC–ICPMS. Having obtained a suitable chromatographic separation, we then proceeded to develop and validate an HPLC–ICPMS-based assay for ZD0473. For this, the various argon gas flows were optimized to produce the best sensitivity for platinum in the ICPMS, and the hydrogen and helium collision cell gases were set to produce the lowest background,[14,15] as detailed in Table 1. For successful HPLC–ICPMS, efficient removal of the organic modifier prior to the introduction of the sample as an aerosol into the plasma is important. Various devices are available to achieve this,[16] and in this study, two types of nebulizer were investigated, namely, an ultrasonic nebulizer and a concentric nebulizer with a double-pass spray chamber. The latter was water-cooled to −4 °C. The ultrasonic nebulizer is far more efficient than the concentric nebulizer in producing an ultrafine aerosol, which is desolvated prior to its introduction into the ICP flame. This increased efficiency leads to an increased sensitivity (5 pg on column limit of detection) and low backgrounds but at the expense of linear range as a result of losses of the analyte at high concentration in the desolvating step. However, in use, it has proven more time-consuming to set up and was found to be less reliable than the concentric nebulizer/double pass spray chamber combination. The latter provides an extended linear range (but with higher background levels) in comparison with the ultrasonic nebulizer and has also proved to be more robust. The higher backgrounds observed with the concentric nebulizer result from its poorer efficiency, as compared to the ultrasonic nebulizer, in removing the organic solvent from the mobile phase. However, this combination still offered a limit of quantification of 0.1 ng/mL (10 pg on column), with a signal-to-noise (S/N) of 10, combined with a linear range extending over eight orders of magnitude. Because of the extended linear range afforded by this method of sample introduction, further method development employed this nebulizer. If greater sensitivity was to be required and reduction in the calibration range was acceptable, the increased setup time for the ultrasonic nebulizer would then

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(16) Feldmann, I. N.; Stuewer, J. D.; Jakubowski, N. *Fresenius' J. Anal. Chem.* **1999**, *365*, 415–421.

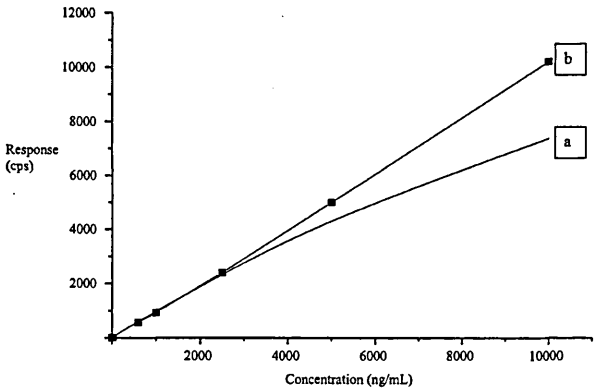

Figure 3. Examples of the calibration curves produced using two types of nebulizing sources: (a) ultrasonic nebulizer and (b) Meinhard double-pass spray chamber.

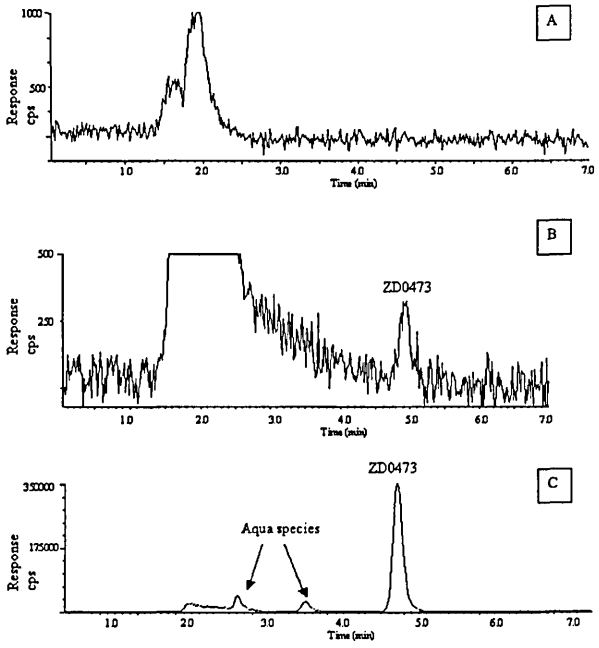

Figure 4. Example chromatograms for HPLC–ICPMS: (A) blank, (B) limit of quantification 10 pg on column, and (C) 0.5-h sample.

become beneficial. Examples of the calibration curves for HPLC–ICPMS using both nebulizer systems are given in Figure 3, with representative chromatograms obtained using the concentric nebulizer illustrated in Figure 4. The equation of the calibration line for the concentric nebulizer is given by $y = 653.5x + 335.7$, which was calculated by least-squares linear regression for a range of 0.1–10000 ng/mL.

On the basis of these conditions using the concentric nebulizer, the method was validated for spiked plasma samples over the range 0.1–10000 ng/mL. The results, shown in Table 3, clearly indicate that the method is suitable for use over the concentration range examined. The excellent accuracy and precision data obtained in the absence of an internal standard are noteworthy. No reduction in response throughout the course of the analysis was noted, as shown by QC sample sets at the start and finish of the individual runs. The limit of quantification of the method using these conditions was 0.1 ng/mL (10 pg on column) using

Table 3. Accuracy and Precision Data for HPLC–ICPMS[a]

quality control samples	mean (ng/mL)	accuracy (%)	coefficient of variation (%)
LLQ[b] (0.1 ng/mL)	0.101	101	19.9
LQC[c] (0.5 ng/mL)	0.47	95	9.3
MQC[d] (5 ng/mL)	4.6	92	1.8
HQC[e] (1000 ng/mL)	913	91	2.5
UQC[f] (10000 ng/mL)	9949	99	3.2

[a] $n = 6$. [b] Limit of quantification. [c] Lower quality control. [d] Mid quality control. [e] High quality control. [f] Upper quality control.

arguments based on either a S/N of 10:1 or the precision being better than 20%. A limit of detection based on a criterion of the signal-to-noise ratio being 3:1 would allow the detection of ZD0473 at 0.05 ng/mL.

HPLC–MS/MS. ZD0473 is not a particularly good candidate for HPLC–MS/MS because as a result of decomposition in the source, the compound could not be isolated in a Q1 scan. The method developed by Oe et al.[6] overcame this problem by making use of the presence of ammonium acetate in the mobile phase to produce an ammonium adduct, which was then fragmented to form product ions, one of which was then used in the multiple-reaction-monitoring transition (Figure 2).

For tuning purposes on the API-3000 mass spectrometer to achieve the required sensitivity, the resolution setting in the Q1 region was changed from unit resolution (peak width 0.7 ± 0.1 amu) to low resolution (peak width 0.8 ± 0.1 amu). This process allowed more ions through to the Q3 region, thereby increasing sensitivity, albeit at the expense of reducing the selectivity of the assay. The linear range of the assay was, however, limited to 5–1000 ng/mL, with the equation of the calibration line given by $y = 0.00136x + 0.00101$ calculated by least-squares linear regression. Above this concentration, the assay showed signs of saturation, and the coefficients of variation were unacceptability large. Typical mass chromatograms are illustrated in Figure 5. These conditions were used to develop an HPLC–MS/MS method for ZD0473 over the range 5–1000 ng/mL. The resulting data are summarized in Table 4. These results show the method to be suitable for the analysis of ZD0473, with a limit of quantification of 5 ng/mL (corresponding to an on-column loading of 125 pg). This limit of quantification was calculated on the basis of a signal-to-noise ratio of 10:1. Using a signal-to-noise ratio of 3:1 would result in a limit of detection of ~2 ng/mL.

The method, as developed, has proved to be robust in practice and has been used to run numerous samples from animal studies.

As indicated above, the HPLC–ICPMS method was able to provide accurate and precise results in the absence of an internal standard. For comparison, we therefore evaluated the data from the HPLC–MS/MS method without taking the internal standard into account to see if a similar approach could be adopted. However, this analysis showed that there was a gradual decrease in the response of the MS/MS during the course of the run of 150 samples, with a difference of ~24% for QC samples analyzed at the start compared to those at the end of the batch. A buildup of deposits on the source probably caused this effect, thus reducing the response of the HPLC–MS/MS. The use of a

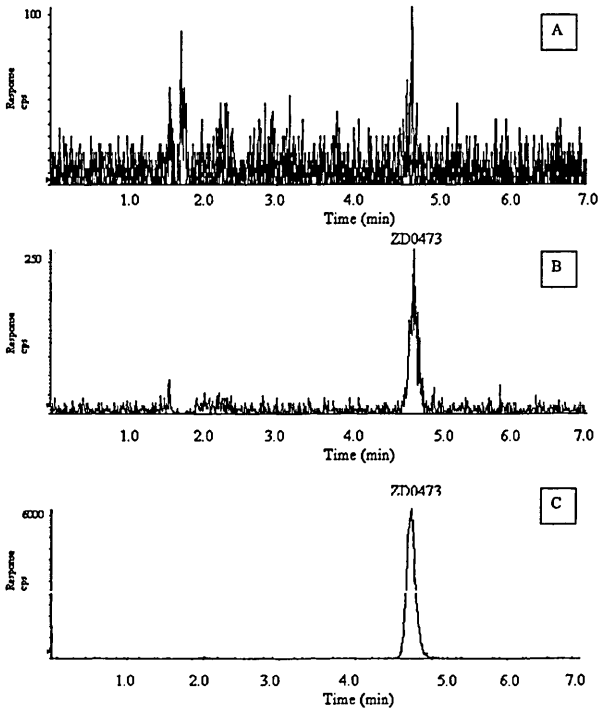

Figure 5. Example chromatograms obtained from the LC–MS/MS assay with MRM transition 393–303 amu: (A) blank, (B) 5 ng/mL standard of ZD0473, and (C) real sample taken at 0.5 h.

Table 4. Accuracy and Precision Data for LC–MS/MS[a]

quality control samples	mean (ng/mL)	accuracy (%)	coefficient of variation (%)
LLQ (5 ng/mL)	5.2	104	12.3
MQC (100 ng/mL)	109	109	5.3
HQC (1000 ng/mL)	1076	107	5.4

[a] $n = 6$.

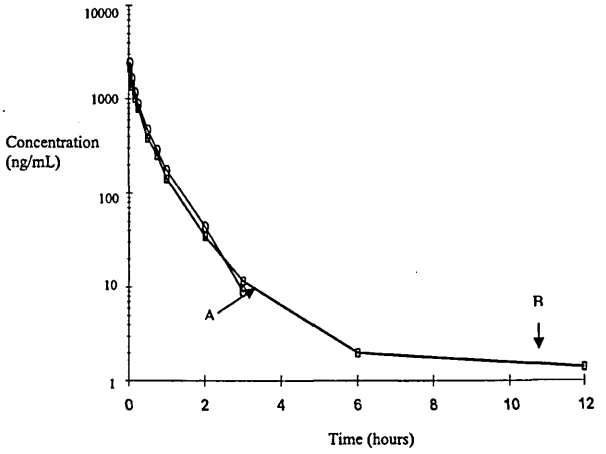

Figure 6. Example of the intravenous profiles gained from a male dog at dose of 0.5 mg/kg: (A) relates to the trace obtained from the HPLC–MS/MS, and (B) relates to the trace obtained from the HPLC–ICPMS.

Table 5. Quality Control Data for Both HPLC–MS/MS and HPLC–ICPMS Obtained during the Analysis of Plasma Samples from a Single Dog Dosed Intravenously at 0.5 mg/kg with ZD0473[a]

	QC levels (ng/mL)			
	5	10	400	2500
HPLC–MS/MS QC set 1	4.5	9.0	419	2670
HPLC–MS/MS QC set 2	4.3	8.8	393	2490
mean	4.4	8.9	406	2580
accuracy (%)	88	89	101	103
HPLC–ICPMS QC set 3	5.6	9.1	321	2700
HPLC–ICPMS QC set 4	5.6	9.3	320	2568
mean	5.6	9.2	320	2634
accuracy (%)	112	92	80	105

[a] N.B. The HPLC–MS/MS and HPLC–ICPMS were carried out on different days.

suitable internal standard is, therefore, mandatory for the assay of ZD0473 by HPLC–MS/MS.

Application to the Determination of ZD0473 in Dog Plasma. To enable a comparison of the two methods to be performed on study-derived rather than spiked samples, both the HPLC–MS/MS and the HPLC–ICPMS methods were used to analyze plasma ultrafiltrate obtained following iv administration of ZD0473 at 0.5 mg/kg to a single dog. The data obtained using both methods are graphically represented in Figure 6, with the corresponding QC data in Table 5. Peak observed plasma concentrations were a little over 2000 ng/mL, falling rapidly to ~10 ng/mL by 3 h post dose. Using HPLC–ICPMS, the concentrations of ZD0473 in the 6- and 12-h post-dose samples could be determined (2.0 and 1.4 ng/mL, respectively). HPLC–MS/MS was, in contrast, unable to provide quantitative data for these samples. For those samples in which both methods were able to provide results, the correlation between the two sets of data was good, even though a small bias was observed. The HPLC–MS/MS method generally shows a 10–20% higher value as compared to the HPLC–ICPMS method. However, when the data was put through statistical analysis (see below), when QC (validation study) and intravenous study sample data were included, the methods were shown to be equivalent.

The HPLC–ICPMS and HPLC–MS/MS-derived intravenous plasma profiles as illustrated in Figure 6 show the differences in the extent to which each technique can follow the plasma concentration of the drug leads to different plasma profiles being calculated. A two-compartment pharmacokinetic model consisting of a distribution phase and an elimination phase best fit the HPLC–MS/MS method, which followed the curve out to 3 h when modeled. The $t_{1/2}$ for the terminal phase was 0.46 h. When the HPLC–ICPMS data were modeled, the best fit was obtained using a three-compartment model. This consisted of a distribution phase followed by two apparent elimination phases. The first elimination phase gave a $t_{1/2}$ of 0.48 h, which is comparable with the data obtained from the HPLC–MS/MS system. The second apparent elimination phase gave a $t_{1/2}$ of 13.3 h; however, more data points would be required to increase the degree of confidence in this value because it is based on data over a limited sampling period (12 h). The AUC value for the HPLC–ICPMS method was calculated to be 745 h·ng/mL, as compared with 820 h·ng/mL for the HPLC–MS/MS method. The difference is primarily due to the slight bias observed in the methods with the HPLC–

MS/MS system having higher results; the longer terminal-phase $t_{1/2}$ value for the HPLC–ICPMS method has a much smaller impact.

Overall, the pharmacokinetic data obtained for the two systems were shown to be comparable, with additional information gained from the HPLC–ICPMS method as a result of the increased sensitivity of the instrument.

Statistical Comparison of Methods. To compare results statistically from two different instruments, it is necessary to look at random error and systematic error. The statistical method used to investigate these parameters was based on the methodology of Gilbert et al.,[17] which requires at least 30 data points made up of QC and real samples. The data used for statistical comparison was, therefore, made up of the QC samples obtained from the validation experiments, together with the samples obtained from a single dog dosed intravenously. A total of 35 data pairs were included in this comparison.

Random error was compared by examining the differences between the two sets of results normalized to the reference concentration. The standard deviation was calculated, and then the range of the mean ± 2 × s.d. If this range includes the value of 0, then there is no significant random error between the two sets of results. The data from the two systems showed +2 s.d. of 272 and −2 s.d. −131, thereby showing that there was no random error associated with the data.

If the ratio between each pair of results is calculated, the standard deviation is then calculated from these ratios and then the range of the mean ± 2 × s.d. If this range includes the value of 1.0, then there is no significant systematic error between the two sets of results. The data from the two systems showed + 2 s.d. of 1.4 and −2 s.d. of 0.8. These data therefore confirm that the error is within acceptable limits.

Even with the apparent bias observed between the two analytical techniques when the single dose intravenous data were subjected to statistical analysis, the QC and real sample data sets were comparable within the limits of the test, indicating that the methods are equivalent.

Aqua Species Comparison of HPLC–ICPMS and HPLC–MS/MS. Additional data were obtained from the HPLC–ICPMS method, which enabled the determination of the aqua species in addition to ZD0473. Thus, the response for platinum remains constant no matter which compound contains the element, and therefore, the concentrations of platinum-containing analytes such as the aqua species can be determined (Table 6), even in the absence of an authentic standard in plasma samples. These data show that, even though acid was added to the samples to force the equilibrium in favor of ZD0473, the aqua species were still present to some extent (e.g., see Figure 4C). In the case of MS/MS, when the relative ionization efficiencies of the aqua species were not known, only qualitative data for these compounds could be obtained in the absence of response factor data. To determine the relative responses of for these aqua compounds, as compared to ZD0473, a hypernated HPLC–ICPMS/MS/MS system was constructed, and samples from an oral dog study were analyzed. From the chromatograms illustrated for the combined system (Figure 7), it can be seen that the integration of the two spectrometers into a single system readily enables the determination of the MS/MS responses for the aqua species by comparison with the values obtained simultaneously by ICPMS. Thus, the relative responses for the aqua species, calculated by comparing the ratio of the responses for the ICPMS based assay to the MS/MS assay, were 1.1 ± 0.1 for the aqua species at Rt 2.3 min and 0.9 ± 0.2 for the aqua species at Rt 3.2 min ($N = 4$). In this instance, therefore, the relative MS/MS response for the aqua species was, in fact, essentially the same as that of ZD0473. The results for the aqua species present in the oral samples provided by both HPLC–ICPMS and HPLC–MS/MS (calculated using the above response factors) are given in Table 6.

As shown elsewhere, such combined systems also provide a powerful technique in metabolite identification studies[9] in which

Table 6. Data Obtained from HPLC–ICPMS and HPLC–MS/MS Run in Parallel after Ultrafiltrate Plasma Samples Were Collected from a Single Male Dog Dosed Orally at 6 mg/kg

sample (hour)	ZD0473 (ng/mL)	aqua species at 2.3 min (ng/mL)	aqua species at 3.2 min (ng/mL)
		HPLC–ICPMS	
0.25	200	14.1	9.4
0.5	326	25.9	21.8
0.75	230	17.9	12.0
1	196	20.8	14.2
		HPLC–MS/MS[a]	
0.25	224	15.8	10.6
0.5	352	28.1	22.8
0.75	253	19.7	15.7
1	210	21.4	14.8

[a] MS/MS data obtained by using response factors for the aqua species at Rt 2.3 min of 1.1 ± 0.1 and for the aqua species at Rt of 3.2 min 0.9 ± 0.2.

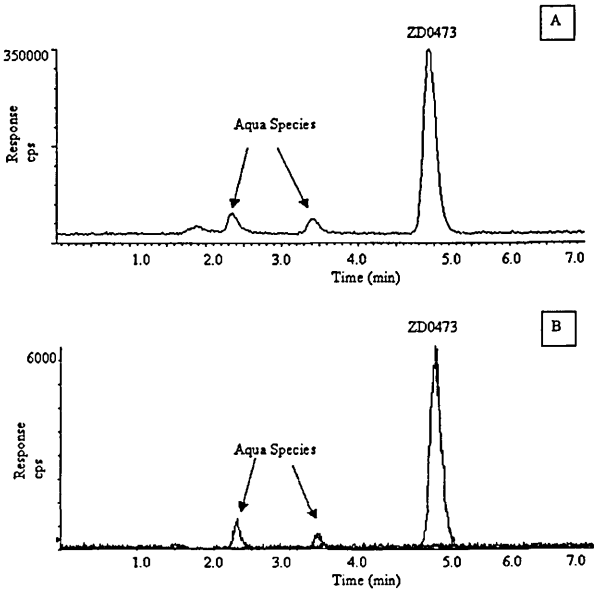

Figure 7. Example chromatograms obtained from the HPLC–ICPMS and HPLC–MS/MS assay run in parallel on a sample taken at 0.5 h from a single dog dosed orally at 6 mg/kg: (A) HPLC–ICPMS trace and (B) HPLC–MS/MS traces showing the two aqua species and the parent component ZD0473 obtained for the sample.

(17) Gilbert, M. T.; Barinov-Colligon, I.; Miksic, J. R. *J. Pharm. Biomed. Anal.* **1995**, *13*, 385–394.

the ICPMS data can be used to direct MS/MS analysis to compound-related materials.

CONCLUSION

Both HPLC–ICPMS and HPLC–MS/MS enabled the detection and quantification of ZD0473 in plasma samples and provided the basis for robust bioanalytical methods. However, the HPLC–ICPMS system was found to have greater sensitivity, with a limit of quantification for ZD0473 of 0.1 ng/mL (0.05ng/mL of platinum), as compared to 5 ng/mL for the HPLC–MS/MS method. Because it is based on the quantification of platinum, the HPLC–ICPMS method also enables the facile quantification of the platinum-containing aqua species, even in the absence of standards. HPLC–ICPMS provided an assay with a much greater linear range than HPLC–MS/MS, being linear over 8 orders of magnitude, as compared to just over 3 orders of magnitude for HPLC–MS/MS. These studies show that HPLC–ICPMS provides the basis for a routine method for the sensitive determination of ZD0473 and the related aqua species in biofluid samples.

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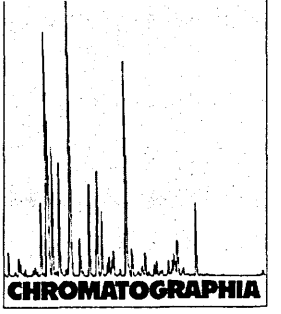
Analysis of a [^{14}C]-Labelled Platinum Anticancer Compound in Dosing Formulations and Urine Using a Combination of HPLC-ICPMS and Flow Scintillation Counting



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C. J. Smith[1]* / I. D. Wilson[1] / F. Abou-Shakra[2] / R. Payne[1] / H. Grisedale[1] / A. Long[1] / D. Roberts[1] / M. Malone[1]

[1] Department of Drug Metabolism and Pharmacokinetics, AstraZeneca Pharmaceuticals, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, UK; E-Mail: Christopher.Smith1@AstraZeneca.com
[2] Micromass UK Ltd, Floats Rd, Wythenshawe, Manchester M23 9LZ, UK

Key Words

Column liquid chromatography
Inductively coupled plasma mass spectrometry
Platinum anticancer drugs
ZD0473

Summary

The use of high performance liquid chromatography with inductively coupled mass spectrometry (HPLC-ICPMS) in combination with on-line radioactivity determination is described for the analysis of ZD0473, a platinum anticancer drug. The technique was used to determine the purity of formulated material in oral and IV dosing solutions, and for the detection of Pt-containing metabolites in urine following IV administration to the dog. In addition to Pt, it was found possible to detect ^{14}C directly by ICPMS when analysing solutions containing [^{14}C]-D0473.

ntroduction

:D0473 {cis-[amminedichloro(2-methyl-yridine)] platinum(II)} is a new genera-'on platinum compound designed to deli-er an extended spectrum of antitumour ctivity and overcome platinum (Pt) resis-ınce [1]. The structure of ZD0473 and re-ıted compounds, including the biologi-ılly active "aqua" compounds, is shown elow (Figure 1). Traditionally the analy-s of Pt drugs has relied upon either omic absorption spectrophotometry, ICPMS or electrochemical detection [2]. However, in the absence of a separation method these techniques do not provide an adequate procedure as they fail to distinguish between active compound and inactive metabolites. Historically metabolite/impurity profiles have been obtained by fraction collection following separation and then determining the Pt content of the individual fractions. This procedure is both slow and tedious. However, the advent of HPLC-ICPMS [3–5] offers the potential to analyse such samples without the need for fraction collection. Indeed, Galettis et al. [5] have used HPLC-ICPMS for the quantitative analysis of the platinum-containing drug JM216 in human plasma. We have, therefore, attempted to use this methodology (cf. p. S-9) to provide a rapid, efficient and sensitive method of analysis for Pt in samples containing ZD0473 and related materials. Here we describe the use of HPLC-ICPMS coupled with radio flow scintillation counter and ultra violet detector to provide a method of analysis for [^{14}C]-labelled ZD0473. The two techniques were used in combination to determine if the profile for [^{14}C]-labelled compound matched the profile obtained for the Pt in chromatograms obtained for dose formulations and urine samples from a metabolism study.

Experimental

Chemicals

Trifluoroacetic acid was purchased from Fisher (Fisher Scientific UK Ltd, Loughborough). Solvents for chromatography were of HPLC grade and were obtained from Fisher Scientific. The physiological saline used in the study for the dilution of samples and for the preparation of IV formulations, together with the Hydroxypropylmethyl cellulose (HPMC) used in the preparation of the oral dose solution, was obtained from the Media Preparation Laboratory at AstraZeneca Pharmaceuticals. The scintillant used was Ultima-Flo M from Packard Ltd (Berkshire, UK).

ZD0473 was obtained from AstraZeneca Pharmaceuticals (Alderley Park, Macclesfield, UK) whilst [^{14}C]-labelled ZD0473 was synthesised in the Isotope Chemistry Laboratory at AstraZeneca Pharmaceuticals at a specific activity of

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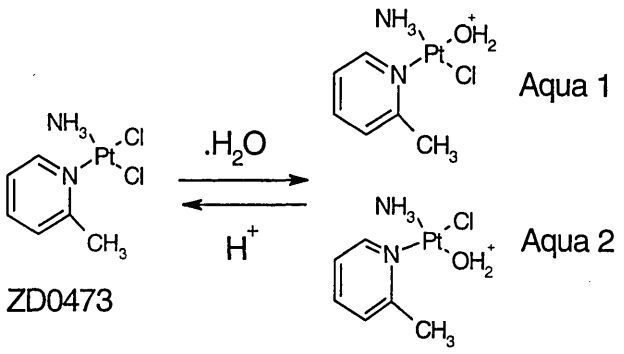
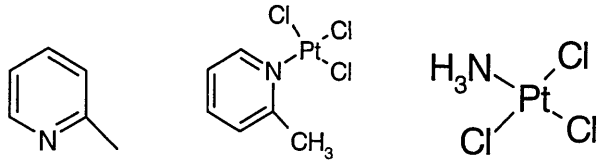


Known impurities



2-Picoline ZD0473 TCPP ZD0473 TCAP

Figure 1. The structure of ZD0473 with the associated aqua species, and known impurities.

Table I. Chromatographic gradient conditions.

Time (min)	Pump programming	Mobile Phase A %	Mobile Phase B %
0–4	Isocratic	98	2
4–11	Linear gradient	98 to 80	2 to 20
11–14	Isocratic	80	20
14–20	Linear Gradient	80 to 98	20 to 2

Table II. Instrument operating conditions for the ICPMS.

Cooling gas	16.00 L.min^{-1}	Plasma Power	1350 w
Plasma gas	0.65 L.min^{-1}	Acquisition mode	SIR
Nebuliser gas	0.75 L.min^{-1}	Dwell time	200 ms
Helium gas	1 mL.min^{-1}	Masses monitored	195, 14
Hydrogen gas	4.0 mL.min^{-1}	Total analysis time	20 min

55 μCi.mg^{-1}. The ^{14}C labelled isotope was laced in the aromatic ring of ZD0473.

amples

)ral and IV Dose Formulations of ZD0473

:D0473 was formulated in physiological aline or 0.5% HPMC for IV and oral ad- iinistration respectively at a concentra- on of 0.5 mg mL^{-1} and a specific activity f 55 μCi.mg^{-1}. To ensure that ZD0473 as completely dissolved the formula- ons were made up and stirred for 3 h rior to analysis.

rine

rine was collected for the period 6–12 h llowing IV administration to a male bea- gle dog (age 11 months, weight 12.8 kg, 0.5 mg.kg^{-1}, 55 μCi.mg^{-1}) of [^{14}C]-ZD0473. At the end of the sample period the weight was recorded and the urine split into two 20 mL portions and stored at −70 °C in a freezer. An aliquot (25 μL) was injected on to the system after centrifuging in an Eppendorf 5417C centrifuge (Eppendorf, Hamburg, D) for 5 minutes at 14000 rpm.

Chromatography

Reversed-phase HPLC was performed using a Jasco gradient HPLC system (Jasco Ltd, Great Dunmow, UK) with samples introduced via a PE 200 autosampler (PerkinElmer Ltd, Beaconsfield, UK). Chromatography was performed on Inertsil ODS-3 150 mm × 4.6 mm 5 μm (Hichrom Ltd, Reading, UK) at a flow rate of 1.0 mL.min^{-1}. The solvent system used for these separations was a gradient formed from 0.2% trifluoroacetic acid in water (solvent A) and methanol (solvent B). The gradient conditions were as follows. For the period 0–4 min isocratic elution was performed with 98% A: 2% B. Thereafter, a linear gradient was performed up to 20% B over 7 min. This was followed by an isocratic period at this solvent composition for 3 min, at which point a further linear gradient was performed over 6 min to regain the starting conditions (Table I).

UV and [^{14}C]-Detection

The eluent from the column was directed via an accurate splitter (LC Packings, Presearch, Hitchin, UK) to either a Micromass Platform ICPMS (Micromass UK Ltd, Wythenshawe, UK) (250 μL.min^{-1}) or to a Jasco UV-1575 intelligent vis/UV detector (265 nm) (Jasco Jasco Ltd, Great Dunmow, UK) and to a Flow One radio scintillation analyser (Packard, Berkshire, UK) (750 μL.min^{-1}) connected in series. The Flow One detector was fitted with a low volume (50 μL) flow cell in order to preserve the resolution between peaks. For both UV and [^{14}C]-detection Masslynx software (Micromass UK, Wythenshawe, UK) was used for data analysis.

ICPMS

The Platform ICPMS instrument employed in these studies used a hexapole collision/reaction cell-based ICP mass spectrometer [6] for the simultaneous measurement of the ^{14}C and ^{195}Pt elements. The eluent from the column was introduced to the ICPMS via a concentric nebuliser and a double pass spray chamber, which was cooled to −3 °C to allow an increase in the percentage of methanol to be used within the system. Masslynx software was used for the instrument control, data acquisition and data handling. The flow of the nebuliser gases and the operating and acquisition conditions of the ICPMS are shown in Table II.

The isotope envelope for Pt contains masses at 194, 195, 196 and 198 (at percentage abundances of 32.9, 33.8, 25.3, 8.0%). Although it is possible to run the instrument in such away as to sum these ions, which would theoretically give rise to an increased signal, in practice the in-

crease in the background caused the process not to be beneficial. Therefore the ion 195 was monitored because it is the most abundant isotope of Pt.

Results and Discussion

Assay Development

The chromatographic system designed for this assay had to achieve the objectives of separation of the components in Figure 1, whilst under the constraints of solvent composition imposed by the ICPMS [7, 8]. Thus, with the present set-up the percentage of organic modifier needs to be kept below approximately 40% or otherwise the plasma may be compromised and/or extinguished. To overcome this limitation ion exchange buffers with a low percentage of methanol were used to obtain the required separation (data not shown). This approach provided an adequate separation but was ultimately unsuccessful due to the buffer creating blockages within the ICPMS system and poor chromatographic reproducibility between runs. The buffers were therefore, replaced with a water-methanol gradient system, which had a final methanol composition of 30%. The instrument was able to run at this percentage of methanol due to the low flow rate (250 μL.min^{-1}) and the spray chamber being cooled to −3 °C. The system was further optimised and the final chromatographic conditions were as described in Table I; a standard chromatogram is shown in Figure 2. The change of HPLC system resulted in improved equipment reliability and more robust chromatographic separation.

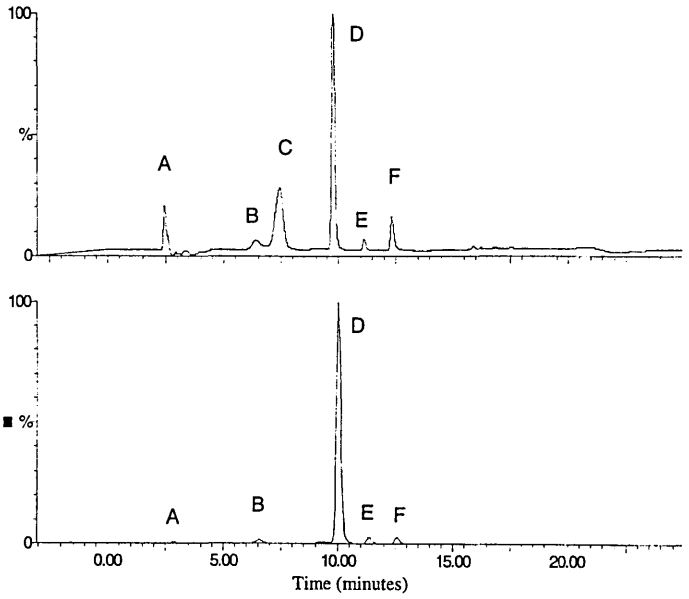


Figure 2. A typical example of the methanol-water gradient chromatography developed for the separation of the aqua species and impurity components: A: TCAP B: Aqua species 1 C: 2-Picoline D: ZD0473 E: Aqua species 2 F: TCPP. **a)** uv strace, **b)** ^{195}Pt trace.

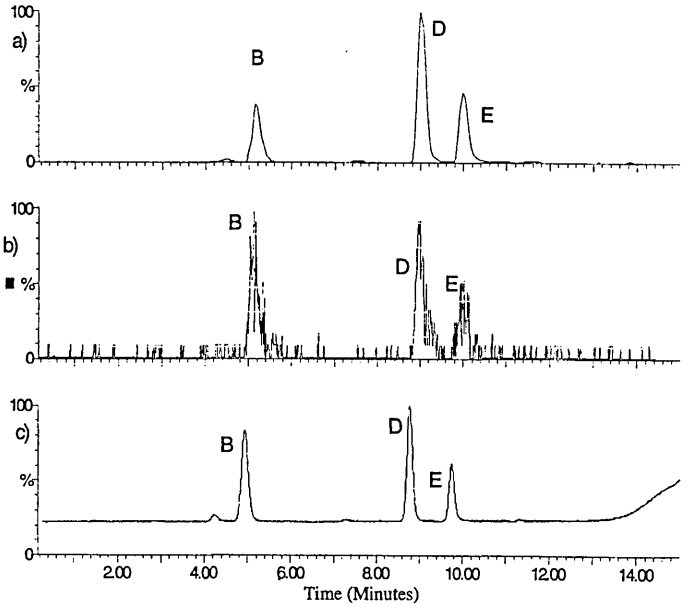


Figure 3. Typical **a)** platinum, **b)** radio and **c)** UV traces obtained for the oral dose solution. Key as for Figure 2.

Oral and IV Dose Formulation Purity Determination

The dose solutions prepared for the IV and oral formulations were analysed to see if they were of the required purity before dosing was carried out. Typical results for the UV, ^{195}Pt, and [^{14}C]-profiles generated for the oral and IV formulations are shown in Figures 3 and 4 respectively. The dose purity can be calculated by summing the ZD0473 peak area with those for the aqua species to give the total percentage of active drug (ZD0473/ ZD0473 total area of other analysed peaks *100). In this instance this was found to be 91% for the oral dose solution and 93% for the IV dose by ICPMS. These results show that for the dose solutions there is a good degree of correspondence between the chromatograms obtained for the 3 modes of detection.

The method demonstrated that the ratio of aqua species was different for each sample analysed. The reason for the increased levels and different ratio of aqua species in the oral sample was due to the formulation having a higher water component and therefore driving the equilibrium in the direction of the aqua species.

Analysis of Urine Samples Obtained from IV Dosing

The chromatographic system developed for the analysis of the formulation samples was also applied to urines obtained from a radiolabelled excretion balance study carried out in the dog, following intravenous administration of [^{14}C]-ZD 0473. The UV, ^{14}C and ^{195}Pt traces obtained for a single dog (6–12 h post dose) are shown in Figure 5. From the ^{195}Pt trace, unchanged ZD0473 accounted for 6.95% of the total based on the retention

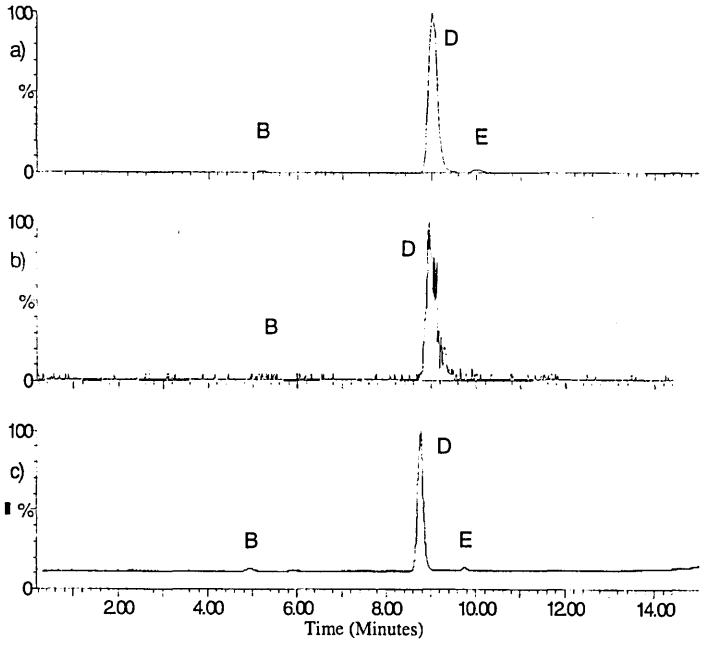


Figure 4. Typical **a)** platinum, **b)** radio and **c)** UV traces obtained for the IV dose solution. Key as for Figure 2.

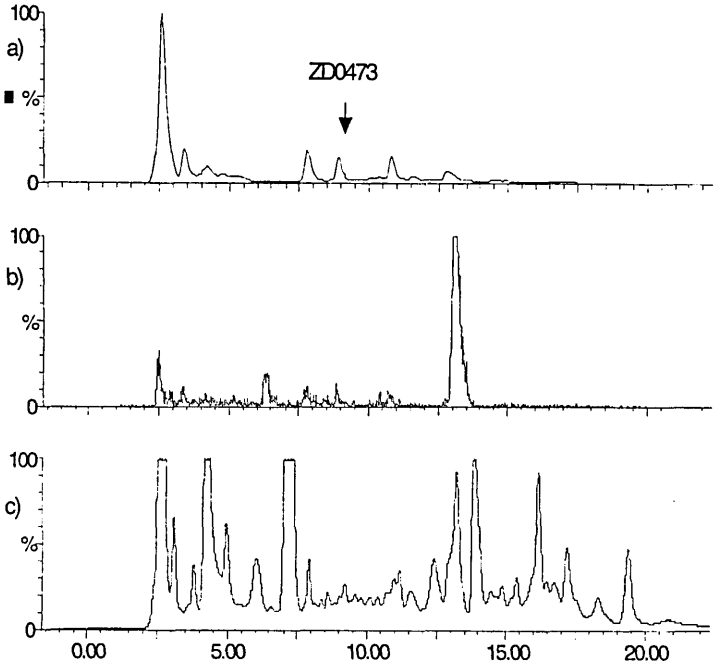


Figure 5. A typical set of traces for **A)** platinum, **B)** radio and **C)** UV obtained for a dog urine sample.

ime. The remainder consisted of 8 Pt-containing peaks, eluting between 2 and 4 minutes. None of these peaks corresponded to any of the degradation products represented in Figure 1. Comparison of the ^{195}Pt trace with the radioactivity profile revealed several noticeable differences. In particular a large radiolabelled peak was observed at 13.10 min, which was not present in the ^{195}Pt trace. This discrepancy in the profiles obtained for the ^{195}Pt and ^{14}C traces indicates that at some stage in the metabolism of the compound by the dog, the 2-picoline moiety, which contains the radiolabel, has been detached from the platinum. The use of both detectors, therefore, provides valuable and more robust metabolic information that would not have been obtained had only one been used. The identity of all of these metabolites is currently being pursued. Due to the presence of endogenous material interfering in the UV trace, no useful information could be gained.

Detection of ^{14}C by ICPMS

Whilst ICPMS has been used predominantly to determine metal species such as e. g. Pt, Se, Cd and As, it is also capable of analysing samples for many other elements. We have recently demonstrated applications of HPLC-ICPMS for the detection of Br, Cl and S-containing xenobiotics or their metabolites [9–11].[1)] In addition to the detection of ^{195}Pt in the present study, we also undertook an investigation of the potential of HPLC-ICPMS to detect the ^{14}C isotope present in [^{14}C]-ZD0473. Detection of $^{14}C^+$ is subject to two major difficulties namely; 1) high background at mass 14 from the presence of $^{14}N^+$ ions, and 2) relatively low sensitivity due to the high ionisation energy of carbon.

The atomic mass of ^{14}N is 14.00307 amu whereas the atomic mass of ^{14}C is 14.00324 amu. Therefore, the resolution required to separate these two ions by mass spectrometry is $m/\Delta m$ = 82371. Commercially available high-resolution ICP-MS systems do not offer much higher resolution than $m/\Delta m$ = 10000. As such, it is impossible to separate $^{14}N^+$ from $^{14}C^+$ by high resolution ICP-MS. However, the ICP-MS instrument used in this study, albeit being a quadrupole-based system with a nominal resolution of $m/\Delta m$ = 400, is equipped with a hexapole collision/reaction cell. It was therefore decided to investigate the potential of using this cell to chemically separate the $^{14}C^+$ and $^{14}N^+$ ions. Literature data of ion/molecule reactions clearly show that C^+ ions do not react with H_2 whilst the reaction of N^+ and H_2 ($N^+ + H_2 \rightarrow NH^+ + H$) is very efficient [12]. In agreement with these data, we observed that the addition of 4 ml/min of H_2 in the cell resulted in a virtually complete conversion of the N^+ ions into NH^+ without significantly affecting the sensitivity for $^{14}C^+$.

Owing to the high first ionisation energy of C (11.3 eV) the population of C^+ ions formed in the ICP is relatively low (c.a. 5%). Therefore, the sensitivity of ICP-MS for $^{14}C^+$ is about 20 times lower than it is for elements with low ionisation

[1)] See also the opening article in this supplement (F. Abou-Shakra et al., p. S-9).

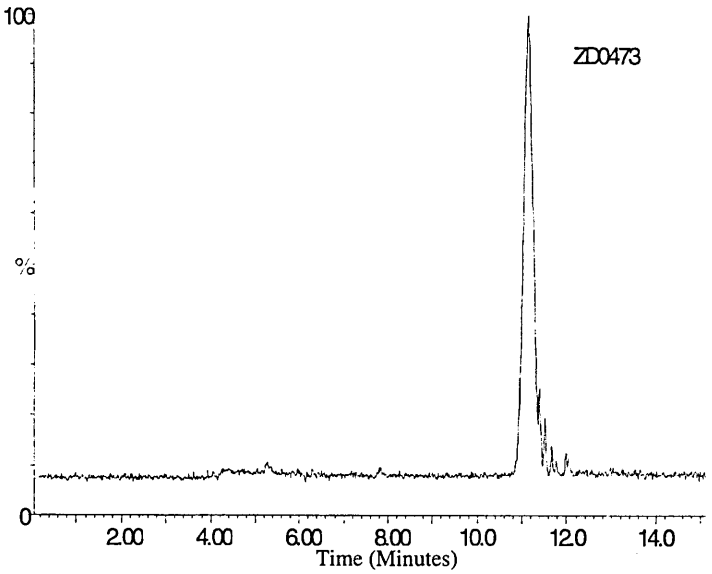


Figure 6. An example of the ^{14}C trace obtained from the ICP-MS after 100 µL injection of 0.5 mg mL^{-1} standard containing 25,6000 dpm.

energies (where the ionisation efficiency is nearly 100%). However, it is possible to optimise for maximum sensitivity for $^{14}C^+$, by adjusting the plasma operating conditions and the ion extraction conditions.

As a result of tuning the instrument and using H_2 in the collision cell, it was possible to detect $^{14}C^+$ in the samples under investigation. The chromatogram shown in Figure 6 was obtained for radiolabelled ZD0473 in the IV formulation. This quantity of material represented 25,600 dpm injected on-column. It is apparent from this chromatogram that minor peaks were not observed and that in its current state of development ICPMS detection could not be used alone for the determination of both ^{195}Pt and $^{14}C^+$. Nevertheless the detection of the radiolabel associated with the main peak of ZD0473 is promising, and strategies aimed at improving the sensitivity of the technique are currently being investigated.

Conclusion

HPLC-ICPMS provided a sensitive and specific method for the detection of the anticancer drug ZD0473 and its Pt-containing impurities and metabolites in formulations and urine. The use of ICPMS and a radio detector in parallel enabled the simultaneous detection of radioactivity and Pt, providing a more complete and robust profile of components in samples than would have been possible using each technique on its own. This combination showed clear benefits when components were produced which contained only Pt or ^{14}C.

We also demonstrated that it was possible to detect $^{14}C^+$using ICPMS. To our knowledge this is the first report to show such analytical capability of the technique. The full potential of the HPLC-ICPMS for this type of work is still being assessed, and new modes of operation such as the use of the technique for $^{14}C^+$ detection for qualitative and quantitative analysis will be investigated further.

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RCM

High-performance liquid chromatography/inductively coupled plasma mass spectrometry and tandem mass spectrometry for the detection of carbon-containing compounds

Christopher Smith[1]*, Berit Packert Jensen[1], Ian D. Wilson[1], Fadi Abou-Shakra[2] and David Crowther[3]

[1]Department of Drug Metabolism and Pharmacokinetics, AstraZeneca, Mereside, Alderley Park, Macclesfield SK10 4TG, UK
[2]GV Instruments Ltd., Crewe Rd, Wythenshawe, Manchester M23 9BE, UK
[3]Sheffield Hallam University, City Campus, Howard Street, Sheffield S1 1WB, UK

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High-performance liquid chromatography (HPLC) combined with inductively coupled plasma mass spectrometry (ICPMS) has been studied as a means for the detection of carbon to provide a 'universal' method for detecting organic compounds in chromatographic eluents. Carbon is particularly difficult to ionise and the amount of carbon present in normal chromatographic systems leads to high backgrounds, making detection a challenge. Novel separation approaches were therefore employed, using either entirely aqueous eluents (at temperatures of 60 and 160°C, dependent on the column used) to eliminate the organic modifier completely, or isotopically enriched solvents. For the aqueous eluents, detection limits for sulphanilamide were found to be 2.26 μg, corresponding to 1.13 μmol (0.47 μmol of carbon), injected on a conventional 4.6 mm i.d. column. The use of a narrow bore column with highly isotopically enriched ^{12}C-methanol (99.95 atom%) as organic modifier for the mobile phase enabled the detection of 86 μmol for ^{13}C-triple-labelled caffeine and 79 μmol for ^{13}C-double-labelled phenacetin. The sensitive detection of ^{12}C-compounds with ^{13}C-enriched methanol as organic modifier proved impractical due to a lower level of isotopic enrichment (99 atom%) of this solvent, with the residual ^{12}C-methanol resulting in significant interference. Copyright © 2004 John Wiley & Sons, Ltd.

The hyphenation of inductively coupled plasma mass spectrometry (ICPMS) with liquid chromatography has resulted in renewed interest in element specific detection in high-performance liquid chromatography (HPLC) within the pharmaceutical industry. In addition to the detection of metals such as platinum,[1,2] the technique has been found to be suitable for the detection of elements such as bromine,[3,4] chlorine,[5] iodine,[6,7] phosphorus[8,9] and sulphur[10–12] for both quantitative and qualitative analysis.

As part of our investigations on the use of HPLC/ICPMS for drug analysis we investigated the ability of HPLC/ICPMS to detect a [^{14}C]-labelled anti-cancer compound in a formulation,[13] based on the presence of the ^{14}C-isotope. The specific detection of the ^{14}C-isotope by ICPMS was possible because of the low natural abundance of this isotope. This meant that interference from endogenous carbon, for example from the HPLC mobile phase, was negligible. Despite the fact that the resolving power of quadrupole ICPMS instruments is insufficient to separate the masses of the element $^{14}N^+$ (14.0031) and the $^{14}C^+$ (14.001) isotope, the addition of H_2 to the collision cell to remove $^{14}N^+$ allowed $^{14}C^+$ to be detected with good specificity. These results led us to consider the possible use of ICPMS to provide a 'universal' method for the detection of organic compounds based on the detection of the more abundant isotopes of carbon (as has been investigated to some extent in HPLC/AES[14]). Such a technique would potentially be useful in that it would enable the amounts of individual components in a mixture (e.g. from combinatorial synthesis) to be determined in the absence of authentic standards or knowledge of e.g. molar UV extinction coefficients, etc. Such information is currently sought using e.g. chemiluminescent nitrogen detectors (CLND) for N- and S-containing compounds, or evaporative light scattering detectors (ELSD),[15] but similar results should be obtained using HPLC/ICPMS with carbon detection. Such information might be especially valuable when combined with some means, such as mass spectrometry, of determining the molecular mass of analytes to enable molar responses to be calculated. The problem with the detection of ^{12}C and ^{13}C is

*Correspondence to: C. Smith, Department of Drug Metabolism and Pharmacokinetics, AstraZeneca, Mereside, Alderley Park, Macclesfield SK10 4TG, UK.
E-mail: Christopher.smith1@astrazeneca.com

that they are present in the organic modifiers used for conventional chromatography. This leads to high background interferences, so that sensitive detection is not possible. However, analysis of the more common isotopes of carbon might be possible if such interferences could be removed from the system. One possible answer (explored here) is the use of entirely aqueous eluents, including superheated water,[16] where the mobile phase consists entirely of water, with separation controlled by changing the column temperature. Indeed, in many ways, water provides the perfect chromatographic eluent to use with ICPMS since there are no organic components present to quench the plasma. Hydrogen and helium are normally employed in the collision cell for the reduction of isobaric interferences.[17] In this instance the gases were used to improve transfer of the elements through the hexapole. An alternative way of reducing background interferences to manageable levels, also investigated here, is the use of ^{12}C- or ^{13}C-enriched solvents for the detection of ^{13}C-enriched and normal ^{12}C-containing compounds, respectively.

EXPERIMENTAL

Chemicals

Sulphanilamide, caffeine, antipyrine, and paracetamol (acetaminophen) used in this study were obtained from Sigma-Aldrich (Poole, UK) at a purity of 99%. The ^{13}C-enriched caffeine (^{13}C-triple-labelled, 99 atom%), phenacetin (^{13}C-double-labelled, 99 atom%) and the ^{12}C-enriched methanol (99.95 atom%) were also supplied by Sigma-Aldrich. The ^{13}C-enriched methanol (99 atom%) was obtained from Cambridge Isotope Laboratories (Andover, MA, USA).

Standard solution preparation

Caffeine, antipyrine and paracetamol stock solutions were prepared in water at a concentration of 5 mg/mL in 10 mL volumetric flasks with gentle heating to aid dissolution. Further dilutions were prepared in water to the concentrations of 1 and 0.1 mg/mL. ^{13}C-enriched caffeine and phenacetin were dissolved in ^{12}C-enriched methanol/water (1:1) mixture to a concentration of 1 mg/mL.

Preparation of standard curve and quality control samples

A standard curve was constructed for caffeine with quality control data also obtained at each calibration point by injecting the sample six times. The caffeine standard curve was prepared at concentrations of 10, 5, 2, 1, 0.75, 0.5, 0.25, 0.1 and 0.05 mg/mL. A similar experiment was carried out for the ^{13}C-labelled caffeine with concentrations of 1, 0.5, 0.3, 0.15 and 0.075 mg/mL used to construct the standard curve.

HPLC/ICPMS and HPLC/ICPMS/MS

The HPLC/ICPMS system was set up in three different configurations as described below.

Chromatography for ICPMS with entirely aqueous solvent systems was performed using a Jasco PU-1580 HPLC pump (Jasco Ltd, Great Dunmow, UK) with samples introduced via a PE 200 series autosampler (Perkin Elmer Ltd., Beaconsfield, UK). Column heating was carried out using an Eppendorf TC-50 column heater (Presearch, Hitchin, UK). ICPMS was performed on a Platform ICP mass spectrometer, which used a hexapole collision/reaction cell for simultaneous measurement of the carbon isotopes (GV Instruments, Manchester, UK). MassLynx software was used for instrument control, data acquisition and data handling. Flow rates of the nebuliser gases and operating conditions of the ICPMS system are shown in Table 1.

Table 1. Instrument operating conditions for ICPMS

Cooling gas	16.00 L/min	Plasma power	1350 W
Plasma gas	0.65 L/min	Acquisition mode	SIR
Nebuliser gas	0.75 L/min	Dwell time	200 ms
Helium gas	1 mL/min	Masses monitored	12, 13 Da
Hydrogen gas	4.0 mL/min	Total analysis time	7 min

For some experiments, see below, an API-365 mass spectrometer (Applied Biosystems, Warrington UK) was connected in parallel via an in-line splitter with a turbo ionspray inlet source used in scan mode over the range *m/z* 100–400. Analyst software was used for the instrument control, data acquisition and data handling. The system was connected to the HPLC/ICPMS system via PEEK tubing (0.13 mm) with the flow controlled by an accurate splitter (Presearch).

Aqueous separations at 60°C

The separation of caffeine, antipyrine and paracetamol with an aqueous mobile phase at 60°C was performed on a Discovery Zirconia-PDB 150 × 4.6 mm, 5 µm column (Supelco, Bellefonte PA, USA) at a flow rate of 1 mL/min. The flow was split using an accurate splitter with 700 µL to an API-365 mass spectrometer and 300 µL to the ICP mass spectrometer using a concentric double pass spray chamber (GV Instruments), for sample introduction. The flow into the API-365 was adjusted to allow 200 µL to enter the turbo ionspray source. The injection volume used in the experiments was 20 µL.

Aqueous separations at 160°C

The separation of caffeine, paracetamol and phenacetin was also performed using superheated water at 160°C as the mobile phase on an XTerra C8 150 × 4.6 mm 5 µm column (Waters, Watford, UK) at a flow rate of 1 mL/min with an IS6000 ultrasonic nebuliser (Cetec Technologies, Omaha, NE, USA) for sample introduction into the ICP mass spectrometer. The injector was flushed with 100 µL of water between samples with an injection volume of 20 µL.

Application to caffeine determination in coffee

Coffee (20 mg, Carte Noire), purchased from a local supermarket, was dissolved in water (1 mL, with gentle heating to aid dissolution). An aliquot (100 µL) of this sample was analysed by HPLC/ICPMS/MS using an XTerra column operated at 160°C and a flow rate of 1 mL/min as described above.

Separations with isotopically enriched solvents

For both the ^{12}C- or ^{13}C-isotopically enriched mobile phases a microbore chromatographic system was used. Jasco PU-1580 pumps were used for solvent delivery at a flow rate of 0.1 mL/min. Separations were performed on a Polaris 50 × 1 mm i.d., 3 µ column (Varian, Lake Forest, USA) with

injection volumes of 10 μL from a PE 200 series autosampler. Mobile phases were made up of the enriched ^{12}C- or ^{13}C-methanol with water (proportion of enriched solvent to water was 20:80, v/v).

Gradient elution was also employed for the chromatography of phenacetin (using 95% methanol/water to 50% methanol/water (v/v) linearly over 10 min).

RESULTS AND DISCUSSION

HPLC/ICPMS with entirely aqueous mobile phases

Initial experiments with sulphanilamide, using superheated water chromatography at 160°C on the XTerra phase, confirmed the principle and indicated that a detection limit of ca. 2.26 μg, or 1.13 μmol, of material could be achieved (470 nmol of carbon). Under these conditions sulphanilamide eluted rapidly from the column, with a retention time of just over 2 min, as shown in Fig. 1(A). In addition these early investigations highlighted the critical importance of eliminating organic solvents from the system. Thus, in one experiment, the compound was taken up in methanol/water (5:95% v/v) and then diluted with water (1:10) for injection onto the system. As shown in Fig. 1(B), whilst the peak corresponding to the analyte is still clearly visible, it is superimposed on a much larger, and very broad, peak due to the residual methanol present in the injection solvent. It is noteworthy that the methanol peak required ca. 40 min of elution before it returned to baseline again (Fig. 1(C)). Thus the required solvent for both sample dissolution and chromatography is clearly water, though this does place constraints on the type of compounds for which this strategy would be suitable.

We also noted that when the PEEK tubing was used at these high operating temperatures it showed signs of degradation, which caused an elevated background level. Stainless steel tubing was therefore substituted to minimise this problem.

Chromatography, based on previously developed separations,[18] was then performed on a mixture of three model compounds, comprising caffeine, paracetamol and antipyrine on Zirconia-based and XTerra stationary phases, with pure water as the mobile phase, and column oven temperatures of 60 and 160°C, respectively. Representative chromatograms are shown in Figs. 2(A) and 2(B) for XTerra and Zirconica with the phases producing similar separation and peak shape.

The response of the ICPMS system to ^{12}C for caffeine was found to be linear over the range examined (25–500 μg on-column, corresponding to 16–320 μmol) with the equation of the calibration line given by $y = 774x + 568$ and correlation coefficient $r = 0.999$. The detection limits were down to 2.5 μg (1.28 μmol) (0.64 μmol of carbon) back-calculated from the standard calibration curve for both columns and temperatures. Whilst not investigated exhaustively, reproducibility was examined by multiple injections of standards (six replicates) at a concentration of 25 μg on-column (e.g. 16 μmol of caffeine) for the Zirconia column at 60°C. The standard deviations obtained for this experiment were ca. 6.8, 7.8 and 6.5% for paracetamol, caffeine and antipyrine, respectively.

Clearly, as detection in the ICPMS system is proportional to the amount of carbon in each compound, the potential exists for the quantitative analysis of compounds in a mixture as, if

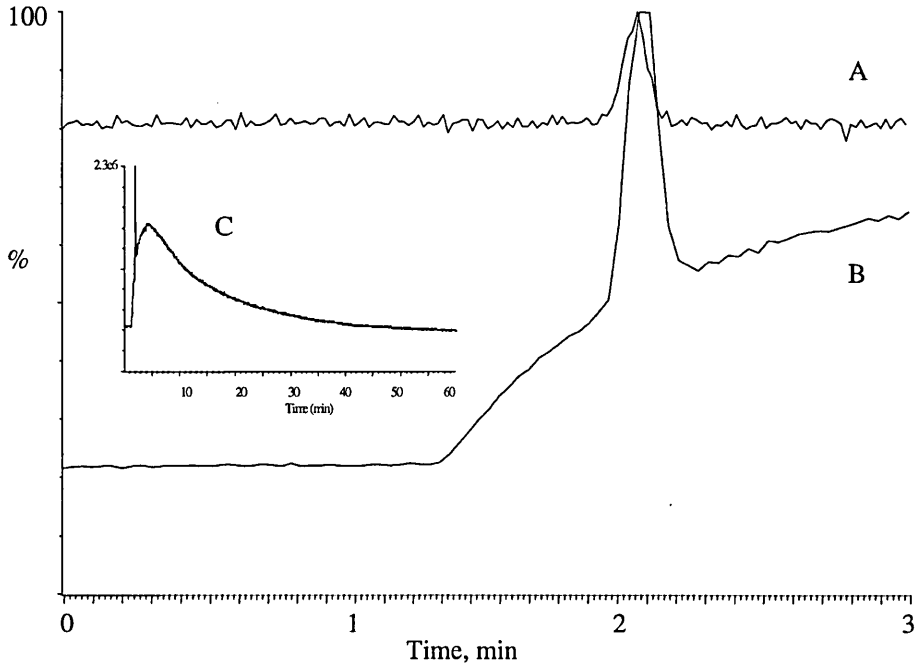

Figure 1. (A) Chromatography of 2.26 μg of sulphanilamide (1.13 μmol, 470 nmol of carbon) on an XTerra column at 160°C and 1 mL/min. (B) The same analysis for a sulphanilamide sample made up in 0.5% methanol on the same time span for comparison. The insert, chromatogram (C), shows the baseline disturbance due to the methanol produced in chromatogram (B).

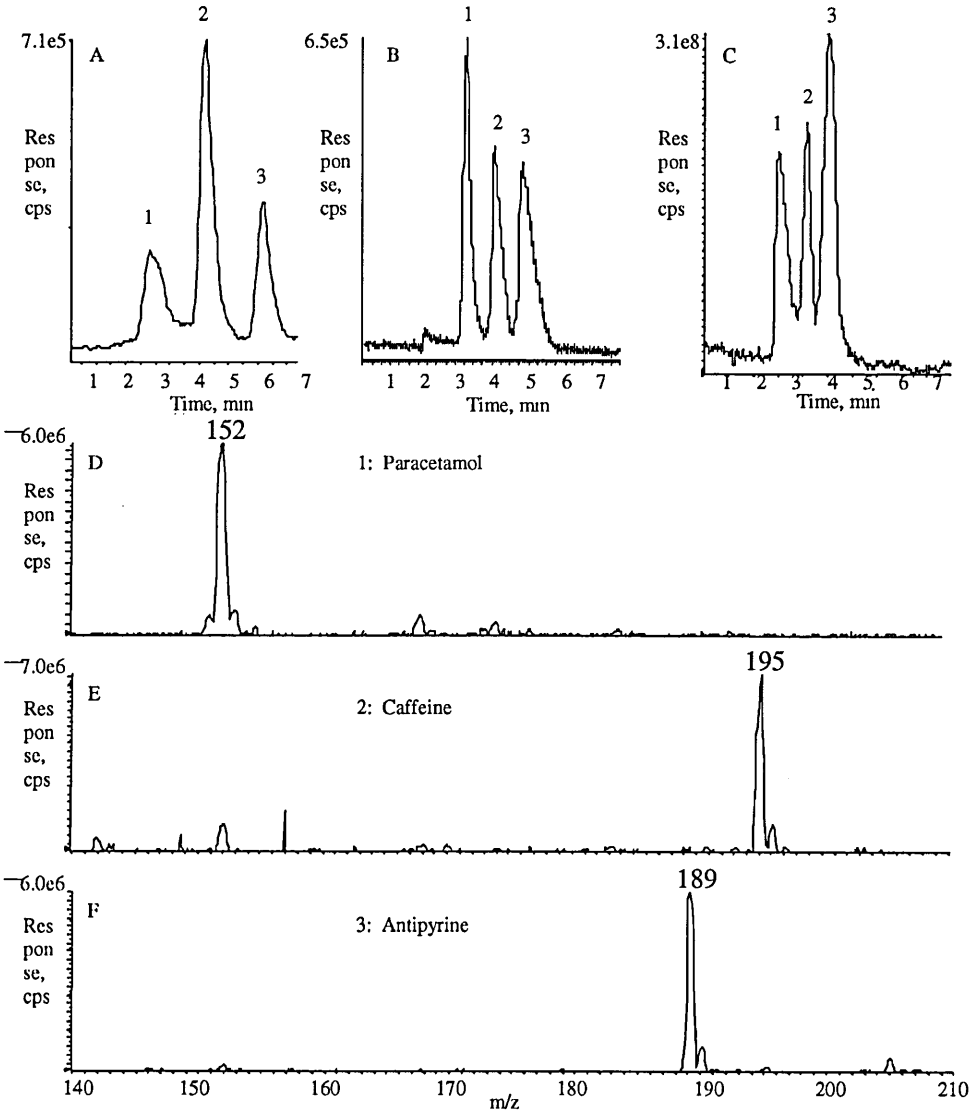

Figure 2. (A) Chromatogram showing the separation of paracetamol (1), caffeine (2) and antipyrine (3) on an XTerra column at 160°C. (B, C) Chromatograms showing the detection of the same compounds, separated on a Zirconica column at 60°C, detected by ICPMS and MS, respectively. (D, E, F) Mass spectra obtained for the three compounds.

the atomic composition of the compound is known, an absolute value can be obtained for the response, which relates to the amount of carbon present in the molecule. Table 2 shows an example of this with the area response obtained proportional to the amount of carbon present in each compound. This information allowed the amount of compound to be quantified relative to an external standard. Where unknowns were present the molecular masses of the individual components could be obtained by dividing the effluent from the column between the ICP and a conventional mass spectrometer. This approach has been demonstrated elsewhere.[19] To illustrate this, an API-365 triple quadrupole mass spectrometer was connected into the system by splitting the eluent from the column to the ICP mass spectrometer as described in the Experimental section. This set up was used to obtain simultaneous HPLC/ICPMS and MS data from the mixture of paracetamol, caffeine and phenacetin, separated on the Zirconia-HBD phase. Figure 2(C) shows the total ion chromatogram obtained from a scan over the range *m/z* 140–210, representing the three peaks of paracetamol, caffeine and antipyrine. The resulting mass spectra are shown in Figs. 2(D), 2(E) and 2(F), giving the $[M+H]^+$ ions (and thus the molecular weights) of the components.

Table 2. Area response obtained proportional to the amount of carbon present in each compound

Compound	No. of carbons present	MW with formulae	Carbon atoms/ MW ratio	Area response
Paracetamol	8	151 $C_8H_9NO_2$	0.053	18278
Caffeine	8	194 $C_8H_{10}N_4O_2$	0.041	14885
Antipyrine	11	188 $C_{11}H_{12}N_2O$	0.058	21109

The XTerra column, operated at 160°C, was used to determine the amount of caffeine in a coffee sample. The chromatogram presented in Fig. 3 shows a large endogenous

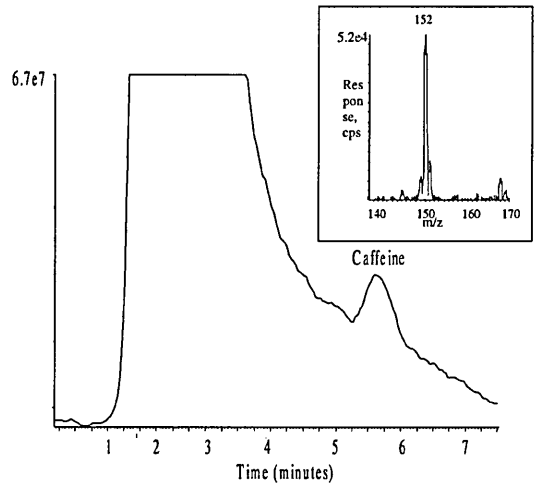

Figure 3. Coffee sample run on an XTerra column at 160°C and 1 mL/min, ca. 2 μg on-column of caffeine (100 μg/g 'Instant Coffee'). The insert shows the $[M+H]^+$ ion obtained for caffeine obtained by parallel MS.

peak, with the caffeine peak detected on the tailing edge, at a retention time of 5.5 min. This was confirmed by parallel work with an API-365 mass spectrometer obtaining a scan over the range *m/z* 140–170 showing the ion for the protonated caffeine molecule. By comparison with an external standard, an amount of ca. 2 μg of caffeine on-column was calculated for the response (100 μg/g of 'Instant Coffee'). For a quantitative method further work would clearly be required to separate the caffeine peak from the endogenous interferences.

HPLC/ICPMS with isotopically enriched eluents

Although the aqueous mobile phases described above have many advantages for ICPMS there are clear limitations as to the types of compound that can be separated in this way. With this in mind alternative chromatographic separations were investigated using isotopically enriched methanol as the organic modifier for chromatography. The enriched ^{12}C- and ^{13}C-methanol can be obtained commercially and we therefore investigated the detection of ^{13}C-labelled analytes in a ^{12}C-solvent as well as ^{12}C detection of compounds in a ^{13}C mobile phase. As the cost of such enriched solvents is not insignificant, chromatography was performed on a narrow bore column in order to minimise solvent consumption. This had the added benefit that the low flow rates employed reduced the amount of methanol in the plasma flame thereby aiding detection.

Highly isotopically enriched ^{12}C-methanol (99.95 atom%) was used as the organic modifier for the detection of ^{13}C-triple-labelled caffeine and ^{13}C-double-labelled phenacetin. The high degree of enrichment of the ^{12}C-labelled methanol used to make up the organic modifier meant that the interference on the *m/z* 13 channel was negligible. An example of this is shown in Figs. 4(A) and 4(B), where the peaks for 5 μg on-column of each of the ^{13}C-labelled caffeine (86 μmol of ^{13}C) and phenacetin (79 μmol of ^{13}C) are shown.

When gradient chromatography was investigated an increase in the background was noted as the proportion of

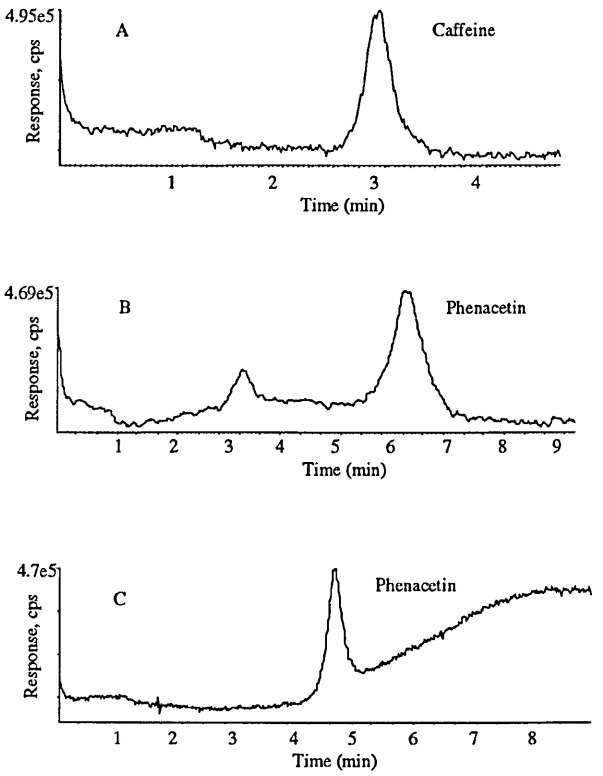

Figure 4. Chromatograms showing (A) a ^{13}C caffeine peak at concentration of 86 μmol for the three ^{13}C labels; (B) a ^{13}C phenacetin peak at a concentration of 79 μmol for the two ^{13}C labels; and (C) the chromatography of phenacetin using gradient elution.

organic modifier was increased, despite the high degree of isotopic purity of the solvent. An example of the use of gradient HPLC/ICPMS for the detection of ^{13}C-labelled phenacetin is shown in Fig. 4(C).

These preliminary experiments clearly demonstrate the potential for the detection of ^{13}C-enriched analytes in reversed-phase eluents. However, when ^{13}C-enriched methanol was used in an attempt to detect un-enriched ^{12}C-carbon compounds, similar success was not achieved. This was due to the lower degree of isotopic enrichment in the ^{13}C-methanol (99 atom%) than in the ^{12}C-methanol. Thus, the residual 1% of ^{12}C-methanol that remained in the ^{13}C-enriched methanol ensured that there was a significant ^{12}C-signal that precluded detection of the analytes at these concentrations.

CONCLUSIONS

The results presented here demonstrate that HPLC/ICPMS can be used for the detection of carbon under a limited set of analytical conditions, and under these conditions HPLC/ICPMS can provide a 'universal' detector for organic compounds. In practice, given both the expense and lack of high enough levels of isotopic enrichment, only those separations based on entirely aqueous eluents are likely to be economically viable. Carbon detection via HPLC/ICPMS/MS, for molecular mass determination and structure elucidation,

may prove attractive for the quantification and identification of unknowns.

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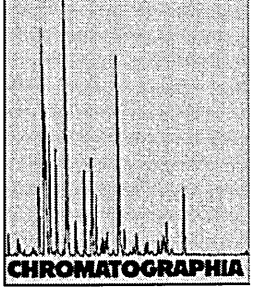
Enhanced Detection of Sulphur and Phosphorous Containing Compounds in HPLC-Inductively Coupled Plasma Mass Spectrometry Using Chemical Resolution via Hexapole-Based Reaction with Oxygen



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C. J. Smith[1,✉], I. D. Wilson[1], L. Weidolf[2], F. Abou-Shakra[3], M. Thomsen[4]

[1] Department of Drug Metabolism and Pharmacokinetics, AstraZeneca, Mereside, Alderley Park, Macclesfield, Cheshire, SK10 4TG, UK; E-Mail: Christopher.smith1@astrazeneca.com
[2] Department of Drug Metabolism and Pharmacokinetics and Bioanalytical Chemistry, AstraZeneca Pharmaceuticals, Pepparedsleden 1, 431 83 Mölndal, Sweden
[3] GV Instruments, Crewe Road, Wythenshawe, Manchester, M23 9BE, UK
[4] PerkinElmer Instruments, Chalfont Road, Seer Green, Beaconsfield, HP9 2FX, UK

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Abstract

The sensitive detection of sulphur and phosphorous-containing compounds using HPLC-ICPMS is difficult due to the high background caused by polyatomic interferences. One potential solution to the problem of polyatomic interferences for S and P is to react the latter with oxygen in the collision hexapole to separate them from interferences eg; (O_2 on S and NOH on P). This has the effect of moving the detection mass from 31 and 32 to 47 and 48 *m/z* for P and S respectively. This region is clear from interferences thereby providing an increase in the sensitivity of detection for the analytes of approximately 50–100 fold. For omeprazole, a model S containing compound, a limit of detection (LOD) of 800 pg on column was achieved, an increase of ca. 100 fold in sensitivity. Similarly in the case of ZD6126 a phosphorous-containing pro-drug, an increase in sensitivity of 50 times was observed with an LOD of 1 ng on column.

Keywords

Column liquid chromatography
Inductively coupled plasma mass spectrometry
Phosphorous-containing pro-drugs
Sulphur-containing drugs

Introduction

Inductively coupled plasma mass spectrometry (ICPMS) as an analytical technique has been around since the 1980's [1–2], however, the use of the instrument in combination with high performance liquid chromatography (HPLC) is more recent.

Whilst mainly used for the analysis of metals [3–5] (e.g. Pt) HPLC-ICPMS has also found some applications in drug and xenobiotic metabolism for halogen-containing compounds (Cl, Br and I) [6–9], as well as some limited investigations for S and P-containing compounds [10, 11]. However, in the case of S and P, sensitive detection is compromised by the presence of a range of polyatomic interferences such as $^{14}N^{18}O^+$ and $^{15}N^{16}O^1H^+$ which, lead to elemental overlap [12]. A specific process to improve the specificity of detection for S and P, and thereby enhance sensitivity, is to make use of chemical resolution with the reaction of S and P with O_2 to move them from the region of polyatomic interference [13]. The main difference between the analytes and the interferences is seen in the O-atom affinity. O-atom transfer from O_2 to P^+ and S^+ is thermodynamically allowed while for NO^+, NOH^+, and O_2^+ it is endothermic and forbidden under thermal conditions. When oxygen is reacted with an S element to produce a sulphoxide (SO^+) the detection mass changes from 32 to 48 *m/z*. Similarly for P, the production of PO^+ results in an increase in mass from 31 to 47 *m/z*. Table 1 shows the reaction enthalpy change and thermal reaction rate constant for S and P with O with the corresponding interference ion processes [12].

In this paper we have evaluated the utility of this approach for the quantitative analysis of sulphur compounds (omeprazole and sulphanilamide) and phosphorous-containing compound (ZD6126).

Experimental

Chemicals

Chromatographic organic solvents were obtained from Riedel-de Haën (Sigma-Aldrich Ltd, UK) with formic acid purchased from Fisher (Fisher Scientific Ltd, UK), which were of analytical or

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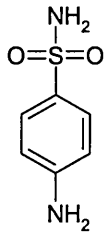
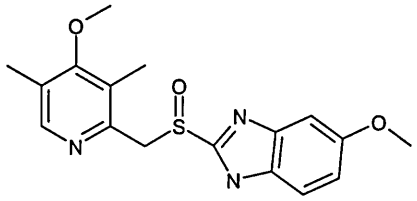
Table 1. Reaction of oxygen with sulphur and phosphate elements and relevant interfering ions

Reaction	Reaction enthalpy change Kcal mol^{-1}	Thermal reaction rate constant K_r, molecule^{-1} cm^3s^{-1}
$P^+ + O_2 \rightarrow PO^+ + O$	−71.4	5.3×10^{-10}
$S^+ + O_2 \rightarrow SO^+ + O$	−6.2	1.8×10^{-11}
$CO^+ + O_2 \rightarrow CO_2^+ + O$	−13.5	$<2 \times 10^{-14}$ (no reaction)
$HCO^+ + O_2 \rightarrow COOH^+ + O$	3.3	$<2 \times 10^{-14}$ (no reaction)
$NO^+ + O_2 \rightarrow NO_2^+ + O$	57.4	$<1 \times 10^{-11}$ (no reaction)
$NOH^+ + O_2 \rightarrow NO_2H^+ + O$	19.3	no data
$O_2^+ + O_2 \rightarrow O_3^+ + O$	102.1	no data

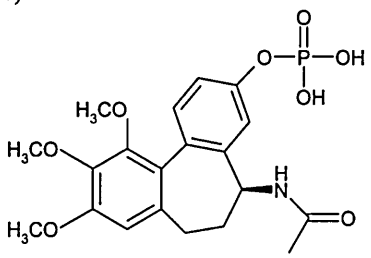
Table taken from ref [12]



Sulphanilamide (Mwt = 172)



Omeprazole (Mwt = 440)



Phosphate pro-drug (Mwt = 453)

Fig. 1. Structures of the compounds used in the evaluation

Table 2. Instrument operating conditions for the Elan DRC II

Nebulizer Gas Flow	0.77 L min^{-1}	Analog Stage Voltage	−2000
Auxiliary Gas Flow	0.8 L min^{-1}	ICP RF Power	1500 w
Plasma Gas Flow	20.00 L min^{-1}	O_2 gas	0.5 mL min^{-1}
Lens voltage	6.5	Mass monitored	31, 32, 47, and 48

HPLC grade. Sulphanilamide and omeprazole were purchased from Sigma (Sigma-Aldrich Ltd, UK) and the phosphorous-containing pro-drug, ZD6126, was supplied by AstraZeneca Pharmaceuticals (Alderley Park, Macclesfield, UK). Structures for test compounds are shown in Fig. 1.

Standard and Calibration Solution Preparation for Sulphur and Phosphorous

A standard solution of sulphanilamide was prepared at a concentration of 10 μg mL^{-1} by dissolution in water aided by sonication for 10 min in an ultrasonic water bath (Ultrawave Ltd, Cardiff UK).

Omeprazole stock solution was prepared in mobile phase at a concentration of 1 mg mL^{-1} in a 10 mL volumetric flask. The dissolution was aided by sonication for 10 min in an ultrasonic water bath. The stock solution was then serially diluted with mobile phase to give final concentrations of 1000, 700, 500, 400, 300, 100, 50 20 and 10 ng mL^{-1}.

ZD6126 stock solution was prepared by dissolving the compound in mobile phase at a concentration of 1 mg mL^{-1}, and sonication for 10 min to aid dissolution. Serial dilutions were prepared by diluting the stock solution in to mobile phase to give final concentrations of 1000, 700, 500, 400, 300, 100, 50 and 10 ng mL^{-1}.

Plasma Samples

200 μL samples of control plasma were spiked with either sulphanilamide or the phosphate pro-drug at a concentration of 100 ng mL^{-1}. These samples were then diluted with 100 μL of water and 50 μL aliquots were injected on to the HPLC-ICPMS system.

Chromatography

Sulphur and Phosphorous Detection

An isocratic HPLC system was used for the analysis of sulphanilamide and the phosphate pro-drug with a Synergi Polar Rp column, 150 × 4.6 mm, from Phenomenex (Macclesfield, UK). The mobile phase consisted of acetoniltrile, water and formic acid (40:60 0.1), which was delivered at a flow rate of 1 mL.min^{-1}. An injection volume of 50 μL was used.

An isocratic HPLC system was also used for the analysis of omeprazole with a C18 Xterra 150 × 2.1 mm, column (Waters, Milford, USA). The mobile phase consisted of actonitrile: water

(20:80) and was delivered at a flow rate of 200 μL.min^{-1}. The injection volume was 50 μL.

HPLC-ICPMS

Initial work using this technique for sulphur-specific detection using sulphanilamide as the test analyte was carried out on an Elan DRC II ICPMS (Perkin Elmer, Beaconsfield, UK) equipped with a PE series 200 pump and a PE series 200 autosampler. The sample was introduced into the plasma via a concentric nebuliser. Interactive software was used for the instrument control and analysis (Perkin-Elmer, Beaconfield, UK). The flow of the nebuliser gases and the operating acquisition conditions of the ICPMS are shown in Table 2.

Chromatography for the HPLC-ICPMS of omeprazole and the phosphate-pro-drug was performed using a Jasco HPLC system (Jasco Ltd, Great Dunmow, UK) with samples introduced via a PE 200 autosampler (PerkinElmer). The injector was flushed with mobile phase. ICPMS was also performed on a GVI Platform ICPMS (GV Instruments Ltd, Wythenshawe, UK) and the eluent was introduced to the ICPMS via an Aridus nebuliser (Cetac Technologies, Omaha, Nebraska, USA). Masslynx software (GV Instruments, Wythenshawe, UK) was used for data analysis and instrument control. The flow of the nebuliser gases and the operating acquisition conditions of the ICPMS are shown in Table 3.

Table 3. Instrument operating conditions for the Platform ICPMS

Cooling gas	17.00 L min^{-1}	Plasma Power	1700 w
Plasma gas	0.65 L min^{-1}	Acquisition mode	SIR
Nebuliser gas	0.6 L min^{-1}	Dwell time	200 ms
Helium gas	0 mL min^{-1}	Masses monitored	31, 32, 34, 47 and 48
Hydrogen gas	0 mL min^{-1}	Argon/Oxygen (95/5 %)	0.2 mL min^{-1}
Oxygen gas	0.8 mL min^{-1}	Total analysis time	6 min

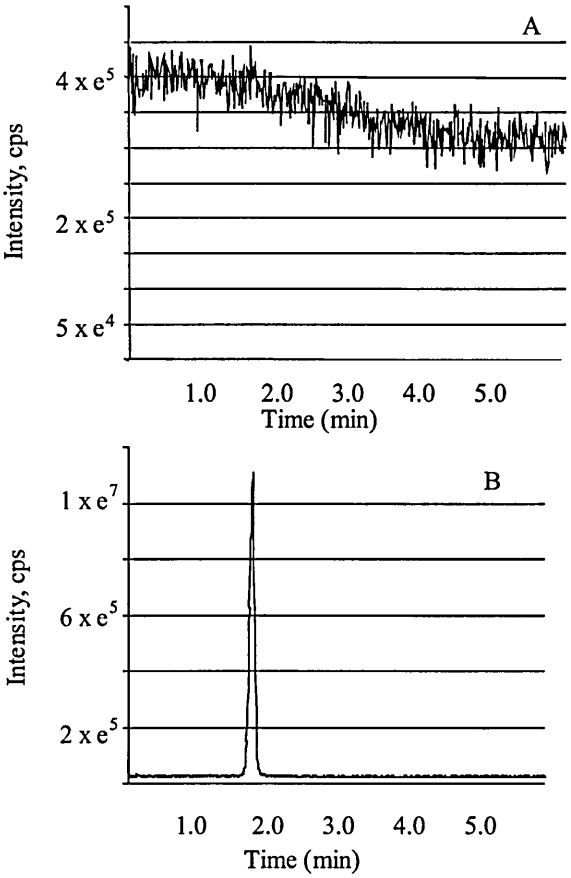


Fig. 2. Trace A shows the response of a sulphanilamide (1 μg on column) and trace B shows the improvement of the same sample after the addition of oxygen

Table 4. Sulphur precision and accuracy data

Quality control samples (pg on column)	mean (pg on column)	accuracy (%)	coefficient of variation (%) (n=6)
800	784	98	7.2
40000	35160	88	5.6
400000	438240	109	9.1

Results and Discussion

In order to perform "chemical resolution" a small amount of oxygen (Tables 2, 3) was introduced into the dymanic reaction cell of the Elan DRC II or the hexapole collision cell of the GVI platform through which the analytes are passed after the inductively coupled plasma. Prior to their exit from the reaction area into the MS detector chemical reaction occurs to produce the oxidised species for detection.

Sulphur-Detection

Initial studies to investigate the potential of chemical resolution were first performed for S using sulphanilamide as a model compound on an Elan DRC II ICPMS. The resulting increase in sensitivity obtained using such an approach is shown in Fig. 2 A and B. Thus in Fig. 2A when the compound was monitored at

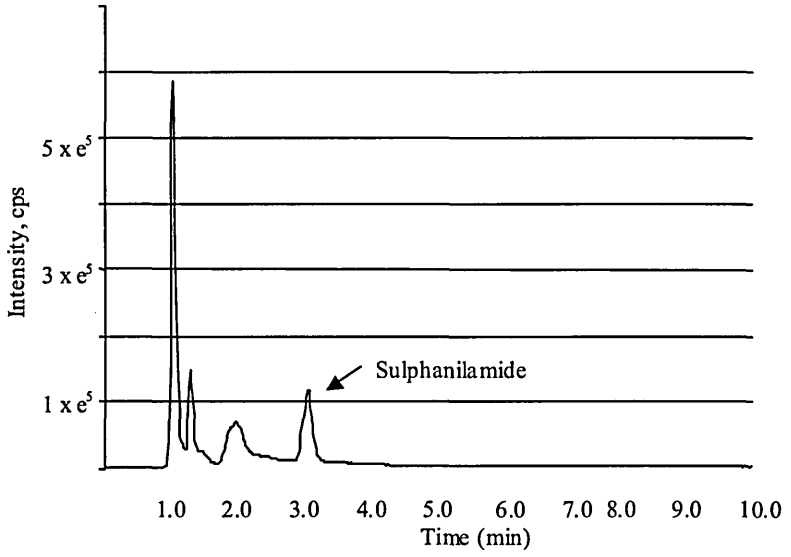


Fig. 3. An example of a diluted plasma sample of sulphanilamide (4 ng on column)

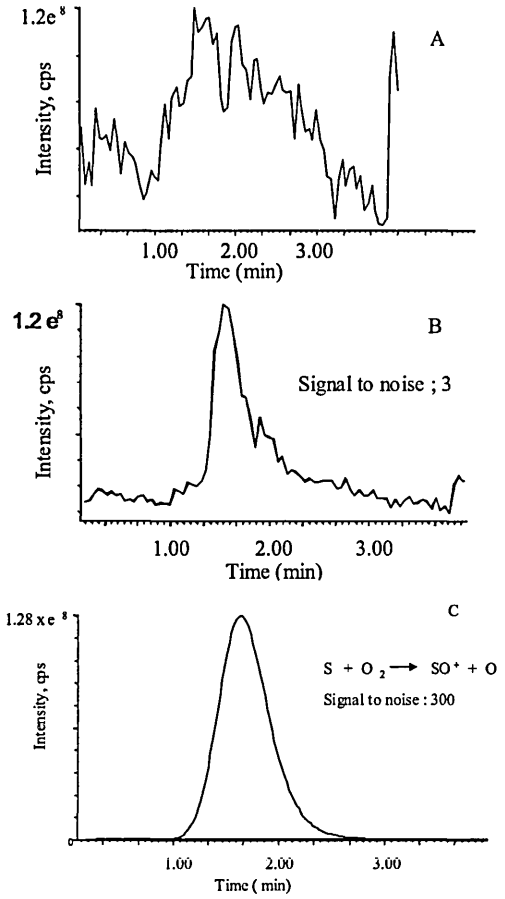


Fig. 4. Example chromatograms for omeprazole at 5 μg on column: (A) S monitored at 32 *m/z*, (B) S monitored at 34 *m/z* and (C) SO^+ monitored at 48 *m/z*

32 *m/z*, only a relatively small signal was observed. In contrast, monitoring the signal for 48 *m/z* after introduction of oxygen in to the dynamic reaction cell resulted in a dramatic increase in signal to noise as shown in Fig. 2B. The process was found to be linear over a range of 0.2 ng to 19 ng (coefficient regression 0.9998). In Fig. 3 the chromatogram obtained following the injection of spiked plasma sample (100 ng mL^{-1} corresponding to 5 ng of analyte on-column) is shown.

Similar data were subsequently obtained for another sulphur-containing compound, omeprazole, on a Platform ICPMS. Given the promising nature of this result we then progressed to a more in-depth evaluation of the potential of this methodology. Thus in Fig. 4A–C the results obtained for 5 μg on column of omeprazole are shown with detection either of ^{32}S, ^{34}S or $^{32}S^{16}O$. Although a minor isotope of sulphur the detection of ^{34}S has been employed previously as a means of avoiding polyatomic interferences [14] and, as shown in Figure 4B there is a useful increase in sensitivity, for this isotope compared to ^{32}S. However, comparison with the result shown in Figure 4C for the response obtained when oxygen is infused in the hexapole reaction cell clearly indicates the superiority of the latter approach. The improvement in response was found to be ca. a 100 times over the response found for ^{34}S isotope and a 1000 times for the ^{32}S isotope.

We therefore proceeded to investigate the properties of the system with respect to sensitivity, accuracy, reproducibility and linear range. Table 4 shows the accuracy and precision data collected using omeprazole and the chromatographic conditions described earlier at three concentrations with six replicates at each concentration. This experiment was carried out on aqueous standards to show that the S oxygen reaction in the collision cell could meet the requirements of an analytical method. The system was linear over the range 800 pg to 400000 pg on column (coefficient regression 0.9998) with an accuracy at the lowest level (800 pg on column) of 98 % and coefficient of variation of 7.2%. The data collected indicates that the use of oxygen in this manner was a reliable means of increasing sensitivity for S-containing compounds. Chromatographic traces obtained from the precision and accuracy

experiment can be seen in Fig. 5 where examples of the limit of quantification (LOQ) (800 pg on column) and a blank are represented. The amount of the S element detected corresponds to 80 pg on column since there is only one S present in the compound (Fig. 1).

Phosphorous-Detection

Evaluation of this methodology for the phosphate pro-drug ZD6126 was carried out on the Platform ICPMS. The gains in (Fig. 6) reveal an increase in response of ca. 50 times. In Table 5 accuracy and precision for this analyte are shown. The data indicates an accuracy of 109 % at the lowest point of detection (1000 pg on column corresponding to 100 pg of P), with a coefficient of variation of 9.7. The run was linear over the range 10 ng to 100 ng on column, with a coefficient of regression 0.999. The tested linear range was small in this evaluation however, the characteristics of the ICPMS would allow for a much large range to be used.

Similar improvements in sensitivity to those seen for S were also evaluated with this approach using the Elan DRC II for the detection of P. When P is reacted with oxygen, moving the detection mass from 31 to 47 *m/z*, polyatomic interference such as $^{15}N^{16}O^+$, $^{14}N^{16}O^1H$ and $^{12}C^1H_3{}^{16}O^+$ [12] are avoided. This is due to the laws of thermodynamics which allow the reaction of P and O as an exothermic reaction, however the reaction of N and O is an endothermic reaction and therefore not seen. The initial results for the PO^+ showed the process to be linear over the range 100 ng to 1900 ng on column with (regression coefficient of 0.999) for directly injected standards. A plasma sample was analysed by HPLC-ICPMS (Fig. 7) and this shows the P-containing pro-drug (5 ng on column) resolved from endogenous phosphate.

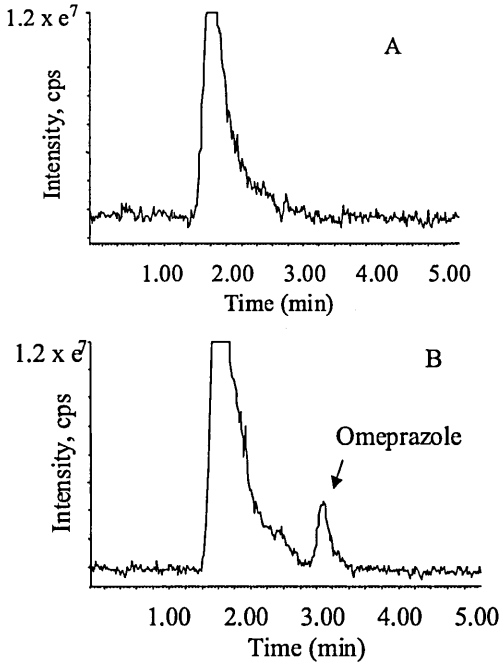


Fig. 5. Example of chromatograms for HPLC-ICPMS from omeprazole evaluation: (A) Blank and (B) lower limit of quantification 800 pg on column

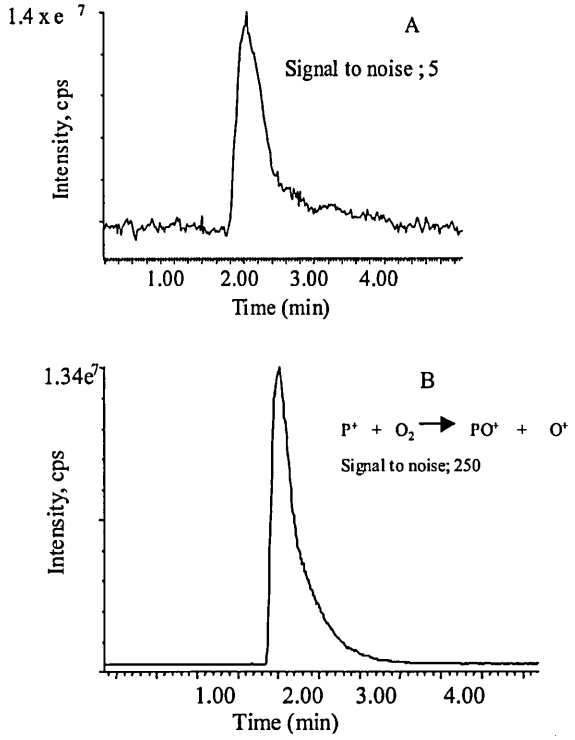


Fig. 6. Example chromatograms for ZD6126 at 5 µg on column: (A) P monitored at 31 *m/z* and (B) P monitored at 47 *m/z*

Conclusion

The use of the ICPMS with the addition of oxygen to the collision cell to increase the sensitivity of methods for compounds containing S and P elements has been demonstrated. An increase in response for S of 100 times for the SO^+ compared to the ^{34}S isotope, and 1000 times compared to ^{32}S, was shown. Similar gains in sensitivity were achieved for P. This type of increase in sensitivity should enable the use of HPLC-ICPMS for the analysis of compounds containing these elements at trace levels in complex matrices such as biological fluids.

Table 5. Phosphorous precision and accuracy data

Quality control samples (ng on column)	mean (ng on column)	accuracy (%)	coefficient of variation (%) ($n=4$)
10	10.9	109	9.7
50	49.9	99.8	9.0
100	102.1	102.1	3.0

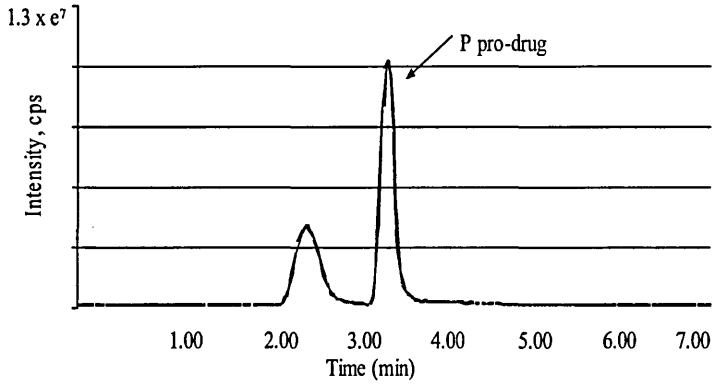


Fig. 7. An example of a diluted plasma sample of ZD6126 (100 ng on column)

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RCM

Sensitive sulphur-specific detection of omeprazole metabolites in rat urine by high-performance liquid chromatography/inductively coupled plasma mass spectrometry

Berit Packert Jensen[1†], Christopher Smith[1]*, Ian D. Wilson[1] and Lars Weidolf[2]

[1]Department of Drug Metabolism and Pharmacokinetics, AstraZeneca, Mereside, Alderley Park, Macclesfield SK10 4TG, UK
[2]Department of Drug Metabolism and Pharmacokinetics and Bioanalytical Chemistry, AstraZeneca R&D, S-431 83 Mölndal, Sweden

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The use of high-performance liquid chromatography/inductively coupled plasma mass spectrometry (HPLC/ICPMS) with sulphur-specific detection was investigated as a method for obtaining metabolite profiles for the drug omeprazole administered as a 1:1 mixture of ^{32}S- and ^{34}S-labelled material. Analysis based on the monitoring of the chromatographic eluent at either *m/z* 32 or 34 was not successful due to insufficient sensitivity caused by interferences from polyatomic ions. However, reaction of sulphur with oxygen in the hexapole collision cell, combined with monitoring at *m/z* 48 (for ^{32}S) or *m/z* 50 (for ^{34}S), provided a facile method for metabolite profiling. Detection of *m/z* 48 was superior in sensitivity to detection of *m/z* 50. Copyright © 2003 John Wiley & Sons, Ltd.

In the pharmaceutical industry, the detection, profiling and identification of metabolites of drugs are essential components of drug discovery and development. Conventional methods use HPLC coupled to mass spectrometry for detection and identification and this approach works well for both early stage discovery and later development studies. However, in the absence of authentic standards, the production of accurate, quantitative, metabolite profiles usually requires the time-consuming and expensive synthesis of isotopically labelled compounds and then HPLC with radiochemical detection. The ability to short circuit this process and obtain the required metabolite profiles based on some feature of the molecule under study could therefore provide a significant saving in time and resources, with environmental benefits resulting from the avoidance of radioactivity. To this end, we have extensively investigated the use of ^{19}F NMR (e.g. see Ref. 1) and have more recently begun to investigate approaches based on element-specific detection using HPLC/ICPMS. For drug substances containing heteroatoms such as sulphur, element-specific detection can be obtained using HPLC/ICPMS without the need for labelling.

However, sulphur detection by ICPMS is compromised by a range of interfering polyatomic ions, leading to high backgrounds and poor sensitivity. This is especially the case for the major isotope ^{32}S (95.0% abundance), which suffers from interference by $^{16}O_2^+$, and to a minor extent for ^{34}S (4.2% abundance), from, e.g., $^{15}N^{18}O^1H^+$ and $^{16}O^{18}O^+$.[2] Detection of ^{34}S using HPLC/ICPMS on a quadrupole-based instrument has been applied[3] and sulphur-containing metabolites of bromoaniline and diclofenac have been detected by monitoring ^{32}S.[4,5] Increased sensitivity can be obtained by using a sector field instrument to resolve sulphur from the interferences and the effectiveness of this approach has been demonstrated on studies of the impurities of the sulphur-containing drug cimetidine.[6] However, such instruments are expensive and less widely available than the quadrupole-based ICPMS systems. For these spectrometers, another approach to improve the sensitivity of sulphur is to make use of chemical resolution by reacting sulphur with oxygen in a collision/reaction cell and thereby move to a region with less interference.[7] The reaction of O_2 with S^+ to form sulphoxide (SO^+) is thermodynamically allowed, which is not the case for the interfering polyatomic ions, and hence sensitivity is increased.[7] By using an oxygen reaction in a hexapole collision cell and measuring SO^+ at *m/z* 48, Smith *et al.*[8] obtained an increase in response of ca. 100 times over the response found for ^{34}S and a 1000 times for ^{32}S. The method was used for the quantitative analysis of omeprazole and was found to be linear over the range 0.8–400 ng omeprazole on-column with good precision and accuracy.

This work describes the use of sulphur-specific detection to study the metabolite profile of [^{32}S]/[^{34}S]omeprazole in rat urine following oral administration.

*Correspondence to: C. Smith, Department of Drug Metabolism and Pharmacokinetics, AstraZeneca, Mereside, Alderley Park, Macclesfield SK10 4TG, UK.
E-mail: Christopher.Smith1@astrazeneca.com
†Present address: Department of Analytical Chemistry, Danish University of Pharmaceutical Sciences, Universitetsparken 2, DK-2100 Copenhagen ø, Denmark

EXPERIMENTAL

Chemicals

Omeprazole was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and [^{34}S]omeprazole was synthesised at AstraZeneca R&D (Mölndal, Sweden) using $C^{34}S_2$.[9] The

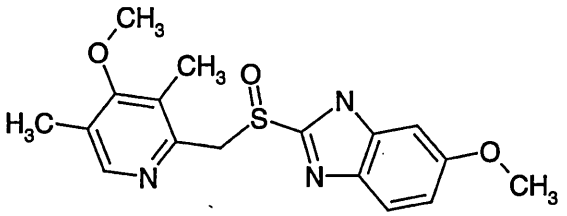

Figure 1. Structure of omeprazole (MW 345).

structure of omeprazole is shown in Fig. 1. Methanol (HPLC-grade) and ammonium acetate were purchased from Fisher Scientific UK Ltd (Loughborough, UK). Water was obtained from an Elga water purification system (Elgastat Maxima, Elga, High Wycombe, UK).

Urine samples

Pooled urine samples (0–9 h post-dose), concentrated using C_{18} solid-phase extraction, were obtained from three male Sprague-Dawley rats dosed orally with 200 μmol/kg of a 1:1 mixture of [^{34}S]-labelled and unlabelled omeprazole as described in Weidolf *et al.*[10] 100 μL were injected on column.

Instrumental

Chromatography was performed using a Jasco gradient HPLC system (Jasco Ltd, Great Dunmow, UK) with samples introduced via a PE 200 autosampler (Perkin-Elmer Ltd, Beaconsfield, UK). A C_{18} Hichrom column (HI-RPB; 5 μm, 150 × 4.6 mm) was used at a temperature of 40°C. The flow rate of 1 mL/min was split with 250 μL/min introduced to the ICPMS system. 0.13 mm i.d. PEEK tubing was used throughout. A gradient was employed based on (A) methanol/H_2O (5:95), 2 mM ammonium acetate, pH 7.0, and (B) methanol/H_2O (95:5), 2 mM ammonium acetate, pH 7.0, as follows: 0–5 min 95% A, 5–20 min 95–50% A, maintained from 20 to 25 min, 25–30 min 50–95% A, remaining at 95% A until 35 min.

ICPMS was performed on a GVI Platform instrument (GV Instruments Ltd., Manchester, UK) equipped with a Meinhard concentric nebuliser coupled to a cooled (−7°C) double pass spray chamber. Masslynx software (GV Instruments Ltd.) was used for data analysis and instrument control. The operating conditions of the instrument are shown in Table 1.

Table 1. Instrumental operating conditions for ICPMS

Parameter	Without oxygen reaction	With oxygen reaction
Cooling gas	17.0 L/min	15.0 L/min
Plasma gas	0.8 L/min	0.8 L/min
Nebuliser gas	0.8 L/min	0.8 L/min
Helium gas	9.8 mL/min	0 mL/min
Hydrogen gas	2.0 mL/min	0 mL/min
Oxygen gas	0 mL/min	0.60 mL/min
Plasma power	1700 W	1700 W
Acquisition mode	SIR	SIR
Dwell time	300 ms	300 ms
Masses monitored	32, 34	48, 50

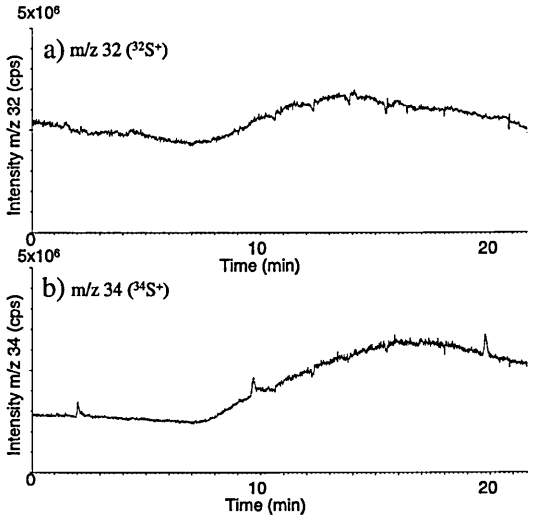

Figure 2. HPLC/ICPMS chromatograms of omeprazole metabolites in a rat urine extract detected at (a) *m/z* 32 and (b) *m/z* 34.

RESULTS AND DISCUSSION

Previous studies have shown that, with suitably concentrated samples, sulphur-containing compounds can be monitored in chromatographic eluents without the need for special measures such as reaction with oxygen.[4,5] For completeness therefore, the urine extract sample was initially analysed by ICPMS for both ^{32}S and ^{34}S without using the oxygen reaction to move from the region of polyatomic interferences. However, as Fig. 2 shows, adequate sensitivity was not obtained for either isotope of sulphur. Thus, in the case of ^{32}S, the high background due to polyatomic interferences meant that no S-containing peaks for the compound or its metabolites could be detected by monitoring at *m/z* 32. At *m/z* 34 it was possible to detect a few peaks but it is clear, even with the use of an isotopically labelled compound, that sensitive detection would not be obtained and that minor metabolites present in this sample would not be detected. The addition of oxygen to the hexapole collision cell with monitoring at *m/z* 48 and 50 was then attempted. As shown in Fig. 3, the result was a dramatic increase in signal-to-noise (S/N) ratio, and many S-containing peaks appeared.

Thus, in the case of ^{32}S, where no peaks were detected by conventional ICPMS, more than 30 resolved S-containing peaks were observed following addition of oxygen to the collision cell. In this study, omeprazole was dosed in a 1:1 ratio of ^{32}S and ^{34}S but, as seen in Fig. 3, a higher sensitivity was obtained at *m/z* 48 (i.e. detection of ^{32}S) than at *m/z* 50 (^{34}S) with respect to the S/N ratios obtained. It is also noteworthy that, due to the generally higher background at *m/z* 50, the increase in background as an effect of the methanol gradient becomes more apparent at *m/z* 50. However, whilst these results show that there is no advantage in terms of sensitivity to be obtained from isotopically labelling a compound with ^{34}S for HPLC/ICPMS, a comparison of the two profiles is instructive. This examination of the two profiles shows that the use of the labelled compound readily enables the omeprazole-related peaks to be distinguished from endogenous S-containing substances, as the former requires peaks

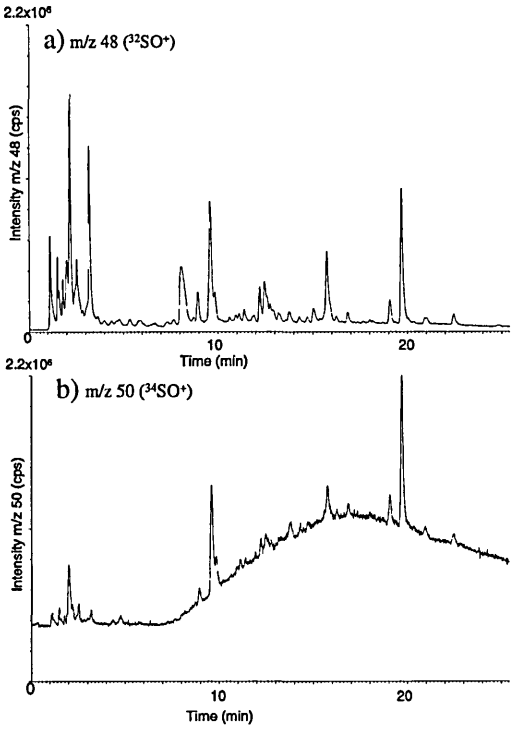

Figure 3. HPLC/ICPMS chromatograms of omeprazole metabolites in a rat urine extract detected at (a) *m/z* 48 ($^{32}SO^+$) and (b) *m/z* 50 ($^{34}SO^+$).

to be present in both the ^{32}S- and ^{34}S-chromatograms. From the results obtained here, the bulk of the endogenous S-containing material would seem to elute early in the gradient. Whilst sensitivity *per se* was not studied in this preliminary work, previous studies[8] have shown that the detection of 800 pg on-column of unlabelled omeprazole is possible by this approach (approximately 80 pg S). This represented an increase in sensitivity of 100-fold compared with the detection limits attainable without addition of oxygen to the hexapole collision cell.

ICPMS provides no structural information so the identity of the various peaks cannot be determined by this method. However, a peak corresponding to unchanged omeprazole was not detected (the drug would have eluted at 23.4 min in this system) clearly showing that the compound was extensively metabolised. Should structural information be required, this can be obtained by simultaneously monitoring the chromatographic eluent by conventional MS.

CONCLUSIONS

The poor sensitivity of ICPMS for ^{32}S and ^{34}S meant that metabolite profiling for omeprazole in rat urine could not be performed. However, moving to a region with less polyatomic interference by reaction with oxygen provided a readily implemented means of increasing the sensitivity of conventional quadrupole-based instruments for sulphur-containing compounds enabling good-quality metabolite profiles to be obtained.

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