Development of novel extraction and separation methods for the determination of anthracyclines and taxanes simultaneously from biological matrices

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Declaration

I, Shane Bermingham, hereby declare that the work carried out in this thesis was completed by me and that the work is original and in no way falsified.

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Table of Contents

Abstract

For certain types of advanced cancers patients may be given a combination of an anthracycline and a taxane for chemotherapy treatment. Despite the fact that a mixture of anthracyclines and taxanes may be administered simultaneously to patients and that accurate measurement of drug levels during chemotherapy is proven to be more beneficial to patients, currently no chromatographic method exists for the measurement of any anthracycline and taxane drugs in a single assay. It would be useful to carry out therapeutic drug monitoring (TDM) in order to assess how the patient is metabolising the drugs and to see whether the dosage is working or should be altered. Such information during therapy can be a very important clinical tool for the oncologist, enabling them to change dosage or even drug regimen on an individual basis for patients, improving outcome in the long run.

This thesis describes the development of two different analytical methods capable of quantifying both drug types in a single assay. The first method employed the use of on-line SPE-LC-UV and this method was then transferred to a mass spectrometric detector for more sensitive monitoring of the drugs. The on-line SPE-LC-MS assay did result in lower limits of detection and was fully automated. The entire method of extraction, separation and detection was achieved on-line by column-switching between an SPE column and an analytical column. The optimum type of switching valve, extraction conditions and separating conditions were evaluated for both methods in order to obtain the highest recovery possible for each target analyte in human serum. Recoveries ranged from 86 to 117% for each analyte in the LC-UV method and from 95 to 113% for most analytes in the LC-MS assay. This research could potentially lead to the introduction of therapeutic drug monitoring in Ireland for cancer patients being treated with anthracyclines or taxanes or both.

Abbreviations

ADS	Alkyl diol silica
APCI	Atmospheric pressure chemical ionisation
BSA	Bovine serum albumin
D	Distribution ratio
DNA	Deoxyribonucleic acid
Dnr	Daunorubicin
Doc	Docetaxel
Dox	Doxorubicin
Epi	Epirubicin
ESI	Electrospray ionisation
HPLC	High performance liquid chromatography
I.D.	Internal diameter
I.S.	Internal standard
LC	Liquid chromatography
LLE	Liquid liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
m/z	Mass to charge ratio
MALDI	Matrix assisted laser desorption ionisation
MRM	Multiple reaction monitoring
MS	Mass spectrometer/mass spectrometry
MS/MS	Tandem mass spectrometry
Pac	Paclitaxel
PBS	Phosphate buffer solution
PDA	Photodiode array
PP	Protein precipitation
QC	Quality control
R^2	Regression coefficient
RNA	Ribonucleic acid
RP	Reversed phase
RSD	Relative standard deviation
S/N	Signal to noise ratio
SIM	Selected ion monitoring
SRM	Selected reaction monitoring
SPE	Solid phase extraction
TOPO 2	Topoisomerase II
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible

Acknowledgements

I would like to thank all the people who took the time to train me on all the instrumentation I used and to thank those responsible for organising the trips to various analytical conferences and seminars which I found were very useful in helping me gain an insight into the analytical work that is carried out on a day to day basis throughout Ireland and Europe. I would especially like to thank my supervisors Dr. Gillian McMahon and Dr. Fiona Regan for their help and funding throughout this research. I would also like to thank Dr Robert O'Connor for allowing me access to the NICB to carry out research.

Chapter 1

General Introduction

1.1 Research objectives and relevance of work

1.1.1 Introduction

The main objectives of this work were to:

- Develop and validate a novel, fast SPE-LC-UV method for the quantitative determination of anthracyclines (doxorubicin, epirubicin and daunorubicin) and taxanes (docetaxel and paclitaxel) in serum.
- Develop and validate a novel, sensitive SPE-LC-MS/MS method for the quantitative determination of anthracyclines (doxorubicin, epirubicin and daunorubicin) and taxanes (docetaxel and paclitaxel) in serum.

Work was carried out in Dublin City University (DCU) School of Chemical Sciences and in the National Institute for Cellular Biotechnology in collaboration with the National Centre for Sensor Research (NCSR).

1.1.2 Research aims

The main aim of this project was to be able to quantify two types of cancer chemotherapy drugs in serum in a fast reliable way. The drugs under investigation are from the anthracycline and taxane classes of chemotherapy agents. These drug types can be given in tandem to treat certain types of cancer such as advanced breast or stomach cancer. To date, there are no methods available that can directly measure the concentration of both types of drug together.

The entire method of extraction, separation and detection was to be achieved on-line by column-switching between a SPE column and analytical column. The aim was to keep run times short and recoveries of both drug class high, which was a formidable challenge as the anthracyclines and taxanes are chemically and structurally so different. The limits of detection should ideally be very low (low ng/mL range) for therapeutic drug monitoring. This should be easily achieved with a MS detector but may be difficult to attain with a UV detector.

1.2 Cancer and chemotherapy

1.2.1 Cancer and its treatment

Cancer is one of the most traumatic diseases in our world. It involves the uncontrolled division of cells and the ability of these cells to spread to different parts of the body by invasion or by metastasis. The uncontrolled growth of cells is caused by damage to deoxyribonucleic acid (DNA) resulting in gene mutations that code for proteins controlling cell division. There are many different causes for these mutations e.g. exposure to radiation, smoking, hereditary factors etc. In 2004, cancer accounted for 13% of all deaths worldwide and it is still the leading cause of death around the globe[1]. It is second only to heart disease as the largest cause of mortality in Western society[2].

There are many types of cancer, the severity of which depends on the location of the cancer in the body and whether there is metastasis. Many cancers can be treated or cured, depending on the type, location and the stage. The different types of cancers are either metastasis, hypoxic or proliferating cells. Metastasis is the invasion of tumour cells elsewhere in the body. The majority of cancer related deaths result from metastasis. Cancer cells leave the main tumour mass and relocate elsewhere in the body. The cancer cells have special proteins that can permeate a protein matrix. No cell death is observed with metastasis as the malignant cells are put into a state of stasis. It is a challenge for doctors and scientists to bring new drugs to market when apoptosis is not observed as clinical trials are ineffective. Hypoxic cells do not replicate and they exist in oxygen deficient regions of the body. Hypoxic cells remain inactive in the body until proliferating cells are acted upon. Once proliferating cells have been removed, hypoxic cells become active. There two types of hypoxia - chronic hypoxia and transient hypoxia. With chronic hypoxia the cells are kept a long distance away from the nearest vein or artery and it is permanent. Transient hypoxia on the other hand is only a temporary state of oxygen deficiency. The third type of cancer cells are proliferating cells which are rapidly dividing. They are the easiest to target. Cell replication can be prevented by inhibiting DNA synthesis or by microtubulin inhibition to kill the cells.

Typical treatments for cancer include surgery, radiotherapy or chemotherapy. The course of treatment is usually dependant on the type of cancer. The aim of surgery is to attempt to completely remove the tumour. However, if the cancer has metastasised to different parts of the body before the surgery, complete removal of the tumour is often impossible. Radiation therapy uses ionising radiation to kill and reduce the size of the tumour. The effects of radiotherapy are localised to the site of the tumour site but the treatment can be used to treat just about every type of solid tumour. Chemotherapy involves the treatment of cancer with anti-cancer agents that can destroy cancer cells. These type of drugs attack rapidly proliferating cells. Unfortunately however, healthy tissue is often damaged alongside the cancerous cells resulting in unwanted side effects for the patient. With chemotherapy drugs there are also serious issues around toxicity, allergic reactions and intolerance. Being so cytotoxic, chemotherapeutic agents are usually administered at low concentrations and in small doses. In order to minimise adverse effects and maximise patient response, therapeutic drug monitoring (TDM) is sometimes exploited in the chemotherapy setting so that the concentrations of the drugs and/or their metabolites can be monitored in the individual patient. Inter patient variation in pharmacology is the primary rationale for TDM. This information can be used to tailor dosing, minimise side effects and even allows early intervention by the clinician for change of regimen if required. Modern studies have suggested that an improved chemotherapeutic effect is observed (efficacy, morbidity toxicity) when chemotherapy treatment is coupled with an accurate measurement of the required drugs [3]. To this end, reliable, sensitive and accurate analytical techniques are needed to support this effort.

In patients with advanced cancers such as those of the brain, breast and stomach, it is common that a patient be administered a combination of anti-cancer agents. TDM in these cases is even more challenging since the chemotherapeutic agents normally have different physiological characteristics and clinical behaviours. This makes it very difficult for the analytical chemist to develop one assay that is capable of determining the drugs simultaneously under the same conditions of extraction and separation.

1.2.2 Anthracycline chemotherapy

Anthracyclines are broad spectrum chemotherapy drugs. They were originally developed as antibiotics but are far too toxic to be used as such. They are aminoglycosides bearing a tetracyclic quinine structure. They were originally isolated from a pigment-producing Streptomyces and are among the most widely used anti-cancer agents [3]. The principal anthracyclines are epirubicin (Epi) doxorubicin (Dox) and daunorubicin (Dnr). As molecules, anthracyclines are polar and weakly basic drugs (See Figure 1.1).

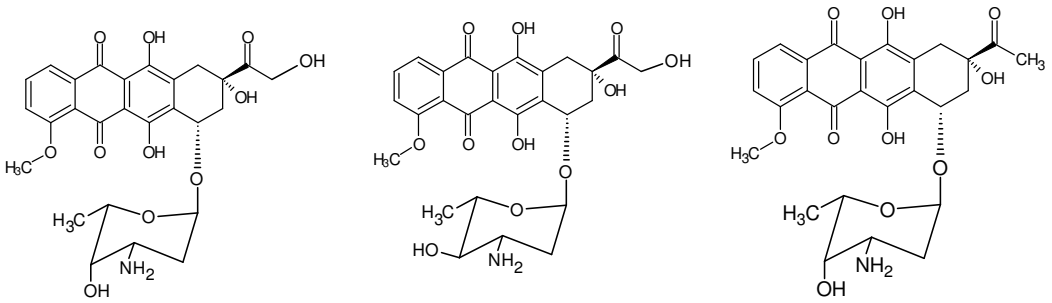


Figure 1.1: Structures of three main Anthracyclines

They are an important group of chemotherapy agents and have been used in cancer chemotherapy for more than 30 years and are considered to be among the most useful antineoplastics. Dnr was the first anthracycline to be isolated (1963) and found to be active against a variety of cancer cell lines. Dox and Epi were the next two anthracyclines to be discovered in 1968 and 1975 respectively. Idarubicin, a fully synthetic Dnr analogue was isolated in 1976 but it is rarely used in chemotherapy. Most advanced breast or stomach cancer patients treated with systemic cancer chemotherapy are given an anthracycline at some point during therapy [4]. Anthracyclines all typically have a common 7, 8, 9, 10-tetrahydrotetracene-5, 12 quinone structure, which is tailored to a sugar to form an antibiotic with anticancer activity [5]. Epi is the 4′-epimer of Dox and a semi-synthetic derivative of Dnr. At equimolar doses, Epi is less myelotoxic than Dnr and Dox and has a lower incidence of cardiotoxicity [6].

As a result of the reorientation of the hydroxyl group in the 4'-position of the daunosamine ring, Epi has several different pharmacological properties than Dox. It has a lower pKa than Dox. (7.7 versus 8.4) Consequently, Epi is more lipophilic and better able to penetrate cells Epi also has a more favourable therapeutic index than Dox and can be given at higher doses[3].

Anthracyclines have an almost unlimited potential for structural modification due to their complex structures. Epi and Dox only differ structurally at the 4’ C position with the hydroxyl group (OH) in the axial orientation for Dox and in the equatorial orientation for Epi. Despite this minor structural difference the resulting product 4-epi-doxorubicin (Epi) has significantly less toxicity to bone marrow and

myocardium[6] but comparable efficacy in a variety of solid and hematologic malignancies, including non-Hodgkin's lymphoma, carcinoma breast, ovarian cancer, small- and non small-cell lung cancer, gastric cancer, and nonresectable hepatocellular carcinoma[8].

Anthracyclines have a broad range of activity against a variety of solid cell malignancies, such as tumours of the breast, lung, ovary, head and bladder. They are the most effective single agents against soft tissue sarcomas in adults [7].

The side effects of anthracyclines include:

- Myelosuppression (the most severe side effect)
- Stomatitis
- Nausea and vomiting
- Cumulative dose-related cardiotoxicity [2,7] (the most serious side effect)

Despite their high toxicity and multidrug resistance, the anthracyclines have a narrow therapeutic range. Many efforts are now ongoing to reduce the side effects in patients by using novel formulations which are able to release the drug in the most appropriate way in the body and by monitoring the quantity of anthracyclines and their metabolites in the body fluids or tissues frequently and in every patient to maintain the drug concentration within the expected range.

The antineoplastic effects of Epi occur through numerous mechanisms. First, it intercalates between DNA nucleotide base pairs resulting in the inhibition of DNA, RNA, and protein synthesis. Second, intercalation leads to topoisomerase II cleavage of DNA, which results in cytocidal activity. Epi inhibits DNA helicase activity, which ultimately interferes with replication and transcription [7].

Anthracyclines prevent cell division by disrupting the structure and function of DNA. They are classed as topoisomerase II (TOPO-2) inhibitors. TOPO-2 can induce double stranded DNA cleavage which is essential for the knotting or unknotting of circular DNA and the introduction or removal of supercoils. TOPO-2 is essential for proper chromosome structure and segregation. TOPO-2 promotes cell survival, because of this it is an excellent target for anticancer agents. The anthracyclines fall into the category of TOPO-2 poisons. They stabilise the cleavable complex. The anthracyclines act as a type of TOPO-2 poison work by binding to the enzyme DNA complex at the strand cleavage stage forming a cleavable complex which results in a permanent strand break in the DNA strand which ultimately leads to cell death [8].

Maximum anthracycline activity is seen in the S-phase of the cell cycle: [9, 10]

- Intercalation of the chromophore between DNA strands and binding of the amino sugar with the DNA backbone causing disruption of the TOPO II function resulting in DNA strand breaks and apoptosis.
- Free radical formation. Microsomal enzymes form alkylating free radicals such as hydrogen peroxide.
- Hydroxyl radicals are formed by metal ion chelation which damages DNA and is thought to be responsible for cardiotoxicity.
- Direct effects on cell membranes resulting in a disruption in intracellular transport.

The most commonly used dosage of Dox or Dnr when used as a single agent is between 60 and 75 mg/m^2 (Epi can be given at the higher dosage of 60-105 mg/m^2 because it is less toxic) as a single intravenous injection every 3 weeks. The dose

administered depends on age, bone marrow density and possible prior therapy[11]. Great care should be taken when anthracyclines are administered so as to reduce the chance of perivenous infiltration and reduce the chances of local reactions such as blistering, ulceration or urticaria.

Immediate gastrointestinal toxicity with nausea is observed soon after administration of anthracyclines. An early clinical study of Dnr in adults and children with acute leukaemia found that bone marrow suppression was the predominant toxicity. Other toxic effects described were severe local reaction following extravasation, infectious complications, oral ulceration and alopecia[10]. Bone marrow suppression caused by anthracycline administration limits dose intensity. The major dose limiting toxicity however is cardiotoxicity. This cardiac damage is believed to be caused by metabolic free radical formation. The most significant form of cardiotoxicity seen from anthracycline administration is a cardiomyopathy which can lead to congestive heart failure in some cases [9].

Despite the cumulative toxicity associated with anthracyclines they are among the most active chemotherapeutic drugs and can be administered in a safe, well tolerated way providing lifetime exposure to the drug is monitored and alternative drugs used when the threshold for cardiotoxicity is reached [9].

Doses of Epi appear to be less cardiotoxic than equivalent cumulative doses of Dox. In a radomised study of adjuvant chemotherapy in Canada including 380 patients who received 300 – 400 mg/m^2 of Epi in the FEC (5-Fluorouracil, epirubicin and cyclophosphamide) regimen, only one case of cumulative heart failure (CHF) was reported. In another where patients received up to 608 mg/m^2 no cases of CHF were reported out of 351 patients on an FEC regimen. These and other similar data resulted in the safe threshold dose of 900 mg/m^2 for Epi which is considerably higher than the safe threshold does for Dox (450 – 600 mg/m^2) [9].

A possible explanation for the differing cardiotoxities between Epi and Dox is their pharmacokinetics. Both drugs are eliminated by a three compartment model but there is evidence that Epi is cleared more rapidly. Plasma half life is between 8 and 25 and 1.5 to 10 hr for Dox and between 3.1 and 4.8 and 1.1 to 2.6 hr for Epi respectively[9].

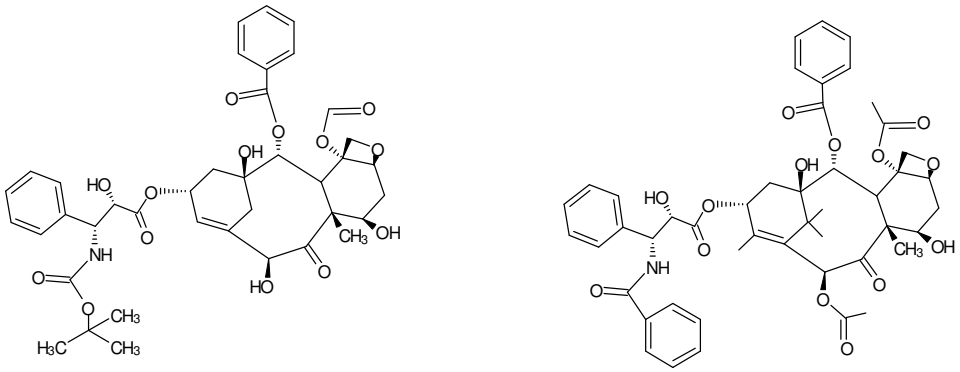
Anthracyclines may be administered to patients suffering from tumours of the head, neck, ovary, lung and breast. Anthracyclines are very cytotoxic agents and an accurate measurement of their circulating concentrations and metabolically transformed compounds is required by the clinic [7]. Often metabolites formed are active and toxic themselves. Anthracycline drugs are metabolised differently by person to person so their actual concentration has to be determined for everyone. Between 3.5–5.7% of administered doxorubicin, 11% of Epi, and 13–15% of Dnr are excreted unmetabolised via urine [12]. Doxorubicinol, the main metabolite of doxorubicin is 10 times more toxic that doxorubicin itself and is believed to cause the cardiotoxicity related to Dox[12]. With the development of increasingly more potent molecules the need for robust accurate and sensitive methods to quantify them is required so that their concentration levels in the body can be monitored. Many analytical methods have been described that are capable of monitoring the amount of anthracyclines present after administration.

As a consequence of the lack of new anthracyclines coming on stream into common clinical practice, the main interest area is actually in the determination of the concentration of the drugs in biological samples using fast and reliable methods[4]. It has been shown that the clinical efficacy of the anthracyclines is related to their actual

concentration in the tumour tissue. This parameter varies from patient to patient and should be evaluated for everyone individually.

1.2.3 Taxane chemotherapy

The taxanes are also widely used as chemotherapeutic agents. The taxane docetaxel (also known as Taxotere) and paclitaxel (also known as Taxol) are the best known of this class. The naturally occurring paclitaxel (Pac) was the first taxane to be isolated from the bark of the Pacific yew tree in the 1960s. It was approved for use in December 1992. Docetaxel (Doc), a semi synthetic analogue of Pac, was first synthesised starting from 10-deacetyl baccatin III, a non-toxic precursor found in the European yew (*Taxus baccata*) in 1986. Pac and Doc are both types of diterpene alkaloid plant extract. These drugs have contributed significantly to the treatment of a variety of malignancies, such as ovarian, breast, and non small cell lung cancers, as well as head and neck cancer and some cancers of the digestive system[13]. Taxanes are of relatively large molecular weight (>800) and non-polar in nature (See Figure 1.2).



Docetaxel (Mass 808) Paclitaxel (Mass 854)

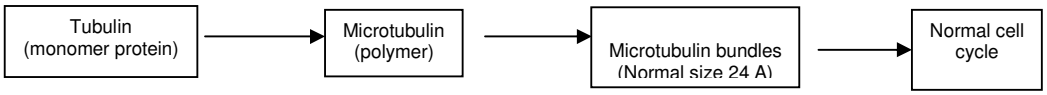
Figure 1.2: Structures of two main Taxanes

Taxanes are inhibitors of microtubule depolymerisation[14]. Microtubules are amongst the most strategic subcellular targets for anticancer agents and they are found in all eukaryotic cells. Microtubules are composed of tubulin dimers and there is a continuous dynamic equilibrium between the dimers and the microtubules. Any disruption of the equilibrium within the microtubule system disrupts cell division and the normal cellular activities in which microtubules are involved [15]. The taxanes promote the polymerisation of tubulin into stable microtubules and inhibit microtubule depolymerisation, thereby inducing the formation of stable microtubule bundles which in turn leads to cell apoptosis (see Figure 1.3). Once the microtubules are not broken down the cancer cells become so clogged with microtubules that they cannot grow and divide.

Microtubules consist of long, filamentous protein polymers having important functions in cellular activities such as, maintenance of cell shape, cellular movement, cell signaling, division and mitosis. These roles make microtubules a highly effective cancer target. Both Pac and Doc bind to the beta subunit of tubulin but the microtubules produced by Doc are larger than those produced by Pac. Doc binds more avidly to tubulin than Pac does and is retained intracellulary for longer. This could be

a possible explanation as to why Doc appears to be up to 2 or 4 times more potent than Pac in antitumour efficiency studies [16].

Normal Case:



In presence of Taxane:

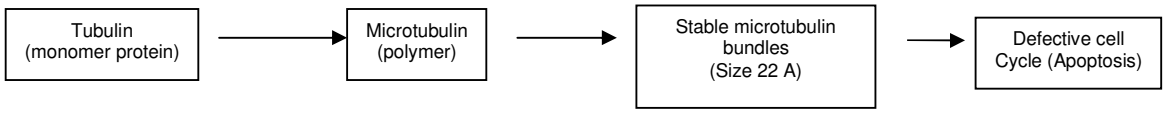


Figure 1.3: Effect of Taxanes on cancer cells

The side effects of taxanes include:

- Myelosupression
- Dose limiting neuteropenia
- Fluid retention
- Hypersensitivity reaction

Taxanes are lipophilic and very hydrophobic. A safe method of getting the taxanes into solution had to be found for intravenous injection or its chemotherapeutic effect would be of no use. Often taxanes are dissolved in an elixir of castor oil known as Cremophor EL. The castor oil is believed to cause many of the unwanted side-effects associated with taking Pac including nausea, vomiting, joint pain, appetite loss, brittle hair and tingling sensations in hands and feet (neuropathy) [17]. The observation of hypersensitivity reactions when taxanes are administered in early phase one trials led to a prolonging of infusion time to 6 or 24 hrs. These reactions may be attributed to the taxane itself or to the formulation vehicle. In later trials involving Pac the infusion time was reduced to 3 or 1 hours. Neuropathy and myalgia were the most significant side effects observed at this lower infusion time. Incidences of hypersensitivity did not differ significantly between short or long infusion times. Neutropenia is the major dose limiting factor when administering taxanes (85% of patients given 100 mg/m^2 of Doc and 75% of patients given 60 mg/m^2 of Doc). Patient blood count must be frequently monitored so that drug dosage can be altered.

Most commonly, Pac and Doc are administered intravenously as a single agent or in combination with other chemotherapeutic drugs depending on the type and stage of cancer in doses of between 75-135 and 75-100 mg/m^2 over a particular time scale. Patients treated with taxanes are often pre-medicated with a dose of corticosteroids to ease the side effects of hypersensitivity reactions and fluid retention [18].

Taxanes are highly protein bound (Pac 95%, Doc > 90%)[16]. Tissue distribution and binding influence the rate of plasma clearance. Pac plasma clearance follows non-linear kinetics; hence, the severity and duration of toxicity increase disproportionately with dose escalation. In contrast, Doc follows linear kinetics within the clinical dose range of (55 to 115 mg/m^2); its concentration changes linearly with changes in dosage[16].

Taxanes are metabolised primarily in the liver by cytochrome P450 enzymes[19]. These P450 enzymes metabolise Pac to different forms of hydroxypaclitaxel; 6α-hydroxypaclitaxel, and p-3'-hydroxypaclitaxel[20] (depending on the enzyme) which in turn is further oxidised to dihydroxypaclitaxel. Pac and its metabolites are excreted in the faeces, but studies have shown that only Pac is toxic, its metabolites are inactive [19].

As with the anthracyclines, much work is being put into developing novel formulations for drug release and into the determination of the concentration of these drugs in biological samples using fast and reliable methods.

1.2.4 Combination chemotherapy

For certain types of cancer, especially for cancers at an advanced stage, regimens containing multiple anti-cancer drugs are typically employed. For example, the combination of an anthracycline and a taxane (mainly Epi and Doc) is one of several standard treatments administered to patients suffering from advanced breast or stomach cancers [21], [22]. In recent years, the need has grown for an individualised patient dosage system as modern studies have suggested that an improved chemotherapeutic effect is observed (efficacy and morbidity) when chemotherapy treatment is coupled with an accurate measurement of the required drugs and/or their metabolites [22]. This is even more important when drugs are given in combination since there may be further interactions between them resulting in more metabolites [22] and they can also affect each other's action and metabolism. However, assays capable of determining different classes of drugs simultaneously are not common due to the difficulties encountered during analytical development in terms of extraction, separation and detection.

1.3 Analytical separation and extraction techniques

1.3.1 Separation techniques

There are a number of separation techniques which can be used for determination of drugs in biological matrices. A separation technique which offers certain advantages for bioanalysis is capillary electrophoresis (CE). Some of the best known are based on chromatography and include gas chromatography (GC) and liquid chromatography (LC).

Electrophoresis is based on the movement of electrically charged particles under the influence of an electric field. The separation is based on the different rates of migration of solutes. When an external electric field is applied to a solution of charged particles, each ion moves towards the electrode of opposite charge. Their individual rates of migration depend on their environment, their size and the strength of electric field.

In GC a sample is vaporised and injected onto an analytical column. The injected sample is then transported through the column by the flow of an inert gas acting as mobile phase. Commonly used carrier gases include nitrogen, argon carbon dioxide and helium. Columns are generally coated with a liquid stationary phase. Column temperature depends on the boiling point of the sample vaporised. There are

many different types of detector that can be used in conjunction with GC including flame ionisation, flame photometric, electron capture and thermal conductivity detectors. The type of detector used depends on the sample and the separation conditions.

By far the most commonly employed separation technique for bioanalysis is that of LC. LC, often called high performance liquid chromatography (HPLC), is an analytical process that separates a mixture into its individual components and often enables both their identification and quantitation. The basic components of any LC system are a pump/solvent delivery system, injector, analytical column (stationary phase), detector and a PC (see Figure 4). The mobile phase, which contains the sample, is pumped through the system and interacts with the stationary phase which is the 'heart' of the system. The column packed with stationary phase is where the separation occurs. The separated components are then 'seen' by the detector before flowing to waste or a fraction collector. There are many different types of detector that can be in LC including UV-Vis, fluorescence and mass spectroscopic detectors.

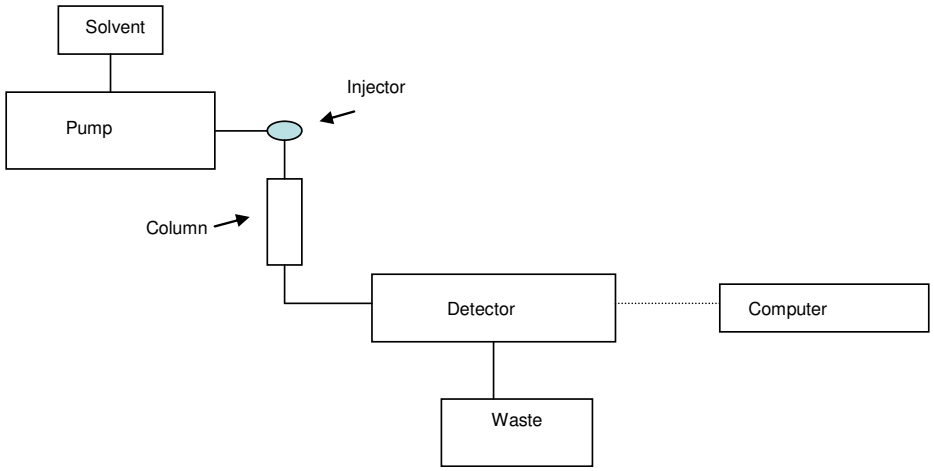


Figure 1.4: Schematic of a HPLC system

There are many different modes of LC including size exclusion, affinity chromatography, chiral chromatography, normal phase and reversed phase.

Size exclusion chromatography (SEC) is a useful technique for separating components with a significant difference in molecular weight. There are two types of SEC, gel filtration chromatography (GFC) and gel permeation chromatography (GPC). GFC uses an aqueous mobile phase and hydrophilic packings used to separate biological macromolecules. In GPC an organic mobile phase and hydrophobic stationary phase is used. One of the major roles of SEC is in the separation of polymers.

Affinity chromatography is based on the ability of biological molecules to recognise and bind to other molecules in a specific way. It takes advantage of a 'lock and key' binding model (see Figure 1.5). An affinity ligand e.g. an antibody, that is specific for a single type of biological molecule e.g. an antigen, is covalently bound to an inert support. A sample mixture containing the biomolecule of interest is applied to the column where only the biomolecule binds to the affinity ligand while the rest of

the sample flows through the column. Interactions between the ligand and target molecule can occur through hydrogen bonding, van der Waal's forces, electrostatic or hydrophobic interactions. The target molecule can be eluted simply by reversing the interaction between ligand and target molecule, by changing the pH or polarity or by introducing a competitive ligand. The technique is often used for protein purification and acts as a preconcentrating step also.

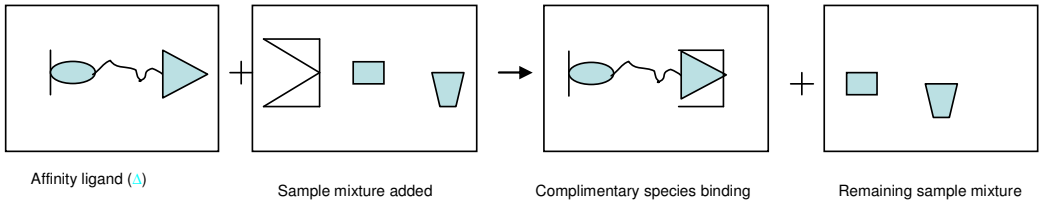
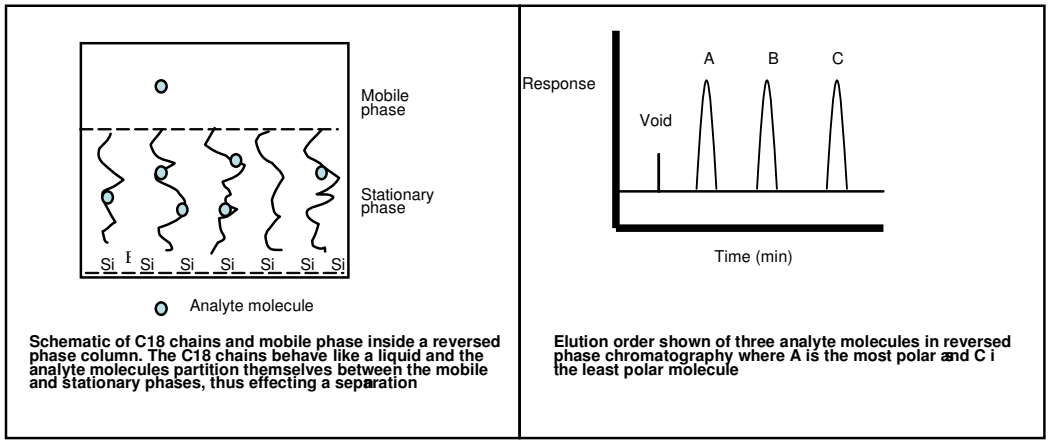


Figure 1.5: Affinity chromatography retention mechanism

In chiral chromatography enantiomers are separated based on their stereoselectivity. To separate enantiomers they must be changed to diastereomers. Enantiomers can be derivatised to diastereomers with a chiral selector. Chiral LC can be performed in three different ways. There is a pre-separation derivatisation approach which creates diastereomers before separation, a chiral stationary phase and an achiral mobile phase can be used or chiral selectors can be added to the mobile phase in conjunction with a regular stationary phase.

With normal phase chromatography the dominant mechanism at work is adsorption. It utilises a polar stationary phase and a non-polar mobile phase. This type of chromatography is used to separate very hydrophobic compounds which would have a very weak interaction on a highly polar stationary phase and hence be eluted quickly. Normal phase chromatography is best suited to the analysis of compounds that are soluble in non-polar solvents.

Reversed phase chromatography is the most commonly used due to its versatility and suitability for drug compounds. In the reversed phase mode of LC, the stationary phase is non-polar while the mobile phase is polar. Based on the interaction of 'like dissolves like', the more polar compounds will spend more time in the mobile phase and will flow through the system quickly, reaching the detector quickly. The less polar compounds will spend more time interacting with the stationary phase and will be retarded on their journey through the column, reaching the detector more slowly. This difference in solubility between the analytes in the mixture effects their separation. From the analytical point of view, judicious selection of stationary phase and correct choice of mobile phase composition and proportion will be the cornerstone of good method development. Silica is most commonly used as the base stationary phase in LC columns as it can cope with the high pressures involved, it is chemically inert, it is abundant and cheap and functional groups can be easily bonded to its surface creating a huge array of LC columns. The most popular columns for reverse phase LC are the bonded phases of C8 and C18, which are chains of eight and eighteen carbons length respectively. See Figure 1.6 for a schematic of reversed phase chromatography.



Schematic of C18 chains and mobile phase inside a reversed phase column. The C18 chains behave like a liquid and the analyte molecules partition themselves between the mobile and stationary phases, thus effecting a separation

Elution order shown of three analyte molecules in reversed phase chromatography where A is the most polar and C is the least polar molecule

Figure 1.6: Schematic of reversed phase chromatography

In LC, the mobile phase may be pumped through the column isocratically or by gradient elution. Isocratic pumping, the simpler of the two, involves the use of a single mobile phase being pumped through the system and the solvent composition remains constant throughout the analysis. Gradient elution, on the other hand, is useful if the solvent composition needs to change during a run. This might be required if there are a large number of compounds to separate, if peaks are not well resolved, from each other, or if the analytes of interest are structurally very different.

1.3.2 Detection techniques

The choice of detector used in LC is very important both qualitatively and quantitatively. The most readily available detector is the UV-Vis detector. Any beam of electromagnetic radiation passing through the detectors flow-cell will cause a change in its intensity. A UV-Vis detector uses light from the visible and UV regions of the electromagnetic spectrum. It has almost universal applicability, responding to any compound which absorbs radiation in the region of approximately 200-800nm. Absorbance of electromagnetic radiation in this range corresponds to the excitation of the relatively low energy electrons such as pi-electrons, or non-paired electrons of some functional groups. For example, any compound with a benzene ring will have an absorbance at around 210nm and 250nm. If a parent compound has a good response using this type of detector, it may also be very useful for the detection of metabolites, impurities and other related substances since the spectra of these compounds will be similar enough to be detected. Advantages of this detector include wide linear range, wide applicability and ease of use. Disadvantages include lack of sensitivity and selectivity.

Approximately 10-15% of compounds are thought to fluoresce and for these molecules, a fluorescence detector can be very useful offering low detection limits due to low background. In fact, laser induced fluorescence (LIF), an expensive version of this detector, is the most sensitive detection technique available. Disadvantages include narrow linear range and narrow applicability, meaning that even if it works well for a parent compound, it may not respond to its related substances.

A more recent detection tool for LC is the mass spectrometer (MS). MS measures the mass to charge (*m/z*) ratio of ions so any molecule which can be ionised will respond to this detector. A mass spectrometer must create charged particles (ions) from molecules entering from the LC instrument. It then examines those ions to obtain information about the molecular weight of the compound and its chemical structure. The principal components of a mass spectrometer are a sample introduction device (interface), an ionisation source, a mass analyser and an ion detector (see Figure 1.7).

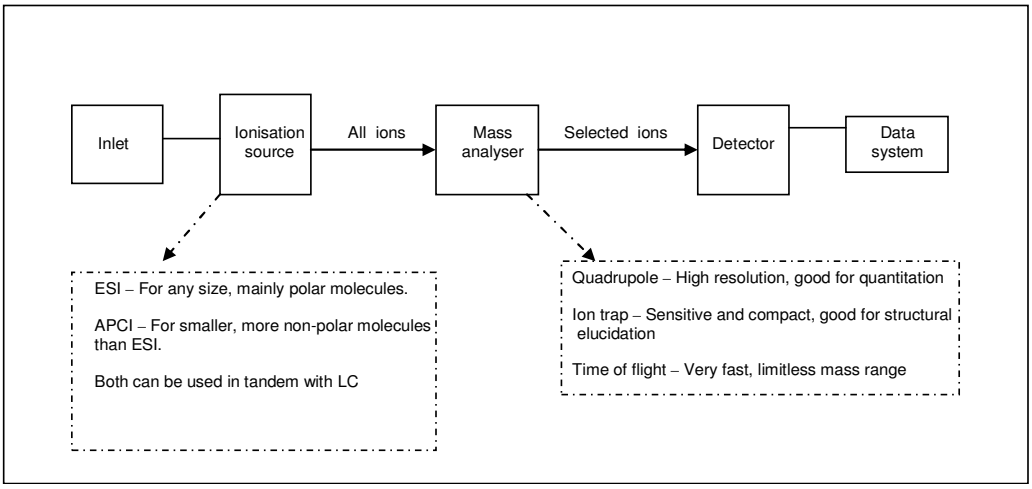


Figure 1.7: A schematic of the principle components of a MS

There are a number of types of ionisation sources and mass analysers available, depending on application. All LC-MS interfaces have to be able to evaporate liquids to gases, ionise neutral molecules to charged species and maintain the required vacuum for mass analysis.

The three main types of ionisation sources used prior to an LC instrument are electrospray ionisation (ESI), atmospheric pressure chemical ionisation (APCI) and matrix assisted laser desorption ionisation (MALDI). However MALDI is not very easily hyphenated to an LC system and it is generally used as an off-line detection system for LC.

ESI is one of the most exciting ionisation techniques to arrive. ESI generates ions directly from solution (usually an aqueous or aqueous/organic solvent system) by creating a fine spray of highly charged droplets in the presence of a strong electric field (typically 3.5 kV). As the droplet decreases in size, the electric charge density on its surface increases. The mutual repulsion between like charges on this surface becomes so great that it exceeds the forces of surface tension, and ions begin to leave the droplet. The ions are then electrostatically directed into the mass analyser by use of applying the opposite charge to the capillary which pulls the ions into the mass analyser. Vaporisation of these charged droplets results in the production of gaseous ions. As well as forming a gas from the liquid phase, the ESI chamber evaporates the LC mobile phase, both processes aiding the ionisation process. See Figure 1.8 below for a schematic of an ESI chamber.

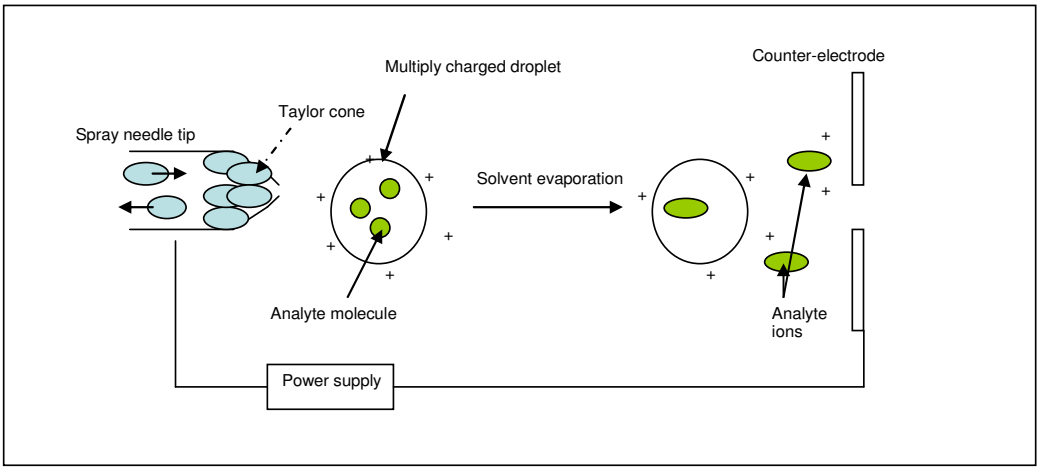


Figure 1.8: Schematic of an ESI chamber

The number of charges retained by an analyte can depend on such factors as the composition and pH of the electrosprayed solvent as well as the chemical nature of the sample. For small molecules (< 1000 Da) ESI typically generates singly charged ions, for medium sized molecules of 1000-2000 Da, ESI generates single or double charged ions while for large molecules (> 2000 Da) the ESI process typically gives rise to a series of multiply-charged species. Because mass spectrometers measure the mass-to-charge (*m/z*) ratio, the resultant ESI mass spectrum contains multiple peaks corresponding to the different charged states. Such spectra can be deconvoluted to yield a molecular weight even for large proteins and other biomolecules.

ESI allows for very sensitive analysis of small, large and labile molecules such as peptides, proteins, organometallics, oligosaccharides, and polymers. Another advantage of ESI-MS is that ions are formed directly from solution, a feature that has established the technique as a convenient mass detector for LC. While past attempts to couple liquid chromatography with mass spectrometry resulted in limited success, ESI has made the technique of LC-MS much more prevalent in analytical laboratories and research centres.

APCI is another very commonly used ionisation technique. In APCI the eluent is introduced to the interface using a capillary of a similar design of that used in ESI. In APCI the emerging liquid from the capillary is surrounded by a heated inert nebulising gas instead of a potential applied to the capillary like in ESI. The nebulising gas and heat form an aerosol that begins to evaporate. There is a pin placed in the heated region with a high potential applied to it that produces an electrical discharge that can ionise solvent molecules. Ionised gas plasma is formed by a combination of collisions and charge transfer processes. Molecules eluting into this gas plasma can become ionised by proton transfer. The rate of evaporation required to ensure that all species are in the gas phase as they reach the discharge pin is determined by the rate of flow of eluent into the heated region. Stable droplet formation is required to ensure that a stable plasma of ionised solvent molecules exists around the discharge pin so that the same discharge conditions affect all analytes within a sample. High flow rates are used in APCI (over 1.0 mL/min) because at lower flow rates an unstable analyte signal is observed due to the instability of the gas plasma by a non-reproducible discharge process. See Figure 1.9 for a schematic representation of the APCI process.

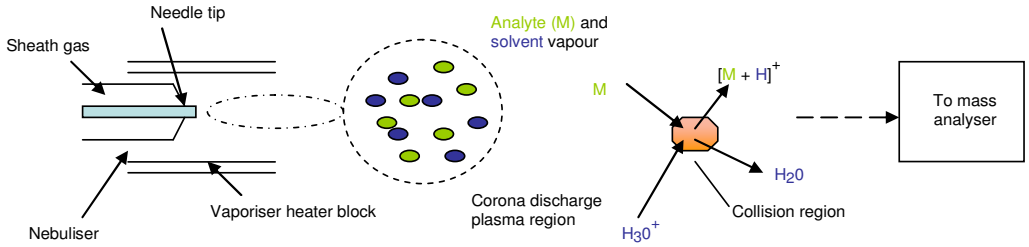


Figure 1.9: Schematic of APCI ionisation

Another type of ionisation technique is MALDI where a special matrix which is easily energised by a laser beam is mixed with a sample of interest on a target spot. The energised matrix molecules in turn ionise the sample molecules they are mixed with and the ionised analytes of interest pass into the mass analyser. MALDI has had its biggest impact on the field of protein research. The ability to generate MALDI-MS data on whole proteins and proteolytic fragments is extremely useful for protein identification and characterisation.

MALDI-ESI can be coupled to nanoscale capillary LC separations. It can be coupled as LC-ESI-MS/MS for more expansive protein characterisation by performing a post column flow split, with an aliquot going to the ESI source and an aliquot spotted onto MALDI targets off-line. This has been done by Bodnar *et al* [23] to evaluate the improvement of proteome coverage of protein mixtures when both MALDI and ESI are combined.

Depending on the information required from the ionised analytes, there are a number of types of mass analysers. For structural information, an ion trap would probably be the analyser of choice. An ion trap is a small chamber that uses RF and DC voltage to 'trap' ions. Using the electric field ions can be stored, isolated, fragmented, and then scanned out of the device to create a mass spectrum. Ion traps can carry out tandem mass spectrometric steps (MS^n) but the number of successive fragmentation steps performed can be limited by the sensitivity and software of the MS. Some ion traps can perform up to MS^{12}. This can be extremely useful for structural elucidation of unknown or novel compounds by sequential MS/MS experiments.

For accurate molecular weight information of large molecules, a time-of-flight (TOF) analyser would probably be chosen. This type of mass analyser is mostly associated with ESI ionisation. TOF mass analysers measure the mass-to-charge ratio based on the length of time it takes for ions to reach the detector (their time of flight) in a tube. TOF analysers can be either linear or reflector based on the ions flight path. TOFs provide a full scan MS spectrum and high mass accuracy and resolution. TOF/TOF instruments are now available.

For quantitative information, a quadrupole analyser would normally be favoured. The triple quadrupole is a tandem mass analyser. It consists of three quadrupoles in series. The first functions as a mass filter, the second as a collision cell and the third as a mass analyser for the chosen fragment ions. By altering the voltage and current, the instrument is tuned to allow only ions of a certain mass-to-charge ratio to travel through the first quadrupole. The second quadrupole acts as a holding

chamber in which a single tandem mass spectrometric experiment can be carried out. The final quadrupole is tuned to allow only the daughter ion of choice to reach the detector. Triple quadrupoles are used for determining the absolute amount of an ion in a sample and can be extremely sensitive.

Advantages of the MS detector include sensitivity and almost universal applicability. Disadvantages include the fact that the MS is a highly technical instrument in its own right so an increased level of skill is required to operate this detector with its many types of ionisation techniques and mass analysers, and ever-increasing array of hybrid instruments.

1.3.3 Sample preparation techniques

Before a biological sample can be analysed by LC it must first be 'cleaned up'. This requires removing the drug to be analysed from the complex matrix it is mixed with the best recovery possible. There are many different sample preparation/extraction techniques available including protein precipitation (PP), liquid-liquid extraction (LLE) and solid phase extraction (SPE).

PP is a well established type of extraction procedure. In essence, a solvent or strong acid or base are added to the biological sample which causes biological material, in particular proteins, to 'crash out' of solution creating a pellet. Following centrifugation, the supernatant, which should contain the majority of the analyte of interest, can be removed for analysis. Protein solubility in aqueous buffers depends on the nature of the polar and non-polar amino acids that make up the protein. A protein with a high content of hydrophobic amino acids on its surface will not be very soluble in an aqueous solution. Proteins feel both attractive and repulsive electrostatic processes depending on the environment they are in. PP then occurs by the addition of a precipitation agent (for e.g. gel beads in a gel filtration technique) to the protein solution. Disadvantages of PP include the fact that the extracts can still contain matrix components and also that the supernatant can continue to form a precipitate on standing.

LLE is an extraction method that is based on mixing two immiscible liquids. The liquid-liquid dispersion created by mixing is then separated by gravity or by centrifugal force [24]. For biological samples, which are aqueous based, generally an organic solvent is added and the mixture shaken. The drug of interest is extracted from the aqueous phase into the organic phase in which it has preferential solubility, leaving behind the proteins and lipids etc. One of the major disadvantages of LLE is that it is difficult to automate and organic solvent consumption tends to be high. Evaporation to dryness and reconstitution are also normally required (see Figure 1.10).

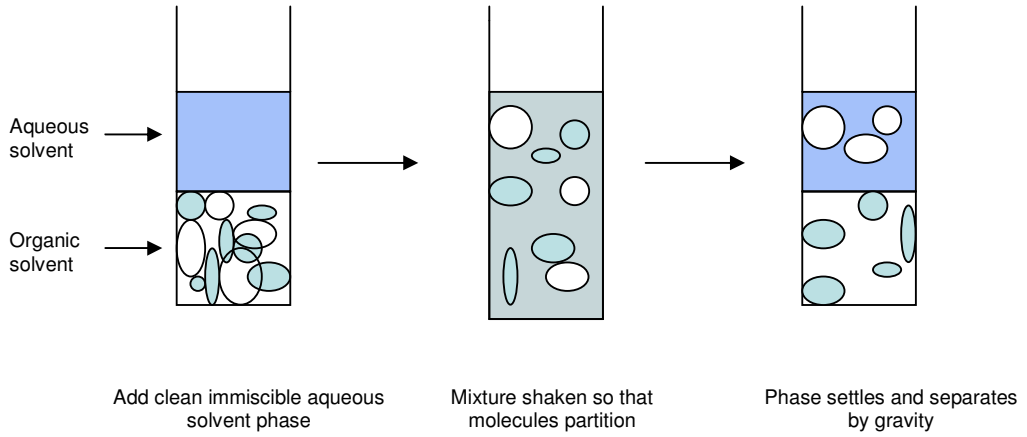


Figure 1.10: Schematic showing the main steps in an LLE procedure

SPE is one of the most common and least expensive extraction techniques. SPE uses a solid and a liquid phase to isolate the analyte of interest from a biosample. The general procedure of SPE involves four steps: conditioning, loading, washing and eluting (see Figure 1.11). The SPE cartridge or disc is conditioned with solvent (wetted) and then the sample is loaded onto the phase and allowed to pass through. If the phase has been correctly chosen for the analyte, the analyte will be retained by the phase while the rest of the biological matrix will wash through to waste. A solvent with an affinity for the analyte is then passed through the cartridge to elute the analyte in as small a volume as possible. One of the major advantages of SPE is that it can be automated which greatly reduces analysis time and increases throughput. The above three extraction procedures are usually carried out off-line, which involve a number of manual and sometimes labour-intensive steps. Each step can introduce errors and so precision can be poor. Of the three processes, SPE does lend itself to automation and hence on-line SPE methods have been reported in the literature. On-line approaches to sample clean-up offer enormous advantages of speed, improved precision, higher throughput of samples and unattended operation. Complex biofluids such as serum or plasma can be directly injected onto an on-line SPE column which is connected to the LC column. When ready, the extract is swept onto the LC column and the mixture separated and detected as normal. The same principles as off-line SPE are used except that instead of using an SPE cartridge (generally single use), an SPE column is used which allows repeated injections and larger volumes of biosample can be accepted. On-line methods offer higher percentage recoveries, quicker analysis time, reduced solvent consumption and no sample pre-treatment is required [25]. However, extensive method development is required since there are many experimental variables to be investigated.

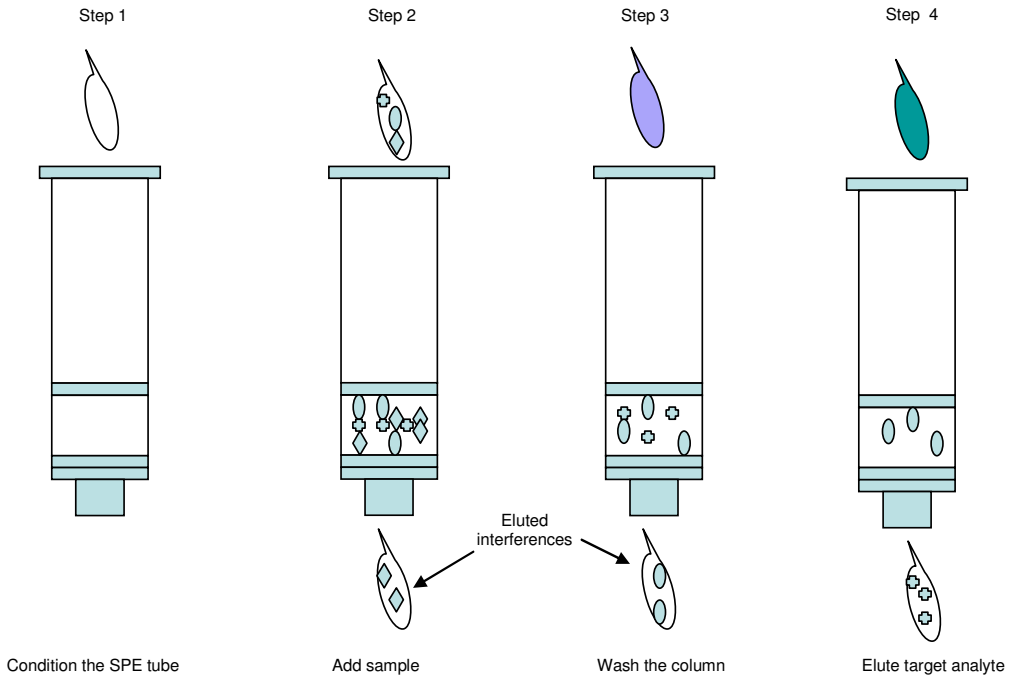


Figure 1.11: Schematic showing the four main steps in an SPE procedure

1.4 Validation of analytical methods

Once any analytical method has been developed, it is important to validate it according to recognised protocols to ensure that the specific test is suitable for its intended use. Reliable analytical results are required to comply with international regulations and to ensure patient safety. FDA regulations require that an analytical method be validated before and during regular routine use. While there are many guidelines for validation of analytical assays such as those published by the FDA, Institute of Validation, EU...etc, [26, 27] the most relevant protocols for bioanalytical work are those laid down at the Washington Conference of 1990 by Shah *et al* [28]. Authors prioritise different parameters during a validation but all would agree that the following are all important: precision, accuracy, sensitivity, linearity and range, recovery and stability. In this thesis, it was decided to follow the guidelines set out by Shah *et a*l [28] as this paper is highly regarded and deals with the validation of analytical methods. This included intra-day precision (repeatability) and inter-day precision (intermediate precision), accuracy, sensitivity, limits of detection and quantitation (LOD and LOQ), linearity and range, recovery and stability to freeze-thaw cycles. Each of these parameters is briefly described below.

1.4.1 Precision

Precision is the closeness of agreement between obtained results. It is sometimes described as the degree of scatter around the mean. It is expressed as a percentage of the relative standard deviation (%RSD) of a set of results. Precision in this thesis was evaluated by analysing intra-day and inter-day results.

1.4.2 Accuracy

Accuracy is a measure of the closeness of the result to true value. It is measured by comparing the calculated concentration to the true (actual) concentration and expressing the difference between them as a percentage error.

1.4.3 Sensitivity

Sensitivity was evaluated by measuring the limit of detection (LOD) and the limit of quantitation (LOQ). The LOD in this work was defined as the analyte concentration that gave a signal to noise (S/N) ratio of 3:1. The LOQ was defined as the analyte concentration that gave a S/N ratio of 5:1. The LOD and LOQ values were obtained by measuring the signal-to-noise ratio at and nearby where a target analyte eluted and by measuring the concentration of that analyte which was either three or five times that baseline noise

1.4.4 Linearity and range

It is important to assess the useful analytical range of the method. A wider range means that more samples can be measured directly and that fewer samples will require dilution or pre-concentration in order to bring their concentrations within the limits of the calibration curve. Linearity and range is determined by preparing a calibration curve and determining its regression coefficient. The calibration curve of an analytical method is, within the range, a monotonic relationship between the analytical signal (response) and the concentration of an analyte [29].

1.4.5 Recovery

Recovery is a useful way to assess how efficient an extraction procedure is. It is universally accepted that recovery values will be lower from biological matrices than from other cleaner matrices. Certainly, drugs often strongly attach to proteins and are therefore lost to some extent during the sample pretreatment steps due to this protein binding phenomenon. However, the closer the recovery value is to 100%, the better the sensitivity will be. Recovery values have to be reproducible to prove that they are accurate.

1.4.6 Stability

Storage conditions can have a big effect on sample integrity. For biofluids, the usual method of storage is freezing and so the ability of the samples to remain stable to freeze-thaw cycles was investigated.

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Chapter 2

Literature review of current methods to quantify anthracyclines and taxanes

2.1 Physiochemical properties

2.1.1 Anthracyclines

Anthracyclines are polar, slightly basic chemotherapeutic drugs. The anthracycline ring is lipophilic but there are abundant hydroxyl groups on the ring so that they are readily dissolved in water. Their molecular weights are 543, 543 and 527 Da respectively for doxorubicin (Dox), epirubicin (Epi) and daunorubicin (Dnr). They have a pKa of between 7.5 and 8.5. Anthracyclines are highly protein bound (>80%).

2.1.2 Taxanes

Taxanes are also relatively low molecular weight cytotoxic chemotherapeutic agents - 854 and 808 Da for paclitaxel (Pac) and doetaxel (Doc) respectively. Taxanes are neutral to slightly basic agents and are lipophilic in nature. They are mainly soluble only in organic solvents. Taxanes are also highly protein bound (Pac 95%, Doc > 90%).

2.2 Sample extraction/clean-up procedures

2.2.1 Anthracyclines

There are three main sample extraction techniques that are usually employed to extract anthracyclines from biological fluids. They are liquid-liquid extraction (LLE), protein precipitation (PP) and solid-phase extraction (SPE). Many examples of these are listed in Table 2.1.

Wall *et al* [1] used LLE to extract Epi and its metabolites from serum. Epi and the internal standard (IS), in this case Dnr, were dissolved in human serum and water with ice-cold isopropanol with ammonium formate buffer for pH control. Once this mixture had been mixed, chloroform was added and the solution was centrifuged so that the layers could be separated. The organic layer containing the drugs was evaporated to dryness and then reconstituted in mobile phase prior to analysis. Recoveries of Epi from this extraction procedure were in the range of 97-122%.

Van Asperen *et al* [2] also used an LLE procedure in the clean-up of Dox and metabolites in murine specimens. In their LLE procedure Dox and Dnr (IS) were added to diluted plasma and a borate buffer for pH control. Analytes were extracted with chloroform/1-propanol (4:1 v/v) by mixing followed by centrifugation. The organic layer containing the drugs was evaporated to dryness and the residue was then reconstituted in acetonitrile/tetrahydrofuran (40:1 v/v) with acidified water prior to HPLC analysis. Recoveries with this method were poorer, ranging from 66-77%.

Based on a review of some papers that used LLE as a method of extraction for anthracyclines from biofluids this type of extraction technique can be time consuming with different recovery values being obtained. A lot of time may be required in the laborious sample preparation stages of an LLE method. Generally, LLE is used for measuring cellular levels, but it's not ideal for serum. There is no easy way that an LLE extraction method can be automated. In some cases, copious amounts of organic solvents are consumed. Judging by the literature, LLE is not used very frequently as an extraction technique for anthracyclines. It may be better in terms of analysis time

and recovery values to avoid using an LLE extraction protocol for isolation of anthracyclines from biological matrices.

Yang *et al* [3] developed a PP protocol prior to the determination of Dnr in rat plasma. A methanol/acetone (1:1 v/v) PP step was developed using 70% (w/v) zinc sulphate. Quality control (QC) samples were added to thawed rat plasma with doxorubicinol (IS). The zinc sulphate and methanol/acetone mix were added and the mixture vortexed and centrifuged. The supernatant was transferred to a 96 well plate where it was evaporated to dryness and the reconstituted in acetonitrile/water/formic acid (25:75:0.1 v/v/v) prior to LC-MS analysis. Recovery of Dnr after this PP step was about 93%. Yang reports that some PP steps can render a sample un-clean for mass spectroscopic analysis (in this case, the methanol/zinc sulphate washing step) because of severe $ZnSO_4$ contamination. It has also been reported that some PP washes do not precipitate proteins fully.

Zhou *et al* [4] also employed PP for the determination of Dox in rat serum and bile. In this method a small aliquot of the biofluid was pipetted into a polypropylene eppendorf with Dnr (IS) and some ice-cold methanol. The tube was then mixed and centrifuged before the supernatant was transferred to an autosampler vial and analysed by LC. Zhou *et al* achieved recoveries of >89% with this PP step for Dox.

PP seems to be quite an efficient extraction technique in terms of recovery for the anthracyclines. Anthracycline recovery can be very high based on the papers discussed in Table 2.1 below. The major drawbacks with PP as an extraction technique are the extraction time, solvent consumption and that it is not possible to automate the process. While high recovery values for anthracyclines make PP an attractive possibility, it may be difficult to find a set of precipitating conditions for both anthracyclines and taxanes together.

SPE has been the most popular method for anthracyclines to date. Mahnik *et al* [5] developed an off-line SPE procedure for the determination of these agents in hospital effluent. Hospital effluent and sewage water were spiked with Dox, Epi and Dnr in DMSO/0.9% physiological NaCl solution (1:1 v/v) to obtain samples of known concentrations. A C8 SPE cartridge was preconditioned with 5 mL of methanol, water and phosphate buffer solution (PBS) containing 2% bovine serum albumin (BSA). The samples were then applied to the cartridges (flow rate of about 2 mL/min). The cartridge was then washed with 5 mL water with any residual water washed with n-hexane. The anthracyclines could then be eluted with MeOH/$CHCL_3$ (1:2 v/v). The solvent was then evaporated to dryness and the eluate reconstituted in mobile phase and injected for analysis. Recovery values for all anthracyclines were >80% in the concentration range of 0.1 to 1.4 µg/mL and 0.1 to 0.5 µg/mL for Epi and Dox respectively. Using off-line SPE their extraction protocol is estimated to have taken about 50 min, a similar time to LLE and slightly quicker than with PP.

Sottani *et al* [6] developed a different off-line SPE method prior to LC-MS-MS analysis of Dox, Epi, Dnr and idarubicin in human urine with epi-daunorubicin as an IS. A C18 SPE cartridge was used as it was found to be the best sorbent in previous work. The C18 cartridge was conditioned with MeOH and equilibrated with deionised water. An aliquot of a urine and PBS solution was then passed through the cartridge at a flow rate of 0.05 mL/min with PBS. The cartridge was then vacuum dried for 20 min before eluting the analytes with a 2-propanol/methanol chloride (50:50 v/v) mixture. This eluted solution was then dried and reconstituted in mobile phase before analysis. Recoveries for the anthracyclines ranged from 79-102% with this off-line SPE procedure.

Off-line SPE seems to be the most commonly used extraction technique in the analysis of anthracyclines. It is slightly quicker than both LLE and PP with similar, if not higher, recoveries than the alternative extraction methods. There is less solvent consumption and it can be semi- or fully automated.

All three clean-up procedures offer good recovery values but each of these processes is time consuming, especially when evaporation to dryness and reconstitution is required. Overall sample analysis time can be greatly reduced and precision improved if the extraction step is automated. LLE and PP cannot really be automated but SPE can.

Rudolphi *et al* [7] developed an on-line SPE approach to sample clean-up using an alkyl-diol silica (ADS) SPE column. The porous ADS was specifically designed for the repeated injection of biological samples, eliminating biological proteins, macromolecules, lipids, etc. and retaining the drugs of interest. Rudolphi used a C4 ADS SPE column in an automated column switching LC system for the analysis of Epi in biological matrices. The ADS precolumn (25 x 4 mm i.d.) had bimodal chromatographic properties. The external (particle) bonded phase was hydrophilic and was non-adsorptive towards proteins. The alkyl chains on the internal surface were hydrophobic and allowed for RP chromatographic interactions. The sample aliquot (Epi in plasma) was directly injected onto the ADS by the extraction pump delivering extraction mobile phase of water/methanol (95:5 v/v) at a flow rate of 1.0 mL/min. The column was left in the 'extraction position' for 10 min before the valve was switched and an analytical mobile phase back-flushed the ADS column for 5 min forcing the retained anthracycline off the ADS column onto the analytical column where it was separated and detected by fluorescence. Recoveries of Epi ranged from 98-106% with this on-line SPE method and extraction time was only 10 min.

Despite all the advantages of using on-line SPE such as the potential for high sample throughput, improved recoveries, better precision, reduced solvent consumption, Rudolphi's paper was the only one found for the anthracyclines. This is probably a reflection on the increased challenges of developing such a method. However, once optimised, the benefits outweigh the increased development effort.

See Table 2.1 below for a summary of extraction techniques used for anthracyclines in biofluids from the past fifteen years.

Table 1: Reported extraction and LC detection techniques for anthracyclines from biological samples.

Year	Reference	Anthracyclines	Extraction Mode	On- or off-line extraction	Extraction Recoveries (%)	Approx Extraction time (min)	Detection
1995	[7]	Epi + Met	SPE	On-line	98-106	10	Fluorescence
1996	[8]	Epi + Met	PP	Off-line	94-104	>45	Fluorescence
1998	[9]	Dox, Epi + Met	SPE	Off-line	89-93	n/a	Electrochemical
1998	[2]	Dox + Met	LLE	Off-line	64-77	>40	Fluorescence
1999	[10]	Dox + Met	PP	Off-line	112 ± 6	90	Fluorescence
2000	[11]	Dox, Epi, Dnr, Idarubicin + Met	SPE	Off-line	71-105	>45	MS
		Dox + Met					
2002	[4]	Dox	PP	Off-line	>89	>45	Fluorescence
2003	[12]	Dox + Met	PP	Off-line	n/a	>60	Fluorescence
2004	[13]	Dox, Epi, Dnr, Idarubicin	SPE	Off-line	84-112	>20	MS/MS
2004	[6]	Dox, Epi, Dnr	SPE	Off-line	79-102	>50	MS/MS
		Epi + Met					
2006	[5]	Dox, Epi + Dnr	SPE	Off-line	>80	>45	Fluorescence
2006	[1]	Epi + Met	LLE	Off-line	97-122	>45	MS/MS
2007	[3]	Dnr	PP	Off-line	93	n/a	MS/MS

Key: SPE =Solid phase extraction, PP = Protein precipitation, LLE = Liquid liquid extraction, Met = Metabolites

2.2.2 Taxanes

SPE and LLE are the two most commonly used sample clean-up techniques in the analysis of taxane drug molecules. PP has also been used as an extraction technique in taxane analysis but examples of PP sample clean-up in the literature are few and far between relative to SPE and LLE.

Vainchtein *et al* [14] developed an assay for the determination of Pac and its metabolites in human plasma using LLE as the extraction technique. Samples were prepared in methanol with subsequent addition of tert-butylmethylether. The samples were then vortexed, shaken and centrifuged before separation of the organic and aqueous layers. The aqueous layer was frozen in an ethanol/dry ice mixture. The organic layer was poured into 1.5mL eppendorf tubes. The organic solvent was then evaporated under nitrogen gas at 40 ^{0}C and the residue reconstituted in 100 µL mobile phase, 0.1M ammonium acetate/acetonitrile (1:1 v/v). The sample was then further cleaned by vortexing and centrifuging before 25 µL of clean supernatant was injected onto an LC-MS system. Recoveries ranged from 85-89% with this LLE extraction procedure for Pac.

Mortier *et al* [15] developed an assay for both Doc and Pac determination in human plasma and saliva using LLE extraction. Sample preparation was slightly different depending on the sample matrix (plasma or saliva). For plasma sample clean-up an aliquot of sample was dissolved in a water/plasma mixture (2:1 v/v). LLE was performed with tert-butyl ether by placing on a rotary device and centrifuging. The organic layer was evaporated to dryness under nitrogen at 30 ^{0}C and the residue redissolved in mobile phase, water/methanol/acetic acid (50:50:0.1 v/v/v) before an aliquot was injected for analysis by LC-MS/MS. Analysis of the saliva samples was complicated further by the fact that a saliva collection device had to be centrifuged, washed and swab centrifuged to obtain the oral fluid before the procedure outlined above for plasma could be implemented. Recoveries ranged from 70-101% for Doc and Pac in this method.

Recovery values of taxanes from biofluids are relatively high in the above two methods and in many of the LLE methods listed in Table 2.2 due to the lipophilicity of these drugs. However, performing the sample clean-up takes up to an hour or more and quite a large volume of solvent is consumed in the extraction process. Much time is lost mixing, shaking, vortexing, centrifuging and drying the samples.

Hou *et al* [16] developed a PP method for the determination of Doc in mouse plasma by LC-MS using Pac as IS. A small aliquot of plasma (40 µl) and 10 µl of IS were added to a micro-centrifuge tube. This mixture was vortexed before 100 µl of the methanol/acetonitrile mix was added. The mixture was then vortexed and centrifuged before the supernatant was transferred to vials for LC-MS analysis. Recoveries of between 91-100% were obtained for Doc using this simple PP extraction step. This PP process was quite efficient taking about 15 min to perform. However, throughput is low and it does not lend itself to automation. Also, finding suitable solvents for extracting both taxanes and anthracyclines would be difficult.

Andersen *et al* [17] developed an off-line SPE assay for the determination of Doc and Pac in plasma. SPE was performed on a column containing 100mg of cyano (CN) packing material with a 10 mL reservoir capacity (LRC-SPE columns, Varian). SPE columns were conditioned with 10 mL methanol and ammonium acetate (pH 5.0) buffer solution. Once conditioned the plasma samples could then be loaded onto the column. The plasma samples were then washed with ammonium acetate buffer solutions before being vacuum dried and hexane washed before drying again. Taxanes

were eluted with two washes of ethyl acetate before the samples were evaporated to dryness and then dissolved in a 40% acetonitrile solution. Samples could then be injected and analysed by LC-UV. Recoveries ranged from 76-118% for both taxanes with this off-line SPE method.

Suno *et al* [18] worked on an off-line SPE-LC-UV method in an assay to determine Pac in plasma with Doc as the IS. A Sep-Pak C18 SPE cartridge was chosen as the SPE sorbent. This cartridge was preconditioned with 10 mL methanol and 10 mL of 20 mM ammonium acetate buffer pH 5.0 before the taxane sample (prepared in methanol and added to plasma diluted with the ammonium acetate buffer) was applied. The C18 cartridge was then rinsed with ammonium acetate buffer, a diluted methanol buffer and hexane before the cartridge was vacuum dried. The taxanes were eluted with acetonitrile and the solvent evaporated under dry nitrogen at 50 ^{0}C. The residue was then re-dissolved in mobile phase before an aliquot was injected and analysed by LC-UV. Recoveries obtained from the C18 SPE sorbent are >90%.

The off-line SPE procedures used by Suno and Anderson *et al* are very similar. One noticeable difference is in the choice of SPE sorbent. Suno *et al* achieve recoveries of over 90% whereas Anderson obtains values in the range from 76-118%, perhaps because of the CN SPE sorbent. They were published relatively recently (2006 and 2007 respectively) and represent the most up to date off-line SPE methods for extraction of taxanes from biological matrices prior to the start of this project.

Recovery of taxane drugs from biological matrices using SPE in the above two methods is comparable to that when LLE is used. Sample extraction time is slightly quicker with an SPE extraction and less solvent is used. The main advantage of SPE, as discussed earlier, is that it can be automated.

One on-line SPE method has also been developed for the determination of taxanes from biological fluids. Grozav *et al* [19] published an assay for the determination of Doc and Pac (IS) in human plasma using on-line SPE prior to LC-MS analysis. An Oasis HLB (2.1 mm × 20 mm) extraction column was used for sample clean-up. The packing material was poly(divinylbenzene-co-*N*-vinylpyrrolidone), a macroporous copolymer, which can retain both hydrophilic and hydrophobic compounds under various conditions. On-line SPE was performed by column switching using a 2-position 6-port automated switching valve. The SPE column was first equilibrated for 1.0 min with the extraction solvent (water). Then an aliquot of the taxane in plasma sample was injected via an autosampler. It was carried to the extraction column by the extraction solvent where the plasma proteins were washed to waste leaving the taxanes retained on the SPE column. The valve was then switched and the elution mobile phase, methanol/water (95:5 v/v) back-flushed the drugs off the SPE column onto the head of the analytical column where separation could occur. The SPE column was reconditioned with the extraction mobile phase prior to the next injection.

The flow rates of the analytical and extraction mobile phases were considerably different in this protocol. During the extraction stage, a flow rate of 2.0 mL/min was used while the analytical flow rate was set to 0.25 mL/min. Total run time from injection to detection for this on-line taxane assay was just 7.0 min. Recoveries were in the range of 86 to 95% for Doc. This would suggest that on-line SPE is an efficient extraction technique for taxanes in terms of recovery, speed and sample throughput.

See Table 2.2 below for a summary of extraction techniques for taxanes from recently published journals.

Table 2.2: Reported extraction and LC detection techniques for taxanes from biological samples.

Year	Reference	Taxanes	Extraction Mode	On- or off-line extraction	Extraction Recoveries (%)	Approx Extraction Time (min)	Detection
1998	[20]	Doc	SPE	On-line	92	>30	UV
1998	[21]	Pac + Met	LLE + SPE	Off-line	76-85	>100	UV
2000	[22]	Doc	SPE	Off-line	99 ± 2	>30	UV
2003	[23]	Pac	SPE	Off-line	83-98	30	MS
2003	[24]	Doc, Pac	SPE	Off-line	96-111	>60	MS
2003	[25]	Doc	LLE	Off-line	93± 8	>25	MS/MS
2003	[26]	Pac + Met	LLE	Off-line	92-105	>90	MS/MS
2004	[19]	Doc	SPE	On-line	86-95	1.4	MS/MS
2004	[27]	Doc	PP	Off-line	91-100	15	MS/MS
2005	[15]	Doc, Pac + Met	LLE	Off-line	70-101	>60	MS/MS
2006	[17]	Doc, Pac	SPE	Off-line	76-118	>50	UV
2006	[14]	Pac + Met	LLE	Off-line	85-89	>60	MS/MS
2007	[18]	Pac + Doc	SPE	Off-line	>90	>45	UV
2008	[28]	Pac	SPE	Off-line	n/a	>45	MS/MS

Key: SPE =Solid phase extraction, PP = Protein precipitation, LLE = Liquid liquid extraction, Met = Metabolites

Sample extraction by SPE took only 1.5 min in the method published by Grozav in 2004 for taxane analysis whereas SPE took about 10 min in the paper published by Rudolphi in 1995 for anthracycline clean-up. It would appear that an on-line SPE approach for the extraction of both anthracyclines and taxanes would be approapriate. No method has ever been published that can extract or separate both drug classes in a single assay, hence it was the aim of this project.

2.3 Sample separation and detection approaches

2.3.1 Anthracyclines

Many different types of separation and detection methods have been applied to the determination of anthracyclines. Chromatographic variables include the dimensions and type of analytical column used, the mobile phase selection, flow rates etc. The detection mode also varies from author to author, with the majority employing MS or utilising the natural fluorescence of the anthracyclines.

Mass spectrometry is a modern detection technique which is becoming more accessible to analysts as its cost decreases. It is a more popular choice of detector than UV because of its specificity and range and its detection limits are far greater. It has been applied to the detection of anthracyclines by Arnold [13], Sottani [6] and Yang [3] with good sensitivity.

Wall *et al* [1] reported an LC-MS method for the determination of Epi in serum. Following extraction, chromatography was performed on a C18 column. A mixture of acetonitrile/ water/formic acid (72:28:0.1 v/v/v) at 0.2 mL/min was used for separation. Epi was analysed by atmospheric pressure chemical ionization (APCI) MS and data collected in selected reaction monitoring (SRM) mode and over the scan range *m/z* 200–700. The breakdown patterns of the Epi and Dnr (IS) adducts were from 544 → 397 [$M+H^+$] and from 528 → 363 [$M+H^+$] respectively. Even though a mass spectrometer was used for detection, a photodiode array (PDA) was also interfaced to the LC-MS system to monitor the UV signal of Epi at 254 nm as it eluted. As anthracyclines are weakly basic drugs with pKa values in the range of 7.5-9.0, in order to maximise stability, ensure protonation and ionisation, improve resolution and control peak tailing, a mobile phase with an acidic pH was required. Mobile phase pH values between 3.0 and 4.5 were tested by Wall *et al* with a pH of 3.2 delivering the optimal results for all anthracyclines in terms of selectivity.

Both APCI and electrospray ionisation (ESI) were tested and it was found that APCI was four and seven times more sensitive than ESI for Dnr and Epi respectively. Dox was also tested in this research. Dox and Epi are epimers of each other so their analysis by SRM MS may be difficult unless they have different fragmentation patterns. It was found that while both Dox and Epi have a fragmentation from 544 → 397 [$M+H^+$], Epi also fragments from 544 → 526 [$M+H^+$] whereas Dox does not. See Figure 2.1 below (taken from Wall *et al* [1]) for the fragmentation patterns of the epimers Dox and Epi.

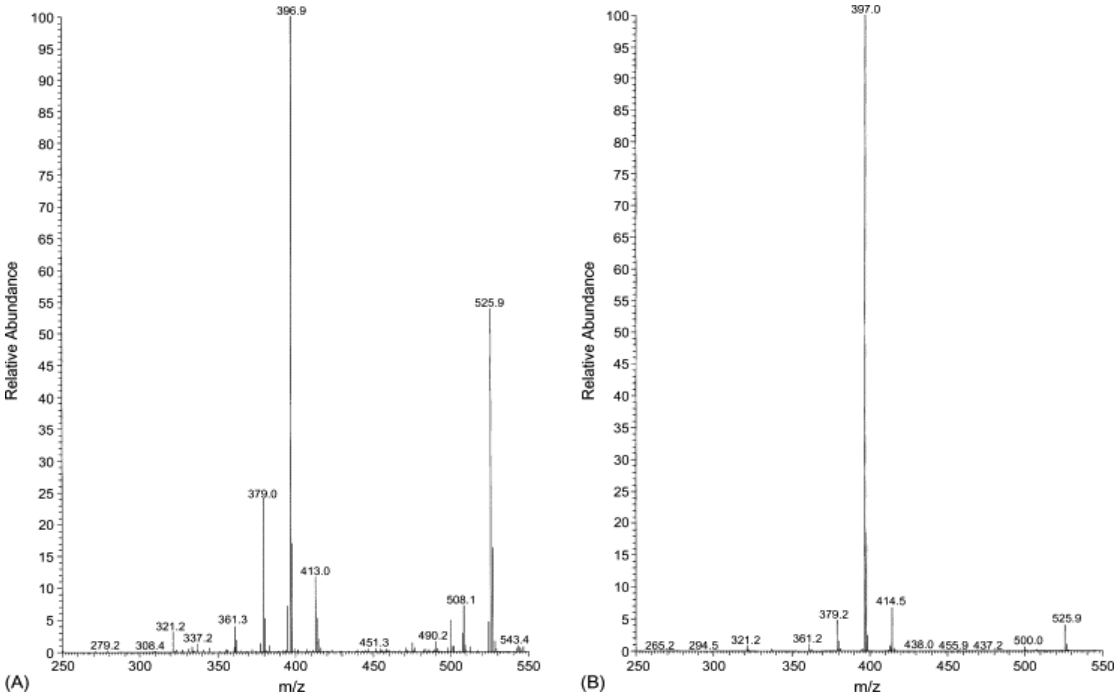

Figure 2.1: Fragmentation patterns of (A) Epi and (B) Dox

The epimers Dox and Epi can be identified and distinguished by selected reaction monitoring (SRM)-MS in the same run [1]. SRM is used with tandem MS where the first mass analyser selectively only allows a single mass through to the second mass analyser for a defined product ion. Multiple reaction monitoring (MRM)-MS allows multiple mass ions through to the second mass analyser. The method was linear in the concentration range 2.5–2000 ng/ml. Detection limits were 1.0 and 2.5 ng/ml for limit of detection (LOD) and limit of quantitation (LOQ).

Lachatre *et al* [11] developed a method for the simultaneous determination of four anthracyclines and their metabolites by LC-MS. Once the sample had been extracted from its biological matrix it was analysed on a column. The mobile phase was composed of 5 mM ammonium formate (pH 3.0)/acetonitrile (70:30 v/v) and the flowrate was 50 µL/min. ESI-MS was used to detect the anthracyclines. Each anthracycline and the metabolites (Dox, Epi, Dnr, idarubicin, aclarubicin, doxorubicinol, daunorubicinol and idarubicinol) were directly infused into the MS to determine their daughter ions so as to distinguish between them. The LOD for the anthracyclines ranged from 0.5 to 2.5 ng/mL. Retention times ranged from 10.5 to 21 min implying that the polarity of the anthracyclines and metabolites were quite different. Lachatre [11] also found that, under their experimental conditions, the optimum pH for stability was between 4.5 and 5.5 for Dnr, 3.0 and 7.0 for Dox, 4.0 and 5.0 for Epi and 4.0 and 5.0 for aclarubicin.

The use of a fluorescent detector is quite common in the analysis of anthracyclines as they are naturally fluorescent molecules and low detection limits can be obtained easily without much interference from biological material as demonstrated by Zhou [4], Van Asperen [2], Rudolphi [7], de Bruijn [10] and Barker *et al* [8].

Anthracyclines are also red in colour and hence absorb strongly in the visible region (see Figure 2.2) of the electromagnetic spectrum but no papers were found exploiting this mode of

detection**.** This detection mode may be very useful however if anthracyclines were to be determined in tandem with another drug class that did not naturally fluoresce.

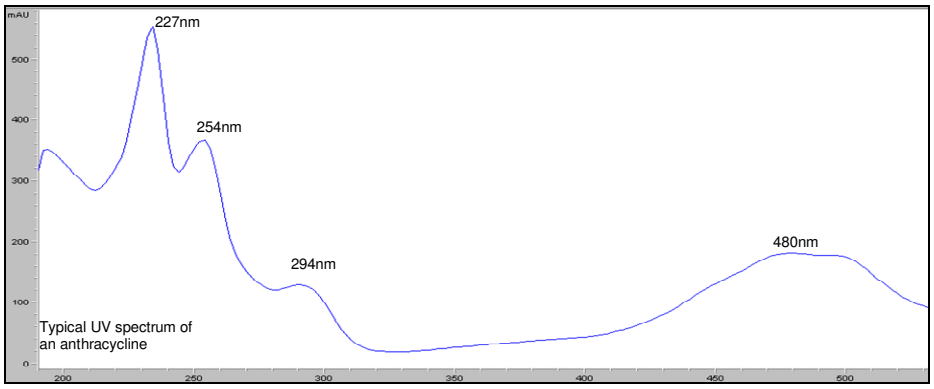

Figure 2.2: UV-Vis spectrum of Dox showing strong absorbances at 227, 254 and 294nm.

Mahnik *et al* [5] developed an LC-fluorescence method for the determination of anthracyclines in hospital effluents. Following extraction from biological material, 20 µL of sample was separated on a narrow bore C18 column. The mobile phase consisted of a water/acetonitrile gradient adjusted to pH 2.0 with a 10 mM K-di-hydrogenphosphate buffer. The flow rate was set to 0.6 mL/min. Dox and Dnr were monitored fluorescently (excitation and emission wavelengths: 480 and 550nm respectively) with retention times of between 13.0 and 16.0 min. The anthracyclines could be detected at concentrations as low as 0.1 µg/L.

During the method development process it was discovered that while there was good linearity for the standard curves in plasma, the same was not true for water. Hence, waste water samples were spiked with bovine serum to take advantage of the high protein binding (~70%) of anthracyclines when performing sample clean-up.

Kümmerle *el al* [12] also employed fluorescence as the mode of detection in the analysis of Dox in biological fluids and tissues in an isolated lung perfusion model. After sample clean-up, a 100 µL Dox sample was analysed on a C18 column. The mobile phase consisted of a water/acetonitrile, 1-heptanesulfonic acid 0.2% pH 4, gradient. Dox was monitored fluorescently with excitation and emission wavelengths of 482 and 550 nm respectively. The method was linear over the range 2–1000 ng/ml in many different matrices with R^2 values >0.997. LOQ was determined to be about 2.0 ng/mL in plasma but only about 0.1 µg/mL from tissue samples.

Electrochemical detection following LC has also been reported for the anthracyclines. Ricciarello *et al* [9] determined Epi and Dox and their metabolites in this manner. After SPE extraction the drug sample was analysed using a C18 column. A mobile phase of water/acetonitrile (71:29 v/v) containing 0.05M Na_2HPO_4 and 0.05% v/v triethylamine at pH 4.6 was run isocratically at 1.0 mL/min to separate the anthracyclines. This method was found to be linear over the range of 1–500 ng/ml for all the analytes.

All of the methods described above for separating and detecting anthracyclines employed a C18 reverse phase column and used an acidic mobile phase in their separation. It is common practice to choose a mobile phase pH that is about 2 units above or below the pKa value of your target analyte. Since anthracyclines are slightly basic with a pKa value of between 7.5 and 9.0, a high pH mobile phase could not be used as this would destroy the silica in a conventional analytical column. An acidic mobile phase (pH 2.5 to 4.5) would most likely be used in the

separation for optimal anthracycline resolution. This is because this pH is far enough away from the pKa so as to ensure the anthracyclines are in one form only and the low pH also ensures stability for these drugs. Since anthracyclines are naturally fluorescent a fluorescent detector would be a sensible choice of detector as it is very sensitive and highly selective. From Table 2.1, it can be seen that a fluorescence detector has been used quite often in anthracycline assays but taxanes are not fluorescent and so this detector would not be useful in a combination assay.

2.3.2 Taxanes

Numerous different LC chromatographic methods have been applied to the separation of taxanes. The majority of these use either UV or MS as a mode of detection.

UV detection is very common in the analysis of taxanes. It was used successfully by Sparreboom [21], Rouini [20] and Garg *et al* [22] to determine either Doc or Pac or both simultaneously in plasma as shown in Table 2.2.

Suno *et al* [18] developed a recent LC-UV method for Pac determination in human plasma. After SPE sample clean-up the taxanes were separated on columns packed with pentafluorophenyl material which is commercially recommended for taxane analysis. A 20 mM potassium phosphate buffer (pH 3.0)/acetonitrile (55:45 v/v) mixture was delivered at a flow rate of 0.25 mL/min at 45 °C for separation. The drugs were detected using UV at 230nm.

Andersen *et al* [17] also developed an LC-UV method for the determination of Doc and Pac in plasma. Once the drugs had been extracted from the plasma matrix they were separated on a C18 column. Andersen used a 20 mM potassium phosphate buffer (pH 3.0)/acetonitrile (55:45 v/v) as mobile phase pumped at 1.0 mL/min through the column. The column temperature was maintained at 40 ^{o}C throughout the separation. UV detection of the taxanes was at 227nm. The LOQ was 0.85 ng/mL for Doc and 0.89 ng/mL for Pac. Both Anderson and Suno used the exact same mobile phase (at different flow rates) to separate taxanes.

Garg *et al* [22] developed an LC-UV method for the determination of Doc in urine or plasma. Separation was performed isocratically with an acetonitrile/0.02 M ammonium acetate buffer (pH 5), (43:57 v/v) mobile phase pumped through a C18 radial compression RP column at 1.0 mL/min. All separations were performed at room temperature. The eluent was monitored by a UV detector at 227nm. Total run time in this LC-UV method was 13 min with retention times of 8.5 and 10.5 min for Doc and Pac respectively (see Figure 2.3 below taken from Garg *et al* [22]). Doc is the eluted at 8.5 min, 2.0 min before Pac is eluted. This shows that Doc is the more polar of the two taxane molecules.

MS is fast becoming the detection mode of choice amongst analysts working with taxanes because of the sensitivity associated with it, and has been used successfully by Alexander [26], Guo [23], Wang [25] and Gaspar *et al* [28] (Table 2.2) to name a few.

Vainchtein *et al* [14] developed a simple and sensitive LC-MS method for determination of Pac and metabolites in plasma. Once Pac had been extracted (LLE) from the plasma it was separated on a C18 column. The mobile phase used in the separation was 10 mm ammonium hydroxide in water/methanol (30:70 v/v). It was pumped at a flow-rate of 0.2 mL/min. Injected samples were kept cool at 10 ^{o}C. Pac was detected by positive mode ESI-MS. MS data was collected in multiple reaction monitoring (MRM) mode with mass transitions of *m/z* 854 → 509 optimised for Pac, 854 being the protonated Pac adduct $[M + H]^+$. Ammonium $[M + NH_4]^+$, sodium $[M + Na]^+$ and potassium $[M + K]^+$ adducts were also detected at 871, 876 and 892

respectively but at a lower intensity. Mass transitions for metabolites were also optimised. According to Vainchtein *et al* an alkaline mobile phase appeared to be most appropriate for a sensitive and selective detection of the Pac and its metabolites. The assay proved to be linear for Pac from 0.25 to 1000 ng/mL.

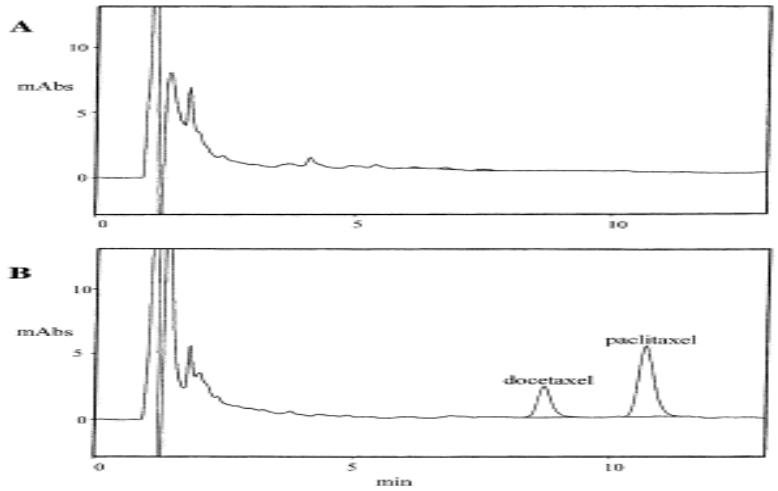

Figure 2.3: LC chromatograms of (A) blank plasma and (B) a patient plasma sample containing 210 ng/ml of Doc and 500 ng/ml of added Pac (internal standard) taken 15 min after a 1hr intravenous infusion of docetaxel (60 mg/m^2) [22].

Mortier *et al* [15] also developed and validated an LC-MS method for the quantification of Doc and Pac in plasma. Once the drugs had been extracted by LLE they were separated on a C18 column. The column and autosampler were thermostatically controlled at 25 and 10 ^oC respectively. The mobile phases composed of (A) 2 mM acetic acid/0.2 mM ammonium acetate in water and (B) 2 mM acetic acid/0.2 mM ammonium acetate in methanol. The gradient mobile phase was pumped at 0.4 mL/min. Once the taxanes were separated they were then detected by positive mode ESI-MS. The MRM transitions (*m/z*) for Doc and Pac were determined to be 808.4 → 226.1, 526.9 and from 854.4 → 509.3, 569.0 respectively. MRM transitions of some metabolites were also determined. The method was found to be linear over the range of 2.0-1000 ng/mL. Total run time from injection to detection was 11 min but the LLE sample pre-treatment protocol was time consuming.

Mortier *et al* found they were getting a mixture of sodium and protonated adducts during initial method development infusion experiments. They determined that additives such as acetic acid and ammonium acetate aided the formation of the protonated adduct. They also looked at the effect of solvent type (acetonitrile versus methanol) and additives (ammonium acetate, ammonium formate, acetic acid, and formic acid) on sensitivity and reproducibility.

Parise *et al* [24] also developed an LC-MS assay for Doc and Pac analysis. Separation of the taxanes was achieved on a C18 analytical column after the drugs had been isolated from biological material. The mobile phase used in the separation of the two taxanes consisted of 0.1% formic acid in methanol/water (70:30 v/v), was pumped at 0.2 ml/min. The column eluate was then analysed by positive mode ESI-MS. The monitored *m/z* adducts were 808.1 and 854.0 for Doc and Pac respectively. Total run time from injection to detection was 7 min with this

method. An off-line SPE method was used for sample clean-up. The assay was linear over the concentration range of 0.3 to 1000 ng/mL for Doc and 1.0 to 1000 ng/mL for Pac.

Reviewing methods for taxane separation, a C18 column is most frequently used. An acidic mobile phase is also favoured. Column dimensions and mobile phase flow rate vary widely among the listed methods in Table 2.2. A slower flow rate would be required if mass spectrometry was used as the mode of detection.

2.4 Conclusions

The literature reveals that there are many different methods of analysis for the determination of anthracyclines in biological material. The best method of drug extraction from complex matrices seems to be SPE. The major advantages of SPE over LLE and PP is that SPE can be automated providing potential for higher sample throughput, greater sensitivity, higher recoveries, less sample handling and reduced solvent waste. On-line SPE can greatly reduce analysis time. Sample clean-up can be done easily in less than 5min compared to off-line SPE, LLE or PP procedures that can take hours.

There is some variety in the type (sorbent) and dimensions of analytical column that can be used in the separation of anthracyclines. Judging by the majority of reviewed papers in the scientific literature the most commonly used LC column sorbent is C18. Most journals state that the anthracyclines are most stable in an acidic pH and all of the papers reviewed here chose a mobile phase with a pH in the range of 3.0-4.5. Although the choice of eluent varies, the organic solvent was predominantly acetonitrile. The majority of methods that quantify anthracyclines use a fluorescence detector. This is possible only because the anthracyclines fluoresce naturally. Unfortunately the taxanes do not. Therefore it is not possible for fluorescence to be used in a method to determine anthracyclines and taxanes simultaneously. The other detectors used are UV or MS.

The most common way of extracting taxanes from biofluids varies considerably. In the literature SPE or LLE are the most commonly used. PP can also be used but has been reported less frequently. Automated SPE is much quicker than off-line extraction methods as proven by Grozav *et al* [10] who reported an on-line SPE-LC-MS method for taxane analysis with a sample clean-up time of approximately 90 sec. For the taxanes, a C18 sorbent seems to be the column of choice and UV or MS the detectors of choice.

For the purpose of this research, it was decided that on-line SPE should be used as the sample pre-treatment step. It was anticipated that a major decision would be the type of SPE sorbent as these cartridges are generally manufactured to suit a particular type of drug (hydrophilic or hydrophobic for example) and anthracyclines and taxanes are chemically very different molecules. The many types of SPE sorbents commercially available operate under different principles (size exclusion, chemical interactions, etc…) and there are many different parameters that can be adjusted (extraction mobile phase, pH of the mobile phase, length of extraction, sample volume, etc…) to offer optimal recoveries for both drug classes. In terms of LC, it would appear that use of a C18 column with an acidified mobile phase would be a good starting point for separating a mixture of both anthracycline and taxane drug classes simultaneously.

As UV is one of the most readily available detectors coupled to LC and since both anthracyclines and taxanes could both be very easily detected in this way, it was decided to start method development with a UV detector.

MS is one of the most powerful and selective detectors available for drug analysis. It has been used in recent papers to quantify anthracyclines and taxanes individually and it offers low limits of detection. It could easily be investigated for measuring the levels of both anthracyclines and taxanes in serum simultaneously. The two different drug classes have very different ionisation conditions and fragmentation patterns but this can be accommodated in one assay using a state-of-the-art LC-MS system.

2.5 References

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Chapter 3

Development of an on-line SPE-LC-UV method for the simultaneous determination of anthracyclines and taxanes in human serum

3.1 Introduction

Both anthracyclines and taxanes are very widely used classes of chemotherapeutic drugs. Anthracyclines have been used in cancer chemotherapy for more than 30 years, and are considered to be among the most useful antineoplastics. They are originally isolated from a pigment-producing *Streptomyces*[1]. The most commonly administered anthracyclines are doxorubicin, epirubicin and daunorubicin. Taxanes are also widely used. The two most commonly administered taxanes include paclitaxel and docetaxel. Paclitaxel was the first naturally occurring taxane to be discovered. It was isolated from the bark of the pacific yew tree[2]. Docetaxel is a semi-synthetic derivative of paclitaxel and was first synthesised from 10-deacetyl baccatin III, a non-toxic precursor found in the European yew.

Both drug types may be used in tandem to treat certain types of cancer such as advanced cancers of the breast or stomach[3]. The drugs are administered normalised to body surface area but despite the fact that these drugs can be administered simultaneously, no methods currently exist that are capable of quantifying both drug types in a single assay. Therefore it is impossible to get an accurate measurement of how a patient is metabolising and reacting to this combination treatment. Every patient reacts differently after administration of such toxic drugs. A combination regimen like this may be too potent for some individuals, not potent enough for others and may not have the desired effect at all on others. It is for this reason that individualised or tailored patient dosing is recommended where possible. But in order to support this system, blood monitoring is required to monitor the serum levels of the administered drugs.

Analytically, combination chemotherapy presents significant challenges since the different physiochemical properties of the drugs employed typically requires individual methods of determination. In order to develop a single assay for combinations of drugs, more extensive method development is needed in order to find a set of conditions that works for all analytes. While chromatographic methods have been reported for the determination of either anthracycline[4,5] or taxane[6,7,8] drug molecules from biological material individually, none have yet been developed for the two together. In this chapter, the development of an assay to extract, separate and detect both drug classes in a simple, precise and accurate manner is described.

3.2 Scope of work

The objective of this work was to develop for the first time an automated, quantitative method for the simultaneous determination of both anthracycline and taxane drug molecules from human serum using an on-line SPE-LC-UV method. Initially a UV detector was used. All five drugs chosen have strong UV chromophores and one advantage of UV detection is that it is readily available in analytical laboratories and easy to use.

The first step involved finding a suitable analytical column and appropriate mobile phase to adequately chromatographically separate the polar anthracyclines and non-polar taxanes. Once the analytical method had been optimised, the sample extraction part of the method was developed. This involved choosing an SPE column and set of extraction conditions that offered a high recovery for both drug classes. Then, finally the overall method could be validated.

3.3 Experimental

3.3.1 Materials and reagents

Water, methanol, formic acid, ammonium formate, isopropanol, (LC grade or higher) and human serum were all bought from Sigma (Dublin, Ireland).Acetonitrile was bought from Labscan (Ireland). Dox, Epi, Dnr, Doc and Pac were all bought from Fluka Chemicals (Ireland). Patient serum samples containing either Doc or Epi were kindly supplied by Saint Vincent's Hospital (Dublin). SPE cartridges were obtained from Phenomenex, UK (Strata-X, Strata X-C, Strata X-CW, Strata-X-AW, Strata-C18, Strata-C18-E and Strata-SDB-L) and Chromtech, UK (Biotrap columns C8, C18 and MS).

3.3.2 Instrumentation

The HPLC system used in this work consisted of two Agilent 1050 pumps (one for the extraction solvent and one for the analytical mobile phase), a model 1050 autosampler with a 100 µL injection loop, a model 1050 VWA UV-Vis detector and an on-line Agilent 1200 degasser. The system was equipped with chemstation software version A10.01 [1635] for system control and data collection. A gradient LC system using an acidified water/acetonitrile mixture was used to separate the analytes on an Agilent Zorbax column (150 x 4.6mm I.D, particle size 5.0µm) packed with XDB C18 material. The extraction column used was a Biotrap 500 MS SPE column (20 x 4mm ID) packed with a hydrophobic polymer material and was coupled to the LC system via a Rheodyne 10-port 2-way switching valve (see Figure 3.1).

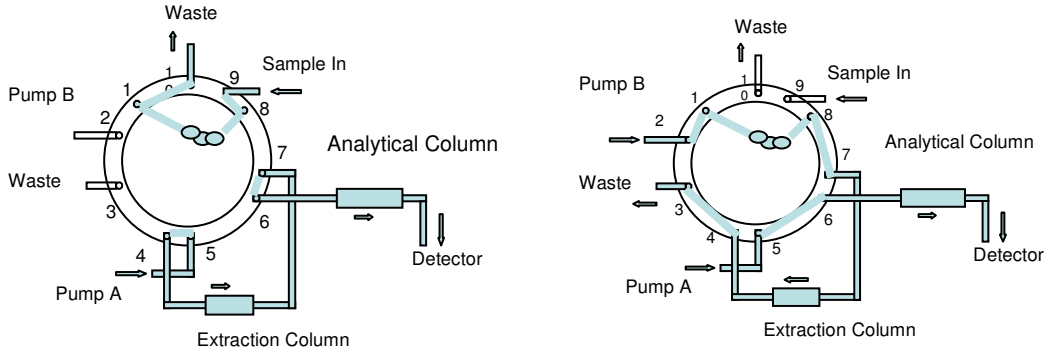

Figure 3.1: Schematic representation of the 10-port switching valve used for on-line sample extraction and the resulting flowpaths in the two valve positions.

3.3.3 Preparation of stock and standard solutions

Stock solutions of epirubicin (Epi), doxorubicin (Dox), daunorubicin (Dnr), docetaxel (Doc) and paclitaxel (Pac), all 2 mg/mL, were weighed in a glove box and prepared by dissolving the

compound in acidified water (adjusted to pH 3.5 with formic acid) for the anthracyclines and in neat methanol for the taxanes. All stock and standard solutions were stored in the refrigerator and protected from light. Standard solutions of the drugs were prepared (250 µg/mL) monthly by dilution with appropriate solution. Fresh working standard solutions were prepared daily by dilution of the standard solution(s) in the aqueous mobile phase to the concentrations required.

3.3.4 Preparation of serum samples

Thawed drug-free human sera samples were spiked with stock solutions of the drugs to achieve the final concentrations required in undiluted serum. The samples were spiked just before analysis.

Patient venous blood samples were obtained from patients undergoing chemotherapy in St. Vincent's Hospital, Dublin, Ireland by informed consent at 10mins after administration. The whole blood samples were immediately centrifuged to yield serum. The samples were kept frozen at -20 ^{0}C until analysed.

3.3.5 Sample extraction and clean-up procedure

Sample clean-up was performed on-line using a Biotrap 500 MS SPE column. This biocompatible sample extraction column allows for repeat injections of serum, plasma or other complex matrices directly onto a HPLC system without prior clean-up. This type of SPE column has a biocompatible external surface and hydrophobic internal surface. Biocompatibility is obtained by the attachment of a plasma protein (alpha$_1$ acid glycoprotein (AGP)) on the external surface of the particles. The hydrophobic internal surface is provided by a hydrophobic polymer coating. The pores of the SPE column are small enough for drug molecules to permeate but exclude proteins and macromolecular compounds commonly found in serum or plasma that must be removed prior to LC analysis. The MS SPE column is stable over a broad pH range (2-11).

The sample (standard solution or human serum) was injected onto the 100 µL loop while the valve was set to position one (see Figure 3.1). In this configuration, the analytical mobile phase was rinsing both the extraction and analytical columns. The valve was then switched to position two where the extraction solvent (30 mM ammonium formate/acetonitrile, 98/2 v/v, pH 6.8) washed any macromolecular proteins in the serum to waste leaving the drugs retained on the extraction column. The extraction solvent was set to flow at 2.0 mL/min for 150 sec. The valve was then switched back to position one and an increasing gradient of acetonitrile with a flow rate of 1.0 mL/min back-flushed the retained drugs off the extraction column onto the analytical column where they were separated and detected.

Firstly, with the valve in position one, the serum sample is introduced into the loop. Then the valve is turned to position two whereby extraction solvent, pumped by pump B, picks up the sample from loop and carries it onto the extraction column where the drugs of interest are retained and serum components flow to waste. During this time, pump A is rinsing the analytical column. The valve is switched back to position one and now analytical mobile phase, pumped by pump A, sweeps the retained drugs from the extraction column in back-flush mode onto the analytical column for separation. During this time, pump B is moving extraction solvent to waste.

3.3.6 Chromatographic conditions

The gradient programme was set up as shown in Table 3.1. The extraction solvent was composed of 30mM ammonium formate in water/acetonitrile (98/2 v/v), pH 6.8. Mobile phase A was composed of 0.1% formic acid in water/acetonitrile (90/10 v/v), pH 3.5 with ammonium formate. Mobile phase B was composed of 0.1% formic acid in acetonitrile/water (90/10 v/v).

Time (min)	(Pump B) Extraction solvent (%)	(Pump A) Mobile phase A (%)	(Pump A) Mobile phase B (%)
0	100	85	15
2.5	100	85	15
3.0	0	85	15
16	0	25	75
21	0	85	15
23	100	85	15

Table 3.1: Extraction and analytical solvent gradient timetable

The monitoring wavelength was held at 254nm for the anthracyclines (13 min) and then switched to 234nm for detection of the taxanes until the end of the run. Separations were performed at room temperature. After each serum sample injected the SPE column had to be washed with a high percentage organic solvent to remove any retained sample. An acetonitrile/water (90/10 v/v) mixture was passed through the SPE column for 2.0 min to clean the SPE column prior to the next sample injection. This washing stage could not be built into the method because of software limitations.

3.3.7 Calibration

Calibration curves were prepared in drug-free serum spiked with stock solutions of each analyte. The concentration range tested was between 0.5 µg/mL and 25 µg/mL. Standards were prepared at concentrations of 0, 0.5, 1.0, 5, 10 and 25 µg/mL in water/acetonitrile (90/10 v/v). Calibration curves were determined by plotting peak area versus spiked drug concentrations.

3.3.8 Method validation

A full validation procedure was performed on the method including intra-day precision (repeatability) and inter-day precision (intermediate precision), accuracy, sensitivity (LOD and LOQ), linearity and range and recovery according to accepted guidelines [9]. Some further parameters were also monitored such as stability following freeze-thaw cycles.

3.4 Results and Discussion

3.4.1 Method development

The determination of anthracyclines and taxanes simultaneously in a single assay had not been reported to date in any literature. A method for their extraction, separation and detection had to be created. Firstly, an LC column and mobile phase had to be found that was capable of adequately separating the five different drugs. The epimers of Dox and Epi represented a significant challenge here as they differed in structure only by the direction (axial or equatorial) of a hydroxyl group. Once a suitable set of analytical conditions had been determined, the on-line sample clean-up part of the method had to be investigated. This involved finding an SPE sorbent that could extract both drug types efficiently and with high recoveries. A suitable extraction solvent also had to be determined, as well as timings.

3.4.2 Choice of LC column

Most published articles on work with anthracyclines or taxanes used a C18 column for their separation. Three different types/brands of C18 columns were tested in an attempt to separate the five drug compounds within a reasonable time. Columns tested included Phenomonex Prodigy C18 (150 x 4.6 mm, 5 µm), Agilent Zorbax XDB-C18 (150 x 4.6 mm, 5 µm) and an Agilent Zorbax Plus C18 rapid resolution (4.6 x 50 mm, 1.8 µm). All three columns were capable of separating the five different drugs but the Zorbax XDB-C18 was chosen as it offered the best compromise between resolution, separation time and acceptable pressure. The analytical runtime was 20 min with analyte retention times of around 5.9, 61, 7.0, 12.5 and 13.2 min for Dox, Epi, Dnr, Doc and Pac respectively. The retention factors (k) were found to be 1.3, 1.4, 1.8, 4.0 and 4.2 for Dox, Epi, Dnr, Doc and Pac respectively with this column. Retention factors of between 1 and 10 are considered acceptable.

3.4.3 Selection of analytical mobile phase and UV detection

Many different organic and aqueous compositions were tested in order to find a suitable separation mixture. Various combinations of acetonitrile and methanol in water were investigated at length. Eventually, a water/acetonitrile gradient was chosen as most suitable for the separation and resolution of all five drugs. The pH of the analytical mobile phase was found to control analyte retention time since the anthracyclines are extremely sensitive to pH and light. It is generally regarded as good practice to choose a pH that is at least two units above or below the pKa of the drugs under investigation. Since the pKa values of the anthracyclines range from about 6.5 – 8.5, it was decided to evaluate a range of pH values between 2.5 and 4.5. Further pH tests showed good separation of all analytes at pH 2.5, 3.5 and 4.5 (see Figure 3.2). Anthracyclines become less stable as pH rises towards neutral pH and some of the analytes were obscured by low molecular weight endogenous serum components that survived extraction at pH 2.5 and 4.5. So for this reason a pH of 3.5 was chosen.

Although there was no major difference in the resolution of separation between the analytes, there were some differences in peak areas however for each peak depending on the pH of mobile phase used. This is illustrated in Table 3.2 below.

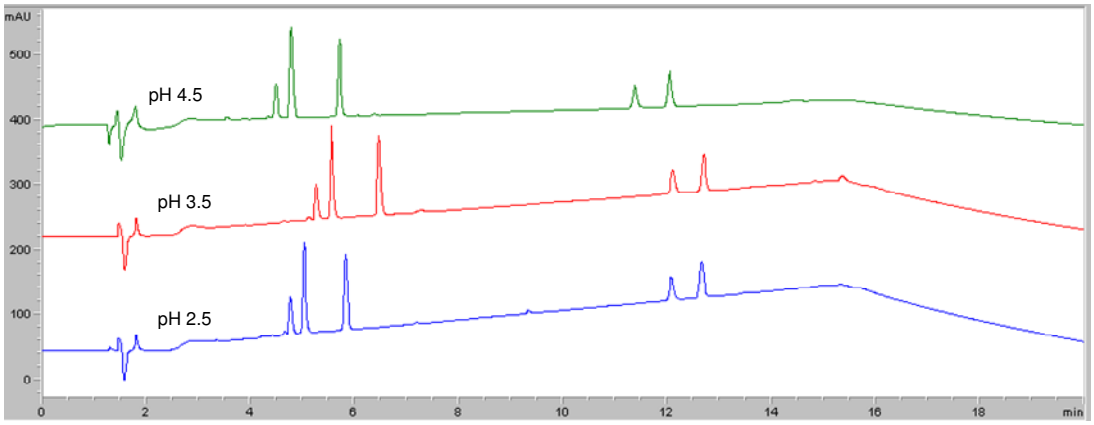

Figure 3.2: A 5 µg/mL sample of a combination of the five drugs in mobile phase. The same sample was injected and separated under the same conditions except for the pH of the mobile phase which was varied from 2.5 (blue) to 3.5 (red) to 4.5 (green).

Drug	Peak area at pH 2.5	%RSD @ pH 2..5	Peak area at pH 3.5	%RSD @ pH 3.5	Peak area at pH 4.5	%RSD @ pH 4.5
Dox	305	1.64	334	2.37	258	4.35
Epi	666	2.31	666	1.89	634	2.11
Dnr	583	2.89	616	2.33	587	1.74
Doc	272	3.59	339	4.53	257	3.47
Pac	417	2.11	420	2.94	401	2.56

Table 3.2: Peak Area of each analyte at different mobile phase pH values

The 5 µg/mL sample was injected in triplicate at each pH and the average peak areas are shown in the table above. The best response for each analyte was achieved when a pH of 3.5 is used in the mobile phase.

The λ_{max} values for the anthracyclines were at 227 and 254nm as shown in Figure 3.3. While it was clear that 227nm would have been best wavelength for anthracycline detection, interference from endogenous compounds at this wavelength was too great so the second λ_{max} was used – 254nm – which proved to be successful at monitoring Dox, Epi and Dnr. After elution of the anthracyclines, the detection wavelength was then changed to 234nm for optimal monitoring of the taxanes.

3.4.4 Choice of SPE column

An extraction column was sought that could give the greatest recoveries for both the polar anthracyclines and the non-polar taxanes from the complicated serum matrix. Numerous commercial SPE columns are available with different modes of sample clean-up. The restricted access material (RAM) exclusion barrier can be either chemical or physical and the surface

topochemistry of the columns can be composed of a single or dual type substance. A number of different brands and types of extraction sorbents were tested in terms of the recovery they could yield for each of the analytes from human serum. A number of different types of SPE sorbents were tested for these drugs to see which was most suitable.

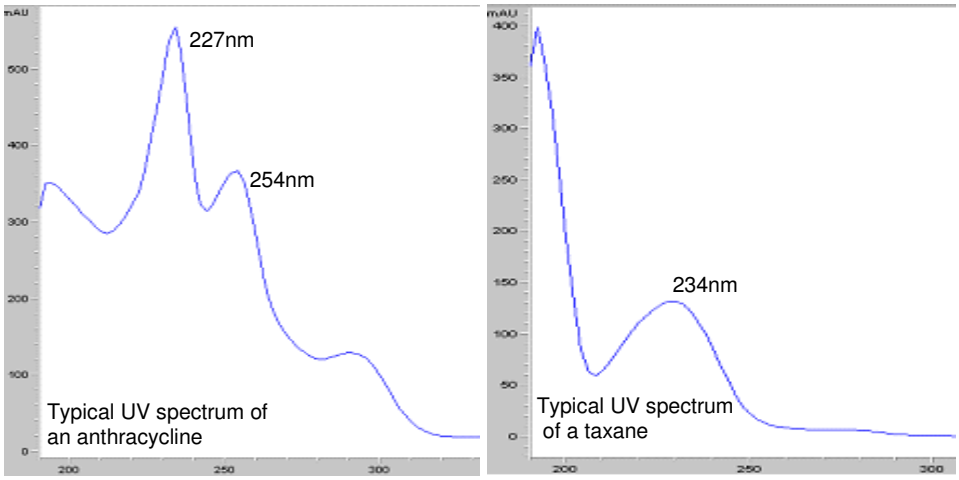

Figure 3.3: UV spectra of anthracyclines and taxanes

The following Phenomonex SPE cartridges (Strata-X, Strata X-C, Strata X-CW, Strata-X-AW, Strata-C18, Strata-C18-E and Strata-SDB-L) were evaluated off-line in a vacuum manifold in terms of their ability to selectively retain anthracyclines and taxanes. These SPE cartridges were thoroughly investigated in terms of their abilty to extract both drug classes. Each type was evaluated at a range of different pH values from pH 2.5 to pH 10.5. Some of the SPE cartridges were only able to extract a single drug class - for example the Strata X-C and Strata X-CW could only extract taxane drugs with recoveries of up to 90%. Other more promising cartridges such as the Strata X-AW and Strata X could extract quantities of both drug classes. The Strata X was the more efficient of the two with anthracycline recoveries of up to 60% and taxane recoveries of up to 106% [10].

Also investigated were three types of Biotrap 500 SPE column (C8, C18 and MS). The SPE MS column from Biotrap was found to give the highest recoveries for all five analytes and so was used in the final on-line method. With the Biotrap SPE columns recovery of both anthracycline and taxane drugs was >85%. The Biotrap MS SPE showed the most potential of the three SPE columns. Despite the Strata X cartridge showing high taxane recovery, its low anthracycline recovery led to the choice of the Biotrap MS SPE column for sample extraction.

3.4.5 Choice of SPE solvent

Many different extraction solvents were tested including mixtures of water in acetonitrile, isopropanol and methanol. The amount of organic modifier added to the extraction solvent varied from 2-4% v/v and the solvents were tested at different pH values ranging from 3-10 adjusted with formic acid or ammonium hydroxide in water to obtain the required pH. Different buffers were also tested and these included ammonium formate, sodium phosphate and formic acid. It

was determined based on analyte recovery of all five cancer drugs that the optimal extraction solvent was 30 mM ammonium formate/acetonitrile, 98/2 v/v, pH 6.8.

The conditions and timing for loading, washing and eluting were critical and were optimised in terms of recovery of analytes from the serum sample. The length of extraction time and the extraction solvent flow rate were determined to be the most important factors. Extraction times were varied from 1 to 5 min at flow rates of between 1.0 – 4.0 mL/min. Optimal extraction conditions for both drug classes in terms of recovery were determined to be a flow rate of 2.0 mL/min for 150 sec. It was determined that at an extraction time of 1.0 min was not sufficient at eluting all retained drugs from the SPE column. After 2.0 min extraction time there was still some target analytes adhering to the SPE column. After between 2.5 and 3.0 min it seemed that all injected analytes had been extracted from the SPE column onto the analytical column. At extraction times greater than 3.0 min no extra amounts of analytes were extracted from the SPE column. Sample recovery increased from 1.0 min extraction time to 2.5 min extraction time with no further increase after this. The flow rate of the extraction solvent was also varied. At 1.0 mL/min it took too long for all target analytes to be washed from the SPE column. A flow rate of 2.0 mL/min was determined to be the optimal flow rate for the extraction solvent as it was capable of eluting all retained drugs from the SPE column within 2.5 min. There was no increased analyte recovery using flow rates quicker than 2.0 ml/min.

The final method allowed for the extraction, separation and detection of five drugs spiked into neat human serum (see Figure 3.4).

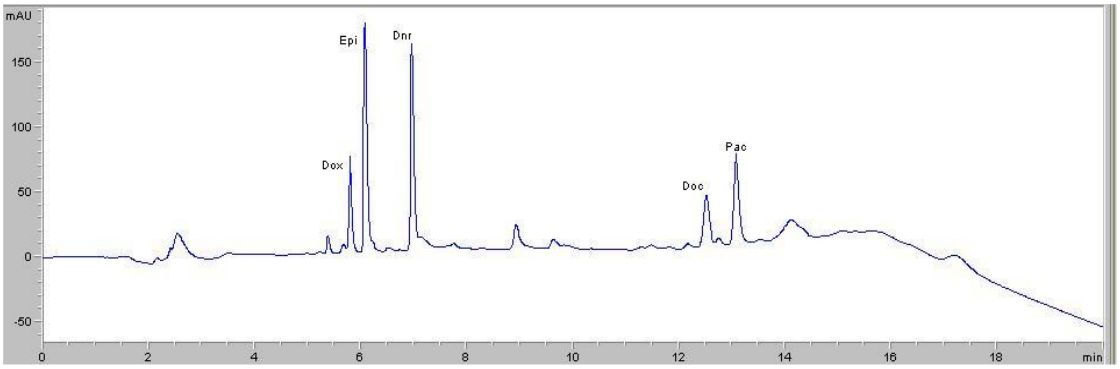

Figure 3.4: Chromatogram showing the separation of a spiked serum sample containing all five drugs at 5.0 µg/mL following on-line SPE. The anthracyclines elute first in the order Dox, Epi and Dnr. The taxanes elute later in the order Doc and Pac. There is little or no matrix interference evident from the serum.

3.5 Method validation

A full validation procedure was performed on the method including intra-day and inter-day precision, accuracy, sensitivity, linearity and range and recovery. Stability following freeze-thaw cycles was also investigated.

3.5.1 Precision and accuracy

Intra- and inter-day accuracy and precision were determined from standards prepared in serum over a concentration range of 0.5 µg/mL to 25 µg/mL. Precision was expressed as percentage relative standard deviation (%RSD). Accuracy was expressed as the ratio of the mean calculated concentration against the spiked concentration for each standard multiplied by 100%. For intra-day precision and accuracy, five different standards (0.5,1.,5,10 and 25 µg/mL) were analysed in triplicate. Inter-day precision and accuracy were determined by analysing the standards on five different days in triplicate. Table 3.3 displays the data for intra- and inter-day precision and accuracy respectively. Intra-day precision ranged from 2.0 to 10.8 % RSD and inter-day precision ranged from 2.0 to 14.9 % RSD. All precision values were ≤ 15%. The accuracies ranged from 88.0 to 136.0% and these values compare quite well with those reported by other authors such as Barker [11], Ricciarello [12] and Garg *et al* [13]. It is quite likely that these precision and accuracy values could be improved if automated switching was used instead of manual switching.

3.5.2 Sensitivity

The limit of detection (LOD) was set as the lowest concentration of the compounds (Dox, Epi, Dnr, Doc and Pac) that could be detected from samples in drug free serum with a signal-to-noise ratio of 3:1. The limit of quantification (LOQ) was defined as that concentration which had a signal-to-noise ratio of at least 5:1 [9]. LOD and LOQ values were obtained by measuring the signal-to-noise ratio at and nearby where target analytes eluted and by measuring the concentration of that analyte which was either three or five times that baseline noise. The LOD values were found to be 0.1 µg/mL for Epi and Dnr and 0.15 µg/mL for Dox, Doc and Pac. The LOQ was 0.5 µg/mL for all five compounds using 0.1 mL serum. The method is not as sensitive as the methods developed by either Garg [13] or Rouini *et al* [14] for taxane analysis with UV detection. The methods sensitivity is affected by the fact that both polar and non-polar drugs are extracted, separated and detected in the same single assay. A compromise had to be made between recovery and sensitivity because of the combination of drugs being studied. Most SPE columns are designed for either polar or non-polar drugs, not both simultaneously.

To obtain high recoveries of all analytes the sensitivity of the method suffered. The current sensitivity of the method was adequate for determination of anthracyclines and taxanes immediately or very soon after administration of the drugs to patients but detection limits would need to be lower (≤ 25 ng/mL) for therapeutic drug monitoring. Replacement of the UV detector with a mass spectrometer would significantly lower the detection and quantitation limits for this purpose. Bearing this in mind, sensitivity was increased in this assay by using a larger (200 µL) sample loop thereby doubling the amount of sample injected. However, after repeated serum injections at this larger volume small blockages occurred at the top of the analytical column due to some excess macromolecular material in the serum surviving the extraction process. This increased system backpressure and affected reproducibility. Prolonging the extraction time did not sufficiently eliminate the blockage.

		INTRA-DAY			INTER-DAY		
Compound	**Spiked Conc. (µg/mL)**	**Calculated Conc. (µg/mL)**	**Accuracy %**	**Precision %RSD**	**Calculated Conc. (µg/mL)**	**Accuracy %**	**Precision %RSD**
Dox	0.5	0.68	135.6	8.0	0.64	128.0	3.9
	1.0	1.13	113.2	5.5	1.11	111.1	4.5
	5.0	4.82	96.3	5.0	4.33	86.6	5.1
	10.0	9.74	97.4	3.2	8.82	88.2	12.9
	25.0	25.13	100.5	4.1	21.54	86.2	12.6
Epi	0.5	0.66	132.4	7.4	0.63	126.4	7.9
	1.0	1.04	104.4	8.7	0.99	98.7	2.0
	5.0	4.84	96.8	3.9	4.60	92.0	3.8
	10.0	9.88	98.8	4.1	9.72	97.2	8.8
	25.0	25.08	100.3	2.0	23.29	93.2	8.0
Dnr	0.5	0.57	114.0	10.8	0.55	110.0	10.1
	1.0	0.96	96.0	7.1	0.87	87.0	6.3
	5.0	4.57	91.4	2.4	4.27	85.4	5.9
	10.0	10.50	105.0	5.0	9.14	91.4	10.8
	25.0	24.85	99.4	4.1	22.66	90.6	10.8
Doc	0.5	0.44	88.0	7.0	0.46	92.0	14.9
	1.0	0.86	86.0	2.3	0.92	92.0	6.6
	5.0	4.80	96.0	5.6	4.39	87.8	3.9
	10.0	10.90	109.0	5.3	9.68	96.8	14.3
	25.0	25.15	100.6	8.0	23.79	95.2	11.2
Pac	0.5	0.68	136.0	2.8	0.68	136.0	11.8
	1.0	1.13	113.0	2.4	1.07	107.0	8.6
	5.0	4.56	91.2	5.2	4.47	89.4	5.9
	10.0	10.04	100.4	2.1	9.63	96.3	11.7
	25.0	25.08	100.3	8.4	24.28	97.1	11.2

Table 3.3: Intra-Day and Inter-Day precision and accuracy

3.5.3 Linearity and range

Calibration curves were prepared in drug free serum spiked with stock solutions of each analyte. The concentration range tested was from 0.5 µg/mL to 25 µg/mL. Three external standard calibration curves were analysed by plotting peak area versus drug concentration. All calibration curves proved to be linear over the range of 0.5 µg/mL to 25 µg/mL. The regression coefficients (R^2) values for each calibration curve were > 0.998 in all cases.

3.5.4 Recovery

Recovery is the principle criterion by which to judge whether an extraction is successful or not. Recovery is a percentage of the ratio of actual concentration found versus expected concentration. The closer the recovery is to 100% the more efficient the extraction process.

On the one day, standards at concentrations of 1.0, 10 and 25 µg/mL were prepared three times for each concentration and then analysed in triplicate. The analyte peak areas obtained for spiked serum samples were compared to analyte peak areas obtained for spiked samples in

mobile phase after SPE extraction. Recovery data for each drug from serum is presented in Table 3.4. Recovery values ranged from 85.8% to 117.2%. These values compare very well with previously obtained recoveries in single drug class assays for taxanes of between 86 to 95% [15] and > 90% [16] and for anthracyclines of between 80 to 102% [17] and > 80% [18].

Compound	1µg/ml Spiked Conc.	10µg/ml Spiked Conc.	25µg/ml Spiked Conc.
Dox	117.2	98.1	107.8
Epi	100.9	102.0	102.8
Dnr	101.2	93.6	110.1
Doc	85.8	94.0	101.6
Pac	104.6	108.9	94.1

Table 3.4: Recovery data for drugs from serum (n = 3)

3.5.5 Stability

The stability of each of the five chemotherapy drugs investigated was determined following one, two and three freeze-thaw cycles. It is important to check the stability of samples in order to determine their optimal storage conditions and expiration dates, to see how many times a sample is stable under freeze-thaw cycles. Spiked serum samples were analysed in triplicate at three concentrations (1.0, 10 and 25 µg/mL). On the first day the serum samples were spiked and each sample extracted and analysed. The remaining samples were then frozen and analysed a further three times following successive freeze-thaw cycles.

Stability data on the anthracyclines and taxanes over three freeze-thaw cycles is presented in Table 3.5. Cycle 0 represents a freshly prepared spiked serum sample; cycle 1 represents a sample injected from the remaining stock after a single freeze-thaw cycle etc. The anthracyclines remained quite stable after all three freeze-thaw cycles showing only a 10% decrease in signal response while the taxanes appeared to be less stable showing a significant 20% decrease in signal response by the third cycle. A 20% signal decrease is higher than a previously quoted signal response following freeze thaw cycles in the literature for taxanes. Grozav *et al* [15] and Mortier *et al* [8] both found that freeze thaw stability of taxanes fell within a 15% deviation range. However Mortier *et al* found that the stability of p'-3-hydroxypaclitaxel in plasma suffered a 25% degradation following freeze thaw cycles.

3.5.6 Patient Samples

Patient serum samples were obtained and analysed from patients treated with epirubicin (Epi) and with docetaxel (Dox) to test method performance. The samples were obtained 10 min after completion of administration to the patient.

Unspiked serum samples from patients who were administered Epi (100 mg/m^2) or Doc (75 mg/m^2) were subjected to the full assay (extraction and separation) in order to test the method's performance. Figure 3.5 displays overlaid chromatograms showing patient sample

chromatograms. The peaks correlating to Epi and Doc are evident from their retention times (6.0 and 12.2 min respectively) which shows that the method could be applied to the analysis of serum samples containing these drugs (or indeed Dox, Dnr and Pac) in the concentration range 0.5 µg/mL to 25 µg/mL. Patients may have drug levels at the lower end of this linear range in their system after drug administration but for therapeutic drug monitoring of patient samples the linear range would have to be lower as the drugs are metabolised.

Compound	Spiked Conc. (µg/mL)	Cycle 0	Cycle 1	Cycle 2	Cycle 3
Dox	1.0	100.0	93.7	87.3	86.3
	10.0	100.0	101.0	98.3	90.7
	25.0	100.0	93.0	88.0	87.3
Epi	1.0	100.0	98.0	96.3	93.7
	10.0	100.0	93.3	92.7	83.3
	25.0	100.0	94.3	94.3	87.3
Dnr	1.0	100.0	99.7	97.3	94.0
	10.0	100.0	99.7	101.3	92.3
	25.0	100.0	93.7	97.3	93.0
Doc	1.0	100.0	95.0	80.0	72.7
	10.0	100.0	97.7	91.3	80.7
	25.0	100.0	91.3	89.0	81.7
Pac	1.0	100.0	91.3	89.0	81.8
	10.0	100.0	100.0	95.7	87.3
	25.0	100.0	89.7	82.7	81.7

Table 3.5: Stability of drugs to freeze-thaw cycles (n = 3)

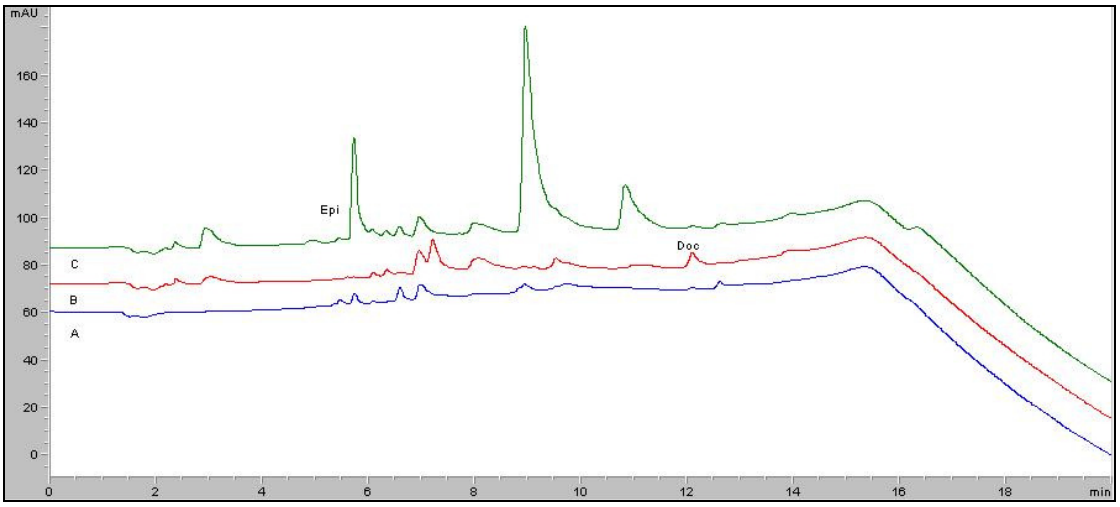

Figure 3.5: Chromatograms of (a) blank human serum, (b) serum taken from a patient 10 min after administration of Doc and (c) serum taken from a patient 10 min after administration of Epi. Time offset between signals at 10%.

3.6 Conclusions

In conclusion, a simple, fast, reliable on-line SPE-LC-UV method for the simultaneous determination of serum levels of two very important and different classes of chemotherapeutic drugs has been developed and validated. This is the first method reported that can extract, separate and detect these five drugs in a single assay. Although the method is not sensitive enough for therapeutic drug monitoring at low serum concentrations using UV detection, the method demonstrates high recoveries for all five drugs from serum, the run time is fast and there are negligible matrix interference effects. The on-line SPE extraction procedure offers many advantages over current off-line extraction procedures in terms of speed (it takes only 150 seconds), recovery (high and reproducible) and reduction of human error. If the sensitivity of the method could be improved, either by injecting a larger sample volume or by incorporating a mass spectrometer as the mode of detection, the method could be applied as a means to ensure patients undergoing chemotherapy treatment with these drugs are receiving adequate concentrations of the drug at all times.

3.7 References

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Chapter 4

Development of an on-line SPE-LC-MS method for the simultaneous determination of anthracyclines and taxanes in human serum

4.1 Introduction

Both anthracyclines and taxanes may be used in tandem to treat certain types of cancer such as advanced cancers of the breast or stomach [1]. Despite the fact that these drugs can be administered simultaneously, until now, no methods have been able to quantify both drug types in a single assay. The previous chapter described work carried out to develop and on-line SPE-LC-UV method for determination of a number of drugs from both classes in a single assay. While the method worked well on all fronts of extracting, separating and detection, it lacked the necessary sensitivity for real therapeutic drug monitoring of such drugs. Detection limits were in the low µg/mL range, whereas ideally sensitivity in the ng/mL range is required for patient samples [2]. Hence it was decided that a mass spectroscopic detector would be required for this purpose and so an on-line SPE-LC-MS method was developed for the determination of anthracyclines and taxanes in human serum.

4.2 Scope of work

The objective of this research was to develop a simple, automated, quantitative method for the simultaneous determination of both anthracycline and taxane drug molecules from human serum using an on-line SPE-LC-MS method. The method of extraction and separation used was similar to that used in the SPE-LC-UV method described in the previous chapter.

The first step involved transferring the extraction and separation parts of the assay over to the new LC-MS instrument. This involved scaling down the analytical column and flow rate. Also, the type of switching valve used in this work was different to the manual valve employed in the UV method. The built-in internal 6-port valve could be triggered electronically by the LC software which enabled an improved level of automation. The development and optimisation of the ionisation and other MS parameters for each drug assayed proved challenging and was not straightforward. However, finally the overall method was developed and patient samples were analysed.

4.3 Experimental

4.3.1 Materials and reagents

Water, acetonitrile, formic acid, ammonium formate, isopropanol (all MS grade) and human serum were purchased from Sigma Aldrich. Doxorubicin, epirubicin, daunorubicin, docetaxel and paclitaxel were all bought from Fluka Chemicals (Ireland). Patient serum samples containing either docetaxel or epirubicin were kindly supplied by Saint Vincent's Hospital (Dublin). Ammonium formate buffer (1M) was prepared by dissolving 3.15g of ammonium formate in 50 mL water. This buffer was used to adjust the aqueous mobile phase to pH 3.5.

4.3.2 Instrumentation

The LC-MS system used in this work consisted of two Agilent binary 1200 (SL) pumps (one for the extraction solvent and one for the analytical mobile phase), a model 1200 autosampler (ALS SL) with a 100 µL injection loop, a thermostatted column compartment (TCCSL), an Agilent 6410 triple quad LC-MS and an on-line Agilent 1200 degasser. A model 1100 PDA UV-Vis detector was coupled to the MS system to monitor the elution of the chemotherapeutic drugs from the analytical column during the transfer of the LC-UV method to the LC-MS. A T-piece and syringe pump, Agilent KD scientific (KDS 100) model were used for direct infusion. The system was equipped with Agilent Masshunter Workstation Software Version B01.03/04 for system control and data collection. A gradient LC system using an acidified water/acetonitrile mixture was used to separate the analytes on an Agilent Zorbax column (50 x 2.1mm i.d, particle size 3.5µm) packed with XDB C18 material. The extraction column used was a Biotrap 500 MS SPE column (20 x 4mm i.d) packed with a hydrophobic polymer material and was coupled to the LC system via a built-in six port switching valve (see Figure 4.1).

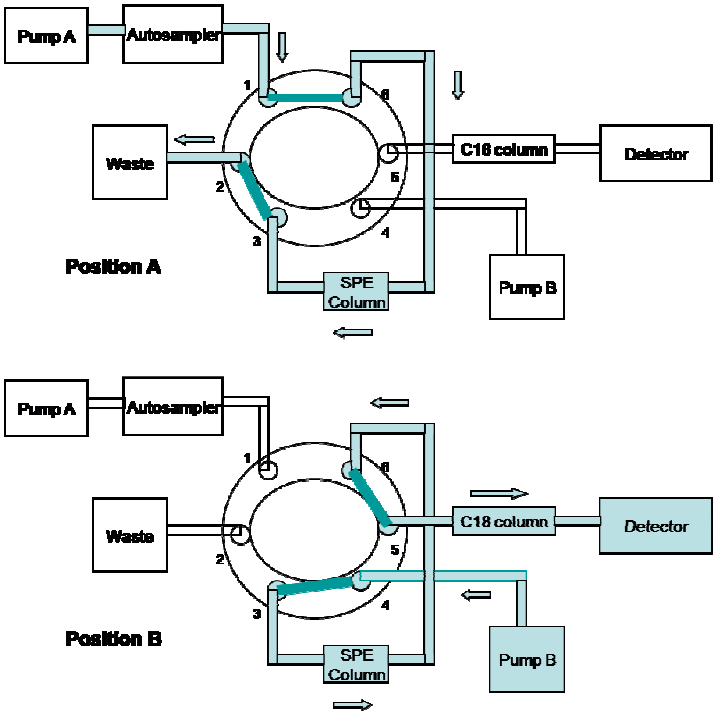

Figure 4.1: Schematic representation of the 6-port switching valve used for on-line sample extraction and the resulting flow paths in the two valve positions.

4.3.3 Preparation of stock and standard solutions

Stock solutions of Epi, Dox, Dnr, Doc and Pac, all 2 mg/mL, were weighed in a glove box and prepared by dissolving the compound in acidified water (adjusted to pH 3.5 with formic acid) for the anthracyclines and in neat methanol for the taxanes. All stock and standard solutions were stored in the refrigerator and protected from light. Standard solutions of the drugs were prepared

(250 µg/mL) monthly by dilution with appropriate solution. Fresh working standard solutions were prepared daily by dilution of the standard solution(s) in the aqueous mobile phase to the concentrations required.

4.3.4 Preparation of serum samples

The 2 mg/mL stock anthracyclines and taxanes were dissolved in thawed drug free serum samples to obtain the required concentrations in undiluted serum. The samples were spiked prior to analysis and were kept cool at 4^0C in the autosampler.

Patient venous blood samples were obtained from patients undergoing chemotherapy in St. Vincent's Hospital, Dublin, Ireland by informed consent at 10 min after administration. The whole blood samples were immediately centrifuged to yield serum. The samples were kept frozen at -20 ^{0}C until analysed.

4.3.5 Sample extraction and clean-up procedure

Sample clean-up was performed on-line using the same Biotrap 500 MS SPE column as in the previous assay. The 10 µL sample (standard solution or human serum) was injected by the autosampler while the valve was set to position A (see Figure 4.1). In this configuration, the extraction solvent (30 mM ammonium formate/acetonitrile, 98/2 v/v, pH 6.8) flowing at 2.0 mL/min for 2.0 min, picked up the injected sample forcing it onto the SPE column and washed any proteinaceous material from the serum to waste leaving the drugs retained on the extraction column. The analytical mobile phase was rinsing the analytical column during this time. The valve was then switched to position B where the analytical mobile phase back-washed the SPE column flushing the retained drugs onto the analytical column where they were separated and detected. The extraction solvent then flowed directly to waste. The valve was then switched back to position A where the extraction solvent washed the SPE column removing any remaining proteinaceous material still on the SPE column readying the system for the next sample injection.

4.3.6 Chromatographic conditions

The gradient programme was set up as shown in Table 4.1**.** The extraction solvent was composed of 30 mM ammonium formate in water/acetonitrile (98/2 v/v), pH 6.8. Mobile phase A was composed of 0.1% formic acid in water/acetonitrile (90/10 v/v), pH 3.5 with ammonium formate. Mobile phase B was composed of 0.1% formic acid in acetonitrile/water (90/10 v/v).

After each serum sample injected the SPE column had to be washed with a high percentage organic solvent to remove any retained sample. An acetonitrile/water (90/10 v/v) mixture was passed through the SPE column for 4.0 min to clean the SPE column prior to the next sample injection. This washing step was incorporated into the method.

Time (min)	Extraction solvent (%B)	Extraction flow (mL/min)	Analytical solvent (%B)	Analytical flow (mL/min)	Valve position
0.00	0	2.0	10	0.3	A
2.00	0	2.0	10	0.3	A
2.01	0	0.3	10	0.3	B
10.00	0	0.3	75	0.3	B
10.10	100	2.0	75	0.3	A
12.00	100	2.0	75	0.3	A
16.00	100	2.0	75	0.3	A
17.00	0	2.0	10	0.3	A
20.00	0	2.0	10	0.3	A

Table 4.1: Extraction and analytical solvent gradient timetable

4.3.7 Mass spectrometric conditions

Different time segments needed to be implemented in the method because the anthracyclines and taxanes were optimally ionised under different conditions. The three anthracyclines eluted first in the same order as they did in the LC-UV method and their ionisation conditions were more gentle than those needed for the taxanes. The gas temperature, fragmentor voltage and collision energies were different depending on which drug class was eluting. The first time segment (Segment 1) was set up with the ionisation conditions for anthracyclines, the second (Segment 2) for the taxanes. For the third time segment (Segment 3), ionisation conditions were reverted back to those used in the first time segment so that all of the conditions were ready for the next sample injection. The ESI (+) mode MS conditions (time segments and source conditions) were set up as described in Table 4.2.

Time Segments	Start time (min)	Scan type	Valve (to)	Delta EMV(+)	Delta EMV (-)	Transitions	Gas Temp (^{0}C)	Collision energy	Fragmentor voltage
1	0.0	MRM	MS	400	0	544 → 526 +397 and 528 → 321	300	10	80
2	8.5	MRM	MS	400	0	846 → 565 +320 and 892 → 607 + 324	350	25	180
3	16.0	MRM	MS	400	0	544 → 526 +397 and 528 → 321	300	10	80

Table 4.2: Time segment conditions for each drug class

<u>Mass spectral conditions that remained the same throughout the run were as follows:</u>

Gas flow: 10 L/min
Nebuliser: 50psi
Capillary (+): 4000

Capillary (-): 4000

4.3.8 Calibration

Calibration curves were prepared in drug-free serum spiked with stock solutions of each analyte. The concentration range tested was between 2.0 ng/mL and 500 ng/mL. Standards were prepared at concentrations of 0, 2.0, 5, 10, 50, 100 and 500 ng/mL in water/acetonitrile (90/10 v/v). Calibration curves were determined by plotting peak area versus spiked drug concentrations.

4.3.9 Method validation

A full validation procedure was planned for the method including intra-day precision (repeatability) and inter-day precision (intermediate precision), accuracy, sensitivity (LOD and LOQ), linearity and range and recovery according to accepted guidelines [3]. Some further parameters were also monitored such as stability following freeze-thaw cycles [4].

4.4 Results and discussion

4.4.1 Method development

A method that could extract, separate and detect both anthracyclines and taxanes in a single assay had been developed using a UV detector but required transfer over to a different instrument with a mass spectrometric detector. The extraction parameters required further changes on the new instrument and separation conditions were modified due to the reduced dimensions of the analytical column used. The ionisation conditions and fragmentation patterns of the chemotherapeutic drugs had to be determined in a logical format so that the best sensitivity could be achieved by tandem MS.

4.4.2 Extraction conditions

4.4.2.1. SPE cartridge

There was no change to the SPE column or its dimensions in the method transfer. The SPE protocol was essentially the same prior to the LC-MS method as it was when used prior to the LC-UV method. The high extraction flow rate of 2.0 mL/min determined to be the optimal extraction solvent flow in terms of recovery was also suitable following method transfer.

4.4.2.2. Extraction solvent

There were no changes to the composition of the extraction solvent used to extract the chemotherapy agents, but a cleaning step was introduced so as to rinse the SPE column of any adhering serum components following an injection. For 4 min after a run, once all the drugs had

been back-washed off the SPE column onto the analytical column, the SPE column was rinsed with a high percentage organic solvent (acetonitrile/water 90:10 v/v). The other important change that was implemented was a 2.0 min extraction time was used in the MS method (as opposed to 2.5 min) because of the fully automated SPE protocol with 6-port column switching.

To perform on-line SPE on the Agilent 1200 system, two Agilent 1200 binary pump were needed, one to pump the analytical mobile phase and the other to pump the extraction mobile phase. The original LC valve configuration had to be completely changed to accommodate the on-line extraction. A built-in 6-port valve was used to extract and separate the drugs. A schematic of the switching valves is shown in Figure 4.1 above.

4.4.3 Separation conditions

4.4.3.1. LC column

A scaled-down version of the Agilent Zorbax XDB (150 x 4.6mm i.d, particle size 5µm) column used in the LC-UV method was used to separate the target analytes in the MS method. The downsized column had the dimensions (50 x 2.1mm i.d. particle size 3.5µm)**.** It was assumed that this column would be capable of separating the five chemotherapy drugs sufficiently when used at a lower flow rate and by scaling the gradient appropriately. This type of column already proved appropriate for separating all drugs including the epimers; Dox and Epi which were well resolved. If the Agilent Zorbax XDB column used in the UV method had been used here in the MS method**,** the pump would have had to deliver 1.0 mL/min to maintain the same separation as before. At this high flow rate it would have been necessary to split the flow before entering the MS detector. This setup would also have resulted in unnecessary wastage of solvent.

4.4.3.2. Analytical mobile phase and UV detection

The same analytical mobile phase that was used in the UV method was used here in the MS method and it proved to be very effective in separating the five drugs. The pH stayed the same (pH 3.5) as it was determined from the UV method that the anthracyclines were most stable at this pH and there was good resolution of all analytes at this pH. A PDA UV detector was used in series with the MS initially and it was very useful during initial method development to ensure that all drugs were being well extracted and well separated. It provided reassurance during the method transfer process.

Once each of the five drugs were optimised in terms of finding their optimal ionisation conditions, the analytical column was coupled to the system so that the gradient for their separation could be optimised and the time segments for changing the mass spectrometric conditions for analysis of the different drug types determined.

Many of the conditions employed in the LC-UV method were used as a starting point for the LC-MS method. The gradient conditions did need to be changed however, as did the flow rate through the narrow bore column. A useful approximation when converting flow rates from 4.6 mm i.d. at 1.0 mL/min to lower dimension columns is:

$$\text{Flow} = (\text{new column i.d.} / \text{old column i.d.})^2$$

Example: $\text{Flow} = (2.1 / 4.6)^2 = 208$ µL/min

This relationship can be used to estimate the flow rate required for an equivalent separation on a downscaled column. A range of flow rates from between 0.1 and 0.3 mL/min were evaluated. It was determined that the optimal flow rate in terms of separation and speed was obtained at 0.3 mL/min.

Figure 4.2 below shows the separation of a 0.25 µg/mL mixture of all five drug compounds dissolved in mobile phase, injected onto the Zorbax XDB (50 x 2.1mm i.d, particle size 3.5µm) column and separated using the gradient shown in Table 4.1. It can be seen that all drugs are well resolved from one another. Although closely eluting, the epimers Dox and Epi can also be easily distinguished from one another (and from any other closely eluting compound) by examining their unique fragmentation patterns discussed in section 4.4.4.2.

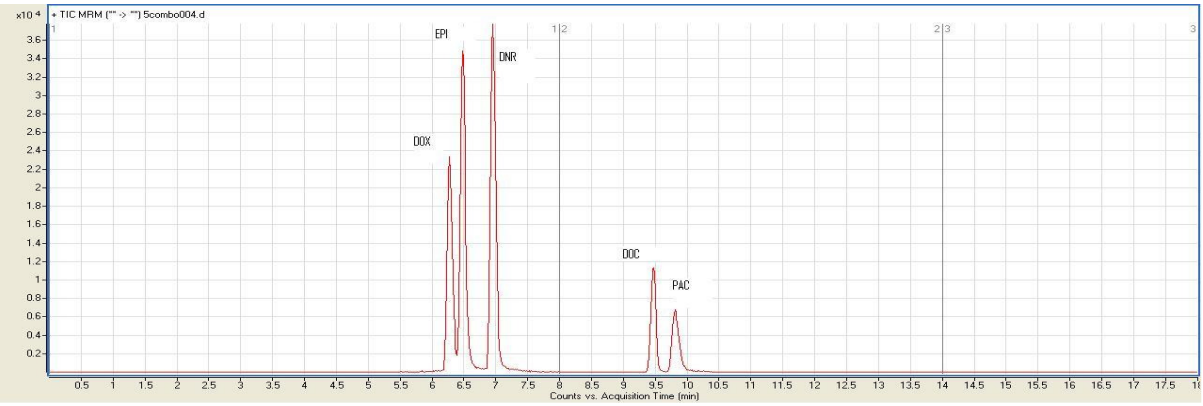

Figure 4.2: A total ion current (TIC) scan of the separation of a mixture of all five drugs (each at 0.25 µg/mL) prepared in mobile phase

4.4.4 Mass spectrometric conditions

There are a number of steps to follow when developing a method for the determination of drugs in biofluids using an MS detector. Different drugs have different ionisation properties so in order to determine their optimum ionisation conditions correctly these steps have to be followed.

Initial work on the development of the MS part of the method involved directly infusing a small quantity of a single chemotherapeutic drug prepared in mobile phase (MS grade) to determine its precursor ion, its optimum conditions for fragmentation (fragmenter voltage), to determine its product (daughter) ions and to determine its optimum collision energy and gas temperature. This process was repeated for each of the individual drugs tested (Dox, Epi, Dnr, Doc and Pac). See Table 4.3 for the final ionisation conditions.

Once these had all been accurately determined it was prudent to determine whether there were any ion enhancement or suppression effects in the sample matrix.

4.4.4.1. Precursor ion scan

A single chemotherapeutic drug agent was made up in mobile phase at a concentration of 0.5 µg/mL (very concentrated for MS analysis so that the precursor (parent) mass ion could be easily established) and this sample was then infused directly from the autosampler to the mass

spectrometer where a TIC scan was run in order to establish the predominant precursor ion. The range was set so that it bracketed the molecular weight of the compound under analysis. The range used in this work was from *m/z* 100 to 1000. Once a large peak was seen at the expected *m/z* value (protonated, sodiated or potassiated adduct), this was deemed to be the precursor ion.

4.4.4.2. Optimising fragmentation voltages for precursor ions

Once the precursor ion had been established the next step was to determine the optimum fragmentation conditions for that precursor ion. The mass spectrometer was programmed to perform an MS selected ion monitoring (SIM) scan which detects only the selected precursor ion so that the optimum fragmentor voltage for that ion could be determined. These SIM scans were done in series where the fragmentor voltage was varied over a range of 0 to 300V. Initially tested in large fragmentor voltage increments of 40V, when the approximate optimum fragmentor voltage was known (between 70 and 90V and between 170 and 190V for anthracyclines and taxanes respectively), the increments were lowered to get a more accurate fragmentor voltage for each drug analysed (80 and 180V for anthracyclines and taxanes respectively).

The taxanes required higher fragmentation voltages compared to the anthracyclines. This was expected as taxanes are more lipophilic and are quite difficult to ionise unless high voltages are used. Anthracyclines, on the other hand are quite fragile molecules with stability issues (anthracyclines are very sensitive to light and pH) and hence needed lower voltages for ionisation to occur. There is a sugar moiety present in all anthracycline molecules and this moiety easily falls off at high voltages and temperatures so it was important to have strong enough conditions to enable efficient ionisation but not so strong as to allow in-source fragmentation.

4.4.4.3. Product ion scan

When the optimum fragmentor voltage for that drug had been established based on ion sensitivity, a product ion scan was carried out in order to obtain the product (daughter) ions of that selected precursor ion. The product ion scan test worked by firstly isolating and then fragmenting the chosen precursor ion with varying collision energy values. This was achieved in a similar fashion to the way the precursor fragmentor voltage was acquired but the collision energy range was much lower (0 to 80V). The product ions were determined by analysing the fragments produced on collision and again determining which were predominant and specific for the precursor ion under examination. See Figure 4.3 (A-E) for examples of precursor and product ion scans for each chemotherapeutic drug tested during direct infusion method development experiments.

Figure 4.3 shows a typical precursor ion scan and a product ion scan for each of the five drugs analysed in this work. This was an important step in the method development stage of this on-line SPE-LC-MS method.

It can be seen from Figures 4.3(A) and 4.3(B) that the epimers Dox and Epi produce different product ions in the collision cell. Epi produced products ions at *m/z* 397 and 526 whereas Dox only has a product ion of *m/z* 397. It would have been impossible to distinguish between them in an MRM scan if their product ions were the same but this difference in their fragmentation patterns allowed for unambiguous identification. Hence, *m/z* 397 was used as the

product ion for Dox identification and *m/z* 526 was used as the product ion for Epi identification. The product ion *m/z* 544 corresponds to the [M+H$^+$] adduct of both Dox and Epi.

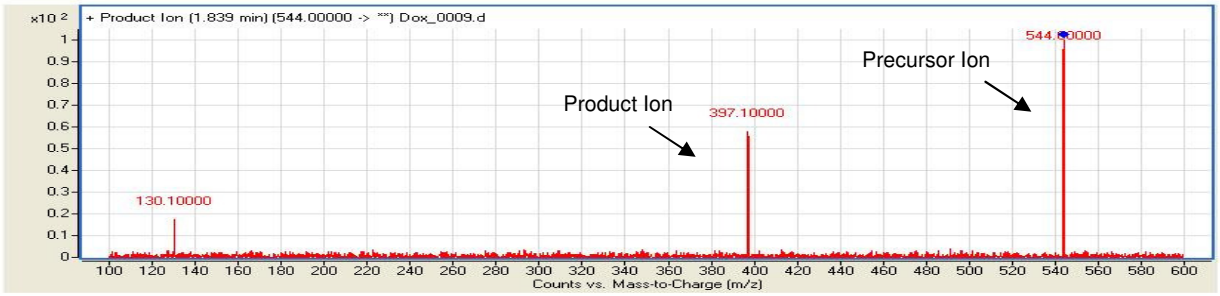

Figure 4.3 (A): MRM scan showing the precursor ion and product ion [M+H$^+$] of Dox

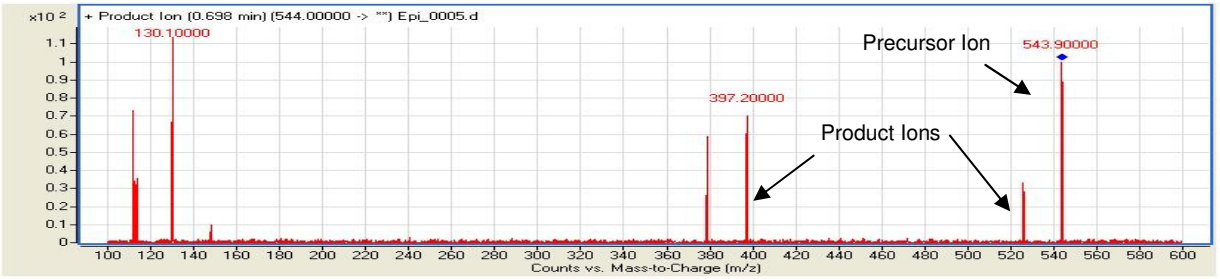

Figure 4.3 (B): MRM scan showing the precursor ion and product ions [M+H$^+$] of Epi

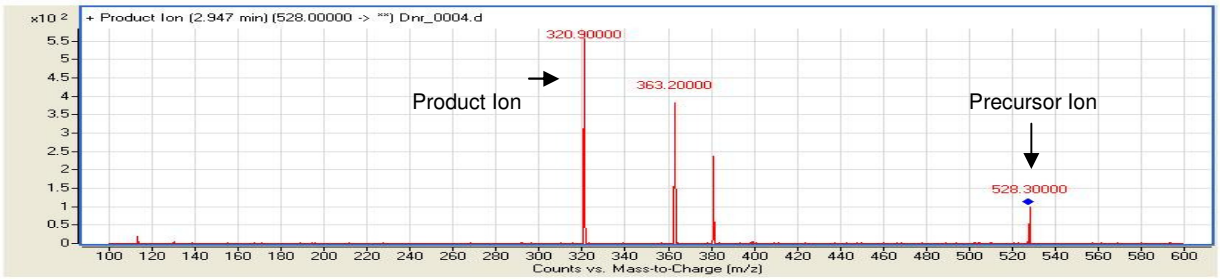

Figure 4.3 (C): MRM scan showing the precursor ion and product ion [M+H$^+$] of Dnr

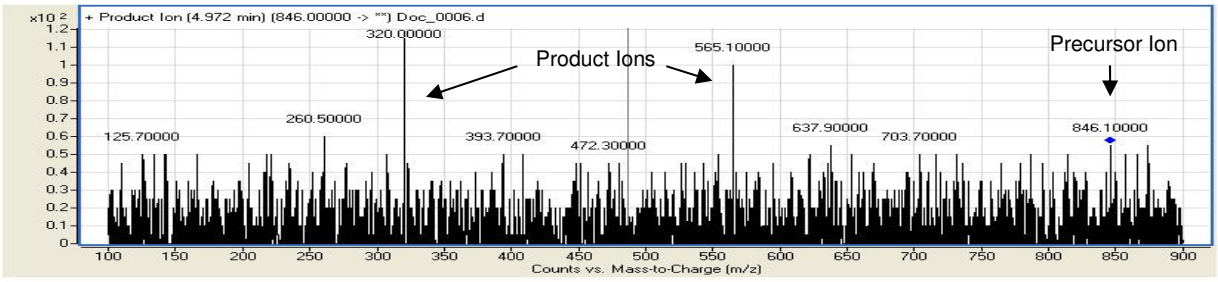

Figure 4.3 (D): MRM scan showing the precursor ion and product ions [M+K$^+$] of Doc

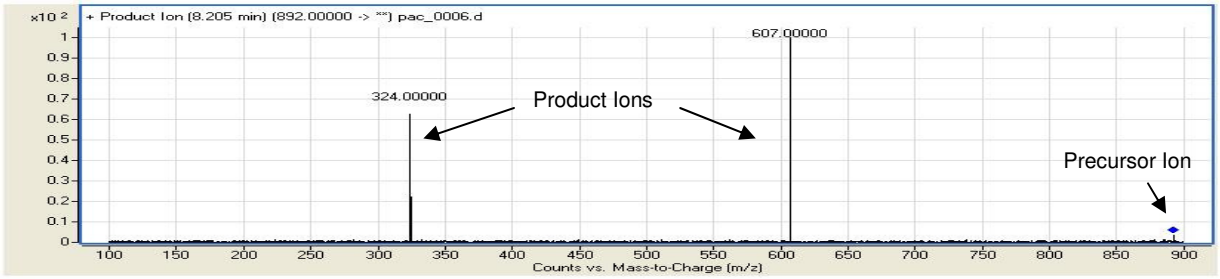

Figure 4.3 (E): MRM scan showing the precursor ion and product ions [M+K^+] of Pac

Figure 4.3 (C) shows the precursor and product ions for Dnr. The protonated precursor ion for Dnr was found at *m/z* 528 with the major product ion determined to be *m/z* 321.

It is evident from Figures 4.3(D) and 4.3(E) that Doc and Pac are not easy to ionise even with high voltages. The intensities of the main precursor ions at 846 and 892 respectively (both K^+ adduct ions) are relatively low compared with the background ions. However, the isolation step prior to fragmentation aids sensitivity for tandem MS. Doc produced two major product ions at *m/z* 565 and 328. Pac also produced two major product ions at *m/z* 607 and 324. Both products ions were easily identified in every separation.

These fragmentation patterns can also be described molecularly. Dox has a product ion at *m/z* 397; therefore the fragmented piece has a molecular mass of 147. This can be explained by the loss of the sugar moiety and a hydroxyl group. Similarly, Epi has a product ion at *m/z* 526 which would result in the loss of a water molecule from the precursor ion. Dnr transition is from *m/z* 528 to 381 which suggests a fragmented piece with a mass of 147 could be explained by the loss of the sugar moiety and a hydroxyl group. Doc and Pac have transitions of *m/z* 846 to 526 and *m/z* 892 to 607 respectively. These can be explained by the cleavage of the large side chains at C^{15} position of the 10 membered carbon ring. The side chain on Doc has a molecular mass of 280 which explains the transitions of *m/z* 846 to 526 and the side chain on Pac has a molecular mass of 284 which explains the transitions of *m/z* 892 to 607.

4.4.4.4. Optimising collision energy conditions for product ions

Repeated multiple reaction monitoring (MRM) scans which detected the MS/MS transitions were carried out in order to obtain the optimum collision energy for fragmentation of a particular drug to a particular product (daughter) ion. Unlike the product ion scan protocol, in this case collision energy was raised in increments of 5V (from 0 to 5 to 10 etc…) so that a more accurate collision energy could be established. The collision energy that gave the most intense peak for the chosen product (daughter) ion was deemed to be the optimum value.

The taxane product ions required higher collision voltages compared to the anthracycline ones as taxanes are more difficult to ionise and it was predicted that their product ions would behave in a similar manner. The collision energy, fragmentor voltage and gas temperature were all significantly higher for taxane analysis showing that it was much harder to fragment the larger molecular weight molecules.

The sugar moiety that often fragments from anthracycline molecules can be observed in Figure 4.3 above (the product ion with an *m/z* of 130). This molecular weight corresponds

exactly to the base sugar moiety present in many anthracycline drugs. The gas temperature had to be lowered to 300 ^{0}C for the anthracyclines because they were being broken down at the higher temperature before the precursor ions could be established. The final product ions of choice for Dox, Epi and Dnr respectively were at *m/z* 397, 526 and 321 (see Figure 4.4).

In Figure 4.4 the first scan is of a TIC MRM scan showing the separation of each of the five chemotherapy drugs analysed. Directly below it (A) shows the transition from 544 to 397 shared by both Dox and Epi ((A) is the same as (C) transition from 544 to 397 as it is shared by both Dox and Epi). Transition (B) is from 544 to 526, related only to Epi and it can be seen from figure 4.4 that is directly below the Epi peak in the TIC MRM. Transition (D) is from 528 and 321 which correlates to Dnr. Transitions (E and F) are from 892 to 324 and 607 respectively, these transitions are from Pac and finally the transitions from 846 to 320 and 565 respectively are related to Doc and it can be seen from the figure that they are directly under the Doc signal in the TIC MRM.

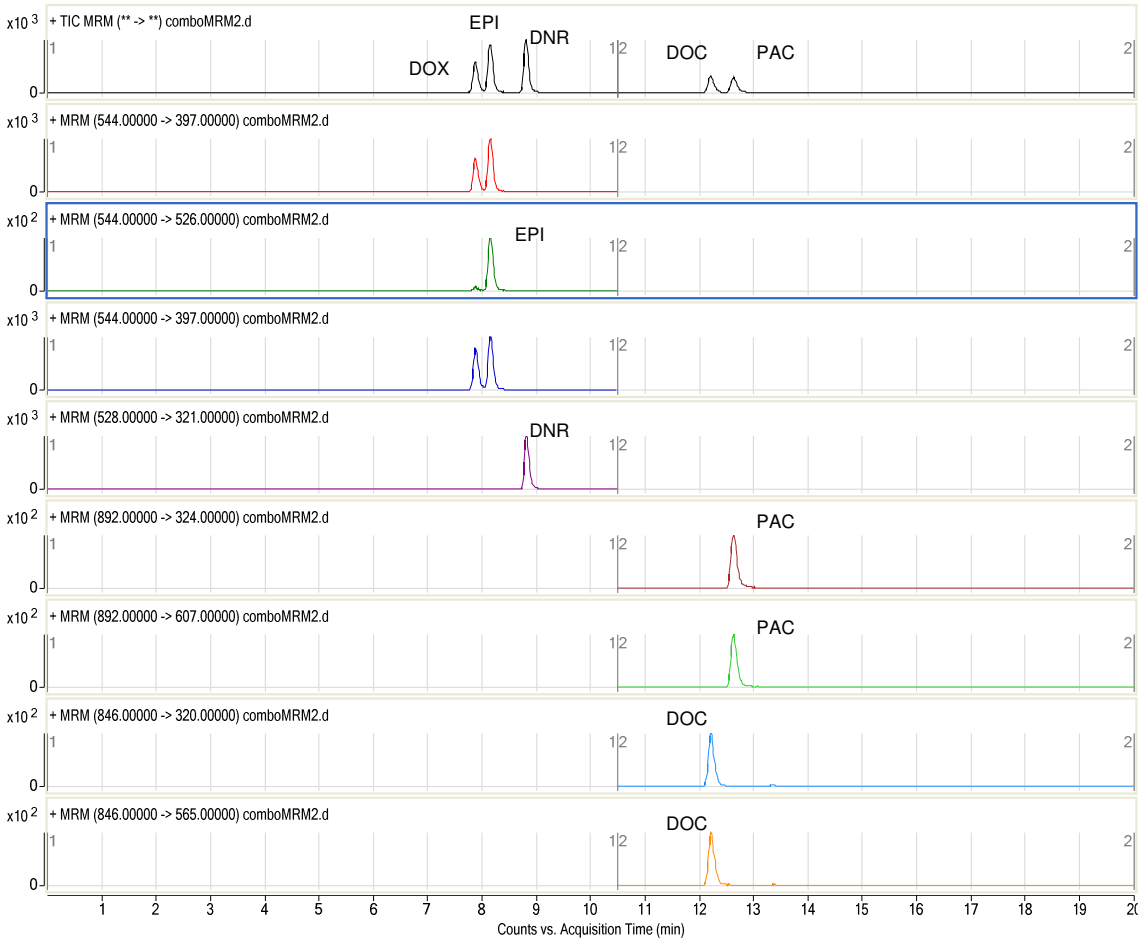

Figure 4.4: Shows an MS scan of the five separated drug compounds and the resultant MS^2 (product ion scan) for each drug under the optimal experimental conditions.

4.4.4.5. Gas temperature

An optimum gas temperature within the ionisation source had to be established for each drug. For fragile compounds, it may be appropriate to keep this low while raising it can improve LC

solvent evaporation and the ionisation process. The optimum gas temperature for the anthracyclines (300 ^{o}C) was found to be slightly lower than the gas temperature required for the taxanes 350 ^{o}C. This is because the fragile sugar moiety could easily break off the anthracycline structure. The final conditions used in terms of collision energies, fragmentation volatages, gas temperatures and precursor and product ions is shown in Table 4.3.

Compound	Precursor ion	Product ion	Collision energy	Fragmentor voltage	Gas temp (^{0}C)
Dox	543/[M+H^{+}] 544	397	10	80	300
Epi	543/[M+H^{+}] 544	526 + 397	10	80	300
Dnr	527/[M+H^{+}] 528	321	10	80	300
Doc	808/[M+K^{+}] 846	565 + 320	25	180	350
Pac	854/[M+K^{+}] 892	607 + 324	25	180	350

Table 4.3: Optimum positive mode ESI conditions for each target analyte

4.4.4.6. Ion enhancement issues

Biological matrices such as plasma, serum and urine have been known to cause ion suppression or enhancement effects in mass spectrometric experiments. The main cause of ion suppression with ESI-MS is a change in the spray droplet solution properties caused by the presence of non-volatile or less volatile solutes. Non-volatile materials (endogenous serum components) cause a change in the efficiency of droplet formation which can affect the amount of charged ion in the gas phase that will reach the detector [5] To test for such effects, a direct infusion experiment [6] was performed to see what effect (if any) the serum matrix was having on an MRM scan. An aliquot of a single drug sample in serum had to be directly infused into the MS chamber to determine what effect, if any, the sample matrix had on the MRM scan for that particular drug.

An aliquot of human serum (the sample matrix used) was injected from the autosampler going directly into the MS. A T-piece was put in place before the MS chamber so that an aliquot of a single chemotherapy agent used in the separation (e.g. Dox) could be directly infused (see Figure 4.5) to mix with the serum just prior to the MS when required.

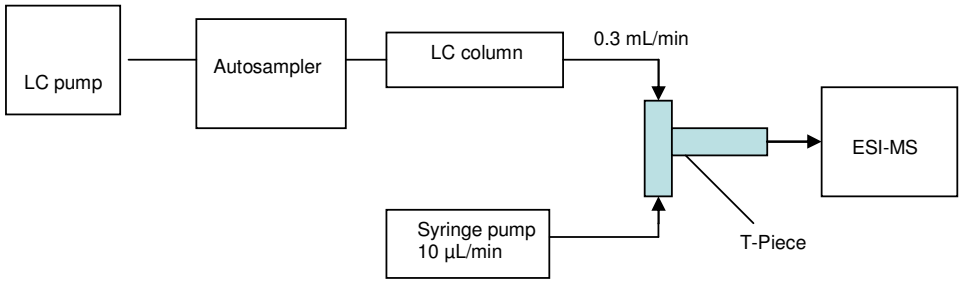

Figure 4.5: Schematic representation of experimental set-up to test for ion enhancement or suppression effects

The matrix profile should remain relatively constant after an initial ‘solvent front’. A large dip in the matrix profile at any point after the ‘solvent front’ indicates that there is an ion suppression effect at that point; a large increase in the matrix profile at any point after the ‘solvent front’ indicates that there is an ion enhancement effect at that point. Ion suppression

would result in an unexpectedly low concentration of analyte in the matrix sample compared to the sample in mobile phase and vice versa for ion enhancement. This experiment was carried out for all five drugs being studied and an ion enhancement effect was found only for Dnr (see Figure 4.6). No ion suppression effects were found.

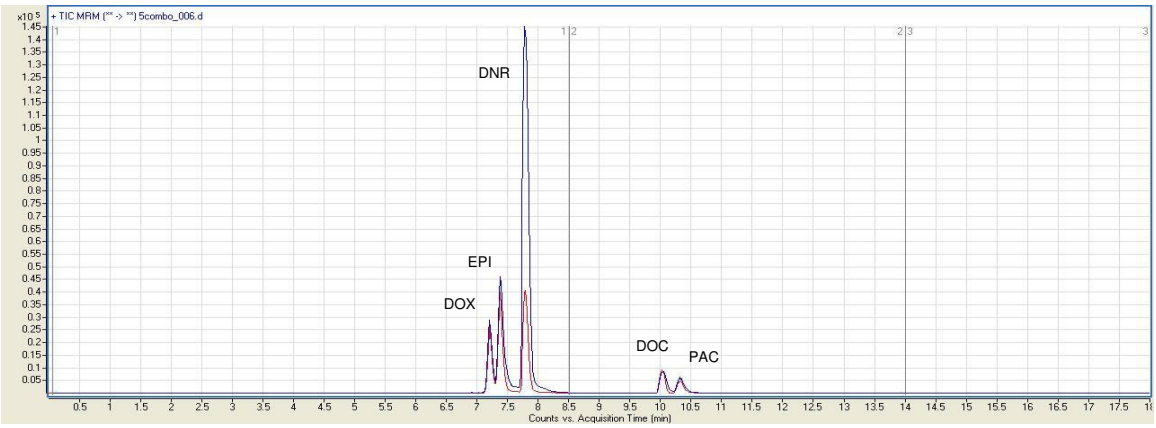

Figure 4.6: MRM scan of a mixture of the five drugs prepared in mobile phase (red trace) overlaid on a mixture of the five drugs prepared in serum (blue trace). It is clear that the peak for Dnr is higher than it should be in the serum matrix.

It can be seen from Figure 4.6 that each of the five drugs are well separated from each other but that the concentration of Dnr in the serum sample appears to be about twice that of Dnr in the mobile phase sample even though both mixtures were made up to the same concentration of 0.25 µg/mL. Many modifications were made to the solvent gradient to see if the ion enhancement of Dnr could be avoided but no gradient could reduce this effect.

4.5 Method Validation

A full validation procedure was planned for the method according to the guidelines in Shah *et al*[3] including intra-day and inter-day precision, accuracy, sensitivity, linearity and range and recovery. Stability following freeze-thaw cycles was also investigated.

4.5.1 Precision and accuracy

Intra- and inter-day accuracy and precision were to be determined from standards prepared in serum over a concentration range of 2.0 ng/mL to 500 ng/mL. Precision would be expressed as a (%RSD) and accuracy would be expressed as the ratio of the mean calculated concentration against the spiked concentration for each standard multiplied by 100% in the same way as calculated in chapter 3. Intra day precision and accuracy was to be determined by preparing seven different standards (0, 2.0, 5, 10, 50, 100 and 500 ng/mL) and analysing in triplicate.

A problem occurred within the SPE-LC-MS system each time this method validation step was attempted. There was a gradual increase in system backpressure with every injected serum sample. Initially the pressure increase was minimal (1 or 2 Bar increase per injection) and did not affect system operation but with more spiked serum samples injected the pressure increase became more pronounced. Eventually this backpressure increase caused distortions in analyte retention time and finally caused the system to shut down because system pressure limits had been reached. Most likely there was a problem with the sample clean-up stage, some serum components must have survived the extraction step and were being flushed onto the head of the analytical column where it gradually built up causing the blockage and retention time shift. Initial pressure on the column before validation was ~90 Bar, when the pump was restarted the pressure had risen by almost 100 Bar to ~190 Bar.

The theory that something was surviving SPE extraction seemed to be confirmed when the head of the analytical column used in this validation attempt was cleaned before injecting another serum sample onto the cleaned column. The top end of the analytical column was screwed off and sonnicated for 45 min before being tightly reattached. The column was then put back into the LC-MS setup and a serum sample injected through it. The pressure through the analytical column had reverted back to about 100 Bar. Column performance after this had unfortunately been changed with a retention shift for all analytes. This problem occurred even after extensive troubleshooting and the purchase of two more new analytical columns and a new extraction column.

4.5.2 Sensitivity

The LOD and LOQ were defined as the lowest concentration of the five target analytes that could be detected by the MS in serum samples. The LOD was set at a concentration that gave a signal to noise ratio of 3:1. The LOQ was set at a concentration that gave a signal to noise ratio of 5:1. These values were obtained by determining the level of background noise at and nearby where each target analyte eluted and determining the concentration of that drug that gave a signal to noise ratio of at least 3:1 or 5:1 for LOD and LOQ respectively. LOD values were 1.0, 1.0, 1.5, 1.0 and 1.0 ng/mL for Dox, Epi, Dnr, Doc and Pac respectively. The LOQ for each drug was determined to be 2.0 ng/mL. These values compare very well with Yang [7] and Alexander [8] *et al* who used tandem MS in the determination of either anthracyclines (Yang) or taxanes (Alexander) individually.. The limiting factor in determining the LOQ was determining the precursor ions of Dox and Epi. The transition from 544 to 526 is unique to Epi and is the only way to distinguish between the two epimers. At concentrations lower than 2.0 ng/mL this transition was not observed.

4.5.3 Linearity and range

Linearity and range was determined by running calibration curves. Calibration curves were prepared in drug free serum spiked with stock solutions of each analyte at concentrations in the range of 2.0 to 500 ng/mL. All calibration curves proved to be linear over the tested concentration range. The regression coefficients (R^2) values for each calibration curve were >0.99 in all cases. A single calibration curve could be run over the concentration range without

the backpressure increasing substantially by injecting each sample individually. There was still a backpressure increase but it was less dramatic and not so severe when only seven serum samples were injected.

4.5.4 Recovery

Analyte recovery was determined in the same way as it was in the LC-UV method described in chapter 3. On the same day, standards at concentrations of 5, 100 and 500 ng/mL were prepared three times for each concentration and then analysed in triplicate. The analyte peak areas obtained for spiked serum samples were compared to analyte peak areas obtained for spiked samples in mobile phase after SPE extraction. Recovery of Dox, Epi, Doc and Pac were very good ranging from 95 to 113% and compared well with work done by Arnold [9] and Parise [10] *et al* who obtained high recovery in their methods to extract anthracyclines (Arnold) and taxanes (Parise) from complex biological matrices. Recovery of Dnr was about twice that of every other analyte investigated. This can be explained by the ion enhancement effect as described in Figure 4.6. See Table 4.4 for recovery data.

Compound	5 ng/mL Spiked Conc.	100 ng/ml Spiked Conc.	500 ng/ml Spiked Conc.
Dox	105.4	110.6	102.3
Epi	114.7	108.1	112.7
Dnr	210.5	218.2	205.3
Doc	98.3	104.1	97.6
Pac	94.6	98.3	94.9

Table 4.4: Recovery data for the five drugs in human serum

4.5.5 Stability

The stability of each drug was to be determined by freeze-thaw cycles as it was in the LC-UV method. The concentrations of the drugs that were to be analysed under freeze-thaw cycles were 5, 100 and 500 ng/mL. These samples were run once and frozen (samples ran on the first day of the 'precision and accuracy' testing. After precision and accuracy tests failed (blocked column) the column performance had diminished so stability testing was not completed.

4.5.6 Patient samples

Patient samples containing both Epi and Doc were kindly supplied from St. Vincents Hospital, Dublin. For the same reasons as those explained under the stability section of this validation protocol, despite repeated attempts at preparation and analysis, the patient samples were never fully analysed. Column performance had diminished and due to time constraints the assay was not able to be tested fully with clinical samples.

4.6 Conclusions

An on-line SPE-LC-MS method was developed that was capable of quantifying two very different types of chemotherapeutic agents in a single assay. The developed method is capable of extracting, separating and detecting the five chemotherapy drugs of interest in a rapid (20 min) assay. The detection limits of this method were an order of magnitude lower than the limits obtained with the UV method. The LOQ was determined to be 2.0 ng/mL. Rates of detection this low are suitable for therapeutic drug monitoring. Detection limits could have been lower if either Dox or Epi were omitted as quantitation of both the epimers Dox and Epi in the one assay was challenging. A patient would never be given a combination of these two drugs during therapy however so one of these anthracyclines could be left out in order to get lower detection limits for the other epimer.

There appeared to be a problem with the extraction protocol when coupled to the LC-MS in this method which resulted in a pressure increase over time with multiple injections. Endogenous compounds in the serum sample seemed to survive the extraction wash and caused a blockage at the analytical column. This problem may possibly be overcome by increasing the length of SPE rinses or by further investigating the column switching timetable. Extensive troubleshooting carried out at the time of the problem did not fix the pressure issue. A pre-column was placed in front of the analytical column to see if it could 'catch' whatever was causing the blockage but it seemed to be too porous because the pressure problem persisted. One possible solution that could be tried in the future would be to change the analytical column to one with similar dimensions to the column used in the LC-UV method (Zorbax XDB (150 x 4.6 mm, 5 µm i.d.)) and use the same gradient and flow rate as that used in the LC-UV method. As 1.0 mL/min would be too fast a flow rate to enter the MS, the flow could be split before entering the MS chamber so that a slower flow would go to the MS. A second option for avoiding the pressure problem would be to change the type of SPE column but this would obviously involve a lot more method development work.

Although the SPE-LC-MS method could not be fully validated due to pressure problems, initial data was very good. Accuracy and precision values could not be evaluated but LOD, LOQ and recovery results were excellent. Also, the overall assay was found to be easy due to being fully automated, fast since it took only 20 min for extraction, separation and detection and less prone to error due to removing labour-intensive sample pretreatment steps. Finally, this is the first time to the author's knowledge that one assay can determine both classes of chemotherapeutic drug simultaneously.

4.7 References

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Chapter 5

Overall conclusions and further work

5.1 Overall conclusions and further work

Both anthracyclines and taxanes may be used in tandem to treat certain types of cancer such as This thesis has discussed the development and validation of methodology for the determination of the anthracycline and taxane classes of chemotherapeutic drugs. The aim was to create reliable, efficient and fast methods for analysis of these drugs in human serum and to apply, where possible, novel, fast extraction and detection techniques in the determination of these drugs. The main research objectives at the outset were:

- To develop and validate an on-line SPE-LC-UV method for the simultaneous determination of anthracyclines and taxanes in human serum
- To develop and validate an on-line SPE-LC-MS/MS method for the simultaneous determination of anthracyclines and taxanes in human serum

Most of these objectives were achieved. In chapter 3, the first reported SPE-LC-UV method for the determination of anthracyclines and taxanes in human serum has been described. The method was successful in that it could quantify both drug types in a quick reliable manner. Analyte recovery from human serum was high (86 to 117 %) within the concentration range tested. The method also has the potential for high sample throughput. The only drawback of this method was its sensitivity. With detection limits only in the low µg/mL range, therapeutic drug monitoring of these drugs would not be an even though Epi and Doc could be seen in patient samples taken directly after administration.

The SPE-LC-UV method was developed, optimised and fully validated. A manuscript describing this work has been submitted to the *Journal of Separation Science* and is currently under review.

The above method was then transferred to a system with a mass spectrometric detector and this work was described in chapter 4. Sensitivity with this method was about three orders of magnitude lower (low ng/mL) in serum than that achieved when the UV detector was used. Detection limits with the MS detector where at about 2.0 ng/mL for drug samples in serum. Therapeutic drug monitoring of both drug class would be easily achieved at this level of detection. This method also offered more reliable identification of parent drugs and fragment ions and could be used in the future for monitoring metabolites in pharmacokinetic and pharmacodynamic studies.

Unfortunately, this method could not be validated due to a pressure increase on the analytical column after repeated serum injections. Exactly what was causing this pressure increase could not be determined in time before this research was completed. It seemed as if a component of the serum was surviving the extraction process and blocking the top of the analytical column. However, during pre-validation work on this method this blockage did not occur when numerous serum samples were passed through the system.

Future work would start with full validation of the SPE-LC-MS method and evaluation of clinical patient samples for therapeutic drug monitoring.

It would be interesting to investigate some newer extraction approaches for the anthracyclines and taxanes such as magnetic beads. A recent preliminary study in the group yielded very promising results for simultaneous extraction of both drug classes in a very short time with high recoveries[1]. Another approach could be the use of 96 well plates for high

throughput supported liquid membrane extraction of the compounds. As the drugs have been previously isolated successfully from serum using liquid-liquid extraction, this protocol should be effective.

It would also be worthwhile to look at other separation techniques for the anthracyclines and taxanes, in particular capillary electrophoresis. This would have the advantages of smaller sample volume requirements, fewer issues with blockages of the capillary from biofluids and less need for organic solvents.

Ultimately, these methods and their speed and automation are important in the cancer treatment arena since their availability offers more possibilities to clinicians in terms of therapeutic drug monitoring, pharmacokinetic studies, clinical trials and indeed, individualisation of patient dosage. While there is still much to do in the area of cancer research, any new analytical research, such as that described in this thesis, will add to the knowledge already there and hopefully will benefit patients.

5.2 References

1. Olayinka A, Hernandez-Santana A, Bermingham S and McMahon G. Development of a novel method for magnetic bead extraction of Taxanes from serum followed by separation and detection by liquid chromatography. *Journal of Undegraduate Chemsitry Research*, currently under review

Appendices

Appendix 1: Publications

1. Published paper

- Callaghan A, Bermingham S and McMahon G. Development of an on-line clean-up method for extraction of two anti-cancer drugs from serum, *Journal of Undergraduate Chemistry Research*, **2008, 7(1), 152**

2. Submitted papers

- Bermingham S, O'Connor R, Regan F and McMahon G. Simultaneous determination of anthracyclines and taxanes in human serum using on-line sample extraction coupled to high performance liquid chromatography with UV detection. Submitted to the *Journal of Separation Science*, currently awaiting review.

- Olayinka A, Hernandez-Santana A, Bermingham S and McMahon G. Development of a novel method for magnetic bead extraction of Taxanes from serum followed by separation and detection by liquid chromatography. *Journal of Undergraduate Chemsitry Research*, currently under review

Appendix 2: Conference presentations

Work carried out throughout this thesis was presented in poster form at numerous scientific conferences throughout Europe:

1. Early research was presented at the *Analytical Research Forum (ARF)* on 16-19th July, 2007. This conference took place in University of Strathclyde, Glasgow
   Poster title: Development of a novel LC/MS method for anthracycline and taxane drugs with on-line sample extraction.
   Authors: Zalma C, Bermingham S, Regan F, McMahon G.

2. A poster was presented at the *HTC, ExTech 10* conference held in Bruges, Belgium on Jan 28th to Feb 1st , 2008.
   The poster title was: On line sample preparation of biological samples for determination of cancer chemotherapy drugs.
   Authors: Bermingham S, McMahon G and Regan F.

3. Another poster was presented at the *5th Biennial Conference on Analytical Sciences in Ireland.* This analytical conference was held at the Waterford Institute of Technology, Waterford on the 7th May, 2008.
   Poster title: Advanced analytical methods for separation of cancer chemotherapy drugs.
   Authors: Bermingham S, O'Connor R, McMahon G and Regan F.

4. The latest research was presented at the *60th Irish Universities Chemistry Research Colloquium* on 11- 13th June 2008. The conference was held at University College Cork, Cork.
   Poster title: Column switching for extraction and separation of cancer chemotherapy drugs.
   Authors: Bermingham S, O'Connor R, McMahon G and Regan F.

5. This research was publically presented was at the *Analytical Research Forum* (ARF) on 21- 23rd July, 2008. The ARF was held at the University of Hull, UK.
   Title: On-line SPE-LC analysis of cancer chemotherapy drugs.
   Authors: Bermingham S, O'Connor R, McMahon G and Regan F. Callaghan A.

Each conference and seminar attended proved very useful and insightful. It provided a great opportunity to network with fellow professionals and to gain an understanding of what type of scientific research, especially analytical research was being developed in Ireland and around Europe. Presented posters were well received at each conference attended

Appendix 3: Training received

Throughout this research masters degree I received both formal and in-house training on a variety of analytical instruments. I was also trained a little on my teaching skills.

- There was an in house LC training programme given by my supervisor Dr. McMahon in Sep 2007 at Dublin City University which was designed to familiarise myself with the LC software and basic running of the instrument.

- I was trained in Dublin City University on a CE instrument in Jan 2008 by an in house technician. This training was designed to show the basic running of CE and how it can be used as a separation technique. If a suitable LC method could provide the separation required in the anthracycline/taxane method then perhaps a CE method for their separation could be developed.

- A course was attended in the National Centre for Sensor Research, Dublin City University where the basic principles of operation of a MS were explained and a demonstration performed on MS operation. This three-day course was put together by an expert consultant in the field. It was run as a training module for people starting off in MS analysis.

- I attended a 12 week tutor training programme in Dublin City University from March until May 2008 which was designed to help new research students perform their demonstrating duties successfully.

- I attended some tutorials and a short course at the HTC, Ex-Tech 10 (Jan 28th until Feb1st) conference in Brugge, Belgium. The short course was entitled 'On-line SPE upfront to bioanalytical LC-MS'. The tutorials were entitled: 'Coupling SPE to LC-MS in bioanalysis', 'Hyphenated techniques in forensic analysis' and 'On-column injection: Why not the most accurate and most simple injection technique?'

  The tutorials and especially the short course attended were very relevant to the sort of analytical research I was working on throughout my project and I found it extremely beneficial and helpful.

Appendix 4: Relevant experience gained

Every scientific research student in Dublin City University has to take part in demonstrating duties to help out in practical scientific laboratories for undergraduate students. During my time in Dublin City University I taught:

- First year Science Education students between Oct and Dec 2008. I had to demonstrate in a classroom environment where the students worked together in groups solving scientific problems and generating weekly reports. I would answer any questions a student might have and help the students to think logically about any problems they found difficult. I also had to grade class reports.

- Masters students doing the MSc in Instrumental Analysis. These mature students took night classes in analytical science. This was a lab based practical course in the analysis of organic and inorganic species. My duties were to parole the laboratory making sure experiments were performed safely and correctly. If a student had a problem with instrumentation or its software I would try to help them. I also had to correct weekly reports.

I also attended safety courses, lone working training and seminars and talks given by guest and visiting lecturers who came to DCU to talk on chemistry and biology topics.