The Development and Validation of a Quantitative Liquid Chromatography-Tandem Mass Spectrometry Method for the Detection of Cremophor EL in Human Plasma

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Declaration

I declare that the research and any lab work included in this document is the result of the independent work of Tom Cull carried out under direct supervision, with guidance and advice only.

I confirm that the material presented in this project work is the result of my own efforts and that where material is derived from other authors / investigators has been used, it has been acknowledged in the text of the document and listed in the reference section.

There are many figures which contain photographic media within this thesis. Most of the photographs have been taken by me in the laboratory setting. Any images which I have not photographed are referenced accordingly.

Signed _____

Name _____

Date _____

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Abbreviations

API	Active pharmaceutical ingredients
APCI	Atmospheric pressure chemical ionisation
BHT	Butylated hydroxytoluene
CE	Capillary electrophoresis
CI	Chemical Ionisation
CRM	Certified reference material
CrEL	Cremophor EL
EDTA	Ethylenediaminetetraacetic acid
ER	Electromagnetic radiation
ESI	Electrospray ionisation
ES-	Electrospray negative
FID	Flame ionisation detector
GC	Gas Chromatography
GC-MS	Gas Chromatography – Mass Spectrometry
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
LC-MS	Liquid Chromatography – Mass Spectrometry
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
m/z	Mass to charge ratio
MOhm	Megohm
MRM	Multiple reaction monitoring
MS	Mass spectrometer/mass spectrometry
MS/MS	Tandem mass spectrometry
PK	Pharmacokinetic(s)
PP	Protein precipitation
Py-MS	Pyrolysis – mass spectrometry
QC	Quality control
R2	Regression coefficient
RPM	Revolutions per minute
S/N	Signal to noise ratio
SIM	Selected ion monitoring
SRM	Selected reaction monitoring
SPE	Solid phase extraction
UPLC	Ultra Performance Liquid Chromatography
UV-Vis	Ultraviolet-Visible
TDM	Therapeutic drug monitoring

Glossary of terms used in this thesis

Accuracy - the nearness of an experimental measurement to the true value. The accuracy of a method is linked to the use of certified reference materials.

Analyte - the chemical species to be identified or quantified.

Baseline - similar to the background, but usually a reference to plotted data in a spectrum or chromatogram. The average value of blank measurements or the average minimum where there are no peaks.

Bioanalysis - is the sub-discipline of analytical chemistry covering the quantitative measurement of xenobiotics (drugs and their metabolites, and biological molecules in unnatural locations or concentrations) and biotics (macromolecules, proteins, DNA, large molecule drugs, metabolites) in biological systems.

Blank - a standard containing no analyte, for example a concentration of 0.0.

Calibration - the process of measuring a known quantity to determine the relationship between the measurement signal and the analyte amount or concentration.

Calibration curve – a plot of signal versus analyte amount or concentration. Used to calibrate a measurement over an extended range. Good practice is to measure five to ten standards that are equally spaced through the measurement range.

Cannabinoid- a cannabinoid is one of a class of diverse chemical compounds that act on cannabinoid receptors in cells that alter neurotransmitter release in the brain.

Carry over - residual analyte in a sample preparation or measurement step that causes the measurement to be higher that the true value.

Certified Reference Material – a material that is verified to contain a known amount of analyte.

Chemotherapy – the treatment of disease with chemical agents.

Chromatography - a technique for analysing or separating mixtures of gases, liquids or dissolved substances, into their individual components. Separation is achieved by passing the components in solution or suspension through a medium in which the components move at different rates.

Contaminant - a substance, which can include the analyte itself, which is introduced unintentionally into a sample during collection, processing or measurement.

Drift - the gradual change in blank measurements over time.

Duplicate Sample – a sample that is split in to two portions to monitor method variability.

Error, Random – the spread in replicate measurements due to random fluctuations. Will be both higher and lower than true value.

Error, Systematic – a consistent difference either higher or lower between an experimental measurement and the true value. Can differ from sample to sample depending on variability in sample matrix effects.

Excipient - an inactive substance that serves as a vehicle or medium for a drug or other active substance.

Internal Standard – a known standard added directly to the sample or blank. The internal standard is then measured simultaneously with the analyte.

Limit of Detection (LOD) – the minimum measured concentration at which an analyte may be reported as being detected in a sample. There are several accepted methods to determine an LOD. A simple method is to calculate the concentration that corresponds to a signal level that equals the baseline plus 3 times the noise.

Limit of Quantitation (LOQ) – the minimum measured concentration at which an analyte concentration may be reported. A simple method is to calculate the concentration that corresponds to a signal level that equals the baseline plus 10 times the noise.

Mass-to-charge ratio (M/Z) – is the ratio of the mass number (m) of a given particle to the number (z) of electrostatic charge units (e) carried by the particle. Thus, m/z is mass divided by charge number.

Matrix Effect – the effect on a measurement resulting from components in the sample. Can vary sample to sample and impact the result higher or lower. Specific species that are that are identified as causing a systematic error are called interferences.

Memory effect – an apparent signal in an instrumental measurement that occurs due to contamination or carry over from a previous test sample.

Noise - random fluctuations in the signal. Usually quantified using the standard deviation of multiple measurements of the blank.

Precision - the repeatability in making replicate measurements. Imprecision, or the lack of precision, is probably a better term to describe the repeatability of measurements, but precision is the more common term. Qualitative measures include standard deviation, standard error and confidence limits.

Repeatability – comparison of replicate measurements made on the same sample and performed under identical conditions.

Replicate Measurements – multiple measurements of the same sample. Replicate measurements can be made by dividing the sample in to several test portions and testing each portion separately. Doing such a test can provide a measure of precision of the method and can identify outliers due to gross errors (blunders) such as omitting a step in a protocol, instrument glitches or incorrectly recorded values.

Reproducibility – comparison of replicate measurements made on the same test sample made by different analysts. The calculation of precision is the same for repeatability and reproducibility, the difference is the source of the measurement results.

Resolution - the minimum difference between separated peaks in a chromatogram or spectrum.

Ruggedness - the degree to which variable experimental conditions such as temperature, pH, ionic strength, will affect the accuracy and precision of a measured result.

Selectivity - the ability of a method or instrument to measure an analyte in the presence of other constituents of a sample.

Sensitivity - the slope of the calibration function, i.e., the change in the detector signal versus the change in the amount of analyte.

Signal-to-Noise ratio (S/N) – the ratio of the signal to the baseline noise.

Smoothing - averaging adjacent points in a spectrum to reduce the apparent noise.

Spike - an internal standard or standard addition added to a sample or blank.

Stability - retention of analyte over time or during sample preparation and analysis steps.

Standard Operating Procedure (SOP) – a document containing the instructions for a specific analytical procedure or instrument.

Therapeutic Drug Monitoring (TDM) – is a branch of clinical chemistry and clinical pharmacology that specialises in the measurement of medication concentrations in blood. Its main focus is on drugs with a narrow therapeutic window.

Thermally Unstable – if a substance is thermally unstable it means it breaks down at high temperatures. Therefore, it cannot withstand high temperatures.

Volatile - the word volatile refers to a substance that vaporizes readily. Volatility is a measure of how readily a substance vaporizes or transitions from a liquid phase to a gas phase.

Abstract

Cremophor EL (CrEL) is an excipient widely used in the pharmaceutical industry, which is derived from the castor oil plant (Ricinus communis). CrEL is produced by reacting castor oil with ethylene oxide at a molar ratio of 1:35. Patients who have advanced or multiple cancer sites are commonly treated using chemotherapeutic agents. Such mixtures are comprised of a drug compound and an excipient carrier which enable the agent to reach its intended site more efficiently. In high concentration, there is evidence to suggest that CrEL may be toxic to those undergoing chemotherapy. Therefore, CrEL should be monitored in order to reduce the incidence of adverse reactions. The measurement of CrEL in human plasma has been previously reported using a number of different analytical techniques ranging from the simple colorimetric dye assays to High Performance Liquid Chromatography with Ultraviolet detection. Throughout many of these techniques CrEL proved particularly difficult to measure using labour intensive sample preparation, which lacked sensitivity, reproducibility and linearity.

The purpose of this study was to develop and validate a Liquid Chromatography tandem Mass Spectrometry (LC-MS/MS) method for the determination of CrEL in human plasma, which could be applied to a Phase 1 Pharmacokinetically (PK) guided, dose escalation study for patients with advanced solid tumours. CrEL was extracted from plasma by liquid-liquid extraction using a mixture of Chloroform: Methanol (2:1 v/v) and butylated hydroxy toluene (BHT). Sodium hydroxide was added to the samples to release Ricinoleic acid. The resulting extracts were then analysed using the Waters Ultra Performance Liquid Chromatography and the Quattro Premier Tandem Mass Spectrometry. Separation of CrEL in the LC was achieved using an HSS T3 column 1.8 µm (hybrid particle) 2.1 mm x 100 mm and ionised using electrospray in negative mode. Multiple reaction monitoring (MRM) was carried out with a transition of m/z 279.3 → 182.9. The assay performed well with no evidence of ion suppression and enhancement or carry over.

The lower limit of quantification was 0.07 µl/mL and the calibration curve in plasma was linear over the range 0.07 to 10.0µg/L with an excellent correlation coefficient (r^2) >0.99. The assay precision was also excellent, intra assay and day to day co-efficient of variation (CV) reporting 17% for the low level and 10% on the high level.

This assay is now in routine use in a Hampshire laboratory where a PK study of patients with advanced solid tumours is being investigated. Patients are dosed with Dexanabinol a synthetically altered cannabinoid drug in combination with the excipient CrEL. Plasma samples are then analysed to evaluate the pharmacokinetics and assess the safety of the prescribed formulation.

Chapter 1

1.1. Research Aims and Relevance of Work

1.1.1. Introduction

The main objectives of this study were to:

- Develop and validate a fast, selective and sensitive method for the quantitative determination of CrEL in human plasma using a chromatography platform within the laboratory.
- To provide a method that is both robust and accurate, whilst still enabling the high throughput of clinical trial samples. CrEL levels across cohorts must be quickly analysed and assessed, before the next dose is prescribed.

1.1.2 Research Aims

During the initial stages of this study many of the previous methods were researched in order to understand how CrEL has been analysed and what analytical platforms had been used. The principal purpose of this study was to develop and validate a fast and accurate method, capable of monitoring CrEL concentrations and giving a reliable value. The method would need to complement the existing Dexanabinol extraction, which was already in place. Dexanabinol is being investigated to establish the maximum safe dose that can be prescribed to patients with advanced solid tumours. Another important factor was to keep the run times short and the analyte recoveries high. The limit of detection (LOD) should be low to satisfy the normal criteria for therapeutic drug monitoring (TDM), but the analytical range should be sufficient allowing for both high and low levels to be quantified.

A selection of papers were researched at the start of the study, but only a handful of methods used the technique of Liquid Chromatography – tandem mass spectrometry (LC-MS/MS), many relied upon Gas Chromatography – Mass spectrometry (GC-MS). Both platforms are available within the laboratory to use in this study.

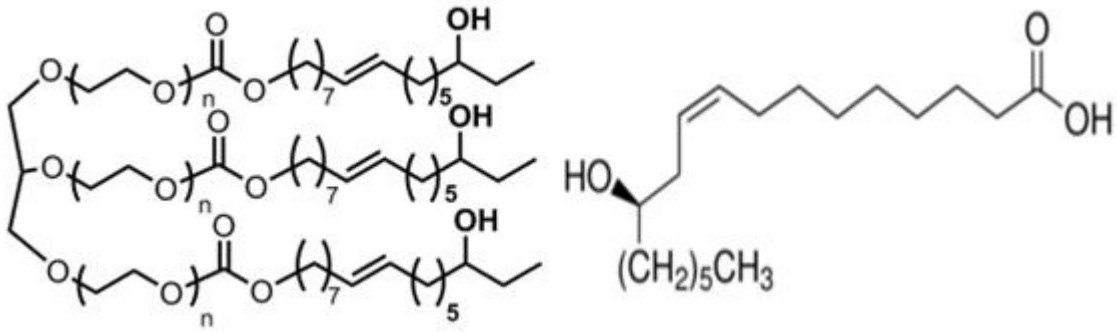
The two platforms have many differences in relation to sample extraction and the way in which the analyte is carried through the system. GC-MS relies heavily upon the analyte being volatile and thermally stable, whereas LC-MS/MS is useful for analytes that are thermally unstable and non-volatile. Having access to the two platforms and understanding the type of compounds that works best on each, will be beneficial during the method development process.

1.2. Cremophor, Cancer and Chemotherapy

1.2.1. Cremophor

Cremophor (Polyoxyethyleneglycerol triricoleate 35), also commonly known as Cremophor EL (CrEL) or more recently Kolliphor EL, is a synthetic solubiliser and emulsifying agent currently used as an excipient or carrier for the active ingredients of a drug.[1] CrEL use is well established in the pharmaceutical industry, where it has been used in combination with a wide variety of hydrophobic compounds including anaesthetics, sedatives, immunosuppressant agents and anticancer drugs.[2] CrEL is produced by reacting castor oil with ethylene oxide at a molar ratio of 1:35.[1] Castor oil is derived from the plant Ricinus communis (Figure 1) by pressing the seeds (Figure 2)[3] The oily substance is mainly composed of triglycerides of which 87% is Ricinoleic acid the primary component[4]. It is presently undergoing clinical trials as an excipient to Dexanabinol, a potential chemotherapeutic agent.

Figure 1: Ricinus communis plant[5] and castor oil seeds[6].



Cremophor MW[7] = 2560.0 Ricinoleic acid MW[8] = 298.46

Figure 2: The chemical structures of CrEL and Ricinoleic acid

Evidence suggests that CrEL may be toxic at high doses. It is therefore important to monitor plasma drug levels in order to reduce the incidence of these side effects.[9] Common side effects include nausea, vomiting, joint pain, appetite loss, brittle hair and tingling sensations in hands and feet or neuropathy.[10]

The side effects that can potentially be life-threatening are immunological hypersensitivity reactions such as anaphylaxis, where the body becomes hypersensitive to a specific antigen causing an acute response.[11] Other factors to consider during the administration of cytotoxic drugs and their excipients are the suitability of the infusion set used or storage container.

Previous studies have noted that CrEL is known to leach plasticizers such as di(2-ethylhexyl)phthalate from polyvinylchloride bags and polyethylene lined tubing.[10] Such occurrences could lead to additional hypersensitivity reactions or an allergic response if the components are released in to the patient's bloodstream.

1.2.2. Excipients

Excipients are typically inactive substances that have no medicinal properties, they can act as a medium to bulk, a substance to reduce absorption during admission or as a vehicle to aid in drug delivery. During the development of pharmaceutical agents many obstacles have to be overcome, one of which is ensuring that a compound reaches its intended site of action. Excipients are crucial to drug delivery within the body and to facilitate physiological absorption of the drug. Additional capabilities served by excipients products are; lubricity, flow-ability, solubility, reducing viscosity, disintegration, reduced degradation, taste and possibly some antimicrobial function.[12, 13]

In this study CrEL is combined with Dexanabinol to act as a carrier as seen in Table 1, thus preventing absorption into only the intravenous site during drug admission. Other excipients are also added to the formulation including ethanol, EDTA and Tocopherol, to improve drug solubility and long term stability. Dexanabinol as a single compound is extremely lipophilic and hydrophobic meaning it strongly combines with fats and lipids but repels water.

Carrier excipients are designed to interact with and enhance the properties of the active pharmaceutical ingredients (APIs), while not having any chemical effect on the action of the agent.

The ingredient qualities are promoted by the carrier excipients and have become useful tools for drug formulators. Long term stabilisation of low quantity ingredients and bulking up solid formulations is also possible now with excipients.[14]

Active component	Treatment dosage vial contains Dexanabinol plus
Excipients	DL-Tocopherol Ethylenediaminetetraacetic acid (EDTA) Ethanol Absolute Cremophor EL

Table 1: Excipients present in the Dexanabinol formulation that will be prescribed to study patients.

1.2.3. Cancer

In the modern world, the known incidence of cancer has risen from 12.7 million in 2008 to 14.1 million in 2012.[15] The current incidence projection rates estimate that within two decades that figure could reach 25 million.[15] The human body of an average sized person is comprised of roughly one thousand billion cells. Cells are recognised as the general building blocks of life and form our tissues and organs. The term cancer is best described as the continual, uncontrolled production of cells that are no benefit to the body.[16] Cancer is commonly recognised as a growth or lump caused by the proliferation of cells that spread beyond their normal space if left untreated. A common misconception is that cancer is a single disease, when it is more accurately classified as a group of disorders with differing symptoms.

Additionally, proliferation without swelling can also occur and is typically found in cases of leukaemia and lymphoma, where the blood forming cells found in bone marrow are affected.[17]

Cancer cells differ from other normal cells because they can multiply indefinitely and have the ability to migrate to other sites in the body resulting in metastases.[17] Metastasis is the formation of secondary tumours in other tissues or organs that differ from the primary origin.[18] The affected cells also differ from normal other cells because they cease to perform their intended function for a particular site in the body. Liver cells for example remove toxic chemicals from blood and nerve cells transmit electrical signals.

Specific cell functions help to keep an organism in equilibrium, and cells without a function are seen as independent cells, still requiring nutrition and support from the host at the expense of other healthy cells which benefit the host.[17]

Cancer can be classified in to two categories benign and malignant (Figure 3). Cancers that spread from one site to other body areas are normally classified as malignant and recognised as cancerous. Benign growths differ because they divide quickly but remain in one place and are commonly non-cancerous. Benign cells often remain in a capsule structure and unlike malignant cells do not invade other tissues facilitating the possibility of surgical removal.[19] Malignant growths manifest in other tissues by producing chemicals which break down healthy tissue. This makes malignant tumours particularly difficult to separate surgically because it is almost impossible to differentiate between healthy and cancerous tissue. When malignant tissue is bound to healthy tissue or organs, complete removal is impossible without serious damage to the system or organ. Surgeons faced with this scenario have to adopt a different technique called cytoreduction surgery or more commonly tumour debulking.[20]

The principle of the surgery procedure focuses upon the removal of the tumour and some healthy tissue while still allowing the system to function. Some patients who have undergone such procedures have responded better to radiotherapy and chemotherapy, have lived longer and had some symptom relieved. [20]

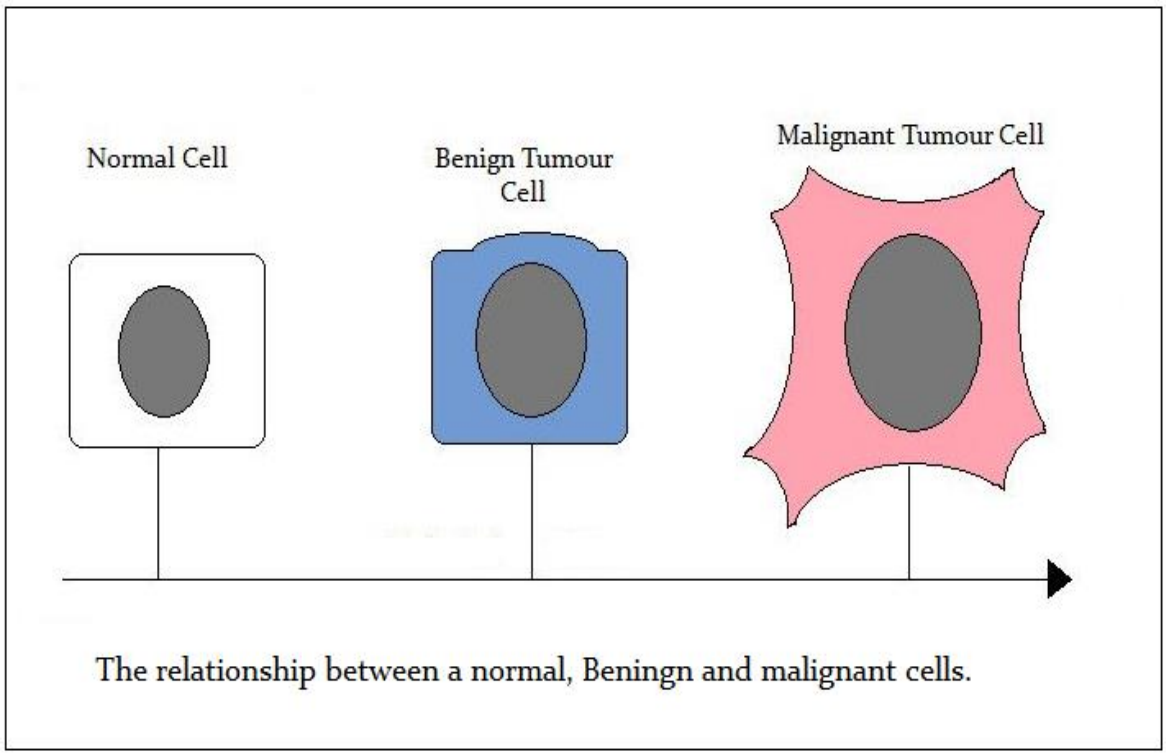


Figure 3: image showing the major differences between normal cells when compared to benign and malignant cells. http://stomach.cancertheory.jp/cancer_theory/outline/carcinogenesis/index.php (Accessed August and September 2017).

Malignant tumours are generally the most likely to cause harm, but if benign growths establish in confined spaces such as the skull they too have potential to cause harm by blocking important structures such as blood vessels or nerves. Malignant growths commonly have an irregular or ulcerated surface and can quickly spread to other sites if the cells enter the lymphatic or circulatory systems.[16]

The cancer type is normally determined by obtaining a biopsy which is viewed under a microscope to understand its behaviour.

Certain cancers can be identified by checking for chemical markers present in the blood. Prostate-Specific Antigen (PSA) for example is a good indicator of prostate cancer when high levels are present. Cancers are categorized in to further types as seen in Table 2.

Cancer group	**Description**	**Incidence rate in UK**
Carcinoma	Cancer form that begins in the skin or tissues that line/cover internal organs. Many further subtypes.	Common type (85 out of every 100 cancers 85%).
Lymphomas and Myeloma	Cancers that start in the cells of the immune system.	Uncommon form (5 out of 100 cases 5%).
Leukaemia	Cancer form that initiates from the blood forming tissues such as bone marrow. This can result in the production of vast quantities of abnormal cells which go directly into the bloodstream.	Uncommon (3 out of 100 cancer cases 3%). The most common form of cancer in children.
Brain and Spinal Cord tumours	These cancers are commonly known as central nervous system cancers.	Uncommon (3 out of 100 cancer cases 3%).
Sarcomas	Cancer that starts in the connective or supportive tissues including bone, cartilage, fat, muscle or blood vessels.	Uncommon (1 in every 100 cancers 1%).

Table 2: the five main cancer groups. (Cancer Research UK, (accessed July 2017)

Cancer is commonly triggered by carcinogens or cancer causing agents, which damage the deoxyribonucleic acid (DNA). Carcinogens are recognised in many forms, radiation from sunlight, toxins in the smoke from pyrolysed cigarettes and other toxic chemicals such as alcohol. In addition to these sources chemicals within our own bodies such as sex hormones can overstimulate cells and provoke the formation of cancers.[17] Some viruses such as Hepatitis C can also damage DNA. A series of events have to take place for cancer to occur.[17]

The first stage normally involves damage to the DNA of genes called oncogenes. Oncogenes are responsible for the programming of cell behaviour, when damaged or mutated the DNA is repaired. If the repair is impossible cell death or apoptosis should occur and the cell is eliminated. The genes fail to act normally and prevent apoptosis happening, instead the cells are encouraged to remain in place and divide. Once normal tissue regulation is disrupted tumour development follows.[17]

1.2.4.Treatment of Cancer

Cancer cells can be treated using a number of different techniques, the simplest being surgery to remove the specific growth or complex therapies which use radiation or chemicals to destroy specific cells.[16,17] The treatment of cancer depends upon a number of factors: the type of cancer, the location in the body and the form it appears in. The principal aim of a surgical intervention is to remove all or some of the tumour while still retaining bodily function. As previously stated this is almost impossible in the majority of most patient cases.[17]

Radiotherapy uses localised high energy ionising radiation to reduce the tumour size or kill the tumour cells. Patients treated using this medium, are exposed to short wavelength radiation in the form of x-rays, gamma rays and charged particles. The frequency of the treatment and the strength of the radiation dosage are determined by the tumour site and size.

Although radiotherapy is suitable for most solid tumour types, and can reduce tumour size it does have its disadvantages. The radiation used is not specific at only killing cancer cells and can additionally damage normal cells leading to potential side effects.[23]

Chemotherapy utilises chemotherapeutic agents or cytotoxic compounds which target and destroy cancer cells.[24] Patients who have advanced or multiple cancer sites are commonly treated using chemotherapeutic agents. The drugs used do have a significant effect on the tumour cells by either completely stopping or changing their growth rate. Their usage however can have considerable side effects which include sores of the mouth, nausea and hair loss. This disadvantage is partly due to the fact that although the drugs used are selective to cancer cells, normal healthy cells which grow and divide quickly are also targeted.[24] The initial side effects of using cytotoxic compounds are less serious, but long term exposure or high dosage can lead to allergic reaction, intolerance and possible toxicity. TDM is used to monitor long term usage and to confirm that an effective dosage has been prescribed, which can minimise the side effects experienced. The effective measurement of the drug or its metabolite allows clinicians to intervene if a dosage needs to be raised or a necessary antidote given if required. The prescribed drugs are commonly given in low concentrations and small doses to prevent adverse events occurring. The frequency and strength of the treatment is again dependent upon the cancer type, size and location.

It is common practice now for patients to receive combination therapies, such as surgery with a programme of either radiotherapy or chemotherapy. In some instances, this treatment regime can eliminate cancer or prevent it manifesting in a new area of the body.[23] Combination therapies also present an analytical challenge as any developed method must allow for any potential interference from other compounds involved in the treatment of the patient. Specific, accurate and reliable results are needed to support TDM.

Single methods that can simultaneously detect and quantify all the compounds used in some treatments are hard to develop due to the nature and characteristics of certain cancer agents. Patients involved in this study received Dexanabinol combined with excipients CrEL and ethanol. In addition to the primary research compound the patients also receive Dexamethasone, Ranitidine and Chlorphenamine to try and prevent potential side effects including hypersensitivity. These compounds are chemically different for example Dexanabinol is a cannabinoid and CrEL is an oily hydrophobic compound. Both compounds can be isolated from EDTA plasma samples but require completely different extraction methods. Each method allows for the complete removal and purification of a particular analyte, but sadly does not ensure the absolute removal of the other.

Chapter 2

2.1. Analytical Separation Theory

A vast range of analytical separation tools exist for the development of bioanalytical methods from biological matrices. Commonly used separation tools include capillary electrophoresis (CE), gas chromatography (GC), liquid chromatography (LC) and more recently ultra performance liquid chromatography (UPLC).

Chromatography is the general term for a group of techniques which have the ability to separate mixtures into their individual components.[25] When separated, the evaluation of each component occurs. The main principal remains the same for all chromatography techniques; separation occurs when the sample is combined with the mobile phase. The mobile phase is a liquid or gas which suspends the compounds then carries the sample mixture through a structure or stationary phase.[26, 27] A stationary phase is typically a chemical contained within a tube that has the ability to attract the compound of interest from the sample mixture. Each compound within the mixture will interact with the stationary phase at a different amount. Compounds that interact weakly and do not adhere to the column stationary medium will be the first to elute from the column, while those interacting and demonstrating strong adhesion will elute at a slower rate eluting last. There are lots of different stationary and mobile phases that can be used depending upon the characteristics of the mixture needing to be separated.[26] Further conditions can be changed including the temperature in the stationary phase and pH or pressure in the mobile phase. Each characteristic which is altered has an effect upon the partition coefficient which is the simple ratio of the concentration of a solute in two immiscible or slightly immiscible phases, when it is in equilibrium across the interface between them.[27]

GC is a powerful chromatographic tool which uses an inert gas as its mobile phase typically helium, argon or nitrogen. Samples are injected and vaporised on to an analytical capillary column containing the stationary phase, typically made of silica and varying in both overall length and internal bore size. The column is situated inside an oven compartment which is used to increase the temperature gradually or ramped up, this procedure aids the rate of separation.[28, 29] Typically compounds with a high molecular weight take longer to elute from the column and compounds with low boiling points elute first and compounds with high boiling points elute last. The gas mobile phase carries the mixture through the heated column where it interacts with the stationary phase, separates and elutes from the column and diverts into a detector. The time it takes for the compound to travel through the capillary column from initial injection until elution is called the retention time.[29] When a compound is detected the detector creates an electronic signal, which is then processed by a computer (Figure 4). Many different types of detectors can be used with GC and include: mass spectrometry, flame photometry, flame ionisation, electron capture and thermal conductivity. The detector used is typically related to the sample type and its chemical characteristics.[29]

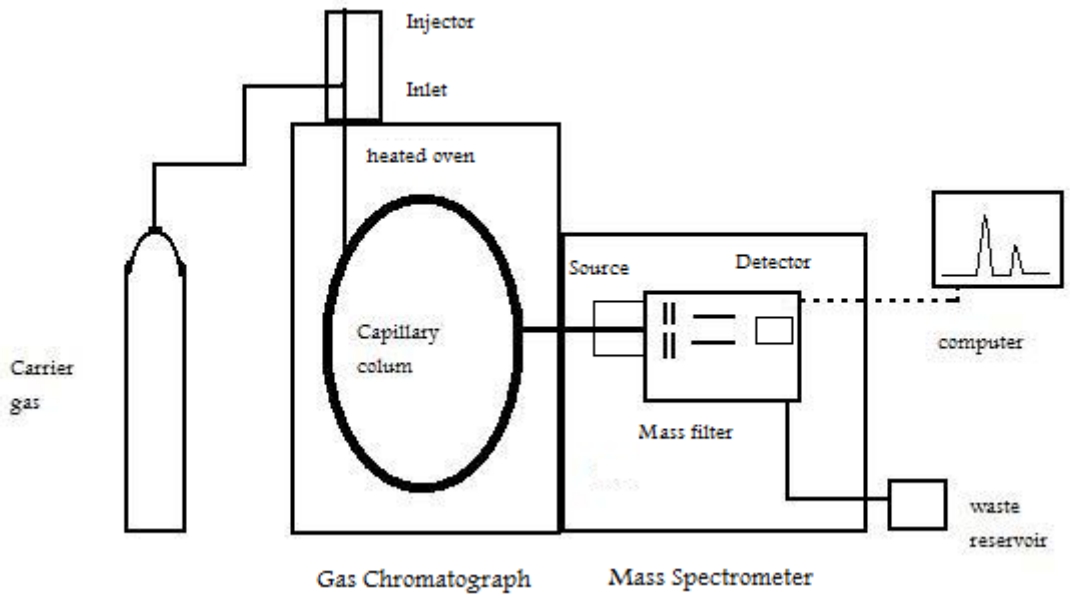


Figure 4: Simple schematic of a GC combined to an MS detector drawn by Tom Cull.

LC or HPLC is another chromatographic tool still following similar principle to GC, but using a solvent as its mobile phase. The solvent is pumped through the system using a high-pressure pump which is controlled by the solvent manager.

An injector introduces the sample to the mobile flow stream which leads directly into the analytical column. The analytical column used typically appears as a stainless-steel tube and comes in a variety of lengths and packing compositions, a common packing material is C18 or octadecylsilane.[30] The packing material is the stationary phase in the system and is the mechanism for separating the mixture of compounds into its components. As the components elute from the column a detector is again required to identify the compound bands and translate them into signals. A computer translates these signals in to a visible form or chromatogram (Figure 5). After the detector, any remaining liquid is diverted to a waste reservoir. Detectors which are commonly combined with LC are Ultraviolet-Visible (UV-Vis), fluorescence and mass spectroscopic. The detector used will relate to the compound of interest and its chemical nature.[31]

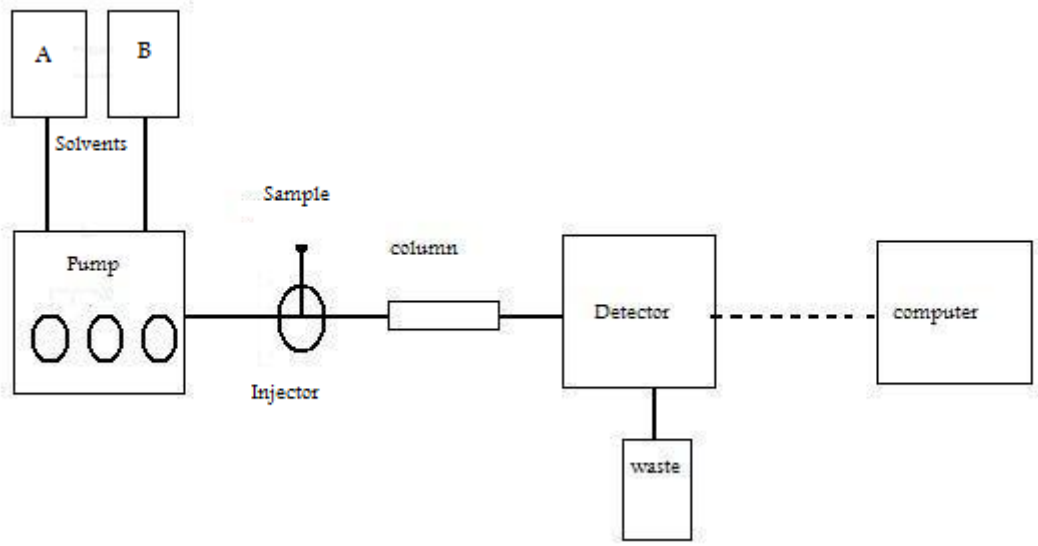


Figure 5: Simple schematic of an LC system drawn by Tom Cull

Within the last decade, HPLC has led to the development of UPLC systems which have the capacity to offer quicker separations and data analysis without compromising accuracy or data quality.[31] The demand for such analytical tools has come from analytical arenas such as pharmaceutical development, clinical trials and forensic toxicology where faster analytical data was desired. The benefit of faster separation relates to shorter method run time, meaning higher throughput of samples when compared to most HPLC run times.[30]

Newer UPLC systems also mean improved technology in relation to columns, detectors and system hardware. UPLC columns are very different when compared to their HPLC counterparts. The column packing mediums are reduced also, from 10µm in HPLC to 2.5 µm or smaller in UPLC. Small particle columns cannot be used on typical HPLC modules because their pumps cannot generate the desired operating pressures which can be in excess of 13,000 psi.[30] Downsides to the advanced technology are the initial cost of the equipment, the column lifespan when used at high pressures and the internal bore size of the tubing used is less forgiving than that of a conventional HPLC system. Increased working pressure and reduced tube bore sizes could potentially lead to blockages of the system if clean extracts are not used. Additionally, regular preventative maintenance is also required to ensure the chromatographic system works robustly.

2.1.1. Detectors

Many different types of detector exist and the three most commonly used with LC are UV-vis, fluorescence and mass spectrometry including tandem mass spectrometry.

A UV-vis detector focuses upon the visible and UV regions of the electromagnetic spectrum around the 200-800nm (Table 3 and Figure 6). The technique is useful for determining heavy metals and organic materials in water and as a quality control technique in the dye, ink and paint industry.

Samples are exposed to a beam of electromagnetic radiation (ER) from a light source. The spectrometer has two light sources, a high intensity tungsten bulb which provides the visible region and a deuterium lamp for the UV region. These lamps together provide the entire range of the instrument. If the wavelength range is used, the source is changed over at the designated point. The radiation from the source is split into two equal intensity beams.

One beam is passed through a dilute sample solution and the other passes through a pure solvent acting as the reference against which the first is compared after transmittance. Advantages of UV-vis include its wide linear range, but disadvantages are its lack of selectivity and sensitivity.[31]

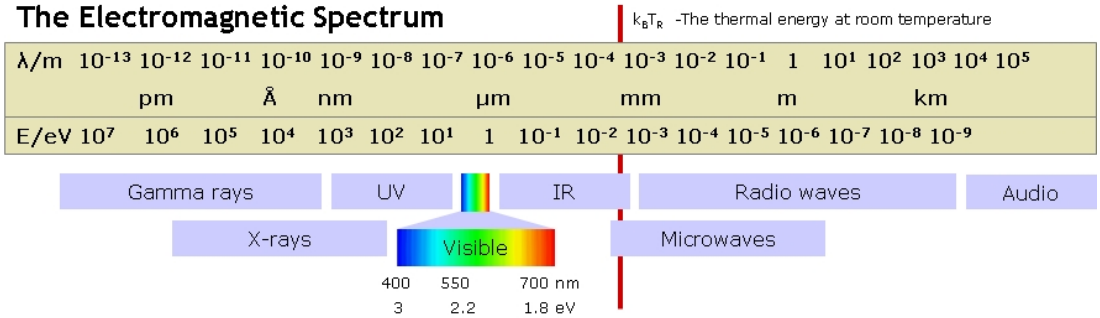


Figure 6: Regions of the Electromagnetic Spectrum (http://arstechnica.co.uk/science/2016/03/james-webb-telescope-details/)

Region	Wavelength (nm)
Infrared (IR)	800 – 50,000 nm
Visible	400 – 800 nm
Ultraviolet (UV)	190 – 400 nm

Table 3: Identified regions for visible, infrared and ultraviolet from the electromagnetic spectrum.

A Fluorescence detector focuses upon a narrow window of only 200-700nm (see Table 3 and Figure 6). The system works on a slightly different principle.[32] Molecules whose electrons have absorbed ER and have been raised to a higher energy level soon expel some of the energy and return to their ground state.

However not all the molecules lose all their energy, only losing some of the energy by non-radiant routes. The remaining energy that is then emitted as ER and this is fluorescence. This means that because only some of the absorbed energy is emitted, the wavelength of the fluorescent light is longer than the absorbed light (longer wavelength = lower energy). [31]

So, a fluorescent molecule has two spectra; an emission and an absorption or excitation spectra. The wavelengths of the emission and absorption are two different values. The wavelengths values are measured in nm and the difference between the two can be compared using a tool known as Stokes shift. The existence of this tool means that emitted light can be detected against a low background. Advantages include higher sensitivity looking at two wavelengths, but its disadvantages are that only 10-15% of compounds actually fluoresce and may require coupling with fluorescent dye. Further negative points include quenching that can occur when other substances in a mixture interfere with the energy transfer.[33]

2.1.2. Mass Spectrometry

Mass Spectrometry is a sensitive and specific technique which offers valuable information about the molecular weight of a compound and potentially its chemical structure. Mass spectrometry utilises the process of the ionisation of a sample, where positive and negative ions are produced. There ions are then accelerated into a high vacuum region where electric and magnetic fields are present.[34]

The fields serve to deflect and focus the ions to the detector. The fields can be controlled and adjusted so to allow different ions to reach the detector. The resulting image of the detected ions is known as a spectrum and is a series of peaks at variable intensities.

From this the mass to charge (m/z) ratio can be assigned. Typically, different compounds can be identified by the specific pattern of peaks, which some refer to as the chemical fingerprint. The main components of an MS are the interface samples inlet, an ionisation source, a mass analyser and an ion detector (Figure 4).[34]

2.1.3. Tandem mass spectrometry

Tandem mass spectrometry is now increasingly used routinely in the clinical biochemistry laboratory in the measurement of various analytes. The major aspects include the ionisation of the compound either in a positive or negative mode. The ionised compounds are detected and separated in the first mass spectrometer as molecular, precursor or parent ions.[34] These are then passed through a collision cell where fragmentation takes place resulting in the formation of many daughter or fragment ions. These are separated in the second mass spectrometer according to their mass/charge (m/z) ratio and passed into the detector for detection and determination of the intensity of the signal, a measure of the concentration of the compound. A schematic diagram showing the principles of tandem mass spectrometry is shown in (Figure 7 and Figure 8).[35]

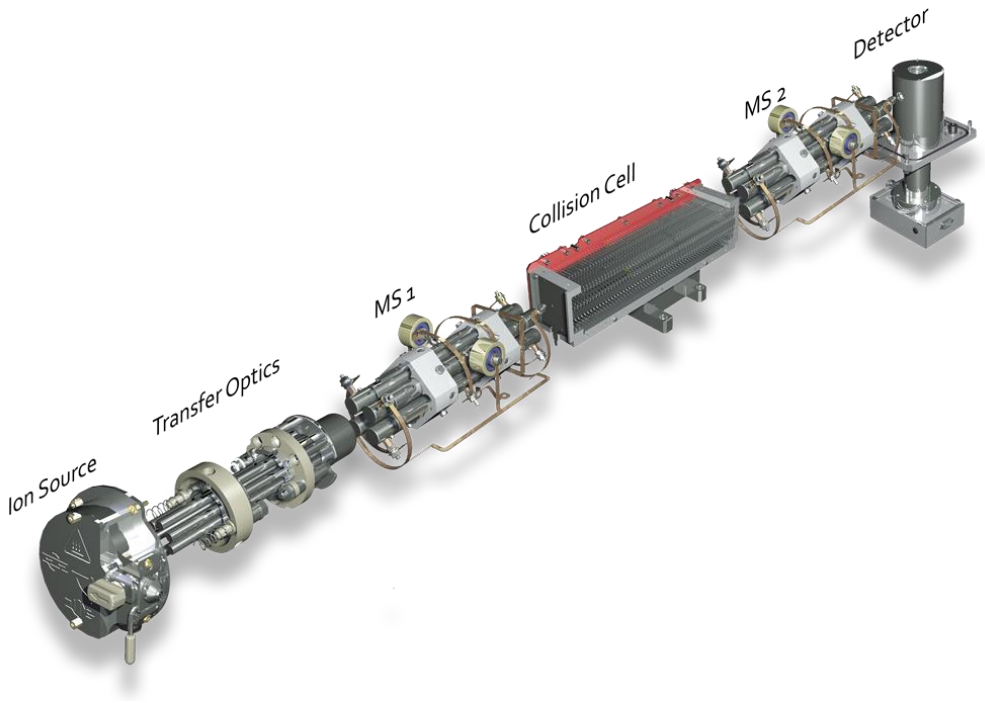


Figure 7: Schematic diagrams of tandem mass spectrometry indicating the individual components. Image belongs to Waters, Manchester, but given permission to use in this thesis.

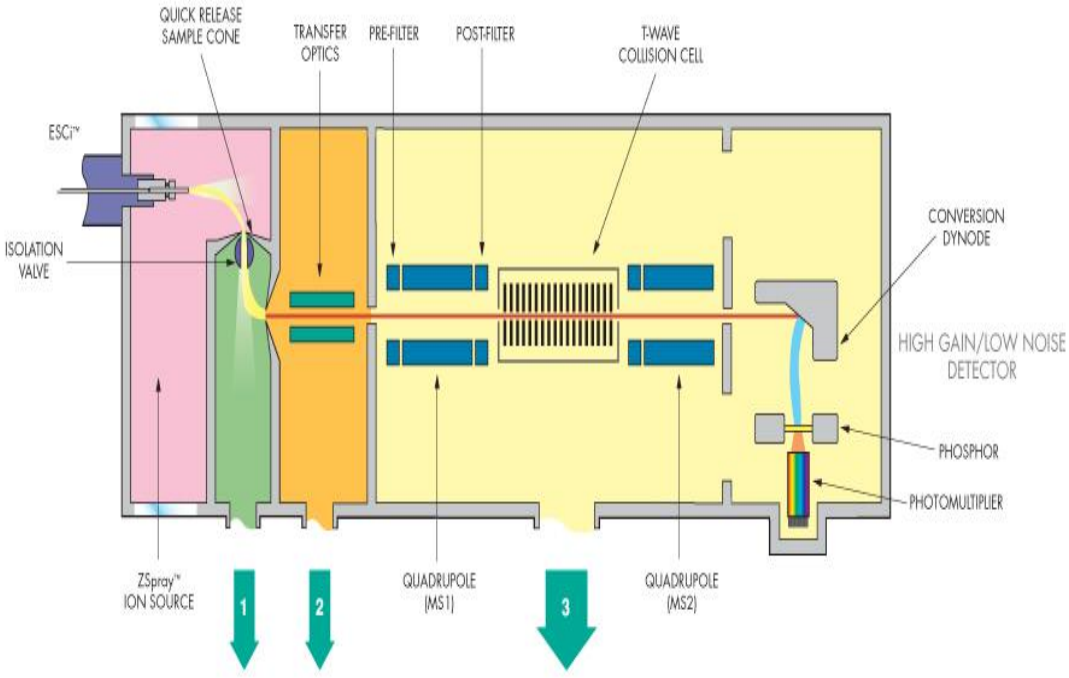


Figure 8: Tandem mass spectrometry schematic including an ESI interface. Image belongs to Waters, Manchester, but given permission to use in thesis.

2.1.4.Ionisation

Many forms of interface source are present in the modern laboratory setting, but the two most popular types are electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI). Although ESI and APCI are different techniques they can still be achieved using the same instrument source. However, they do require different components to achieve the same chemical objective. [34]

In ESI mode, ions are generated from the solution entering the inlet. The sample is dispersed via the capillary in the nebuliser, which has a high-energy potential applied to it of around 3-5 kV. The high-energy field causes the solution to form a mist of highly charged droplets. The droplets reduce in size as the solvent evaporates and the electric charge density on the surface increases, this effect is called the Coulomb explosion.[34] The droplets then further subdivide when they are exposed to a potential and pressure gradient. Positive and negative ions are separated using a positive charge which is applied to the capillary tip. Positive ions because of their nature then repel the capillary and form a cone called a (Taylor cone) where they experience reduced surface tension due to their mutual repulsion of like charges (Figure 9).[34] The combination of solvent evaporation and charge density eventually causes the ions to be ejected from the droplet's surface. During the final stage, it is believed that the analyte abstracts one or more protons from the solvent to give a positively charged gas phase ion. The ion is directed into the mass analyser using electrostatic direction, where an opposite charge to that used on the capillary is applied to the skimmer cones resulting in the redirection of analyte ions.[31]

Ion generation from analyte solutions follows two path directions: charge separation or adduct formation. Examples of ion formation are shown below:

Ion formation in positive mode:

$R\text{-}NH_2 + H^+ \rightarrow R\text{-}NH_3^+$ (protonation)

$C_6H_{12}O_6 + Na^+ \rightarrow C_6H_{12}O_6\ Na^+$ (adduct formation)

Ion formation in negative mode:

$R\text{-}COOH \rightarrow R\text{-}COO^- + H^+$ (deprotonation)

$C_6H_{12}O_6 \rightarrow + A^- \rightarrow C_6H_{12}O_6\ A^-$ (adduct formation, A=Cl^-, CH_3COO^-, $HCOO^-$.)

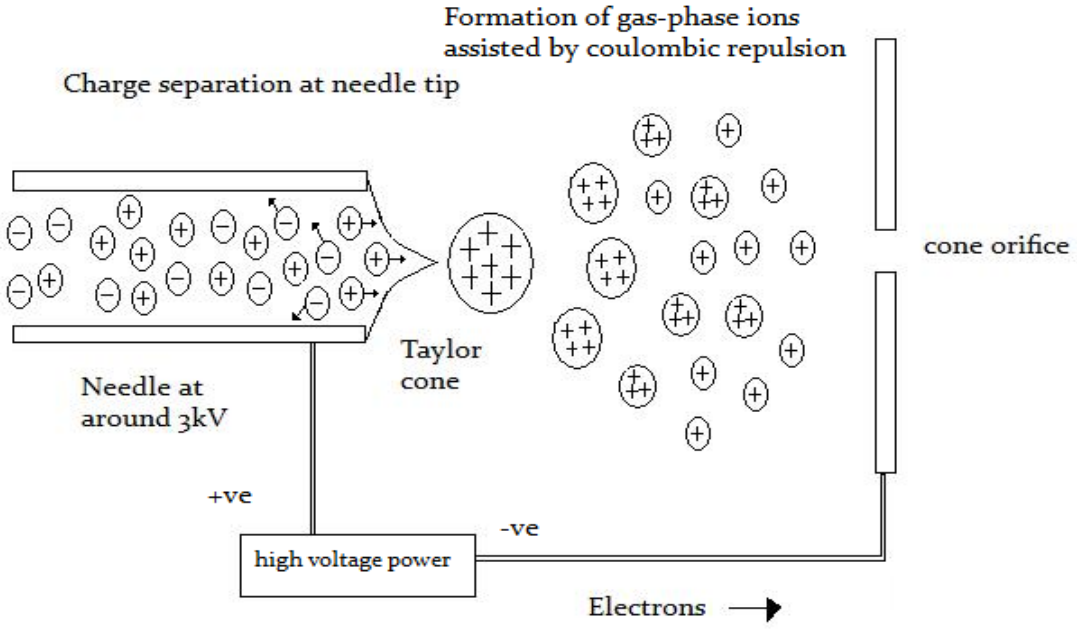


Figure 9: ESI schematic showing the main components and sample conversion into ions including the Taylor cone and the Coulomb explosion. Image drawn by Tom Cull, replicated from D. Watson, in Clarke's Analytical Forensic Toxicology, ed. S. Jickells and A. Negrusz [35].

APCI as previously mentioned can be undertaken using the same instrument source used for ESI, but it is reconfigured with the addition of a corona discharge pin.

ESI promotes the production of ions by evaporation, but APCI differs because it uses chemical ionisation (CI), where the solvent acts as the reagent gas to ionise the sample.[31] The eluent enters the interface again using the same capillary system used in ESI, but the major difference is the way the in which the aerosol is formed. The sample emerges from the capillary and is instantly heated by an inert nebulising gas causing the liquid to evaporate. The corona discharge pin is placed directly in the path of the heated region of the source. The corona pin again has a high-energy potential applied to it which can ionise solvent droplets using its electrical discharge. A gas plasma is produced by the chemical ionisation and proton transfer (Figure 10). Ions that promote ionisation using this form of ionisation include N_2^+ and H_3O^+. [34]

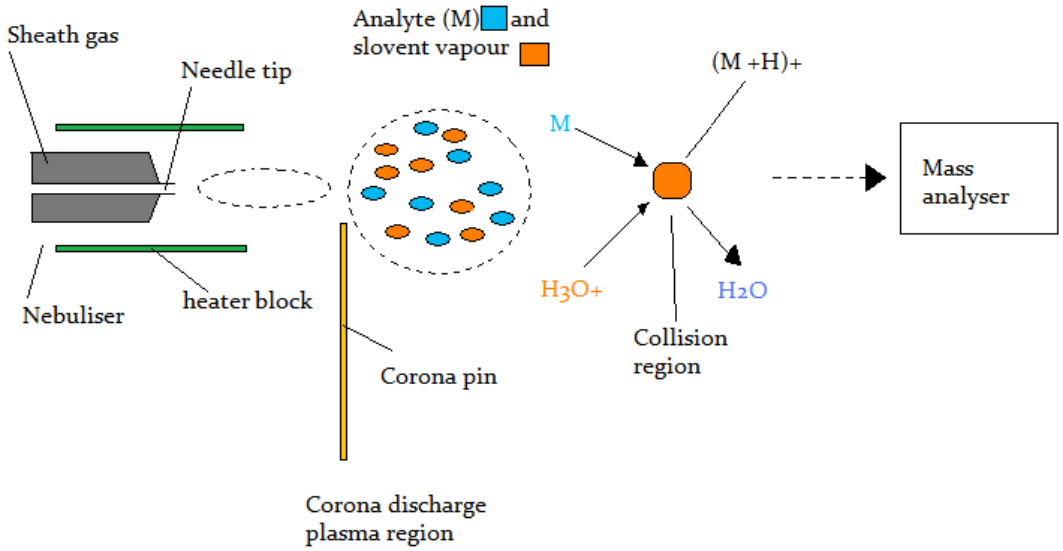


Figure 10: APCI schematic showing the main components and how ions are formed. Image replicated by Tom Cull from http://www.chm.bris.ac.uk/ms/apci-ionisation.xhtml viewed on 25/09/2018.

The key concept to understand about APCI is how the reagent gas forms in both positive and negative modes.[34] The positive and negative reagent gas formations are shown below:

Reagent gas in positive mode:

$N_2^{+\cdot} + H_2O \rightarrow N_2 + H_2O^{+\cdot}$

$H_3O^+ + M \rightarrow MH^+ + H_2O$ (protonation)

Reagent gas in negative mode:

$H_2O + e^-$ (cold electron) $\rightarrow H2O^{-\cdot} \rightarrow HO^- + H^{\cdot}$

$M\text{-}H + HO^- \rightarrow M^- + H_2O$ (deprotonation)

APCI lacks the sensitivity of ESI, but can be used in the application of molecules such as neutral drugs which will not easily ionise using ESI.[34] ESI however is useful for analysing compounds that have large masses, but is susceptible to over contamination from previous experiment residues and is not easy to clean.

2.2. Literature review

Year	**Principal investigator**	**Analytical techniques used**	**Simplicity of method - disadvantages**
1996	J.M. Challinor	GC-MS and Curie-point pyrolyser/ Fatty acid extraction	Simple method/ unsuitable equipment
1996	A. Sparreboom	HPLC / Fatty acid extraction/ derivatization	Long procedure – many steps.
1998-A	A. Sparreboom	Colorimetric dye binding assay/protein precipitation	Relies on dye binding

1998	E. Brouwer	Colorimetric dye binding assay/protein precipitation	Relies on dye binding
1998-B	A. Sparreboom	Colorimetric dye binding assay/ protein precipitation	Relies on dye binding
2000	L. van Zuylen	HPLC with UV detection / dye binding assay	Relies on dye binding
2001	H. Gelderblom	Discussion of CrEL drawbacks/advantages	Only a discussion paper
2003	A. Ghassempour	Pyrolysis mass spectrometry compared against HPLC / Fatty acid extraction / derivatization	Equipment not readily available
2004	H.J.G. Desiree van den Bongard	Liquid scintillation counting	Only suitable for determining unbound fraction of drug
2008	K. Yamamoto	GC-MS / Fatty acid extraction / derivatization	Method developed for oils not plasma
2009	V. Coopman	GC-MS and LC-MS/MS / SPE	
2011	T.Cull	LC-MS/MS / Fatty acid extraction	No internal standard
2013	V. Vijaya Bhaskar	LC-MS/MS / protein precipitation	Only specific to PEG

Table 4: Summary table of publications and journal papers focussing upon the measurement of CrEL in human plasma and other matrices, specifically CrEL.

The measurement of fatty acids has been investigated since the 1920's, specific acids such as CrEL were looked at in later years. The first recorded journal paper from 1922 was by Bloor et al.

This paper describes some of the extraction techniques appropriate for the removal of fatty acids from a blood matrix, which include alcohol/ether extraction, saponification using a suitable alkali and a technique called a nephelometer. [36]

A nephelometer is an instrument for measuring the size and concentration of particles suspended in a liquid or gas, especially by means of the light they scatter. During these early experiments, it was also established that it was important to have pure uncontaminated solvents such as chloroform which should be free from moisture and alcohol. An addition point stated in the paper is that accurate determinations cannot be made if the standard and the test solutions are more than 30% apart, showing that even then analysts had grasped the concepts of validity and reproducibility.[36] Later papers started to use analytical equipment to perform similar experiments previously undertaken using basic tools.

The equipment available to these early scientific pioneers was limited. Chromatography was basic with only thin layer chromatography.
The findings of these scientific pioneers led to the development of future methods, which when combined with advanced technology led to further breakthroughs into lipid profiling.

Challinor et al (1996) developed a simple pyrolysis GC-MS method for profiling fatty acids in a range of vegetable oils and animal fats. The precise equipment used was a Curie- point pyrolyser connected to a Hewlett-Packard gas chromatograph equipped with a flame ionisation detector (FID). Samples were run in duplicate using flattened pyrolysis wire, which after the addition of aqueous tetramethyl ammonium hydroxide (TMAH) was placed directly into the pyrolyser to act as a high temperature reactor converting the triglycerides into fatty acid methyl esters (FAME). The paper discusses in depth the recognised standard procedure for isolating fatty acids in triglycerides.

The steps include saponification using a suitable alkali, and acid catalysed methylation using boron trifluoride followed by analysis using GC. Although this method is very quick and simple, sadly the equipment is very uncommon and the method only profiles fatty acids rather than quantifying them.[37]

Sparreboom et al (1996) developed a different method using HPLC with ultra violet detection (UV), which focuses upon the quantitation of the major component of CrEL, ricinoleic acid. The internal standard used was Magaric acid a saturated fatty acid. The samples were extracted using the following procedure: saponification using alcoholic potassium hydroxide (KOH), lipid isolated using chloroform and finally derivatization using 1-naphthylamine. The original abstract of this paper described a simple method, however the actual method when viewed in detail has many separate steps including sample preparation and involves the uses of some highly carcinogenic chemicals, benzene and α-naphthylamine. The use of HPLC often means long run times and in this assay each sample takes at least 35 minutes in order to retain good resolution even though the analyte elutes at 10 minutes.[38]

Sparreboom et al (1998) developed another assay utilising Colourimetric dye binding. This technique relies upon the binding of Coomassie brilliant blue G-250 to CrEL and in doing so causing a shift in the absorption maximum from 465 to 624 nm. The samples do require sample preparation, drying and reconstitution, before the final step where the dye can be added. Specifically, 50 µl of plasma was added to 500 µl of acetonitrile in the sample deproteinizing step. Samples and calibrators were extracted in duplicate, and each single extract analysed twice. QC samples however had quintuplicate measurements taken. The assay does have selectivity, but according to other written papers using the same method it may have interference from non-ionic surfactants such as Triton x-100 and Tween 80. After further testing, these chemicals did not appear to have any effect upon the produced spectra.

Potential problems with this assay relate to its detectable range which is only 0.05 – 1.00% (v/v), the range should be greater to allow for higher sample concentrations. Further dilution of samples is not mentioned and would need to be undertaken during the sample preparation steps. This method does appear simple, but still relies upon specific equipment in the form of an automated microplate-absorbance reader.[39]

A discussion in this paper reviews alternative methods previously used to measure CrEL including reverse HPLC which it refers to as complex, expensive and time consuming when compared to a rapid extraction followed by Colorimetric analysis. However, something overlooked in this method is the variability of acetonitrile quality and the potential contamination from purified water. The grade of solvent used is of particular importance during analysis, especially for Colourimetry where the solvent used can influence the shape and quality of the spectrum. An HPLC grade was used in the assay and supplied by Sigma Aldrich, but other HPLC grades from different suppliers could have been tested and compared to confirm any possible quenching or enhancement. Water quality can also affect Colorimetry, depending upon the type of water purifying system used and the cartridge type.

Some cartridges have the potential to leave ion-exchange resin fragments in the cleaned product, which can lead to non-specific absorption at low wavelengths [40].

Brouwer et al (1998) also uses a Colourimetric dye binding assay, but states that the assay of Sparreboom et al (1998-A) has a major disadvantage which is its lack of linearity. The plot of the peak wavelength of CrEL, versus the concentration of the CrEL is non-linear. Brouwer et al (1998) has shown in this paper that the nonlinearity is associated with a decrease in the free dye concentration and a reduction in complex formation by increasing CrEL concentration. This assay also covers the wider range of 0.500 – 10.0 µl/ml and samples can be analysed as single extracts rather that in duplicate.

The method also uses a different version of the typical Coomassie dye, using the red form which turns blue when binding to CrEL.[41] The method is more specific and does cover a greater range, but still uses the automated microplate-absorbance reader. The authors of this research still make no provision for any potential interferences related to acetonitrile grade between different suppliers. In addition deionised water is used rather than double-distilled water, which still could contain ion-exchange resin particulates which could lead to non-specific absorption at low wavelengths.

Sparreboom et al (1998-B) published a later paper still using the Colourimetric dye binding assay, but this time the aim was to establish how quickly the human body eliminates CrEL. Patients receiving the taxane Piclitaxel as part of their treatment plan had plasma samples collected on regular intervals during drug infusion. This is a similar method to a previous paper by Sparreboom et al (1998-A), but using some of the modifications suggested by Brouwer et al (1998). A further addition not mentioned in previous Colourimetric assays is how to deal with samples above the analytical range, they are diluted in drug free plasma and reanalysed.

This assay still utilises the automated microplate-absorbance reader, but did establish that CrEL is a slow clearance compound remaining in the body for an extended time within the central plasma compartment.[42] This paper has some interesting findings regarding CrEL, but is still reliant upon the automated microplate-absorbance reader. The potential problems of acetonitrile still remain and are not allowed for [40].

Zuylen et al (2000) focussed again upon the chromatographic principles of HPLC to understand the clinical PK of CrEL. This method differed from Sparreboom et al 1 because UV detection was used to analyse piclitaxel and CrEL, levels were again established in plasma using Colourimetric dye binding. The collected data was then applied to response models to determine the relationship between the two analytes.

The determined relationship shows that as CrEL concentration increases the speed at which piclitaxel is expelled from the body slows down.[43] This paper again has useful findings, but still uses Colorimetry as its main analytical tool.

Gelderblom et al (2001) is a discussion paper around the usage of CrEL as an excipient vehicle and as a drug formulation additive. Throughout the paper no analytical procedures are discussed, but important clinical details are discussed around the biological effects of CrEL.[10] This paper outlines the importance of extensive testing when developing drugs and their formulation compounds.

Ghassempour et al (2003) returned to the initial technique of pyrolysis – mass spectrometry (Py-MS). However, this time it was specific to the determination of CrEL in both drugs and plasma and also facilitated the comparison of Py-MS and HPLC. For Py-MS sample underwent a simple preparation step before being dried and analysed in triplicate. The sample preparation for HPLC analysis was more detailed and included saponification, lipid removal and reconstitution steps. The conclusion of the methodology comparison was better for the Py-MS method than the HPLC method.[43]

The sample volumes required, the extraction times and the reliance on highly carcinogenic chemicals, gave Py-MS a number of advantages over the HPLC method. However, the Py-MS equipment is not readily available and has limited usage in a clinical setting.

Desiree van den Bongard et al (2004) used ultra-filtration and liquid scintillation counting to determine the unbound fraction of piclitaxel in human plasma.[44] This paper does comment on the usage of CrEL in relation to piclitaxel concentration. This technique might not be a standard practice used in a clinical laboratory and relies upon radio labelled tracers being available.

Yamamoto et al (2008) looked at ricinoleic acid in both common vegetable oils and oil seed using GC-MS. The method used a common FAMES extraction to isolate the ricinoleic acid before finally derivatising the extract to be analysed on the GC-MS. The method was successful in detecting ricinoleic acid, but the authors were unaware of how many vegetable oils contain ricinoleic acid. Its application is suitable for industries such as food and cosmetics where trace quantities are required.[45]

Coopman et al (2009) is a case report from Belgium on the death of a man who injected himself with a castor bean and acetone extract. Ricinine is a piperidine alkaloid toxin with a low molecular weight which is present in the castor leaves and beans. The paper is particularly interesting because it utilises two techniques in the determination of the compound Ricinine. Solid phase extraction (SPE) in combination with GC-MS in full scan mode was used to identify the compound and SPE in combination with LC-MS/MS in positive mode was used to confirm Ricinine in three biological matrices. The methods discussed in this paper appear to be effective and even mention the detection of ricinoleic acid as the sample pre-treatment did not completely remove the castor oil present in the samples. This is one of the first papers to discuss the use of LC-MS/MS, for the detection of a CrEL related compound.[46]
The major disadvantage of this method is the uses of SPE as a sample preparation step. SPE offers high recovery, but is normally an expensive consumable.

At this stage, no other methods existed that involved CrEL analysis and HPLC was the most advanced technology used. The assay developed from this research was used in 2011 and was at that time was the first non-published method for the measurement of CrEL using LC-MS/MS. However, by 2014 when the study ended another researcher Bhaskar et al had already published an LC-MS/MS method for the measurement of CrEL in rat plasma.

Bhaskar et al (2013) used LC-MS/MS with atmospheric pressure chemical ionisation (APCI) to measure CrEL in rat plasma. The sample extraction stage is very rapid and uses a polypropylene 96 well plate, which allows for small sample volumes and reduced usage of glass tubes which are commonly an expensive medium.[47] The disadvantages of this method are firstly it measures free Polyethylene glycol (PEG) which only makes up 7% of CrEL, so it does not focus upon the main constituent of CrEL. Secondly the method has only been used on rat plasma, no human plasma appears to have been tested. Many formulations routinely used contain PEG, so the specificity of CrEL in human samples could be compromised and the analysed data values could be significantly greater or invalid due to this unforeseen internal contaminant.

In conclusion, the literature indicates that only a fraction of instruments have been used to measure CrEL and other compounds found in the fatty acid species. The majority of the sources reviewed have a strong links to the use of colorimetric equipment combined with use of the dye agent Coomassie Blue which binds to the CrEL compound.[39, 41, 42] Most of the methods discussed seem to have major pitfalls, due to either a complicated or long extraction procedure or reliance on dated or uncommon equipment.

The number of extraction methods that did not involve Coomassie Blue and colorimetry were limited, but the original paper by Challinor et al, mentioned the FAME extraction principles.[37] After further research around this topic a paper from Burdge et al (2000) presented a potential method for the extraction of fatty acids from plasma.[47]

The sample preparation steps included lipid isolation using LLE with chloroform: methanol: BHT and sodium chloride, the eluted solvent fraction was collected. The remaining protein disc was further extracted again using chloroform: methanol: BHT and sodium chloride, the previous collected faction and the second fraction were combined and then dried under nitrogen.

The dried extracts were then reconstituted in chloroform before being added to SPE cartridges (aminopropyl silica column 100 mg). Separate columns were used for different fatty acid species, some needed preconditioning with methanol while others required hexane. The pre-sample preparation steps up to the drying down of the extracts could be applied to the isolation of CrEL from human plasma.[48]

2.2.1. Extraction techniques

All types of sample matrix will generally require some form of sample pre-treatment before they can be analysed on an instrument platform. Many pre-treatment methods exist and each is best suited to a particular form of matrix. The principal aims of all sample pre-treatments in the context of chromatography are to provide an aliquot that recovers the majority of the analyte while being free of interferences, it will not damage the analytical column and is in a compatible form to be separated and detected on the intended platform.[28, 49]

Different pre-treatments available for biological liquid samples are protein precipitation (PP), liquid-liquid extraction (LLE) and solid phase extraction (SPE).

PP is a common extraction procedure involving a solvent, acid or base which is added to the biological matrix. Commonly used examples include zinc sulphate, methanol or acetonitrile. The solution is normally agitated and centrifuged causing the proteins to 'crash out' of the solution and form a pellet. The supernatant layer above the pellet should contain the analyte and can be injected directly into some analytical systems such as LC-MS. This particular extraction method is quick, non-expensive and uses relatively simple equipment. However, it only removes some of the biological components in the matrix, meaning that some protein components still remain.[28]

LLE is the application of separating analytes from interferences using two immiscible liquid phases, one aqueous and the other a solvent (Figure 11). Analyte compounds which are hydrophilic in nature will partition themselves in the polar aqueous phase, while the hydrophobic ones will be attracted to the organic solvent. The sample is added to the container, the aqueous phase is added followed by the solvent.[28,50]

The mixture is again agitated using a mechanical shaker and is then centrifuged to separate the defined layers (Figure 12). The analyte at this stage should have partitioned in the solvent phase, leaving any biological material in the aqueous layer. This form of extraction is also quick to undertake and removes more biological interferences than PP, but consumes vast quantities of organic solvents. Depending upon the nature of the analyte further steps to dry and reconstitute may be necessary. LLE is also suitable for compounds which are non-volatile and heat sensitive.[50]

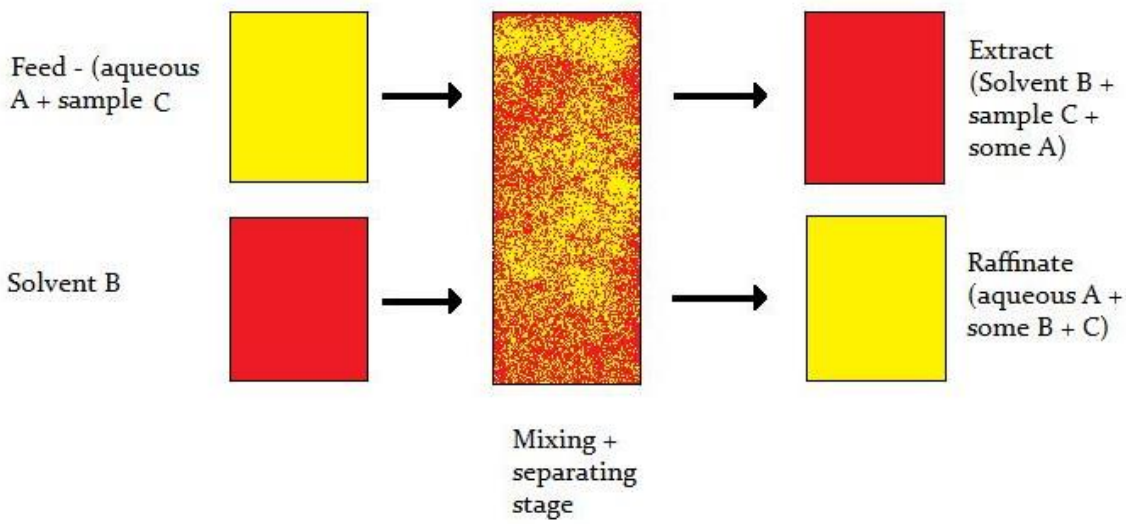


Figure 11: Process map of the main stages of LLE.

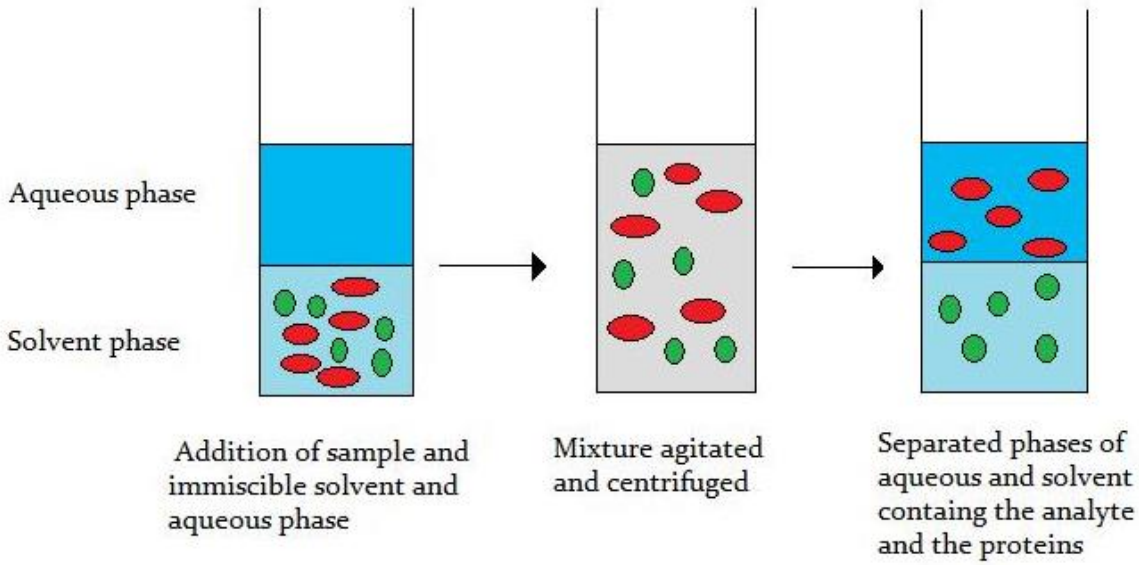


Figure 12: Schematic of the LLE extraction process

SPE is another form of sample preparation which uses solid and liquid phases to remove the analyte from the biological matrix. The application is typically four steps which are: conditioning, sample loading, washing and eluting (Figure 13).

The SPE column contains a sorbent disc, which is chemically able to retain the analyte while allowing all other biological material to be washed through. The column must be conditioned/wetted using a suitable solvent. The biological sample can then be added to the column, as the sample flows through the column the sorbent binds to the analyte allowing any other material including proteins to be washed off and elute the column. Finally, a suitable elution solvent is then added which has a stronger affinity to the analyte removing it from the sorbent. At this point the sample may require further steps to dry and reconstitute. SPE offers high recovery of analytes, but can be expensive and labour intensive if performed manually.

The technique can be automated and is sometimes available as an on-line component on instrument allowing samples to be extracted and injected directly onto the analytical system. [28, 49, 50]

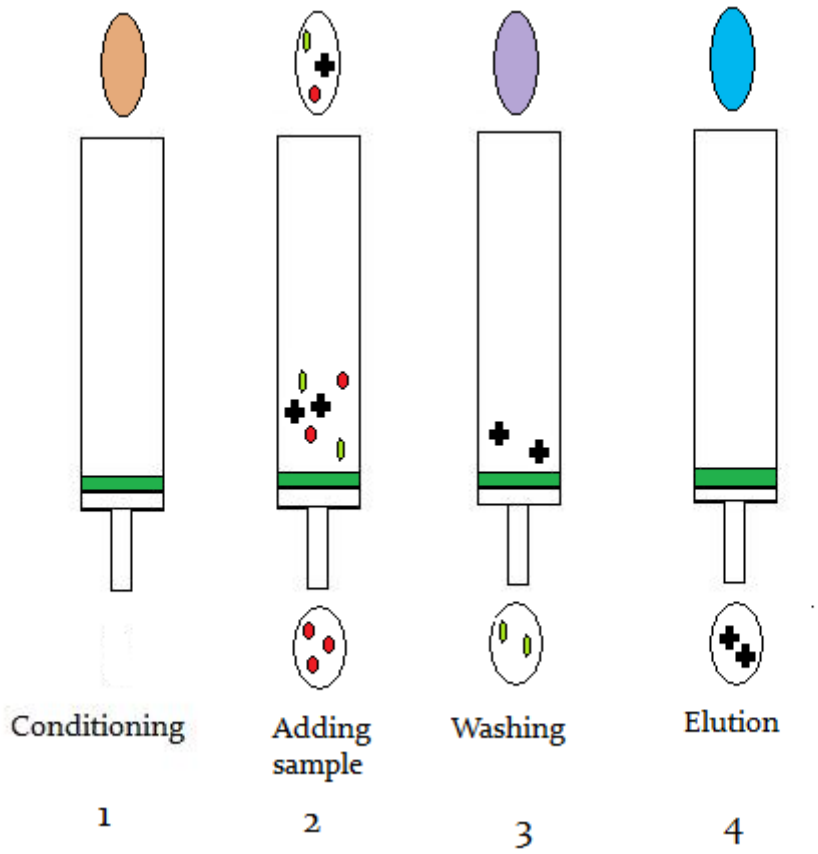


Figure 13: Schematic of SPE analysis steps including: conditioning, sample addition, washing and elution.

2.3. Current equipment in the laboratory

2.3.1. Analytical System 1

Liquid Chromatography

Liquid chromatography was performed using a (Waters, Manchester, UK) Acquity UPLC (serial number: L08UPH779M) with a HSS T3 column (100 mm x 2.1 mm, 1.8 µm) (186003539) also from Waters UK.

The Acquity UPLC system consists of an auto sampler, a binary solvent pump an injection loop and a column compartment (Figure 14). The Acquity is a high pressure, ultra performance liquid chromatography system. The high pressure generated up to 15,000 psi compared to other liquid chromatography systems results in a better separation of compounds in a small amount of time.

Mass Spectrometry

Detection of analytes was performed using a tandem quadrupole mass spectrometer also a Waters instrument, a Quattro Premier XE (serial number VAB 1255). The instrument contains a Z-Spray ionisation source and a T-wave collision cell. The high speed scanning T wave technology of the Quattro Premier makes it suitable to ionise and scan the compounds presented to it at the high pressures of the Acquity. Ionisation is achieved by electrospray in either a positive (ESI+) or negative mode (ESI-). A photograph showing the Acquity and the Quattro Premier is shown in (Figure 14).

Figure 14: the Acquity and Quattro Premier LC-MS/MS systems. Refer to comment in declaration regarding photographs.

Software

Operation of both the Acquity and the Quattro Premier XE, was controlled through Masslynx 4.1, supplied by Waters. Data processing was achieved with Targetlynx.

2.3.2. Analytical System 2

Gas Chromatography

Gas Chromatography (GC) was performed using an (Agilent, West Lothian, UK) 6890N Network GC system (serial number: CN10542008). The GC uses a DB 17 MS (Agilent Technologies) column (15 M x 0.25 mm, 0.25µm). The GC system also consists of 7683B series Injector (serial number: CN55330443) and a 7683 series Auto sampler (serial number: CN55137795). The carrier gas employed by the system is helium. The GC system is joined to a Mass Spectrometer (MS) which enables the detection and identification of ions within the samples analysed. The Agilent GC system is a state of the art gas chromatograph with superb performance over a range of applications (Figure 15).

Mass Spectrometry

Mass Spectrometry (MS) was performed using an (Agilent, West Lothian, UK) 5975 Inert mass selective detector (serial number: US52420874).
An image of the instrument can be seen in (Figure 15).

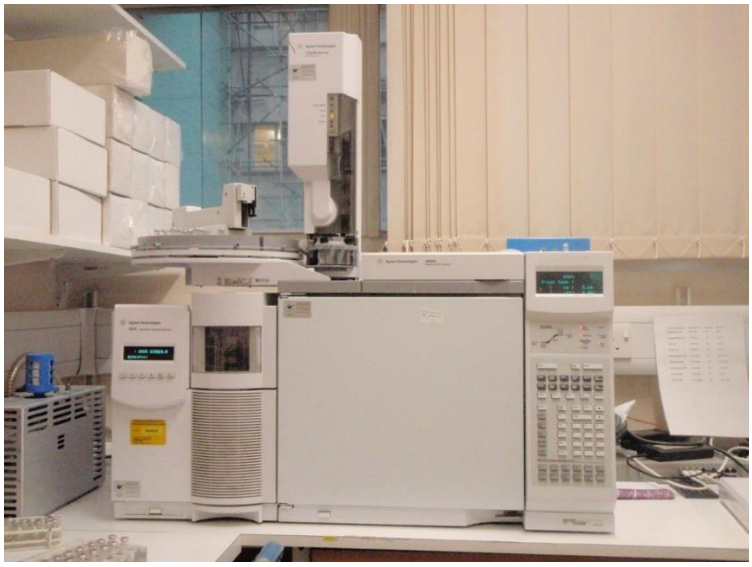


Figure 15: Agilent GC-MS. Refer to comment in declaration regarding photographs.

Software

The GC-MS system was controlled using the Agilent MSD Chem Station software, which also served to analyse the data collected during analysis.

Chapter 3

3.1. Experimental Section

3.1.1. Equipment

3.1.1.1. Tandem mass spectrometry was performed on the Quattro Premier XE (serial number VAB 1255) Waters Ltd.

3.1.1.2. Liquid chromatography was performed by the Acquity UPLC (serial number DO9 UPA 880M) Waters Ltd.

3.1.1.3. Column: Acquity UPLC® HSS T3 1.8 µm, 2.1 x 100 mm column Part No: 186003539, Lot No: 0114391761

3.1.1.4. Techne Driblock - FDB03AD (serial number 154625 - A) and Sample Concentrator – FSC400D (serial number 155395 - 2)

3.1.1.5. IKA Vibrax VXR Basic - mechanical shaker (serial number 01.798796)

3.1.1.6. Beckman Spinchron™ DLX Centrifuge (serial number GHY98F29)

3.1.2. Reagents / Solutions

3.1.2.1. Cremophor EL 500 g standard (product number: C5145-500G) was purchased from Sigma-Aldrich, UK.

3.1.2.2. Ricinoleic acid 1 g standard (product number: R7257) was obtained from Sigma-Aldrich, UK.

3.1.2.3. Mobile Phase (UPLC) and system reagents

Methanol (LC-MS grade)	Fisher, UK
Methanol (HPLC grade)	Fisher, UK
Ethanol (HPLC grade)	Fisher, UK
Acetonitrile (HPLC grade)	Fisher, UK
Isopropanol (HPLC grade)	Fisher, UK
Formic acid, 98/100% (Optima, LC grade)	Fisher, UK

Millipore deionised water – filtered daily to a recommended quality of 18.2 MΩ resistivity.

3.1.3. Extraction Solvents and chemicals

Hexane (HPLC grade)	Fisher, UK
Chloroform (HPLC grade)	Fisher, UK
Methanol (LCMS grade, part no: 34966)	Sigma, UK
Ethanol (HPLC grade)	Fisher, UK
Diethyl ether (HPLC grade)	Fisher, UK

Millipore deionised water – filtered daily to a recommended quality of 18.2 MΩ resistivity.

The water purity value and grade relate to the removal of bacteria and possible particles from the deionizer.

Butylated hydroxytoluene (BHT)	Sigma, UK
Molecular sieves (AW-300)	Sigma, UK
Sodium chloride	Fisher, UK
Sodium hydroxide	Sigma, UK
Sodium sulphate	Sigma, UK

3.1.4. Working Reagents

Mobile phase A2: 0.1% Formic Acid in DW

Mobile phase B2: ethanol

Extraction reagents:

1. Chloroform: methanol (2:1,v/v) BHT (50mg/L):

   50mg of BHT was accurately weighed out, 667ml of chloroform and 333ml of methanol were mixed. The materials were then added together and mixed thoroughly. The resulting solution was stable for 6 months at room temperature.

2. 1 M Sodium Chloride in deionised water:

   58.44g of sodium chloride was added to 1 litre of deionised water. The produced solution was stable for 6 months at room temperature.

3. 2 M Sodium hydroxide in methanol (LC-MS) grade (40g in 500 ml):

   40g of sodium hydroxide pellets was accurately weighed and added to 500ml of LCMS grade methanol. The prepared solution was thoroughly mixed and heated at 50°C in an ultrasonic bath until all the pellets had dissolved. The resulting solution was stable for 3 months at room temperature.

3.1.5. Materials and consumables

3.1.5.1. Fresh frozen plasma (FFP) was supplied by a Haematology Department, in Hampshire.

3.1.5.2. 10 ml glass LabCo tubes were obtained from Fisher Scientific, UK.

3.1.5.3. Pyrex – disposable 12 x 75 mm glass culture tubes (LP4), glass Pasteur pipette and Chromacol – glass analysis vials and crimp caps were also bought from Fisher Scientific, UK.

3.1.6. Sample Preparation

Cremophor (Polyoxyethyleneglycerol triricoleate 35), undergoes alkaline hydrolysis to form ricinoleic acid which is measured by Liquid chromatography tandem mass spectrometry (LC-MS/MS). Test samples were prepared by spiking Cremophor into fresh frozen plasma (FFP) and extracted using chloroform: methanol Lipid extraction procedure as shown below.

3.1.7. Preparation of Cremophor Standard Curve

3.1.7.1. Cremophor EL stock (Ready to use) Stored in lockable cupboard at room temperature.

3.1.8. Preparation of Top Standard (20 µL/ml)

3.1.8.1. 2ml of cremophor EL stock was added to 98ml of FFP and thoroughly mixed using a magnetic stirrer at room temperature at a constant speed until the lipid cremophor solution had dissolved into the FFP.

3.1.9.Working standard curve

3.1.9.1. Double dilute 1ml 20 µL/ml mixed top standard with 1 ml FFP in a series of LabCo tubes to produce a range of standards of the following concentrations: 10, 5,2.5, 1.25, 0.625,0.313 µL/ml. Vortex mix briefly. Previous pharmacokinetic studies of the disposition of Cremophor EL after infusion of Paclitaxel an anticancer drug shows that the concentration of Cremophor EL rarely peaks beyond 10 µL/ml.

3.1.10. Preparation of Samples for Analysis

Before running the assay it was important to ensure that a sufficient stock of sodium sulphate columns were made (see 3.1.10.1).

3.1.10.1. Column preparation

Sodium sulphate columns were made from glass Pasteur pipettes, cotton wool and sodium sulphate crystals (Figure 16). The cotton wool acted as a plug to prevent the crystals from leaving the pipette. Only a pinch of cotton wool was required and this was carefully pushed inside the glass shaft to the neck of the pipette. The cotton wool plug was gently eased down to the neck of the pipette using a thin spatula. Care was taken not compact the cotton wool plug as this could have created problems during the extraction process.
To each glass Pasteur six spatula scoops of sodium sulphate crystals were added. The columns were stored in an airtight container away from moisture with a silica desiccant. The sodium sulphate used in the columns acted as a desiccant. The purpose of the desiccant is to remove any remaining water left in the solution once the Chloroform: methanol LLE was performed.

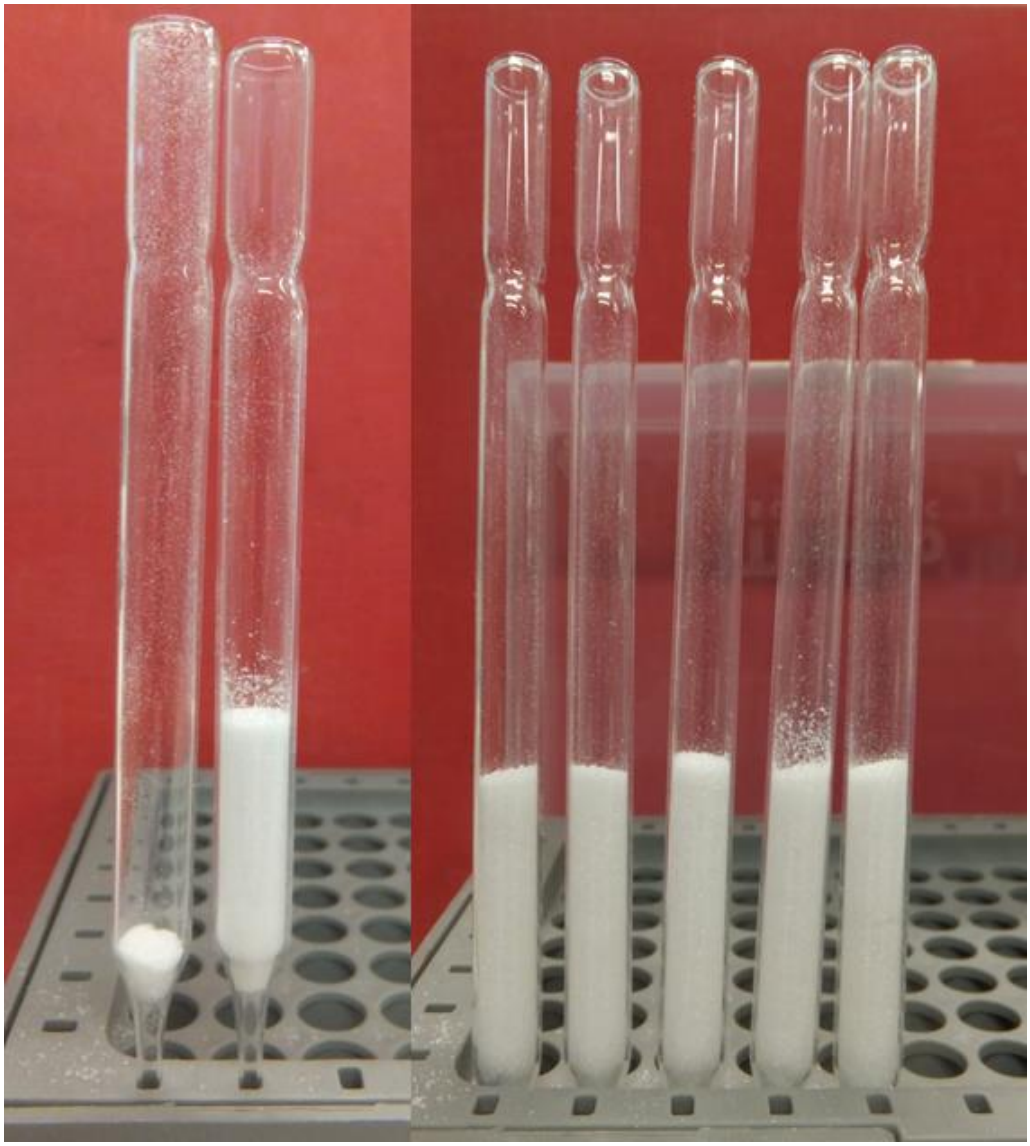


Figure 16: Sodium sulphate columns. Image on right shows column with cotton wool bung and wool with sodium sulphate. Image on left shows prepared columns. Refer to comment in declaration regarding photographs.

50µL plasma / QC/ Standard samples were added to a 10 ml LabCo tube and vibra-mixed.

The samples were then ready for extraction.

Lipid Extraction

- 5 mls of Chloroform: Methanol (2:1,v/v) with BHT (50mg/L), and 1 ml sodium chloride were added to the prepared samples and mechanically shaken for 5 minutes at 1000 rpm. (Figure 17 and Figure 18)

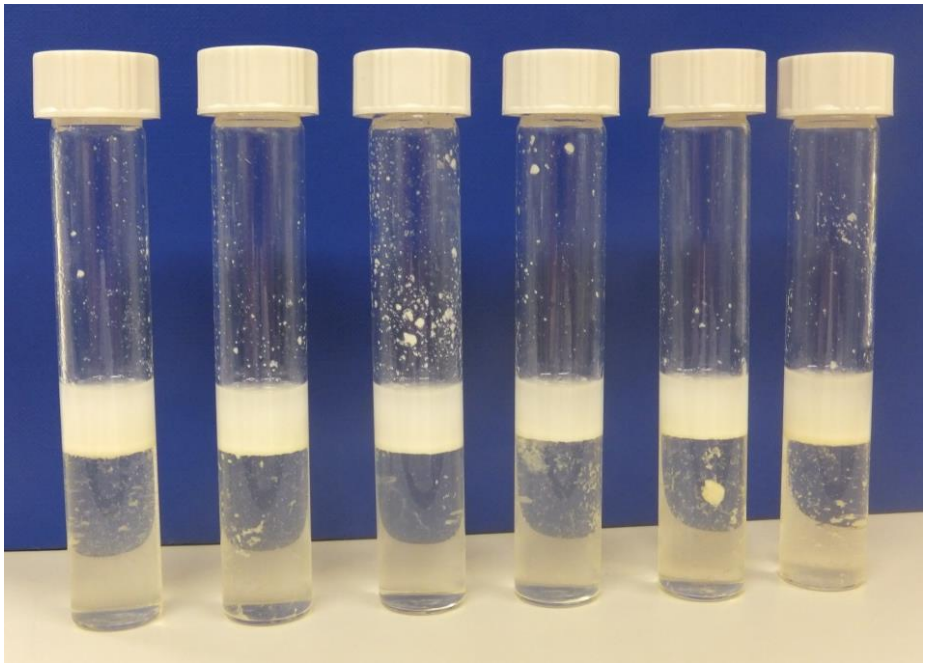


Figure 17: 10 mL glass tubes containing 50 µl plasma, 5 mL chloroform:methanol: BHT and 1 mL sodium chloride. Refer to comment in declaration regarding photographs.

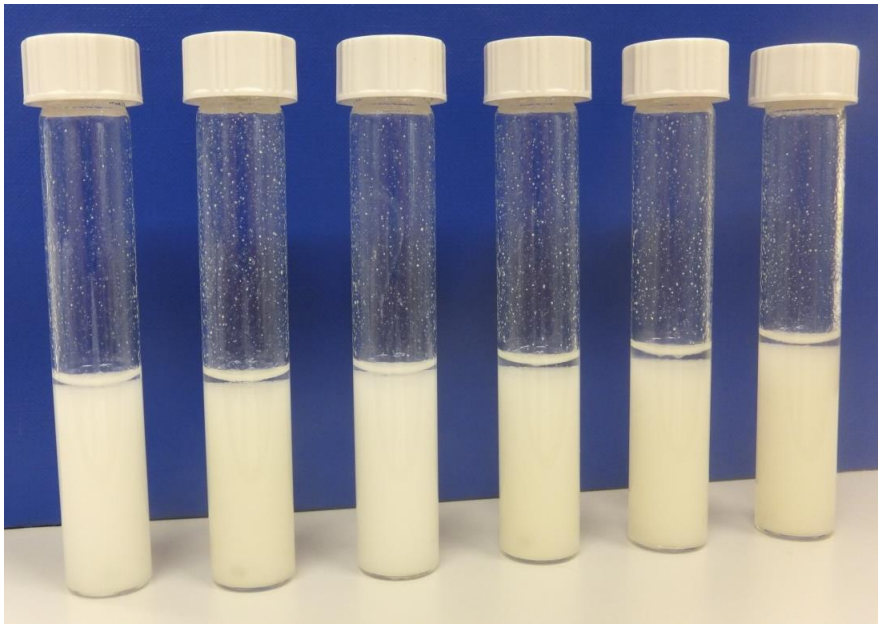


Figure 18: 10 mL glass tubes after mechanical shaking for 5 minutes at 1000 rpm. Refer to comment in declaration regarding photographs.

- The samples were then centrifuged for 10 minutes at 3000 rpm. Two defined phases were then visible. Figure 19

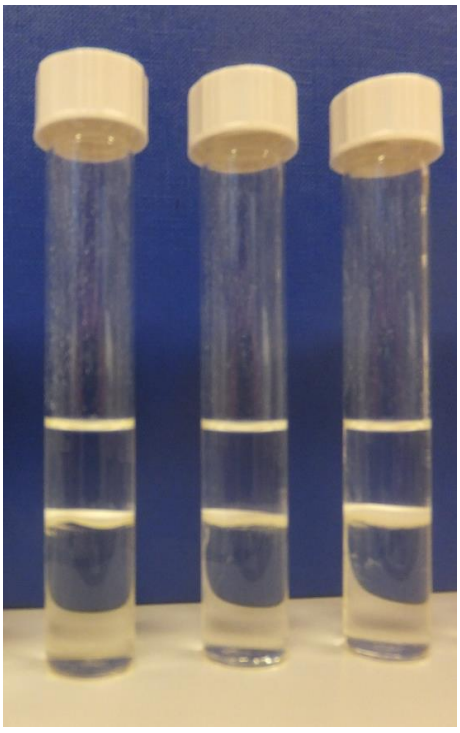


Figure 19: 10 mL glass tubes after centrifugation at 3000 rpm for 10 minutes. Two layers are clearly defined layers with a protein disc sandwiched between the two. Refer to comment in declaration regarding photographs.

- The lower phase was collected using a glass Pasteur pipette and transferred to a 10 ml Labco tube. When extracting the lower phase, the tube was tilted slightly to allow the glass pipette to slip past the upper phase and protein band. The collected lower phase was then processed using sodium sulphate columns. Sodium sulphate columns were prepared in advance of the assay.

Column conditioning

- The column was conditioned with 1 ml diethyl ether, the ether was allowed to drain to waste in a beaker. The sample was the added to the conditioned column to collect the filtrate in a 10 ml LabCo tube. The columns were washed with a further 1 ml diethyl ether and collected.
- The filtrate and ether mixture was then evaporated using a gentle air flow in a laminar flow fume cabinet. This stage took approximately 30 minutes.

Release of Ricinoleic acid

- To each 10 ml LabCo tube, 2 ml of 2 M sodium hydroxide in methanol was added and mechanically shaken for 5 minutes at 1600 rpm.
- A further 2.5 ml of deionised water and 2.5 ml of hexane was then added. This was mixed thoroughly and the layers allowed to settle. The supernatant phase containing ricinoleic acid was transferred to a glass (LP4) tube using a Pasteur pipette.
- The supernatant was then evaporated using a gentle flow of air in a laminar flow fume cabinet.

Reconstitution

- 500 µL of Ethanol was added to each dry eluate, it was then vortex mixed and then 300 µL was transferred to glass analysis vials (auto-sampler vials) and capped. The samples were then ready for analysis.

3.1.11. Discussion

At the start of the development process a Gas Chromatography Mass Spectrometry (GC-MS) method was tried (Figure 210 and Figure 21). The GC-MS was fitted with a lipid specific column a (DB 17 MS) and the GC-MS parameters used are present in appendix 1.

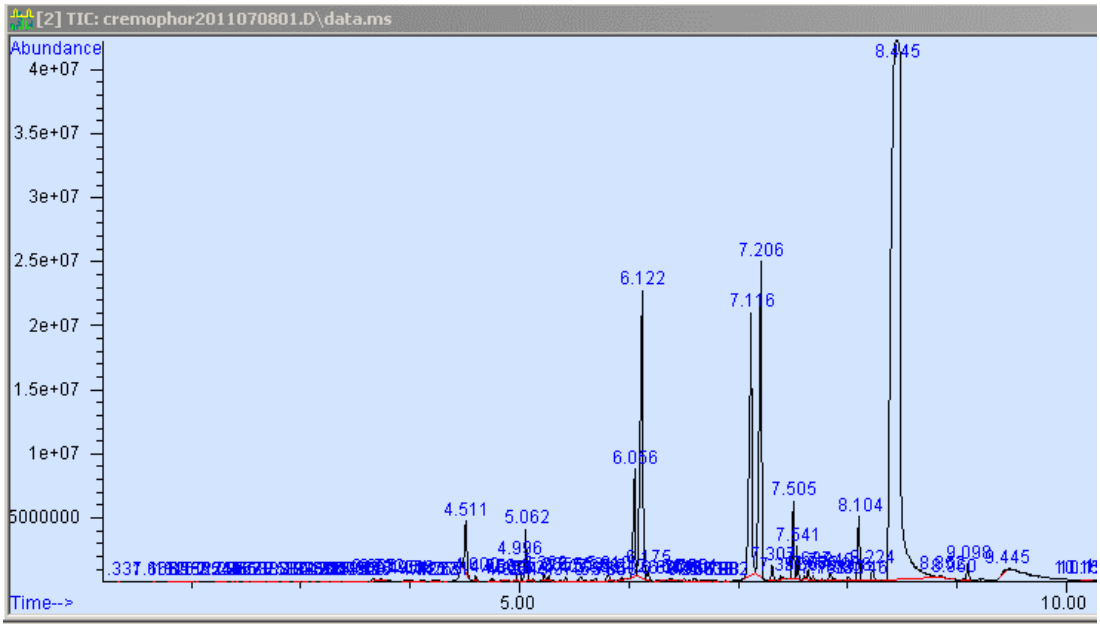


Figure 20: Image showing a chromatogram of cremophor peak at 8.445.

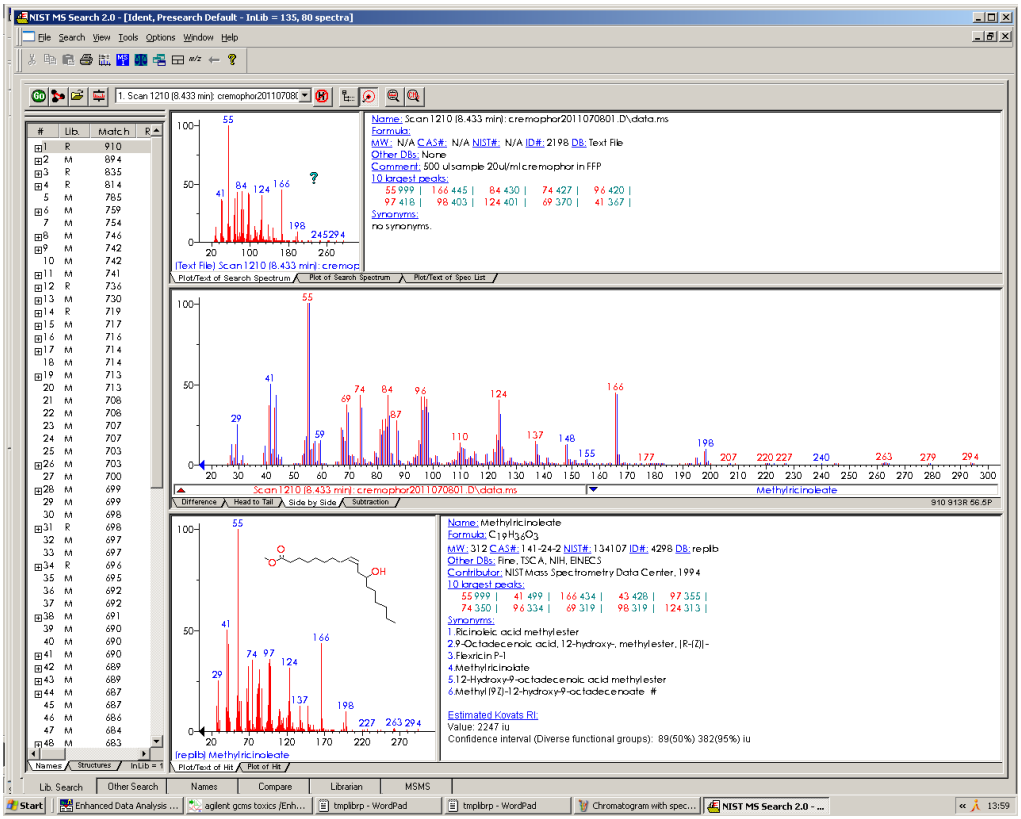


Figure 21: NIST (National institute of Standards and Technology) Library identification tool-identifying CrEL during the first analysis run.

The sample preparation involved a chloroform-methanol extraction, hydrolysis using sodium hydroxide and methylation using boron trifluoride (Figure 22).

GC-MS Cremophor assay extraction protocol

Cremophor Stocks

Original cremophor stock = 20 µl/mL
Add 2 mL of 20 µl/mL cremophor to 98 mL FFP = 20 µl/mL in FFP(1x QC + 1x STD).
Dispense out 100 mL of QC or STD into 95 x 1 mL aliquots of 20 µl/mL cremophor.
Using 1 mL of 20 µl/mL cremophor, dilute out to form 0.5, 1.0, 2.0, 5.0, 10.0 µl/mL of cremophor in 1 mL FFP.

Lipid Extraction

- Add 100 µl of sample/standard plasma to a 10 mL glass tube, add 100 µl of 0.1 mg/mL internal standard.
- Add 5 mL of chloroform : methanol (2:1, v/v) with BHT (50 mg/L).
- Add 1 mL of 1M sodium chloride.
- Mix mechanically for 5 minutes at 1000 RPM.
- Centrifuge at 3000G for 10 minutes.
- Collect lower phase with Pasteur pipette and transfer to another 10 mL tube.
- Make solid sodium sulphate columns using more glass Pasteur pipettes.
- Pre- wash columns with 2 mL of diethyl ether, drain into a waste beaker.
- Apply sample to column and collect into another 10 mL tube.
- Wash column with 2 mL of diethyl ether, collect in sample tube.
- Dry under stream of air (approximately 30 minutes).

Release of Fatty Acid

- Add 2 mL of 2M sodium hydroxide in methanol (8 g in 100 mL).
- Incubate at 100 °C for 1 hour.

Fatty acid Derivitisation

- Add 2ml of 25% borontrifluoride solution in methanol
- Incubate at 80 °C for 1 hour.
- Add 2.5 mL of deionised water and add 2.5 mL of Hexane.
- Mix mechanically for 2 minutes at 1000 RPM.
- Collect supernatant phase with FAME into a 10 mm LP4 tube.
- Dry under stream of air (approximately 15 minutes).
- Reconstitute in 100 µl hexane, vortex mix briefly on a vortexer.
- Add the 100 µl reconstitution mixture to an analysis vial and crimp.

Figure 22: The original GC-MS extraction method.

Initial results appeared promising with 100% recovery of CrEL and the internal standard heneicosanoic acid, but subsequently the signal was lost. After a chemical reaction it is possible to calculate the remaining original substance, this result is calculated as a percentage and is termed percentage recovery. Values can either be less than 100% or greater, in the case of percentages exceeding 100% the results are inaccurate due to incorrect measurement or calculation. Values below 100% are more desirable.

The reason the signal was lost was not fully apparent, and the experiments were repeated using new columns but still proved unsuccessful. The loss of signal at this time may have been due to column saturation as a result of the chemical nature of the CrEL compound leading to a likely blockage (Figure 23). On reflection and after reading GC-MS troubleshooting guides a potential root cause could have been a reaction between the stationary phase in the column and the analyte (however the extraction method used had been previously used to quantify other FAMEs) or it was caused by a blockage of the detector jet.

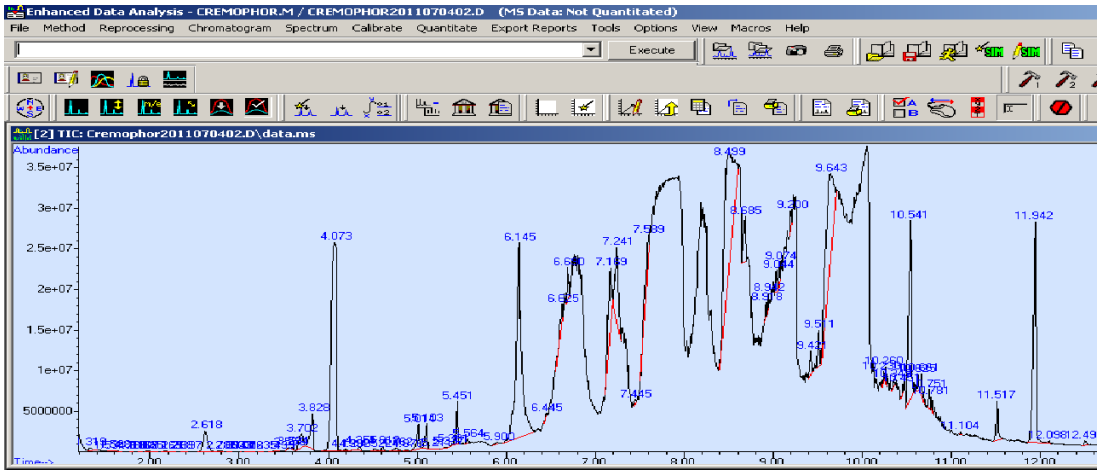


Figure 23: Image showing the GC-MS spectrum produced after the injection of an extracted CrEL standard. Peaks such as these occurred when the column began to saturate and eventually block.

Chapter 4

4.1. Method Validation

After the development of an analytical method, the validation is the next key step to running the method routinely. Many validation guides are available for LC-MS/MS platforms. Reliable analytical data is of the foremost importance when treating patients.

The Cremophor assay was developed and validated according to the methods published by J W Honour in 2011.[51] This method covers all aspects of method development as recommended by the FDA and EMA guidelines on bioanalytical method validation.[52, 53]

The various steps include:

4.1 Tuning and Selectivity of Ricinoleic acid
4.2 Interference by ion suppression or enhancement
4.3 Linearity
4.4 Accuracy and Recovery
4.5 Imprecision
4.6 Sensitivity
4.8 Carry-Over
4.8 Stability Studies
4.9 Summary & Conclusions

4.2. Tuning

The first step in LC-MS/MS method development is to determine the optimal parameters for ionisation of the parent compound. The instrument must be adjusted to establish the best resolution which relates to the width of the peak and the highest sensitivity which is the height of the peak.

It is advisable where possible to obtain quality chemical products or Certified reference materials (CRM).

Waters recommend in their operators guide a set of starting parameters for each stage of tuning from parent ion through to daughter and qualifier ions. The following suggested conditions in tables 5 and 6 were used when Ricinoleic acid was tuned. During the tuning of the parent ion the parameters remain low or wide in range to allow the conditions for the ion to be optimised. The only gas applied to the compound at this point is the API gas nitrogen. Once the best conditions are applied to the parent ion and the resulting peak width and height has been seen the conditions are then changed to allow the fragmentation of the parent ion to occur (see figure 24). A collision gas in the form of argon is applied and the parent ion is subjected to a series of scans to help establish potential daughter ion peaks. Once the individual scans at 15 eV, 30eV and 45eV have been executed it is then possible to anticipate which potential fragments could be suitable daughter ions. Each daughter ion is then optimised to check its suitability.

Quadruple 1	Quadruple 2	Quadruple 3
MS 1	Collision cell	MS 2
Sorts ions	Fragments ions	Sorts fragments

Figure 24: Schematic of tandem mass spectrometer

Parameter	Recommended value
Voltages	
Capillary (kV)	3.00
Cone (V)	50
Extractor (V)	5
RF Lens (V)	0.0
Temperatures	
Source Temp ()	120
Desolvation Temp ()	300
Gas Flow	
Desolvation (L/hr)	700.0
Cone (L/hr)	50

Table 5: Recommended ES+ Source Page Parameter values – starting conditions to establish the parent ion.

Parameter	Recommended value
Analyser	
LM Resolution 1	15.0
HM Resolution 1	15.0
Ion Energy 1	0.5
Entrance	50
Collision	3
Exit	50.0
LM Resolution 2	15.0
HM Resolution 2	15.0
Ion Energy 2	3.0
Multiplier	550

Table 6: Recommended Analyser Page Parameter values – starting conditions to establish the parent ion.

For Ricinoleic acid, the product of alkaline hydrolysis of Cremophor EL, electrospray ionisation was used. In this method, as the compound elutes from the LC a gas in this case nitrogen was used to nebulize the eluent as it enters the mass spectrometer through a narrow capillary. A potential is applied to the capillary resulting in ionisation of the compound of interest. A heated desolvation gas is applied to the spray generated in order to help solvent evaporation. Ions can either form as pronated hydrogen adducts (M + 1) or as negative ions (M – 1) or as other adducts. In the case of Ricinoleic acid, ions were formed in the negative mode.

Ionisation of Ricinoleic acid was achieved by infusing 1000 ug/L of the fatty acid in ethanol via post column (flow rate 20 µL/min) with 0.1% formic acid in DW/ ethanol (neat) (50:50) at a flow rate of 0.4 ml/min. The optimised capillary and cone voltages were 1kV and 32 V respectively. The source and desolvation temperatures were 150°C and 400°C respectively. The optimised molecular ion of Ricinoleic acid 297.3 and its principal daughter ion (quantifier 182.9) are shown in Figure 25 and Figure 26.

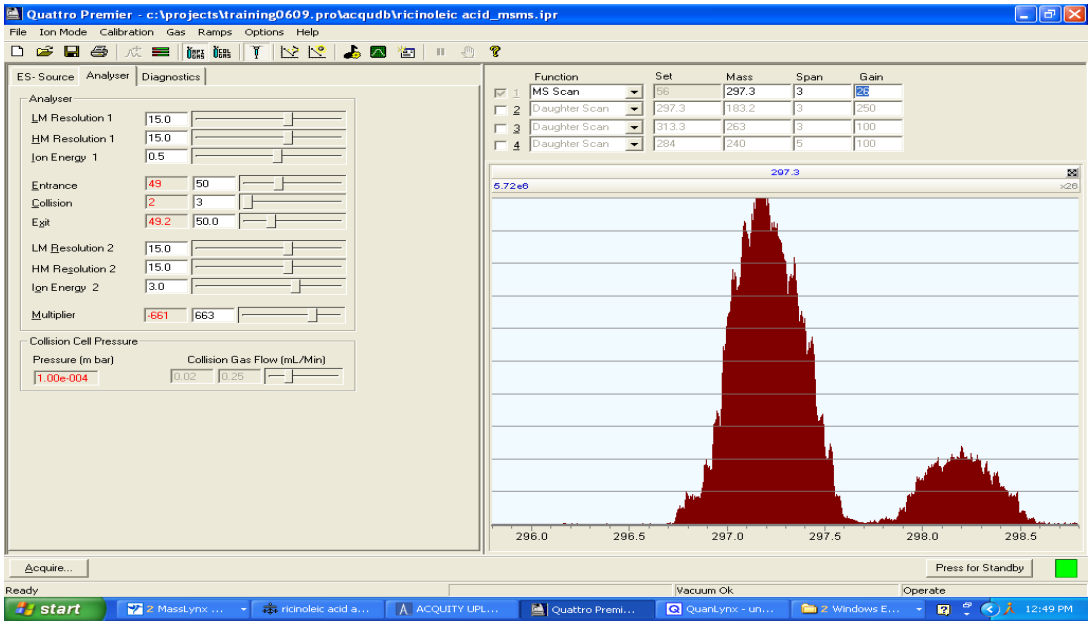


Figure 25: Parent molecular ion of Ricinoleic acid at 297.3. The image was obtained during the first stage of the tuning process when only the Cone and Desolvation gas (nitrogen) was applied.

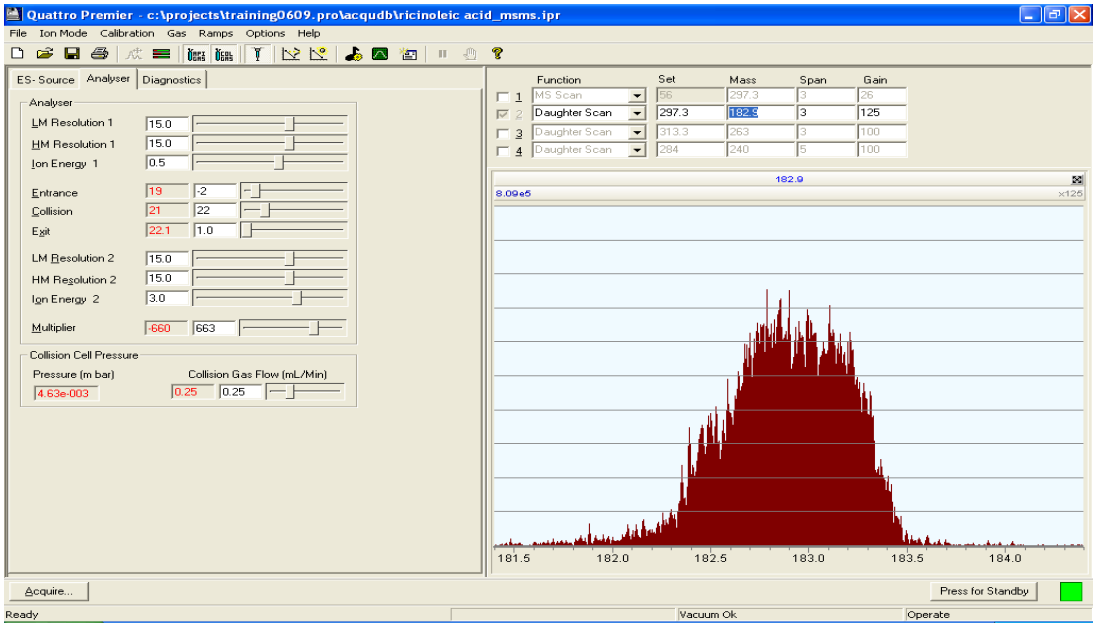


Figure 26: Quantifier daughter 182.9 ion of Ricinoleic acid. This image was obtained during the second stage of tuning when the parameters differ. The collision gas (argon) was applied allowing fragmentation to occur. The conditions seen are the optimum parameters for the daughter fragment.

The fragmentation pattern for Ricinoleic acid at 15, 30 and 45 electron volts (eV) can be seen in Figure 27. Each scan at 15, 30 and 45 electron volts was performed independently of each other and compared. At 15 eV the scan indicates that more voltage is required to fully fragment the parent compound at 297.4. At 30 eV the scan indicates more complete fragmentation, the parent ion at 297.4 has reduced and the daughter fragmentation peak has appeared at 183.2.

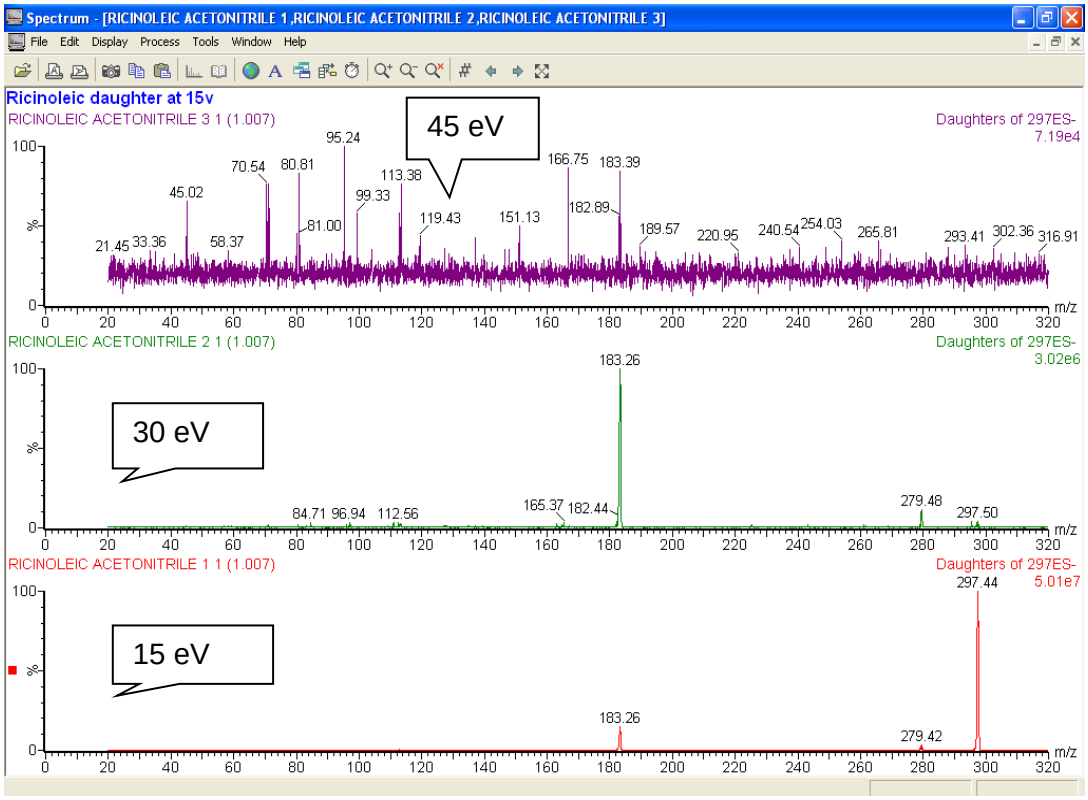


Figure 27: Ricinoleic acid fragmentation patterns at 15, 30 and 45 eV.

During method development, a suitable internal standard will be obtained and optimised using exactly the same procedure as the analyte of interest. Typically, an analogue form of the analyte that has three or more isotopic labels added to it are regarded as effective when possible. After tuning many internal standard compounds successfully, the internal standards were tested in an analytical batch. However, the internal standard showed poor reproducibility and lacked precision or accuracy. On some occasions, the internal standard peak did not appear in the chromatogram.

Therefore, a suitable internal standard was not obtained that could survive the lipid extraction method or maintain precision.

The final set of optimised ionisation parameters are shown in Table 7

MS tune conditions

Parameter	**Value/Condition**
Polarity	ES-
Capillary (kV)	1.0
Cone (V)	60
Extractor (V)	3
RF (V)	0
Source Temperature (°C)	150
Desolvation Temperature (°C)	400
Cone Gas Flow (L/Hr)	800
Desolvation Gas Flow (L/Hr)	1000
Collision Gas Flow (mL/min)	0.25
LM 1 Resolution	15
HM 1 Resolution	15
Ion Energy 1	0.5
MS-MS Mode Entrance	-2
MS-MS Mode Collision Energy	15
MS-MS Mode Exit	1
LM 2 Resolution	15
HM 2 Resolution	15
Ion Energy 2	3.0
Multiplier(V)	663
Syringe pump flow(µL/min)	20
Collision cell pressure(mbar)	2.70e-3
Collision gas flow(mL/min)	0.25
Cone + Desolvation Gas	Nitrogen
Collision Gas	Argon

Table 7: MS tune parameters indicating the settings for each independently controlled quadrupole, the source condition and gas flow rates and temperatures.

After tuning the compound, the LC Isocratic gradient (Table 8 and Table 9*: LC Isocratic Settings*

) was used to run aqueous standards of 1000 ug/L of Ricinoleic acid in order to determine the retention times.

An isocratic elution remains at a constant uniform composition, whereas a gradient elution varies in the solvent strength typically low at the start leading to high near the end of the overall gradient.

Option	Setting
Run time	5.00
Load Ahead	Enabled
Loop Option	Partial loop with needle overfill
Loop Offline	Disable
Weak wash solvent	95/5 water:methanol
Srong wash solvent	IPA:ACN:MeOH:H20:FA
Weak needle wash volume	1800 uL
Strong needle wash volume	600 uL
Column temperature	50.0 C
Sample temperature	off

Table 8: Autosampler method

	Time (min)	Flow (ml/min)	%A 0.1% Formic Acid	%B Ethanol	Curve
1		0.400	3.0	97.0	
2	5.00	0.400	3.0	97.0	6

Table 9: LC Isocratic Settings

A chromatogram showing the retention time of Ricinoleic acid Figure 28.

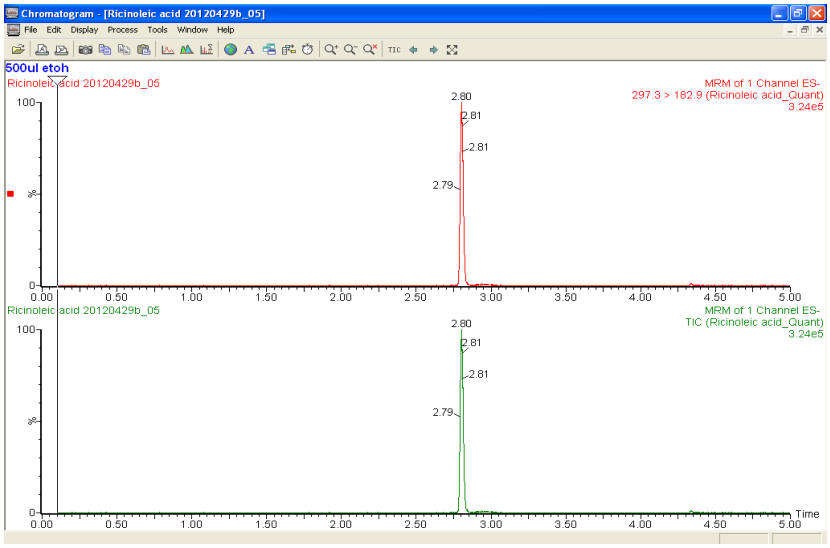


Figure 28: Chromatogram of aqueous standard of Ricinoleic acid eluting at 2.80 minutes.

4.3. Ion Suppression and Enhancement

Ion suppression and enhancement is a potential problem in the development of LC-MS/MS methods relating to matrix effects. It is related to the efficiency of the ionisation from likely phospholidids found in human samples. In order to exclude significant ion suppression or enhancement from matrix effect 1000 ug/ml of aqueous Ricinoleic acid was infused post column whilst an extracted FFP without Ricinoleic acid was injected into the LC. There was evidence of about 40% ion suppression at about 0.6 minutes but before the eluting time of Ricinoleic acid at 0.72 mins as shown in Figure 29.

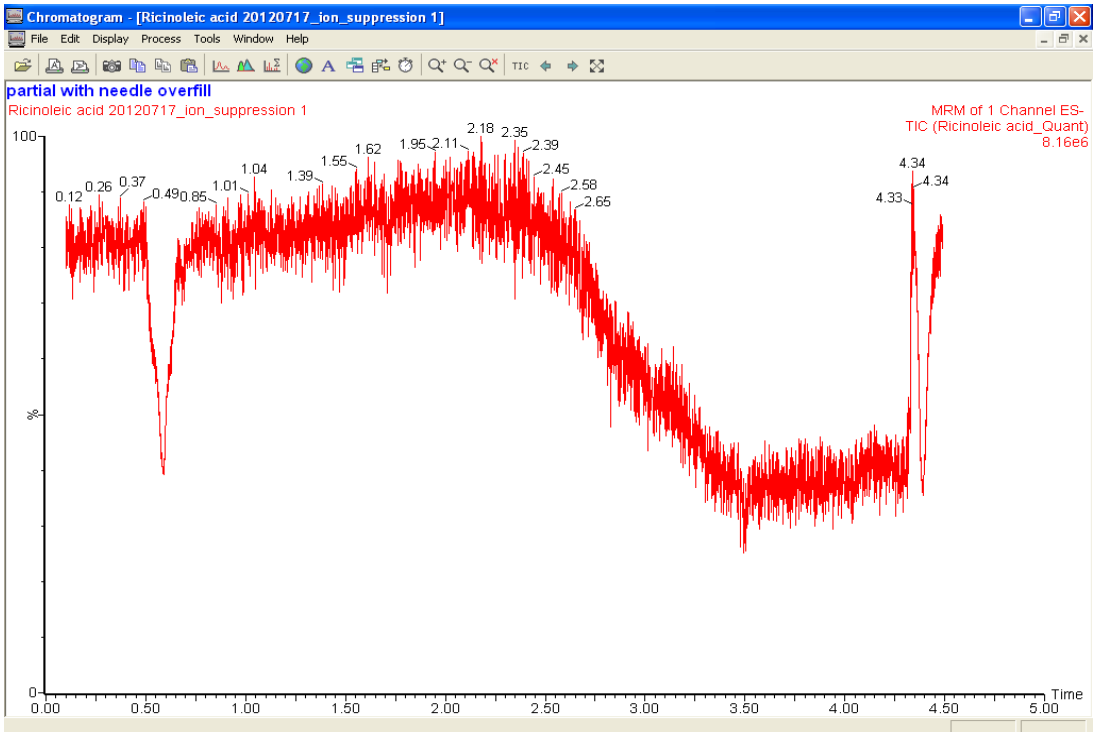


Figure 29: Chromatogram showing about 40% ion suppression at 0.6 minutes.

As shown in Figure 30 and subsequent experiments, there was no evidence of any significant effect on linearity, precision and accuracy.

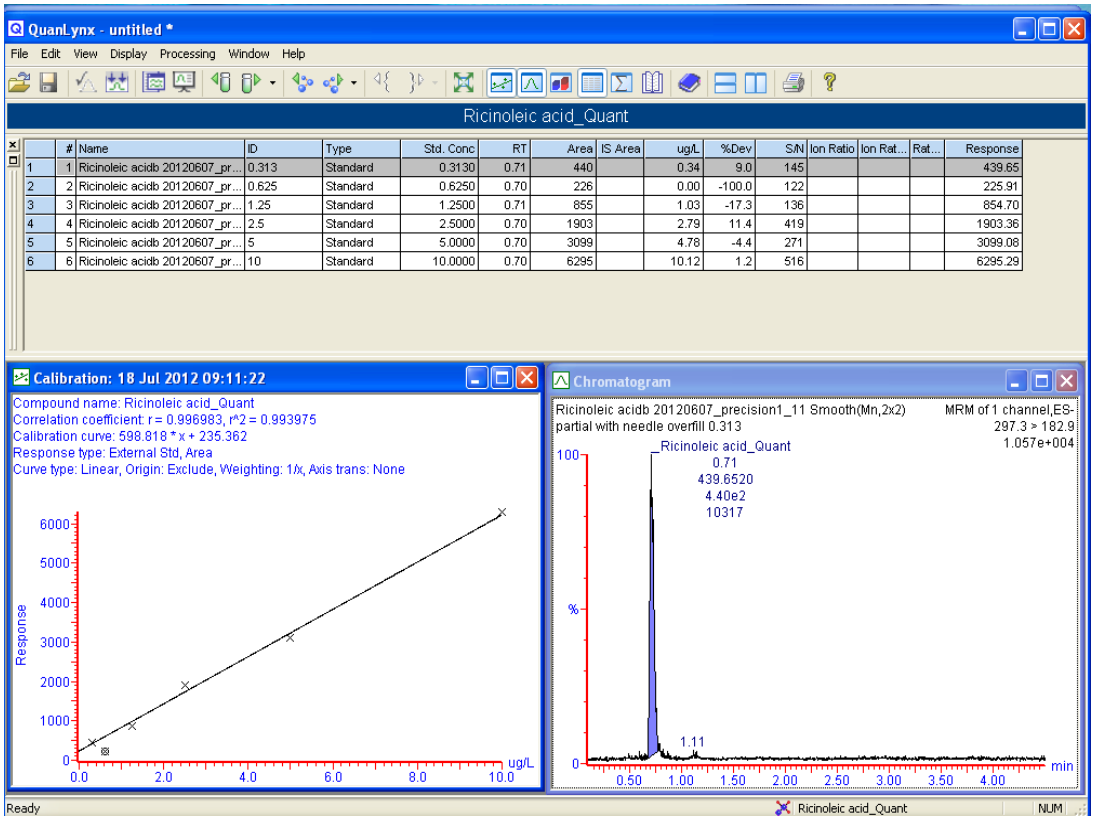


Figure 30: Standard curve of extracted Ricinoleic acid

4.4. Linearity

A standard curve of Ricinoleic acid was prepared in FFP with concentrations ranging from 0.313 µL/ ml to 10 µL/ ml. Previous pharmacokinetic studies of the disposition of Cremophor EL after infusion of Paclitaxel an anticancer drug shows that the concentration of Cremophor EL rarely peaks beyond 10 ul/ ml. [3] A sample of a standard curve of Cremophor EL after extraction from FFP is shown in Figure 30. Generally, the linearity was greater than $r^2 = 0.98$.

4.5. Accuracy and Recovery

As Cremophor is not a naturally occurring substance in human plasma, relative recovery was determined by spiking blank FFP and determining percentage recovery at two concentrations of Ricinoleic acid at low 0.313 µL/ ml and high 5 µL/ ml (n = 10). Results (Table 10) showing excellent recovery.

Spiked (µL/ ml)	Mean (µL/ ml) (n = 10)	% Recovery (n = 10)	Standard Deviation
0.313	0.277	88.5%	0.04
5	5.015	100%	0

Table 10: Recovery of Ricinoleic acid at 0.313 and 5.0 µL/ ml.

4.6. Imprecision

Intra assay precision of the method was determined by running 9 replicates of low (0.313 µL/ ml), and high (5 µL/ ml) samples. Co-efficient of variation (CV) was calculated. The day to day precision was determined over 3 days at the same concentrations. Results are presented in Table 11. The assay showed excellent precision over 3 consecutive days at both low and high concentrations at 17 and 10% respectively.

Concentration (µL/ ml)	Day 1 (CV%)	Day 2 (CV%)	Day 3 (CV%)	Mean (CV%)
0.313	10	19	22	17
5	8	9	13	10

Table 11: Intra-assay and day to day variation at different concentrations of Cremophor.

4.7. Sensitivity (Lower Limit of Quantification, LLOQ)

Previous pharmacokinetic studies of the disposition of Cremophor EL after infusion of Paclitaxel an anticancer used a standard curve with concentrations of the drug ranging between 0.5 to 10 ul/ ml.[3] In this study we have extended this range from 0.313 to 10 ul/ ml. The intra assay and day to day precision at 0.313 ul/ ml was about 10% (precision table) whilst the signal to noise ratio was constantly above 10 (Table 12). This fits into the acceptance criteria for LLOQ which is the lowest measurable amount at precision of < 20% and signal to noise ratio of > 10.

Sample	0.313 ul/ ml Recovery	S/N ratio at 0.313 ul/ ml
1	0.29	62
2	0.22	90
3	0.26	48
4	0.25	82
5	0.22	77
6	0.29	65
7	0.26	60
8	0.28	76
9	0.26	58
Mean	0.259	
CV %	10%	
%recovery	88.5	
Standard Deviation	0.02	

Table 12: Sensitivity at 0.313 ul/ ml (n = 10)

4.8. Carryover

Carryover is the appearance of the analyte in a run when a blank containing no analyte is injected. Carryover was assessed by injecting a high concentration at 5 µL/ml over 10 consecutive runs and comparing response to another 10 runs at the same concentration injected alternately with 10 FFP blanks.

The chromatograms of the extracted FFP blanks were examined for any evidence of carry over. The results are shown in Figure 31 respectively.

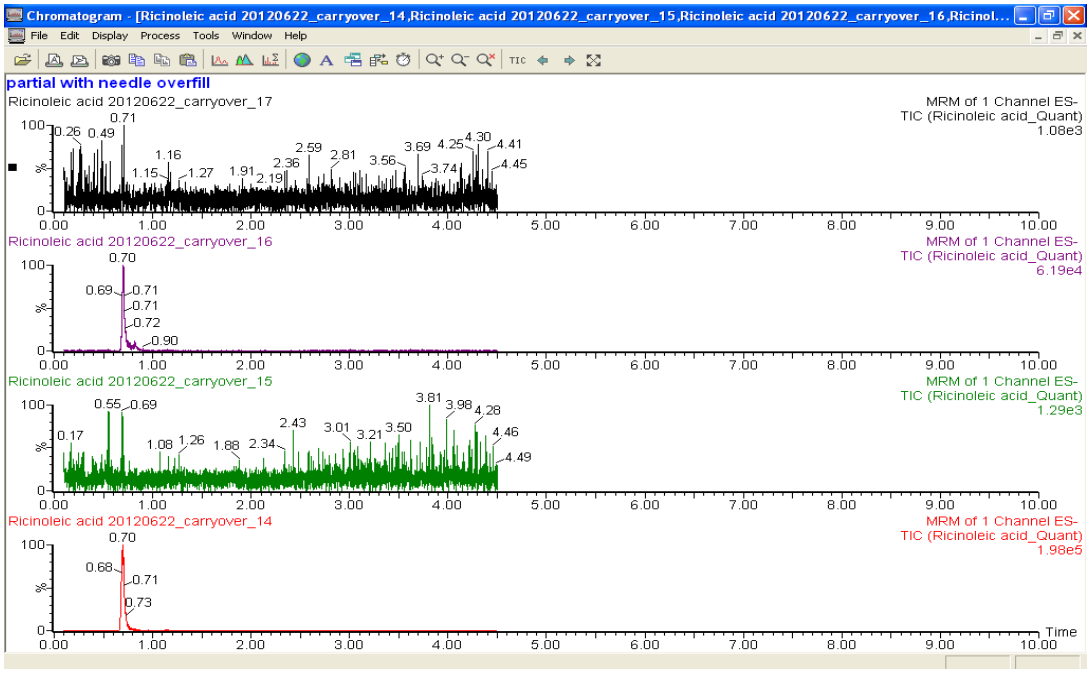


Figure 31: Alternate chromatographic runs of 5 ul/ml of cremophor alternating with extracted FFP blank showing no evidence of carry over of sample into the blanks. Retention time 0.7 min

4.9. Stability Studies

Stability studies are typically conducted in a pharmaceutical setting to ensure that developed medicinal products retain their quality, safety and efficacy throughout a planned shelf life. In the analytical chemistry setting the stability studies serve to establish the most suitable storage conditions for the product, drug or chemical. Usually the studies are planned to replicate the conditions the product or samples would encounter. A suitable range of temperatures is also included in a study to allow for all eventualities. Transportation and pre-analysis storage are factors to consider when devising stability study experiments.

Chemically, it is also important to include the following factors when conducting experiments with active ingredients; interaction between active ingredients and excipients, processes during manufacture, the type of dosage form, the container system used and how the product was protected from heat, light and moisture during transportation. Fluctuations in temperature could result in freezing, thawing or shearing.

Stability studies were undertaken to replicate storage and transportation conditions prior to analysis of Cremophor at 4°C and at -20°C

Aliquots (10) at concentrations of 1.25 µL/ml and 5 µL/ml each, were stored at +4°C and -20°C for 1 week and analysed. Results were compared to aliquots from the same pool analysed at day 1. (Table 13)

Refrigerated Samples

Concentration (µL/ml)	Day 1 (CV%)	Day 7 (CV%)	Mean (CV%)
1.25	9.0	10.0	9.5
5	7.5	9.5	8.5
n	10	10	

Table 13: Sample Stability at 40°C for 1 week

Frozen Samples

Ten aliquots of spiked plasma samples, 0.625 µL/ml and 5 µL/ml were stored at -20°C for 1 week to again replicate storage conditions if the samples were frozen transported before being analysed. Acceptance criteria was a CV of <15%. (Table 14)

Concentration (µL/ml)	Day 1 (CV%)	Day 7 (CV%)	1 Month	Mean (CV%)
0.625	9.5	9.5	10.7	9.9
5	8.0	7.5	6.9	7.5
n	10	10	10	

Table 14: Sample Stability at -20°C for 1 week

Stability of Stock Solutions

Stock solutions and standards were protected from direct sunlight and kept in a locked solvent store.

Stability of Processed Samples

To eliminate the possibility of detrimental effects of delays in analysis due to instrument problems, sample extracts were divided into two sets. The first set was analysed immediately or within the normal time delay from extraction to analysis. The second set was injected after 24 hours storage at 4°C. The UPLC-MS/MS equipment has the added advantage of a refrigerated sample store. This capability prevents sample degradation during analysis and batch runs, ensuring that all the samples remain the same temperature throughout the assay.

The results of the stability studies for Cremophor EL in plasma proved successful throughout all experiments tested at both 4 and -20. The low CV's for all the tested parameters indicates that if patient samples were delayed in transit for up to 7 days they would remain stable with no apparent degradation.

Discussion

CrEL is challenging and difficult compound to quantify in plasma or serum, especially when talking in terms of precision and reproducibility. This challenge was overcome by using UPLC-MS-MS as the analytical tool on the basis that it should give much improved specificity and sensitivity, speed of analysis and cost efficiency.

We reached a stage where we developed a method which could be applied to samples from this study but believed that improvements could still be made over time and we have taken advantage of unexpected delays in the study start to use this extra time to continue to develop the methodology.

There are very few reference methods for the analysis of Cremophor and all report problems with linearity and precision over the expected therapeutic range / concentration detection of 0.5-10.0µm/L.

A modification of the Coomassie Blue method (Sparreboom et al 1998)[39] using dual wavelength spectrophotometry improved precision and reproducibility though the application of a complex software package, transforming the calibration graph. With this method samples have to be analysed in duplicate and each singleton itself analysed twice.

There are also published HPLC (High Performance Liquid Chromatography) methods with UV (Ultraviolet) detection (Ghassempour et al 2003)[2] which require labour intensive sample preparation with long run times. A method using pyrolysis and Gas Chromatography with Mass Spectrometry has also been published but the instrumentation for the former is not readily available.

During the initial method development process, a Gas Chromatography Mass Spectrometry (GC-MS) method with lipid specific columns was tested. This involved chloroform-methanol extraction, hydrolysis using sodium hydroxide and methylation using boron trifluoride.

Initial results were promising but subsequently the signal was lost. The reason for this was not apparent, and the experiments were repeated using new columns but still proved unsuccessful. The loss of signal at this time may have been due to the cremophor compound saturating the column blocking it.

To overcome the encountered problems with GC-MS an Ultra Performance Liquid Chromatography (UPLC) linked to Tandem Mass Spectrometry was investigated. LC-MS/MS is a brilliant analytical tool offering rapid separation and identification of compounds. A method was initially developed using a modified Liquid Chromatography Mass Spectrometry (LC-MS) method using selective ion monitoring (SIM), but it suffered from interference from solvents and buffers. The precision was poor and unacceptable for validation. A subsequent method was developed to a stage where it was sufficiently reliable, where it was then validated to show it was fit for purpose. The current method uses MRM mode.

Chapter 5

5.1. Summary and Conclusion

A UPLC tandem mass spectrometry method has been developed and validated for measuring CrEL. This method showed very good linearity, accuracy, recovery, precision, sensitivity, with little evidence of carry-over. This method will be used in a to analyse patient study samples examining the use of Dexanabinol prepared in CrEL as a chemotherapeutic agent. The current SOP used in the laboratory is in appendix 6.2.

5.2. Future work

Additional work could include finding a suitable internal standard that could survive the extraction process. Many examples currently exist in some of the reviewed literature papers. Examples of these fatty acid compounds are in Table 15 below.

Internal standard used	MW
Margaric acid	270.45
2 hydroxy Oleic acid D17	315.57
10 hydroxy decanoic acid	188.26

Table 15: Additional internal standard options

More recently a potential way to increase the productivity of the CrEl extraction was bought, in the form of an oversized mechanical mixer produced by Lab-tek International (Figure 32) (Christchurch, New Zealand). The mixer was purchased because of its time saving nature and capability to accommodate large batch assays. Glass tubes remain in their original rack and are put directly into the mechanism under a securing arm covered in foam.

When the arm is pressed down the foam compresses around the screw top tubes holding them in place and a screw knob is then tightened to stop the arm rising. The shaker has a speed dial giving a range of 300-2800 rpm and two setting of continuous or timed mixing. The old system for mechanical shaking involved the transfer of each glass tube and pressing them in to a polycarbonate rack fixed on top of the shaker (Figure 32). This system has a maximum capacity of 36 spaces and when undertaking large batches of over 100 tubes can be tiresome. In comparison, the large mechanical shaker can accommodate a rack of 50 -80 samples depending on the type of rack used. Samples are mixing in around two minutes using the Lab-tek system meaning extractions could be accomplished in less time. In particular, this assay has three separate steps where mixing is required and the overall time saving could be close to one hour of saved analyst time.

Figure 32: Comparison images of a Lab-tek international vortexer and a IKA VIBRAX VXR vibramixer. Refer to comment in declaration regarding photographs.

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Chapter 6

Appendices

6.1. Appendix 1: GC-MS parameters

INSTRUMENT CONTROL PARAMETERS: agilent gcms toxics

C:\MSDCHEM\1\METHODS\cremaphor slow.M
Tue Aug 16 16:08:10 2011

Control Information
------- -----------

Sample Inlet : GC
Injection Source : GC ALS
Mass Spectrometer : Enabled

==========
==========
6890 GC METHOD
==========
==========

OVEN
Initial temp: 80 'C (On) Maximum temp: 340 'C
Initial time: 0.00 min Equilibration time: 0.50 min
Ramps:
Rate Final temp Final time
1 10.00 320 5.00
2 0.0(Off)
Post temp: 0 'C
Post time: 0.00 min
Run time: 29.00 min

FRONT INLET (SPLIT/SPLITLESS) BACK INLET (UNKNOWN)
Mode: Pulsed Splitless
Initial temp: 250 'C (On)
Pressure: 9.59 psi (On)
Pulse pressure: 23.0 psi
Pulse time: 2.00 min
Purge flow: 48.8 mL/min
Purge time: 0.25 min

Total flow: 53.8 mL/min
Gas saver: On
Saver flow: 15.0 mL/min
Saver time: 2.00 min
Gas type: Helium

COLUMN 1
Capillary Column
Model Number: J&W 122-4712
DB-17ms
Max temperature: 320 'C
Nominal length: 15.0 m
Nominal diameter: 250.00 um
Nominal film thickness: 0.25 um
Mode: constant pressure
Pressure: 9.59 psi
Nominal initial flow: 2.0 mL/min
Average velocity: 75 cm/sec
Inlet: Front Inlet
Outlet: MSD
Outlet pressure: vacuum

COLUMN 2
(not installed)

FRONT DETECTOR (NO DET)

BACK DETECTOR (NO DET)

SIGNAL 1
Data rate: 20 Hz
Type: test plot
Save Data: Off
Zero: 0.0 (Off)
Range: 0
Fast Peaks: Off
Attenuation: 0

SIGNAL 2
Data rate: 20 Hz
Type: test plot
Save Data: Off
Zero: 0.0 (Off)
Range: 0
Fast Peaks: Off
Attenuation: 0

COLUMN COMP 1
(No Detectors Installed)

COLUMN COMP 2
(No Detectors Installed)

THERMAL AUX 2
Use: MSD Transfer Line Heater
Description:
Initial temp: 280 'C (On)
Initial time: 0.00 min
Rate Final temp Final time

1 0.0(Off)

POST RUN
Post Time: 0.00 min

TIME TABLE
Time Specifier Parameter & Setpoint

GC Injector

Front Injector:
Sample Washes 0
Sample Pumps 3
Injection Volume 2.00 microliters
Syringe Size 10.0 microliters
PreInj Solvent A Washes 4
PreInj Solvent B Washes 4
PostInj Solvent A Washes 3
PostInj Solvent B Washes 3
Viscosity Delay 3 seconds
Plunger Speed Fast
PreInjection Dwell 0.00 minutes
PostInjection Dwell 0.00 minutes

Back Injector:
No parameters specified

Column 1 Inventory Number : js002
Column 2 Inventory Number :

MS ACQUISITION PARAMETERS

General Information
------- -----------

Tune File : atune.u
Acquistion Mode : Scan

MS Information
-- ----------

Solvent Delay : 0.90 min

EM Absolute : False
EM Offset : 0
Resulting EM Voltage : 1564.7

[Scan Parameters]

Low Mass : 25.0
High Mass : 550.0
Threshold : 150
Sample # : 2 A/D Samples 4

[MSZones]

MS Source : 230 C maximum 250 C
MS Quad : 150 C maximum 200 C

END OF MS ACQUISITION PARAMETERS

TUNE PARAMETERS for SN: us52420874

Trace Ion Detection is OFF.

EMISSION : 34.610
ENERGY : 69.922
REPELLER : 25.264
IONFOCUS : 90.157
ENTRANCE_LE : 16.000
EMVOLTS : 1564.706
AMUGAIN : 1680.000
AMUOFFSET : 125.500
FILAMENT : 2.000
DCPOLARITY : 0.000
ENTLENSOFFS : 20.329
MASSGAIN : -768.000
MASSOFFSET : -34.000

END OF TUNE PARAMETERS

END OF INSTRUMENT CONTROL PARAMETERS

6.2. Appendix 2: Standard operation procedure (SOP)

2.05 CREMOPHOR ANALYSIS (FOR RESEARCH PURPOSES ONLY)

Procedure name
Cremophor

Test code
CREM

Units
µL/mL

Clinical significance

To determine and quantify the presence of Cremophor in EDTA human plasma samples. Cremophor is a nonionic solubiliser and emulsifier which is commonly used in the pharmaceutical industry as an additive or excipient. Modern drugs are rarely administered in their pure chemical state and often use an excipient combined with the active compound to enable the transport of active drug to its target site. Examples of drugs using cremophor as an excipient include Dexanabinol, Benzocaine and Hexedetine.There is evidence that Cremophor may exhibit human toxicity and it is therefore necessary to monitor its levels in drug trials.

Principle of method
Cremophor (Polyoxyethyleneglycerol triricoleate 35) undergoes alkaline hydroxylation to form ricinoleic acid which is measured by Liquid chromatography tandem mass spectrometry (LC-MS/MS). This is performed by a Waters Quattro Premier XE system. Analyte separation is by an Acquity UPLC™ HSS T3 1.8µm column followed by gradient elution with 0.1% formic acid in deionised water and neat ethanol delivered at a flow rate of 0.4mL/min. Ionisation is achieved by the transfer of a 1kV capillary voltage to the molecules and protonation from the desolvation gas (nitrogen) delivered at a flow rate of 800L/hr to form negative molecular ions ($M\text{-}H^-$) in an "electrospray$^-$ mode". Molecular ions are fragmented to product ions following the reaction with the collision gas (argon) and monitored by a detector. System operation and data acquisition are controlled using Waters MassLynx V4.1 software. Data processing is controlled by TargetLynx V4.1 software.

Specimen type required
EDTA plasma.

Reagents and equipment

When handling reagents that are identified as a risk, staff MUST take precautionary measures and familiarise themselves with COSHH data and decontamination procedures before continuing.

LCMSMS System
Acquity UPLC® (serial number DO9 UPA 880M, Waters, UK)
HSS T3 1.8µm column (part no: 186003539, Waters, UK)
Waters Quattro Premier XE LC-MS/MS system (serial number VAB 1255, Waters, UK)
IKA Vibrax VXR Basic (mechanical shaker)
Beckman Spinchron™ DLX Centrifuge
Techne – Driblock and Sample Concentrator

Standards	**Supplier**	**Hazard**
Cremophor EL	Sigma	**NOT KNOWN – HANDLE WITH CARE**
Ricinoleic acid	Sigma	**IRRITANT, WEAR GLOVES/GOGGLES**

System fluids

Acetonitrile	Fisher	**IRRITANT, WEAR GLOVES/GOGGLES**
Ethanol	Fisher	**TOXIC, WEAR GLOVES/GOGGLES**
Formic acid, 98/100%	VWR	**IRRITANT, WEAR GLOVES/GOGGLES**
Isopropanol	Fisher	**FLAMMABLE, WEAR GLOVES/GOGGLES**
Methanol	Fisher	**FLAMMABLE, WEAR GLOVES/GOGGLES**
Methanol (LCMS grade)	Fisher	**FLAMMABLE, WEAR GLOVES/GOGGLES**
Millipore deionised water		

Extraction solvents and chemicals

Chloroform	Fisher	**FLAMMABLE, WEAR GLOVES/GOGGLES**
Diethyl ether	Fisher	**FLAMMABLE, HARMFUL**
Ethanol	Fisher	**TOXIC, WEAR GLOVES/GOGGLES**
Ethyl acetate	Fisher	**FLAMMABLE, WEAR GLOVES/GOGGLES**
Hexane	Fisher	**FLAMMABLE, WEAR GLOVES/GOGGLES**
Methanol (LCMS grade)	Fisher	**FLAMMABLE, WEAR GLOVES/GOGGLES**

Sodium chloride	Fisher	**IRRITANT, WEAR GLOVES/GOGGLES**
Sodium hydroxide	Sigma	**CORROSIVE, WEAR GLOVES/GOGGLES**
Sodium sulphate	Sigma	**IRRITANT, WEAR GLOVES/GOGGLES**
Butylated hydroxytoluene	Sigma	**HARMFUL, IRRITANT, WEAR GLOVES/GOGGLES**
Molecular sieves (AW-300)	Sigma	**IRRITANT, WEAR GLOVES/GOGGLES**

(Sodium Sulphate is hygroscopic in nature and must be kept away from moisture in a sealed airtight container)

Consumables
Chromacol - glass analysis vials and caps
LabCo -10mL/5mL glass tubes and screw tops
Fisher scientific - Pasteur pipettes, glass unplugged, (150mm)
Pyrex – disposable 12 x 75mm glass culture tubes

Working reagents
System fluids

Mobile Phase A2: 0.1% formic acid in deionised water
Add 1ml formic acid to 999ml millipore DW. Stable for 1 month at room temperature.

Mobile Phase B2:
Ethanol. Ready to use, kept in lockable solvent bin. Record useage.in ethanol record book.

Strong needle wash
Decant 250mL methanol, 250mL acetonitrile, 250mL isopropanol, 250mL DW and 2mL formic acid 98/100% into a container and mix. Stable for 6 months at room temperature.

Weak needle wash
Add 50mL methanol to 950mL DW and mix. Stable for 1 month at room temperature.

Seal wash

Add 200mL methanol to 800mL DW and mix. Stable for 1 month at room temperature.

Extraction reagents

Chloroform: methanol (2:1 v/v) with 50mg/L Butylated hydroxytoluene (BHT). Accurately weigh out 50mg of BHT, add 667mL of chloroform to 333mL of methanol. Add BHT to solvent mixture and mix thoroughly. Stable for 6 months at room temperature.

2M Sodium hydroxide in methanol

Accurately weigh out 40g of sodium hydroxide pellets and add to 500mL LCMS grade methanol and mix. The prepared solution is then heated in an ultrasonic bath set at 50°C until all the sodium hydroxide pellets have dissolved. Stable for 3 months at room temperature.

1M Sodium chloride in deionised water

Accurately weigh out 58.44g of sodium chloride and add 1 litre of deionised water. Stable for 6 months at room temperature.

.

Preparation of stock standard (20µL/mL)
Separately add 2ml stock Cremophor EL add 98ml FFP. Mix thoroughly using a magnetic stirrer. Decant as 1.5ml aliquots. Store at –20°C.

Preparation of 5µL/mL cremophor internal quality control
Add 1mL 20µL/mL Cremophor EL stock standard to 3mL FFP. Mix thoroughly using a magnetic stirrer. Store at –20°C in LEC lockable Forensic toxicology freezer as 200µL aliquots.

Preparation of 0.6µL/mL cremophor quality control
Add 500µL 5µL/mL cremophor internal quality control to 3.5mL FFP. Mix thoroughly using a magnetic stirrer. Store at –20°C in LEC lockable Forensic toxicology freezer as 200µL aliquots.

Working Standard curve
Double dilute the 20µL/mL cremophor stock standard in FFP to produce a standard curve with the following concentrations: 10, 5, 2.5, 1.25, 0.625 and 0.313 µL/mL

Prepare a worksheet 8.14 Cremophor worksheet

Procedure
Preparation of sodium sulphate extraction columns
Before running the assay ensure that a sufficient stock of Sodium sulphate columns are made.Sodium sulphate columns are constructed from glass Pasteur pipettes, cotton wool and sodium sulphate crystals. The cotton wool acts as a plug to prevent the crystals from leaving the pipette.

Carefully push a pinch of cotton wool inside the glass shaft to the neck of the pipette. Ensure the cotton wool plug is gently eased down to the neck of the pipette using a thin spatula. Do not compact the cotton wool plug as this can create problems during the extraction process.
To each glass Pasteur add six small spatula scoops of sodium sulphate crystals. Store the columns in an airtight container away from moisture. The sodium sulphate acts as a desiccant.

Chloroform: methanol Lipid Extraction
Perform this procedure under forced ventilation
Add 200µL sample/qc/standard to a 10mL Labco tube. Add 5mL of chloroform: methanol:BHT (2:1,v/v) mixture. Add 1mL of 1M sodium chloride and vibramix for 5 minutes at 1000 rpm. Next, centrifuge samples for 10 minutes at 3000rpm. The samples should be clearly separated with two defined layers.

Carefully collect the lower chloroform phase using a glass Pasteur pipette ensuring the layers remain separated and transfer this to another 5mL labco tube.

For each sample wash a sodium sulphate column with 1.5mL diethyl ether using a dispenser bottle. Allow the ether to drain by gravity through the column into a waste beaker.

Apply sample to washed column and collect filtrate into a 10mL labco tube.

Add 1.5mL diethyl ether to the column and collect the filtrate into the 10mL labco tube..
Dry the filtrate and ether mixture under air using the “Dri-Block” setting air flow regulator at about 5 bar. This should take approximately 50 minutes.

Alkaline hydrolysis of Cremophor to Ricinoleic acid
1.Add 2mL of 2M sodium hydroxide in methanol to the dried extract and vibramix for 5 minute at 1600rpm. Heat at 80°C for 60 minutes in the heating block. Allow to cool.

2.Add 2.5mL of Millipore water and 2.5mL of hexane. Vortex mix the solution and transfer the supernatant to an LP4 glass culture tube using a glass Pasteur pipette.

3.Dry down the supernatant under air using the "Dri-Block" setting air flow regulator at 2- 4 bar.

Reconstitution
Add 500µL of ethanol to each dry eluate, vortex mix, transfer 300µL to injection vials and cap. Samples are now ready for analysis by LC-MS/MS.

Analyser Set-up
Open software
At the desktop, select **MassLynx V1 icon**. The sample loader will initialise.
Upload assay method
Select **Instrument** tab followed by the **inlet method** icon.
Select file, open and select method file **ricinoleic acid ethanol. wvhp**.

Pre-run
Check waste containers situated in the spill tray under the instrument and ensure all solutions are in sufficient quantity to complete the assay.
Load the inlet method ricinoleic acid ethanol6.wvhp as shown in Table 1 and click on the load icon. Allow to run for 15 minutes.

Table 1 isocratic elution program for cremophor method

Time (min)	Flow (ml/min)	A2 (%)	B2 (%)
initial	0.4	3.0	97.0
5.00	0.4	3.0	97.0

Flow is confirmed by the green light.
Select the **Launch acquity UPLC console** icon (situated 2nd icon RHS) and ensure delta<100.If not consult a senior member of staff.

Switch on inlet gas and collision gas
In MassLynx, select instrument tab and select **MS Tune icon**
Open **Ricinoleic acid_MSMS** file
Click on **Gas** and select **Inject**
Switch inlet gas ON by toggling the **API gas** icon on top toolbar
Switch collision gas ON by toggling the **COL gas** icon on the top toolbar
Select **Press for Operate** and allow to equilibrate for 15 minutes

Sample loading
Open the door to the sample manager module of the LC-MS/MS system and remove well plate 1 by pressing on the grey tab of the well plate tray holder and withdraw holder. Remove the well plate and load samples according to

the worklist. Reset the well plate (nb cut-off corner orientated towards top RHS) and push holder back into position until assembly clicks into place. Close the sample manager door.

Program worklist
In MassLynx, select file, open and select a previous worklist eg Cremophor20120416 (format is test name yyyy/mm/dd) and edit as follows:
File name
Edit data file name in the format Test-Year-Month-Day-File number eg Cremophor2012041601.To autoincrement, highlight first entry, press shift and highlight your last entry, right **click** and **Fill series**
Sample ID
Enter blk, std, qc or sample lab number
File text
Enter patient surname and sample type
Tune file
Ensure mstune file = Ricinoleic acid MSMS
Method file
Ensure ms file= Ricinoleic acid acetonitrile
Inlet method file
Ensure inlet file= ricinoleic acid ethanol
nb to change these files, double left click field and select desired option
Bottle
Enter rack and position eg 1:1 (rack:position)
Injection volume
Enter 15µL
Sample type
Enter std, qc, analyte
Conc A
Enter the standard concentrations in the appropriate fields

Save the worklist, select **file**, **save as** and save in the Sample DB folder. Format is 'test name yyyy/mm/dd' eg Ricinoleic acid20120416.

Run worklist
Ensure that the source and desolvation temperatures have reached their set points.
Highlight the worklist by holding down the left mouse button and press the ▶ icon on the top toolbar. In *start sample list run* window ensure the number of samples to be analysed corresponds with the worklist and all tabs are ticked except pre- and post-run options and select **OK**. At *Create TargetLynx dataset* window, do not change any settings and select **OK**

To calculate results
In **MassLynx**, select **QuanLynx** tab, highlight the worklist and select **Process samples.**

To view chromatograms/results
In **QuanLynx**, select **view results.** Check that the calibration curve is linear and the fit is good, ie correlation coefficient R^2>0.98.
For all samples, QC and standards check the following:
1, The standard has integrated correctly and the peak area is acceptable.
2, The retention time for the analyte quantifier ion peak is correct and the peak has integrated correctly.
3, There is a corresponding qualifier peak with the same retention time as the quantifier ion peak.
If in doubt, refer to a senior member of staff.
Results are reported as µL/mL.

Compound elution times should be as follows:

Compound	**Approximate retention time (min)**
Ricinoleic acid	0.70

To view ions
Select MS method

MRM transitions are as follows:

Compound	**Parent ions (m/z)**	**Daughter ions (m/z)**
Ricinoleic acid	297.3	182.9

QC Interpretation
Enter data for internal quality control into QCPLUS. Acceptance criteria is stated as being within 2SD of the assigned mean. If QC is outside these limits inform a senior member of staff.

Reporting results
Lower level of quantitation 0.07µL/mL. Report levels as <0.07µL/mL if less than lower level of quantitation.
Upper level of quantiation 10µL/mL. Re-analyse levels >10µL/mL in the next assay as a 1:2 dilution in FFP.

Report results to Masterlab to 2 decimel places. All results are validated by Dr Abu.

Traceability:
The method is traceable to the following standards
Ricinoleic acid , Cat No. R7257 purity >99% Sigma-Aldrich
Cremophor Cat. No C5135, Sigma-Aldrich

Uncertainity:
Uncertainty is defined in Q3.9 Traceability and Uncertainty and values are stored in Q3.9a Uncertainty of measurement values.xlsx.
Where EQA and IQC is available the uncertainty is calculated as 1.96*SD + Absolute Bias.
For binary results the uncertainty of Positive or negative results as a percentage is reported for the process utilising the positive predictive value or the negative predictive value.
If none of these are possible then it is calculated from the constituent uncertainties of the process
.
There are currently no External Quality Assurance (EQA) schemes for Cremophor to compare against.

Risk assessment
Low overall risk if this procedure is performed using good laboratory practice with particular awareness to the hazardous properties of highlighted substances.

Associated Procedures
Toxics validation: FTV7- The Development and Validation of a Liquid Chromatography Tandem Mass Spectrometry Method for Cremophor EL in Human Plasma.

COSHH assessments and MSDS documents are stored in QPulse.