Development and application of novel approaches to quantitate therapeutic drugs in human blood

Deirdre Fox

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Development and application of novel approaches to quantitate therapeutic drugs in human blood

A thesis submitted for the Degree of Doctor of Philosophy

By

Deirdre Fox B.Sc. (Hons)

The research work described in this thesis was carried out under the supervision of

Dr. Gillian McMahon

and

Dr. Robert O'Connor

National Institute for Cellular Biotechnology

Dublin City University

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Abbreviations

%	Percentage
3-D	Three dimensional
α	Selectivity factor
Å	Angstrom
AA	Ascorbic acid
ACN	Acetonitrile
AIDS	Acquired immunodeficiency syndrome
API	Atmospheric pressure ionisation
APCI	Atmospheric pressure chemical ionisation
Atr	Atropine
AUC	Area under the curve
CE	Capillary electrophoresis
C_{max}	Maximum concentration
CNS	Central nervous system
DAD	Diode array detector
DCM	Dichloromethane
DNA	Deoxyribonucleic acid
dRif	Desacetyl rifampicin
Efv	Efavirenz
ESI	Electrospray ionisation
FDA	Food and drug administration
FIA	Flow injection analysis
FV	fragmentor voltage
g	Gram
GC	Gas chromatography
HAART	Highly active antiretroviral therapy
HETP	Height equivalent to theoretical plate
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HLB	Hydrophilic-lipophilic balance
hr	Hour

IS	Internal standard
k'	Capacity factor
L	Litre
LC	Liquid chromatography
LLE	Liquid liquid extraction
LOD	Limit of detection
LOQ	Limit of quantitation
MAX	Mixed-Mode Anion Exchange
MCX	Mixed-Mode Cation Exchange
MeOH	Methanol
min	Minute
mL	Milli litre
MRM	Multiple reaction monitoring
MS	Mass spectrometer/mass spectrometry
MS/MS	Tandem mass spectrometry
MW	Molecular weight
m/z	Mass to charge ratio
NaOH	Sodium hydroxide
NASA	National Aeronautics and Space Administration
NCSR	National Centre for Sensor Research
NICB	National Institute for Cellular Biotechnology
ng	Nano gram
nm	Nano meter
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NSI	Nanospray ionisation
OVAR	Off-vertical axis rotation
PDA	Photo diode array
pg	Pico gram
PP	Protein precipitation
QC	Quality control
QQQ	Triple quadrupole MS
R^2	Correlation coefficient
RI	Refractive index
Rif	Rifampicin

RNA	Ribonucleic acid
RP	Reversed phase
RSD	Relative standard deviation
Scop	Scopolamine
SEC	Size exclusion chromatography
SIM	Selected ion monitoring
SLE	Supported liquid extraction
SLSL	Space Life Sciences Laboratory
S/N	Signal to noise ratio
SPE	Solid phase extraction
SPhEAR	Study on the Pharmacokinetics of Efavirenz And Rifampicin
SRM	Selected reaction monitoring
TB	Tuberculosis
t-BME	*tert*-butyl methyl ether
THF	Tetrahydrofuran
TIC	Total ion count
T_{max}	Time to maximum concentration
TOF	Time-of-flight
t_R	Retention time
µg	Micro gram
µL	Micro litre
µm	Micro meter
UV	Ultraviolet
UV-Vis	Ultraviolet-Visible
v/v	Volume/volume
WAX	Weak Anion Exchange
WCX	Weak Cation Exchange

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Appendices -1-

Abstract

Abstract

The research described in this thesis utilised novel extraction phases and analytical approaches in order to develop new methods for the separation and determination of important pharmaceuticals in complex biological matrices. The project employed bioanalytical chemistry to solve real-world biological and clinical problems.

The first project involved the development and validation of an analytical protocol for the extraction, separation and determination of rifampicin and efavirenz in HIV-positive patients. The protocol was developed to support the hypothesis that 'concomitant treatment with rifampicin-containing anti-tuberculosis (TB) therapy and the anti- HIV therapy, efavirenz, results in a decreased rifampicin half life'. The emphasis in this work was the determination of both drugs simultaneously in one sample in a simple, rapid and cost effective assay. This was achieved using solid phase extraction and LC-UV analysis. The main challenge was developing a low cost assay to detect the drugs within a clinically relevant concentration range using standard chromatography equipment in order to make it applicable to resource-limited settings, in particular in areas where there are high incidences of HIV infections, such as sub-Saharan Africa, Eastern Europe and Asia. Successful development of a LC-UV method preceded by SPE obtained satisfactory results fit for this purpose and was employed to measure drug levels in HIV-positive patients. Using a Zorbax SB-Phenyl reverse-phase analytical column, good separation and detection of the drugs was attained within a 10 minute run time. Intra- and inter-assay precision RSD values were found to be less than 15% at the concentrations examined (0.1-20 μg/mL). The LOQ was found to be 0.1 μg/mL for each agent and the assay was found to generate a linear response up to 20 μg/mL. Drug levels were measurable and significant differences in circulating rifampicin levels were found among the patients.

Having completed the above, a second analytical protocol was developed to support evaluation of an emerging hypothesis that 'cutting a scopolamine transdermal patch in half will reduce the side effects associated with the medication while still having effective protection from motion

sickness'. The validated assay utilised solid-phase extraction coupled to LC-MS to sensitively and accurately estimate the serum drug concentrations evident in subjects who participated in a pilot study undertaken as part of this project. The main challenge met here was developing a fast, isocratic method capable of determining the extremely low concentrations of scopolamine in serum post patch and half patch application. As the circulating level of scopolamine, especially when only half a patch is applied, is very low, enormous challenges had to be overcome to couple extraction and analysis methods and push them to their very limits to reliably and reproducibly measure the levels of drug encountered. This was accomplished by coupling SPE and LC-MS methods to detect the drug at pg/mL levels in blood. Using an Agilent Zorbax SB-Phenyl column, intra- and inter-assay precision RSD values were found to be less than 15% at the concentrations examined (10-200 pg/mL). The assay LOQ was 10 pg/mL with a linear response up to 200 pg/mL. Successful determination of scopolamine levels in circulating blood levels of patients administered the transdermal patch, both full and cut in half was achieved. It was found cutting the patch significantly impacted on the anticipated pharmacokinetics of the drug.

Chapter 1

General introduction to quantitation of pharmaceutical levels in biological samples

1.1 Research objectives and relevance of work

1.1.1 Introduction and research aims

Analytical methods for finished products, raw materials, or active pharmaceutical ingredients have their own development and validation challenges. However, bioanalytical methods, especially in support of pharmacokinetic studies, are among the most challenging to develop and validate (FDA, 2001). The complexity of the sample matrices, the trace levels of drug and metabolites usually encountered, and even the complexity of the instrumentation used for the analyses, combine to present a huge challenge to analysts (FDA, 2001).

Many components in biological matrices influence the result of an analysis and affect assay sensitivity and reproducibility (Chiu *et al.*, 2010). Matrix components present in biological samples can disturb the response of the analyte of interest which can lead to inaccurate quantitation (Burgess, 2000). Careful assessment of matrix effects and judicial use of the appropriate sample preparation coupled with adequate chromatography and detection is essential for successful method development and validation.

The main objectives of this work were to:

- Develop and validate a novel, fast and simple LC-UV method for the quantitative determination of efavirenz and rifampicin in plasma employing SPE.
- Develop and validate a novel, sensitive LC-MS method for the quantitative determination of scopolamine in serum employing SPE.

While each project had their own requirements, overall, it was hoped to keep the assays relatively simple, recoveries of the relevant drugs high and to make the procedure easy to follow such that technology transfer to a hospital, research lab or even into a field clinic could later be achieved.

Work for the first project of the thesis was carried out in Dublin City University (DCU) School of Chemical Sciences and in the National Institute for Cellular Biotechnology in collaboration with the National Centre for Sensor Research (NCSR). Work for the second project of the thesis was carried out in the Space Life Sciences Laboratory (SLSL), Kennedy Space Centre, Florida, and in the National Institute for Cellular Biotechnology.

1.2 Analytical separation and extraction techniques

1.2.1 Separation techniques

There are a number of techniques used for the separation of drugs from biological matrices for analytical purposes. Some of the most widely used are based on chromatography. Chromatography encompasses a diverse group of methods that are utilised for the separation of closely related components of mixtures. Chromatography is a physical method of separation in which the components to be separated are distributed between two phases, one of which is stationary (the stationary phase), while the other (the mobile phase) moves in a definite direction over the stationary phase (Ardrey, 2003b). The phases are chosen such that components of the sample have differing affinities for each phase.

The most commonly employed separation technique for bioanalysis is high performance liquid chromatography (HPLC), also known simply as LC. The method is popular because it is non-destructive, has very broad applicability and may be applied to thermally labile compounds (unlike GC); it is also a very sensitive technique since it can incorporate a wide choice of detection methods. With the use of post-column derivatisation methods to improve selectivity and detection limits, HPLC can easily be extended to trace determination of compounds that do not usually provide adequate detector response. The wide applicability of HPLC as a separation method makes it a valuable separation tool in many scientific and pharmaceutical fields. HPLC is an important tool for the analysis of pharmaceutical drugs, for drug monitoring and for quality assurance.

1.2.2 Theory of liquid chromatography

1.2.2.1 Capacity factor

The time taken for an analyte to elute from a chromatographic column with a particular mobile phase is termed its retention time t_R. Since this will vary with several factors, including, column length and mobile phase flow rate, it is more useful to use the capacity factor, k' (Dong, 2006a). This relates the retention time of an analyte to the time taken by an unretained compound, i.e. one which passes through the column without interacting with the stationary phase, to elute from the column under identical conditions (t_0) (Ardrey, 2003b). The capacity factor, k', (sometimes called the retention factor) is an essential factor in the retention mechanism of analytes. The retention factor for analyte A is defined as a measure of the affinity that an analyte has for the stationary phase (See Eqn. 1.1 and Figure 1.1)

$$k'_A = t_R - t_0 / t_0 \qquad \textit{Eqn 1.1}$$

where: t_R: analyte retention time (min)

t_0: time taken for the mobile phase to pass through the column (min)

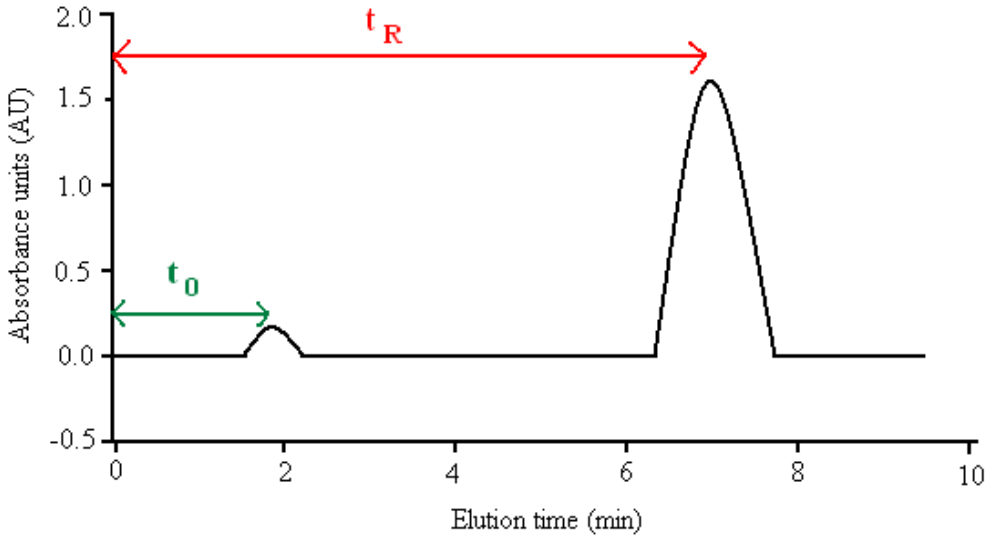

Figure 1.1: Schematic of a typical HPLC chromatogram

Ideally, the capacity factor for an analyte is between two and five. A retention factor less than two indicates the analyte has eluted too quickly and is too close to the void volume so accurate determination of the retention time is difficult. When an analytes retention factor is more than five, the analyte has eluted too slowly so the run time may be excessively long. The *k'* value can be altered by modifying the mobile phase composition or pore size of the column (Hooper, 1992).

1.2.2.2 Separation efficiency

Separation efficiency is a measure of the sharpness of peaks eluting from a specific column. The column efficiency is measured either in terms of the plate height (H), the efficiency of the column per unit length, or the plate number (N), i.e. the number of plates for the column (Ardrey, 2003b). The plate model supposes that the chromatographic column contains a large number of separate layers, called theoretical plates. Separate equilibrations of the sample between the stationary and mobile phase occur in these "plates". The analyte moves down the column by transfer of equilibrated mobile phase from one plate to the next (Dong, 2006a). The number of theoretical plates that a real column

possesses can be found by examining a chromatographic peak after elution, see Eqn. 1.2.

$$N = \frac{5.55\, t_R^{\,2}}{w_{1/2}^{\,2}} \qquad \textit{Eqn. 1.2}$$

where: $w_{1/2}$: Peak width at half-height

The number of theoretical plates is proportional to the column length. The height equivalent to theoretical plate HETP also describes the efficiency of a given column for unit length of column. See Eqn 1.3 (Dong, 2006a). The higher the number of theoretical plates in a column, *N*, the better and the lower the plate height the better.

$$\text{HETP} = L / N \qquad \textit{Eqn. 1.3}$$

where: *L:* Length of the column

The concept of theoretical plates was first introduced by Martin and Synge (Martin *et al.*, 1941); however, Van Deemter (van Deemter *et al.*, 1956) developed a more realistic theory which takes into account the diffusion effects of mass transfer and migration through a packed bed, with the resulting peak shape being affected by the rate of elution (Hooper, 1992). See Eqn. 1.4 for the Van Deemter equation for plate height.

$$\text{HETP} = \frac{A + B}{u + C\,u} \qquad \textit{Eqn. 1.4}$$

where: *u*: Average velocity of the mobile phase
A: Eddy diffusion
B: Longitudinal diffusion
C: Resistance to mass transfer

The mobile phase moves through the column which is packed with stationary phase. Eddy diffusion is caused by the movement of molecules from the same analyte, which migrate through the stationary phase at random. This

will cause broadening of the solute band, because different paths are of different lengths (Braithwaite *et al.*, 1996).

Longitudinal diffusion occurs due to the concentration of analyte being less at the edges of the band than at the centre. Analyte diffuses out from the centre to the edges causing broadening. If the velocity of the mobile phase is high then the analyte spends less time on the column, which decreases the effects of longitudinal diffusion (Braithwaite *et al*., 1996).

The last parameter is due to the amount of time an analyte takes to equilibrate between the stationary and mobile phase. If the velocity of the mobile phase is high, and the analyte has a strong affinity for the stationary phase, then the analyte in the mobile phase will move ahead of the analyte in the stationary phase (Braithwaite *et al.*, 1996). The band of analyte is broadened. The higher the velocity of mobile phase, the worse the broadening becomes.

1.2.2.3 Selectivity factor

The selectivity factor, α, of a chromatographic separation describes the separation of two species (A and B) on the column and is the ratio of their capacity factors (Ardrey, 2003b), see Eqn. 1.5.

$$\alpha = \frac{k'_B}{k'_A}$$

Eqn. 1.5

It is a measure of the band proximity of two adjacent analyte bands. Selectivity is a measurement of the difference in interactions of two analytes with the mobile and stationary phases, and therefore the difference in retention times. The factor is identified with the selectivity of a chromatographic system, i.e., the ability of the system to provide different retention times for two specific analytes. Selectivity factor is always greater than one (Dong, 2006b). As with capacity, the selectivity of a column is determined by the column packing material and the eluents used.

1.2.2.4 Resolution

Arguably the most important concept in HPLC chromatography is resolution (R), which is dependent on all the factors listed above. R is calculated as the difference in retention time of two analytes divided by the average width of the two peaks at the baseline. The most important goal of the chromatographer is to achieve adequate resolution between all peaks in the chromatogram in a reasonable amount of time (Ardrey, 2003b). The resolution, R is calculated using Eqn. 1.6.

$$R = \frac{2\,(t_{R2} - t_{R1})}{W_1 + W_2}$$

Eqn. 1.6

where: W: the baseline peak width of the components

For quantitative analysis, baseline resolution is achieved when R=1.5. It is useful to relate the resolution to the number of plates in the column, the selectivity factor and the retention factors of the two solutes as per Eqn. 1.7;

$$R = \frac{\sqrt{N}}{4}\left(\frac{\alpha - 1}{\alpha}\right)\left(\frac{1 + k'_B}{k'_B}\right)$$

Eqn. 1.7

To obtain high resolution, the three terms must be maximised. The optimum parameter with which to control resolution is α; however, this is primarily achieved by changing the stationary phase. Resolution is also proportional to the square root on N and to *k'*. It may be improved by increasing these parameters; however, these can lead to long analysis times and increased band broadening.

The degree of resolution between two chromatographic peaks is dependent upon three factors. The first term, efficiency can be varied with flow rate and column length. This term reflects how much dispersion takes place within a chromatographic peak. The second term, selectivity, illustrates how

well the chromatographic system chosen can distinguish between sample components.

Selectivity is dependent upon stationary phase selection, mobile phase selection and column temperature, among others. The final term is related to the capacity factor and is primarily influenced by mobile phase composition (Dong, 2006b).

1.2.3 Modes of separation in HPLC

There are various modes of operation of HPLC. The mechanism of interaction of the solutes with the stationary phases determines the classification of the mode of liquid chromatography. Almost any type of solute mixture can be separated by HPLC because of the wide range of stationary phases available. There are many different modes of LC including size exclusion, ion-exchange, normal and reversed phase. Table 1.1 summarises the variety of modes of liquid chromatography, of which Reversed Phase stands out as the most widely used mode in HPLC.

Mode	Normal Phase	Reversed Phase	Ion exchange	Size Exclusion
Stationary Phases chemistry	Polar-hydrophilic	Non-polar-lipophilic	Ion-bonding	Sieving by size
Typical Stationary Phases	Silica, Alumina	Alkylated silica, mostly C18	Ionic functional groups on silica or polymer	Gel type polymers
Typical mobile phase	Hexane; isopropanol; methylene chloride	Water; methanol; acetonitrile; buffers; ion pairing agents	Water; buffers; acid; base	Two modes: aqueous and non-aqueous
Typical solutes	Fatty and oily	Almost all organic compounds	Any ion-charged compounds	Polymers: synthetic or biological

Table 1.1: Summary of the different modes of operation of HPLC

1.2.3.1 Size exclusion LC

Size exclusion chromatography (SEC) is a useful technique for separating components with a significant difference in molecular weight. The solutes are separated on the basis of size, with the larger ones eluting first. Stationary phases contain pores through which compounds are able to diffuse to a certain extent (Rouessac *et al.*, 2007). SEC is of particular value in characterising polymer mixtures and in separating biological macro molecules such as peptides and proteins (Steehler, 2003).

1.2.3.2 Ion exchange LC

Ion-exchange chromatography is used to separate mixtures of ionic solutes such as inorganic cations and anions, amino acids and proteins (Steehler, 2003). The packing material possesses charge bearing functional groups, which are capable of exchanging with ionic analytes in the mobile phase. The functional groups employed are permanently bonded ionic groups associated with counterions of the opposite charge. Separation occurs as a result of interaction between the charged solute and the oppositely charged, solid stationary phase (Rimmer, 2011). There are two modes of Ion exchange chromatography, anion exchange chromatography in which the packing material is a strongly basic quaternary ammonium group, and cation exchange chromatography where the packing material is a strongly acidic sulphonic acid group. Both of these functional groups are totally dissociated, i.e., their exchange properties are independent of pH. There are also exchangers that possess weakly acidic and basic functional groups, such as carboxylate and tertiary amine groups. In this instance, the retention mechanism of analyte ions is dependent upon the pH of the mobile phase (Rimmer, 2011).

Ion exchange chromatography has a wide range of applications including the analysis of amino acids on a cation exchanger to the simultaneous separation of inorganic anions and cations using anion and cation exchange columns in tandem (Rimmer, 2011).

1.2.3.3 Normal phase LC

In normal phase chromatography, separation occurs through the interaction of analytes with a polar stationary phase (usually a cyano or amino bonded stationary phase) and a less polar mobile phase, such as hexane and heptane (Rimmer, 2011). Analytes are retained on the column based upon their polarity; the more polar the analyte, the more it will be retained on the column. Its main application is for the separation of very hydrophobic compounds that would take too long to elute in reversed phase chromatography. Normal phase chromatography is best suited to the analysis of compounds that are soluble in non-polar solvents (Rouessac *et al*., 2007).

1.2.3.4 Reversed phase LC

Reversed phase liquid chromatography is considered as the method of choice for the analysis of pharmaceutical compounds for several reasons, such as its compatibility with aqueous and organic solutions as well as with different detection systems, high consistency and repeatability and the fact that the vast majority of drugs are lipophilic or have some lipophilic moieties and will necessitate organic-based separation strategies. The separation mechanism in reversed phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilised hydrophobic ligand, i.e. the stationary phase (Braithwaite *et al.*, 1996). The solute molecules partition (i.e. an equilibrium is established) between the mobile phase and the stationary phase. The distribution of the solute between the two phases depends on the binding properties of the medium, the hydrophobicity of the solute and the composition of the mobile phase (Smith, 1995). In summary, the more polar the compound, the more time it will spend in the polar mobile phase and will hence flow through the system quickly. The more non-polar the compound, the more time it will spend interacting with the stationary phase and will hence be retained on the column for a longer period of time and elute later.

The stationary phase in the reversed phase chromatographic columns is a hydrophobic support that is consisted mainly of porous particles of silica gel

in various shapes (spherical or irregular) at various diameters (1.8, 3, 5, 7, 10 mm etc.) at various pore sizes (such as 60, 100, 120, 300 Å). The surface of these particles is covered with various chemical entities, such as various hydrocarbons (C1, C6, C4, C8, C18, etc.) (Rouessac *et al.*, 2007). There are also hydrophobic polymeric supports that are used as stationary phases when there is an extreme pH in the mobile phase. In most methods used currently to separate medicinal materials, C18 columns are used.

When the samples contain solutes of ionisable functional groups, such as amines, carboxyls, phosphates, phosphonates, sulfates and sulfonates, it is possible to control their ionisation degree with the help of buffers in the mobile phase. As a rule, the change of an ionisable molecule to an ion makes it more polar and less available to the stationary phase. In most of the traditional silica-gel based stationary phases it is not possible to increase the mobile phase's pH above 8 due to hydrolysis of the silica gel (Rimmer, 2011).

1.2.4 Detection techniques

The detector is the part of the chromatographic system that responds to the presence of the solutes in the mobile phase. In HPLC there are a number of different detectors used today including refractive index, UV/Vis, Mass Spectrometry and Fluorescence. Less common but important detectors are conductivity and Evaporative light scattering. See Table 1.2 for a brief comparison of advantages and limitations of some of the most common detection techniques used with HPLC today.

Advantages	Limitations
UV/Vis detection	
Easy to use Reliable Relatively inexpensive Solvent gradient compatible Non-destructive to sample Relatively sensitive Specific	Poor response if compound doesn't have a good chromophore Solvents limited by UV cutoff Only detects compounds that absorb UV or visible radiation Detection based on extinction coefficient Baseline drift with gradients at low wavelength Solvent front interference at low wavelength
Fluorescence detection	
Very sensitive Generally insensitive to flow and temperature changes Very selective	Limited linearity Complicated to use –understanding of chemical and instrument variables essential Must degas well- chemicals like oxygen may quench fluorescence Not many compounds naturally fluoresce Derivatisation complicates method
Refractive index detection	
Universal nature of the detector response Good linear dynamic range - ~4 orders of magnitude Easy to operate	Poor sensitivity Very sensitive to temperature and pressure fluctuations Slow to equilibrate Cannot be used with solvent gradients Baseline instability due to temperature change Solvent front interference

Mass spectrometry detection	
Very sensitive	Purchase price is very high
Very selective	Complicated operation
Provides structural information	Frequent maintenance needed

Table 1.2: Summary of advantages and limitations of the most commonly used detectors used in HPLC

1.2.4.1 UV-Vis spectrometry detection

UV-Vis spectroscopy examines the electronic transitions of molecules as they absorb light in the UV and visible regions of the electromagnetic spectrum (Settle, 1997). UV–Vis is a relatively easy method that delivers fast results in a non-destructive format where sample remnants can be collected after the detector for further analysis by other applications. Molecular moieties likely to absorb light in the 200 to 800 nm region of the electromagnetic spectrum generally contain pi-electron functions and heteroatoms possessing non-bonding valence-shell electron pairs e.g. all olefins, all aromatics and compounds, for example, containing > C = O, > C = S, – N = N – groups. Such light absorbing groups are known as chromophores (Settle, 1997). Any species with an extended system of alternating double and single bonds will absorb UV light, and anything with colour absorbs visible light, making UV-Vis spectroscopy applicable to a broad variety of samples (Rouessac *et al.*, 2007). The UV-Vis spectra have broad features that are of restricted use for sample identification but are extremely useful for quantitative measurements. The concentration of an analyte in solution is determined by measuring the absorbance at a particular wavelength, usually at a wavelength where absorbance is greatest.

Radiation absorbance depends on the radiation wavelength and the functional groups of the chemical compound. Electromagnetic field depending on its energy (frequency) can interact with electrons causing their excitation and transfer up to a higher energy level, or it can excite molecular bonds causing the vibration or rotation of their functional group. The intensity of the beam with

the energy corresponding to the possible transitions will decrease while it is passing through the flow-cell. Absorbance is the logarithm of the ratio of the intensities of the incident light (I_0) and the transmitted light (I). It is related according to the Beer-Lambert Law to the molar absorptivity (molar extinction coefficient, ε), the thickness of the substance (i.e., the path length of the cell, b) and the molar concentration of the substance (c) as per Eqn. 1.8 (Rimmer, 2011).

$$A = \log (I_0/I) = \varepsilon bc \qquad \textit{Eqn. 1.8}$$

The molar extinction coefficient (ε) corresponds to the absorbance for a molar concentration of the substance with a path length of 1 cm. Molar absorptivity is dependent on the wavelength and chromatographic conditions, (solvent, pH and temperature). It is a constant at a specified wavelength.

UV detectors can be used with gradient elution providing the solvents do not absorb significantly over the wavelength range that is being used for detection. In reversed phase chromatography, the solvents usually employed are water, methanol, acetonitrile and tetrahydrofuran (THF), all of which are relatively transparent to UV light over the total wavelength range normally used by UV detectors (Rimmer, 2011). In normal phase operation more care is needed in solvent selection as many solvents that might be appropriate as the chromatographic phase system are likely to absorb UV light very strongly. The n-paraffins, methylene dichloride, aliphatic alcohols and THF are useful solvents that are fairly transparent in the UV and can be used with normal distribution systems (e.g. a polar stationary phase such as silica gel).

Fixed wavelength detectors are detectors which do not allow change to the wavelength of the radiation measured whereas variable wavelength detectors are detectors which allow selection of the operating wavelength. Monitoring of different wavelengths during a run (one at a time) is possible using a variable wavelength detector (Rimmer, 2011). With a Photo Diode Array (PDA) detector, a single wavelength, a variety of wavelengths or the entire spectrum from the whole photodiode array of the column eluent can be monitored in real time. This often permits identification of compounds as well

as quantitation and is an extremely useful method development tool (Rimmer, 2011). Many organic compounds have characteristic spectra in the UV which can be used to help identify the substance passing though the sensor cell. Thus, when a given substance is eluted through the sensor cell, all the outputs from the array can be acquired and the result used to construct an absorption spectrum that can be compared with standard and/or library spectra for identification purposes. Alternatively, by selecting the appropriate diode, the wavelength of the light at which there is maximum absorption can be selectively monitored to provide maximum detector sensitivity for that substance.

1.2.4.2 Mass spectrometry detection

Mass spectrometry (MS) combined with the separation power of chromatography has revolutionised the way chemical analysis is done today. Liquid chromatography - mass spectrometry (LC-MS) is an extremely versatile instrumental technique. With a selection of LC–MS interfaces now available, a wide range of analytes, from low molecular-weight drugs and metabolites (<1000 Da) to high-molecular-weight biopolymers (>100 000 Da), may be studied (Chen *et al.*, 2006). The mass spectrometer provides the most definitive identification of all of the HPLC detectors. It allows for the molecular weight of the analyte to be determined and with certain mass spectrometers, the structure can be elucidated also. Also the high selectivity of the mass spectrometer often provides identification capability on chromatographically unresolved or partially resolved components. This selectivity allows the use of isotopically labelled analytes as internal standards and this, coupled with high sensitivity, allows very accurate and precise quantitative determinations to be carried out (Ardrey, 2003b).

The equipment comprises a chromatograph, attached via a suitable interface, to a mass spectrometer. As MS measures the mass to charge (*m/z*) ratio of ions, most molecules which can be ionised will be suitable for this detector, hence the detector is considered to be a ‘universal’ one. The MS obtains information regarding molecular weight and the compound’s chemical

structure. Components eluting from the chromatographic column are introduced to the mass spectrometer by means of a specialised ion source (See Figure 1.2).

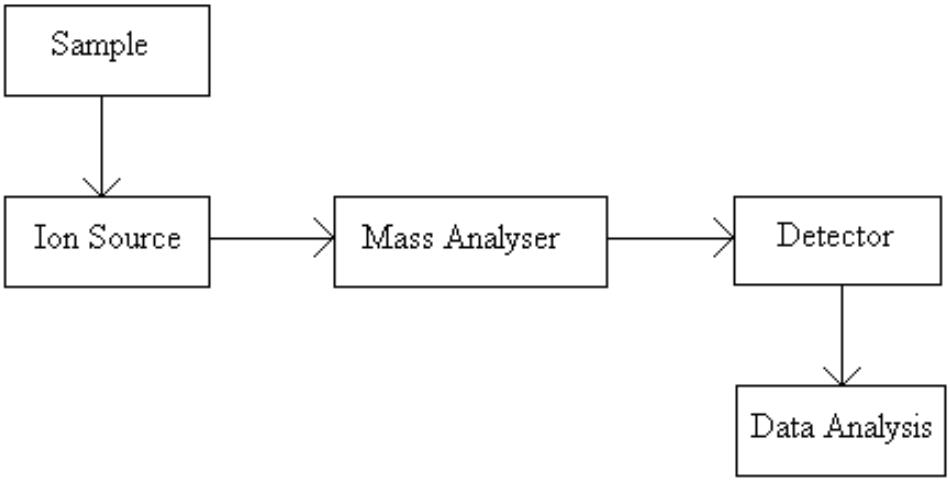

Figure 1.2: A schematic of the principle components of a MS

Interfacing the two techniques is not straightforward as the solutes leaving an LC column are dissolved in mobile phase at atmospheric pressure, whereas the MS is set up to detect gas phase ions in vacuum (Ardrey, 2003a). To ease this transition from the liquid to the gas phase, a number of ion source interfaces have been developed.

1.2.4.2.1 Sample ionisation

The use of Atmospheric Pressure Ionisation (API) techniques allow positive or negative ions to be created and detected. API offers a soft ionisation approach resulting in little or no analyte fragmentation. A typical API spectrum contains only the protonated (positive ion mode) or deprotonated (negative ion mode) molecular ion (Westman-Brinkmalm *et al.*, 2008). The detected ion peaks are generally created through addition or subtraction of a proton adducts of analyte ions of other types such as sodium or potassium can be seen in some circumstances (in positive mode) (Hoffmann, Stroobant 2007). MS spectra provide valuable molecular weight information of singly and multiply charged ions and identification of the charge state of each peak in the charge-state

envelope of a given compound. Software can be used to deconvolute multiply charged mass spectral data of mixtures of proteins and other biopolymers.

The API source can operate using electrospray, nanospray or atmospheric pressure chemical ionisation (APCI) techniques. The vast majority of LC–MS analyses currently in use employ either electrospray ionisation (ESI) or APCI, both which are soft ionisation techniques (Ardrey 2003c). See Figure 1.3 for a broad summary of the general analyte analysis applications of APCI and ESI ionisation techniques.

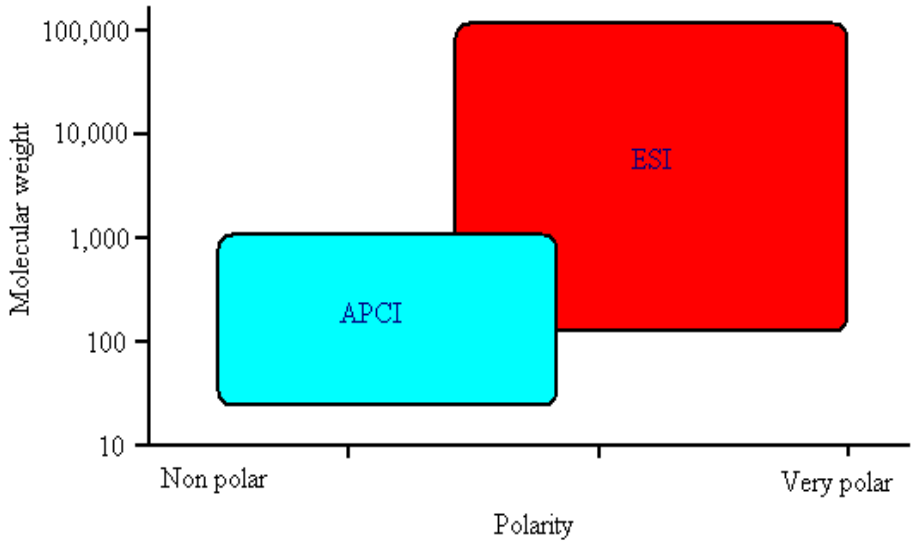

Figure 1.3: Illustration of the applications of different ionisation techniques

In electrospray ionisation (ESI) the analyte is introduced to the source at slow flow rates, typically of the order of 0.5 mL/min. The analyte solution flow passes through the electrospray needle that has a high potential difference (with respect to the counter electrode) applied to it (typically in the range from 2.5 to 4 kV) – see Figure 1.4 (Westman-Brinkmalm *et al.*, 2008). This forces the spraying of charged droplets from the needle with a surface charge of the same polarity to the charge on the needle. The droplets are repelled from the needle towards the source sampling cone on the counter electrode. As the droplets pass through the space between the needle tip and the cone, solvent evaporation also occurs, generally augmented by a flow of inert gas. As the solvent evaporation

occurs, the droplet shrinks until it reaches the point that the surface tension can no longer sustain the charge (the Rayleigh limit) at which point a 'Coulombic explosion' occurs and the droplet is ripped apart. This produces smaller droplets that can repeat the process as well as naked charged analyte molecules. These charged analyte molecules can be singly or multiply charged (Hoffmann *et al.*, 2007). This is a very soft method of ionisation as very little residual energy is retained by the analyte upon ionisation.

In summary, ESI converts the liquid molecules that elute from the LC into charged gaseous analytes by evaporating the LC solvent and charging the analytes and then pulling the ions into the mass analyser by keeping the capillary at a high voltage of opposite charge and at high vacuum. The only disadvantage of the technique is that very little (usually none) fragmentation is produced directly although this may be overcome through the use of tandem mass spectrometric techniques such as MS/MS or MS^n. This ionisation technique is very suitable for the analysis of polar, thermally labile molecules such as drugs, DNA, RNA, sugars, peptides and proteins (Westman-Brinkmalm, 2008).

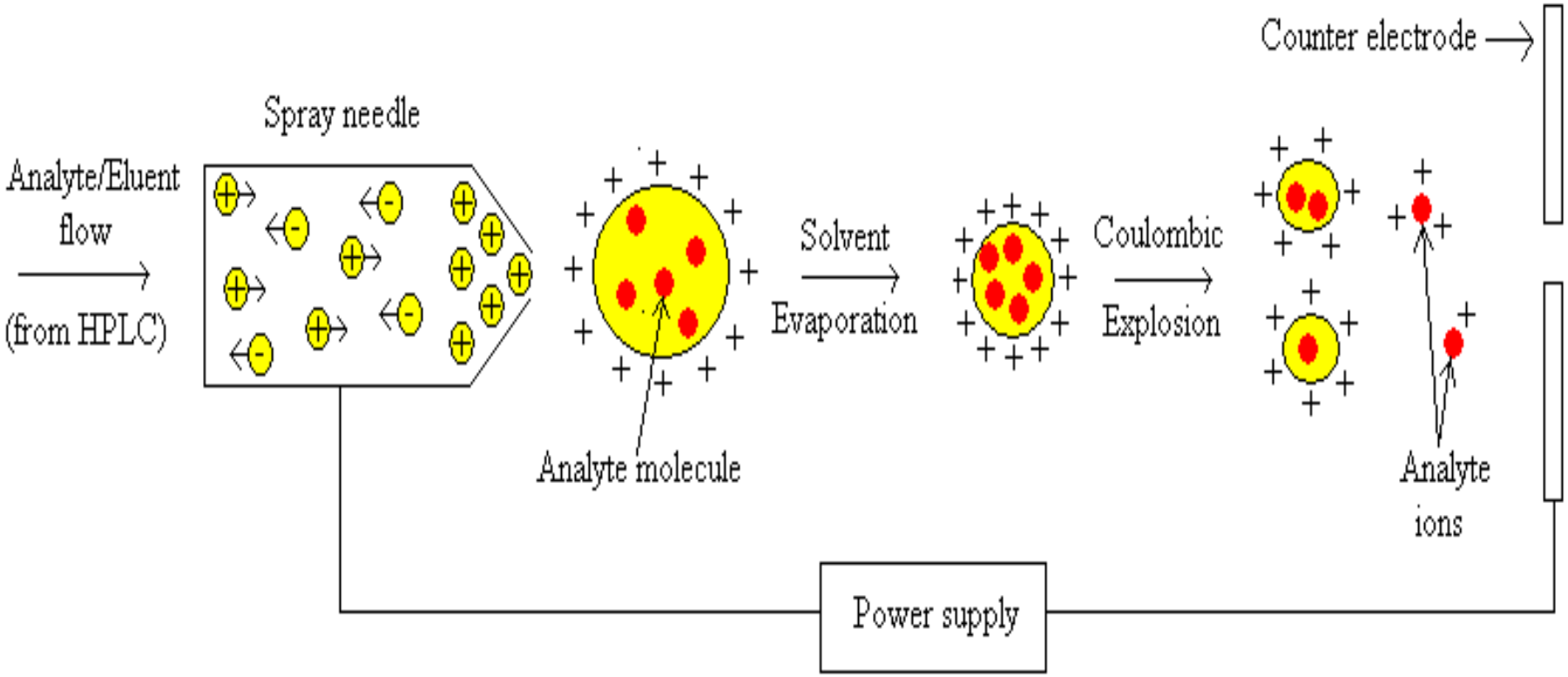

Figure 1.4: Adapted schematic of electrospray ionisation (Gates, 2004)

Nanospray ionisation (NSI) is essentially ESI operating at very low liquid flow rates of 100 nL/min to several μL/min in static or dynamic modes. It is most compatible with capillary LC separations.

Atmospheric pressure chemical ionisation (APCI) vaporises the sample solution at high temperatures up to 600 °C. Application of a high electrical potential produces reagent ion plasma, mainly from the solvent vapour. The sample vapour is formed by ion-molecule reactions with the reagent ions in the plasma. APCI accommodates liquid flows of 100 μL/min to 2 mL/min and is generally employed for larger, poorly ionisable, hydrophobic molecules (Westman-Brinkmalm *et al.*, 2008).

1.2.4.2.2 Mass analysers

A mass analyser measures gas phase molecules with respect to their mass-to-charge ratio (*m/z*), where the charge is produced by the addition or loss of a proton(s), cation(s), anions(s) or electron(s) (Westman-Brinkmalm *et al.*, 2008). The addition of charge allows the molecules to be affected by electric fields thus allowing its mass measurement. There are a number of types of mass analysers available and choice depends on the information required from the ionised analytes.

Quadrupole mass analysers have been important in mass analysis for many decades because they are relatively inexpensive, rugged, and have been implemented in a wide variety of instrumental configurations including triple quadrupole instruments which can do MS/MS experiments. A quadrupole mass analyser consists of four parallel rods that have fixed DC and alternating RF potentials applied to them (see Figure 1.5) (Westman-Brinkmalm *et al.*, 2008). Ions produced in the source are focussed and passed along the middle of the quadrupoles. Motion of these ions will depend on the electric fields so that only ions of a particular mass to charge ratio (*m/z)* will have a stable trajectory and thus pass through to the detector (Chen *et al.*, 2006). Varying the RF brings ions of different *m/z* into focus on the detector and thus builds up a mass spectrum.

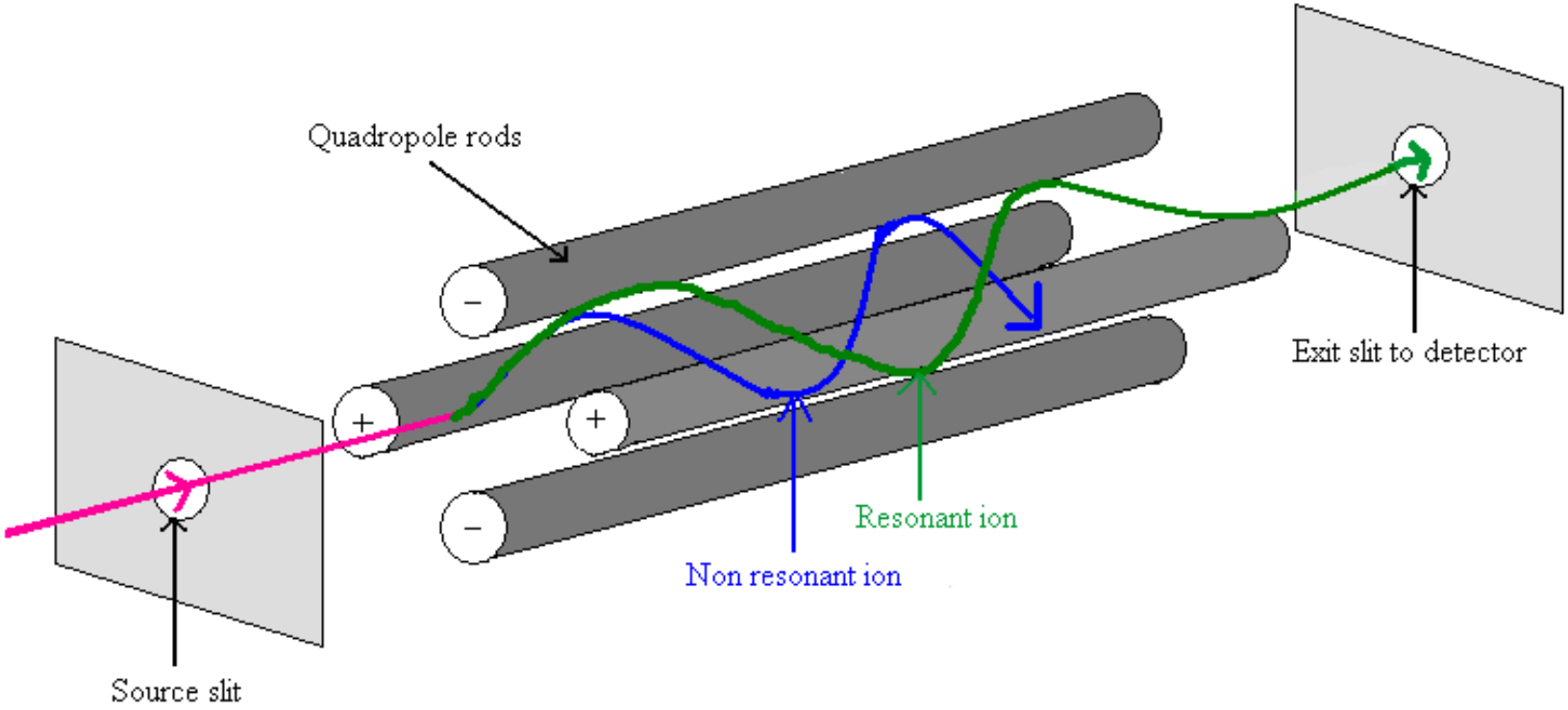

Figure 1.5: Adapted schematic of a Quadrupole Mass Analyser (Gates, 2009)

Quadrupoles can also be placed in tandem to enable them to perform fragmentation studies - the most common set-up is the triple quadrupole (QQQ) (Yost *et al.*, 1978) mass spectrometer which enables basic ion fragmentation studies (tandem mass spectrometry MS/MS) to be performed. These instruments are particularly sensitive in selected ion monitoring modes and hence ideal for trace analysis and pharmacokinetic applications.

In a triple quadrupole, the sample enters the ion source and is usually fragmented by either an electron impact or chemical ionisation process. The first quadrupole acts as a filter for the ion of interest; the various charged ions are separated in the usual way and then pass into the second quadrupole section sometimes called the collision cell. This one acts as a collision chamber for the MS/MS to form the product ions of interest (Hoffmann *et al.*, 2007).

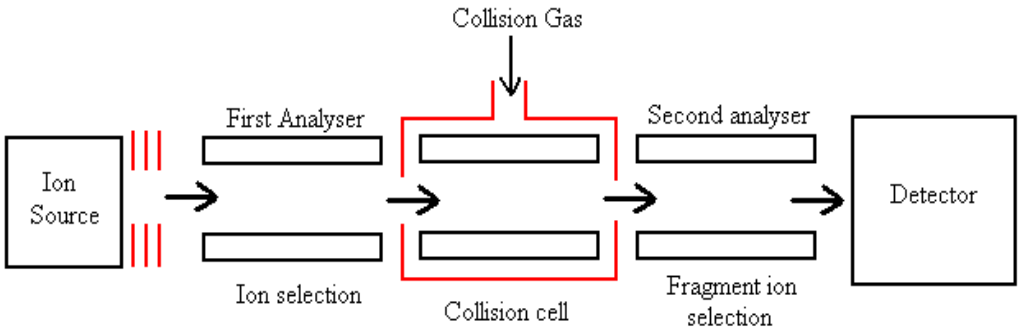

Figure 1.6: Schematic of the Triple Quadrupole Mass Spectrometer

These product ions are then passed into the third quadrupole which functions as a second analyser to detect the aforementioned ions for quantitation. The second analyser segregates the product ions into their individual masses, which are detected by the sensor, producing the mass spectrum (originally from ions of one mass only). In this way, the exclusive mass spectrum of a particular molecular or product ion can be obtained from the myriad of ions that may be produced from the sample in the first analyser. A diagram of a triple Quadrupole Mass Spectrometer is shown in Figure 1.6. MS QQQ is an extremely powerful analytical system that can handle exceedingly complex mixtures and very involved molecular structures. The system has more than adequate resolving power and is valuable for structure

elucidation. The combination of the triple quadrupole mass spectrometer with a separation technique such as a gas chromatograph or a liquid chromatograph is probably one of the most powerful analytical tools available to the contemporary chemist.

Ion traps (which are normally quadrupole ion traps) are popular because they are relatively inexpensive, rugged, and provide MS^n capabilities that enhance selectivity and qualitative analysis. The trap confines the motion of ions in three, rather than two dimensions (Westman-Brinkmalm *et al.*, 2008). Ions are generated outside of the mass analyser in most commercial LC-MS systems, mainly by the ion source and hence sent into the trap. The quadrupole ion-trap consists of a ring electrode with further electrodes, the end-cap electrodes, above and below this. In contrast to the quadrupole, described above, ions, after introduction into the ion-trap, follow a stable (but complex) trajectory, i.e. are trapped, until an RF voltage is applied to the ring electrode. Ions of a particular *m/z* then become unstable and are directed toward the detector. By varying the RF voltage in a systematic way, a complete mass spectrum may be obtained. Again, this is a low-resolution device, capable of fast scanning and tolerant of relatively high operating pressures (Ardrey, 2003c).

The trap can carry out a number of experiments on the trapped ions such as isolation and fragmentation, before their release and detection (Westman-Brinkmalm *et al.*, 2008). While ion traps can be used for quantitation, they are most useful for structural elucidation of novel compounds. This analyser is being widely employed for applications in drug discovery and combinatorial chemistry. An ion trap performs tandem mass spectrometry in time as opposed to space for the triple quadropole (Hoffmann *et al.*, 2007). Tandem in time instruments perform the steps of precursor ion selection, ion activation and acquisition of fragment ion spectra in the very same place. Ion traps have limited dynamic range due to space charge affects. If too many ions are accumulated in the trap their charges repel each other having a detrimental affect on instrument resolution and quantitative analysis (Hoffmann *et al.*, 2007).

The Time-of-flight (TOF) analyser is the simplest type of mass analyser. TOF systems require a pulsed ion source, a flight tube and an ion detector. This

system relies on the fact that if all of the ions produced in the source of a mass spectrometer, by whatever technique, are given the same kinetic energy then the velocity of each will be inversely proportional to the square root of its mass. As a consequence, the time taken for them to traverse a field-free region (the flight tube of the mass spectrometer) will be related in the same way to the *m/z* of the ion (Westman-Brinkmalm *et al.*, 2008). A complete mass spectrum is obtained simply by allowing sufficient time for all of the ions of interest to reach the detector. The operation of this type of device is fundamentally different to those described previously in which ions of one *m/z* ratio at a time enter the mass analyser. By varying the conditions in the mass analyser, e.g. magnetic field, quadrupole field, etc., ions of different *m/z* values are brought to the detector and a corresponding mass spectrum obtained. In the time-of-flight instrument, it is essential that ions of all *m/z* ratios present in the source are transferred, simultaneously and instantaneously, into the mass analyser at a known time so that their times of flight, and thus their *m/z* ratios, may be determined accurately. Were ions to be introduced continuously it would be impossible to determine exactly when each began its passage through the flight tube and therefore to calculate its *m/z* ratio. A complete mass spectrum at a specific time is therefore obtained and when this has been recorded, a matter of milliseconds later, a further set of ions can be transferred from the source.

This is sometimes referred to as a 'pulsed' source. Fast scanning, only limited by the time it takes the heaviest ion to travel from the source to the detector, is possible and any distortion of ion intensity brought about by changes in analyte concentration during the scanning process is removed (Ardrey, 2003c).

The mass of an ion is related to the time it takes that ion to travel through the flight tube to the detector. Recently developed TOF systems have demonstrated high sensitivity and high resolution. Another attribute of TOF is the 'scan speed' benefits. Because TOF scan times for complete mass spectra are so fast, chromatographic techniques that separate complex mixtures in short intervals can be interfaced with this technique. The other great advantage of TOF is the virtually unlimited mass range when sampling in the time domain (Hoffmann *et al.*, 2007).

1.2.4.2.3 Modes of detection

Typically the mass spectrometer is set to scan a specific mass range. This mass scan can be wide as in the full scan analysis or can be very narrow as in selected ion monitoring. A single mass scan can take anywhere from 10 ms to 1 s depending on the type of scan. Many scans are acquired during an LC-MS analysis. LC-MS data is represented by adding up the ion current in the individual mass scans and plotting the 'total' ion current as an intensity against time (Hoffmann *et al.*, 2007). Different modes of acquiring LC-MS data are available including full scan acquisition resulting in the typical total ion current plot, selected ion monitoring and selected reaction monitoring or multiple reaction monitoring. Selected reaction monitoring and multiple reaction monitoring are essentially identical techniques from different manufacturers.

The total ion current (TIC) is a plot of the total number of ions in each MS scan plotted as an intensity point against time. In the TIC plot, ions of every mass over the chosen range are plotted. As many compounds have the same *m/z* it can be difficult finding the compound of interest. A specific mass can later be selectively extracted but sensitivity is not as good as the next technique of selected ion monitoring. TIC plots are often overlaid onto UV or other plots and this can give useful information about the compounds being studied.

With selected ion monitoring (SIM) the mass spectrometer is set to scan over a very small mass range, typically one mass unit. This is obviously most useful when the actual mass is known. Compounds with the selected mass only are detected and plotted. However, some compounds have the same mass and in ESI where there are multiply charged species the likelihood is high for compounds to have the same *m/z* value. As the mass spectrometer can dwell for a longer time over a smaller mass range, this makes SIM more sensitive that TIC.

Selected reaction monitoring (SRM) is the most common method used for performing mass spectrometric quantitation. SRM creates a unique fragment (product) ion that can be monitored and quantified in the midst of complicated matrices, thus enabling more confirmatory identification. SRM plots usually

contain only a single peak rendering it ideal for sensitive and specific quantitation.

1.2.4.2.4 Limitations of MS analysis

Analysing biological fluids is one on the most difficult tasks faced by an analytical chemist. Most challenging are interferences from matrices and decomposition products combined with low concentrations of target analytes. Matrix components present in biological samples can affect the response of the analyte of interest and can lead to inaccurate quantitation. The primary matrix effect associated with LC-MS/MS methods is ion suppression or enhancement caused by the co-eluting matrix components. Matrix effects occur when molecules co-eluting with the compound/s of interest alter the ionisation efficiency of the electrospray interface.

This phenomenon was first described by Tang and Kebarle (Tang *et al.*, 1993) who showed that electrospray responses of organic bases decreased as the concentrations of other organic bases were increased. The exact mechanism of matrix effects is unknown, but it probably originates from the competition between an analyte and the coeluting, undetected matrix components (Taylor, 2005). King *et al.*, 2000, have shown through a series of experiments that matrix effects are the result of competition between nonvolatile matrix components and analyte ions for access to the droplet surface for transfer to the gas phase. Although they conclude that the exact mechanism of the alteration of analyte release into the gas phase by these nonvolatile components is unclear. They postulate "…a likely list of effects relating to the attractive force holding the drop together and keeping smaller droplets from forming should account for a large proportion of the ionisation suppression observed with electrospray ionisation". Depending on the environment in which the ionisation and ion evaporation processes take place, this competition may effectively decrease (commonly known as ion suppression) or increase (ion enhancement) the efficiency of formation of the desired analyte ions present at the same concentrations in the interface. Thus the efficiency of analyte ions to form is very much dependent on the matrix entering the electrospray ion source (Taylor, 2005).

Matrix effects are also compound dependent. Bonfiglio *et al.*, 1999, reported that the chemical nature of a compound has a significant effect on the degree of matrix effects. In a study of four compounds of different polarities under the same mass spectrometric conditions, the most polar was found to have the largest ion suppression and the least polar was affected less by ion suppression. These findings of differential matrix effects have important ramifications particularly when selecting a suitable internal standard for quantification purposes.

The possible origins of ion suppression are multiple. The main problem source commonly reported is the presence of endogenous substances, i.e. organic or inorganic molecules present in the sample and that are retrieved in the final extract. Among this first group of ion suppressor agents, can be included ionic species (inorganic electrolytes, salts), highly polar compounds (phenols, pigments), and various organic molecules including carbohydrates, amines, urea, lipids, peptides, analogous compounds or metabolites with a chemical structure close to the target analyte one. Finally, a wide range of molecules can lead to ion suppression especially when they are present in high concentration in the extract and eluted in the same retention window than the analyte of interest (Antignac *et al.*, 2005). A second problem source, usually less described, is due to the presence of exogenous substances, i.e. molecules not present in the sample but coming from various external sources during the sample preparation. Among this second group of ion suppressor agents, can be included plastic and polymer residues (Mei *et al.*, 2003), phthalates, detergent degradation products (alkylphenols), ion pairing reagents (Gustavsson *et al.*, 2001, Chaimbault *et al.*, 1999, Chaimbault *et al.*, 2000) , proton-exchanges promoting agents such as organic acids (Chaimbault *et al.*, 2000, Keever *et al.*, 1998, Roberts *et al.*, 1998), calibration products, buffers, or material released by the solid phase extraction, LC or GC stationary phases.

Many components in biological matrices influence the result of an analysis, affecting assay sensitivity and reproducibility. Improved matrix management becomes critical as requirements for higher assay sensitivity and increased process throughput become more demanding (Chiu *et al.*, 2010). When ion suppression occurs, the sensitivity and lower limit of quantification of a method may be adversely affected (Buhrman *et al.*, 1996).

1.2.5 Sample preparation techniques

Prior to LC analysis, biological samples must be 'cleaned up'. This involves isolating the drug to be analysed from its matrix with reliable recovery. Three sample preparation techniques are commonly used to extract analytes out of biological matrices: protein precipitation, supported liquid extraction or liquid-liquid extraction and solid phase extraction. Among the three, protein precipitation was the most used sample preparation approach in early drug discovery due to a lesser amount of method development time required which resulted in fast data turnaround time. However, realistically choosing which extraction approach is best is compound dependent. Analysis of highly hydrophilic molecules in biological matrices presents challenges due to low extraction recovery from biological matrices.

1.2.5.1 Liquid-liquid extraction

Classical liquid-liquid extraction (LLE) is one of the simplest techniques used in the preparation of biological fluids such as urine, plasma and blood. Also known as solvent extraction or partitioning, LLE is a method of separating compounds from each other based on their relative solubilities in two different immiscible liquids, usually water and an organic solvent (Steehler, 2004). It involves transfer of a substance from one liquid phase into another liquid phase. The sample solution is mixed thoroughly with an immiscible solvent in which the target analyte is preferentially soluble. The mixing process creates a large interfacial area between the two liquids to facilitate efficient mass transfer of the target analytes from the sample into the extractant. The two phases are allowed to separate, or are separated by centrifugation, and the extractant is removed for analysis. If the drug of interest has preferential solubility for a hydrophobic environment, it will be successfully extracted from the aqueous phase by an organic solvent leaving behind the endogenous compounds such as proteins and lipids etc. For biological fluids, a water-immiscible solvent such as ethyl acetate or chloroform is generally employed (Steehler, 2003).

The success of LLE depends upon the difference in solubility of a compound in various solvents. A judicious choice of solvent leads to clean

extracts although the technique is not applicable to hydrophilic analytes. For example, with LLE, the majority of phospholipids stay in the aqueous layer when using *tert*-butyl methyl ether (*t*-BME) as an extraction solvent regardless if the plasma pH is adjusted or not. Phospholipids are major components that stay in the extracts and elute late in the columns. It may not affect polar compounds as much as they do for late eluting analytes. However, the lipids require high organic composition in the LC gradient to clean them out of the LC column, otherwise it will accumulate inside the column and leading to column deterioration with poor peak shape or retention shifts. By switching to a more polar organic extraction solvent, such as ethyl acetate, more lipids will participate into the organic layer. LLE is preferred by many practicing scientists due to its speed and simplicity compared with solid-phase extraction and protein precipitation, although its green credentials are questionable.

1.2.5.2 Protein precipitation

Protein precipitation (PP) is a well-established sample preparation technique for the removal of unwanted proteins from biological fluid samples. In a PP procedure a solvent (often an equal or higher volume (e.g. 1:3) of acetonitrile (or sometimes methanol) or strong acid or base are added to the biological sample which causes proteins and other biological material to precipitate out of solution. The sample is mixed and centrifuged, resulting in the formation of a protein pellet and its corresponding supernatant. The supernatant, now containing the analyte of interest, is transferred, dried, reconstitiuted, or directly injected into a LC column (Bakhtiar *et al.*, 2006). This procedure is easily amenable to automation and is applicable to a host of structurally diverse group of small analytes. While PP is fast, easy to apply, and applicable to a broad class of small molecules, it also suffers from several disadvantages. PP lacks specificity and selectivity that SPE and LLE can offer. Consequently, significant matrix effects and ion suppression can be observed due to the presence of other endogenous molecules that compete with the analyte(s) during ionisation. PP contributes to the sample extracts a fair amount of phospholipids that concentrate during the dry down and reconstitution step. In

addition, compounds that are highly bound to the protein can yield low sample recovery in the PP procedure (Bakhtiar *et al.*, 2006).

1.2.5.3 Solid phase extraction

Over the last twenty years, solid phase extraction (SPE) has become the most powerful technique available for rapid and selective sample preparation prior to analytical chromatography. Various SPE procedures are used due to the abundance of different retention mechanisms available to retain the desired analytes, and extract them out of bio-matrices. Often this involves time consuming method development, not suitable for quick turnaround of samples.

SPE is one of the most effective and most versatile methods of sample preparation. SPE is a chromatographic technique used to prepare samples for subsequent analysis by removing interfering substances that may be present. Problems associated with LLE such as incomplete phase separations, less-than-quantitative recoveries as much of the target analyte is typically lost during the extraction process, use of expensive, breakable specialty glassware, and disposal of large quantities of organic solvents can be prevented using SPE which is a more efficient clean up method yielding quantitative extractions that are easy to perform. SPE is rapid, can be automated and solvent use and lab time are reduced (Arendale, 1986). SPE switches sample matrices to a form more compatible with chromatographic analyses, has more efficient use of all of the analyte present for increased sensitivity, removes interferences to simplify chromatography and improve quantitation and also protects the analytical column from contaminants.

Common SPE methods use a solid phase, typically in a disposable cartridge, and a liquid solvent to isolate one, or one type, of analyte from a solution. SPE can be used to isolate analytes of interest from a wide variety of matrices, including complex ones such as urine, blood, water samples, beverages, soil and even animal tissue. There are three approaches in which SPE is used to separate compounds of interest from impurities.

In the classic mode, selective extraction, the separating ability of SPE is based on the preferential affinity of desired solutes in a liquid, mobile phase for a solid, stationary phase through which the sample is passed. For interference

laden samples (e.g., biological fluids), samples are generally diluted with buffer. When dealing with ionisable compounds pH manipulation is important. A compound's ionisation state can drastically change its retention and elution characteristics on a given SPE sorbent. Mobile phase pH should be selected so that it is at least ± 2 pH units from the analyte's pKa. This assures that the analytes are either 100% ionised or 100% non-ionised and should help control run to run reproducibility. At high pH, acidic compounds are ionised and are much more hydrophilic than under ion suppression conditions.

The first step involves conditioning and equilibrating the SPE cartridge. Conditioning wets or activates the bonded phases to ensure consistent interaction between the analyte and the sorbent functional groups. Reversed-phase sorbents are often conditioned with a water miscible solvent such as methanol or acetonitrile (Steehler, 2004). Equilibration introduces a solution similar to the sample load in terms of solvent strength and pH in order to maximize retention. Buffer or water are good choices for reversed-phase equilibration (see Figure 1.7).

The second step involves loading the diluted sample at a consistent and reduced flow rate of ~1-2 drops/second to ensure optimal retention. Impurities and irrelevant substances in the sample are then washed away while the analyte of interest is retained on the stationary phase. A wash step is necessary to elute interferences without prematurely eluting compounds of interest. 5-20% methanol in water or sample pre-treatment buffer is typical for wash solvents (Steehler, 2004).

Analytes that are retained on the stationary phase are then eluted from the cartridge with an appropriate solvent. The hydrophobic interactions between the analyte and sorbent functional groups are interrupted with an organic solvent or solvent combination of sufficient non-polar character (Steehler, 2004). Example elution solvents are methanol or acetonitrile. It is often necessary to evaporate and reconstitute the SPE eluate in mobile phase prior to LC analysis.

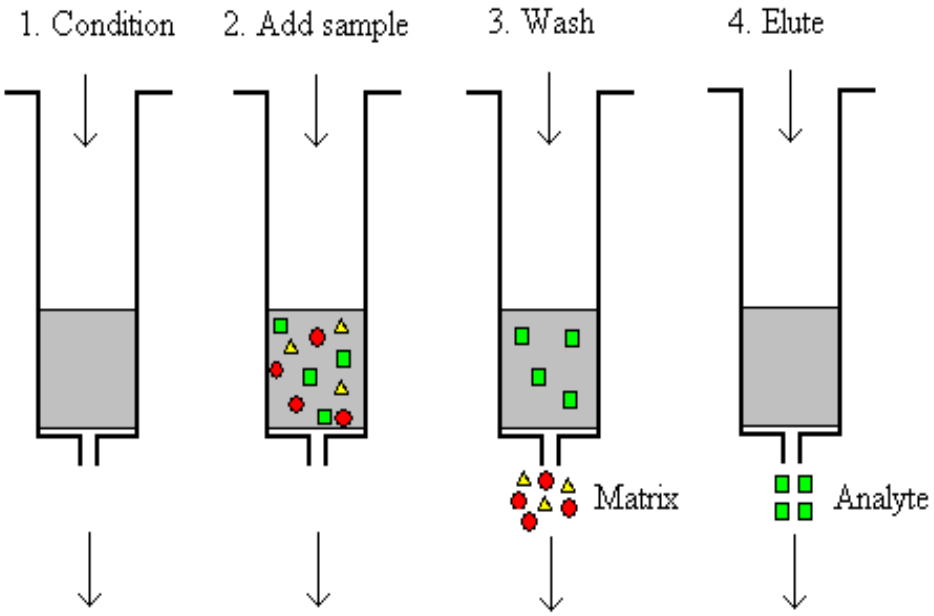

Figure 1.7: Schematic showing the four main steps in a typical SPE procedure

The second mode is selective washing which involves the compounds of interest and the impurities being retained on the SPE packing when the sample passes through. The impurities are rinsed through with wash solutions that are strong enough to remove them, but weak enough to leave the compounds of interest behind. Finally, selective elution works by eluting the adsorbed compounds of interest in a solvent that leaves the strongly retained impurities behind.

SPE cartridges are commercially available with a variety of different stationary phases, but most are based on a bonded silica material that is derivatised with a specific functional group. Some of these functional groups include hydrocarbon chains of variable length (for reversed phase SPE), quaternary ammonium or amino groups (for anion exchange), and sulfonic acid or carboxyl groups (for cation exchange) (Steehler, 2004).

Reversed phase SPE separates analytes based on their polarity. In a reversed phase SPE cartridge compounds of mid to low polarity are retained due to their hydrophobicity and attraction to the stationary phase. Separation is achieved using a polar or moderately polar sample matrix and a nonpolar stationary phase (LoBrutto *et al.*, 2006). Materials for reversed phase SPE include alkyl- and aryl-bonded silicas (LC-18, LC-8, LC-4, and LC-Ph)

whereby the hydrophilic silanol groups at the surface of the raw silica packing are chemically modified with hydrophobic alkyl or aryl functional groups by reaction with the corresponding silanes (Steehler, 2004). Retention of organic analytes from polar solutions onto these materials is due primarily to the attractive forces between the carbon-hydrogen bonds in the analyte and the functional groups on the silica surface. These nonpolar-nonpolar attractive forces are commonly called van der Waals forces, or dispersion forces (Kazakevich *et al.*, 2006). The analyte can be eluted by washing the cartridge with a non-polar solvent, which disrupts the interaction of the analyte and the stationary phase. Reversed-phase SPE is considered the least selective retention mechanism when compared to normal-phase or ion-exchange SPE. In other words, it may be difficult for a reversed-phase method or bonded-chemistry to differentiate between molecules that are structurally similar. However, because reversed-phase will retain most molecules with any hydrophobic character, it is very useful for extracting analytes that are very diverse in structure within the same sample (LoBrutto *et al.*, 2006).

Normal phase SPE typically involves a polar analyte, a mid- to nonpolar matrix (e.g. acetone, chlorinated solvents, and hexane), and a polar stationary phase. Polar-functionalized bonded silicas (e.g. LC-CN, LC-NH_2, and LC-Diol), and polar adsorption media (LC-Si, LC-Florisil and LC-Alumina) typically are used under normal phase conditions (Kazakevich *et al.*, 2006). Retention of an analyte is primarily due to interactions between polar functional groups of the analyte and polar groups on the sorbent surface. These include hydrogen bonding, pi-pi interactions, dipole-dipole interactions, and dipole-induced dipole interactions, among others. A compound adsorbed by these mechanisms is eluted by passing a solvent that disrupts the binding mechanism - usually a solvent that is more polar than the sample's original matrix.

Ion exchange sorbents separate analytes based on electrostatic interactions between the analyte of interest and the positively charged groups on the stationary phase. For ion exchange to occur, both the stationary phase and sample must be at a pH where both are charged to encourage interaction. Anion exchange sorbents are derivatised with positively charged functional groups that interact with and retain negatively charged anions, such as acids. Strong anion exchange sorbents contain quaternary ammonium groups that have a permanent

positive charge in aqueous solutions, and weak anion exchange sorbents use amine groups which are charged when the pH is below about 9. To elute the analyte from either the strong or weak sorbent, the stationary phase is washed with a solvent that neutralises the charge of either the analyte, the stationary phase, or both. Once the charge is neutralised, the analyte will elute from the cartridge. Cation exchange sorbents are derivatised with functional groups that interact and retain positively charged cations, such as bases. Strong cation exchange sorbents contain aliphatic sulfonic acid groups that are always negatively charged in aqueous solution, and weak cation exchange sorbents contain aliphatic carboxylic acids, which are charged when the pH is above about 5. To elute the analyte from either the strong or weak sorbent, the stationary phase is washed with a solvent that neutralises ionic interaction between the analyte and the stationary phase. Once the interaction between analyte and stationary phase is neutralised, the analyte will elute from the cartridge.

Solutions used in SPE procedures can have a very broad pH range. Silica-based packings, such as those used in HPLC columns, usually have a stable pH range of 2 to 7.5. At pH levels above and below this range, the bonded phase can be hydrolyzed and cleaved off the silica surface, or the silica itself can dissolve. In SPE, however, the solutions usually are in contact with the sorbent for short periods of time. The fact that SPE cartridges are disposable, and are meant to be used only once, allows one to use any pH to optimise retention or elution of analytes.

For reversed phase SPE procedures on bonded silicas, the pH of the conditioning solution and sample should be adjusted for optimum analyte retention. If the compound of interest is acidic or basic a pH at which the compound is not charged is used. Retention of neutral compounds (no acidic or basic functional groups) usually is not affected by pH. In normal phase SPE procedures on bonded silicas or adsorption media, pH is usually not an issue, because the solvents used in these processes are typically nonpolar organic solvents, rather than water. Retention in ion exchange SPE procedures depends heavily on the pH of the sample and the conditioning solutions. For retention of the analyte, the pH of the sample must be one at which the analyte and the functional groups on the silica surface are charged oppositely. In order for

electrostatic retention to occur, both analyte and sorbent functional groups must be in their ionised form. This is done through strict pH control of the sample matrix. For basic analytes, the pH should be adjusted to at least 2 pH units below the molecule's pKa. For acidic analytes, the pH should be adjusted to at least 2 pH units above the molecule's pKa. To elute, the opposite is true. By adjusting the pH of the eluant to at least two pH units above or below the analytes' and/or sorbent's pKa, one can effectively neutralize one or both functional groups disrupting the electrostatic

The stationary phase in SPE is packed into a syringe-shaped cartridge, a disc or a 96 well plate, which can be mounted on a commercially available extraction manifold. A typical SPE manifold can accommodate 12 or 24 SPE cartridges so that many samples can be processed simultaneously. Many SPE manifolds are equipped with a vacuum port which speeds up the extraction process by pulling the liquid sample through the stationary phase at a set speed. A sample tube rack inside the manifold collects the samples as they are eluted off the cartridges.

Figure 1.8: Photograph of a typical SPE manifold (above) which can accommodate 12 SPE cartridges and a 96 well sample processing manifold (below)

Reliable, high throughput sample preparation techniques are essential in today's bioanalytical laboratory. A 96 well sample processing manifold increases productivity by allowing the processing of up to 96 samples simultaneously. These manifolds can process SPE, LLE or PP plates hence minimising method development time and enhancing throughput. Figure 1.8 shows a typical SPE manifold accommodating 12 SPE cartridges and a 96 well sample processing manifold. Both types of manifold were used for work in this thesis.

1.2.6 Validation of analytical methods

Before a procedure can provide useful analytical information, it is necessary to demonstrate that it is capable of providing acceptable results. Several articles have been published on the requirements of method validation for analytical methods (Kanarek, 2005, Krause, 2005). Validation is an evaluation of whether the precision and accuracy obtained by following the procedure are appropriate for the problem. In addition, validation ensures that the written procedure has sufficient detail so that different analysts or laboratories following the same procedure obtain comparable results (Goldsmith, 2000). Bioanalytical method validation includes all of the procedures that demonstrate that a particular method used for quantitative measurement of analytes in a given biological matrix, such as blood, plasma, serum, or urine, is reliable and reproducible for the intended use.

Due to the nature of the sample, bioanalytical methods are more complex and more difficult to validate than methods used for quality control or quality assurance of drug products. This is because bioanalytical methods include more complex matrices, a large variety of possible interferences from metabolites, and typically low sample volumes (Garofolo, 2004). In addition, bioanalytical data needs to be accurate and reliable because they are used for critical applications including the calculation of pharmacokinetic parameters that are critical to the review of the performance of a drug or a formulation. Therefore, bioanalytical methods need to be thoroughly validated.

The objective of analytical method validation is to ensure that every future measurement in routine analysis will be close enough to the unknown true value for the content of the analyte in the sample (González *et al.*, 2007). There are various guidelines for validation of analytical assays such as those published by the FDA, Institute of Validation, EU and those laid down at the Washington Conference of 1990 to name a few (Clarke, 1994, Taverniers *et al.*, 2004, Shah *et al.*, 1992).

Precision, accuracy, sensitivity, linearity and range, recovery and stability are the most important parameters during a validation. Where possible, intra-day precision (repeatability) and inter-day precision (intermediate precision), accuracy, sensitivity, limits of detection and quantitation (LOD and

LOQ), linearity and range, recovery and stability to freeze-thaw cycles are tested. After developing a method with desired attributes, the method is validated to establish that it will continue to provide accurate, precise, and reproducible data during study-sample analysis (FDA, 2001). Results from method validation can be used to judge the quality, reliability and consistency of analytical results; it is an integral part of any good analytical practice. Analytical methods need to be validated or revalidated before their introduction into routine use, whenever the conditions change for which the method has been validated (e.g., an instrument with different characteristics or samples with a different matrix) and whenever the method is changed and the change is outside the original scope of the method.

1.2.6.1 Accuracy

Accuracy is a measure of the closeness of test results obtained by a method to the true value (Goldsmith, 2000). Accuracy indicates the deviation between the mean value found and the true value. It is determined by applying the method to samples to which known amounts of analyte have been added. These should be analysed against standard and blank solutions to ensure that no interference exists. The accuracy is then calculated from the test results as a percentage of the analyte recovered by the assay.

Errors in measurement can be divided into two general categories: systematic errors and random errors. Systematic errors result from sources that can be traced to the methodology, the instrument or the operator, and affect both the accuracy and the precision of the measurement. Random errors only affect the precision, and are difficult to eliminate, because they are the result of random fluctuations in the measured signal, due to noise and other factors (Garofolo, 2004). Whilst systematic errors are proportional to the sum of individual contributions, random errors are proportional to the root of the sum of the squares of the individual contributions (Garofolo, 2004). Thus, the precision of the entire procedure is often dominated by the random errors of the most imprecise step.

Accuracy in this thesis was expressed as % error i.e. [the absolute difference between calculated concentration and nominal concentration] /

nominal concentration × 100. Analytical methods may be divided into three groups based on the magnitude of their relative errors. When an experimental result is within 1% of the correct result, the analytical method is highly accurate. Methods resulting in relative errors between 1% and 5% are moderately accurate, but methods of low accuracy produce relative errors greater than 5%. The magnitude of a method's relative error depends on how accurately the signal is measured and the ease of handling the sample without loss or contamination (Goldsmith, 2000).

1.2.6.2 Precision

The precision of an analytical method is the degree of agreement among individual test results obtained when the method is applied to multiple sampling of a homogenous sample (Goldsmith, 2000). The closer the agreement between individual analyses, the more precise the results. Precision is a measure of the reproducibility of the whole analytical method (including sampling, sample preparation and analysis) under normal operating circumstances.

Accuracy is generally the more important characteristic of quantitative data to be assessed, although consistency, as measured by precision, is of particular concern in some circumstances (Steehler, 2003). Precision is a measure of the spread of data about a central value and may be expressed as the range, the standard deviation, or the variance. Intra-day and inter-day precision in this thesis was determined by calculating RSD (relative standard deviation) values. RSD was expressed as [deviation from the mean]/mean concentration × 100.

Precision is commonly divided into two categories: repeatability and reproducibility. Repeatability is the precision obtained when all measurements are made by the same analyst during a single period of laboratory work, using the same solutions and equipment. Reproducibility, on the other hand, is the precision obtained under any other set of conditions, including that between analysts, or between laboratory sessions for a single analyst (Garofolo, 2004). Since reproducibility includes additional sources of variability, the reproducibility of an analysis can be no better than its repeatability. Errors affecting the distribution of measurements around a central value are called

indeterminate and are characterised by a random variation in both magnitude and direction. Indeterminate errors can be traced to several sources, including the collection of samples, the manipulation of samples during the analysis, and the making of measurements.

1.2.6.3 Sensitivity

The ability to demonstrate that two samples have different amounts of analyte is an essential part of many analyses. A method's sensitivity is a measure of its ability to establish that such differences are significant. Sensitivity is often confused with a method's detection limit. The detection limit is the smallest amount of analyte that can be determined with confidence. The detection limit, therefore, is a statistical parameter. Sensitivity is the change in signal per unit change in the amount of analyte (Goldsmith, 2000).

Limit of detection is the lowest concentration in a sample that can be detected, but not necessarily accurately quantitated, under the stated experimental conditions. The limit of detection is important for impurity tests and the assays of dosages containing low drug levels and placebos. The limit of detection is generally quoted as the concentration yielding a signal-to-noise ratio of 3:1 (Garofolo, 2004). Since the limit of detection is dependent on the signal-to-noise ratio; it can be improved by enhancing the analyte signal and reducing the detector noise. Noise is the width of the baseline created by background fluctuations such as the detector electronics, pump oscillations and/or a dirty column or the baseline signal in the absence of the analyte. The signal (i.e. peak height) can be increased by selecting the optimum monitoring wavelength, increasing the injection volume or mass (below signal or column saturation), increasing the peak sharpness with high efficiency columns and by optimising the mobile phase. For absorbance detectors, longer path lengths in the flow cell enhances sensitivity though often to the detriment of post column dispersion. Noise can be reduced by using high sensitivity detectors with low noise and drift characteristics, slower detector response time, mobile phases with low absorbance and pumps with low pulsation. Limit of quantitation is the lowest concentration of analyte in a sample that can be determined with

acceptable precision and accuracy. It is quoted as the concentration yielding a signal-to-noise ratio of 10:1 (Garofolo, 2004).

1.2.6.4 Linearity and range

The linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the sample (Lee, 2004). The range of an analytical procedure is the interval between the upper and lower concentrations (amounts) of analytes in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy, and linearity (Lee, 2004). To determine linearity and range of an assay a calibration curve needs to be prepared over the range of concentrations appropriate to the assay and the regression coefficient determined.

1.2.6.5 Recovery

The recovery is a measure of the efficiency of the method in detecting all the analyte of interest present in the original sample. It is more important that recovery is constant within the calibration range and is hence evaluated close to the extremes of the calibration range and at an intermediate concentration (Braggio *et al.*, 1996). The percentage of drug recovered from the biological samples was determined by comparing the integrated areas of extracted samples spiked before extraction with the response of extracted blank matrix samples spiked just before HPLC assay. This is performed rather than a comparison with pure unextracted standards in order to compensate any effect the matrix may have in the signal response (Peters *et al.*, 2007). Most authors agree, that the value for recovery is not important, as long as the data for LOD, precision and accuracy (bias) are acceptable (Bressolle *et al.*, 1996, Causon *et al.*, 1997, Dadgar *et al.*, 1995, Hartmann *et al.*, 1998, Karnes *et al.*, 1991, Shah *et al.*, 2000).

1.2.6.6 Stability

It is extremely important to perform a stability study of the analyte in biological fluids in order to obtain information concerning the conditions and times of sample storage so that sample integrity before assay is assured (Braggio *et al.*, 1996). Chemical compounds can decompose prior to chromatographic investigations, for example, during the preparation of the sample solutions, extraction, cleanup, phase transfer or storage of prepared vials (in refrigerators or in an automatic sampler). Method development should investigate the stability of the analytes and standards under these circumstances (Bretnall *et al.*, 2011).

Drug stability in a biological fluid is a function of the storage conditions, the chemical properties of the drug, the matrix, and the container system. Stability procedures should evaluate the stability of the analytes during sample collection and handling, after long-term (frozen at the intended storage temperature) and short-term (bench top, room temperature) storage, and after going through freeze and thaw cycles and the analytical process. The procedure should also include an evaluation of analyte stability in stock solution. Conditions used in stability experiments should reflect situations likely to be encountered during actual sample handling, storage and analysis (Peters *et al.*, 1998). Stability of analytes during the whole analytical procedure is a prerequisite for reliable quantification.

1.3 Conclusions

From this introductory chapter, the underlying theories of the different separation techniques along with the various modes of detection and sample preparation methods were outlined and discussed. Due to its wide applicability, high performance liquid chromatography is the most commonly employed separation technique in many scientific and pharmaceutical fields. HPLC is a very sensitive technique seeing as it can incorporate a wide choice of detection methods such as UV/Vis, fluorescence and mass spectrometry, all of which have been successfully applied to the analysis of a wide range of samples.

The aim of this thesis was the development of chromatographic methodologies for the analysis of different therapeutic drugs in biological matrices under challenging conditions representative of the extremes of real world analytical problems. As previously discussed, one of the most difficult tasks faced by the analytical chemist is in the analysis of biological fluids. Analytical methods for pharmaceutical formulations are far less challenging to develop and validate than bioanalytical methods, especially in support of pharmacokinetic studies. Problems encountered with interferences from sample matrices, decomposition products and low levels of drug and metabolites proved the most challenging to overcome in this work. Careful assessment of matrix effects and judicial use of the sample preparation methods coupled with adequate chromatography and detection proved critical to the overall success of the methods developed herein.

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Chapter 2

Simultaneous determination of efavirenz, rifampicin and its main metabolite desacetyl rifampicin levels in human plasma

2.1 Scope of research

The objective of this work was to develop a method for the simultaneous determination of efavirenz (Efv) and rifampicin (Rif) and its metabolite, desacetyl rifampicin (dRif), from human plasma. Appropriate treatment of microbial illness represents a significant pharmaceutical challenge. Nowhere more so than in the management of HIV infection, where patients often present with concurrent tuberculosis infection, thereby necessitating combination drug treatment. We developed a collaboration with Dr Patrick Mallon in the Mater Hospital who routinely treats concurrently HIV and TB positive patients. An international collaboration involving the Mater group hypothesise that the pharmacokinetics of Rif are adversely affected when a patient is administered both drugs at the same time. A clinical study termed the SPhEAR study (Study on the Pharmacokinetics of Efavirenz And Rifampicin) was initiated with the objective of determining the effect of HIV infection and exposure to antiretroviral therapy on the pharmacokinetics of Rif in patients treated for *Mycobacterium tuberculosis* infection. Examination of this hypothesis would necessitate developing a new method capable of convenient, rapid and cost effective quantitation of two unrelated pharmaceuticals the antiviral agent Efv, the anti-TB agent, Rif and its primary metabolite (dRif) and we sought to provide just such a technique.

2.2 Introduction

2.2.1 HIV and its treatment

The human immunodeficiency virus (HIV) pandemic is a complex mix of diverse epidemics within and between countries and regions of the world (Simon *et al.*, 2006). It represents a global problem, estimated to affect over 40 million people with infection rates increasing by nearly five million per year (WHO, 2006). The HIV retrovirus, if left untreated, leads to acquired immunodeficiency syndrome (AIDS). HIV infection occurs through the transfer of blood, semen, vaginal fluid

or breast milk. The four major routes of transmission are unprotected sexual intercourse, contaminated needles, transmission from mother (if infected) to her baby at birth and through breast milk.

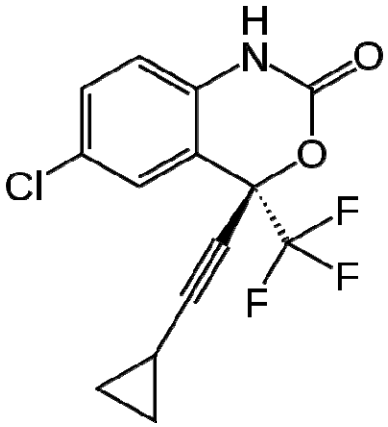
HIV primarily infects vital cells such as helper T cells, macrophages and dendritic cells in the human immune system. It is estimated that AIDS has killed more than 25 million people since it was first recognised in 1981. It is also estimated that 0.6% of the worlds living population is infected with HIV. To date, there is no cure or vaccine for HIV or AIDS. Avoiding exposure to the virus is the only know method of prevention.

Antiretroviral treatment has transformed AIDS from an inevitably fatal condition to a largely chronic, manageable disease (Simon *et al*., 2006). Highly active antiretroviral therapy (HAART) is the current treatment for HIV infection. HAART does not cure the patient but it does allow stabilisation of the patient's symptoms and reduction of viremia. Current HAART options are combinations of at least three drugs belonging to at least two classes of anti-retroviral agents. These classes include nucleoside analogue reverse transcriptase inhibitors, protease inhibitors and non-nucleoside reverse transcriptase inhibitors (NNRTI). Efv is used as part of HAART for the treatment of HIV type 1 - see Figure 2.1 for structure. Efv belongs to the non-nucleoside reverse transcriptase inhibitors group. It works by interrupting the replication of new HIV particles in already infected cells (Rezk *et al*., 2004).

Efv does this by preventing the reverse transcriptase enzyme from properly functioning. This enzyme functions to change the RNA of the virus so that it becomes DNA. Once this occurs, the material which encodes HIV will be inserted into the genetic code of the infected cell.

The recommended dose for Efv is 600 mg daily (some recommend a higher dose of 800 mg daily when used with Rif), usually dosed at night and as the agents do not cure the disease, Efv treatment for HIV will need to be given continuously or with treatment "holidays" for the remainder of the patient's life.

(a)



(b)

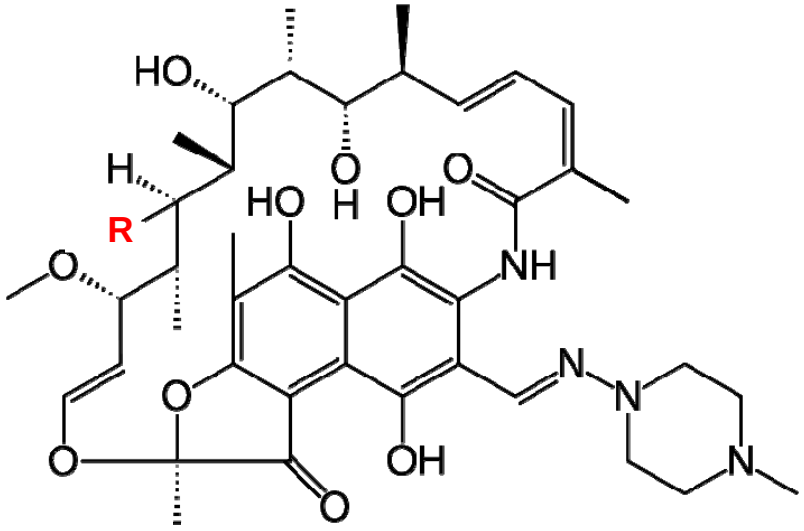


Figure 2.1: Chemical structures of (a) Efv and (b) Rif (R = $OCOCH_3$) and dRif (R = OH)

2.2.2 TB and its treatment

Tuberculosis (TB) is an infection caused by mycobacteria, mainly *Mycobacterium tuberculosis* (Allanson *et al*., 2007). TB most commonly attacks the lungs (as pulmonary TB) but can also affect the central nervous system, the lymphatic system, the circulatory system, bones, joints and even the skin. Among infectious

diseases, TB is globally the leading cause of death, killing around three million people each year (Zumla *et al.*, 1999). There are approximately eight million new cases each year worldwide, almost all of them preventable or treatable (Allanson *et al.*, 2007). When people suffering from active pulmonary TB cough, sneeze, speak, kiss, or spit, they expel infectious aerosol droplets, known as bacilli. Currently one-third of the world's population is infected with the TB bacillus (Allanson *et al.,* 2007). However, TB bacilli-infected people will not necessarily become sick with the disease. TB bacilli are protected with a thick waxy coat and the immune system may form a fibrous granuloma around a site of infection limiting growth hence, the disease can lie dormant for years. However, the chances of becoming sick are increased when a person's immune system is weakened. TB is significantly more common in deprived city areas, among the homeless, alcoholics, older people and patients with HIV/AIDS.

Treatment for TB uses antibiotics to kill the bacteria. Rif is an important first line drug prescribed throughout TB therapy, often as part of fixed dose combination tablets, which may also contain isoniazid and pyrazinamide (Allanson *et al.,* 2007). Rif has both early bactericidal activity and, more importantly, sterilising activity against *Mycobacterium tuberculosis*. See Figure 2.1 for structure. Rif exerts its anti-TB activity by targeting the bacterial DNA dependant RNA polymerase of both intracellular and extracellular *Mycobacterium tuberculosis* (Hartkoorn *et al.*, 2007). The ability to kill *Mycobacterium tuberculosis* is related to the concentration of drug to which the bacterium is exposed (Balbão *et al.,* 2010).

The recommended dose for Rif is 600 mg daily, which can be dosed at any time of the day (usually in the morning). The duration of treatment depends on the type of TB - standard is six months for pulmonary TB and 9-12 months for extrapulmonary TB. WHO recommends a 6-month regimen comprising Rif, isoniazid, pyrazinamide, ethambutol which are given together for the first 2 months followed by Rif and isoniazid therapy for the next 4 months. Rif is mainly eliminated in the bile and then reabsorbed, hence, enterohepatic circulation ensues. During this time the drug is progressively deacylated into its

microbiologically active metabolite, 25-desacetyl rifampicin which is less absorbable as compared to the parent drug (Panchagnula *et al.,* 1999).

2.2.3 Combination treatment

In the decades preceding 1980, rates of TB declined globally. After the appearance of HIV, however, rates in many countries increased markedly and now, the two epidemics are catastrophically intertwined (Myers *et al.,* 2008). TB is a leading cause of death among people who are HIV-positive. There are an estimated 11.5 million people co-infected with HIV and TB (Sehu *et al.*, 2008). A patient who is HIV-positive and infected with TB bacilli is many times more likely to become sick with TB than someone infected with TB bacilli who is HIV-negative. Current guidelines recommend treatment of TB and HIV co-infection with antiretroviral combinations containing NNRTI's (WHO, 2006). Of the two NNRTI drugs currently available, Efv is preferred as it is dosed once daily and is less hepatotoxic than the alternative.

How HIV infection alters Rif pharmacokinetics and any additional effects of introducing antiretroviral therapy have not been closely studied. In particular, the optimal dose of Rif for use in HIV-infected patients treated with Efv-containing antiretroviral regimens has not been determined. If Rif levels were significantly affected by either HIV infection itself or concomitant exposure to Efv therapy then this may limit the effectiveness of Rif in TB therapy and increase the risk of development of multi-drug resistant TB, an increasing cause of morbidity and mortality in resource limited settings with evidence of spread within these populations (Gandhi *et al.,* 2006) and the potential for spread to other countries, including Western Europe (Brennan-Benson, 2005).

Concurrent treatment of HIV and TB is complicated by drug interactions between Rif and antiretrovirals (Ramachandran *et al.*, 2006) and it has been hypothesised that the pharmacokinetics of Rif are adversely affected when a patient is administered both drugs at the same time. In order to study whether the pharmacokinetics of Rif are adversely affected when a patient is also administered

Efv, a clinical study termed the SPhEAR study was initiated at the Mater Hospital, Dublin which required an analytical method capable of extraction, separation and determination of Efv and Rif (and also Rif's metabolite dRif) simultaneously in plasma.

2.2.4 Physiochemical properties of efavirenz and rifampicin

Efv, a white to slightly pink crystalline powder, is a polar, slightly basic anti-HIV drug. It has a molecular weight of 315.7 g/mol (D'Avolio *et al.*, 2010) and a pKa of 10.2. It is practically insoluble in water but soluble in lower alcohols. The aqueous solubility increases as the pH increases above 9.0, consistent with the loss of the proton on the amine of the carbamate.

Rif, a red/orange powder has a molecular weight of 822.94 g/mol. Rif has two pKa values since it is a zwitterion – the pKa at 1.7 relates to the 4-hydroxy moiety and the pKa at 7.9 relates to the 3-piperazine nitrogen (Balbão *et al.*, 2010). It is very slightly soluble in water, freely soluble in chloroform and DMSO and soluble in ethyl acetate, methanol and tetrahydrofuran. Solubility in aqueous solutions is increased at acidic pH.

2.2.5 Sample extraction/clean-up procedures for efavirenz and rifampicin

In order to routinely determine drug concentrations in plasma, it is necessary to have an accurate, precise and selective analytical method, requiring a small sample volume complemented by rapid sample processing methods. The three main sample extraction techniques employed to extract drugs from biological fluids are liquid liquid extraction (LLE), protein precipitation (PP) and solid phase extraction (SPE). All three approaches have been used in the reported methods for Efv and Rif analysis as shown in Tables 2.1 and 2.2.

2.2.5.1 Sample extraction/clean-up procedures for efavirenz

Efv has been successfully isolated from plasma using LLE, PP and SPE. LLE is by far the more popular mode of extraction for Efv. See Table 2.1. However, a number of these methods require large sample volumes of 0.5 mL or higher (Lakshmi Sailaja *et al.*, 2007, Dailly *et al.*, 2004, Matthews *et al.*, 2002, Proust *et al.*, 2000), and LLE is a labour-intensive method which often does not lend itself easily to automation. Also recoveries vary between 78 and 101% (Lakshmi Sailaja *et al.*, 2007, Dailly *et al.*, 2004, Proust *et al.*, 2000, Weller, 2007, Rouzes *et al.*, 2004). Matthews *et al.*, 2002, and Ramachandran *et al.*, 2006, both employed LLE and obtained recoveries between 89 and 101%. Matthews used a mixture of dichloromethane and hexane to extract Efv from 0.5 mL plasma. The procedure involved a number of steps and a large quantity of extraction solvent (8 mL) but analyte recovery was high, greater than 89% at all points on the standard curve. Ramachandran used ethyl acetate for the extraction in a much simpler procedure which involved 0.25 mL plasma and also preconcentrated the sample by a factor of five. The average recovery of Efv from plasma using this extraction procedure was 101%.

PP has also been successful for Efv's extraction. In comparison to LLE the sample volumes required are much lower, in the region of 50 – 100 µL (D'Avolio *et al,.* 2010, Mogatle *et al.*, 2009, Kappelhoff *et al.*, 2003, Volosov *et al.*, 2002) and in one case 250 µL (Veldkamp *et al.*, 1999) and the recoveries obtained are high (85 – 110%). Veldkamp *et al.*, 1999, utilised PP to extract Efv from plasma. Precipitation was achieved by mixing 0.25 mL plasma with acetonitrile. Recovery of Efv was 106%. Efv has also been extracted from serum using PP. Dogan-Topal *et al.*, 2007, achieved excellent recovery of Efv (99.9-100.3%) but the method required a 1 mL serum volume for the extraction.

All reported SPE methods for the extraction of Efv from plasma require plasma volumes of 500 µL or higher (Matthews *et al.*, 2002, Notari *et al.*, 2006, Rezk *et al.*, 2004, Rezk *et al.*, 2002) and up to 1 mL in Rentsch's *et al.*, 2003, case. The only SPE method which required a smaller sample volume was Sarasa-

Nacenta *et al.*, 2001, with 300 μL plasma. However, recovery was much lower than the other SPE methods at 83% in comparison to greater than 90% in all the other SPE methods reported. Sarasa-Nacenta *et al.*, 2001, developed an SPE method for the determination of Efv in human plasma. SPE was performed using Oasis SPE cartridges (30 mg) which were conditioned with methanol and water, loaded with 0.3 mL plasma followed by a water aliquot and then washed with 50% aqueous methanol. Efv was eluted with methanol, dried down and reconstituted with 0.1 mL of mobile phase, preconcentraing the sample by a factor of three.

Reference	Analytes	Matrix	Extraction mode	Extraction recoveries (%)	Detection	Approx retention time (min)	LOQ/ LOD	Column	Sample vol. (µL)
(D'Avolio *et al*., 2010)	Efv and other drugs	Dried plasma spots on glass filter	n/a	>85.0	MS	~16.9	31.2 ng/mL LOQ	Atlantis T3 C18	Not stated
(Martin *et al.*, 2009)	Efv and other drugs	Human plasma	PP & on-line SPE	75-98	MS	1.9	100 ng/mL LOQ	LUNA Phenyl Hexyl	Not stated
(Mogatle *et al.*, 2009)	Efv	Human plasma	PP	92.7-94.1	UV	~8	200 ng/mL LLOQ	LUNA C18	100
(D'Avolio *et al*., 2007)	Efv and other drugs	Human plasma	PP	93-110	MS	16.7	31.2 ng/mL LOQ	Atlantis dC-18	50
(Dogan-Topal *et al.*, 2007)	Efv and other drugs	Human serum	PP	99.9-100.3	UV	11.6	8.96 ng/mL LOQ	Spherisorb	1000
(Lakshmi Sailaja *et al*., 2007)	Efv	Human plasma	LLE	>78.0	UV	6.5	200 ng/mL	Zorbax C18	1000

(Weller *et al.*, 2007)	Efv and other drugs	Human plasma	LLE	83.6-84.4	UV	15	50 ng/mL	Octyl C8	200
(Choi *et al*., 2007)	Efv and other drugs	Human plasma	LLE	>89.0	UV	23.7	10 ng/mL LLOQ	Zorbax C18	200
(Ramachandran *et al.*, 2006)	Efv	Human plasma	LLE	101	UV	6.2	50 ng/mL	Lichrospher C18	250
(Notari *et al.*, 2006)	Efv and other drugs	Human plasma	SPE	89.7-121.5	UV	28.4	100 ng/mL	C18 Symmetry	600
(Rezk *et al.*, 2004)	Efv and other drugs	Human plasma	SPE	>90.0	UV	24.9	10 ng/mL	Zorbax C18	550
(Rouzes *et al.*, 2004)	Efv and other drugs	Human peripheral blood mononuclear cells	LLE	88.6	MS	8.3	2 ng/3 x 10^6 cells	X-Terra MS C18	Not stated
(Dailly *et al.*, 2004)	Efv and other drugs	Human plasma	LLE	80	UV	16.1	100 ng/mL	Symmetry C18	1000

(Kappelhoff *et al.*, 2003)	Efv and Nevirapine	Human plasma	PP	85.0-96.6	UV	7.8	50 ng/mL	Zorbax Extend C18	100
(Rentsch *et al.*, 2003)	Efv and other drugs	Human plasma	SPE	96-6-97.9	MS	16.3	10 ng/mL LOQ	Nucleosil C18 HD	1000
(Turner *et al.*, 2003)	Efv and other drugs	Human plasma	LLE	90.7	UV	24.2	50 ng/mL	Supelguard Discovery C8	200
(Matthews *et al.*, 2002)	Efv and other drugs	Human plasma	LLE	>89.0	Fluorescence	5	50 ng/mL	BDS Hypersil	500
(Volosov *et al.*, 2002)	Efv and other drugs	Human plasma	PP	Not stated	MS	4.5 min runtime	10 ng/mL	Supelco LC-18-DB	80
(Rezk *et al.*, 2002)	Efv	Human plasma	SPE	>99.6	UV	22.5	10 ng/mL	Eclipse XDB C8	500
(Langmann *et al.*, 2001)	Efv	Human plasma	LLE	96.2 ± 3	UV	10.9	25 ng/mL	Xterra RP 18	Not stated
(Sarasa-Nacenta *et al.*, 2001)	Efv	Human plasma	SPE	83	UV	6.9	100 ng/mL	Supelcosil LC8	300

(Aymard *et al.*, 2000)	Efv and other drugs	Human plasma	SLE	97.2 ± 2.7	UV	15.2	50 ng/mL	Symmetry C18	1000
(Proust *et al.*, 2000)	Efv and other drugs	Human plasma	LLE	83.8	UV	28.6	75 ng/mL	Licrospher 100-RP-18	500
(Veldkamp *et al*., 1999)	Efv	Human plasma	PP	106.4	UV	10.2	10 ng/mL	Zorbax SB C18	250
(Marzolini *et al.*, 2000)	Efv and other drugs	Human plasma	SPE	89.0-98.0	UV	28.7	250 ng/mL	ChromCart filled with Nucleosil 100	600

Table 2.1: Summary of the characteristics of reported extraction and LC detection techniques for the determination Efv from biological samples

Reference	Analytes	Matrix	Extraction mode	Extraction recoveries (%)	Detection	Approx retention time (min)	LOQ/LOD	Column	Sample vol. (µL)
(Gikas *et al.*, 2010)	Rif, daptomycin and IS	Rabbit plasma	PP	97.3-102.0	UPLC-UV	2.1	2 µg/mL	Acquity BEH C18	25
(Balbão *et al*., 2010)	Rif	Human plasma	Stir bar sorptive extraction	75.0-80.0	UV	11	125 ng/mL	LiChrocart 100 RP-8	200
(de Velde *et al.*, 2009)	dRif, Rif and other drugs	Human plasma	PP	dRif: 104.1-116.9 Rif: 101.3-109.4	MS	dRif: 2.3 Rif: 2.45	200 ng/mL LLOQ	HyPurity Acquastar C18	10
(Fernández-Torres *et al*., 2008)	Rif	Mouse plasma	PP	85.6-96.4	UV	~7.9	1.46 µg/mL LOQ	Lichrosphere 100 RP-18	50
(Song *et al.*, 2007)	Rif and other drugs	Human serum	PP	94.8	MS	3.4	50 ng/mL	Hydrosphere C18	50

(Hartkoorn *et al*., 2007)	Rif	Human cells and plasma	PP	Plasma -85-95 Cells - 51.73	MS	1.1	100 ng/mL	Betasil Phenyl-Hexyl	50
(Allanson *et al*., 2007)	Rif	Human plasma and blood spots	SPE	Plasma 84.5 Blood spots 65.0	UV	10.9	Plasma: 500 ng/mL Blood spots: 1.5 µg/mL	Kromasil C18	450
(Unsalan *et al*., 2005)	Rif and other drugs	Human plasma	PP	94.6	UV	10.2	700 ng/mL	Nova Pak C18	100
(Hemanth Kumar *et al*., 2004)	Rif and dRif	Human plasma and urine	PP	dRif: 90.0-113.0 Rif: 100.0-104.0	UV	dRif: 2.9 Rif: 4..8	Plasma: 250 ng/mL Urine: 2.5 µg/mL	LUNA C18	Plasma: 200 Urine: 2000
(Calleja *et al*., 2004)	Rif	Rat Plasma and liver	LLE	Liver: 91.0 Plasma: 83.0	UV	4	Plasma: 50 ng/mL Liver: 250 ng/g	Ultrabase-C18	Plasma: 100 Liver: 0.5g

(Panchagnula *et al.*, 1999)	Rif and dRif	Human plasma and urine	LLE	Plasma dRif:79.1 Rif: 91.7	UV	dRif: 3.0 Rif: 6.0	2 µg/mL	Nova Pak C18	100
(Yau Yi Lau *et al.*, 1996)	Rif	Human plasma	SPE	97	UV	4.4	50 ng/mL LOQ	Zorbax Rx C8	500

Table 2.2: Summary of the characteristics of reported extraction and LC detection techniques for the determination of Rif and dRif from biological samples

Reference	**Analytes**	**Matrix**	**Extraction mode**	**Extraction recoveries (%)**	**Detection**	**Approx retention time (min)**	**LOQ/LOD**	**Column**	**Sample vol. (µL)**
(Boffito *et al.*, 2002)	Rif and Efv	Human plasma	LLE	Rif: 70 ± 6 Efv: 98 ± 1	UV	Rif: 4.3 Efv: 13.1	50 ng/mL LOD	Phenomenex Reverse Phase Luna	200

Table 2.3: Summary of the characteristics of reported extraction and LC detection techniques for the simultaneous determination of Efv, Rif and dRif from biological samples

2.2.5.2 Sample extraction/clean-up procedures for rifampicin

Rif has also been successfully isolated from plasma using LLE, PP and SPE. From the literature, PP appears to be the most common approach used for the extraction of Rif from both plasma (Hartkoorn *et al.*, 2007, Gikas *et al.*, 2010, de Velde *et al.*, 2009, Fernández-Torres *et al.*, 2008, Unsalan *et al.*, 2005) and serum (Song *et al.*, 2007). Recoveries obtained for Rif using this approach were high (84.5% or greater) and required small sample volumes (0.2 mL or less).

Both Unsalan *et al.*, 2005, and Hartkoorn *et al.*, 2007, employed ACN for protein precipitation. Unsalan, using 100 µL plasma sample, required a very long evaporation step at room temperature for two days. However, recoveries were in the range of 92–111%. Hartkoorn used 50 µL of sample and achieved 92% recovery of Rif from plasma. Song *et al.*, 2007, used PP for the extraction of Rif from serum. Only a small volume of serum (50 µL) was required and protein precipitation was achieved using methanol. The recovery of Rif in this case was 95%. De Velde *et al.*, 2009, required only 10 µL plasma for the precipitation. The most recent PP used for the extraction of Rif was Gikas *et al.*, 2010. Rif was extracted from 25 µL rabbit plasma using a mixture of ethanol and acetonitrile. Recoveries were high at 97-102%

Both Panchagnula *et al.*, 1999, and Calleja *et al.*, 2004, used LLE for the extraction of Rif from plasma. Both required small sample volumes, with relatively high recoveries. Panchagnula used 95 µL of plasma spiked with Rif which was extracted with 500 µL of methanol. After centrifugation, 300 µL of supernatant was vacuum dried and the residue was reconstituted in 100 µL of mobile phase. Recoveries for drug were greater than 90% in plasma. Calleja used LLE to extract Rif from rat plasma. Dichloromethane and *n*-pentane was used for the extraction from 100 µL rat plasma. Recoveries averaged at 83% for Rif's extraction from plasma.

Allanson *et al.*, 2007, and Yau Yi Lau *et al.*, 1996, used SPE to extract Rif from plasma. Allanson used Strata-X-CW solid phase extraction cartridges, which were firstly washed with methanol and water before loading. Plasma was diluted 1:1 with ammonium acetate (25 mM). Interfering substances were washed away with ammonium acetate and Rif was eluted with methanolic

ammonia. Samples were dried down and reconstituted in 200 µL acetonitrile-water (1:4, v/v). The recovery of Rif from plasma was 85%.

Yau Yi Lau used 1 mL of blank human plasma spiked with Rifabutin. C 8 Bond Elut extraction columns were conditioned with methanol, followed by water. The plasma mixture was loaded onto the column and the column was washed with water. The analytes were eluted with methanol. Recoveries were high at 97%.

Balbão *et al.*, 2010, recently used stir bar sorptive extraction using 200 µL plasma and dichloromethane to extract Rif. In comparison to the other approaches used for the extraction of Rif, the recoveries were low, 75-80%.

2.2.5.3 Sample extraction/clean-up procedures for efavirenz and rifampicin simultaneously

Reported methods for the individual extraction of Rif and Efv have been very successful. The only reported method for the determination of Rif and Efv simultaneously was achieved by Boffito *et al.*, 2002. LLE was used for the simultaneous extraction of Rif and Efv from plasma. Extraction was achieved using ethyl acetate and n-hexane with a plasma sample volume of 200 µL. The recovery of Rif was 70% and Efv was 98%.

2.2.6 Sample separation and detection approaches for efavirenz and rifampicin

Various methods of separation and detection have been applied for the determination of Efv and Rif as can be seen in Table 2.1 and 2.2.

2.2.6.1 Sample separation and detection approaches for efavirenz

UV is by far the most reported detection approach for the analysis of Efv in the literature. In comparison to the MS methods reported for Efv, the sensitivity for Efv using UV is quite comparable and at times better with that achieved using MS. Dogan-Topal *et al.*, 2007, Choi *et al.*, 2007, and Rezk *et al.*, 2004, each achieved extremely good sensitivity using UV to detect Efv. Dogan-

Topal achieved a very low limit of quantitation (LOQ) of just 9 ng/mL using this form of detection. However, the method required a large sample volume of 1 mL serum in order to achieve this sensitivity. Also, Efv had a lengthy retention time of 11.6 mins. Choi achieved an LOQ of 10 ng/mL, again excellent for UV. The sample volume required was low at only 0.2 mL plasma but the method suffered from an extremely long retention time of 23.7 mins for Efv. Rezk also achieved an LOQ of 10 ng/mL for Efv. Again, sample volume was high at 0.55 mL plasma and the retention time of Efv long at 24.9 mins. Veldkamp *et al.*, 1999, reported an LOQ of 10 ng/mL with a relatively small sample volume of 0.25 mL plasma and a retention time of 10.2 mins for Efv. Marzolini *et al.*, 2000, Proust *et al.*, 2000, Aymard *et al.*, 2000 and Notari *et al.*, 2006, each used UV for the detection of Efv. However, large sample volumes and long elution times for Efv were required in all cases. Notari achieved an LOQ of 100 ng/mL, however, 0.6 mL plasma was required and the resultant retention time for Efv was 28.4 mins. Aymard required 1 mL plasma for the extraction of Efv and a retention time of 15.2 mins resulted for Efv with an LOQ of 50 ng/mL. Proust used 0.5 mL plasma and gave a retention time of 28.6 mins but achieved an LOQ of 75 ng/mL. Marzoloni required 0.6 mL plasma which achieved a LOQ of 250 ng/mL but a retention time of 28.7 mins resulted. Dailly *et al.*, 2004, suffered from similar problems. Even though an LOQ of 100 ng/mL was obtained, a 1 mL plasma volume was required and a retention time of 16.1 mins was needed to elute Efv. Lakshmi Sailaja *et al.*, 2007, achieved a fast retention time of 6.2 mins for Efv and a reasonable LOQ of 200 ng/mL but the major disadvantage of this method was that it needed 1 mL plasma to achieve these results.

A 50 ng/mL LOQ was obtained by both Weller *et al.*, 2007, and Turner *et al.*, 2003, using C8 columns. Both required sample volumes of only 0.2 mL plasma however the retention for Efv was relatively long at 15 mins for Weller and 24.2 mins for Turner.

Ramachandran *et al.*, 2006, Kappelhoff *et al.*, 2003, and Veldkamp *et al.*, 1999, were quite successful in their determination of Efv using UV. Ramachandran achieved an LOQ of 50 ng/mL which required only 0.25 mL plasma and retention time was fast at 6.2 mins for Efv. Kappelhoff also achieved an LOQ of 50 ng/mL, required a smaller sample volume of just 0.1

mL and a short retention time of 7.8 mins. Veldkamp achieved an LOQ of just 10 ng/mL using just 0.25 mL plasma and Efv eluted at 10.2 mins which is quite a good result. Mogatle *et al.*, 2009, and Sarasa-Nacenta *et al.*, 2001, both had reasonably short runtimes and small sample volumes. Mogatle required only 0.1 mL sample volume and achieved an LOQ of 200 ng/mL with a retention time of 8 mins for Efv. Sarasa-Nacenta achieved a retention time of 6.9 mins for Efv using 0.3 mL plasma which resulted in an LOQ of 100 ng/mL.

D'Avolio *et al.*, 2010, describes the most recently used MS method for the detection of Efv using a C18 column. The method can detect Efv down to 31 ng/mL but Efv took 16.9 mins to elute. Martin *et al.*, 2009, described a MS method in which Efv was separated using a phenyl column which resulted in an extremely short retention time of just 1.9 mins but the method lacked sensitivity with an LOQ of just 100 ng/mL. Rentsch *et al.*, 2003, obtained an LOQ of 10 ng/mL using MS but again Efv had a high retention time of 16.3 mins using a C18 column. Volosov *et al.*, 2002, use MS for Efv's detection and achieved an LOQ of 10 ng/mL with a short runtime of just 4.5 mins.

Matthews *et al.*, 2002, developed a HPLC method with post-column photochemical derivatisation and fluorescence detection for the determination of Efv and its enantiomer in plasma. Once the drugs had been extracted from the plasma matrix they were separated on a YMC-Pack C8 analytical column. The LOQ achieved was 50 ng/mL; however, 0.5 mL plasma was required.

2.2.6.2 Sample separation and detection approaches for rifampicin

For the determination of Rif, UV is again the predominant method used in the literature. Some of the methods suffer from poor sensitivity including Gikas *et al.*, 2010, Fernández-Torres *et al.*, 2008, and Panchagnula *et al.*, 1999. Panchagnula used a mobile phase composed of sodium phosphate and methanol which, at a flow rate of 1 mL/min had an overall run time of 17 mins and eluted Rif at 6 mins. The resultant LOQ was 2 µg/mL which was poor but the method only required 0.1 mL plasma for the extraction of Rif. Fernández-Torrres achieved an LOQ of 1.46 µg/mL which again was not very low, but sample volume was low at only 50 µL. Rif eluted at 7.9 mins. Gikas reported the most recent UV method in which µHPLC was used for Rif's determination.

As expected, the elution time for Rif was fast at 2.1 mins, however, the sensitivity was poor at 2 μg/mL. On the plus side, only 25 μL rabbit plasma was required.

Some of the reported UV methods have good sensitivity but suffer from a long runtime and/or large sample volumes. Balbão *et al.*, 2010, using 0.2 mL plasma achieved an LOQ of 125 ng/mL with Rif eluting at 11 mins. Allanson *et al.*, 2007, achieved an LOQ of 500 ng/mL but the method required 0.45 mL plasma. The method was carried out at ambient temperature using a mobile phase of ammonium acetate and acetonitrile delivered at 0.7 mL/min on a gradient programme over 18 mins with Rif eluting at 10.9 mins. Unsalan *et al.,* 2003, achieved an LOQ of 700 ng/mL with the method only requiring 0.1 mL plasma. Gradient elution was achieved by using potassium dihydrogen phosphate and acetonitrile. A flow rate of 0.8 mL/min was used. Overall runtime was 20 mins with Rif eluting at 10.2 mins. Calleja *et al.*, 2004, achieved separation with a mobile phase composed of water and acetonitrile run at a flow-rate of 1 mL/min. LOQ from plasma was good at 0.05 μg /mL and Rif eluted at 4 mins.

Hartkoorn *et al.*, 2007, Song *et al.*, 2007, and de Velde *et al.*, 2009, developed LC-MS methods for the determination of Rif. Sample volumes were low, retention times fast, but good sensitivity was difficult to achieve. Hartkoorn achieved separation on a Betasil Phenyl-Hexyl column using an isocratic mobile phase composed of ammonium acetate and acetonitrile at a flow rate of 0.4 mL/min. Mass spectral analysis for Rif was carried out using electrospray ionisation (ESI) in the positive ion mode with a capillary temperature of 250 °C. The LOQ was found to be 100 ng/mL using only 50 uL plasma. Song developed a HPLC/tandem MS method for the determination of first-line anti-TB drugs (including Rif) and their major metabolites. Samples were analysed using a Hydrosphere C18 column. The mobile phase was a gradient of a mixture of methanol and water. Chromatographic separation was performed at room temperature for 4 min. Using a Quattro Micro tandem mass spectrometer, quantification was achieved by multiple reaction monitoring (MRM) in positive ion mode. The LOQ obtained was 50 ng/mL with again only 50 μL serum required. DeVelde required only 10 μL plasma for the

extraction of Rif, however an LOQ of 200 ng/mL was all that could be achieved.

2.2.6.3 Sample separation and detection approaches for efavirenz and rifampicin simultaneously

Because both Efv and Rif are used together in HIV/TB applications, it makes sense to simultaneously quantitate both drugs. Boffito *et al.*, 2002, developed an LC-UV method for the simultaneous determination of Rif and Efv. Separation was achieved on a reversed phase Luna 5 μm column. The mobile phase consisted of sodium phosphate buffer, acetonitrile and methanol run at a flowrate of 1.0 mL/min using a gradient. The UV detector was operated at a wavelength of 254 nm. This one reported method for the simultaneous determination of Efv and Rif exhibits good sensitivity (LOD 0.05 μg/mL) but suffers from a relatively long analysis time (15 mins). See Table 2.3. Boffito, however, did not measure Rif's main metabolite, dRif in the assay they developed. It is important to measure Rif as well as dRif because dRif has an antimycobacterial activity closely similar to Rif (Hemanth Kumar *et al.*, 2004). A method capable of quantifying the metabolism of Rif into its main metabolite dRif in the presence of Efv was required. Developing such a method was both novel and analytically important.

Rif (and its metabolite, dRif) and Efv exhibit very different physiochemical properties, and so developing a simple, low cost assay to detect all three within a clinically relevant concentration range using standard chromatography equipment proved extremely challenging. Choice of column, mobile phase composition and pH, along with choice of detection and sample extraction technique proved vital to the overall success of the assay.

2.3 Experimental

2.3.1 Reagents and materials

HPLC grade acetonitrile (ACN), methanol (MeOH) and water were purchased from Sigma-Aldrich. Ammonium acetate, formic acid and acetic acid, all

analytical grade, were obtained from Sigma-Aldrich, United Kingdom. Drug-free sterile filtered human plasma (EDTA k3) was purchased from Europa Bioproducts, United Kingdom. Efv and Rif and Rif's metabolite, dRif were purchased from Sequoia Research Products Ltd. United Kingdom. Strata X (33 µ) SPE cartridges were from Phenomenex, Ireland and Supelco Discovery SPE cartridges (Phenyl, C18, 18LT, C8, and CN) were from Sigma Aldrich, United Kingdom. Extraction cartridges and plates (SPE, LLE and PP) were purchased from Biotage, Sweden. Analytical columns came from Agilent.

2.3.2 Equipment and HPLC assay conditions

The HPLC system consisted of a 1050 series quaternary pump, 1100 series autosampler, a diode array detector (DAD) and a 1200 series degasser, all from Agilent, United Kingdom. System management and data acquisition were performed by the Agilent Chemstation for LC 3D software. Other instrumentation employed included a pH meter and electronic mass balance both from a Mettler Toledo, USA and a Genevac EZ-2 series personal evaporator from Ipswich, United Kingdom. The VacMaster-96 sample processing manifold was sourced from Biotage, Sweden.

Analytical columns used were Agilent Zorbax SB-C18 (150 x 4.6 mm, 5 µm), Varian Pursuit C18 (150 x 4.6 mm, 5 µm), Varian Polaris C18 (150 x 4.6 mm, 5 µm) from Agilent, United Kingdom, and Agilent Zorbax SB Phenyl, (150 x 4.6 mm, 5 µm) from Phenomenex, United Kingdom. The HPLC mobile phase consisted of ammonium acetate (pH 4.75, adjusted with 1 M acetic acid, 20 mM), ACN and MeOH (40:45:15, v/v/v). A diode array UV detector was employed which monitored Efv at 246 nm and Rif and dRif at 334 nm. The overall runtime was 10 min and the flow rate was 0.8 mL/min at ambient temperature.

2.3.3 Preparation of standards

Stock standard solutions of Efv, Rif and dRif were prepared by weighing out the powders in a glovebox, dissolving them in ACN to 1 mg/mL concentration and storing them in amber vials at 2-4 °C in the dark. Working standards of

Efv, Rif and dRif were prepared both in drug-free human plasma and in mobile phase (ammonium acetate (pH 4.75, adjusted with 1 M acetic acid, 20 mM), ACN and MeOH (40:45:15, v/v/v)) to concentrations over the range 0.1–20 µg/mL (0.1, 0.5, 1, 5, 10 and 20 µg/mL in plasma which corresponded to 0.2, 1, 2, 10, 20 and 40 µg/mL in MP) . This range of the standard curve was chosen to reflect the plasma concentrations expected in a typical 12 hr pharmacokinetic profile post administration of Rif: 0-10 µg/mL (Hartkoorn *et al.*, 2007, Yau Yi Lau *et al.*, 1996) Efv: 0-20 µg/mL (Ramachandran *et al.*, 2006, Mogatle *et al.*, 2009) and both (Boffito *et al.*, 2002).

A 0.3 mL aliquot of each of the working standards was diluted 1:1 (v:v) with a 1% formic acid solution in water. The SPE Evolute Array Wells (25 mg ABN, 1 mL) in the 96 well plate sample processing manifold were conditioned with 1 mL MeOH and then equilibrated with 1 mL of the 0.1% formic acid solution. A 0.6 mL aliquot of the diluted, acidified sample was loaded onto the well, washed with 20% MeOH in water and eluted with a water (1 mg/mL ascorbic acid)-ACN-MeOH mixture (25:50:25, v/v/v). The samples were evaporated to dryness and reconstituted with 150 µL HPLC mobile phase (ammonium acetate (pH 4.75, adjusted with 1 M acetic acid, 20 mM), ACN and MeOH (40:45:15, v/v/v)). The overall clean-up process resulted in the diluted plasma sample being concentrated by a factor of four which equated to an overall enrichment factor of two for the neat plasma. The reconstituted sample was then transferred to an autosampler vial (with insert) and 20 µL was injected into the HPLC system for analysis.

2.3.4 Preparation of patient samples

Blood samples from eleven patients participating in the SPhEAR project were obtained by informed consent in the Infectious Disease Department at the Mater Hospital, Dublin, Ireland. The methodology of this study was approved by the Ethics committee of the Mater Misericordiae Hospital. Bloods samples were taken after at least three weeks on therapy to ensure steady state. Patients were administered a 600 mg dose of Rif. Samples were drawn at 8 different time points over 10 hours. The first sample was taken fasting with a set meal provided for all study participants. Blood samples were taken in EDTA

preservative which were then centrifuged in a chilled centrifuge and the plasma kept frozen at -80 ºC until analysis. A 0.3 mL aliquot of each patient sample was extracted using the same procedure described above for the working standards. Samples were taken and prepared in duplicate.

2.3.5 Method validation

The fundamental parameters for a bioanalytical method validation are accuracy, precision, selectivity, sensitivity and stability. Measurements for each analyte in the biological matrix were validated according to FDA guidelines (FDA, 2001).

2.4 Results and discussion

2.4.1 Method development

Taking a methodical approach to method development is important. Knowledge of the chemistry, structure and solubility of the drugs prior to choosing the column, mobile phase and monitoring wavelength was essential. The choice of the sample preparation step is also imperative to the success of the protocol. It was vital that any methodology developed was better than the current state-of-the-art, had novelty, context and application.

Assay development for this project was challenging due to the very different physiochemical natures of the two drugs (affecting hydrophobicity, solubility, acidity) and so designing one fast on-line assay for both drugs was a difficult task.

2.4.2 Chromatographic procedure

The heart of a HPLC system is the column. Choosing the right column for this assay required consideration of stationary phase chemistry, retention capacity, particle size, and column dimensions. Identifying the best stationary phase for the separation was a critical step of column selection, and was based on the

general chemical principle that "likes dissolves like." The choice of column was challenging due to the widely different polarities of Rif and Efv. A separation mechanism employing differences in the chemical structures of these analytes was required. As discussed in Chapter 1, in reversed phase chromatography, the stationary phase is non-polar and the mobile phase is polar. Analytes are attracted to the surface by their non-polar functional groups. The most polar analyte elutes from the reversed phase column first followed by other analytes in order of decreasing polarity.

Reversed phase columns, both octadecylsilane (C18) and phenyl, were investigated as they work well for water-soluble hydrophobic compounds, see Figure 2.2.

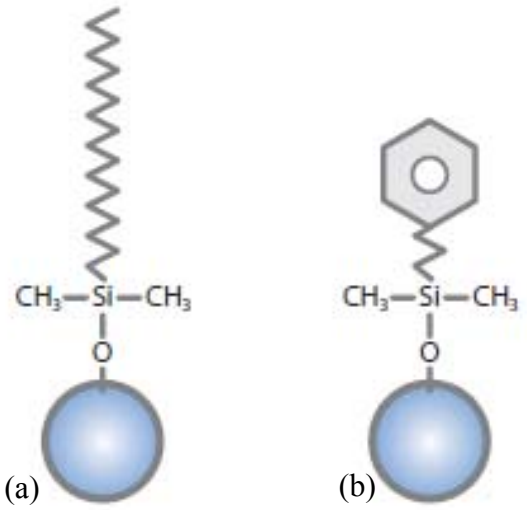


Figure 2.2: Reversed phase stationary phases (a) C18 and (b) phenyl

Alkyl phases such as C18 and C8 are best suited for analysing neutral compounds with a high ratio of carbon:heteroatoms where the major distinction among analytes is their hydrophobicity. C18 is the most popular reversed phase in HPLC and so was the stationary phase we chose to primarily investigate.

An Agilent Zorbax SB-C18 (150 x 4.6 mm, 5 μm) column resulted in poor peak shape for Efv, even under different pH conditions, see Figure 2.3, so it was deemed unsuitable for the analysis. See Table 2.4.

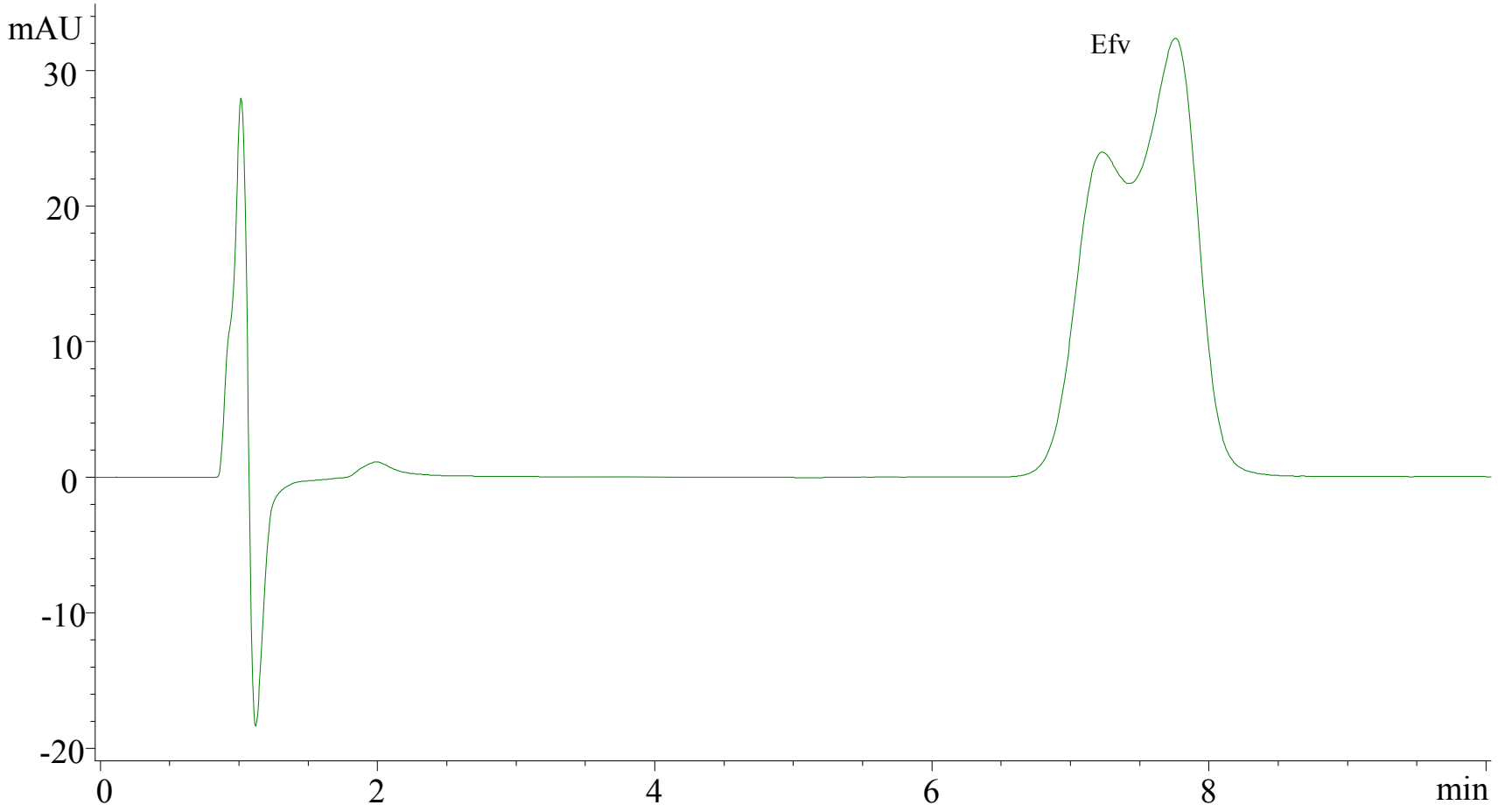


Figure 2.3: Chromatogram of a working standard of Efv using an Agilent Zorbax SB-C18 column

pH	Rif peak area	Efv peak area
3	Negligible	591
4	254	603
5	272	598

Table 2.4: Comparison of different mobile phase pH values for Rif and Efv using an Agilent Zorbax SB-C18 column

Both Varian Pursuit C18 (150 x 4.6 mm, 5 μm) and Varian Polaris C18 (150 x 4.6 mm, 5 μm) columns were also trialled. Better results were achieved with the Varian Polaris C18 column over the Varian Pursuit C18 column. Figure 2.4 shows a chromatogram of a working standard of Rif and Efv using the Varian Pursuit C18 column. Good separation was achieved but at the cost of a lengthy runtime. Again different pH conditions were tested but they yielded no improvement in peak shape or area counts. See Table 2.5.

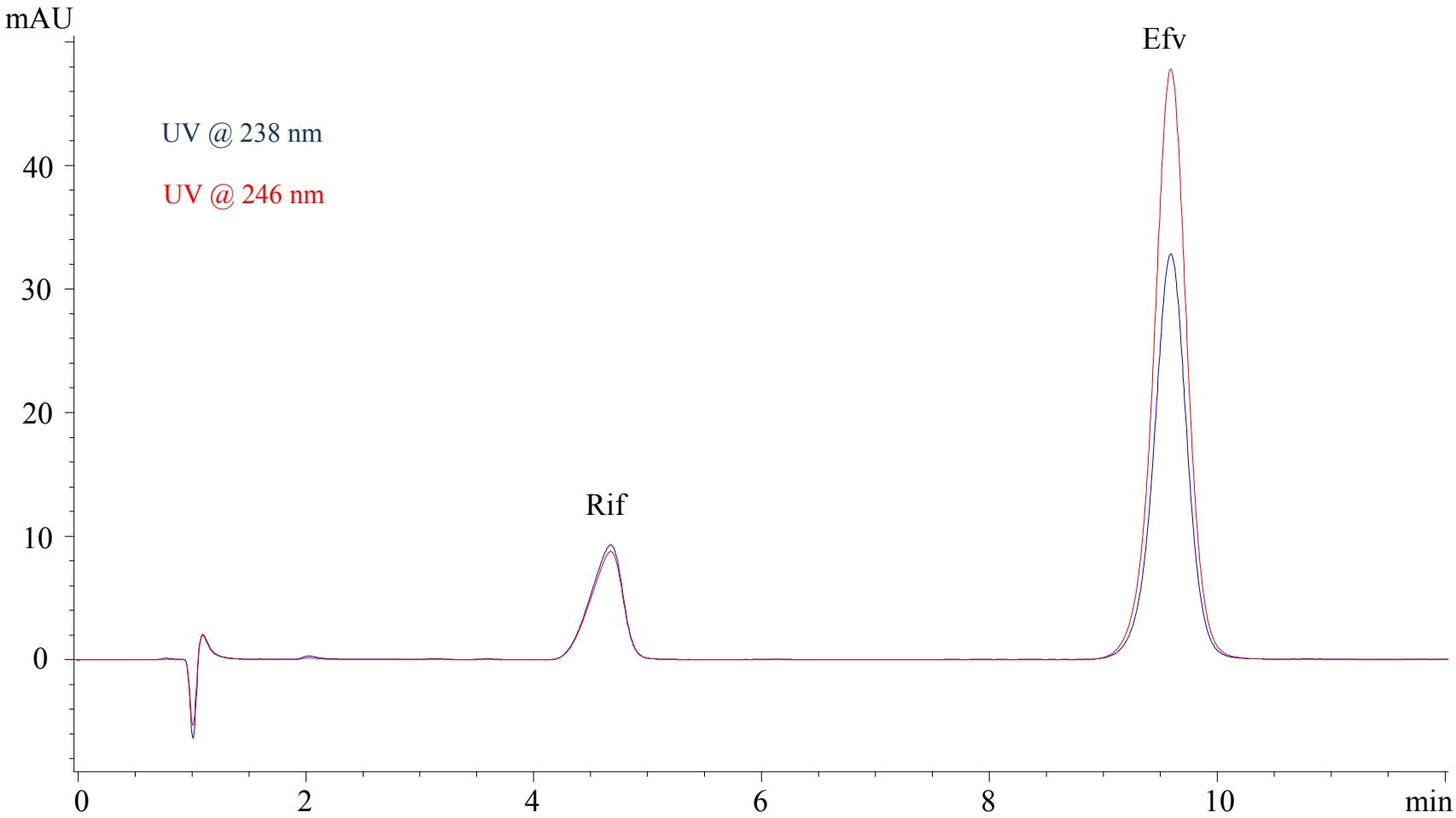


Figure 2.4: Chromatogram of a working standard of Rif and Efv using a Varian pursuit column

pH	Rif peak area	Efv peak area
3	691	1317
4	655	1519
5	714	1509

Table 2.5: Comparison of different mobile phase pH's for Rif and Efv using Varian Polaris C18 Column

The three C18 columns were found to be unsuitable primarily due to the large difference in elution time between Efv and Rif. While C18 columns are effective for nonpolar analytes, these phases often do not provide the desired selectivity for other compounds. In such cases, phenyl columns are the primary alternative to alkyl phases and so this column chemistry was also investigated. Phenyl columns possess useful selectivity for compounds containing phenyl type moieties and so it was postulated that these might be a good alternative to C18 for Rif and Efv. The unique selectivity for the phenyl phase is derived from an interaction of the pi electrons found in the phenyl groups and is therefore useful for the analysis of aromatic-containing compounds.

An Agilent Zorbax SB Phenyl (150 x 4.6 mm, 5 µm) column was found to give the best chromatographic results in terms of good separation and fast runtime. Phenyl columns offer unique selectivity from the alkyl phases and are generally less retentive than the C18 phase. Figure 2.5 shows the separation of both drugs on the Varian Polaris C18 column versus the Agilent Zorbax SB Phenyl, column. It was clear that the phenyl column offered a more efficient separation with less lag time between both peaks.

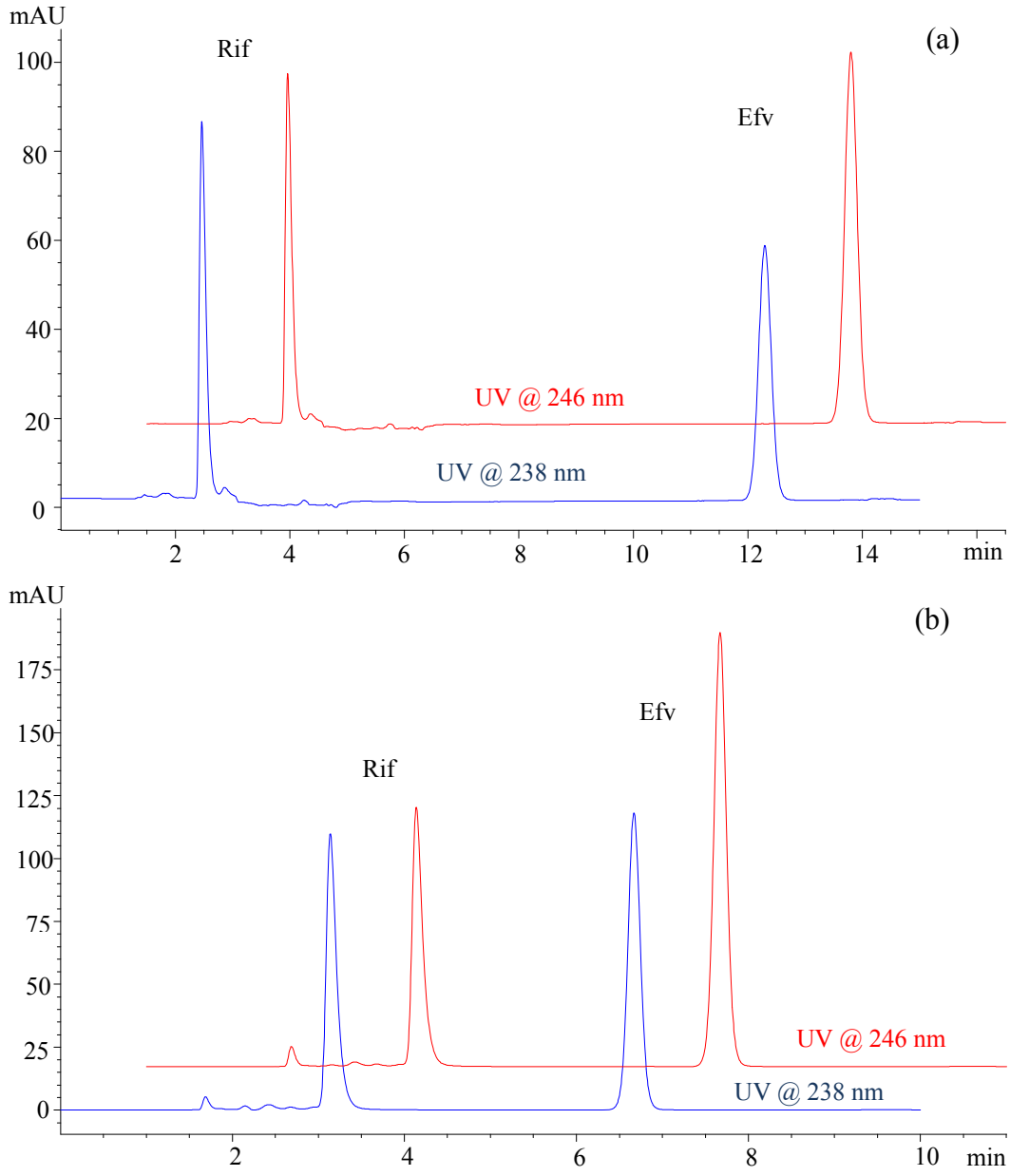


Figure 2.5: Chromatogram of a working standard of Efv and Rif prepared to 10 µg/mL in mobile phase comparing (a) the Varian Polaris C18 column and (b) the Agilent Zorbax SB Phenyl column

Though choice of column has the greatest effect on resolution, mobile phase also effects selectivity and efficiency and is the aspect of chromatography over which analysts have the most control. In reverse phase chromatography, mobile phase consists of an aqueous buffer and a non-UV active water miscible organic solvent. The effect of the organic and aqueous phase and the proportions in which they are mixed are discussed in this section. Common organic solvents in reverse phase chromatography are methanol and acetonitrile. The selection of mobile phase composition and pH in this work required extensive optimisation due to the sensitivity of Rif in particular to small changes in percentage of organic modifier and pH of the mobile phase. As the degree of solubility of the components varies independently in different solvents, the choice of organic solvent can affect selectivity and therefore resolution. Acetonitrile is a highly polar aprotic solvent, providing adequate resolution for many compounds. Due to its ability to form hydrogen bonds, the use of methanol as the organic phase can provide significantly different selectivity. Selectivity is also greatly affected by amount of aqueous solution in the mobile phase, with higher percentages of aqueous phase leading to increased retention and frequently to improved selectivity. It was found necessary to use a ternary mixture of mobile phase containing water and the two organic solvents - ACN and MeOH in order to achieve a fast runtime and the sensitivity required. Figure 2.6 shows some of the combinations of water, acetonitrile and methanol used. Ascorbic acid was required for the assay and its addition is discussed in more detail in section 2.4.4.

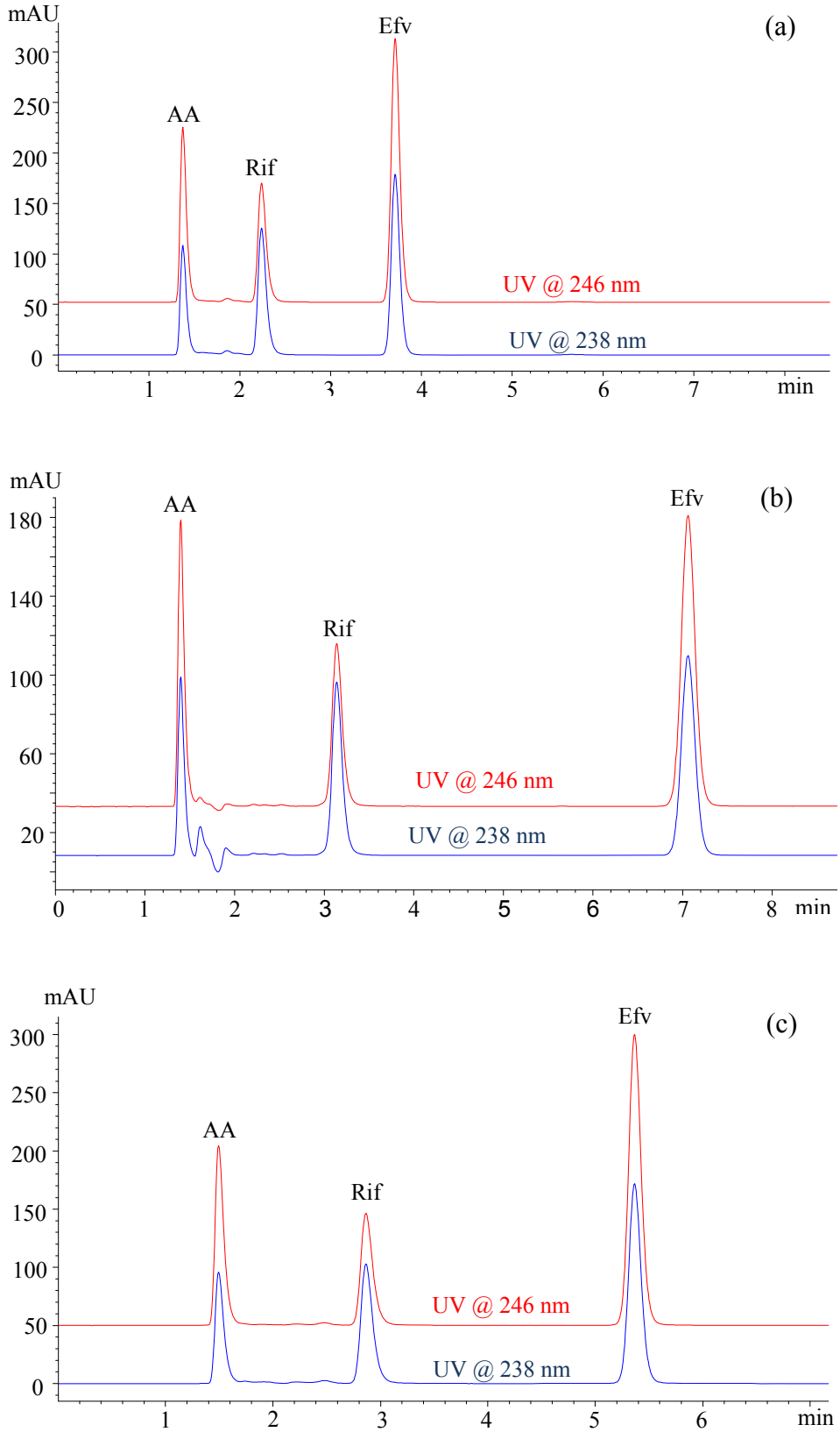


Figure 2.6: Chromatograms of a 10 µg/mL standard of ascorbic acid (AA), Efv and Rif prepared in mobile phase using various mobile phase compositions of H_2O-ACN-MeOH (a) 35:50:20, v/v/v (b) 45:40:15, v/v/v and c) 40:45:15, v/v/v.

In Table 2.6, a comparison of mobile phases investigated are shown using ammonium acetate (0.02 M) at ph 4.5 (using acetic acid). The flowrate was 1 mL/min. The optimal mixture was found to be H_2O-ACN-MeOH; 40:45:15, v/v/v, as shown in Figure 2.6 (c). Although, at first glance, Figure 2.6 (a) appears to be the best (fastest) set of conditions, the endogenous plasma components coeluted with Rif so it was necessary to move the Rif peak away from the solvent front in order to accommodate these early-eluting peaks.

Mobile phase composition (H_2O-ACN-MeOH)	**Rif**		**Efv**	
	Retention time (min)	**Peak area**	**Retention time (min)**	**Peak area**
35:50:15	2.2	800	3.9	1700
40:45:15	2.8	816	5.3	2060
40:35:25	3.0	708	5.2	1626
45:40:15	3.1	721	7.0	1624

Table 2.6: Comparison of different mobile phase compositions using ammonium acetate as buffer

Both pH and ionic strength of the aqueous portion of mobile phases are important parameters in developing analytical methods. The pH of the mobile phase can have an important role on the retention of an analyte and can change the selectivity of certain analytes. Method development proceeded by investigating chromatographic separations firstly at low pH and then at higher pH until optimum results were achieved. A thorough pH study revealed that the water component of the mobile phase should be 20 mM ammonium acetate, adjusted to pH 4.75 with acetic acid for optimal analyte response. See Table 2.7.

Mobile phase pH	Rif		Efv	
	Retention time (min)	Peak area	Retention time (min)	Peak area
4.5	2.9	816	5.5	1549
4.75	2.7	879	5.3	1775
5.0	2.5	785	4.8	1668
5.5	2.8	816	5.3	1860

Table 2.7: Comparison of different mobile phase pH values using ammonium acetate as buffer

Different strengths of ammonium acetate were also looked at using pH 4.75. Best results were achieved using 0.02 M. See Table 2.8.

Ammonium acetate conc. (M)	Rif		Efv	
	Retention time (min)	Peak area	Retention time (min)	Peak area
0.01	2.5	676	5.0	1540
0.02	2.8	816	5.3	2060
0.04	2.6	724	4.9	1362
0.1	2.7	239	5.0	326

Table 2.8: Comparison of different ammonium acetate concentrations

Ammonium phosphate was also investigated using different mobile phase compositions (H_2O-ACN-MeOH) to see if signal could be improved. See Table 2.9.

MP composition (H_2O-ACN-MeOH) using 0.01 M ammonium phosphate	Rif		Efv	
	Retention time (min)	Peak area	Retention time (min)	Peak area
30:55:15	2.0	795	2.9	1646
40:45:15	2.4	850	4.8	1685
35:40:25	2.5	826	4.1	1683

Table 2.9: Comparison of different mobile phase compositions using ammonium phosphate as buffer

From the results it can be seen the peak area was slightly higher for Rif but significantly lower for Efv. It was decided best to use ammonium acetate as buffer mainly due to ease of transfer to a MS method at a later stage. Flowrate was changed from 1 mL/min to 0.8 mL/min which gave higher peak area values for both drugs while increasing the retention times slightly. See Table 2.10.

Flowrate (mL/min)	Rif		Efv	
	Retention time (min)	Peak area	Retention time (min)	Peak area
1.0	2.7	879	5.3	1775
0.8	3.1	970	6.1	1934

Table 2.10: Comparison of different flowrates on Rif and Efv

2.4.3 UV detection

As the circulating plasma concentrations of Rif and Efv, when co-administered to patients for the treatment of both HIV and TB infection, are high (1-20 μg/mL) it was decided adoption of UV detection was likely to be sufficient as these levels are well within the range that UV can detect. The UV detector is also both simple to use and cost effective (employing low price equipment which is cheap to maintain and run) (Hartkoorn *et al.*, 2007, Balbão *et al.*, 2010, Ramachandran *et al.*, 2006, Mogatle *et al.*, 2009, Boffito *et al.*, 2002). Hence it is anticipated that adoption of such methodology in areas where there are high incidences of HIV infections, but limited laboratory budgets, such as sub-Saharan Africa, Eastern Europe and Asia would be more easily achieved.

The λ max wavelength for Efv was 246 nm and for both Rif and dRif, 238 nm and 334 nm gave highest absorption. See Figure 2.7. Under the final chromatographic conditions, the drugs were well separated with retention times for Efv, Rif and dRif of 6.1, 3.1 and 2.7 mins respectively. See Figure 2.8.

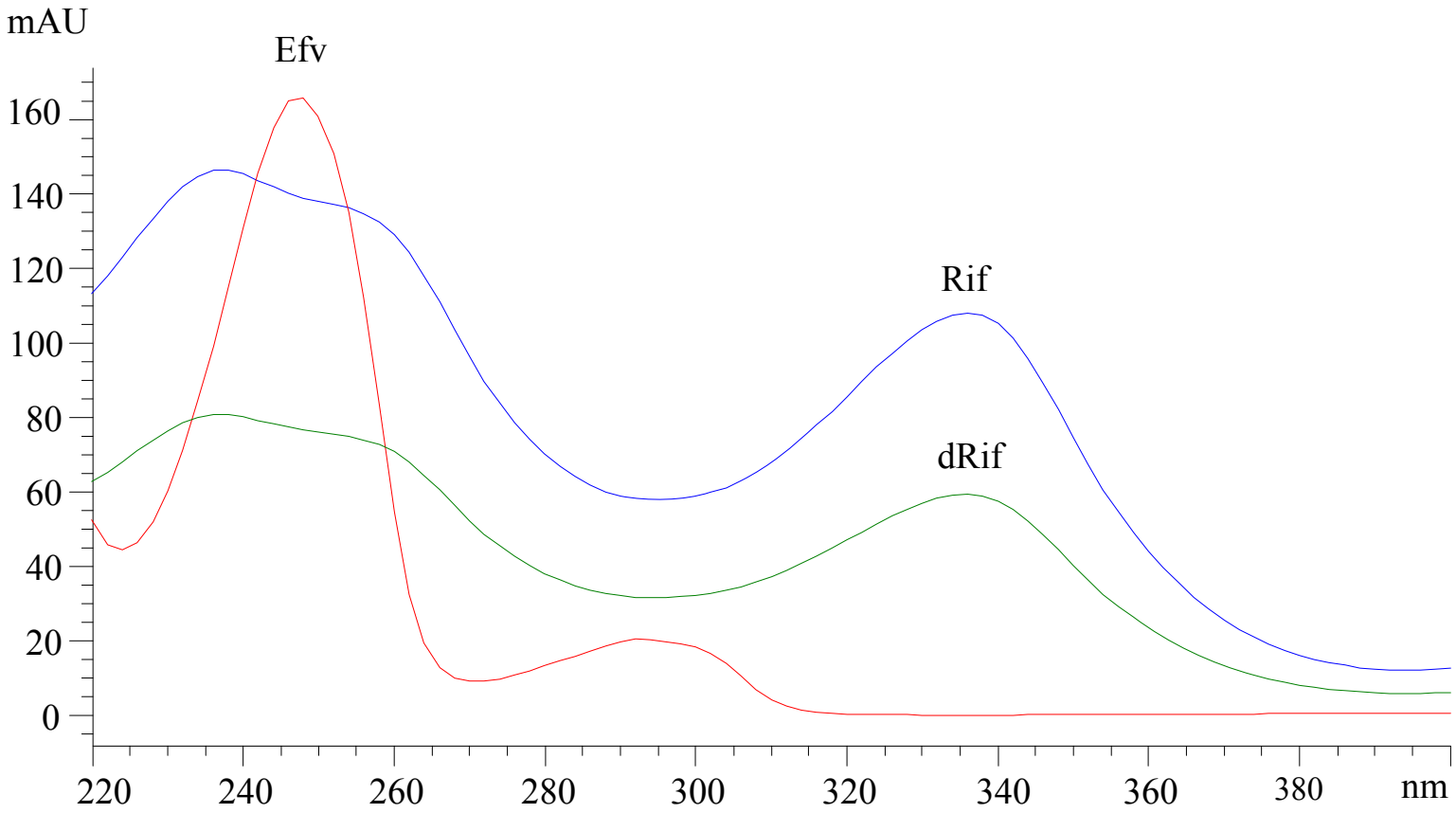


Figure 2.7: UV absorption spectra for Efv, Rif and dRif

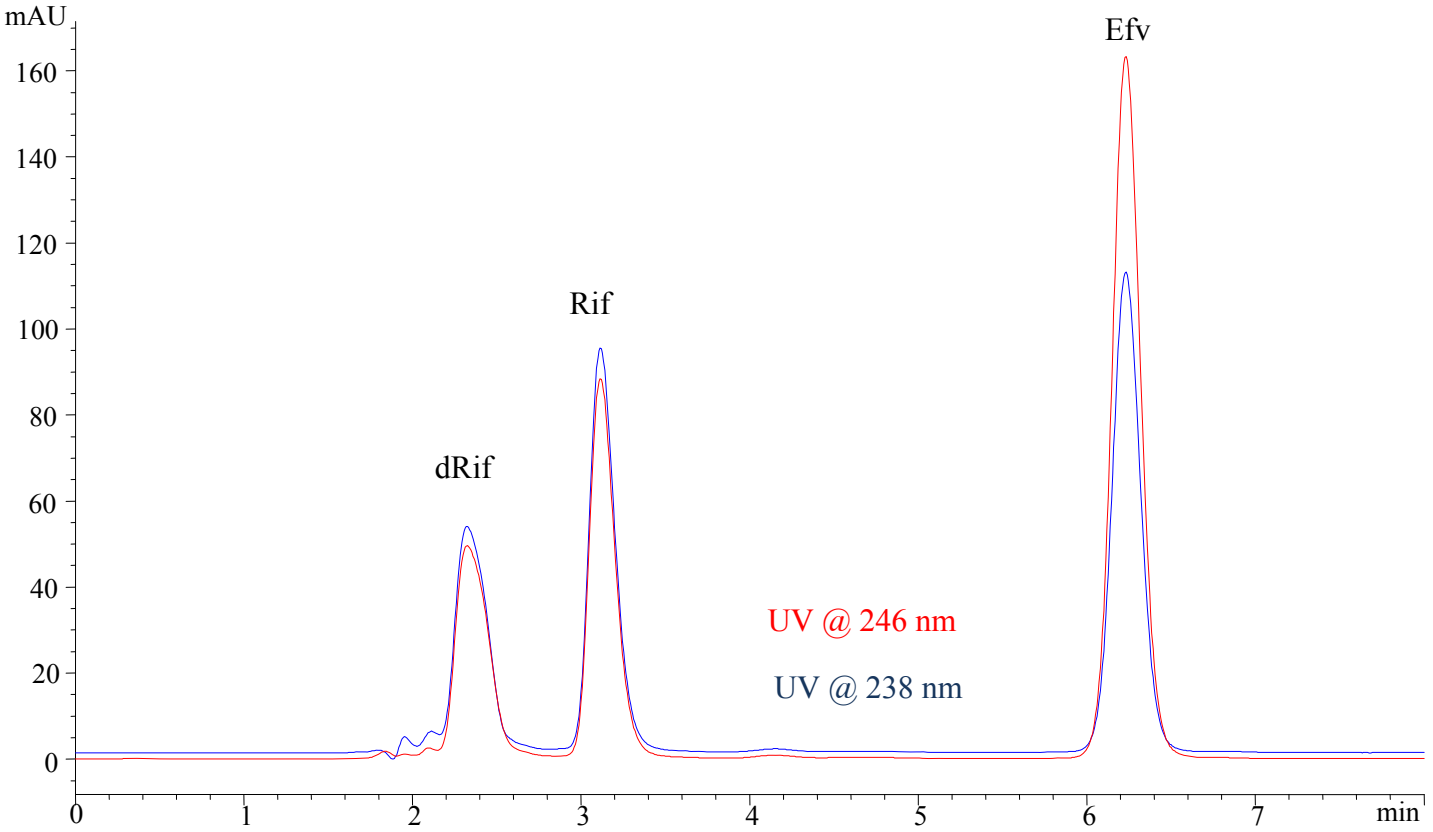


Figure 2.8: Chromatogram of a working standard of Efv and Rif prepared to 10 µg/mL in mobile phase under the final chromatographic conditions

334 nm was chosen as the monitoring wavelength for Rif and dRif as it is in a region of the UV spectrum that is typically more selective (nucleic acids and proteins absorb strongly up to 280 nm (Lam, 2004)) and also at 334 nm it is only slightly less sensitive than the maximum at 238 nm. See Figure 2.9.

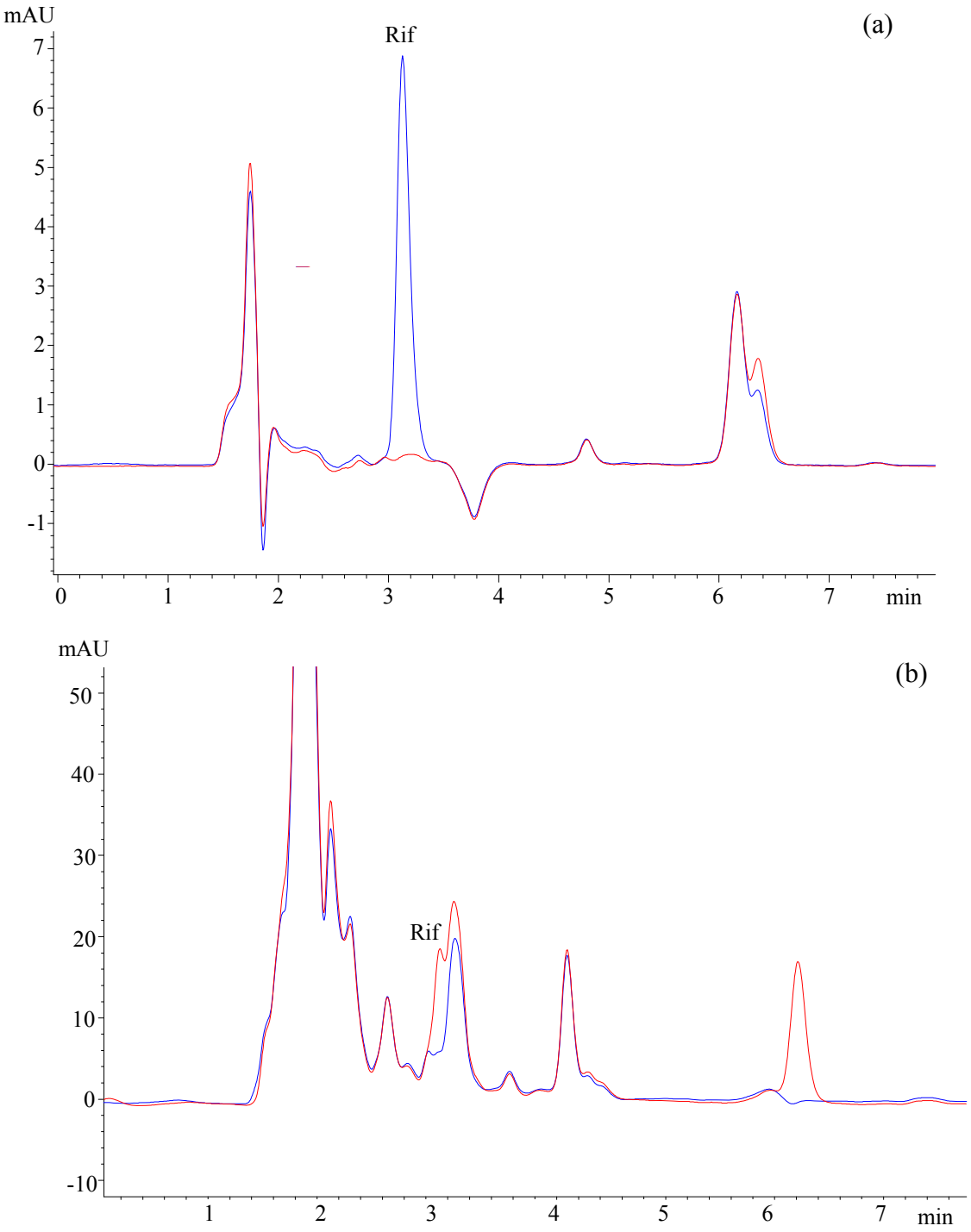


Figure 2.9: Comparison of a 1 µg/mL Rif spiked plasma standard monitored at (a) 334 nm and (b) 238 nm

For quantitation of analytes in biological samples, a usual procedure is to add an internal standard in the calibration samples and real samples. In this work, it was found that the results were sufficiently accurate and robust without an internal standard.

In addition, any routinely available reagent which could act as an internal standard could not be identified and given the aim of having a reasonably uncomplicated broadly applicable analytical method (e.g. one which could be employed with limited infrastructure) the addition of an internal standard would increase the complexity of the study. This is in line with previous published attempts.

2.4.4 Extraction procedure

In today's bioanalytical laboratory, reliable, high throughput sample preparation techniques are essential. For this work, sample preparation was ultimately achieved using the VacMaster-96 sample processing manifold from Biotage. The manifold can support SPE, LLE and PP clean-up of samples in cartridges and/or well plates. All three extraction approaches were investigated in order to find one that could be optimised for extraction of both Efv and Rif quickly and efficiently. The three sample extraction methods examined were:

- supported liquid membrane extraction (SLE), a scaled-down version of liquid liquid extraction (LLE)
- protein precipitation (PP)
- solid phase extraction (SPE)

The SLE plates claim to provide an easier alternative to LLE, with no off-line steps (e.g. mixing or centrifuging) required. Problems including emulsion formation and separation of liquid layers tend to be eliminated. Another big advantage is that the volumes of solvent required are very small. SLE uses columns or 96 well plates packed with an inert support material (a modified form of diatomaceous earth). When the aqueous sample is applied, it spreads over the surface of the support and is absorbed. The analytes remain on the surface of the support which forms the phase interface for the extraction.

When the organic solvent is applied, analytes are efficiently desorbed and the extract is collected. Various extraction solvents were evaluated along with different plasma load conditions. Best results in terms of recoveries were obtained when plasma was loaded 1:1 with ammonium hydroxide and extracted using DCM. See Table 2.11 for all SLE results.

Extraction solvent		% Recovery ± % RSD			
		1:1; plasma: 1% formic acid	1:1; plasma: 0.1% formic acid	1:1; plasma: Water	1:1; plasma: ammonium hydroxide
***t*-BME**	Rif:	<10	41.4 ± 6.0	<10	<10
	Efv:	29.6 ± 8.6	30.6 ± 7.1	26.1 ± 5.6	22.4 ± 5.1
DCM	Rif:	15.9 ± 4.0	62.7 ± 1.9	48.8 ± 4.1	64.1 ± 4.3
	Efv:	41.3 ± 3.6	72.4 ± 2.8	80.0 ± 2.9	81.4 ± 6.2
Ethyl acetate-Hexane (80:20, v/v)	Rif:	<10	80.0 ± 3.4	49.9 ± 2.9	34.8 ± 4.1
	Efv:	49.9 ± 0.9	64.8 ± 1.2	48.9 ± 1.6	49.9 ± 1.5
Ethyl acetate	Rif:	<10	60.0 ± 2.7	34.4 ± 9.5	<10
	Efv:	45.3 ± 2.1	53.4 ± 1.8	80.6 ± 5.1	<10
Hexane	Rif:	<10	<10	<10	<10
	Efv:	32.8 ± 3.0	15.1 ± 8.3	<10	14.8 ± 5.3
DCM-APA (95:5, v/v)	Rif:	<10	<10	40.8 ± 2.0	<10
	Efv:	51.8 ± 5.3	51.0 ± 4.8	42.8 ± 3.2	50.1 ± 1.5

Table 2.11: Comparison of SLE using different load and extraction solvents for a 10 μg/mL standard

PP was carried out in the 96 well format using a 'solvent first' approach. This claims to eliminate the need for time consuming vortex mixing or plate inversion and yield better recoveries when compared with 'plasma first' methods. PP by filtration in the 96 well format is a high throughput alternative to the traditional centrifugation based technique using collection plates. The functionalised bottom frit holds up organic precipitation solvent while plasma is added, maximising contact time and giving the most efficient precipitation effect. No vortex mixing is required. The filtrate does not pass into the collection plate until vacuum is applied.

All three methods gave initial recoveries of greater than 64% for the extraction of both Efv and Rif individually at 10 µg/mL, but the extraction method which gave the best recoveries for both drugs combined was SPE - see Table 2.12. Rif, being highly lipid soluble, is widely distributed in tissues. In circulation, 89% of Rif is bound to plasma proteins. Efv is almost completely protein bound (> 99%), primarily to albumin. Taking this into consideration, the recoveries obtained are very good.

Sample preparation approach	**Recovery (%) ± % RSD**	
	Rif	**Efv**
SLE	64.1 ± 2.0	81.0 ± 2.6
PP	76.3 ± 4.1	73.1 ± 5.7
SPE	65.1 ± 1.1	93.8 ± 0.2

Table 2.12: Comparison of initial sample preparation approaches (n=3)

Phenomenex Strata X 33 µ, polymeric reversed phase 30 mg/1mL and Supelco Discovery SPE, 100 mg/1mL tubes (Phenyl, C18, 18LT, C8, and CN) were also investigated. However, high recoveries were not achieved for the three drugs combined in comparison to those of the Evolute ABN SPE cartridges. The samples were also not as clean and so sensitivity was affected. See Table 2.13 for results.

SPE cartridge	dRif Recovery % ± % RSD	Rif Recovery % ± % RSD	Efv Recovery % ± % RSD
Phenyl	69.1 ± 2.4	52.8 ± 5.5	86.5 ± 0.5
18 LT	0.1 ± 0.0	14.6 ± 28.1	6.0 ± 51.7
C8	0.1 ± 0.0	7.7 ± 40.3	8.9 ± 23.6
CN	71.6 ± 1.5	54.9 ± 9.5	104.6 ± 6.8
Strata X	90.3 ± 5.5	82.1 ± 5.0	3.8 ± 28.9

Table 2.13: Comparison of recoveries obtained for dRif, Rif and Efv using different SPE cartridges (n=2)

Evolute ABN was the SPE cartridge chosen for this assay. The cartridge sorbent consists of a rugged polystyrene-based polymer functionalised with well-defined hydroxyl-functional oligomers which simultaneously give very high water wettability but, through the combination of non-polar (hydrophobic) and polar (hydrophilic) interactions associated with the various functionalities on the column material, allows efficient extraction of analytes of wide ranging polarities. See Figure 2.10.

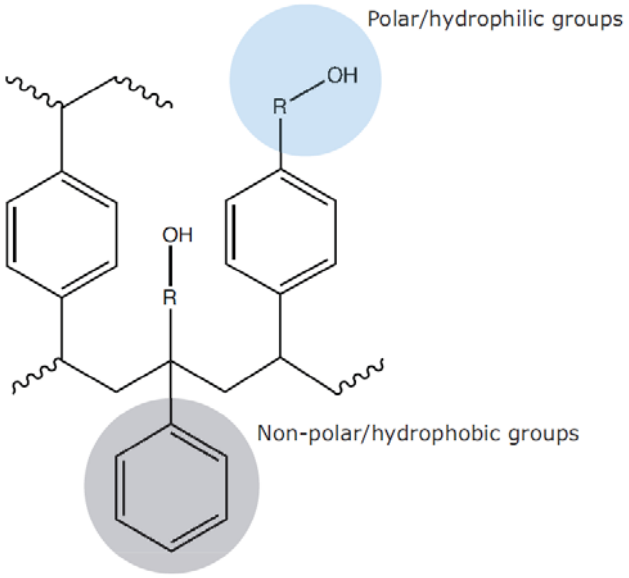


Figure 2.10: Evolute ABN sSorbent

These characteristics result in a versatile sorbent for extraction of acidic, basic and neutral analytes from biological fluids and other aqueous matrices. The stationary phase is packed in a syringe-shaped cartridge or a 96 well plate, which can be mounted on the extraction manifold. Evolute ABN's optimised pore structure minimises the retention of high molecular weight endogenous materials present in complex sample matrices. Efv has a molecular weight of 316 g/mol (D'Avolio *et al.*, 2010) and a pKa of 10.2. Rif has a molecular weight of 823 g/mol and an acidic pKa of 1.7 and a basic pKa of 7.9 (Balbão *et al.*, 2010), it is also very sensitive to light and pH (MSDS Rifampicin). Hence, it was postulated that Efv, Rif and dRif – even with their different physiochemical properties - could be extracted by this one sorbent. This was borne out experimentally. There are six basic steps in SPE which include sample pretreatment, sorbent conditioning, sorbent equilibration, sample loading, washing, and analyte elution. During the SPE method development each of these steps were investigated.

As many SPE sorbents are not water-wettable, analytes applied in an aqueous sample will not partition onto the sorbent, due to lack of a proper phase interface between the dry sorbent surface and the sample. Conditioning with a water-miscible organic solvent, methanol for this work, wets the sorbent surface and allows the analytes to break through the surface tension of the water and move into the sorbent.

Prior to loading the aqueous sample, the conditioning solvent needed to be displaced from the sorbent using an equilibration solvent. The equilibration solvent also prepared the sorbent surface to receive the sample. In order that the chemistry of the sorbent remained constant throughout the sample loading step, the equilibration solvent was similar to the composition of the sample. The sorbent was equilibrated with the same buffer as used to dilute the sample.

Sample pretreatment involved sample dilution and pH adjustment. Dilution reduces the viscosity of the aqueous sample, improving chromatographic mass transfer, and thus improving the efficiency of the extraction. In this work, the plasma sample was diluted 1:1 with an aqueous buffer containing formic acid. A comparison of spiked plasma loaded 1:1 with 0.1% formic acid, 0.01% formic acid and water was evaluated. 1% and 0.1% formic acid showed no significant difference between the recoveries observed

for dRif and Rif. Loading of samples diluted with water showed a slight decrease in the recoveries for both drugs. See Figure 2.11. When Efv was loaded with water a cleaner baseline was observed but at the cost of recovery. See Figure 2.12. A consideration during the sample load step was the speed with which the sample was applied. Care had to be taken to ensure it was slow and consistent. Too rapid an application would not allow sufficient time for the analytes to partition into the sorbent surface, resulting in analyte breakthrough and poor recovery.

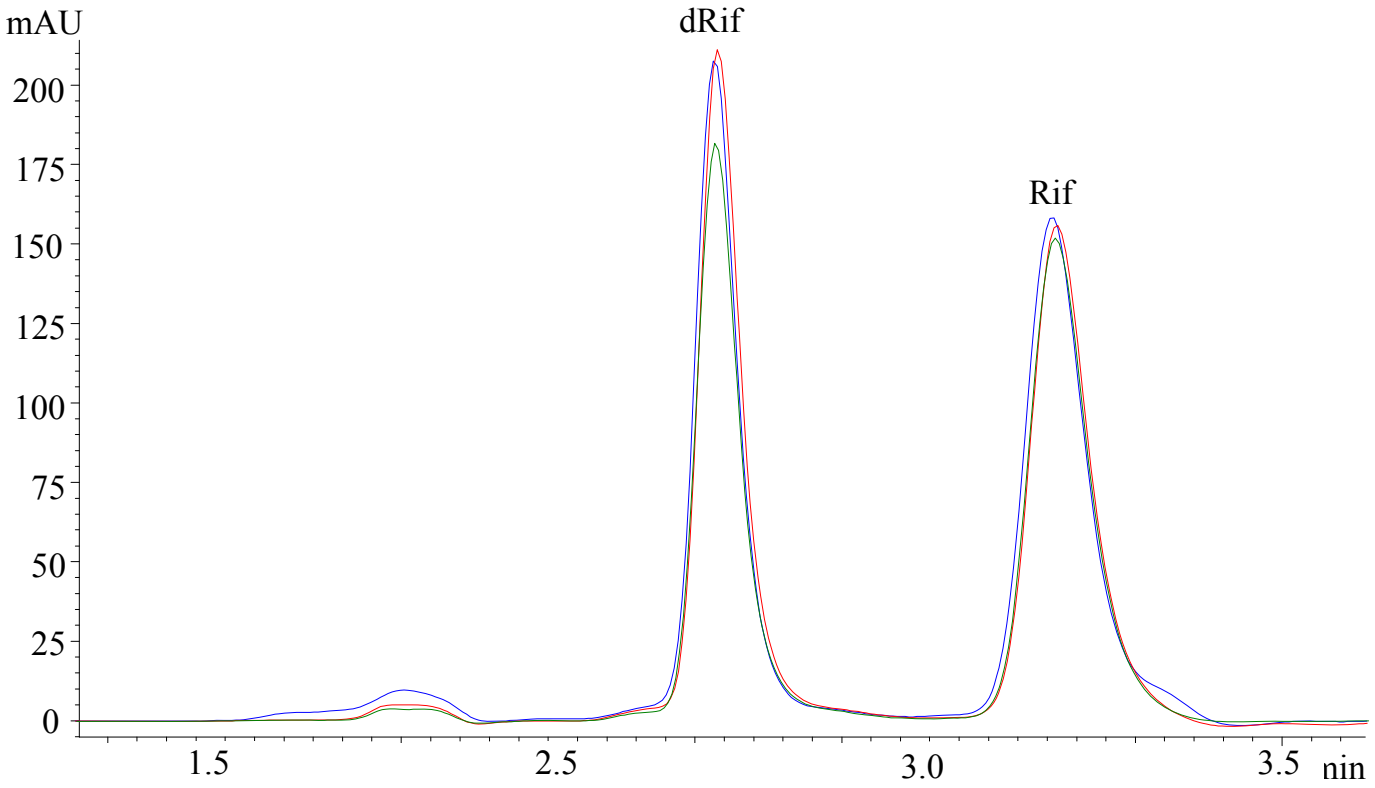


Figure 2.11: Chromatogram comparing different plasma load conditions for dRif and Rif.

Blue: 0.1% FA, Red: 0.01% FA Green: water

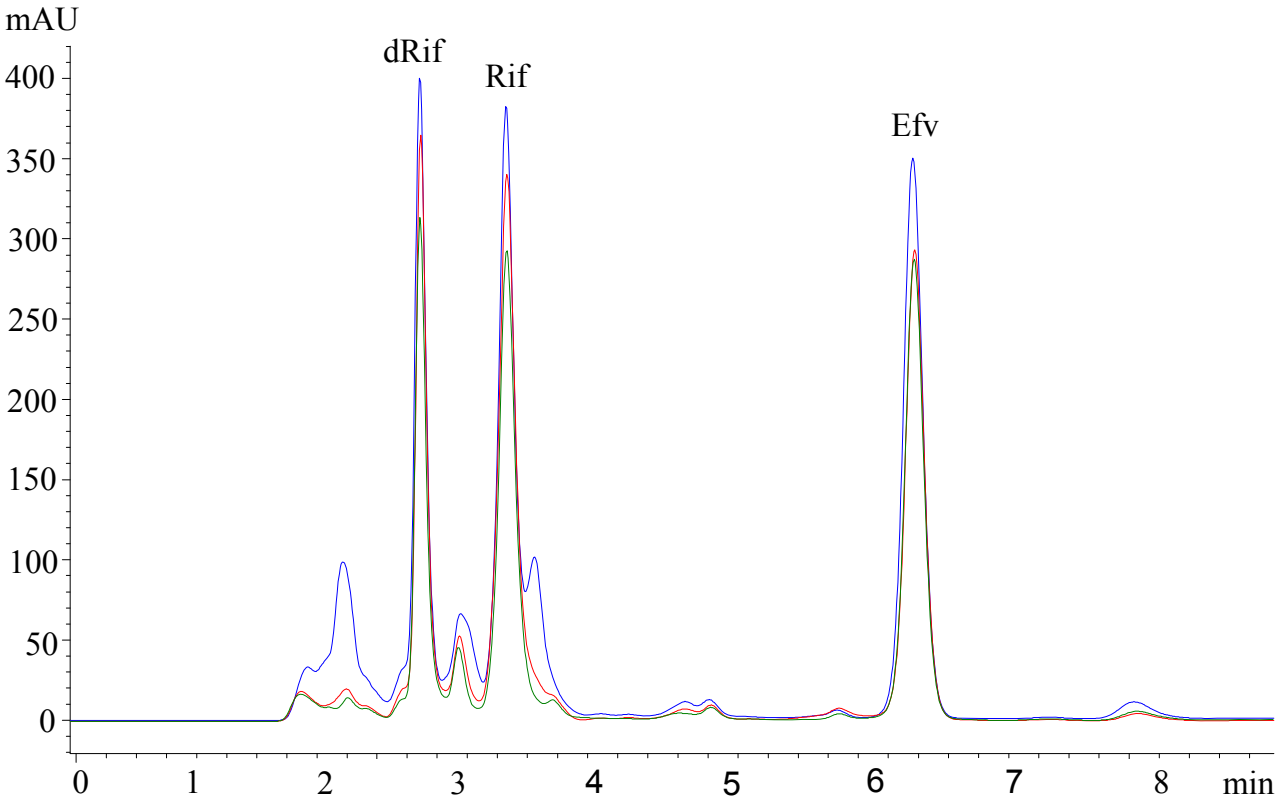


Figure 2.12: Chromatogram comparing different plasma load conditions for Efv.

Blue: 0.1% FA, Red: 0.01% FA, Green: water

The optimum wash solvent needed to be non-polar enough to elute as many interferences as possible, but not sufficiently non-polar to elute the analytes. This solvent was identified by washing the SPE cartridges starting with 100% water, then progressively increasing the proportion of MeOH present. Proportions of 10, 20 and 30% MeOH in the wash solvent were evaluated. Each wash step was collected and analysed for the presence of the drugs. There was a slight loss of recovery when 30% MeOH was employed. However, even with an increase in the percentage organic in the wash step from 10-30% MeOH, the cleanliness of the baseline was not altered a great deal. The wash containing the highest percentage organic but eluted no analytes was at 20%.

For elution, it was required to use the weakest elution solvent that had just enough hydrophobicity to give quantitative analyte elution. Such a solvent, requiring a ternary mixture of H_2O-ACN-MeOH, left behind on the sorbent the maximum number of contaminants. Elution of the drugs with mobile phase (H_2O-ACN-MeOH, 40:45:15, v/v/v) in contrast to H_2O-ACN-MeOH, 25:50:25, v/v/v, significantly affected the recovery of Efv - see Figure 2.13. With a higher % aqueous present in the elution solvent, there was a dramatic decrease in the integrated areas observed for Efv with elution in mobile phase.

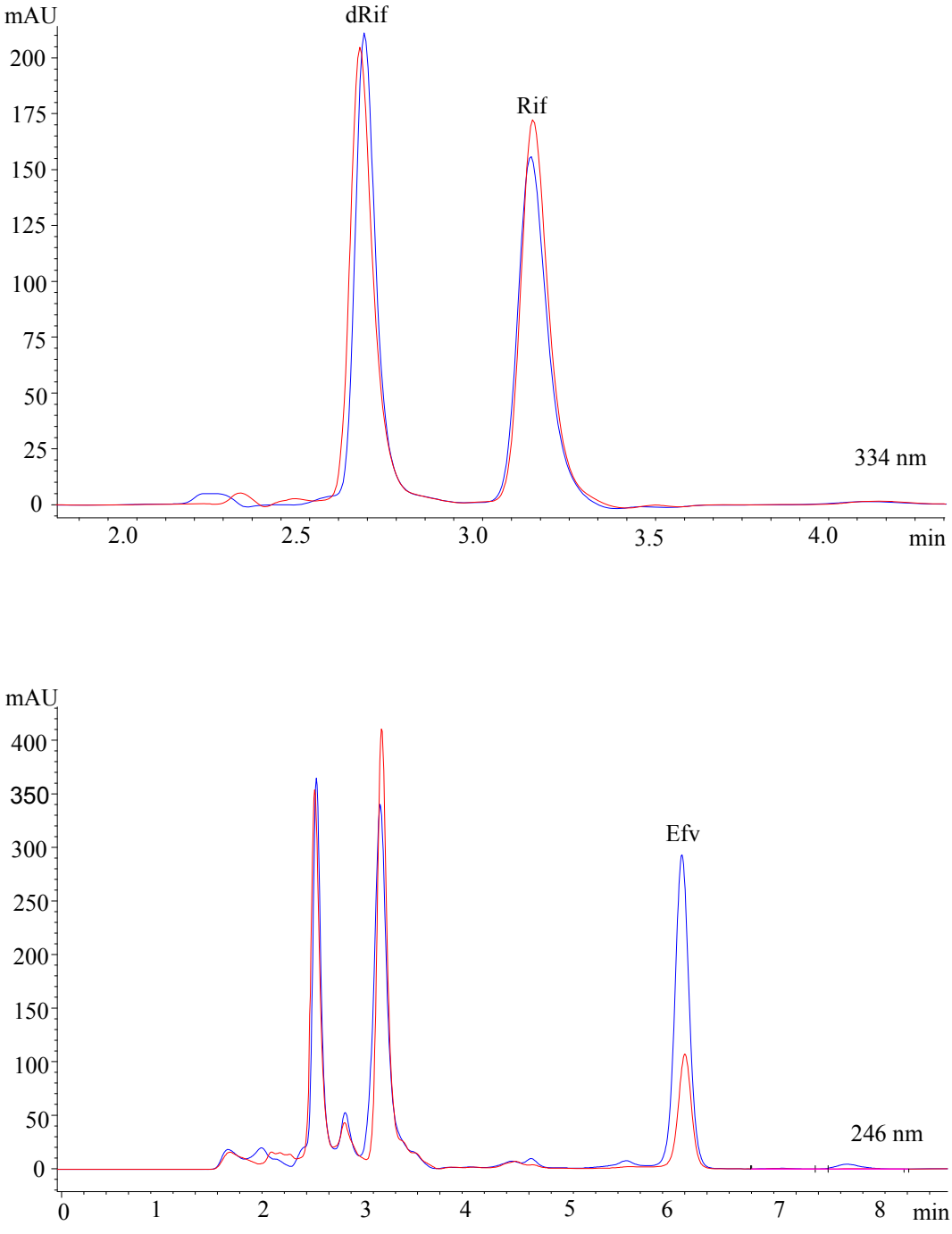


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Figure 2.13: Comparison of elution with (blue) H_2O-ACN-MeOH, 25:50:25 v/v/v, and (red) mobile phase (H_2O-ACN-MeOH, 40:45:15, v/v/v)

Initially, 0.5 mL plasma was loaded and reconstituted in 0.25 mL mobile phase. As a small plasma volume was an objective for this work, it was decided to reduce the amount of plasma loaded to 0.3 mL and reconstitute in 0.15 mL. On doing so, there was no significant difference in the recoveries obtained. See Tables 2.14 and 2.15 for this data.

% MeOH wash	Recovery (%) ± % RSD		
	dRif	Rif	Efv
10	96.7 ± 0.5	95.1 ± 0.4	71.0 ± 0.8
20	96.2 ± 0.9	96.0 ± 0.3	73.1 ± 0.3
30	97.9 ± 0.3	95.8 ± 0.8	72.6 ± 0.3

Table 2.14: Comparison of different wash steps loading 0.5 mL plasma and reconstituting in 0.25 mL mobile phase

% MeOH wash	Recovery (%) ± % RSD		
	dRif	Rif	Efv
10	97.3 ± 0.4	96.5 ± 0.1	77.9 ± 0.1
20	96.9 ± 0.1	96.2 ± 0.2	74.5 ± 0.1
30	99.1 ± 0.1	97.0 ± 0.4	75.5 ± 0.3

Table 2.15: Comparison of different wash steps loading 0.3 mL plasma and reconstituting in 0.15 mL mobile phase

There are safety implications working with material from HIV/TB infected patients. Some literature has described the use of heat treatment to

inactivate HIV in plasma samples making the samples safer to work with (ranges between 56-60 ^{o}C and 30-60 mins have been used (Volosov *et al.*, 2002, Rezk *et al.*, 2004, Sarasa-Nacenta *et al.*, 2001, Marzolini *et al.*, 2000, Boffito *et al.*, 2002)) but more recent research has proven that the process of heat inactivation significantly and adversely affects the stability of Rif (Hartkoorn *et al.*, 2007). When correct health & safety procedures are used, the heating process can be eliminated. It was therefore decided not to heat inactivate the samples used to validate this assay. The addition of ascorbic acid to the elution step of the SPE (1 mg/mL) has been used by some authors to prevent the oxidation of Rif to dRif (Allanson *et al.*, 2007, Panchagnula *et al.*, 1999). In the course of this study, it was found that ascorbic acid had a significant impact in maintaining high recoveries for Rif and was hence employed in this assay. See Figure 2.14.

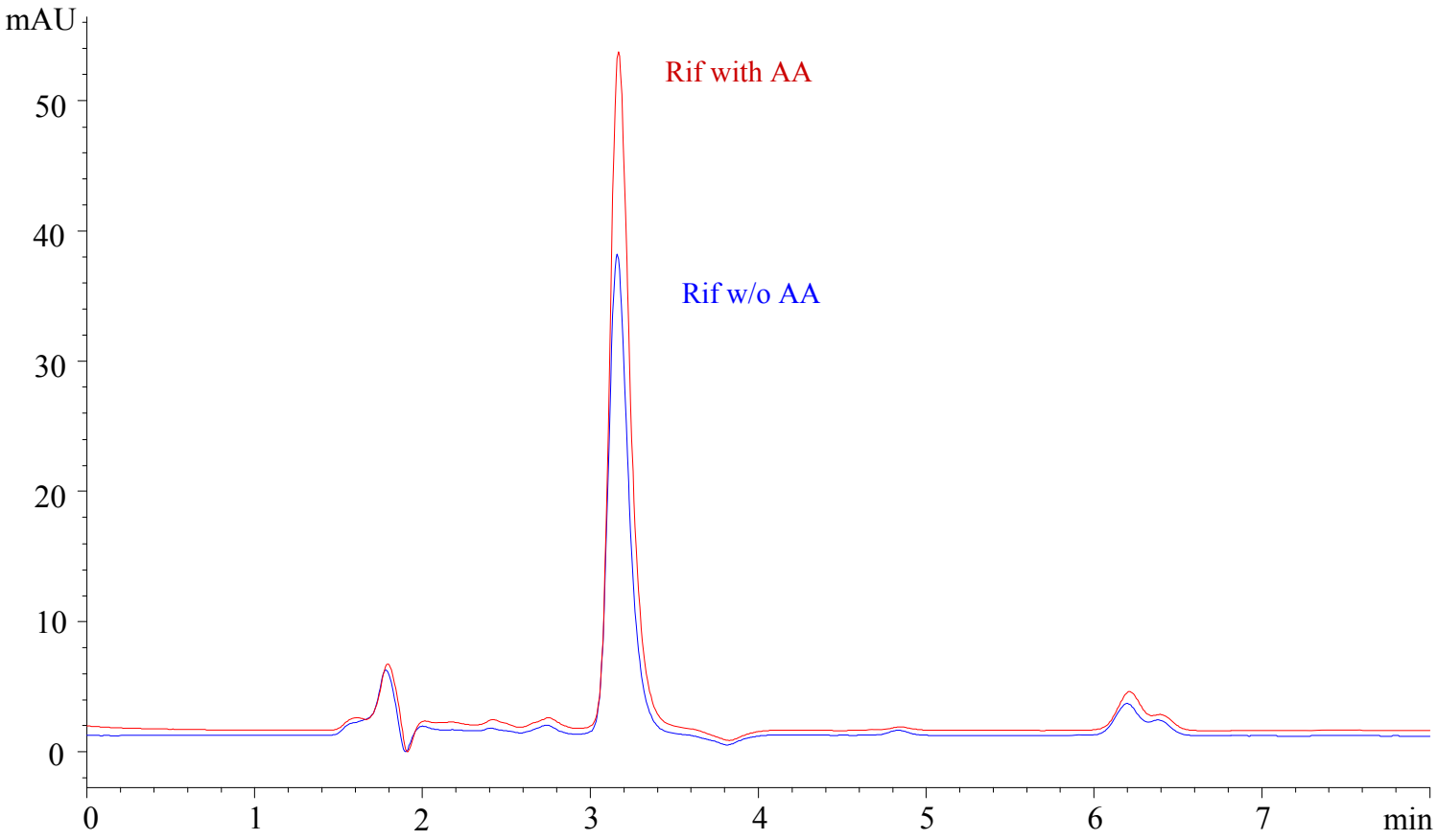

Figure 2.14: Comparison of 10 µg/mL spiked plasma sample with and without addition of ascorbic acid

A mini stability study indicated that Rif stock solution, when prepared in ACN and stored in the fridge, remained stable over time across the entire concentration range analysed. See Table 2.16 for a comparison of the same concentrations of Rif standard prepared from the ACN stock in mobile phase. However, on storing the standards prepared in mobile phase in the fridge and analysing them over three days, loss of analyte signal was evident across the same concentration range tested. Efv proved stable under the same conditions at all concentrations analysed - see again Table 2.16. Stability of Rif varies according to pH due to its amphoteric nature. In acidic solutions, Rif undergoes hydrolysis to yield 3-formyl-rifamycin and 1-amino 4-methylpiperazine. Under alkaline conditions (pH of 7.5 to 9.0) Rif will oxidize if oxygen is present, becoming rifampicin-quinone (Alves *et al.*, 2010). Maximum stability of Rif is reached in near neutral solutions. As discussed, the addition of ascorbic acid to the solution decreases its oxidation (Alves, *et al.,* 2010). Under the slightly acidic conditions of the MP (ammonium acetate (pH 4.75, adjusted with 1 M acetic acid, 20 mM), ACN and MeOH (40:45:15, v/v/v)), hydrolysis of Rif was occurring and hence causing a decrease in its concentration present over the 3 days. When prepared in the more neutral ACN there was no loss of concentration observed for Rif due to it being more stable in near neutral solutions.

Conc. (µg/mL)	Conc. determined Day 1 ± % RSD	Conc. determined Day 3 ± % RSD	% drug determined after day 3
Rif - prepared in MP			
0.5	0.5 ± 3.6	0.2 ± 0.5	41.7
1	0.9 ± 4.1	0.4 ± 0.8	38.0
2	2.1 ± 3.5	0.8 ± 1.5	36.2
5	4.8 ± 2.1	2.1 ± 2.5	43.9
7	7.4 ± 1.4	3.2 ± 2.1	42.7
10	9.8 ± 0.5	4.5 ± 3.2	45.7
Rif - prepared in ACN			
0.5	0.5 ± 1.2	0.4 ± 2.1	90.9
1	0.9 ± 2.7	1.0 ± 0.3	102.9
2	2.1 ± 2.5	2.1 ± 2.1	101.3
5	4.8 ± 0.5	4.8 ± 1.1	101.9
7	7.4 ± 1.1	7.6 ± 1.1	103.0
10	9.8 ± 1.2	9.9 ± 1.3	100.2
Efv - prepared in MP			
0.5	0.5 ± 4.2	0.5 ± 4.1	94.8
1	0.8 ± 3.2	0.8 ± 3.9	97.6
2	2.1 ± 3.2	2.0 ± 1.1	95.3
5	4.8 ± 4.2	5.0 ± 3.2	103.1
7	6.8 ± 1.5	7.0 ± 1.1	103.4
10	9.9 ± 4.5	10.0 ± 1.2	100.5
Efv - prepared in ACN			
0.5	0.5 ± 2.8	0.5 ± 0.9	92.9
1	1.1 ± 2.1	1.1 ± 2.0	106.7
2	2.1 ± 1.1	2.0 ± 1.1	96.2
5	4.8 ± 1.9	5.0 ± 0.3	103.1
7	7.0 ± 1.0	7.1 ± 2.1	101.5
10	9.9 ± 1.1	10.0 ± 1.2	100.7

Table 2.16: Comparison of the stability of Rif and Efv when stored in both mobile phase and ACN

As discussed in Chapter 1, bioanalytical methods, especially in support of pharmacokinetic studies, are among the most challenging to develop and validate (FDA, 2001). The method developed for this work was no exception. Determination of all three drugs simultaneously from such a complex matrix, in a simple, rapid and cost effective assay was the key focus of this work. Developing such a low cost assay to detect the drugs within a clinically relevant concentration range using standard chromatography equipment in order to make it applicable to resource-limited settings was a significant challenge.

In summary, an isocratic high performance liquid chromatography assay employing solid phase extraction for the simultaneous determination of Rif, Efv and dRif from plasma was developed as previously outlined. Using a Zorbax SB-Phenyl reversed-phase analytical column with UV detection, good separation and detection of the drugs was attained within a fast run time of 10 min. The method is suitable for selective estimations of Efv, Rif and its metabolite dRif in plasma matrix. Various features of the developed method include low volumes of plasma required for analysis, simple and fast extraction procedure and cost effective chromatographic equipment. This makes the method very rapid and economical, especially when a large number of samples are to be handled.

2.5 Method validation

A full validation procedure was performed on the method in accordance with FDA guidelines (FDA, 2001) including intra-day and inter-day precision, accuracy, sensitivity, linearity and range and recovery. Stability following freeze-thaw cycles was also investigated.

2.5.1 Accuracy

In order to evaluate the accuracy of the method, six different concentrations of Efv, Rif and dRif (0.1, 0.5, 1, 5, 10 and 20 μg/mL) were prepared in plasma and analysed in sextuplicate on four consecutive days. The measured amounts

were inserted into the equation of the calibration curves and treated as unknown concentrations. The calculated concentrations were compared with the nominal concentrations. Assay accuracy was expressed as % error i.e. [the absolute difference between calculated concentration and spiked concentration] / nominal concentration × 100.

The accuracies obtained at the six concentrations examined were all acceptable with % error values < 10% - see Table 2.17.

2.5.2 Precision

The precision of Efv, Rif and dRif standards were evaluated by analysing six different concentrations of Efv, Rif and dRif (0.1, 0.5, 1, 5, 10 and 20 μg/mL). The intra-day precision values were determined by processing each working standard concentration in octuplicate on the same day and calculating the relative standard deviation (RSD) values. The inter-day precision values were determined by processing each working standard concentration in sextuplicate for five consecutive days and calculating the RSD values. % RSD was expressed as [deviation from the mean]/mean concentration × 100. Intra- and inter-assay precision RSD values were found to be less than 10% in all cases. See Table 2.17 for the summarised data.

Compound	INTRA-DAY (n =8)			INTER-DAY (n = 6)		
	Calculated conc. (µg/mL)	Accuracy (% Error)	Precision % RSD	Calculated conc. (µg/mL)	Accuracy (% Error)	Precision % RSD
dRif	0.1	10.0	0.2	0.1	9.0	0.1
	0.5	-6.0	1.8	0.5	4.3	3.2
	1.0	2.0	1.7	1.1	6.9	1.0
	5.1	1.4	7.2	5.0	0.8	0.4
	9.9	1.3	0.2	9.9	0.2	0.3
	20.0	0.2	0.3	20.0	0.1	0.8
Rif	0.1	10.9	0.8	0.1	10.0	0.9
	0.5	1.7	3.3	0.5	3.5	1.5
	1.1	6.9	2.4	1.1	6.8	0.3
	4.9	1.9	0.6	4.9	1.4	0.3
	9.8	1.6	0.5	9.8	2.1	0.6
	20.1	0.5	0.2	20.1	0.6	0.3
Efv	0.1	8.1	0.0	0.1	9.9	4.2
	0.5	5.8	0.8	0.5	1.0	0.9
	0.9	4.7	1.6	0.9	6.3	1.7
	5.0	0.2	0.2	5.1	0.9	0.8
	9.9	0.8	0.1	10.0	0.1	0.5
	20.0	0.2	0.8	20.0	0.0	0.2

Table 2.17: A summary of the Intra-Day and Inter-Day precision and accuracy determinations

2.5.3 Selectivity

For selectivity, six blank plasma samples from different sources were analysed and checked for peaks interfering with the detection of the analytes. The assay was found to be selective for all drugs analysed. No interfering peaks were observed in the extracts of the different blank plasma samples.

2.5.4 Sensitivity

Both the limits of quantitation (LOQ) and detection (LOD) were determined for the assay. The LOQ was defined as the lowest concentration that produced a peak distinguishable from background noise with a minimum ratio of 10:1. LOD was defined as the lowest concentration that produced a peak distinguishable from background noise with a minimum ratio of 3:1. The LOQ was 0.1 μg/mL for all three drugs. Extracted blank plasma samples did not yield any endogenous peaks at the retention times of the drug compounds – see Figure 2.15. The LOD was found to be 0.075 μg/mL with % error and RSD values less than 15%.

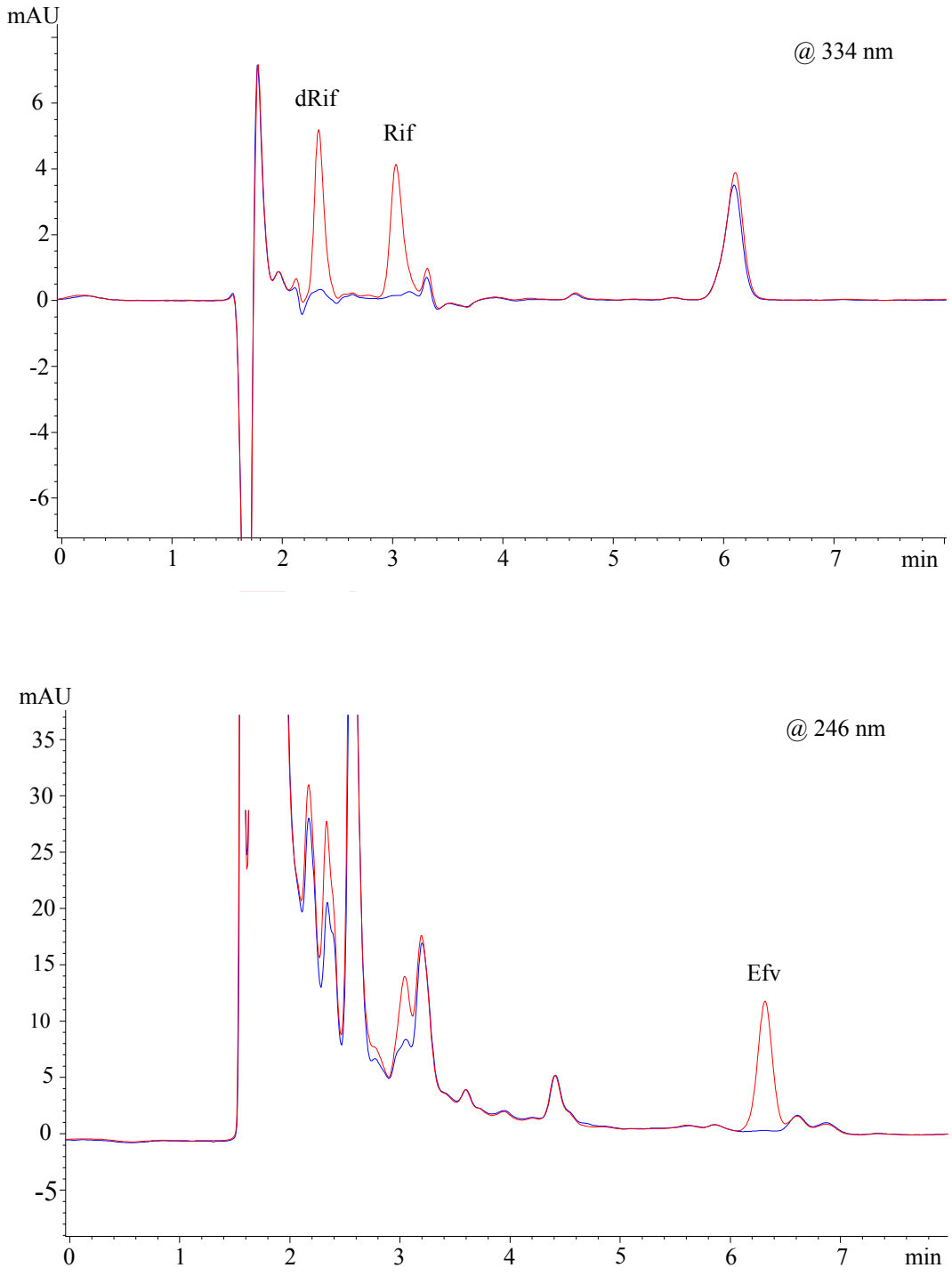

Figure 2.15: Chromatograms of extracted drug-free plasma sample superimposed on extracted plasma spiked with 100 ng/mL dRif, Rif and Efv.

2.5.5 Linearity and range

The calibration curves for Efv, Rif and dRif over the concentration range 0.1 to 20.0 µg/mL exhibited good linearity with correlation coefficients (R^2) for all standard curves above 0.99. See Figures 2.16 - 2.18.

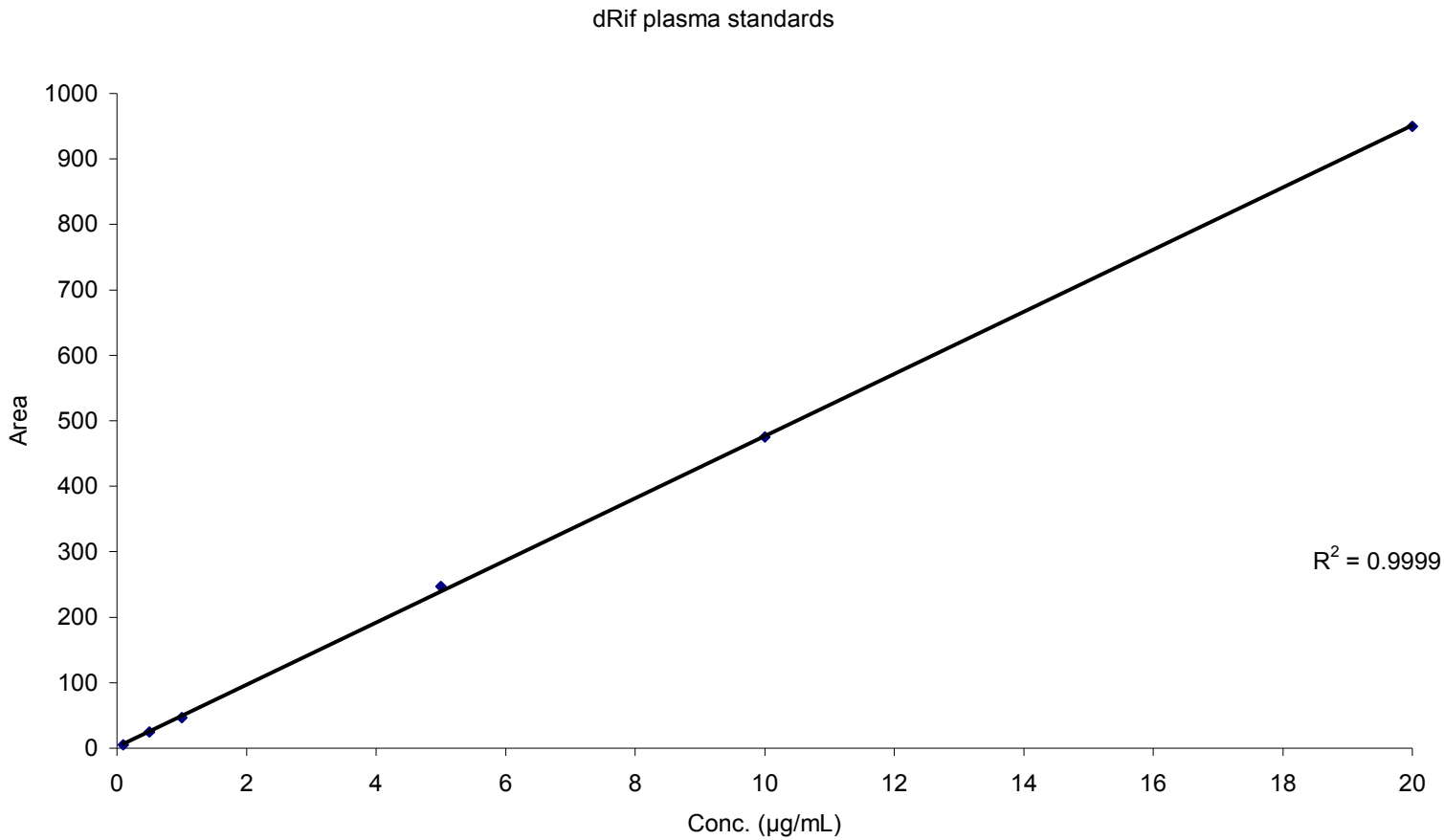

Figure 2.16: Linearity for dRif plasma standards

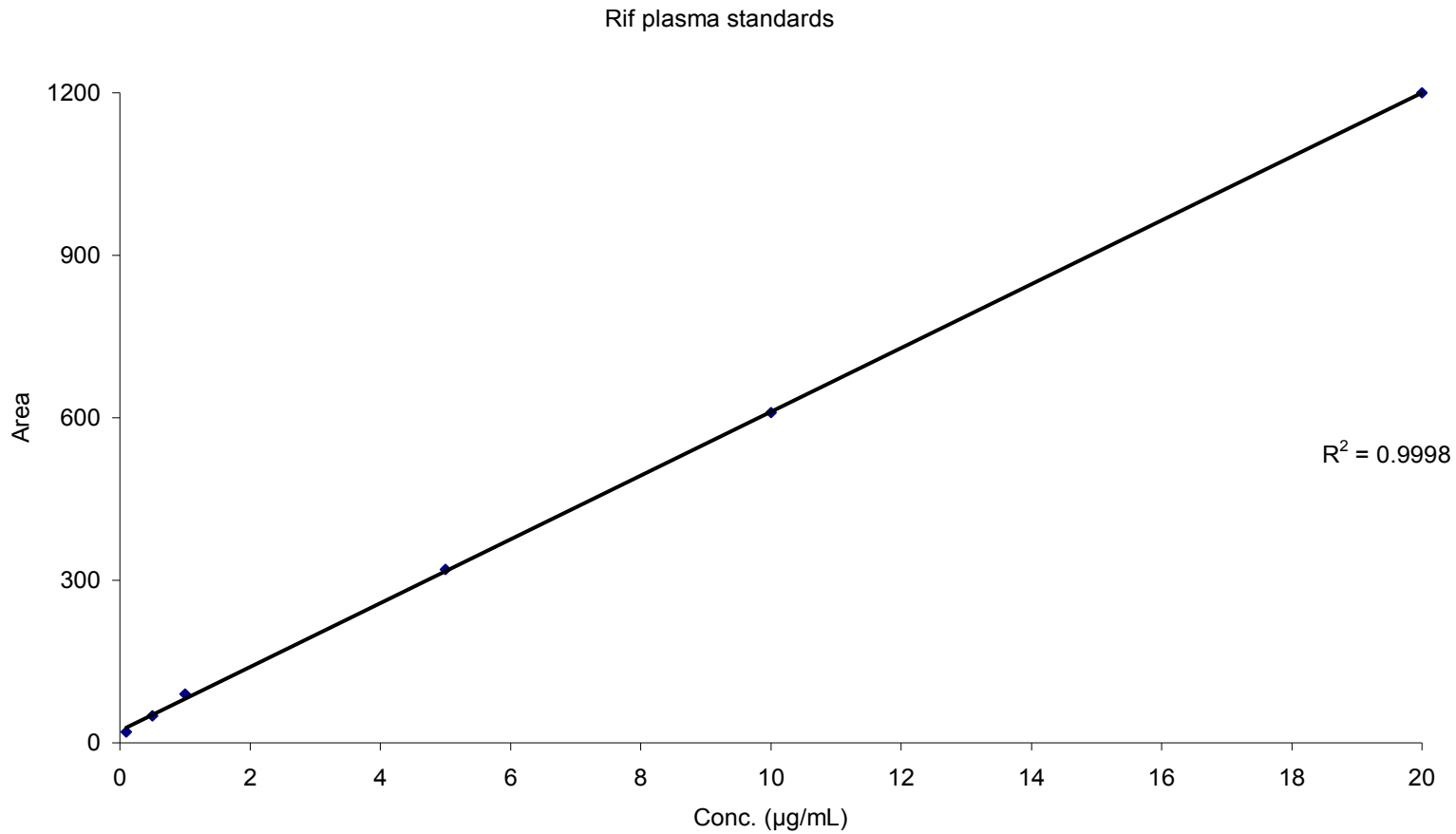

Figure 2.17: Linearity for Rif plasma standards

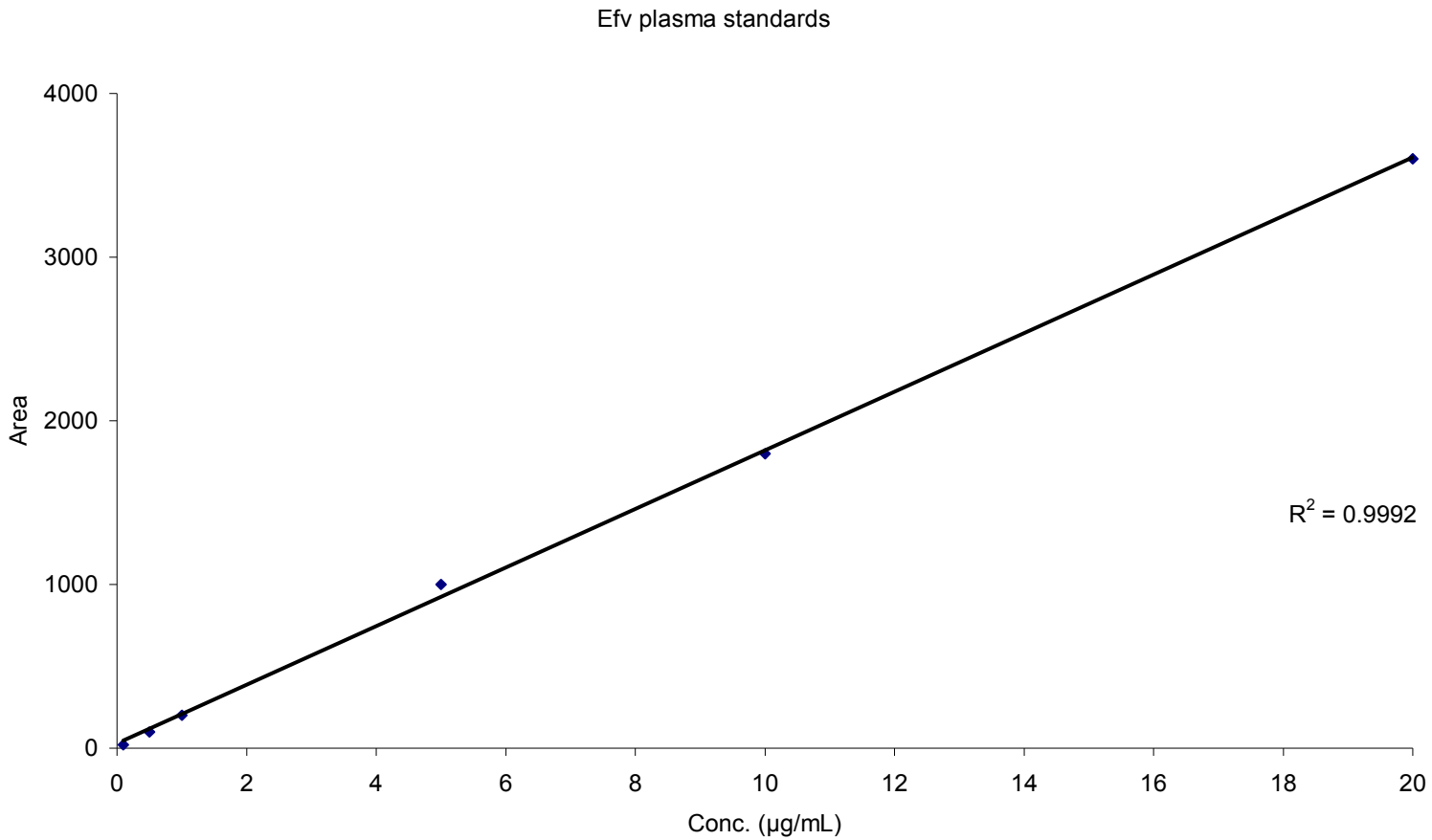

Figure 2.18: Linearity for Efv plasma standards

2.5.6 Recovery

As previously discussed, highest recoveries for the drugs in one extraction step were obtained with the SPE ABN well plates. Varying concentrations of Efv, Rif and dRif (1, 5 and 20 μg/mL) were prepared in drug-free human plasma and extracted using the SPE cartridges. The percentage of drug recovered from these plasma samples was determined by comparing the calculated concentrations following extraction and HPLC assay with the calculated concentrations from unextracted samples in MP of the same concentration after HPLC assay. Recovery experiments were carried out on three consecutive days. Recoveries for plasma samples spiked with 1, 5 and 20 μg/mL of Efv were all ≥ 70% (Table 2.18).

Conc. (μg/mL)	**Mean % recovery ± % RSD**		
	dRif	**Rif**	**Efv**
1.00	96.9 ± 0.9	94.4 ± 0.7	70.0 ± 0.4
5.00	97.1 ± 0.2	94.0 ± 0.1	69.4 ± 0.6
20.00	97.0 ± 0.3	93.8 ± 0.2	69.8 ± 0.4

Table 2.18: Recovery data for drugs from plasma (n = 3)

2.5.7 Stability

To examine relevant variables around storage and long term stability we determined the impact of freeze–thaw cycles on the stability of the drugs in the presence of plasma, fresh drug-free plasma samples were spiked with 0.5, 5 and 20 μg/mL of Efv, Rif and dRif as shown in Table 2.19. Samples underwent four freeze-thaw (−20 °C to room temperature) cycles. The drug concentrations were then determined in triplicate on three separate occasions and compared to plasma samples prepared to the same concentrations that were stored at −20 °C and only thawed once immediately prior to analysis.

Long term stability was evaluated using plasma samples spiked with 0.5, 5 and 20 µg/mL of Efv, Rif and dRif after six months of freezing. This storage time was chosen as is does not exceed the time from when samples are first collected for the SPhEAR study to the time of last sample analysis. Finally, stock solution stability was evaluated by comparing the response obtained from standards prepared in mobile phase at three concentrations (0.5, 5 and 20 µg/mL) left at room temperature for 8 hours to freshly prepared stock solutions.

Compound	Conc. (µg/mL)	Cycle 1 % recovery ± % RSD	Cycle 2 % recovery ± % RSD	Cycle 3 % recovery ± % RSD	Cycle 4 % recovery ± % RSD	Cycle 5 % recovery ± % RSD
dRif	0.5 5 20	99.7 ± 0.7 100.7 ± 2.1 99.7 ±1.1	98.1 ± 2.5 100.8 ± 2.0 99.5 ± 1.2	96.0 ± 0.6 96.8 ± 0.1 95.7 ± 1.2	87.6 ± 2.8 89.5 ± 2.7 85.3 ± 1.1	85.6 ± 2.1 90.3 ± 3.2 83.8 ± 0.5
Rif	0.5 5 20	100.9 ± 2.0 99.9 ± 2.9 99.2 ± 1.1	99.5 ± 0.8 102.0 ± 2.8 100.2 ± 1.7	96.1 ± 1.5 94.3 ± 0.7 96.7 ± 0.3	88.0 ± 1.2 90.4 ± 1.0 90.3 ± 3.1	89.9 ± 1.3 88.4 ± 2.1 93.3 ± 1.9
Efv	0.5 5 20	102.3 ± 1.4 100.6 ± 1.0 99.4 ± 0.5	102.0 ± 1.4 103.5 ± 2.0 99.1 ± 0.5	99.3 ± 1.4 100.3 ± 1.3 96.5 ± 0.1	94.7 ± 0.5 92.9 ± 0.8 97.7 ± 0.1	94.0 ± 0.2 94.8 ± 0.9 96.9 ± 1.2

Table 2.19: Summary of freeze-thaw stability findings for Rif, dRif and Efv (n = 3)

The data indicated that three freeze–thaw cycles had little significant impact on the Efv, Rif and dRif concentrations measured, which means that samples could be repeatedly thawed and re-analysed up to three times if required. However, it was noted that the drug quantiation showed increased evidence of instability in dRif and Rif on the fourth cycle of the freeze-thaw study which suggests that the drugs are less stable after further freeze-thaw cycles. Long term (6 months, -80 ºC) storage at the three different concentrations showed no significant decline in responses obtained for the three drugs. The stability of the three stock solutions prepared in mobile phase was also evaluated. Standards (0.5, 5 and 20 μg/mL) left for eight hours at room temperature showed no decline in detector response at the three concentrations analysed which allowed for daily preparation of daily solutions.

The HPLC–UV method was fully validated with inter- and intra-assay accuracy and precision falling within the FDA guidelines (FDA, 2001). As described in the FDA guideline, quality control samples may not deviate more than 15% from the nominal concentration (and 20% for the LLQC), a guideline followed and adhered to for this assay.

In the literature Calleja *et al.*, 2004, achieved the lowest LOQ for the determination of Rif at just 50 ng/mL, however, this was Rif extracted from rat plasma. Balbão *et al.*, 2010, achieved an LOQ of 125 ng/mL for Rif extracted from human plasma, however, the recovery of drug was low at 75-80% and Rif had a long retention time of 11 mins. Taking into consideration all aspects of the UV method described in this thesis, the method developed and described appears to have many advantages over others reported in the literature to date. With an LOQ of 100 ng/mL, well below the concentrations found in human PK samples, Rif has a usefully short retention time of 3.1 min and a recovery of 94% from plasma. Also our method is capable of determining Rif's metabolite dRif. In the presence of Efv, this has never been investigated and doing so was important in order to determine the extent of Rif's metabolism to dRif in the presence of Efv. There are limited reported methods for the simultaneous determination of dRif and Rif, the most successful was Hemanth Kumar *et al.*, 2004, however, their sensitivity was not as good at 250 ng/mL. Panchagnula *et al.*, 1999, also developed a method for the simultaneous determination of dRif and Rif. Sample volume was low at only 100 μL, however, the sensitivity

obtained was poor at 2 µg/mL. So again, the method described in this thesis is the best to date for the simultaneous determination of both Rif and dRif. Similar to the LOQ for Rif, dRif's LOQ was again 100 ng/mL with a recovery of 97% from plasma was achieved.

For Efv, the lowest LOQ obtained for Efv was by Mogatle *et al.*, 2009, having achieved 8.96 ng/mL in serum. However, 1 mL sample was needed and retention time was 11.6 mins. There are numerous methods reported with LOQs of just 10 ng/mL but all suffer from either extremely long elution times 10.2 – 24.9 min, (Veldkamp *et al.*, 1999, Rezk *et al.*, 2004, Rezk *et al.*, 2002, Choi *et al.*, 2007) high sample volumes 0.5 – 1.0 mL (Rezk *et al.*, 2004, Rezk *et al.*, 2002, Choi *et al.*, 2007) or both (Rezk *et al.*, 2004, Rezk *et al.*, 2002, Choi *et al.*, 2007). The main advantage of the UV method described in this thesis in terms of Efv is the short retention time obtained for Efv at 6.1 min, which was very challenging in the presence of both dRif and Rif.

Boffito *et al.*, 2002, developed the only LC-UV method for the simultaneous determination of Rif and Efv. This one reported method for the simultaneous determination of the two exhibits comparable sensitivity with the method we outline herein (LOD 50 ng/mL) but requires gradient elution and hence suffers from a relatively long analysis time of 22 mins. The authors also did not measure Rif's main metabolite, dRif. So again, the LC-UV method described in this thesis offers excellent sensitivity for each of the drugs, allows for the determination of Rif's metabolite which hasn't been done before in the presence of Efv and the sample volume required is low at only 0.3 mL.

2.6 SPhEAR patient sample results

The blood samples analysed were obtained from eleven patients participating in the SPhEAR study. All patients were administered a 600 mg dose of Rif for the treatment of TB with Patient B administered both Rif and Efv for the simultaneous treatment of HIV and TB. Samples were drawn at eight different time points (0, 1, 2, 3, 4, 6, 8 and 10 hrs) over a 10 hour period. See Figure 2.19 for the chromatograms at 0 and 1 hour post-dosing in Patient A.

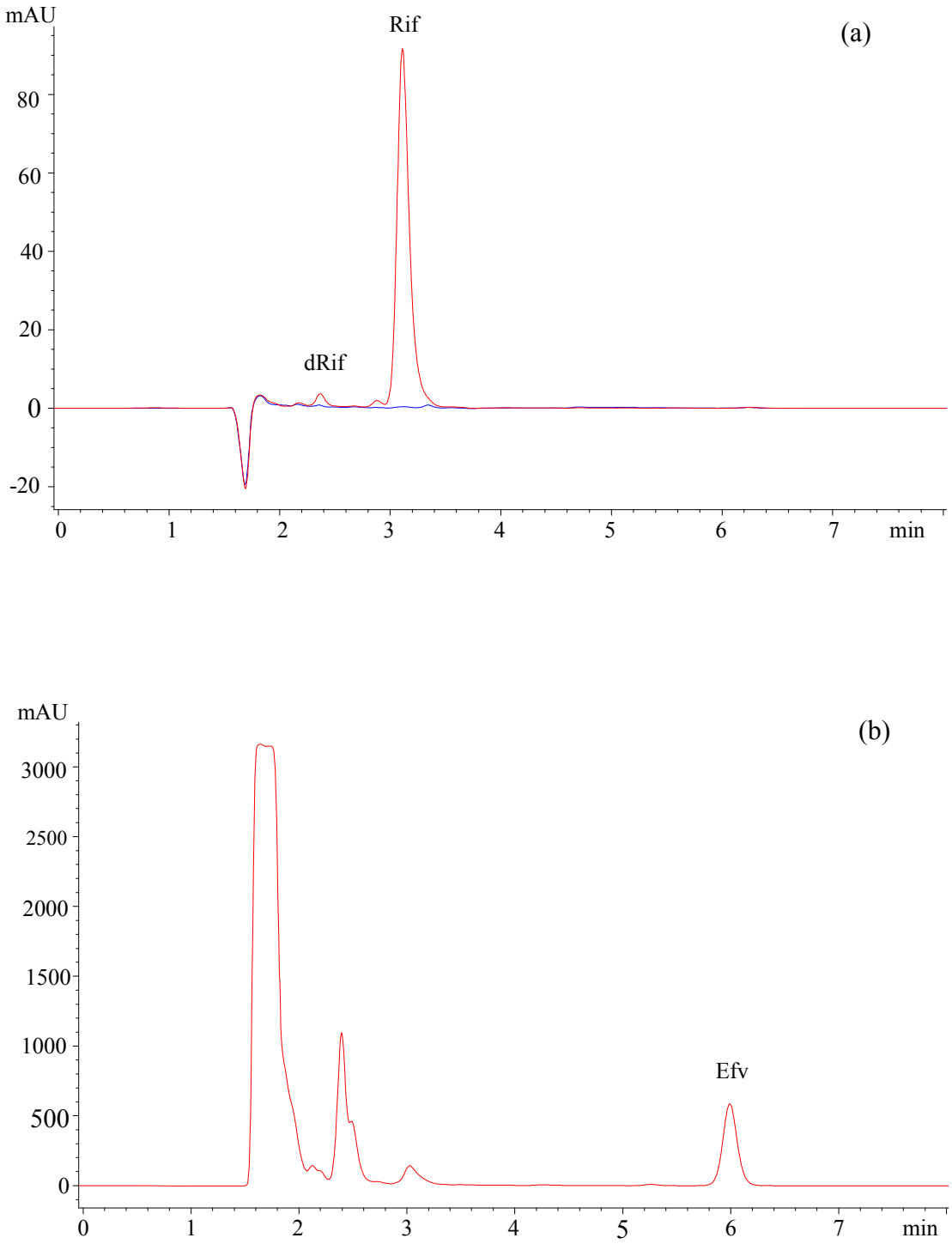

Figure 2.19: (a) Overlaid chromatograms from 0 (blue) and 1 hour (red) timepoints post-dosing in Patient A being treated with Rif and (b) chromatogram from 1 hour timepoint post-dosing in Patient B being treated with Efv

The pharmacokinetic profiles for the drugs in each of the patients can be seen in Figures 2.20 to 2.31. The results obtained agree with previous levels published (Hartkoorn *et al.*, 2007, Boffito *et al.*, 2002). Patient B (HIV positive) was administered Efv 10 hours prior to T0 (treatment with Rif). The long half-life of Efv generates a gradual decline in the blood concentration over time (Ramachandran *et al.*, 2006) and as expected, Efv concentrations showed a slight decrease over the same timepoints with concentrations ranging from 3 to 5 μg/mL (see Figure 2.22). Again, results agree with previous levels published (Mogatle *et al.*, 2009, Boffito *et al.*, 2002).

Rif or dRif were not detected in the plasma of any of the patients pre administration of the drug at t=0. Post administration, absorption of Rif into the blood stream occurred rapidly. Maximum plasma concentrations (C_{max}) of both Rif and dRif and the time taken for the drug to reach its highest concentration in the blood stream (T_{max}) are presented in Table 2.20. Rif concentrations for all patients ranged from up to 19 μg/mL, over the full time period (with levels peaking 2-3 hours post administration) and up to 11 μg/mL for dRif. This again complies with previous levels published (Hartkoorn *et al.*, 2007, Boffito *et al.*, 2002).

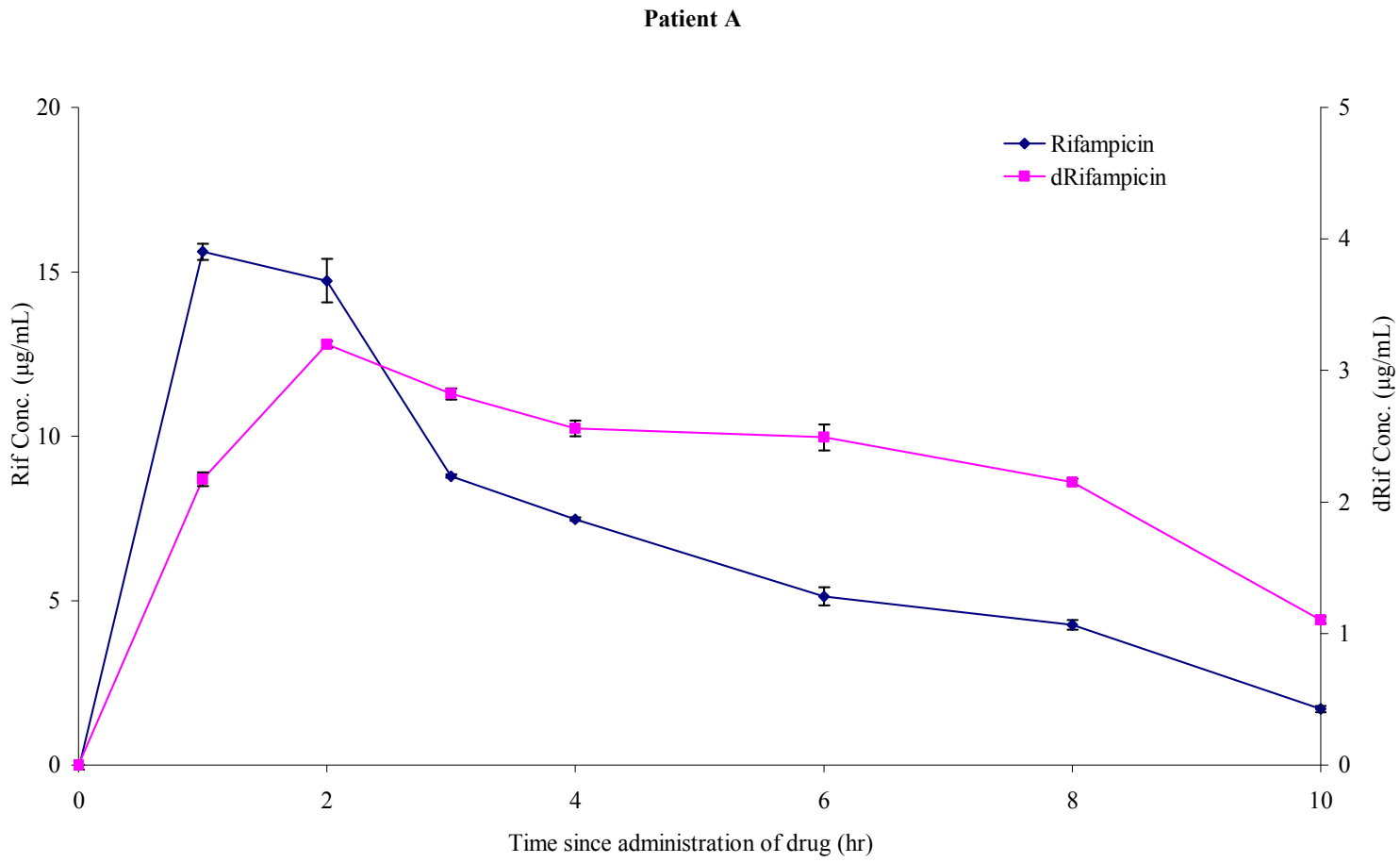

Figure 2.20: Pharmacokinetic profile of Rif and dRif from Patient A participating in the SPhEAR study

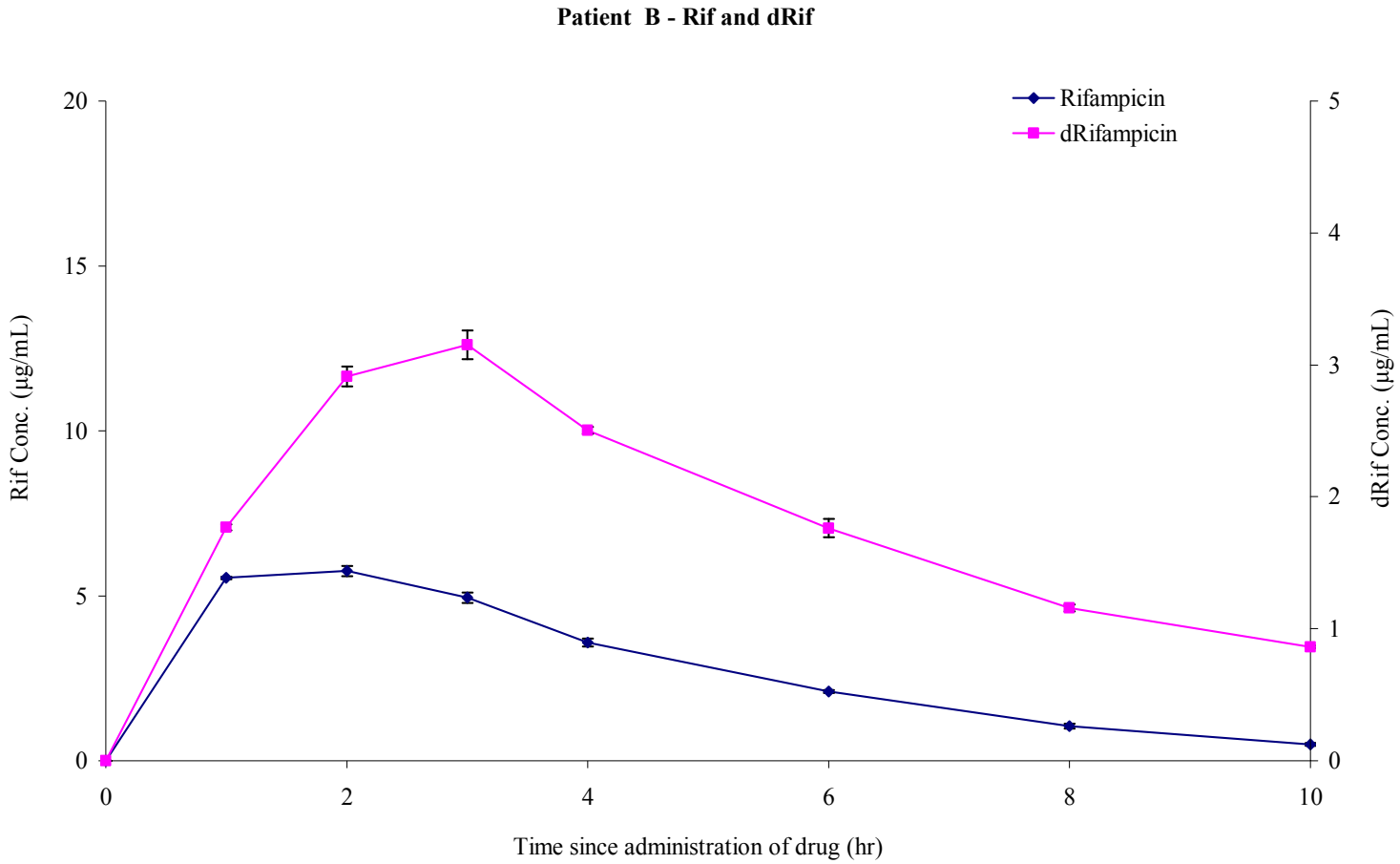

Figure 2.21: Pharmacokinetic profile of Rif and dRif from Patient B participating in the SPhEAR study

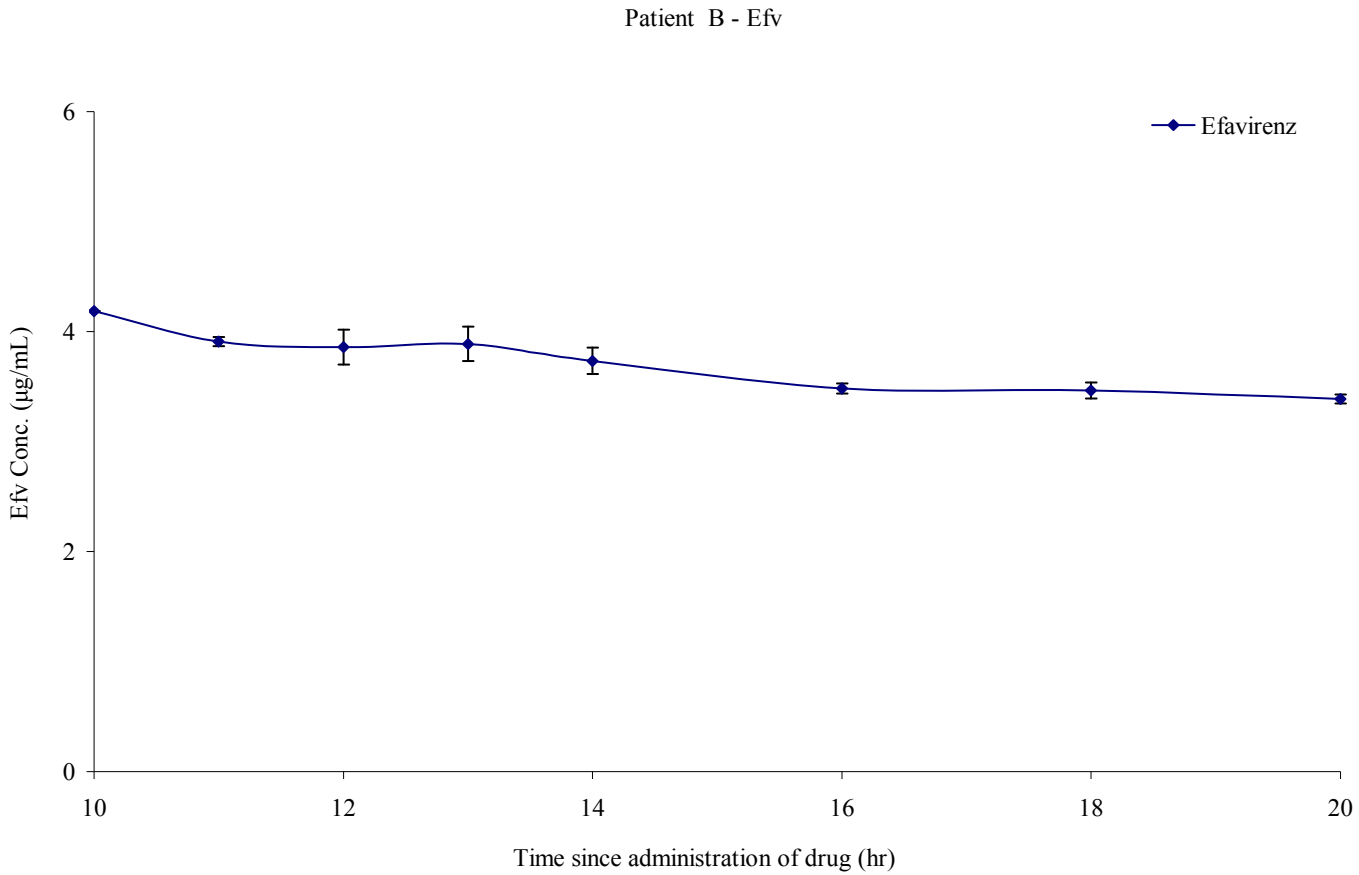

Figure2.22: Pharmacokinetic profile of Efv from Patient B participating in the SPhEAR study

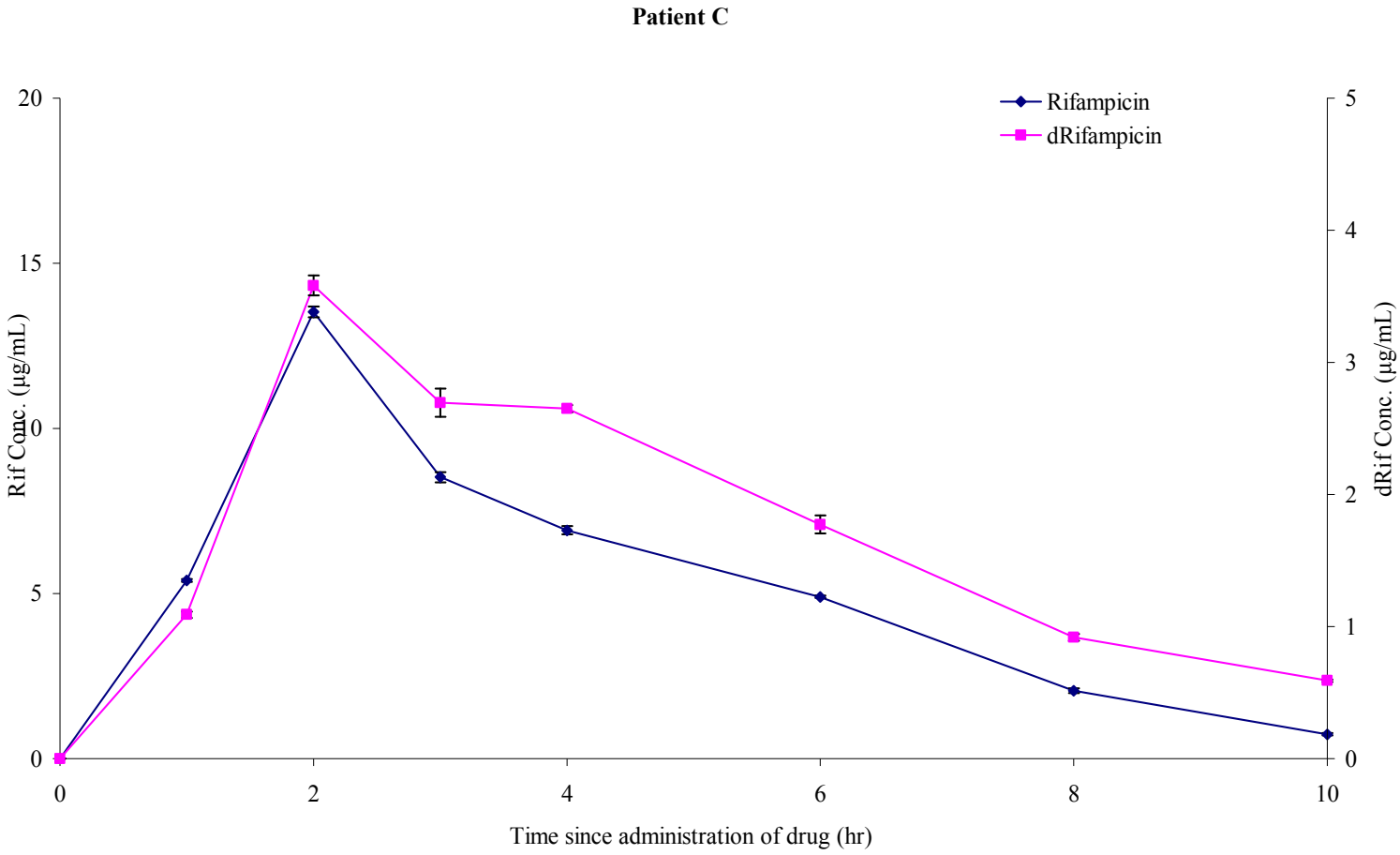

Figure 2.23: Pharmacokinetic profile of Rif and dRif from Patient C participating in the SPhEAR study

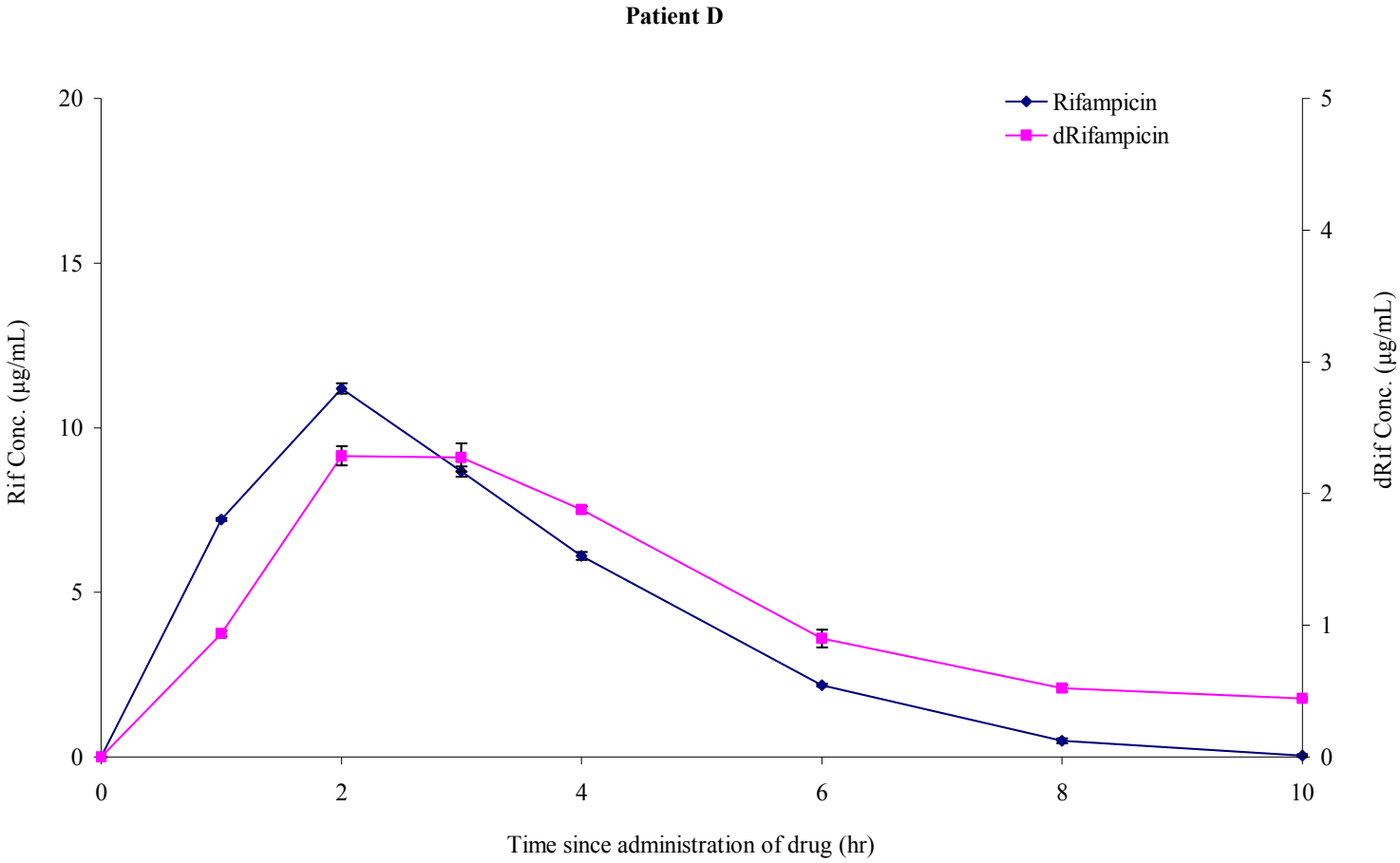

Figure 2.24: Pharmacokinetic profile of Rif and dRif from Patient D participating in the SPhEAR study

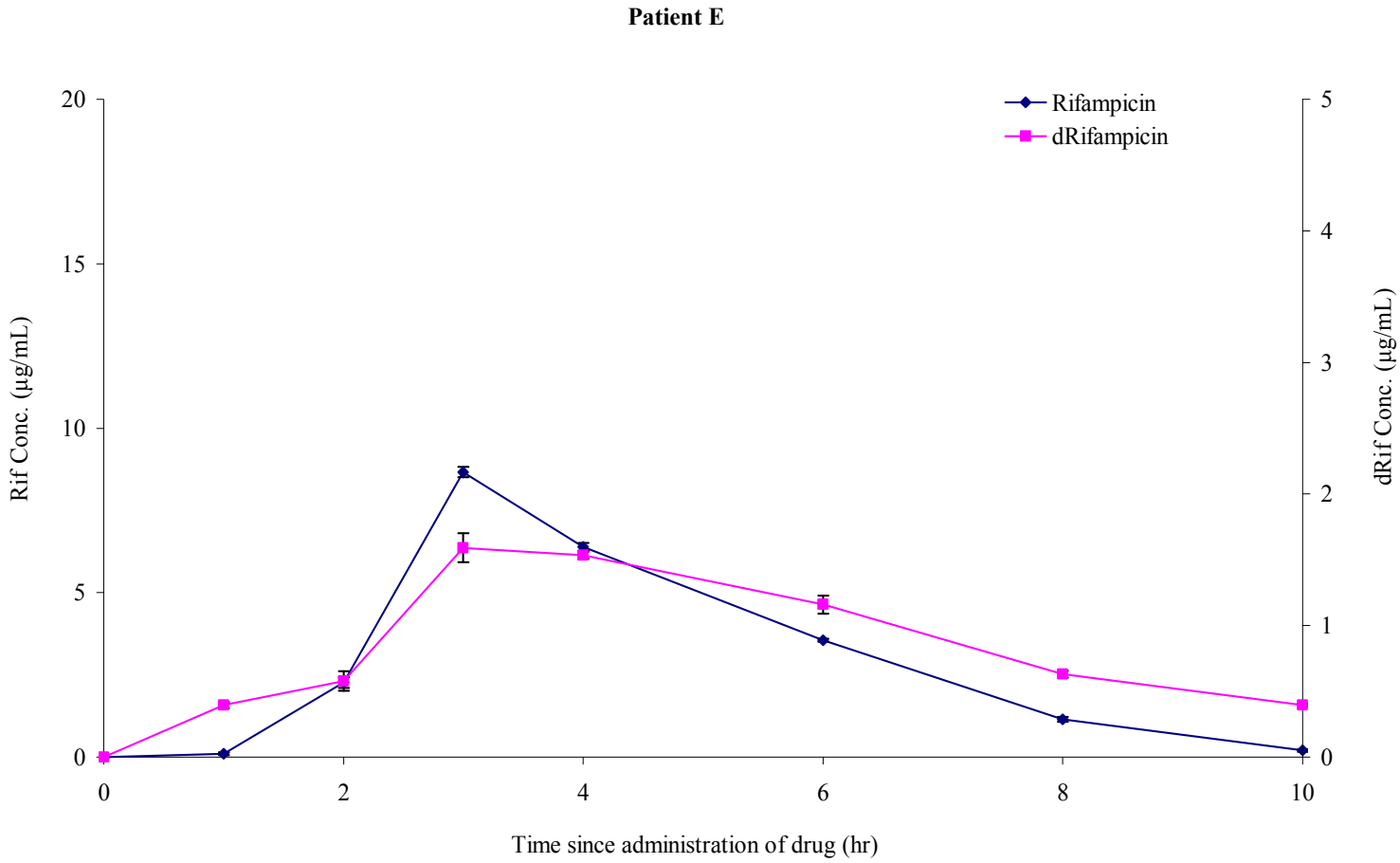

Figure 2.25: Pharmacokinetic profile of Rif and dRif from Patient E participating in the SPhEAR study

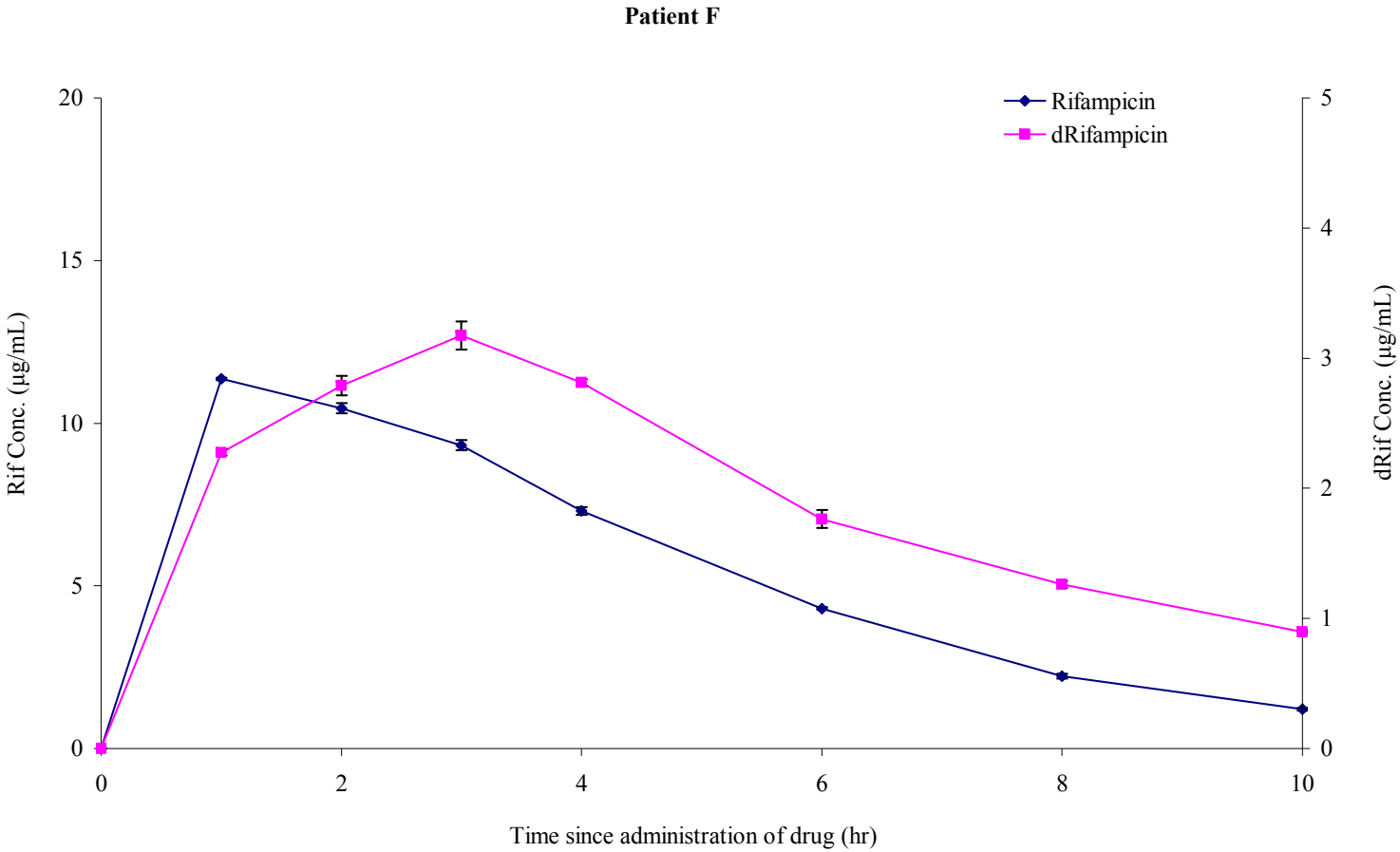

Figure 2.26: Pharmacokinetic profile of Rif and dRif from Patient F participating in the SPhEAR study

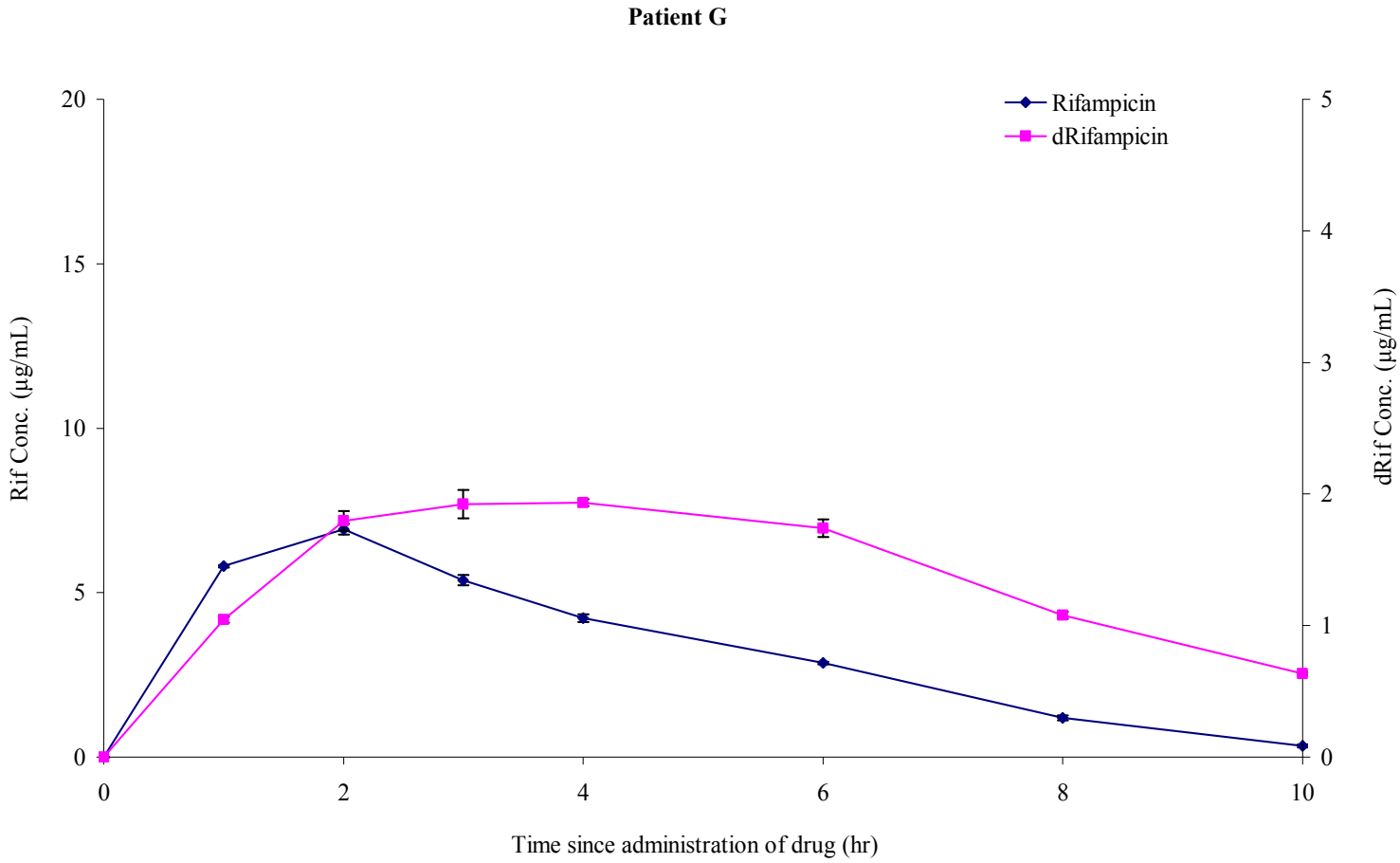

Figure 2.27: Pharmacokinetic profile of Rif and dRif from Patient G participating in the SPhEAR study

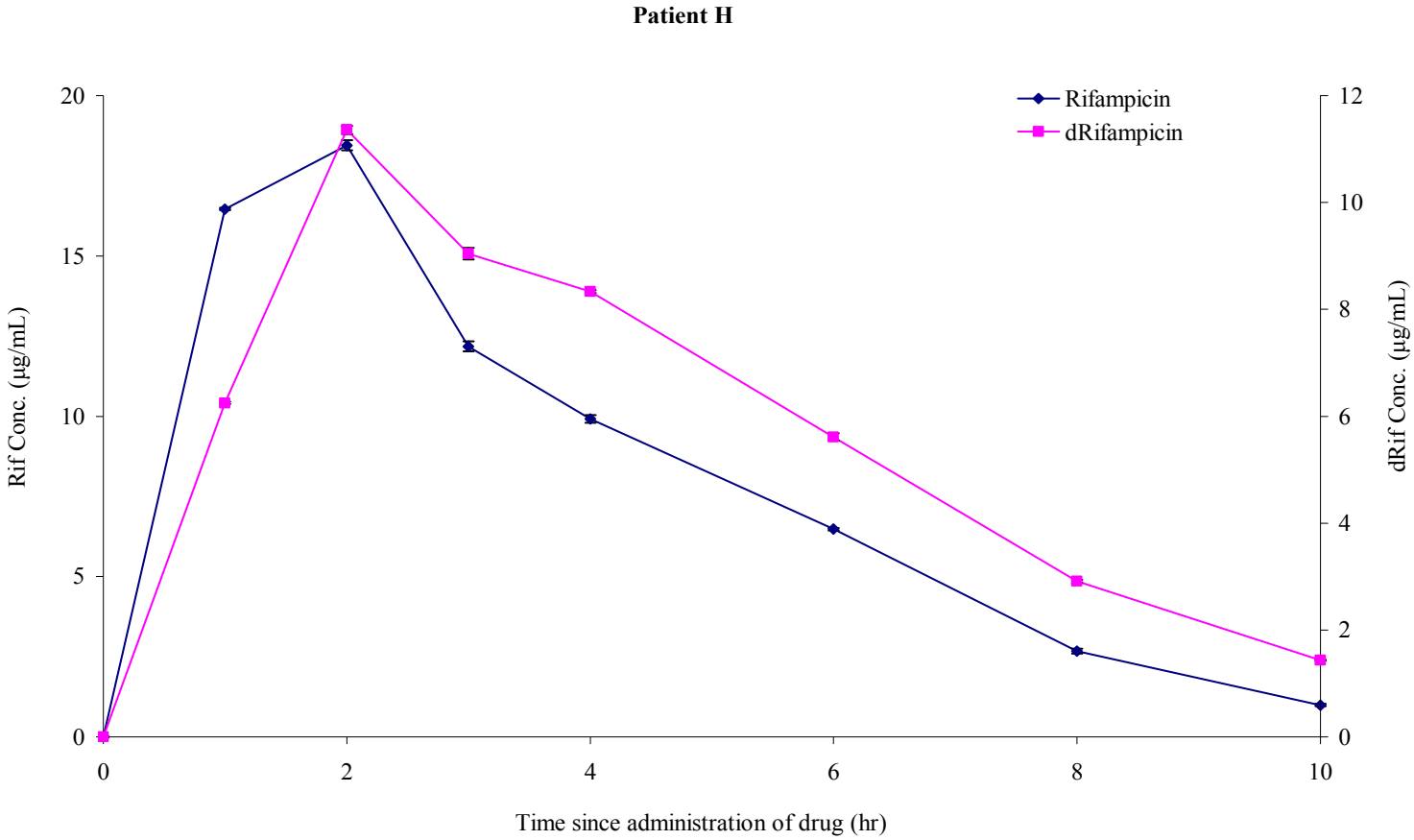

Figure 2.28: Pharmacokinetic profile of Rif and dRif from Patient H participating in the SPhEAR study

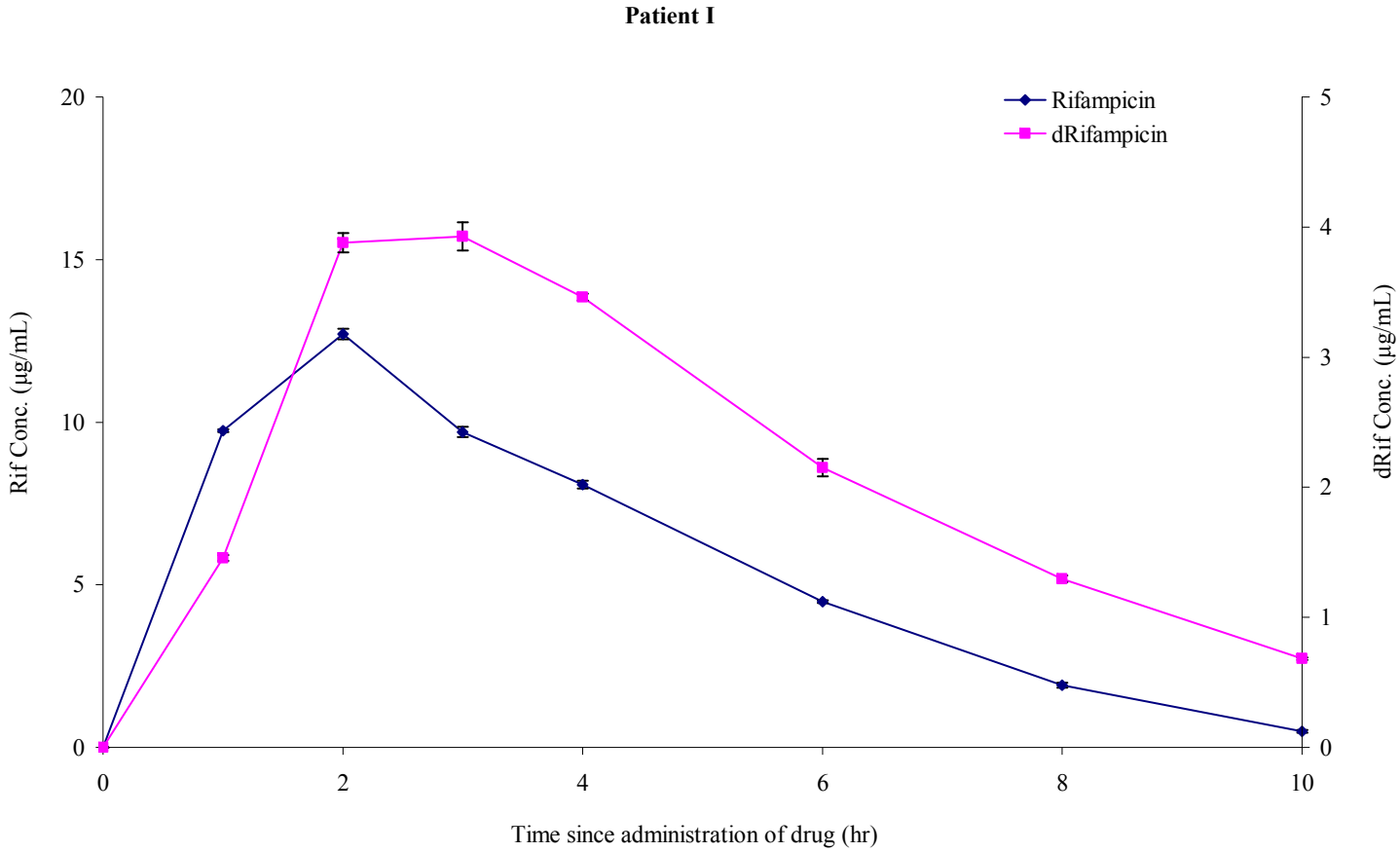

Figure 2.29: Pharmacokinetic profile of Rif and dRif from Patient I participating in the SPhEAR study

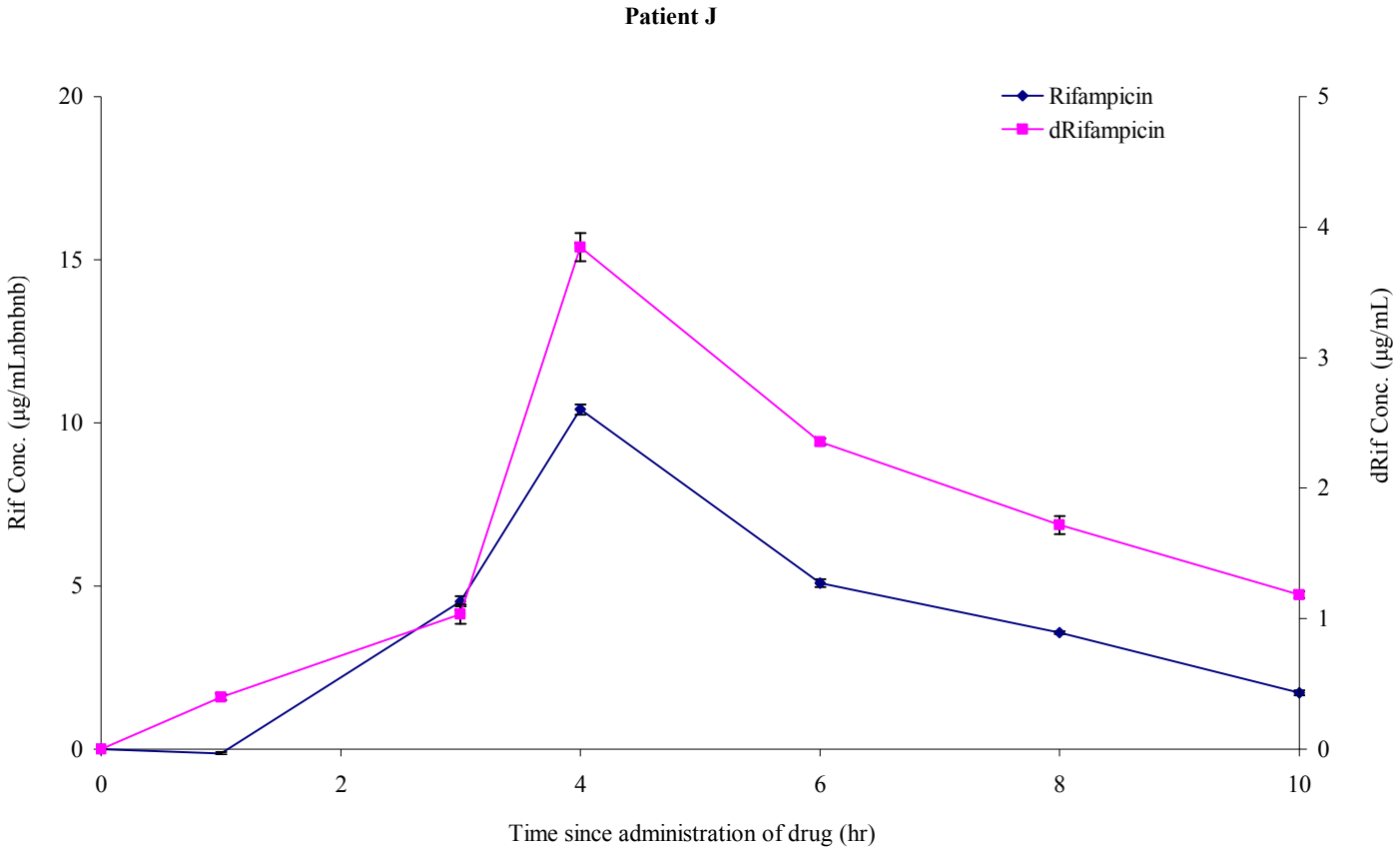

Figure 2.30: Pharmacokinetic profile of Rif and dRif from Patient J participating in the SPhEAR study

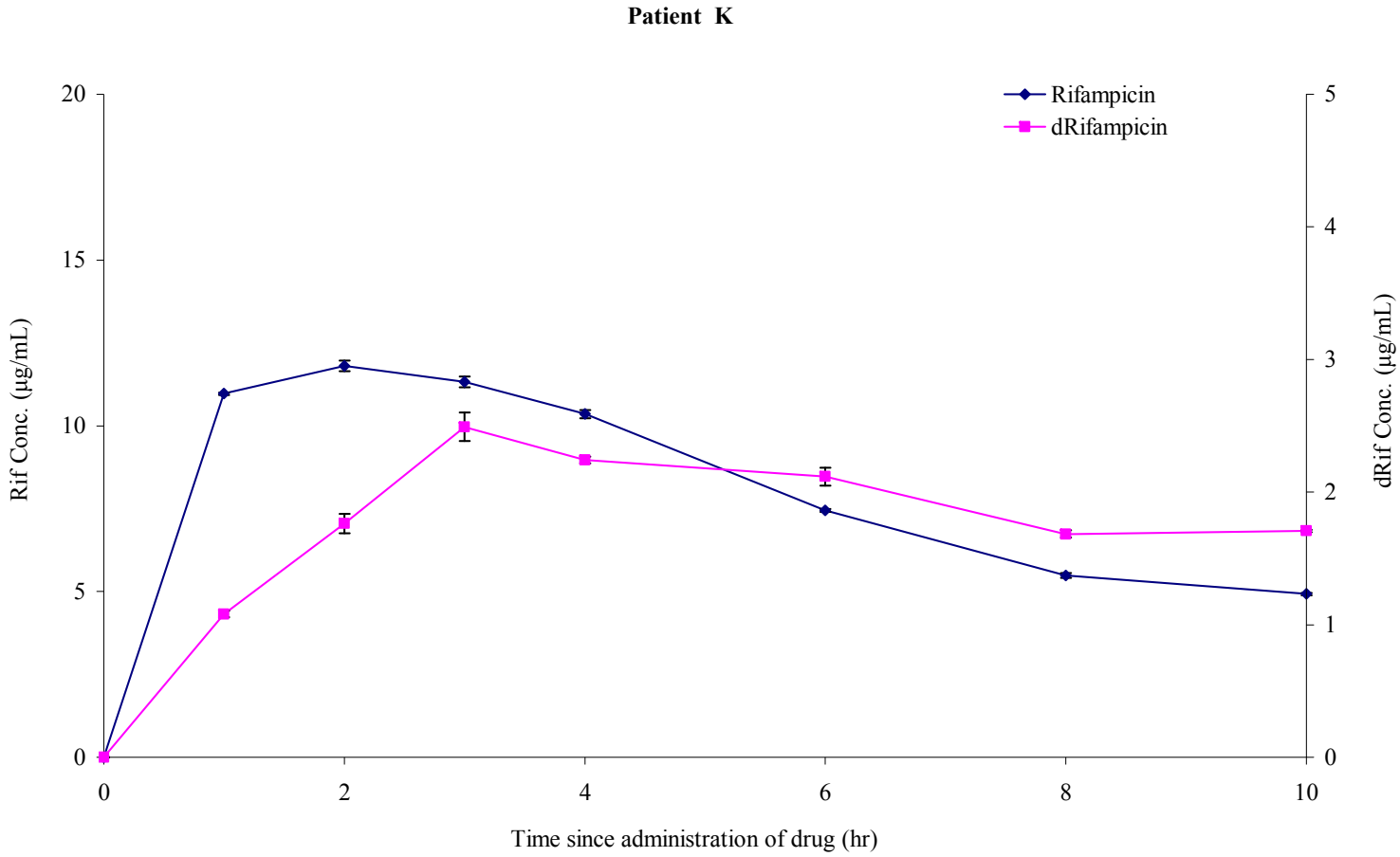

Figure 2.31: Pharmacokinetic profile of Rif and dRif from Patient K participating in the SPhEAR study

Patient	dRif		Rif	
	C_{max} (µg/mL) ± % RSD	T_{max} (hr)	C_{max} (µg/mL) ± % RSD	T_{max} (hr)
A	3.2 ± 3.1	2	15.6 ± 1.9	1
B*	3.1 ± 3.2	3	5.8 ± 1.7	2
C	3.6 ± 2.8	2	13.5 ± 1.5	2
D	2.3 ± 4.3	2	11.2 ± 0.9	2
E	1.6 ± 0.5	3	8.7 ± 2.3	3
F	3.2 ± 3.1	3	11.4 ± 0.9	1
G	1.9 ± 5.3	3	6.9 ± 1.4	2
H	11.4 ± 2.6	2	18.5 ± 1.1	2
I	3.9 ± 0.5	3	12.7 ± 5.5	2
J	3.9 ± 2.6	4	10.4 ± 1.9	4
K	2.5 ± 4.0	3	11.8 ± 0.8	2

Table 2.20: Comparison of Patient's C_{max} and T_{max} following Rif administration

*Co-administered Efv

To date, blood samples obtained from the SPhEAR study were of patients administered Rif only, with the exception of Patient B who was co-administered both Rif and Efv. Examination of the Rif and dRif data from all patients show that there are interpatient pharmacokinetic differences for this drug (as expected from published literature).

The same dose of Rif in patient B generated the lowest circulating blood concentration of drug than in any other patient. As this was the only patient who was co administered the drugs, it cannot be certain if this low level is due to the presence of Efv in the blood or not.

Circulating levels of dRif represent a fraction (approximately 20%-60%) of the levels of Rif found. Conclusive results cannot be made at this early stage with regard to if the pharmacokinetics of Rif are adversely affected when a patient is administered both drugs at the same time.

2.7 Conclusions

In conclusion, a novel isocratic high performance liquid chromatography method for the simultaneous determination of the anti HIV drug, Efv, the anti-TB drug, Rif and the desacetyl metabolite of Rif in plasma has been developed and validated. Using a Zorbax SB-Phenyl reverse-phase analytical column with UV detection, the assay employed solid phase extraction which achieved good separation and detection of the drugs within a fast run time of 10 min. The LOQ was found to be 0.1 μg/mL for each agent.

The described assay is accurate, precise and fast for the determination of these very different drugs in plasma. The easy sample preparation and fast separation makes this assay highly suitable for pharmacokinetic studies and therapeutic drug monitoring in patients with HIV only, TB only or both diseases being treated with Efv and Rif. This new protocol will be used in a SPhEAR project which will examine the effects of Efv medication on the pharmacokinetics of oral Rif in the treatment of TB in HIV infected patients. Analysed to date are blood samples from 11 patients participating in the study. Conclusive results with regard to the hypothesis proposed could not be made due to lack of patient samples from different patient categories of the project. Ten of the eleven patients were administered Rif only, with the exception of one who was co-administered both Rif and Efv. This patient generated the lowest circulating blood concentration of drug than in any other patient. Results to date show there are interpatient pharmacokinetic differences for this drug. Circulating levels of dRif represent a fraction (approximately 20%- 60%) of the levels of Rif found.

This fast, low cost and relatively easy UV-based assay can accurately detect the drug concentrations within a clinically relevant concentration range which renders it broadly applicable, especially in third world countries and in resource limited settings where the burden of disease exists. Using standard LC-UV equipment, it has the advantage of being cost effective and easy to use, especially when compared to MS which may not be available in laboratories in resource limited settings.

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Chapter 3

LC-MS determination of efavirenz and rifampicin levels in human plasma

3.1 Scope of research

The objective of this work was to develop an LC-MS method for the simultaneous determination of efavirenz and rifampicin from human plasma. As discussed in Chapter 2, appropriate treatment of microbial illness represents a significant pharmaceutical challenge. Nowhere is this more so than in the management of HIV infection, where patients often present with concurrent tuberculosis infection, thereby necessitating combination drug treatment. Examination of the SPhEAR hypothesis necessitated developing a new method capable of convenient, rapid and cost effective quantitation of two unrelated pharmaceuticals - the antiviral agent efavirenz (Efv) and the anti-TB agent, rifampicin (Rif) and this was achieved as outlined in Chapter 2. It was decided to further investigate the methodology in order to achieve greater sensitivity for the assay. This was achieved using LC-MS analysis.

3.2 Introduction

The previous chapter described work carried out to develop a LC-UV method preceded by SPE for determination of Rif, its metabolite dRif and Efv. It was decided to transfer the method to LC-MS in order to increase the sensitivity already achieved using the LC-UV method as there are no reported methods in the literature for the simultaneous analysis of Rif and Efv using LC-MS. Due to time constraints it was decided to focus on the primary drugs Rif and Efv for the method development and not optimise for Rif's metabolite dRif.

3.2.1 Sample extraction/clean-up procedures for efavirenz prior to LC-MS analysis

The three main sample extraction techniques employed to extract Efv from biological fluids are liquid-liquid extraction (LLE), protein precipitation (PP) and solid-phase extraction (SPE). All three approaches have been used prior to LC analysis of both drugs as discussed in section 2.2.5. In conjunction with LC-MS, all three sample preparation methods have been investigated as shown in Tables 3.1 and 3.2. Sample volumes required for PP have been reported as

quite low, in the region of 50 – 80 µL ((D'Avolio *et al.*, 2007, Volosov *et al*., 2002) and the recoveries obtained were high (93 – 110%). Rentsch *et al.,* 2003, describe the one reported SPE method for the extraction of Efv from human plasma before LC-MS detection. Recovery for Efv was high in this case (96.6-96.9%) however, the method required a large sample volume of 1 mL. Martin *et al.,* 2009, used PP and online SPE for the extraction of Efv from human plasma. Recovery was good at 75-98%.

3.2.2 Sample extraction/clean-up procedures for rifampicin prior to LC-MS analysis

Rif has also been successfully isolated from plasma/serum prior to LC-MS detection. From the literature, PP appears to be the most common approach used for the extraction of Rif from both plasma (de Velde *et al.,* 2009, Hartkoorn *et al.,* 2007) and serum (Song *et al.,* 2007). Recoveries obtained for Rif using this approach were high (84.5% or greater) and required relatively small sample volumes (0.05 mL or less).

Hartkoorn *et al.*, 2007, and Song *et al.,* 2007, employed ACN for protein precipitation. Hartkoorn used 50 µL of sample and achieved 92% recovery of Rif from plasma. Song *et al.,* 2007, used PP for the extraction of Rif from serum. Only a small volume of serum (50 µL) was required and protein precipitation was achieved using MeOH. The recovery of Rif in this case was 95%. De Velde *et al.,* 2009, required just 10 µL plasma for the precipitation of both Rif and dRif.

There are no reported sample extraction/clean-up procedures for Efv and Rif simultaneously.

Reference	Analytes	Matrix	Extraction mode	Extraction recoveries (%)	Detection	Approx retention time (min)	LOQ	Column	Sample vol. (µL)
(D'Avolio *et al.*, 2010)	Efv and other drug	Dried plasma spots on glass filter	n/a	>85.0	MS	~16.9	31.2 ng/mL	Atlantis T3 C18	Not stated
(Martin *et al.*, 2009)	Efv and other drugs	Human plasma	PP & on-line SPE	75-98	MS	1.9	100 ng/mL	LUNA Phenyl Hexyl	Not stated
(D'Avolio *et al.*, 2007)	Efv and other drugs	Human plasma	PP	93-110	MS	16.7	31.2 ng/mL	Atlantis dC-18	50
(Rouzes *et al.*, 2004)	Efv and other drugs	Human peripheral blood mononuclear cells	LLE	88.6	MS	8.3	2 ng/3 x 10^6 cells	X-Terra MS C18	Not stated
(Rentsch *et al.*, 2003)	Efv and other drugs	Human plasma	SPE	96-6-97.9	MS	16.3	10 ng/mL	Nucleosil C18 HD	1000
(Volosov *et al.*, 2002)	Efv and other drugs	Human plasma	PP	Not stated	MS	4.5 min runtime	10 ng/mL	Supelco LC-18-DB	80

Table 3.1: Reported extraction and LC-MS detection techniques for Rif from biological samples

Reference	Analytes	Matrix	Extraction mode	Extraction recoveries (%)	Detection	Approx retention time (min)	LOQ/LOD	Column	Sample vol. (µL)
(de Velde *et al.*, 2009)	dRif, Rif and other drugs	Human plasma	PP	dRif: 104.1-116.9 Rif: 101.3-109.4	MS	dRif: 2.3 Rif: 2.45	200 ng/mL LLOQ	HyPurity Acquastar C18	10
(Song *et al.*, 2007)	Rif and other drugs	Human serum	PP	94.80	MS	3.4	50 ng/mL	Hydrosphere C18	50
(Hartkoorn *et al.*, 2007)	Rif	Human cells and plasma	PP	Plasma -85-95 Cells - 51.73	MS	1.1	100 ng/mL	Betasil Phenyl-Hexyl	50

Table 3.2: Reported extraction and LC-MS detection techniques for Efv from biological samples

3.2.3 Sample separation and MS detection approaches for efavirenz

D'Avolio *et al.*, 2010, and Simiele *et al.,* 2010, described the most recently used MS method for the detection of Efv using a C18 column. The method can detect Efv down to 31 ng/mL but Efv took 16.9 mins to elute. Martin, *et al.,* 2009, described a MS method in which Efv was separated using a phenyl column which resulted in an extremely short retention time of just 1.9 mins but the method lacked sensitivity with an LOQ of just 100 ng/mL. Rentsch *et al.,* 2003, obtained an LOQ of 10 ng/mL using MS but again Efv had a long retention time of 16.3 mins using a C18 column. Volosov *et al,,* 2002, used MS for Efv's detection and achieved an excellent LOQ of 10 ng/mL with a short runtime of just 4.5 mins.

Matthews *et al.,* 2002, developed a HPLC method with post-column photochemical derivatisation and fluorescence detection for the determination of Efv and its enantiomer in plasma. Once the drugs had been extracted from the plasma matrix they were separated on a YMC-Pack C8 analytical column. The LOQ achieved was 50 ng/mL; however, 0.5 mL plasma was required.

3.2.4 Sample separation and MS detection approaches for rifampicin

Hartkoorn *et al.,* 2007, Song *et al.,* 2007, and de Velde *et al.,* 2009, developed LC-MS methods for the determination of Rif. Sample volumes are low, retention times are fast, but good sensitivity has been difficult to achieve. Hartkoorn achieved separation on a betasil phenyl-hexyl column using an isocratic mobile phase composed of ammonium acetate and acetonitrile at a flow rate of 0.4 mL/min. Mass spectral analysis for Rif was carried out using electrospray ionisation (ESI) in the positive ion mode with a capillary temperature of 250 °C. The LOQ was found to be 100 ng/mL using only 50 µL plasma. Song developed a HPLC/tandem MS method for the determination of first-line anti-tuberculosis drugs (including Rif) and their major metabolites. Samples were analysed using a Hydrosphere C18 column. However, the assay required a gradient with mobile phase containing a mixture of methanol and water. Chromatographic separation

was performed at room temperature for 4 min. Using a Quattro Micro tandem mass spectrometer, quantification was achieved by multiple reaction monitoring (MRM) in positive ion mode. The LOQ obtained was 50 ng/mL with again only 50 µL serum required. De Velde required only 10 µL plasma for the extraction of Rif, however an LOQ of 200 ng/mL was all that could be achieved.

There are no reported LC-MS methods for the simultaneous determination of Efv and Rif.

3.3 Experimental

3.3.1 Reagents and materials

MS grade acetonitrile (ACN), methanol (MeOH), water and ammonium acetate were purchased from Sigma-Aldrich, United Kingdom. Formic acid and acetic acid glacial, analytical grade were obtained from Sigma Aldrich, United Kingdom. Drug-free sterile filtered human plasma (EDTA k3) was purchased from Europa Bioproducts, United Kingdom. Efv and Rif were purchased from Sequoia Research Products Ltd. United Kingdom. SPE extraction cartridges and plates were purchased from Biotage, Sweden. 1.1 mL Chromacol vials were purchased from Fisher Scientific, Ireland.

3.3.2 Equipment and HPLC assay conditions

The LC-MS system consisting of a 1200 quaternary pump, autosampler and degasser and a 6410 QQQ LC/MS, all from Agilent, United Kingdom. System management and data acquisition were performed using Agilent Masshunter B.01.03 WorkStation Data Acquisition software. Other instrumentation employed included a pH meter and electronic mass balance both from a Mettler Toledo, USA a Genevac EZ-2 personal evaporator from Ipswich, United Kingdom. The VacMaster-96 sample processing manifold was sourced from Biotage, Sweden. Chromatographic separation was achieved using an Agilent Zorbax SB Phenyl

column, (150 x 4.6 mm, 5 µm). A mixture of ammonium acetate (pH 4.75, adjusted with 1 M acetic acid, 20 mM), ACN and MeOH (40:45:15, v/v/v) was used as mobile phase, at a flow rate of 0.8 mL/min. The column temperature was maintained at 20 °C and the temperature of the autosampler was maintained 4 °C. The complete chromatographic run time of each sample was 10 min. Peaks were quantified using Agilent Masshunter Software.

The mass spectrometer was operated using an ESI source in the positive ion detection mode. The ionisation temperature was 375 °C, gas flow rate was 11 L/min and nebuliser pressure was 50 psi. Nitrogen was used as the ionisation source gas and ultrapure nitrogen as the collision cell gas. Analysis was performed in MRM mode with the following transitions: *m*/*z* 823.4 → *m*/*z* 791.3 for Rif, *m*/*z* and *m*/*z* 316.1 → *m*/*z* 168.0 for Efv with a dwell time of 200 ms. Transitions and their optimal detector settings are listed in Table 3.3. The quantifier ions are indicated in the table though all transitions were monitored with the qualifier ions adding specificity.

Compound	**Precursor ion**	**Optimum fragmentor voltage**	**Product ion**	**Optimum collision energy**
Rif	823.4	150	791.3*	12.5
			399.2	22.5
Efv	316.1	100	272.1	2.5
			244.1	7.5
			168.0*	22.5
			52.9	17.5

Table 3.3: Optimal fragmentor voltages and collision energy settings for Rif and Efv

* Quantifier ion

Quantification was based on the integrated peak area as determined by the Masshunter quantification analysis software which quantitates the peak areas of the MRM transitions of each analyte.

3.3.3 Preparation of standards

Stock standard solutions of Rif and Efv were prepared by weighing out the powders in a glovebox, dissolving them in MS grade ACN to 1 mg/mL concentration and storing them in amber vials at 2-4 °C in the dark. Working standards of Efv and Rif were prepared both in drug-free human plasma and in mobile phase (ammonium acetate (pH 4.75, adjusted with 1 M acetic acid, 20 mM), ACN and MeOH (40:45:15, v/v/v)) to concentrations over the range 0.01–20 μg/mL (0.01, 0.1, 1, 2, 10 and 20 μg/mL in plasma which corresponded to 0.02, 0.2, 2 and 4, 20 and 40 μg/mL in MP) for Efv and 0.001–20 μg/mL (0.001, 0.01, 0.1, 1, 2, 10 and 20 μg/mL in plasma which corresponded to 0.002, 0.02, 0.2, 2 and 4, 20 and 40 μg/mL in MP) for Rif. 20 μL was injected into the HPLC system for analysis.

A 0.3 mL aliquot of each of the working standards was diluted 1:1 (v:v) with a 1% formic acid solution in water. The SPE Evolute Array Wells (25 mg ABN, 1 mL) in the 96 well plate sample processing manifold were conditioned with 1 mL MeOH and then equilibrated with 1 mL of the 0.1% formic acid solution. A 0.6 mL aliquot of the diluted, acidified sample was loaded onto the well, washed with 20% MeOH in water and eluted with a water (1 mg/mL ascorbic acid)-ACN-MeOH mixture (25:50:25, v/v/v). The samples were evaporated to dryness and reconstituted with 150 μL HPLC mobile phase (ammonium acetate (pH 4.75, adjusted with 1 M acetic acid, 20 mM), ACN and MeOH (40:45:15, v/v/v)). The overall clean-up process resulted in the diluted plasma sample being concentrated by a factor of four which equated to an overall enrichment factor of two for the neat plasma. The reconstituted sample was then transferred to chromacol vial and 20 μL was injected into the HPLC system for analysis.

3.3.4 Method Validation

Due to time constraints, the method was only partially validated. Validation parameters including sensitivity, linearity, range and recovery were determined for the method.

3.4 Results and discussion

3.4.1 Method development

Chapter 2 describes a method capable of extraction, separation and detection of Rif, its metabolite dRif and Efv in a single assay utilising UV detection. The sensitivity achieved was sufficient for its intended purpose in the SPhEAR project, however, there is no method in the literature capable of extracting both Rif and Efv simultaneously using LC-MS and so a transfer to an MS-based analysis method was proposed in order to achieve greater sensitivity, potentially allowing for much smaller sample volumes and less inconvenience for patients having drug levels monitored.

3.4.2 Chromatographic procedure

As the optimised chromatographic conditions developed for the LC-UV method proved excellent in terms of separation of the drugs, it was decided to keep the analytical method in keeping with these conditions and focus method development on optimising the MS conditions. Refer to section 2.4.2.

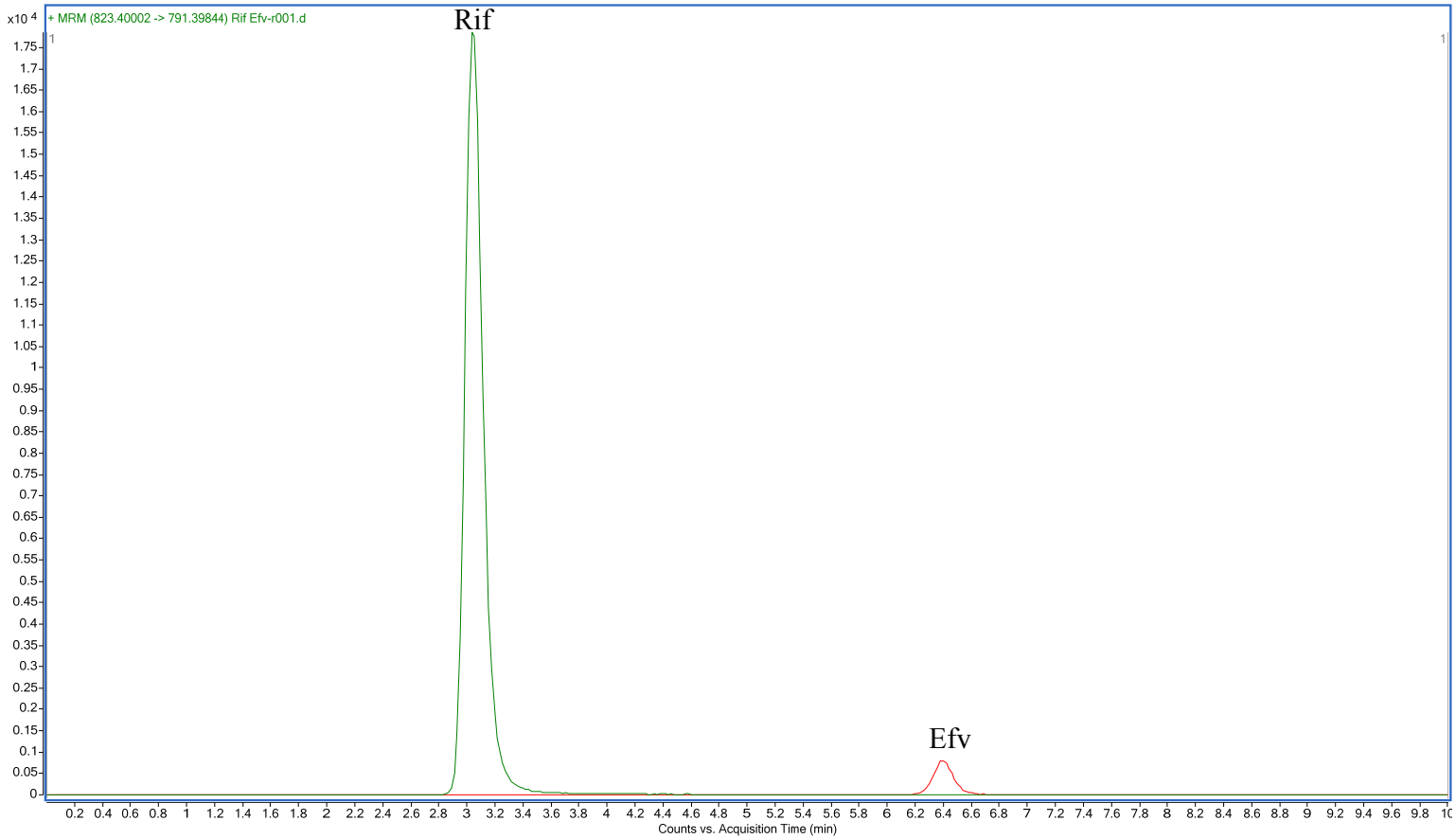


Figure 3.1: Chromatogram of a working standard of Efv and Rif prepared to 500 ng/mL in mobile phase

3.4.3 Extraction procedure

There were no changes required for the SPE column or its dimensions in the method transfer. The SPE protocol using Evolute ABN cartridges was the same as for the LC-UV method discussed in Chapter 2. Refer to section 2.4.4.

3.4.4 MS detection

Briefly discussed in Chapter 1 was the triple quad MS. The MS system utilised for this assay consisted of a 6410 QQQ MS from Agilent, United Kingdom. The quadrupoles are the first and last sections (MS1 and MS2) of the instrument, with a collision cell in the middle section. See Figure 3.2. By creating oscillating electric fields, the quadrupole determines the selectivity of the ions trajectory to the detector. In the collision cell, ions collide with nitrogen gas atoms to give rise to product ions. The fragment ions resulting then proceed to the last quadrupole (MS2) where the mass to charge ratio (*m/z*) is detected. There are various modes in which the triple quad MS can operate.

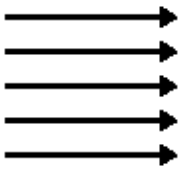
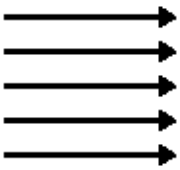
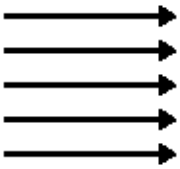
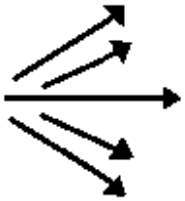
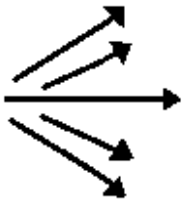
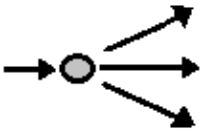
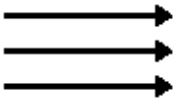
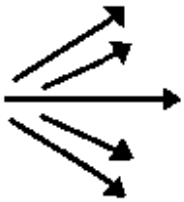
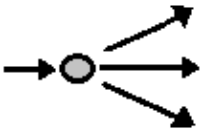
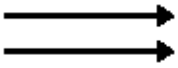
MS mode	MS1	Collision cell	MS2
MS2 scan			 Monitor all ions
SIM	 Select *m/z*		 Monitor single ion
Product ion scan	 Select *m/z*		 Product ion scan
MRM	 Select *m/z*		 Monitor selected fragment ion(s)

Figure 3.2: Schematic diagram of mode of action for each quad in the various MS scan modes using the Agilent 6410 QQQ MS

MS2 Scan

MS2 Scan mode is where the first quadrupole allows all ions unfiltered into the collision cell. The collision cell directs the ions to pass unchanged and enter into the MS2 where all ions are detected. See Figure 3.2.

SIM

In selected ion monitoring the precursor ion is detected without collision in MS2. MS1 filters the ions so that only the ions of a particular *m/z* ratio are permitted into the system. The detector continues to monitor the same single *m/z* value over the entire scan period, as shown in Figure 3.2.

Product Ion Scan

Product ion scan is used to determine the product ions. The ions enter MS1 where only the ions of interested are filtered in and sent to the collision cell. Through collision with nitrogen gas, at operator controlled voltages, the precursor ion is broken into the product ion(s). In this way it is possible to identify the product ions, as shown in Figure 3.2.

MRM

Detection and quantification by MRM mode is undoubtedly the optimum in bio-analytical methods, as the incoming ion stream is filtered for the specific ion of interest. The specific ion undergoes fragmentation in the collision cell, in the presence of an inert gas, in this case pure nitrogen, to yield product ions. The most abundant product ion is used for quantification, while any additional product ions detected are used as qualifier ions. Qualifier ions increase the specificity of the analysis.

Using flow injection analysis (FIA) the optimum MRM settings were determined for Rif and Efv. Optimisation was carried out by FIA injecting 250 ng/mL of the drug, varying each condition sequentially. The use of an injector program allowed for the multiple injection of the same compound, from the same vial, a defined number of times. This permits multiple injections to be viewed and analysed in one data file, which eliminates the majority of error associated with inter-injection variability.

The settings of each ion source/spray chamber depend on the mobile phase composition, flow rate, and sample identity. The nebuliser pressure, drying gas flow, and drying gas temperature are dependent upon the mobile phase composition and flow rate. The settings used were set according to the MS operation manual for a mobile phase flowrate of 0.8 mL/min. Drying gas temperature was set to 375 ^{0}C, drying gas flow to 11 L/min and nebuliser pressure to 50 psi and with these settings, the following experiments were carried out in order to find the best parameters for each drug.

3.4.4.1 Precursor ion determination

The precursor ion is an electrically charged molecular moiety which may dissociate to form fragments (Westman-Brinkmalm *et al.*, 2008). These fragments may be charged or neutral moieties. Charged fragments can be detected and used to further identify the analyte.

Chromatographic conditions were simulated as for the assay developed, a flow rate of 0.8 mL/min and ammonium acetate (pH 4.75, adjusted with 1 M acetic acid, 20 mM), ACN and MeOH (40:45:15, v:v:v) MP were used while optimising the MS conditions. From the autosampler, 10 µL of sample were injected into the mass spectrometer by a FIA setup where a TIC scan (discussed in Chapter 1) was run in order to establish the predominant precursor ion. The range was set from 600 to 850 *m/z* for Rif and from *m/z* 200 to 400 for Efv. These ranges were chosen as they bracketed the molecular weights of Rif and Efv. Once a

prominent peak was seen at the expected *m/z* value, this was deemed to be the precursor ion, Rif had a *m/z* of 823.4 and Efv had a *m/z* of 316.1, see Figure 3.3.

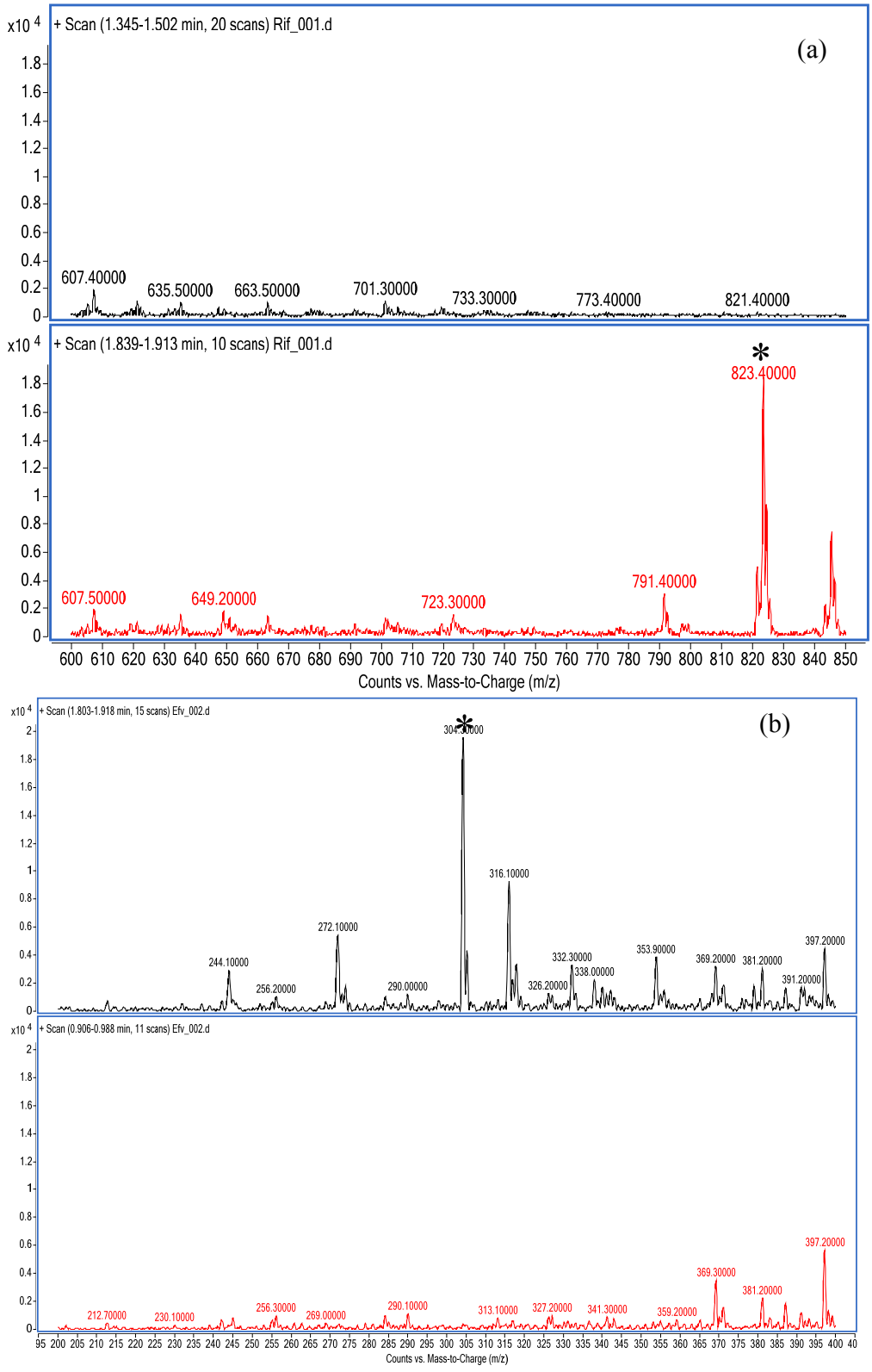


Figure 3.3: Comparison of (a) background and Rif* and (b) background and Efv*

3.4.4.2 Fragmentor voltage optimisation

Once the precursor ion had been established, the next step was to determine the optimum fragmentation conditions for that precursor ion in order to maximise its intensity. The fragmentor voltage (FV) is not actually involved in the fragmentation of the precursor ion to the product ion, rather it acts as a filter for the MS. The purpose of the fragmentor voltage is to preferentially maximise the throughput of precursor ions into the MS.

The mass spectrometer was programmed to perform an MS selected ion monitoring (SIM) scan which detects only the selected precursor ion so that the optimum fragmentor voltage for that ion could be determined. To increase accuracy duplicate injections were performed. These SIM scans were done in series where the fragmentor voltage was varied over a range of 20 to 160 V using voltage increments of 20 V. See Figure 3.4 and Figure 3.5. The aim was to find the highest intensity for the precursor ion as this would maximise sensitivity of the assay. Often, more stable molecules will require higher voltages in order to fragment in source while more fragile molecules will need less voltage to break apart. 150 V achieved optimal results for Rif while 100 V was the best voltage setting for Efv. See Table 3.4. The reduction in peak area at higher fragmentor voltage is due to the precursor ion being broken down before entering the MS.

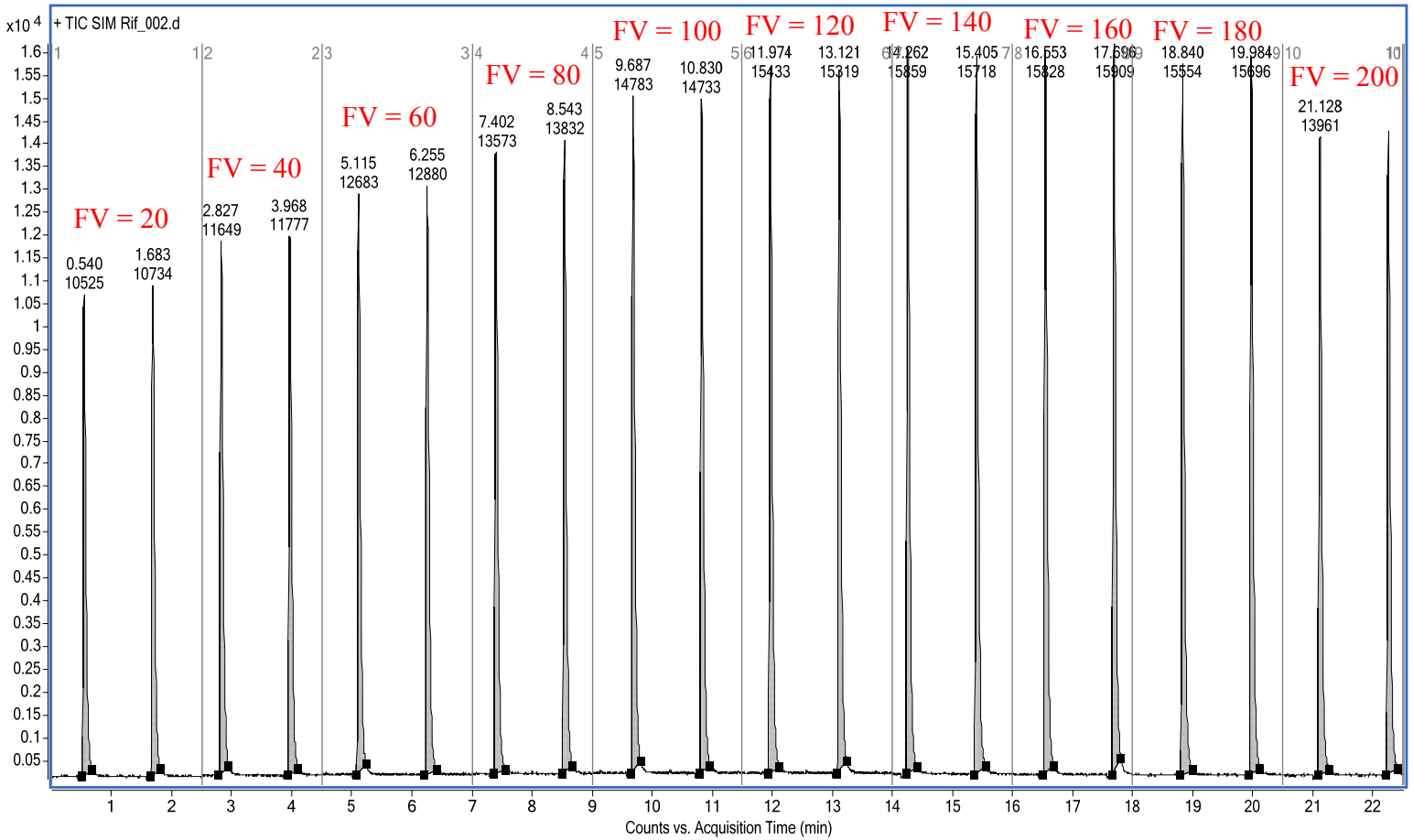


Figure 3.4: TIC for *m/z* of 823.4 for Rif in SIM mode with increasing fragmentor voltage

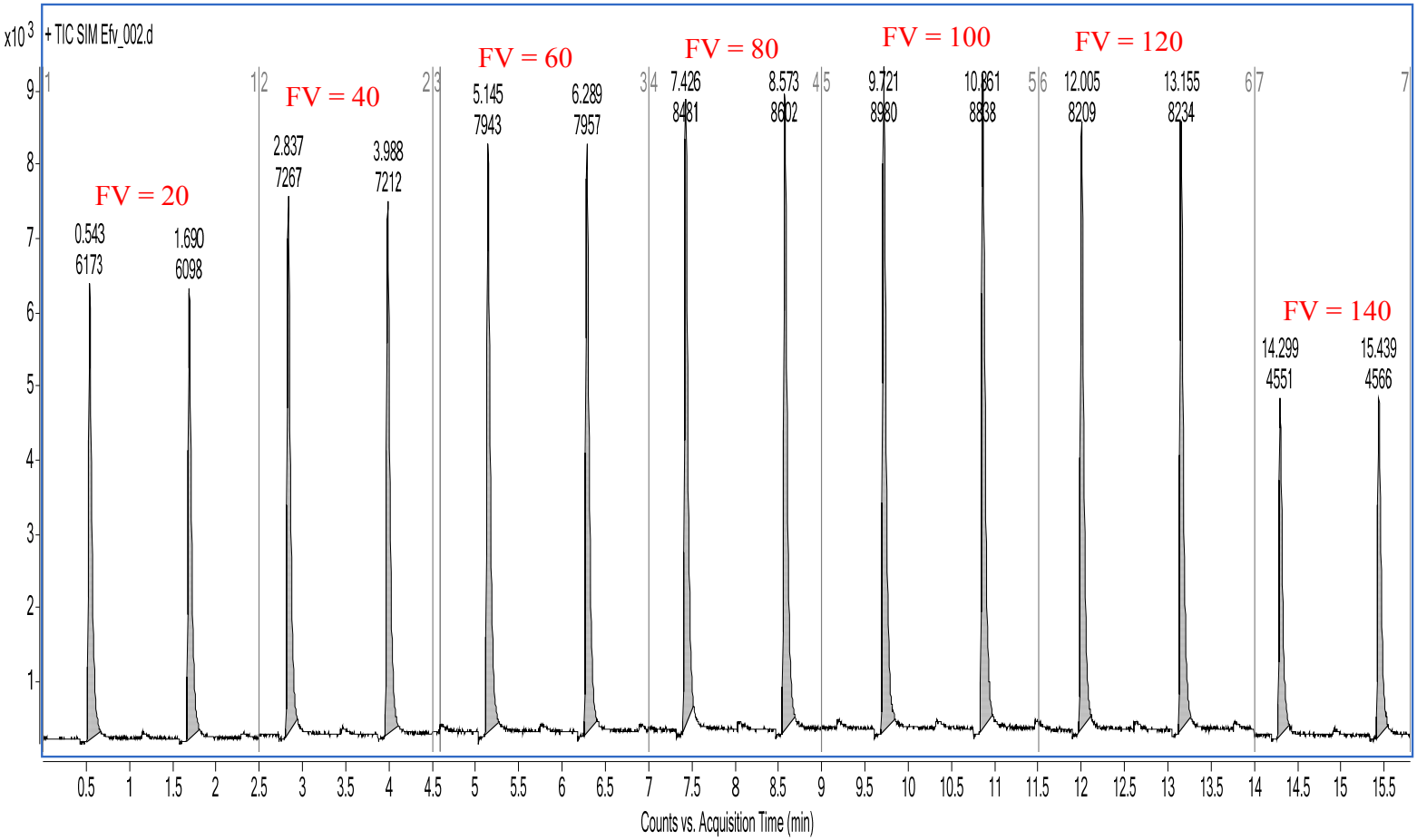


Figure 3.5: TIC for *m/z* of 316.1 for Efv in SIM mode with increasing fragmentor voltage

Voltage	Rif		Efv	
	Area	Area %	Area	Area %
20	33489	66.27	16757	67.02
	33262	65.82	16711	66.84
40	36809	72.84	19477	77.90
	37483	74.17	19943	79.76
60	39812	78.78	22143	88.56
	41063	81.25	22133	88.52
80	44053	87.17	22768	91.06
	43590	86.25	23613	94.44
100	46167	91.35	25003	100.00
	46631	92.27	24431	97.71
120	49027	97.01	22435	88.73
	48022	95.02	22518	90.06
140	50323	99.58	12235	48.93
	50389	99.71	12367	49.46
160	50537	100.00		
	49601	98.15		
180	50082	99.10		
	49385	97.72		
200	44687	88.42		
	44396	87.85		

Table 3.4: Comparison of fragmentation voltages for Rif and Efv on peak area of integrated SIM

3.4.4.3 Product ion determination

Having established the optimum fragmentor voltage for Rif and Efv, the next step involved carrying out a product ion scan in order to identify the product ions of Rif and Efv's precursor ions. The product ions are the ions detected by the detector in the third quadrupole of the mass spec, as a result of the fragmentation of preselected precursor ions in the collision cells.

This was achieved by firstly isolating and then fragmenting the precursor ion with varying collision energy values ranging from 0 to 40, using increments of 10. By exposing the precursor ion to increasing collision energy, fragmentation occurred and the product ions were determined As it is very common for compounds to readily lose water and or ammonia, it's important not to choose a fragment peak too near to the precursor peak. Choosing the ammonia or water loss peaks for the MRM experiment would result in a less than unique transition.

Figure 3.6 shows the extracted MS spectrum of the TIC of the product ion scan for Rif. Figure 3.6 (A) illustrates Rif's precursor ion with no collision energy applied. Figure 3.6 (B) is with a collision energy setting of 10 V applied. The product ion *m/z* 791.3 was seen. A drop in the abundance of the precursor ion was also seen, indicating that the *m/z* 791.3 ion came from *m/z* 823.4. Figure 3.6 (C) showed that a collision energy of 20 V gave a second product ion at m/z 399.2 and left little precursor ion. Figure 3.6 (D) with a collision energy of 30 V applied showed that a high collision energy completely broke down the precursor ion so that it was no longer detectable. The ion with *m/z* of 791.3 was the most abundant ion and so was used for quantification of Rif.

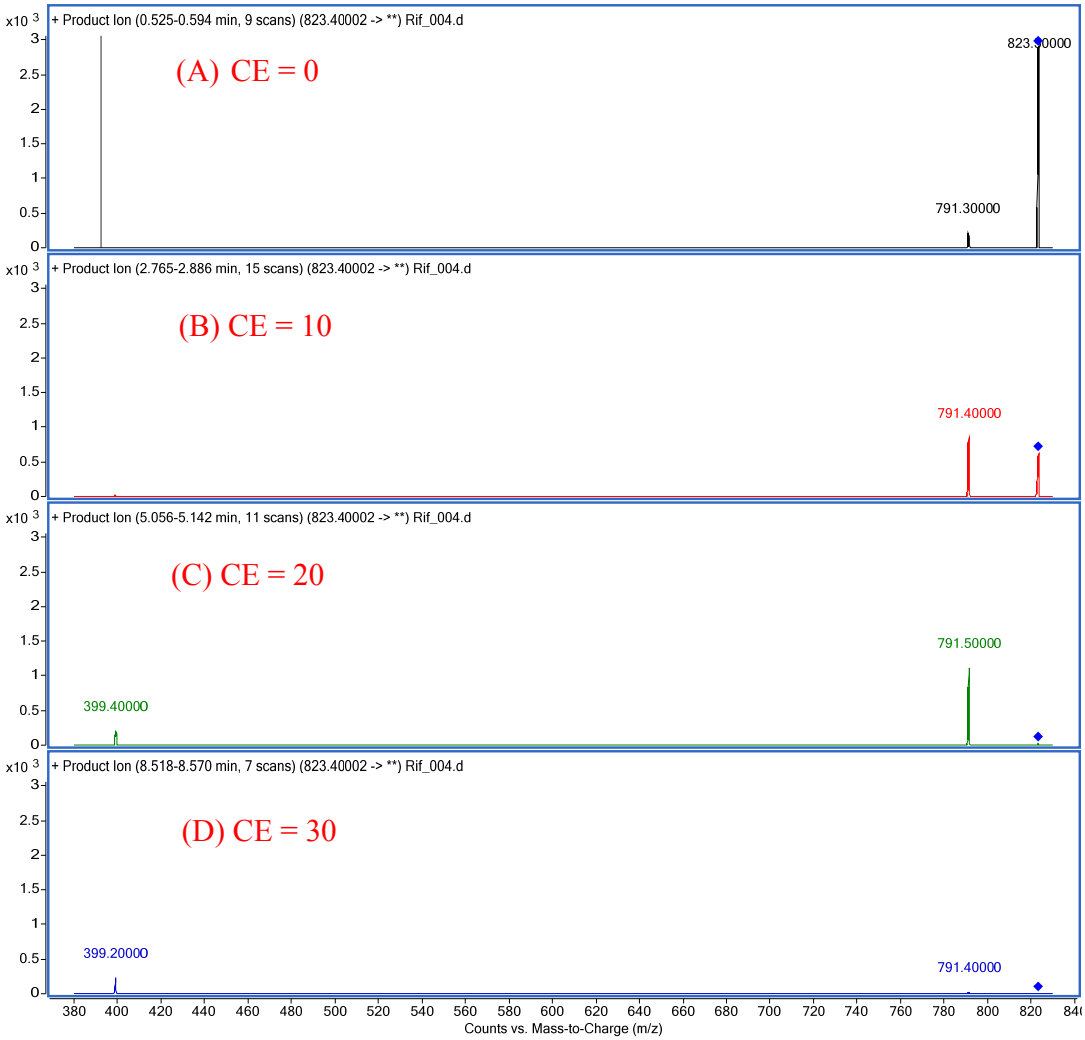


Figure 3.6: TIC for the product ion scan of Rif with increasing collision energies

Figure 3.7 shows the extracted MS spectrum of the TIC of the product ion scan for Efv. Figure 3.7 (A) illustrates Efv's precursor ion with no collision energy applied. Figure 3.7 (B) is with a collision energy setting of 10 V applied. Four product ions of *m/z* 272.1, *m/z* 244.1, *m/z* 168.0 and *m/z* 52.9. were seen and left little precursor ionindicating these ions came from *m/z* 318.1. Figure 3.7 (C, D and E) of collision energies 20, 30 and 40 V respectively showed that a high collision energy completely broke down the precursor ion so that was it was no longer detectable. *M/z* 168.0 was the most abundant ion and so was used for quantification of Efv.

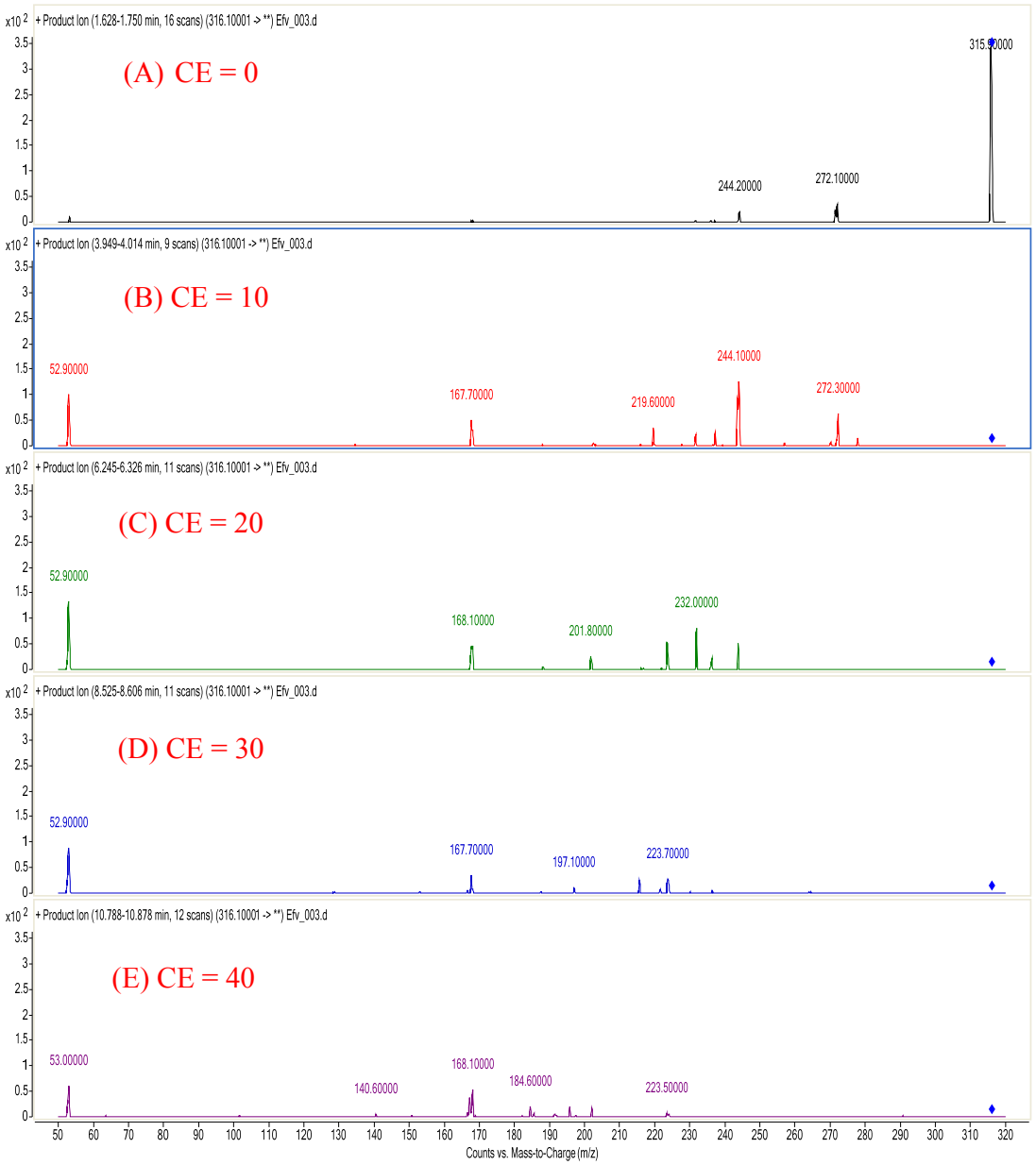


Figure 3.7: TIC for the product ion scan of Efv with increasing collision energies

3.4.4.4 MRM transition optimisation

MRM can track many precursor ions and their multiple product ions through the MS to provide spectra that are quantifiable. Each transition from precursor ion to product ion can have different collision energies. By optimising the MRM transition through FIA, the collision energy for each transition was optimised.

MRM scans which detected the MS/MS transitions were carried out in order to obtain the optimum collision energy (CE) for fragmentation of Rif and Efv into their product ions. Duplicate injections were performed to increase accuracy. Collision energy was raised in increments of 5 V starting from 0 V so that an accurate collision energy could be established. The collision energy that gave the most intense peak for the chosen product ion was deemed to be the optimum value. Figures 3.8 and Figure 3.9 show the determination of the optimum MRM transition setting for Rif and Figures 3.10 through to Figure 3.13 show the determination of the optimum MRM transition setting for Efv. Tables 3.5 and 3.6 display the results obtained for the optimum MRM transition settings for both Rif and Efv.

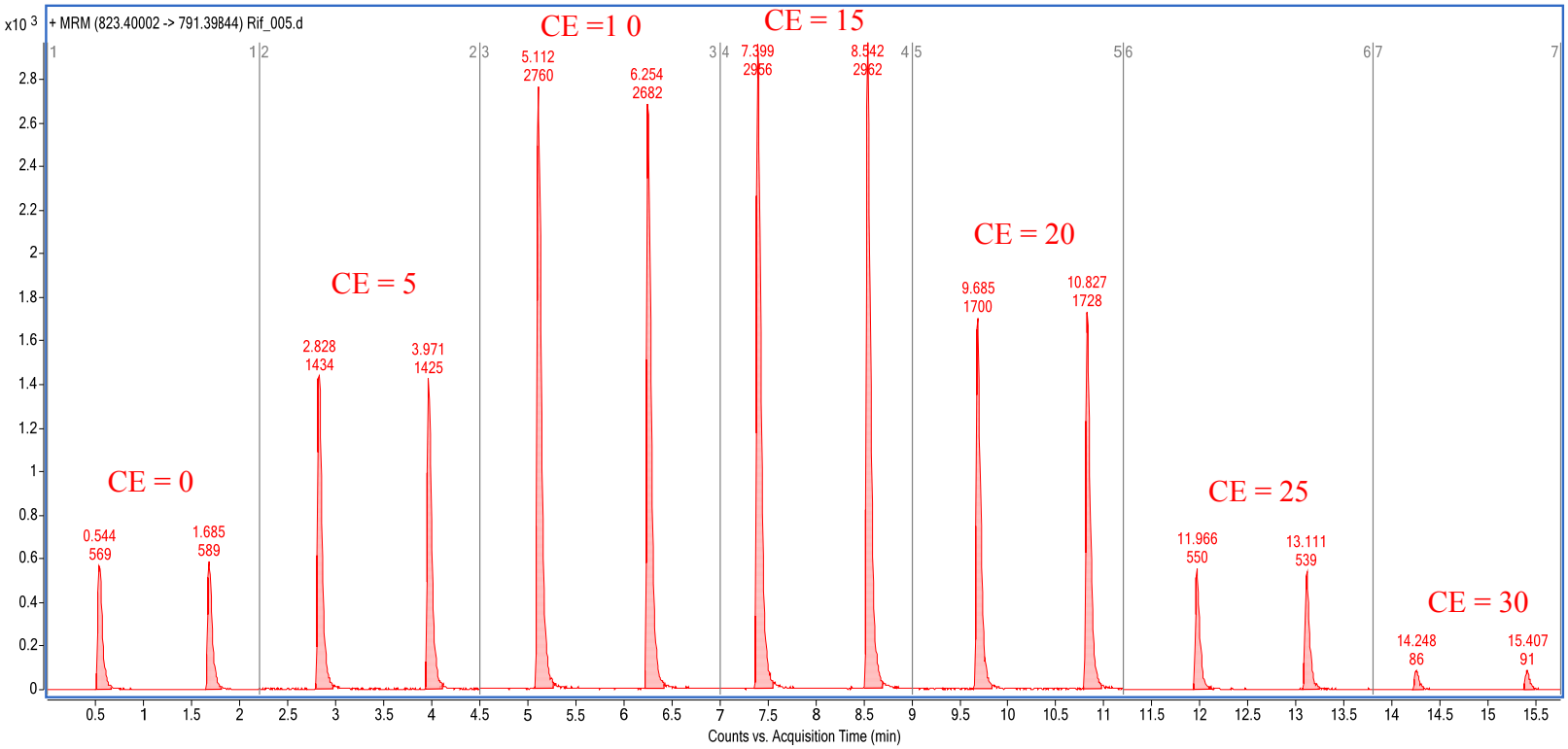


Figure 3.8: Rif product ion *m/z* 791.3 with increasing collision energies

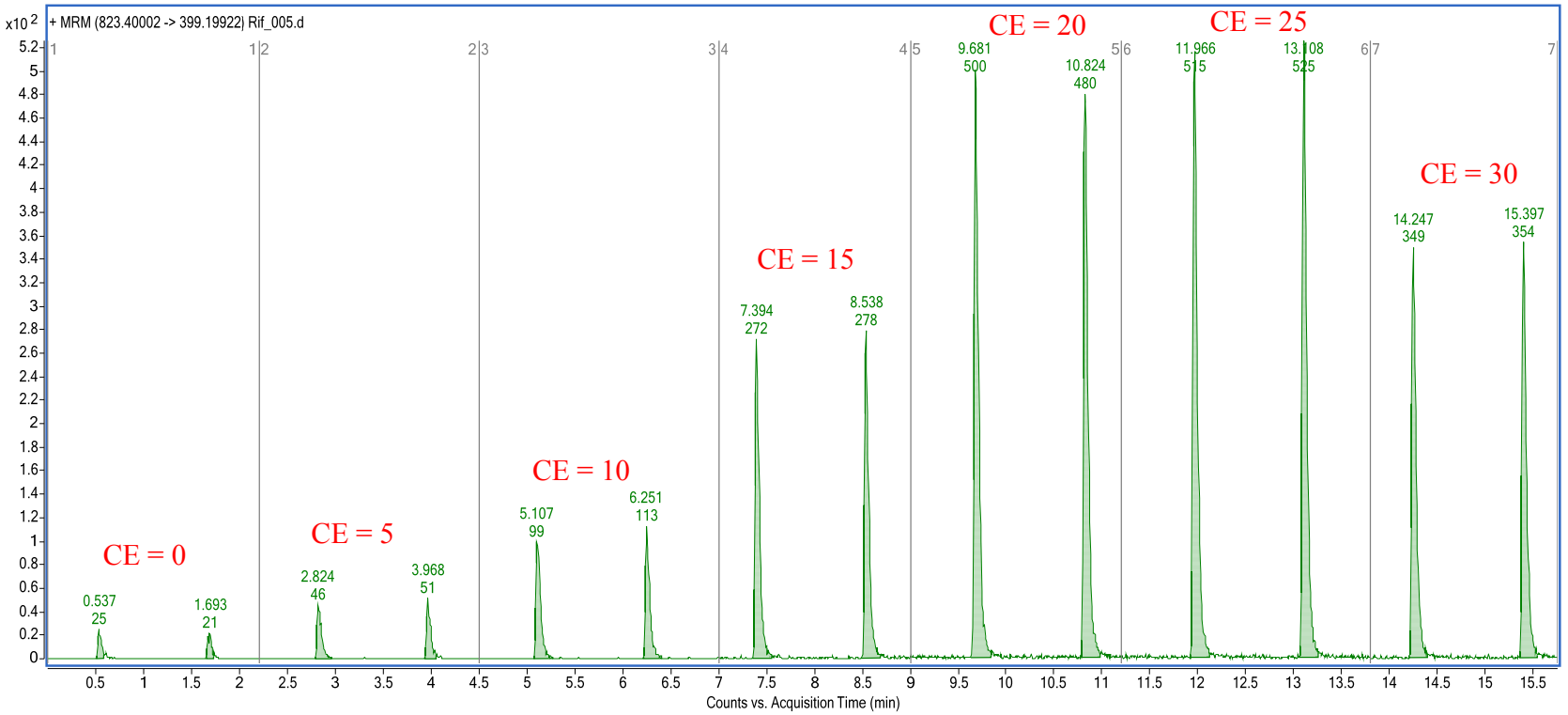


Figure 3.9: Rif product ion *m/z* 399.2 with increasing collision energies

Voltage	791.3		399.2	
	Area	Area %	Area	Area %
0	1908	19.18	57	3.29
	1907	19.17	56	3.23
5	4744	47.68	142	8.19
	4666	46.90	145	8.36
10	9102	91.48	338	19.46
	9050	90.96	348	20.03
15	9949	100.00	880	50.63
	9829	98.80	874	50.30
20	5715	57.44	1663	95.73
	5753	57.82	1610	92.69
25	1761	17.70	1723	99.16
	1736	17.45	1737	100.00
30	282	2.83	1133	62.50
	281	2.82	1151	66.24

Table 3.5: Collision energy results for product ions of Rif

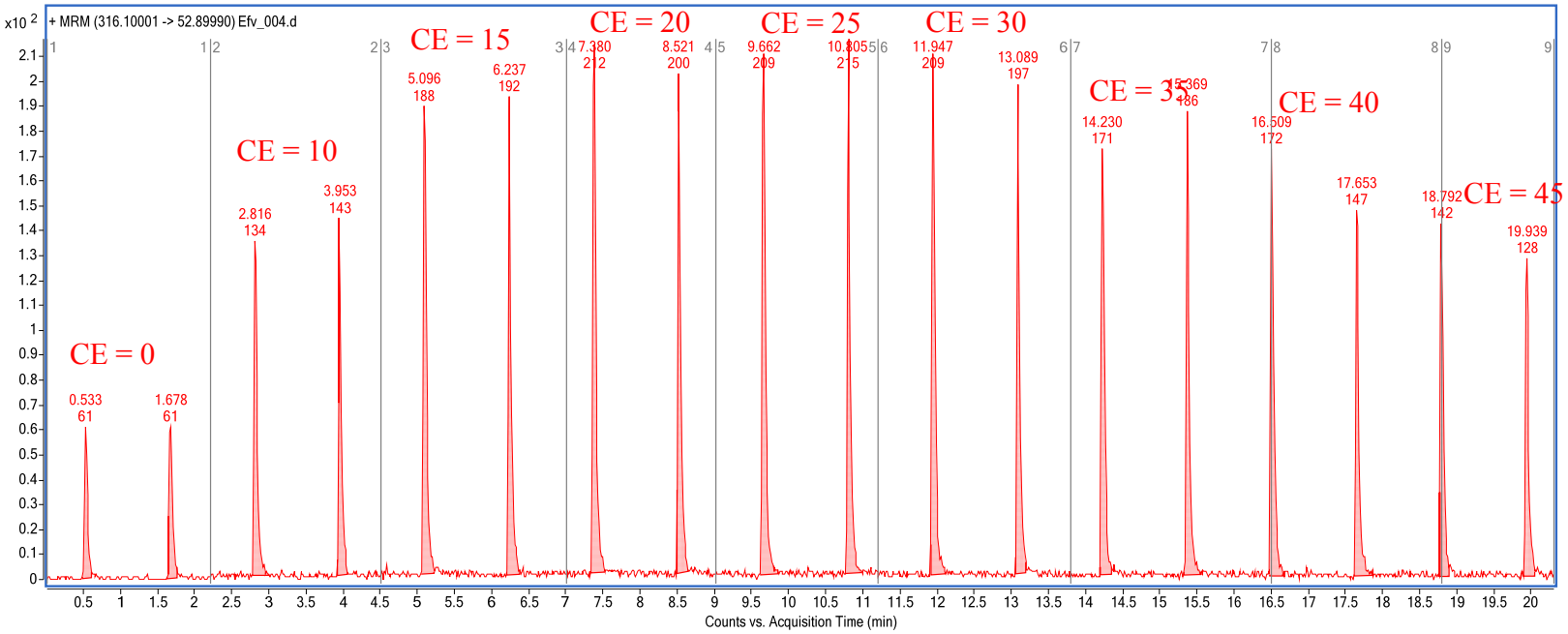


Figure 3.10: Efv product ion *m/z* 52.9 with increasing collision energies

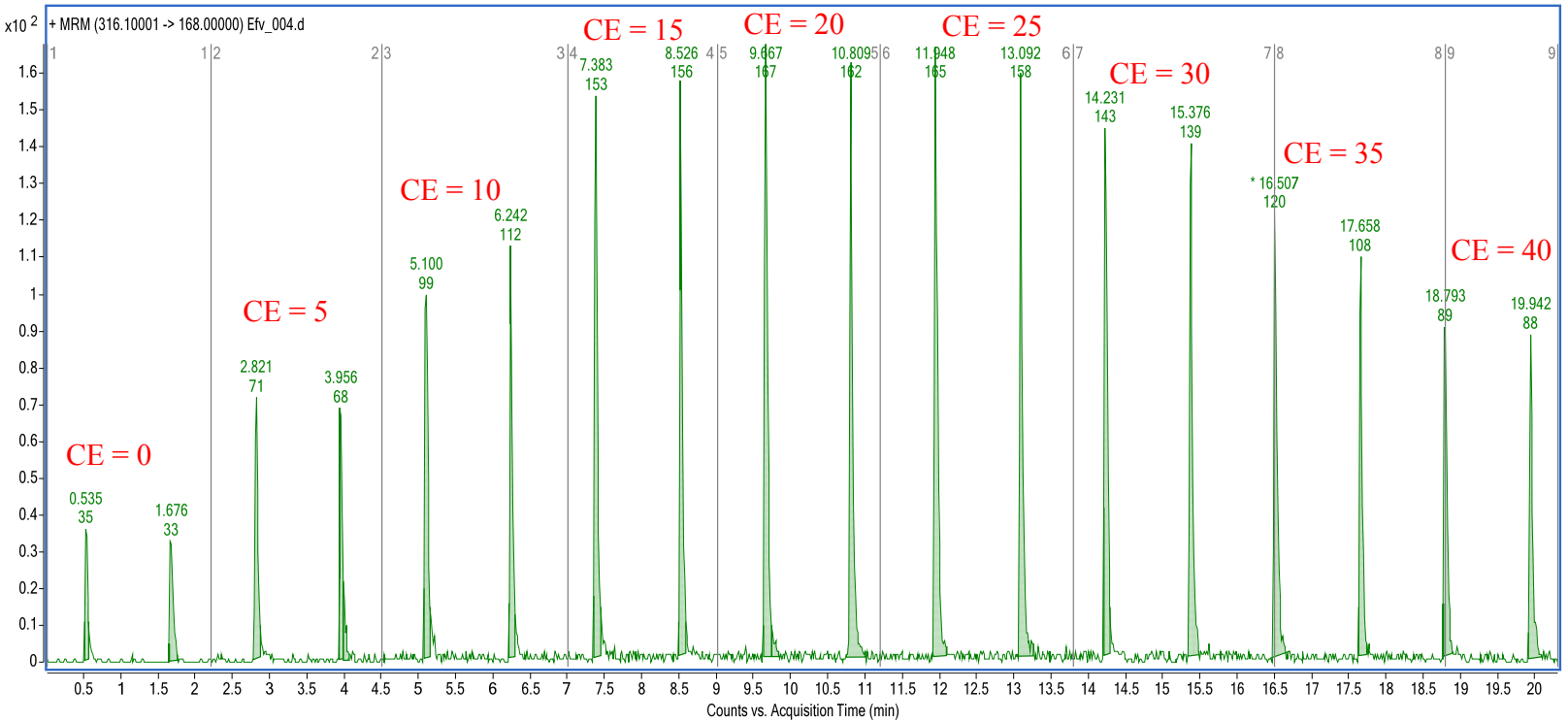


Figure 3.11: Efv product ion *m/z* 168.0 with increasing collision energies

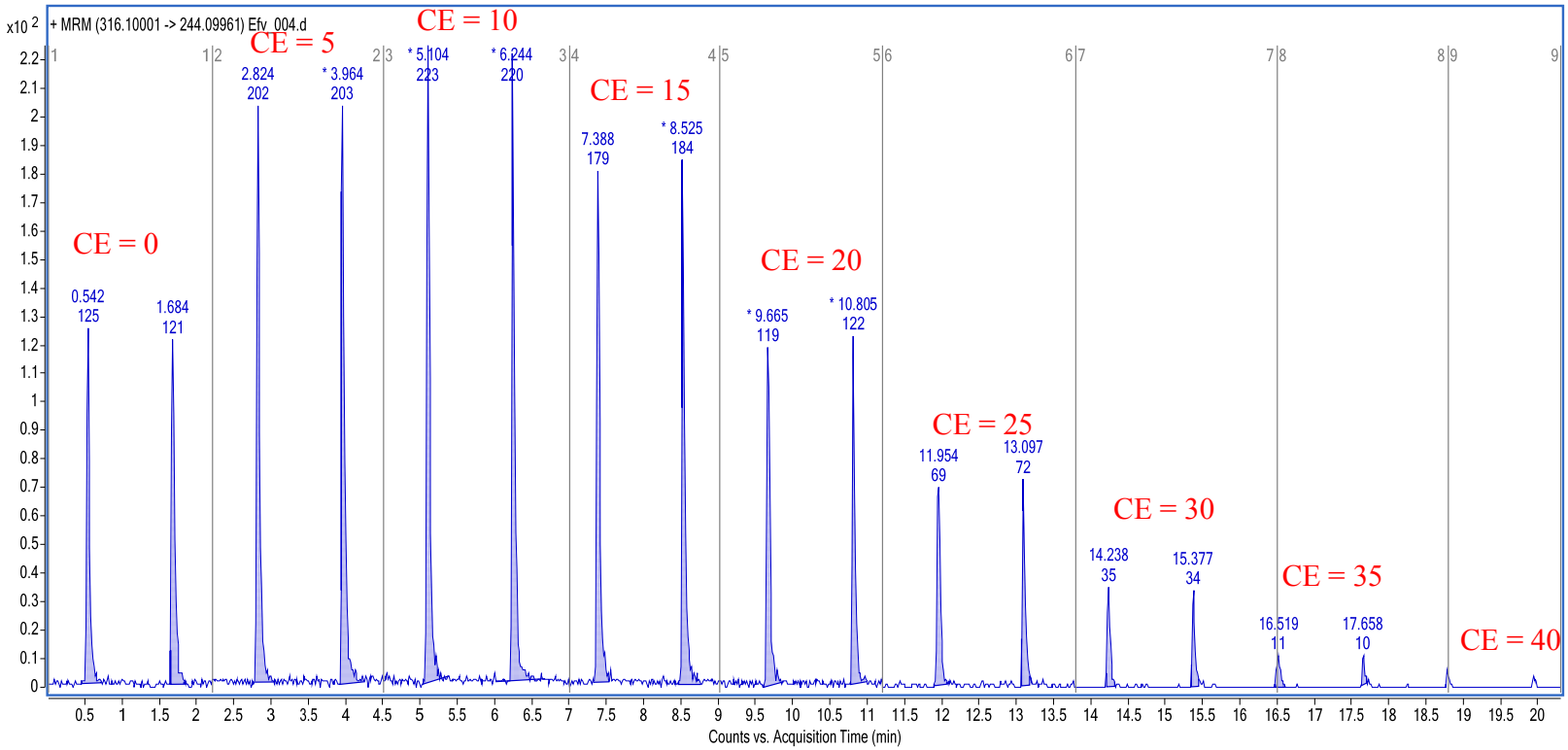


Figure 3.12: Efv product ion *m/z* 244.1 with increasing collision energies

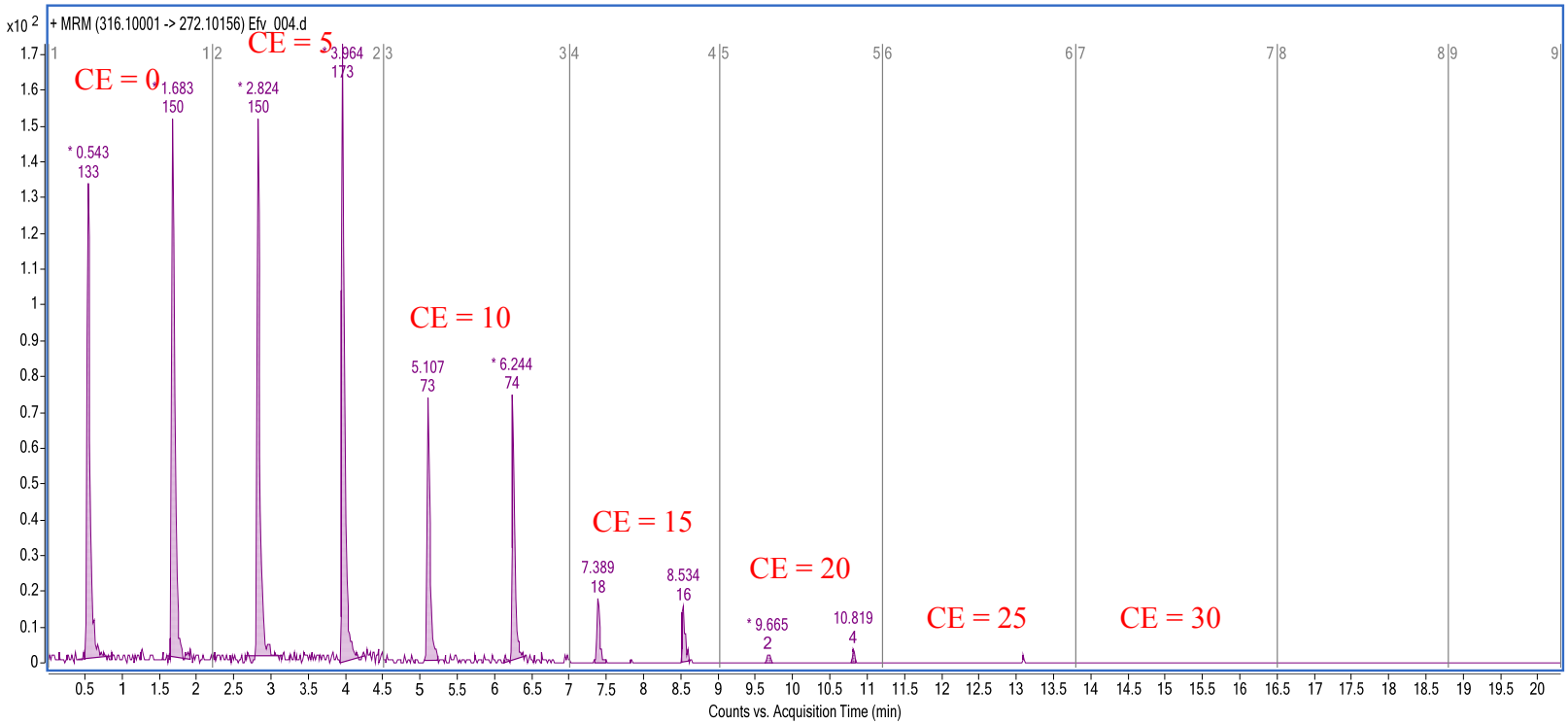


Figure 3.13: Efv product ion *m/z* 272.1 with increasing collision energies

Voltage	Product ion							
	272.1		244.1		168.0		52.9	
	Area	Area %	Area	Area %	Area	Area %	Area	Area %
0	425	88.17	348	52.08	86	17.53	174	27.09
	441	91.49	365	54.51	99	20.12	191	29.74
5	428	88.80	593	88.70	210	42.77	413	64.21
	482	100.00	629	94.02	211	42,97	394	61.29
10	209	43.42	657	98.21	308	62.74	568	88.30
	201	41,70	669	100.00	317	64.56	576	89.59
15	53	10.98	520	77.78	437	89.00	634	98.61
	42	8.7	550	82.21	408	83.11	616	95.79
20	7	1.45	362	54.11	491	100.00	643	100.00
	10	2.03	265	54.56	481	97.97	614	95.53
25			211	31.57	451	91.89	598	93.04
			215	32.06	457	96.79	575	89.40
30					419	85.34	511	79.57
					409	83.27	543	84.54
35					341	69.45	493	76.72
					303	61.68	463	71.96

Table 3.6: Collision energy results for product ions of Efv

Table 3.7 displays a summary of the collision energy results obtained for product ions of Rif and Efv.

Compound	Product ion	Collision energy
Rif:	791.3	12.5
	399.2	22.5
Efv:	272.1	2.5
	244.1	7.5
	168.0	22.5
	52.9	17.5

Table 3.7: Summary of collision energy results for product ions of Rif and Efv

The final MS conditions used in terms of precursor and product ions, collision energies and fragmentation voltages are shown in Table 3.8.

Compound	Precursor ion	Fragmentor voltage	Product ion	Collision energy
Rif	823.4	150	791.3	12.5
Efv	316.1	100	168.0	22.5

Table 3.8: Optimum positive mode ESI conditions for Rif and Efv

The signal intensity of Efv in relation to Rif at a concentration of 500 ng/mL is quite low. On comparing 3 different capillary temperatures, 350 °C remained optimal for Rif but on changing from 350 °C to 250 °C, a slight improvement was observed for Efv and so 250 °C was used as capillary temperature for Efv. See Figure 3.14.

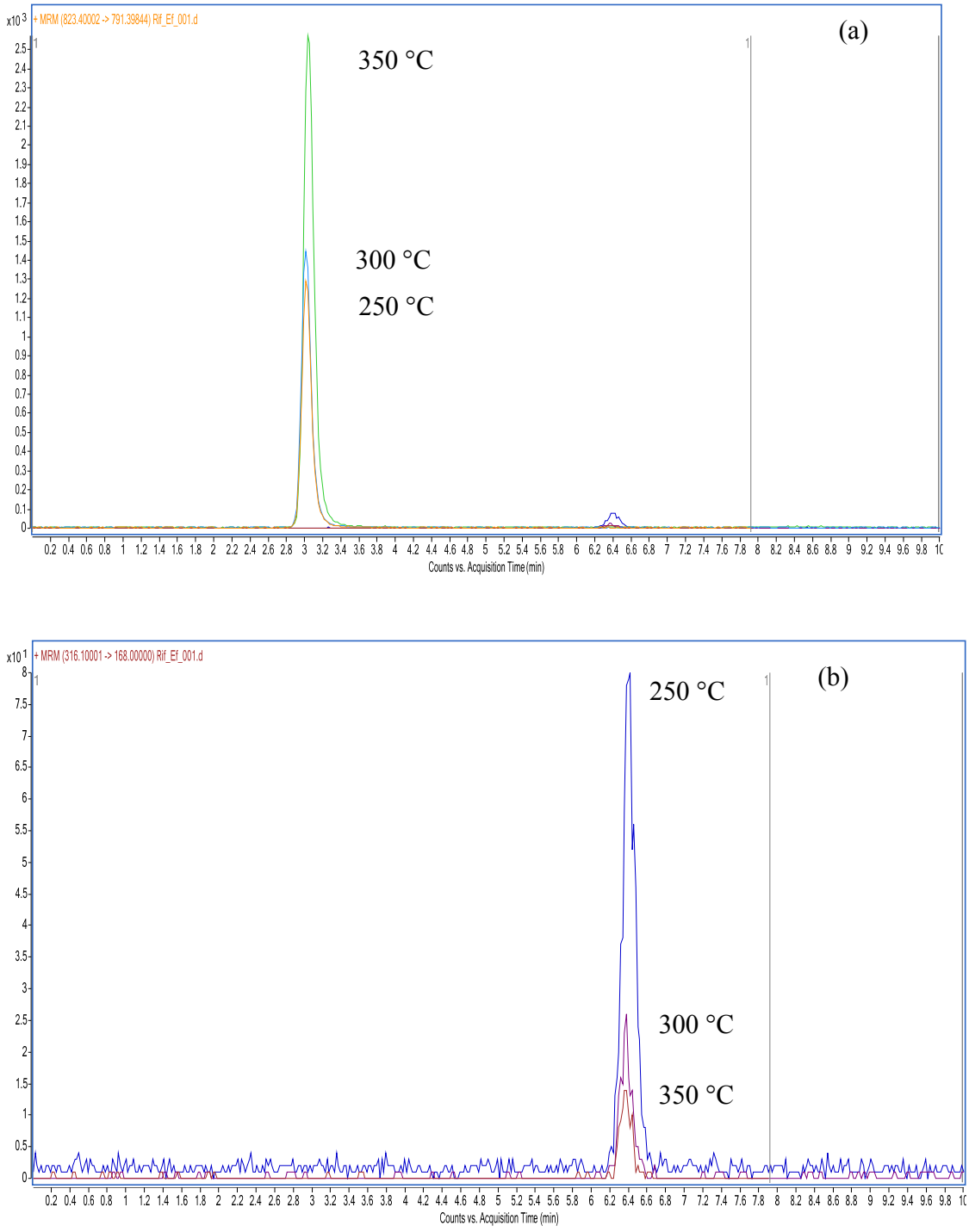


Figure 3.14: Comparison of different capillary temperatures for (a) Rif and (b) Efv

3.5 Method Validation

3.5.1 Sensitivity

LC-MS is inherently more sensitive than LC-UV detection as the technique can monitor individual ions whereas; LC-UV is reliant on an entirely different detection system. MS detectors combine specificity, selectivity and sensitivity. Quantification was based on the mass transitions from precursor to product ion. The increased sensitivity of the Agilent Triple Quadrupole MS is in part due to the orientation of the ionisation. The orthogonal orientation of the nebuliser spray to the sample capillary reduces the background noise from the samples allowing lower LOD and LOQ. This is because any ions that are uncharged in the source do not make it into the MS system.

Both the limits of quantitation (LOQ) and detection (LOD) were determined for the assay, see Figure 3.15 and Figure 3.16. The LOQ was defined as the lowest concentration that produced a peak distinguishable from background noise with a minimum ratio of 10:1. An LOQ of 1 ng/mL was obtained for Rif in plasma. This is an extremely sensitive result as the literature revealed the lowest LOQ for serum spiked with Rif using MS was 50 ng/mL (Song *et al.,* 2007) and in plasma 100 ng/mL (Hartkoorn *et al.,* 2007). Both required a small sample volume of 50 µL.

An LOQ of 10 ng/mL was obtained for Efv prepared in 0.3 mL plasma. Again this is an excellent result and is comparable with the literature which reveals the lowest LOQ obtained was 10 ng/mL in plasma (Rentsch *et al.,* 2003, Volosov *et al.,* 2002). Rentsch *et al.* 2003, required a large sample volume of 1 mL plasma for the analysis and 30 µL of the reconstituted post extracted sample was injected for LC-MS analysis. Comparing the analyses by Rentsch with our own, the mass of drug on column with their method equated to 300 pg in comparison to 200 pg for the assay we describe here. This renders our method superior for the analysis of Efv. Volosov *et al.,* 2002, required only 80 µL plasma, however, the volume injected for analysis was not specified and so a direct

comparison to the method developed here in terms of mass on column could not be made. Extracted blank plasma samples did not yield any endogenous peaks at the retention times of the drug compounds.

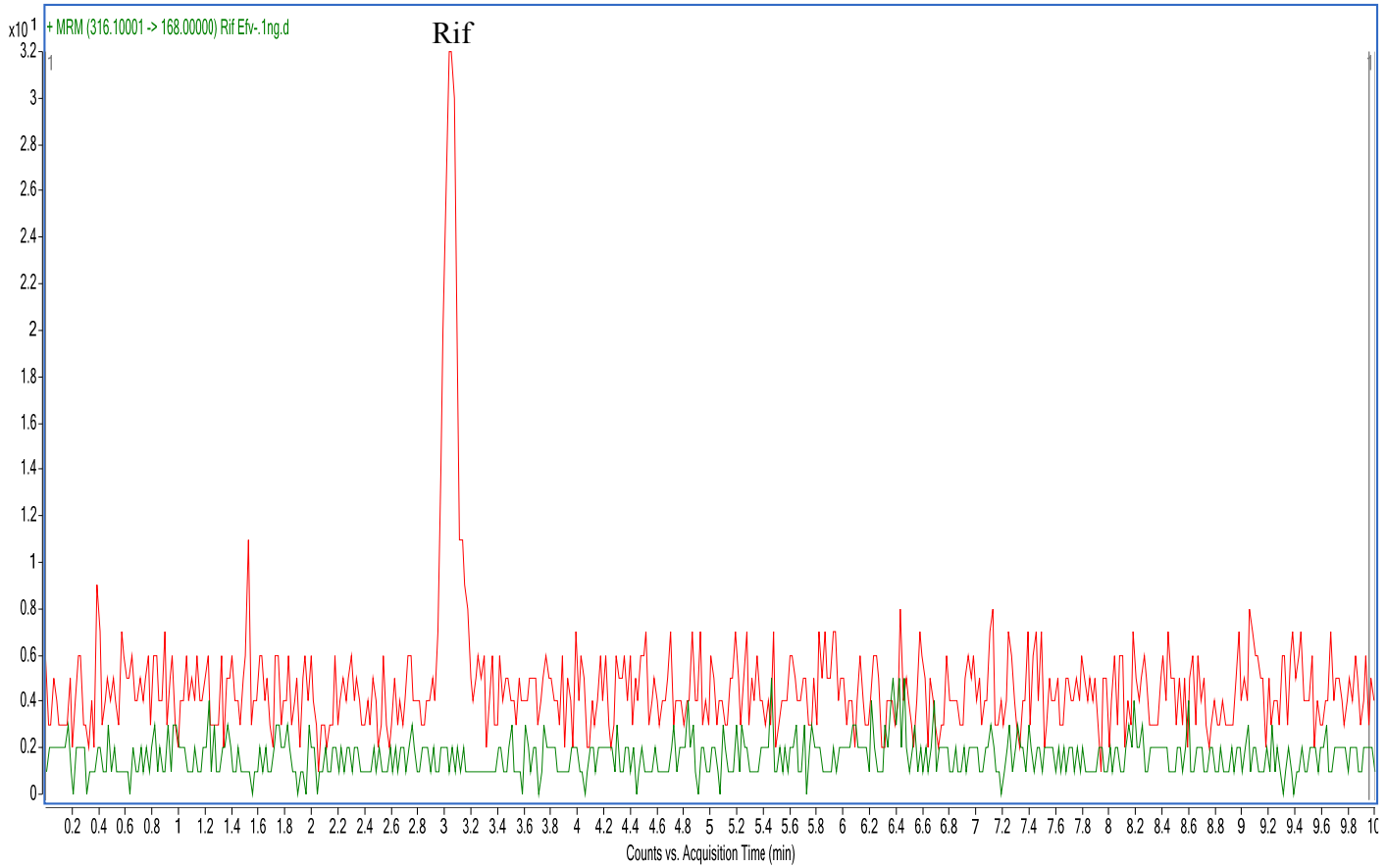


Figure 3.15: Chromatogram of a working standard of Rif prepared to 1 ng/mL in plasma

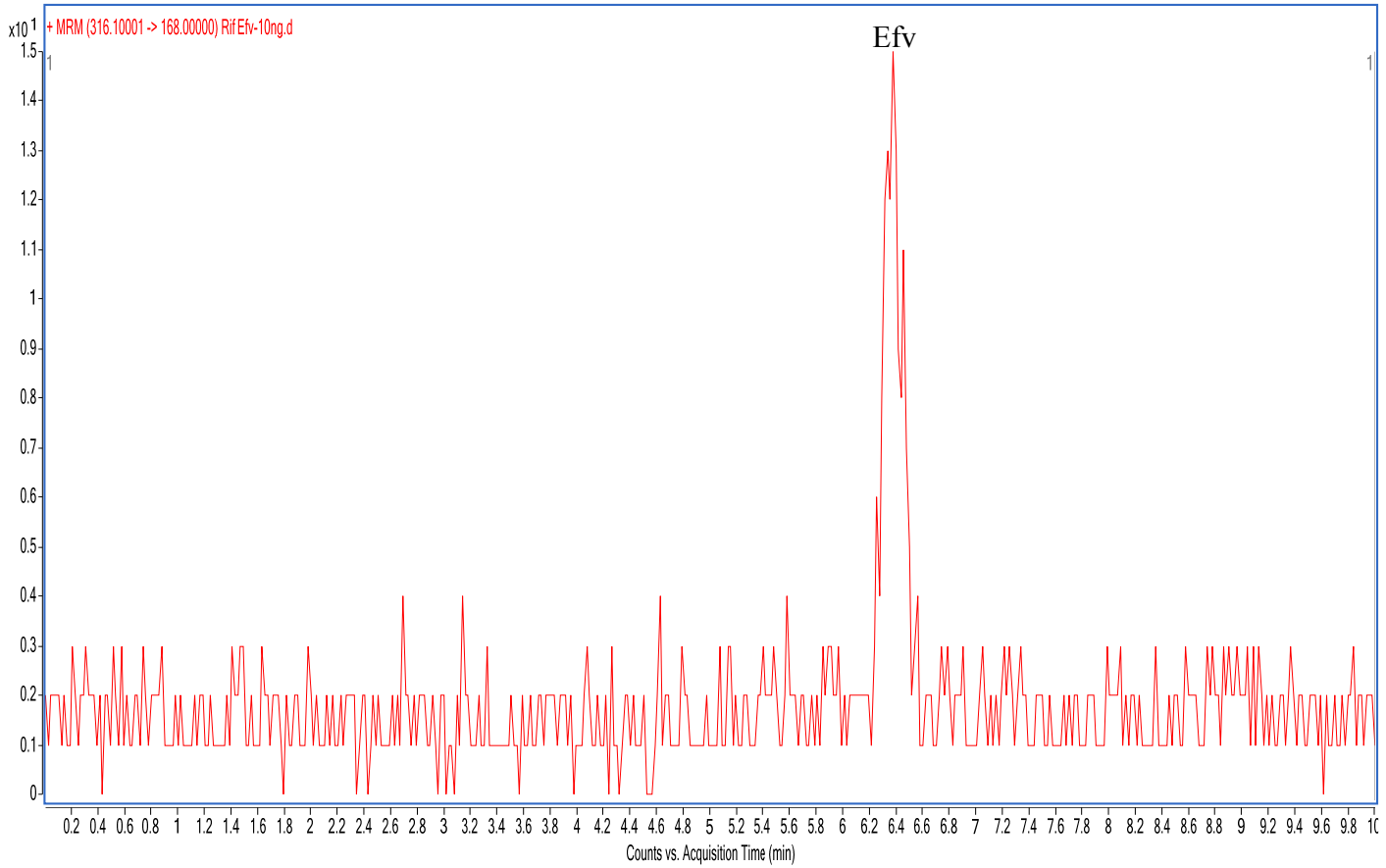


Figure 3.16: Chromatogram of a working standard of Efv prepared to 10 ng/mL in plasma

3.5.2 Linearity and range

The calibration curves for Efv and Rif over the concentration range 0.001 - 20 μg/mL for Rif and 0.01 - 20 μg/mL for Efv exhibited good linearity with correlation coefficients (R^2) for all standard curves above 0.99. See Figure 3.16 and Figure 3.17.

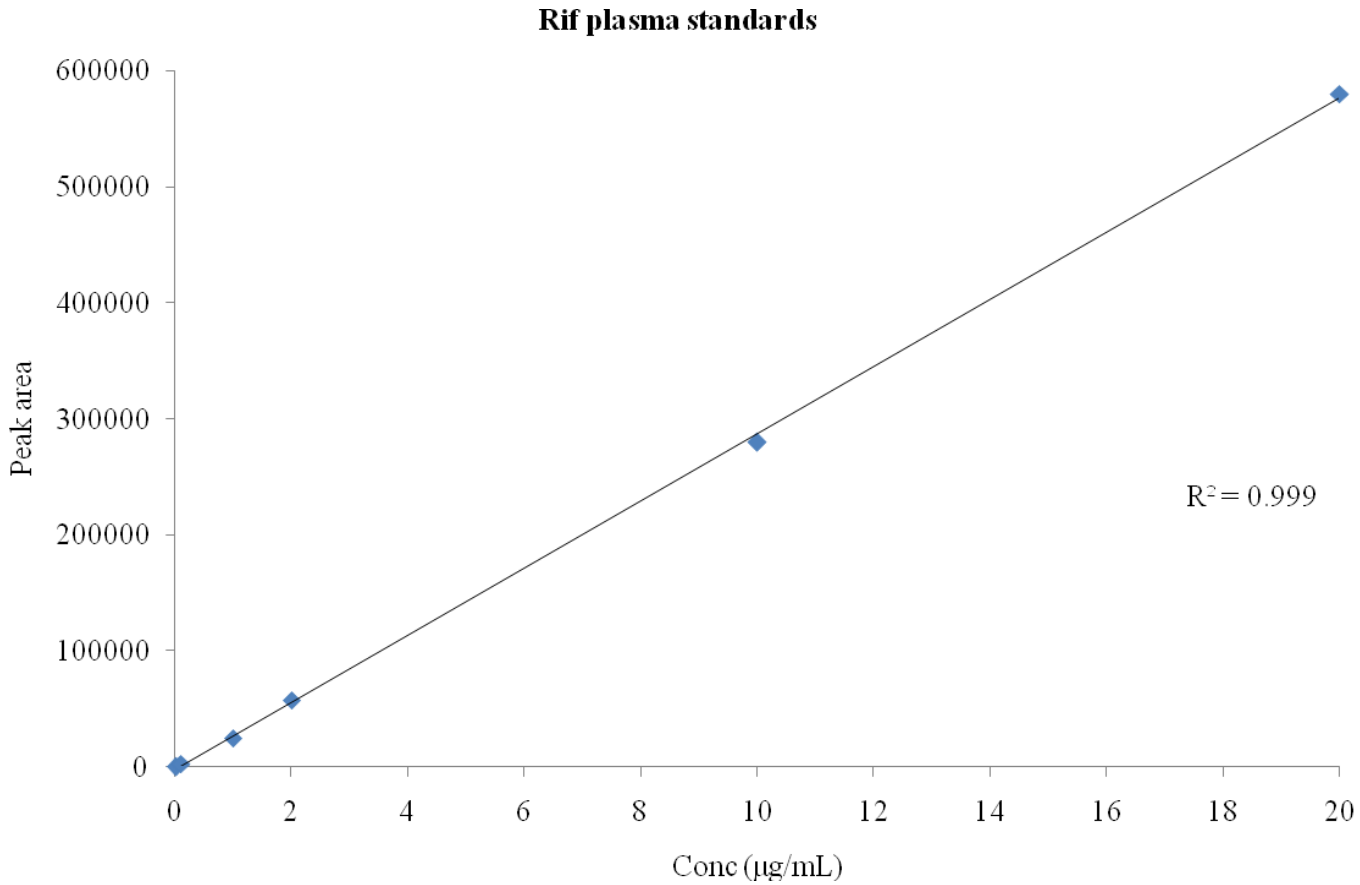


Figure 3.16: Linearity for Rif plasma standards

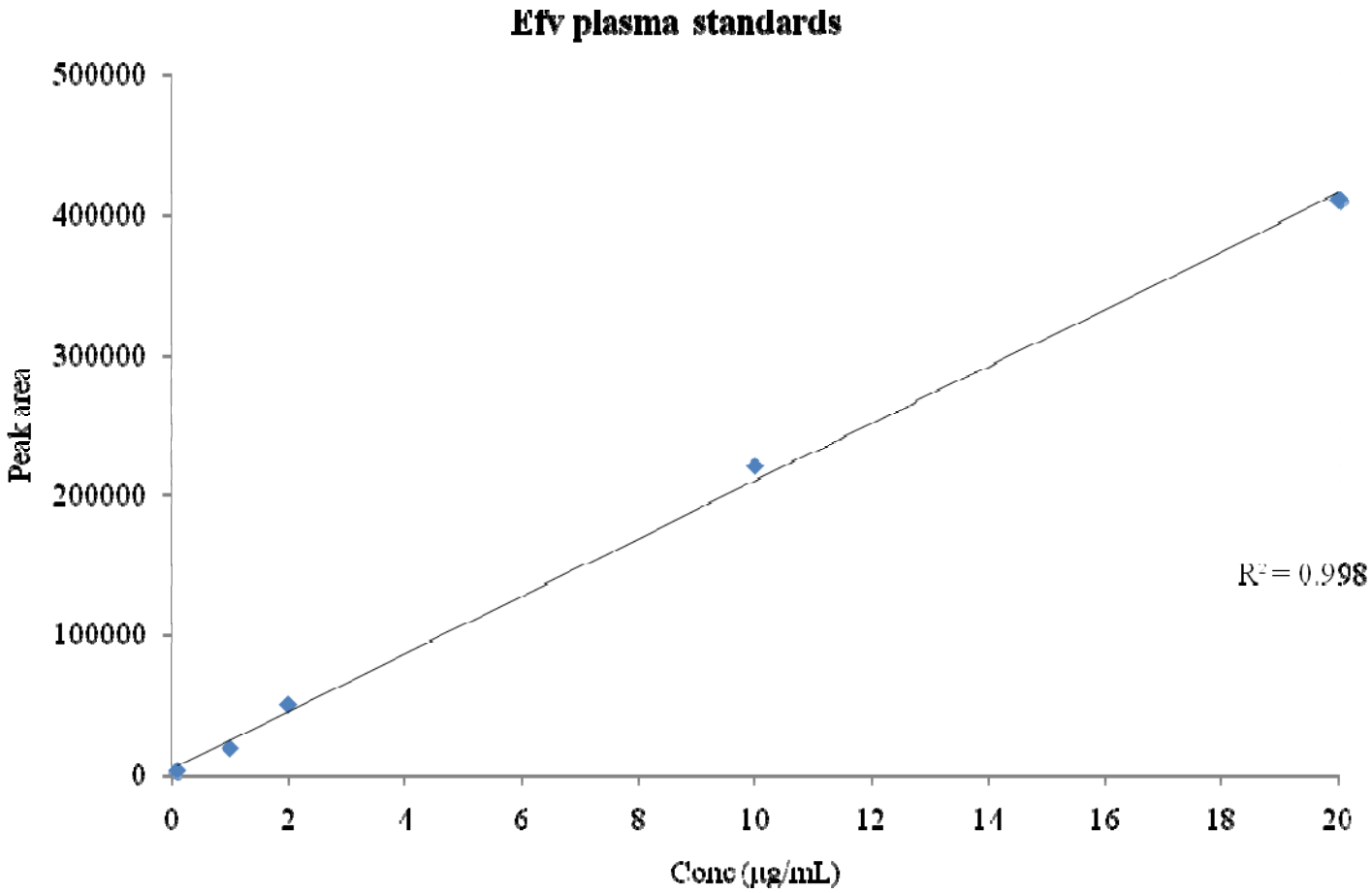


Figure 3.17: Linearity for Efv plasma standards

3.5.3 Recovery

As discussed in Chapter 2, highest recoveries for the drugs in one extraction step were obtained with the SPE ABN well plates. And so, the extraction procedure remained unchanged. Varying concentrations of Efv, Rif and (0.1, 1 and 10 μg/mL) were prepared in drug-free human plasma and extracted using the SPE cartridges. The percentage of drug recovered from these plasma samples was determined by comparing the calculated concentrations following extraction and HPLC assay with the calculated concentrations from unextracted samples in MP of the same concentration after HPLC assay. Recoveries for plasma samples spiked with 0.1, 1 and 10 μg/mL of Efv and Rif were all ≥ 70% (See Table 3.9). This correlates with results obtained in Chapter 2.

Conc. (μg/mL)	**Mean % recovery ± RSD**	
	Rif	**Efv**
0.1	93.1 ± 1.5	71.0 ± 2.4
1	94.0 ± 1.8	70.4 ± 1.9
10	94.8 ± 0.8	71.8 ± 2.1

Table 3.9: Recovery data for drugs from plasma (n = 3)

3.6 Conclusions

The LC-UV method described in Chapter 2 was transferred to MS detection using similar chromatographic parameters as for the UV method. The sensitivity achieved was excellent with an LOQ of just 1 ng/mL for Rif in plasma. As

discussed, it is far superior to what is available in the scientific literature which reveals the lowest LOQ for serum spiked with Rif using MS to be 50 ng/mL (Song *et al.,* 2007) and in plasma 100 ng/mL (Hartkoorn *et al.,* 2007). An LOQ of 10 ng/mL was obtained for Efv prepared in plasma. Again this is an excellent result in comparison with what has previously been reported in the literature which reveals the lowest LOQ obtained for Efv was 10 ng/mL in plasma (Rentsch *et al.,* 2003, Volosov *et al.,* 2002).

The assay described in this chapter is the first capable of achieving such low levels of Rif and Efv from a biological matrix. Furthermore, the fast and easy sample treatment by SPE, as well as the short runtime of the assay allows for rapid sample processing and analysis.

Given more time, a decrease in the plasma sample volume would have been investigated. There would be major advantages of using finger prick assays for blood rather than taking mLs using standard blood collection tubes which would allow for more routine analysis.

Measurements for each analyte in the biological matrix would be validated according to FDA guidelines (FDA, 2001) given more time. Validation parameters including intra- and inter-day precision, accuracy, sensitivity, linearity and range and recovery would be performed on the method. Following a full validation, this method would be capable of detecting extremely low levels of both drugs simultaneously in aqueous samples which would be useful to pharmaceutical companies. It would also be interesting to apply the method to biological samples. It would also be advantageous to modify the assay in order to quantify cellular and tissue levels of the drugs.

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Chapter 4

Development of a sensitive assay to extract, separate and quantitate scopolamine (anti-motion sickness medication) in human serum

4.1 Scope of research

The objective of this work was to develop a method for the quantitative and sensitive determination of the anti-motion sickness drug, scopolamine, in human serum using LC-MS with solid phase extraction. Scopolamine is administered as a transdermal patch loaded with 1.5 mg of agent which is gradually released over many hours into the circulation, as a result the serum concentration resulting from the application of a patch is very low and clearly it would be anticipated that half a patch should generate substantially lower circulating drug levels, well below the anticipated threshold where UV detection might be employed. MS detection was chosen for this work as it is a technique which we felt might be sensitive enough to detect the drug at the required concentrations (pg/mL range) over time in blood samples. It had been hypothesised at NASA that in individuals who appeared adversely affected by the use of a whole transdermal patch, if it is halved and then used by the astronaut, that this might be just as effective but with a lower severity and incidence of side effects. Hence, the rationale for developing this assay was to enable the hypothesis to be tested so that, if we generated pilot information a larger more detailed evaluation could be conducted.

4.2 Introduction

4.2.1 Motion sickness and its treatment

Motion sickness can occur in response to real or apparent motion. When motion sickness occurs in response to real motion, it is often labelled with the related vehicle or situation to more specifically identify the ailment, e.g., car sickness, air sickness, sea sickness, space sickness, etc. Apparent motion refers to a situation in which the individual is stationary, but motion in the visual field causes the individual to experience an illusion of motion. Apparent motion can occur in rotating drums lined with variously contrasting scenes, in large field of view movies that display motion and in computer-generated simulations of real-world environments. When motion sickness occurs in response to computer-

generated simulations, it is often referred to as simulator sickness or virtual environment sickness. All of these terms are somewhat misleading because motion sickness is not really a sickness, but rather a psychophysiological response of healthy individuals to real or apparent motion stimulation of significant intensity and/or duration and where the orientation information being transmitted from the inner ear conflicts with visual and other sensory signals arriving concurrently in the brain (Muth, 2006).

Motion sickness remains a persistent problem for astronauts in spaceflight. Motion sickness first appeared as an operational problem in the second manned Soviet mission in 1961 and became a significant concern in later missions for all nations, especially with increased flight duration. The overall incidence of symptoms of motion sickness in the space shuttle program has been approximately 70% for astronauts in their first space mission but is lower in experienced astronauts. Symptoms of space motion sickness usually start to develop within the first several hours in weightlessness. After 72–96 hr, most astronauts have either recovered or begin to recover and are able to move about freely without eliciting debilitating symptoms. Because the shuttle missions are of relatively brief duration, generally 7–14 days, 3 days of impaired performance represent a severe negative impact on the mission. Symptoms range in severity from drowsiness to nausea and vomiting. Space motion sickness is most severe when the body is actively adjusting to weightlessness and sickness may reoccur upon landing during readaptation to earth gravity (Lackner *et al.,* 2006).

In motion sickness, a person vomits because conflicting sensory information arrives simultaneously in the brain from the inner ear and the eye. A group of nerve fibres deep inside the ear helps people keep their balance. It is thought that, for some people, the motion of ships, airplanes, trains, automobiles, buses and space travel increases the activity of these nerve fibres. This increased activity causes the dizziness, nausea, and vomiting of motion sickness. People may have one, some, or all of these symptoms. The drugs primarily used to treat motion sickness have antihistaminic or anticholinergic actions or a combination thereof. In NASA, scopolamine is one of the primary drugs used to manage motion sickness symptoms in space.

Atropine and scopolamine belong to the alkaloid family. Atropine and scopolamine, also referred to as (+)-hyoscyamine and hyoscine, respectively, are extracted from plant species belonging to the solanaceae family (Cherkaoui *et al.,* 1997). Scopolamine (Scop), is a tropane alkaloid drug (Ceyhan *et al.,* 2001) (see Figure 4.1) obtained from plants of the family solanaceae, such as henbane or jimson weed (datura species) and belladonna. Scop has well known pharmacological properties and can be highly toxic due to its powerful anti-cholinergic properties (Roberge, 2006). Scop acts (1) as a competitive inhibitor at postganglionic muscarinic receptor sites of the parasympathetic nervous system, and (2) on smooth muscles that respond to acetylcholine but lack cholinergic innervation. It has been suggested Scop acts in the central nervous system (CNS) by blocking cholinergic transmission from the vestibular nuclei to higher centers in the CNS and from the reticular formation to the vomiting center. Scop can inhibit the secretion of saliva and sweat, decrease gastrointestinal secretions and motility (Beyer *et al.,* 2009), cause drowsiness, dilate the pupils, increase heart rate, and depress motor function (Steenkamp *et al.,* 2004).

Although Scop has been employed therapeutically for thousands of years, in the 20th century it was purified and its pharmacological actions studied extensively. In the past, Scop has been used as a 'truth' drug, a mind control drug dating back to applications by the Nazis in Germany during World War II, and, subsequently, evaluated by the American CIA. As it depresses the central nervous system, it has been used as an amnesiac for women in labour, producing "Twilight Sleep" (Binotto *et al.,* 2006). Scop has also unfortunately become prevalent as a date rape drug as Scop leaves the victim in a state of compliancy, where their mind is totally controlled so a recipient can participate in the rape and afterwards remember little or nothing at all. The typical maximum dosage use for legal/medicinal applications which include control of motion sickness and postoperative nausea and vomiting (Roberge, 2006) is 0.33 milligrams. High doses of 5-7 milligrams can render the victim into a zombie-like state as described above and at 10 milligrams can produce coma then death.

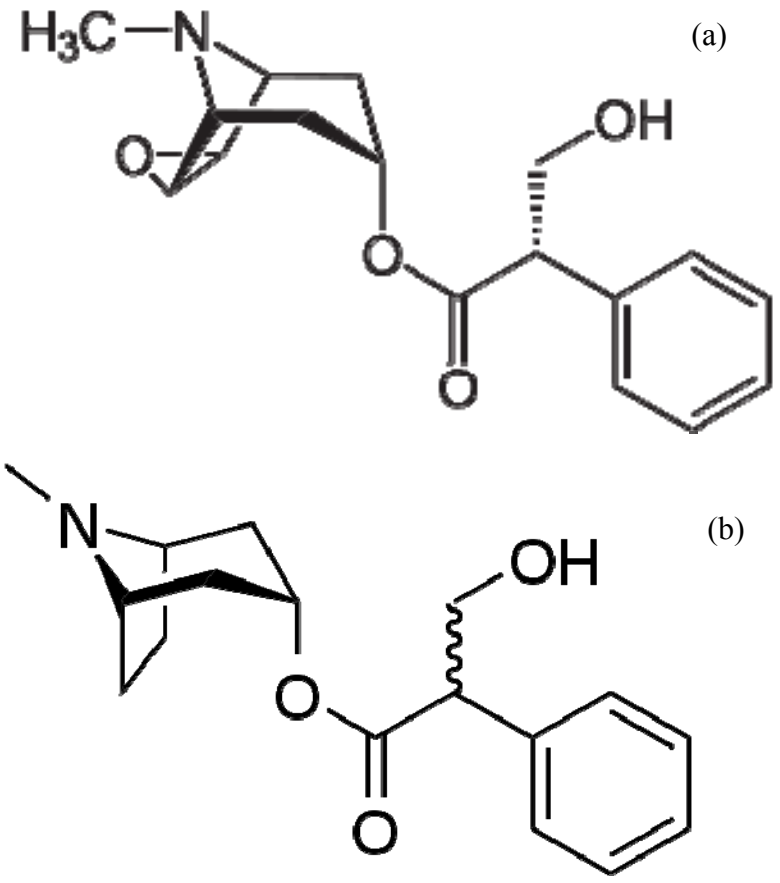


Figure 4.1: Chemical structures of (a) Scop and (b) Atr

Scop can be administered via a transdermal patch, orally, subcutaneously, and via ophthalmic and intravenous routes. The transdermal patch (worn behind the ear) is the most common form of the drug used in the US. However, the following undesirable side effects have been reported while taking the patch: dry mouth, as Scop inhibits saliva secretion, drowsiness, dizziness and depressed motor function (Sandlin, 2002). In NASA, for patients who demonstrate side effects, it has been suggested that using half a transdermal patch (cut in half) gives symptom relief while reducing side effects. A project to evaluate the efficacy and side effects of standard patch versus half patch transdermal Scop in preventing motion sickness induced by off-vertical axis rotation (OVAR) was initiated. The hypothesis is that some subjects who have undesirable levels of side effects with the full dose will have fewer side effects but still have efficacious symptomatic motion sickness management with the lower dose.

4.2.2 Physiochemical properties of scopolamine and atropine

Scop is an antimuscarinic agent and has actions that are qualitatively similar to those of atropine. Quantitatively, Scop is more potent than atropine in its action on the iris, ciliary body and certain secretory (salivary, bronchial and sweat) glands, but less potent in its actions on the heart, and intestinal and bronchial smooth muscle (Renner *et al.,* 2005).

Scop is a heterocyclic tropane alkaloid with a molecular weight of 303.36 g/mol (Steenkamp *et al.,* 2004) and a pKa value of 7.55-7.81 (Cherkaoui *et al.,* 1997). Scop is lipid soluble (Tullberg, 2007) and therefore poorly soluble in water, hence the drug is prepared experimentally in methanol. Because of its many exposed functional groups, Scop is readily subject to chemical and biological decomposition and is thus stored and administered as a hydrated hydro-halogen (typically HCl or HBr) salt for stability.

Atropine (Atr), like Scop, has a tertiary amine structure (Tullberg, 2007), the only difference being an additional epoxy group on the tropane ring of Scop (Oertel *et al.,* 2001) (See Figure 4.1). Atr has a molecular weight of 289.37 g/mol (Steenkamp *et al.,* 2004) and a pKa value of 9.85 (Cherkaoui *et al.,* 1997). Atr, though less lipid soluble than Scop (Tullberg, 2007), is still poorly soluble in water and is also experimentally prepared in methanol.

4.2.3 Sample extraction/clean-up procedures for scopolamine

In order to determine drug concentrations in plasma or serum, it is necessary to have an accurate, precise and selective analytical method, requiring a small sample volume and with rapid sample processing. The three main sample extraction techniques employed to extract drugs from biological fluids tend to be liquid-liquid extraction (LLE), protein precipitation (PP) and solid-phase extraction (SPE). The characteristics of several of the most accepted Scop quantifications methods are summarized below.

In the main, SPE approaches have been exploited for the extraction of Scop from biological matrices such as plasma, serum and urine – see Table 4.1. However, many of these methods demonstrate poor Scop recovery.

To date, there are no reported PP extraction procedures for Scop from biological samples prior to LC analysis. LLE has been used prior to GC-MS- (Oertel *et al.,* 1996*)* or LC-MS- based quantitation strategies (Xu *et al*., 1995). For example, Xu used dichloromethane to extract l-hyoscyamine and Scop from plasma samples. A high recovery resulted for Scop at 93% but the plasma sample volume required was high at 1 mL.

In terms of SPE, Oertel *et al.,* 2002, developed a procedure for the extraction of Scop from serum using Atr as an internal standard and employing Oasis HLB cartridges. Cartridges were conditioned with methanol of water, pushing with air. A sample volume of 0.2 mL serum was required for the extraction which was mixed with 0.2 mL phosphate buffer before loading. Washing solutions were composed of water and 10% methanol in water. Compounds were eluted with methanol. A concentration factor of two resulted but the recovery achieved was low at only 51%.

Chen *et al*., 2005, and Beyer *et al.*, 2007**,** both used SPE for the extraction of Scop. Chen used SPE for the extraction of Scop and its 18 metabolites from rat urine. A 1 mL aliquot of urine samples was loaded onto a C18 solid-phase extraction cartridge that was preconditioned with methanol and water. The SPE cartridge was then washed with water and the analytes eluted with of methanol. In this case, recovery for Scop was satisfactory at 68-79%. Beyer used mixed mode SPE for the extraction of Scop from plasma. Again a high sample volume was required (1 mL) and recovery ranged between 67 and 92% for Scop.

Reference	Analytes	Matrix	Extraction mode	Extraction recoveries (%)	Detection	Approx retention time (min)	LOQ/LOD	Column	Sample vol.	Gradient
(Beyer *et al.*, 2007)	Scop, Atr & other drugs	Plasma	Mixed mode SPE	Atr: 51-85 Scop: 67-92	MS	Atr ~ 5.4 Scop ~ 4.8	APCI: LLOQ: 5ng/mL ESI LLOQ: 0.1ng/mL	C8	1 mL	Yes
(Chen *et al.*, 2005)	Scop & its 18 metabolites	Rat Urine	SPE	20 ng/mL: 68 50 ng/mL: 79	MS	2.9	LOD 5 ng/mL UV; 10 µg/mL MS;	Zorbax-Extend RP C18	1 mL	No
(Steenkamp *et al.*, 2004)	Scop & Atr	Viscera samples	SPE	n/a	UV/MS	Scop: 20.0 Atr: 21.6	Scop: 1 ng/mL Atr: 100 pg/mL	Xterra Phenyl	3g of each sample	Yes
(Oertel *et al.*, 2002)	Scop & other drugs	Human serum	SPE	Not stated	MS	Scop: 2.7	LLOQ 50 pg/mL	C18 Purospher STAR RP	0.2 mL	Yes, dual pump

(Oertel *et al.*, 2001)	Scop & Atr (IS)	Human serum & micro dialysis samples	SPE	51	MS	Scop: 3.3 Atr: 3.6	LLOQ 20 pg/mL	C18 Purospher STAR RP	0.2 mL serum	Yes
(Xu *et al.*, 1995)	l-hyoscyamine & Scop (IS)	Plasma	LLE	Scop: 93 l-hyoscyamine: ~110	MS	Scop: 0.8 l-hyoscyamine: 1.2	l-hyoscyamine 20pg/mL	BDS C18	1 mL	No

Table 4.1: Summary of the characteristics of reported extraction and LC detection techniques for the determination of Scop and Atr from biological samples

4.2.4 Sample separation and detection approaches for scopolamine

There are a number of LC-MS (Auriola *et al*., 1991, Kursinszki *et al.,* 2005, Rancic, *et al*., 2009, Lund *et al*., 1978, Lau *et al*., 1997, Mandal *et al*., 1991, Fliniaux *et al*., 1993) and LC-UV (Ceyhan *et al.,* 2001, Kursinszki *et al.,* 2005) methods in the literature for the analysis of tropane alkaloids in pharmaceutical preparations and in plants. GC-MS (Majlát *et al.,* 1982) , CE (Cherkaoui *et al.,* 1997, Ye *et al.,* 2001, Mateus *et al.,* 1998, Gao *et al.,* 2005, Bo *et al.,* 2003, Eeva *et al.,* 1998, Yuan *et al.,* 2010) and fluorimetry (Pohjola *et al.,* 1994, Takahashi *et al.*, 1997) have also been used for the detection of Scop and Atr.

However, only a limited number of methods for the determination of Scop and Atr in biological material have been published (see Table 4.1). Atr and Scop have been detected in biological samples using GC–MS techniques (Eckert *et al.,* 1981, Oertel *et al.,* 1996, Namera *et al.,* 2002, Bayne *et al.,* 1975, Deutsch *et al.,* 1990) but the drawback of GC-MS in detecting tropane alkaloids is the heat instability of these compounds. Therefore, methods for quantitative determination either convert Atr or Scop to their degradation product (Eckert *et al.,* 1981) or convert Atr or Scop to less unstable derivatives (Namera *et al.*, 2002).

LC–MS is currently the most common technique employed for the detection and quantification of tropane alkaloids as there is little issue with stability and sensitivity is excellent (Steenkamp *et al.,* 2004, Xu *et al.,* 1995, Oertel *et al.,* 2002, Chen *et al.,* 2005, Beyer *et al.,* 2007).

UV detection has been employed for Scop, but does not achieve the sensitivity required for the levels of the drug found in plasma/serum post patch application. As the doses of Scop to be detected are quite low (in the pg/mL range), it was decided that MS detection would be necessary in this project to give the best chance of being able to achieve adequate sensitivity in blood samples.

Oertel *et al.* developed two LC-MS methods for the analysis of Scop and achieved the best sensitivity for Scop published to date. Oertel *et al.,* 2001 firstly developed an LC–tandem mass spectrometry for the determination of Scop in human serum and microdialysis samples. Even though the retention times of the drugs were fast with Scop eluting at 3.3 min, the chromatographic

separation involved a gradient programme which required a 10 min equilibration time between runs. The LC–MS/MS system used was an API 3000 equipped with a turbo ion spray interface which was used in positive ion mode. Multiple reaction monitoring (MRM) was performed giving an excellent LOQ of 20 pg/mL.

Oertel *et al.,* 2002, subsequently investigated increasing sample throughput in pharmacological studies by using dual-column liquid chromatography with tandem MS. The sensitivity achieved using this approach was an excellent at 50 pg/mL. They extracted propiverine and its N-oxide, talinolol and Scop from serum samples using an automated SPE method. Using two C18 Purospher STAR columns, mobile phase gradients were again applied and within 4.2 min analytes and matrix were separated, eluting peaks of the first column were detected and quantitative LC–MS/MS data were acquired while the second column was equilibrated for the next injection. Even though excellent sensitivity was achieved, a gradient was required and two columns were needed which added complexity to the method.

Chen *et al.,* 2005, Beyer *et al.,* 2009, and Xu *et al.* 1995, also developed LC-MS methods for Scop.

Chen *et al.,* 2005, developed a liquid chromatography-tandem mass spectrometry method for the analysis of Scop and its eighteen metabolites in rat urine. After application of the extraction procedure (see previous section), the pre-treated samples were injected into a Zorbax Extend-C18 reversed-phase column and detected by an on-line MS/MS system. Mass spectrometric detection was carried out in positive ion mode. However, the LOQ achieved was poor at 5 ng/mL.

Beyer *et al.,* 2009, used both APCI and ESI approaches for Scop's detection. APCI resulted in a LOQ of 5 ng/mL and ESI 0.1 ng/mL. Although the LOQ using ESI was very low, the method required 1 mL plasma to achieve this sensitivity. Scop eluted at 4.8 min but a gradient programme was required for the method.

Xu *et al.,* 1995, developed an LC-MS method whereby Scop was used as the internal standard. Using a BDS C18 column, Scop eluted at 0.8 min and no gradient was required. Steenkamp *et al.*, 2004, developed an LC-UV-MS method for the identification of Atr and Scop. This method allowed the direct

coupling of an electrospray (ZMD) mass selective detector to the HPLC system. Atr and Scop were well separated from other components and detected on the PDA with a LOD of 1 μg/mL and ZMD giving an LOD for Atr of 10 pg/mL and for Scop of 100 pg/mL. Use of an Xterra Phenyl HPLC column with a mobile phase containing ammonium acetate at 0.2 mL/min resulted in Scop having an extremely long retention time of 20.0 min and Atr a retention time of 21.6 min.

From the literature, many methods for the determination of Scop suffer from having to use a gradient programme or a large sample volume in order to achieve their fast runtimes and low sensitivity. In one reported case the use of two columns was required which complicates the method. And summarising, there is no fast, simple, isocratic method reported which achieves the sensitivity required for the analysis of Scop in serum post patch and half patch application.

4.3 Experimental

4.3.1 Reagents and materials

MS grade acetonitrile (ACN) methanol (MeOH), water, ammonium formate, formic acid, phosphoric acid, scopolamine hydrobromide and atropine were obtained from Sigma Aldrich, United Kingdom. SPE extraction cartridges were purchased from Waters, Ireland and United States (HLB, WAX, MAX, WCX, and MCX) and Supelco (Supelclean LC-18). Scopolamine patches (Transderm Scop, scopolamine 1.5 mg from Novartis) were obtained from a local pharmacy. Human serum was purchased from Sigma Aldrich. 1.1 mL Chromacol vials were purchased from Fisher Scientific, Ireland. Analytical columns used were Supelcosil LC-18 (250 x 2.1 mm, 5 μm), Agilent Zorbax SB-CN (100 x 2.1 mm, 3.5 μm), Agilent Zorbax SB-C18 (50 × 2.1 mm., 3.5 μm), Agilent Zorbax SB-Phenyl (150 × 4.6 mm, 5 μm) and Waters Nova-Pak C18 (150 × 3.9 mm, 4 μm).

4.3.2 Equipment and HPLC assay conditions

4.3.2.1 NASA equipment and assay conditions

The LC-MS systems used in NASA, Kennedy Space Centre, consisted of an Agilent 1050 series quaternary pump and autosampler, an 1100 degasser and a Thermo Finnigan LCQ Deca MS. A T-piece and syringe pump, Agilent KD scientific (KDS 100) model were used for direct infusion. System management and data acquisition were performed using Empower software. The SPE manifold was from Phenomenex. Other instrumentation included a Mettler Toledo pH meter and a Mettler Toledo electronic mass balance.

Chromatographic separation was achieved using an Agilent Zorbax SB-CN (150 x 2.1 mm, 5 μm). A mixture of ammonium formate (pH 4 adjusted with 1 M formic acid; 2 mM) - MeOH, (30:70, v/v) was used as mobile phase, at a flow rate of 0.2 mL/min at ambient temperature. The complete chromatographic run time of each sample was 10 min. Peaks were quantified using Agilent Masshunter Software.

The mass spectrometer was operated using an ESI source in the positive ion detection mode. Analysis was performed in MRM mode with the following transitions: *m*/*z* 304.1 → *m*/*z* 138.1 for Scop and *m*/*z* 290.2 → *m*/*z* 124.1 for Atr. A collision energy value of 34% was used. Sheath gas and auxiliary gas were set to 90% and 10% respectively with a capillary temperature of 270 °C.

4.3.2.2 DCU equipment and assay conditions

Work continued in DCU on a LC-MS system consisting of a 1200 quaternary pump, autosampler and degasser and a 6410 QQQ LC/MS, all from Agilent, United Kingdom. System management and data acquisition were performed using Agilent Masshunter B.01.03 WorkStation Data Acquisition software. Other instrumentation employed included a pH meter and electronic mass balance both from a Mettler Toledo, USA a Genevac EZ-2 personal evaporator from Genevac, Ipswich, United Kingdom and a Cyclone high speed evaporator. The SPE manifold was from Phenomenex.

Chromatographic separation was achieved using an Agilent Zorbax SB-Phenyl (150 × 4.6 mm, 5 μm). A mixture of ammonium formate (pH 8 adjusted with ammonium hydroxide; 10 mM) - MeOH (10:90, v/v) was used as mobile phase, at a flow rate of 0.6 mL/min. The column temperature was maintained at 20 °C and the temperature of the autosampler was maintained 4 °C. The complete chromatographic run time of each sample was 10 min. Peaks were quantified using Agilent Masshunter Software.

The mass spectrometer was operated using an ESI source in the positive ion detection mode. The ionisation temperature was 350 °C, gas flow rate was 11 L/min and nebuliser pressure was 50 psi. Nitrogen was used as the ionisation source gas and ultrapure nitrogen as the collision cell gas. Analysis was performed in MRM mode with the following transitions: *m*/*z* 304.1 → *m*/*z* 138.1 for Scop and *m*/*z* 290.2 → *m*/*z* 124.1 for Atr with a dwell time of 200 ms. Transitions and their optimal detector settings are listed in Table 4.2. The quantifier ions are indicated in the table though all transitions were monitored with the qualifier ions adding specificity.

Compound	Precursor ion	Optimum fragmentor voltage	Product ion	Optimum collision energy
Scop	304.1	120	110.1	30
			121.1	20
			138.1*	20
			156.1	15
Atr	290.2	140	124.1*	22.5
			93.1	35

Table 4.2: Optimal fragmentor voltages and collision energy settings for Scop and Atr

* Quantifier ion

Quantification was based on the integrated peak area as determined by the Masshunter quantification analysis software which quantitates the peak areas of the MRM transitions of each analyte.

4.3.3 Preparation of standards

Stock standard solutions of Scop and Atr were prepared by weighing out the powders in a glovebox, dissolving them in MeOH to 1 mg/mL concentration and storing them at 2 - 4 °C. Working standards of Scop were prepared both in drug-free human serum and in mobile phase (MP) (ammonium formate (pH 8, adjusted with ammonium hydroxide; 10 mM) - MeOH (10:90, v/v)) to concentrations over the range 10–200 pg/mL (10, 20, 50, 100 and 200 pg/mL in serum which corresponded to 100, 200, 500, 1000 and 2000 pg/mL in MP). This range of the standard curve for Scop was chosen to reflect the serum concentrations expected in a typical 72 hr pharmacokinetic profile post administration of Scopderm, as the transdermal system is designed to deliver

approximately 5 µg/hour over the 72 hours (Bosman *et al.,* 1997, Douma *et al.,* 1997). The internal standard Atr was prepared in a similar way to Scop. 100 pg/mL Atr was added to all concentrations of Scop.

Both spiked serum samples and post extracted spiked serum samples were prepared. For the post extracted spiked serum samples, a 500 µL aliquot of diluted blank serum sample diluted 1:1 (v/v) with a 5 mM ammonium formate buffer, pH 8 was loaded onto the SPE well, washed with 20% MeOH in water and eluted with 0.5 mL 75% MeOH. 50 µL of working standard containing Scop and Atr was added to the blank serum extract. For the spiked serum samples, a 500 µL aliquot of spiked blank serum sample diluted 1:1 (v/v) with a 5 mM ammonium formate buffer, pH 8, was loaded onto the SPE well, washed with 20% MeOH in water and eluted with 0.5 mL 75% MeOH, evaporated to dryness and reconstituted with 50 µL mobile phase. The overall clean-up process resulted in the diluted serum sample being concentrated into 50 µL of MP. 20 µL of the reconstituted sample was injected into the HPLC system for analysis.

4.3.4 Preparation of patient samples

Following approval from the Dublin City University Research Ethics Committee, (See Appendix A) a study was carried out on 5 volunteers. Blood samples were obtained by informed consent in Dublin City University. Participants were first administered a whole patch containing 1.5 mg of the drug on the back of the ear with subsequent blood collection and observation. Blood was drawn over 72 hours at different time points. The blood samples were centrifuged in a chilled centrifuge and the serum kept frozen at -80 ºC until analysis. A 500 µL aliquot of each patient sample was diluted 1:1 with a 5 mM ammonium formate buffer, pH 8 and extracted using the same procedure described above for the spiked serum standards. Samples were taken and prepared in duplicate. After a wash out of several days half a patch was applied and the participant's serum sampled as previously.

4.3.5 Method validation

Validation parameters including intra- and inter-day precision, accuracy, sensitivity, linearity and range and recovery were performed on the method. Measurements for each analyte in the biological matrix were validated according to FDA guidelines (FDA, 2001).

4.4 Results and discussion

Taking a methodical approach to choosing the column, mobile phase and mass spectrometer conditions, as well as developing a detailed understanding of the chemistry, structure and solubility of the drugs was essential as part of the initial development of this part of the work. Optimisation of the sample preparation step was also imperative to the likely success of the protocol. Given the practical limitations of the existing literature in this field, coupled with the practical challenges of employing half patches, it was also vital that any methodology developed was better than the current state-of-the-art, had novelty, context and application.

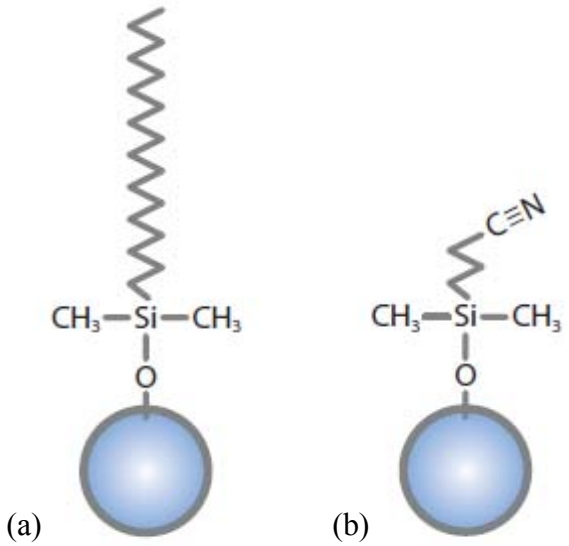
Assay development for this project was challenging due to the fact that over a period of three days, the transdermal patch delivers Scop into the blood stream in the pg/mL range. The main task was to develop a method capable of accurately and reproducible quantitating the anticipated, very low Scop concentrations in blood post patch application which would require high recoveries from the extraction procedure and excellent sensitivity from the detector.

4.4.1 Method development in NASA

4.4.1.1 Chromatographic procedure

Areas focused on for the optimisation of Scop chromatography included choice of column, mobile phase composition, buffer concentration and pH. Agilent Zorbax SB-CN (100 x 2.1 mm, 3.5 μm) and C18 Supelcosil LC-18 (250 x 2.1

mm, 5 μm) columns were investigated using different mobile phase compositions and pH. Alkyl-based stationary phases, such as C18, are best suited for analysing hydrophobic molecules with a high carbon: heteroatom ratio. Cyano-based phases interact strongly with basic, nitrogen-containing and halogenated analytes (Kazakevich *et al.,* 2006). See Figure 4.2.



(a) (b)

Figure 4.2: Reversed phase stationary phases (a) C18 and (b) CN

These columns were investigated using different mobile phase compositions and pH. The column which gave the best result in terms of peak area and shape was the Agilent Zorbax SB-CN. See Fugure 4.3.

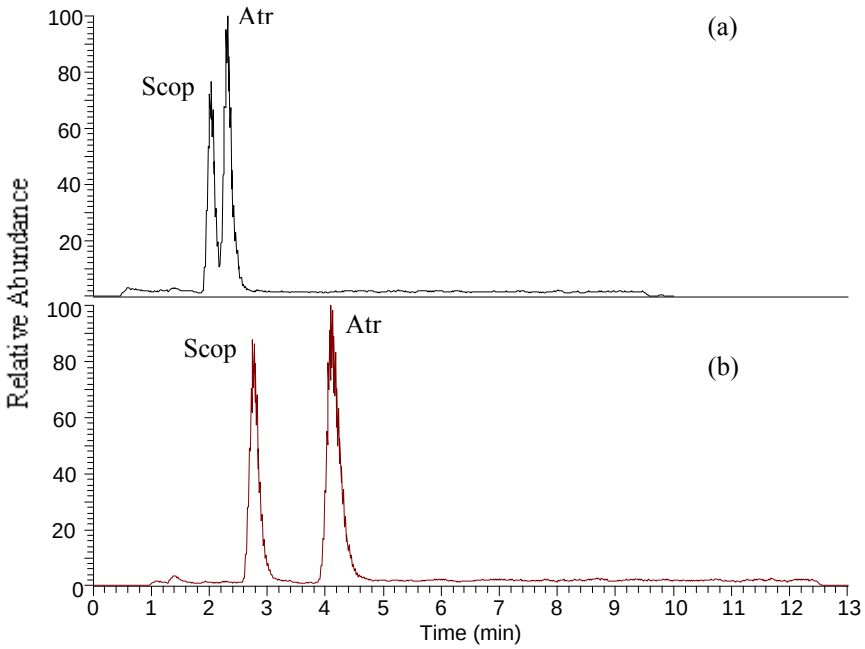


Figure 4.3 Chromatograms comparing the separation of Scop and Atr using (a) a Supelcosil LC-18 column and (b) an Agilent Zorbax SB-CN column

The most common solvents used in HPLC/ESI-MS are water, methanol, acetonitrile, and mixtures of these. The solvent composition (organic/water ratio) is particularly important in the electrospray nebulisation and ionisation process, since it determines surface tension of the droplets formed and the vaporisation efficiency. Because the surface tension of water is much higher than the surface tension of methanol or acetonitrile, the sensitivity is reduced when using more than 70 to 80% of aqueous mobile phases. The organic/water ratio is more significant when working at high flow rates since there is more solvent to be nebulised and vaporised (Chen, 2006).

With reversed phase stationary phases, retention time is longer for molecules which are less polar, while polar molecules elute more readily. By adding more water to the mobile phase the retention time generally increases; as it makes the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase (Kazakevich *et al.,* 2006). Different proportions of methanol-water and acetonitrile-water were investigated during the method development stage. Poor resolution of Scop and Atr was observed using ammonium formate -

MeOH (60:40 v/v) but better results were achieved with ammonium formate - MeOH (70:30 v/v). Also see Figure 4.3 (b).

The influence of the MP pH is important since this can change the hydrophobicity of the analyte. For this reason, a buffering agent was required to control the pH (Neue, 2002). Since hyphenated LC-MS instrumentation has become more and more popular during the last few years, the standard buffers of former times, for example phosphate, have dropped out of favour. The issue is that one would like to use volatile mobile phase additives. Phosphates are not volatile, and with time will clog the LC-MS interface. This results in significant downtime, and more importantly, in a large amount of work to clean up the interface again. Therefore, standard HPLC methods used with MS detection use mobile phase additives that are volatile. Ammonium formate and formic acid were chosen for this reason (Neue, 2002).

Analytical mobile phases of differing pH values (3, 4 and 5) were tested. The optimal pH for the LC-MS analysis was at pH 4. The pH adjustment was achieved by addition of formic acid. In terms of buffer concentration, 2 mM and 5 mM ammonium formate buffers were investigated - see Figure 4.4. On comparing the peak areas (response) for Scop using these two buffers, it was concluded that the 5 mM buffer solution proved considerably better for the peak shape and area of the drug - see Table 4.3.

Conc. (ng/mL)	**5 mM peak area**	**2 mM peak area**
10	2,170,998	2,069,315
20	4,184,275	3,800,601

Table .4.3: Comparison of different ammonium formate buffer concentrations on peak area of Scop using an Agilent Zorbax SB-CN column

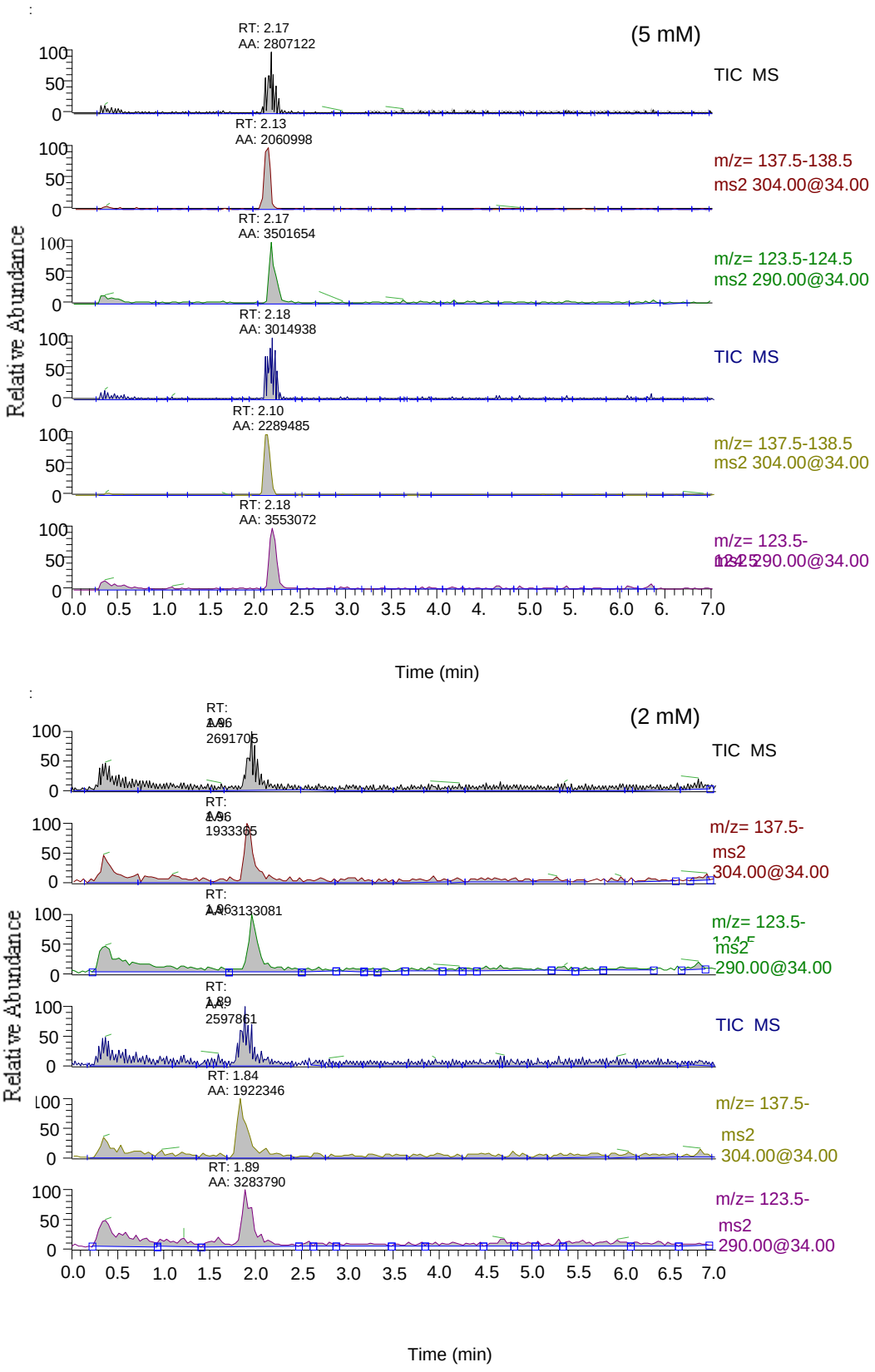


Figure 4.4: LC-MS chromatograms comparing ammonium formate buffer concentrations for a 10 ng/mL Scop and Atr standard using an Agilent Zorbax SB-CN column

4.4.1.2 Extraction procedure

Blood contains an innumerable number of compounds that can interfere with analyte detection. As the number of interferences increase as the analyte concentration decreases, the sample preparation technique becomes an essential part of the chromatographic method. Serum samples contain a significant amount of salt and proteins that can precipitate or adsorb on reversed-phase packings. The adsorbed protein can easily foul the column, resulting in changes in the separation and ultimately clogging the column (Neue, 2002).

As discussed, there are several sample preparation techniques available for the pretreatment of biological samples. Solid phase extraction (SPE) was employed for the sample preparation step as the literature shows this to be successful and appropriate for the extraction of Scop, yielding good recovery of the drug from serum. Two SPE cartridges were investigated - Supelco Supelclean LC-18 and Oasis HLB Supelco Supelclean LC-18 is used for reversed-phase extraction of nonpolar to moderately polar compounds. The Oasis HLB copolymer, with hydrophilic-lipophilic balance can retain both non-polar and polar compounds.

Best extraction efficiency results were achieved with the Oasis HLB cartridge and hence it was the SPE cartridge of choice for this assay. Oasis HLB is a hydrophilic-lipophilic water wettable reversed phase sorbent. It is made from a balanced ratio of two monomers, the hydrophilic N-vinylpyrrolidone and the lipophillic divinylbenzene. See Figure 4.5 and 4.6. It provides superior reversed phase capacity with a special “polar hook” for enhanced retention of polar analytes and so is recommended for the extraction of both non-polar and polar compounds from biological fluids (Waters, 2010). Taking into consideration the structure of Scop, a non-polar tertiary amine, it was an ideal extraction sorbent for the drug.

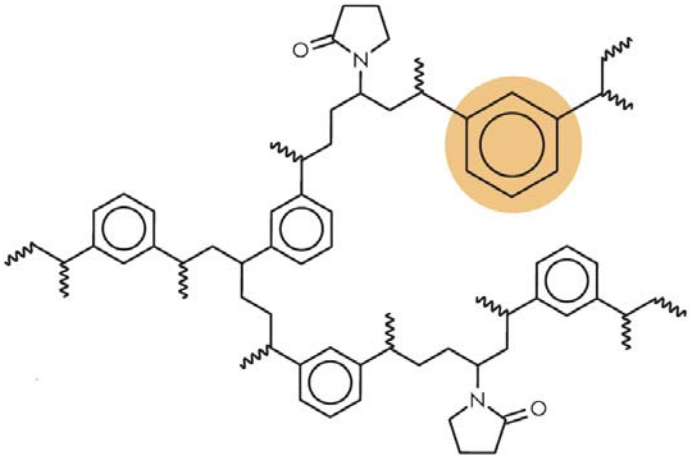


Figure 4.5: Oasis HLB sorbent

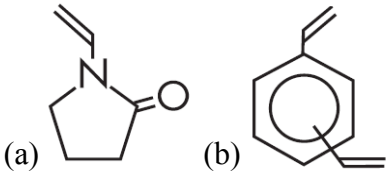


Figure 4.6: (a) The hydrophilic N-vinylpyrrolidone and (b) the lipophillic divinylbenzene of the HLB sorbent

The Oasis cartridge achieved 75% recovery of Scop from serum in comparison with 60% for the Supelco cartridge. See Table 4.4.

SPE cartridge type	**Scop recovery (%) ± % RSD n = 3**
Oasis HLB	75.1 ± 0.7
Supelco Supelclean	60.2 ± 2.3

Table 4.4: Comparison of recoveries obtained for Scop using different SPE cartridges

As discussed in Chapter 2, there are six basic steps in SPE which include sample pretreatment, sorbent conditioning, sorbent equilibration, sample loading, washing, and analyte elution. During the SPE method development each of these steps were investigated.

Methanol was again used to condition the sorbent in order to wet the sorbent surface and allow the analytes to break through the surface tension of the water and move into the sorbent.

The conditioning solvent needed to be displaced from the sorbent using an equilibration solvent, this also prepares the sorbent surface to receive the sample. The sorbent was equilibrated with the same buffer as used to dilute the sample in order that the chemistry of the sorbent remained constant throughout the sample loading step.

Sample pretreatment involved sample dilution and pH adjustment. As discussed, dilution reduces the viscosity of the aqueous sample, improving chromatographic mass transfer, and thus improving the efficiency of the extraction. In this work, the serum sample was diluted 1:1 with an aqueous buffer containing formic acid. The speed with which the sample was applied was closely monitored ensuring a slow consistent rate to allow sufficient time for the analytes to partition into the sorbent surface.

The optimum wash solvent needed to be non-polar enough to elute as many interferences as possible, but not sufficiently non-polar to elute the analytes. The wash solvent was identified by washing the SPE cartridges starting with 100% water, then progressively increasing the proportion of MeOH present. Proportions of 10, 20 and 30% MeOH in the wash solvent were evaluated. Each wash step was collected and analysed for the presence of the drugs. There was a slight loss of recovery at 30% and so, the wash containing the highest percentage organic but no analytes was at 20%.

Under the final optimised SPE conditions a 2 mL aliquot of each of the working standards was diluted 1:1 (v/v) with a 1% formic acid base solution in water. The SPE Oasis HLB (60 mg) cartridges were conditioned with 2 mL MeOH and then equilibrated with 2 mL of H_2O- buffer solution (50:50 v/v.) A 4 mL aliquot of the diluted sample was loaded onto the well, washed with 20% MeOH in water and eluted with 0.5 mL MeOH. The samples were evaporated to dryness and reconstituted with 100 µL MP. The reconstituted sample was

then transferred to an autosampler vial (with insert) and 20 μL was injected into the HPLC system for analysis.

The major setback of this optimised SPE method was the high serum sample volume at 2 mL. Ideally, a smaller sample volume would be desirable but due to the sensitivity achieved from the optimised LC-MS conditions, this high volume was required in order to concentrate the sample enough for it to reach into the pg/mL range required.

4.4.1.3 Mass spectrometric conditions

As mentioned, Atr and Scop have been detected in biological samples using GC–MS techniques (Eckert et al., 1981, Oertel et al., 1996, Namera et al., 2002, Bayne et al., 1975, Deutsch et al., 1990) but the drawback of GC-MS in detecting tropane alkaloids is the heat instability of these compounds (Eckert et al., 1981). UV does not achieve the sensitivity required for the levels of the drug found in plasma/serum post patch application. As the doses of Scop to be detected are quite low (in the pg/mL range), it was decided that MS detection would be necessary to achieve adequate sensitivity in blood samples.

During method development, Scop was identified by directly infusing a known concentration into the MS via the t-piece with mobile phase at a flow of 200 μL/min. Electrospray ionisation (ESI) in the positive ion mode allowed for the detection of the protonated precursor (parent) ion *m/z* 304 – see Figure 4.7. This precursor ion was subsequently isolated and subjected to a collision energy resulting in two predominant fragment (daughter) ions: *m/z* 138 and *m/z* 156 - Figure 4.7 also.

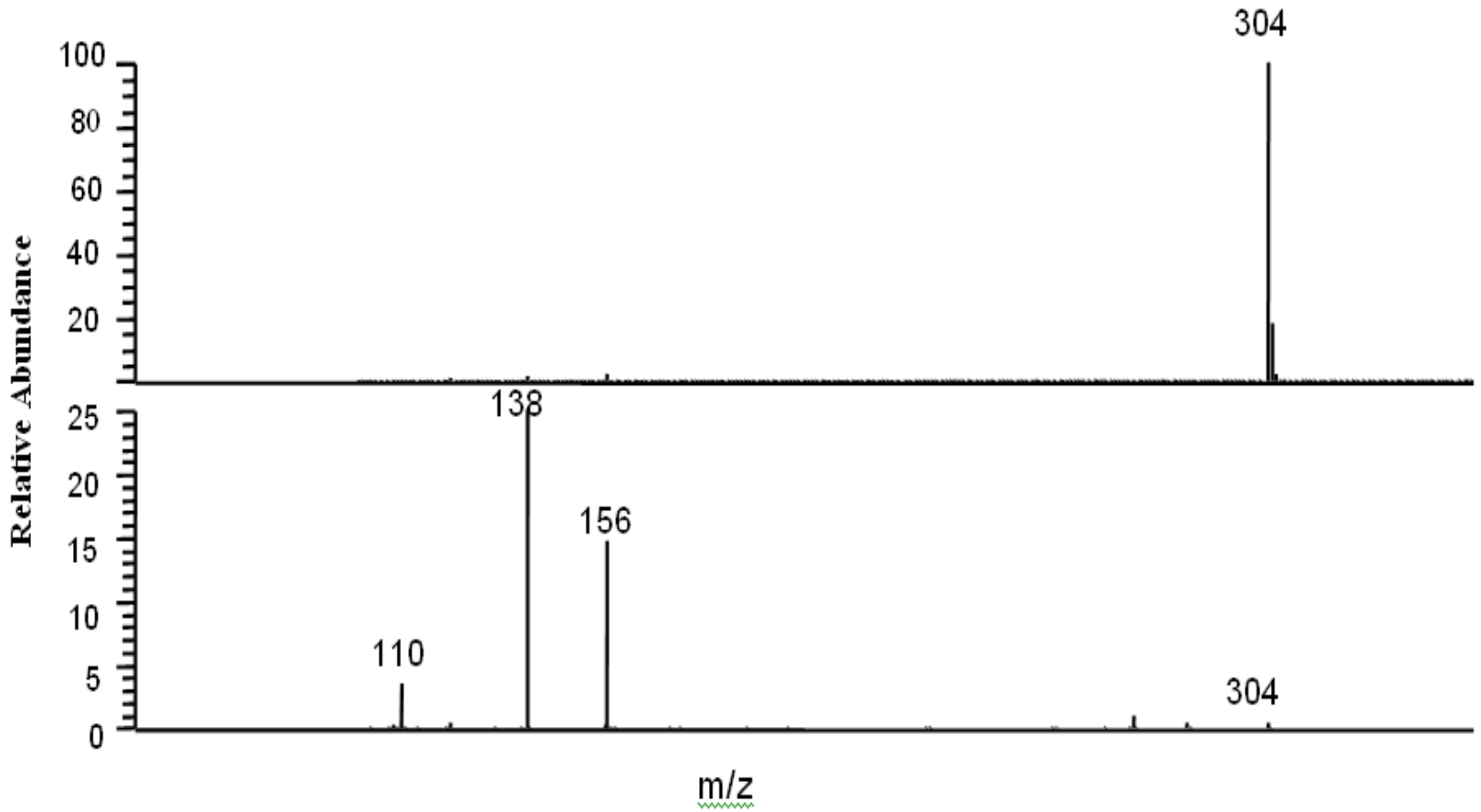


Figure 4.7: The precursor ion and fragment ions of Scop

Following this, Scop was analysed via LC-MS using the multiple reaction monitoring (MRM) mode. MRM involves selection of the precursor ion and fragmenting it in order to deliver unique product ions that are monitored and quantified. MRM was performed on Scop by monitoring the transitions between *m/z* 304 and 138 and between *m/z* 304 and 156. MRM was performed on Atr by monitoring the transition between *m/z* 290 and 124. This approach allowed for sensitive and selective quantitation of Scop. The most important parameters affecting the signal quality during ESI/MS operation are heated capillary temperature, tube lens offset voltage, capillary voltage and sheath gas flow rate and these were all optimised during the development process.

Optimisation of the sensitivity of Scop detection in the MS detector was carried out using both automatic and semi-automatic procedures. The automatic procedure adjusts the tube lens offset voltage, capillary voltage, and voltages applied to the ion optics until the ion transmission of the analyte is maximised. Once this is completed, semi-automatic tuning allows for manual optimisation of sheath and auxiliary gas, collision energy and also the capillary temperature which needs to be optimised in order to maximise the ion transmission to the MS detector. All conditions manually controlled were optimised so as to maximise the intensity of the Scop response. Under final conditions within the LCQ, capillary temperature was set to 270 °C, collision energy to 34% and sheath gas and auxiliary gas to 90% and 10% respectively. The injection time was 800 ms and the number of microscans was three with an isolation width of 3 amu.

4.4.1.4 Validation

The overall LC-MS method was only partially validated by investigating linearity, range, LOD and recovery. Due to time constraints, a full validation could not be performed.

The measured limit of detection (LOD) for Scop in serum was found to be 1.0 ng/mL. The calibration curves for Scop over the concentration range 1.0-15.0 ng/mL in serum exhibited good linearity with correlation coefficients (R^2)

for all standard curves above 0.99 - see Figure 4.8. The instrument showed excellent reproducibility, linearity and signal stability over time.

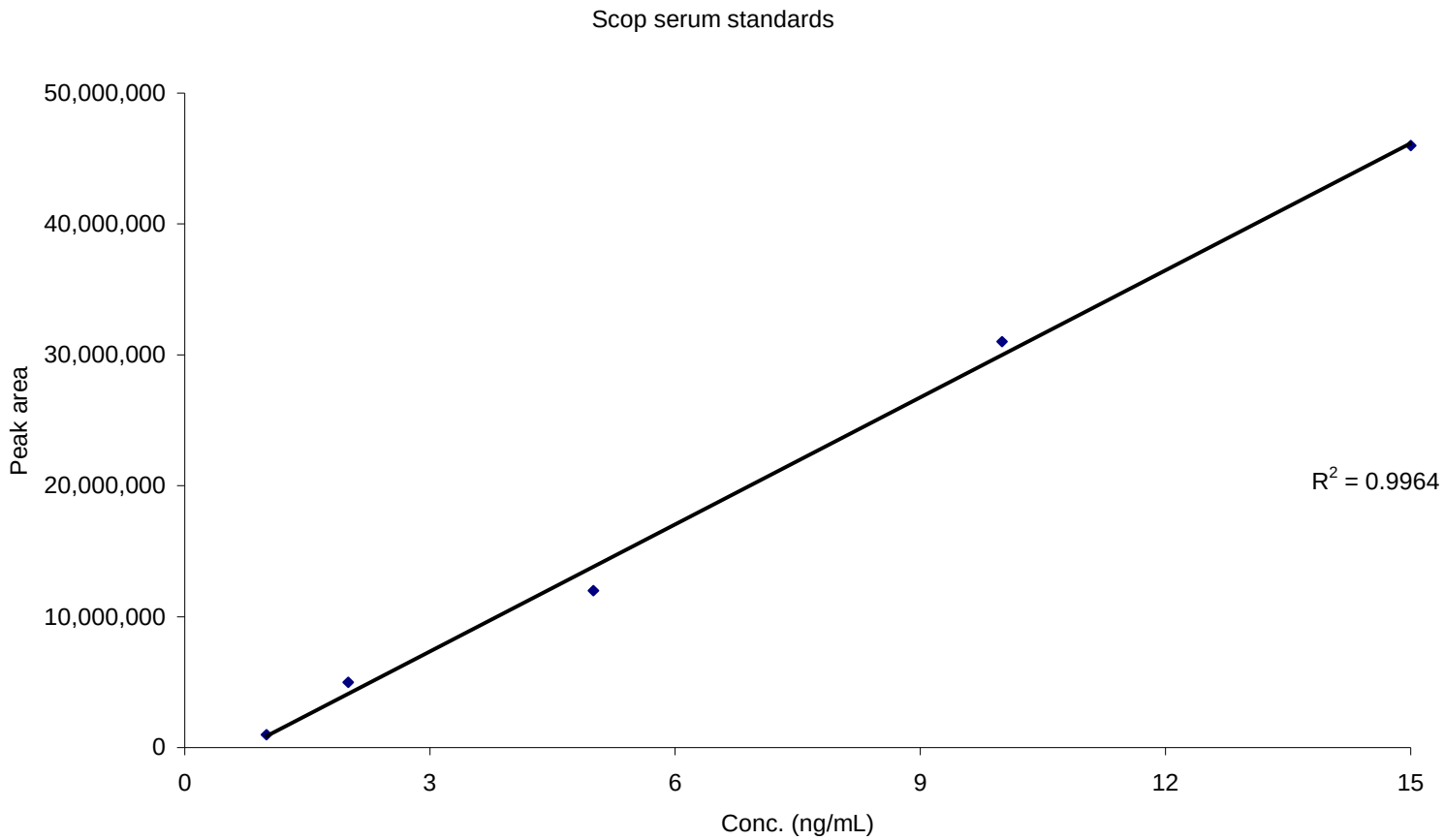


Figure 4.8: Linearity for Scop serum standards using an Agilent Zorbax SB-CN column

Recovery experiments were performed by spiking known amounts of Scop into serum and extracting by SPE. The samples were then analysed using the LC-MS method and compared with the results of the standards of Scop prepared in diluent which were carried through the same process. Recovery was determined in triplicate at concentrations 1, 5 and 10 ng/mL. The extraction recovery for Scop averaged 75% at all concentrations prepared. See Figure 4.9. SPE showed consistent day-to-day reproducibility of Scop spiked in serum.

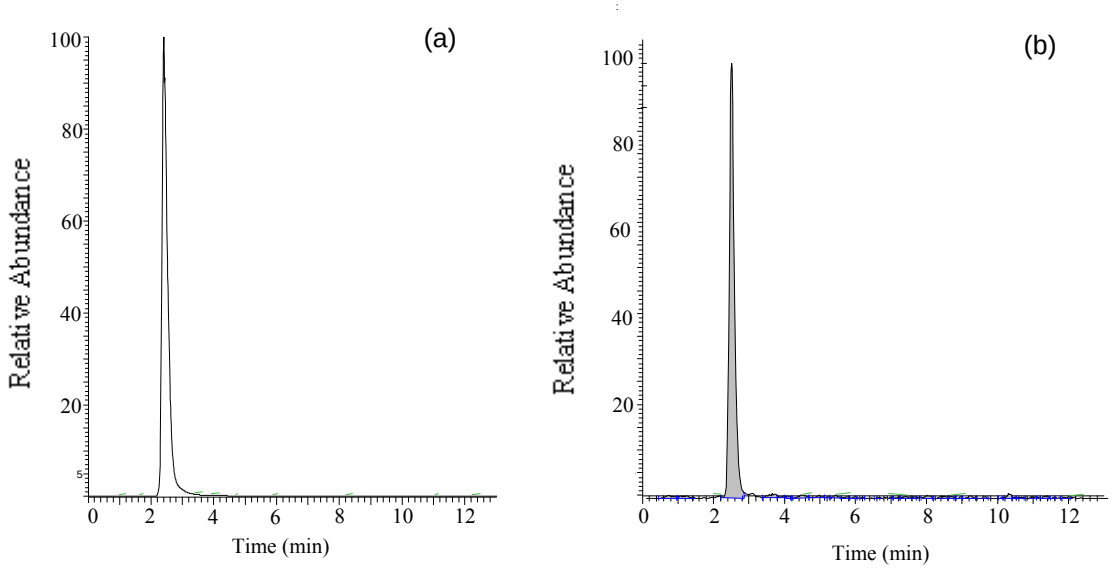


Figure 4.9: MRM chromatograms of a) extracted serum sample spiked with 10 ng/mL Scop and b) a working standard of Scop prepared to 10 ng/mL in mobile phase using an Agilent Zorbax SB-CN column

Due to time constraints, further optimisation and validation of the method could not be carried out and so a migration of the method was proposed in order to continue the research in Ireland.

4.4.2 Method development in DCU

On returning to DCU, it was hoped to continue the above research with a method migration to a laboratory within the NICB and validate the method.

Work continued using an Agilent LC-MS system consisting of a 1200 quaternary pump, autosampler and degasser and a 6410 QQQ LC/MS.

4.4.2.1 Chromatographic and extraction procedures

Agilent Zorbax SB-CN column

Under the same chromatographic conditions developed in NASA, a migration of the method to the DCU equipment proved unsuccessful. The Agilent Zorbax SB-CN (100 × 2.1 mm, 3.5 µm) column previously used was not able to achieve separation between the two drugs. The main variable between the two methods was the LC-MS instrumentation used. This could have been a possible source for differing results obtained upon migrating the methood. Although unlikely, there could also have been variability between the chemistries of the two cyano columns used. Figure 4.10 displays results using the cyano column while varying the % MeOH between 30 and 70%. Buffer used was as before, 5 mM ammonium formate, pH 4. Using the same flowrate of 0.2 mL/min, 20 µL of a Scop and Atr 10 ng/mL mixed standard was injected. As a short runtime was desired, a gradient programme was not considered and so, further optimisation using this column was not undertaken.

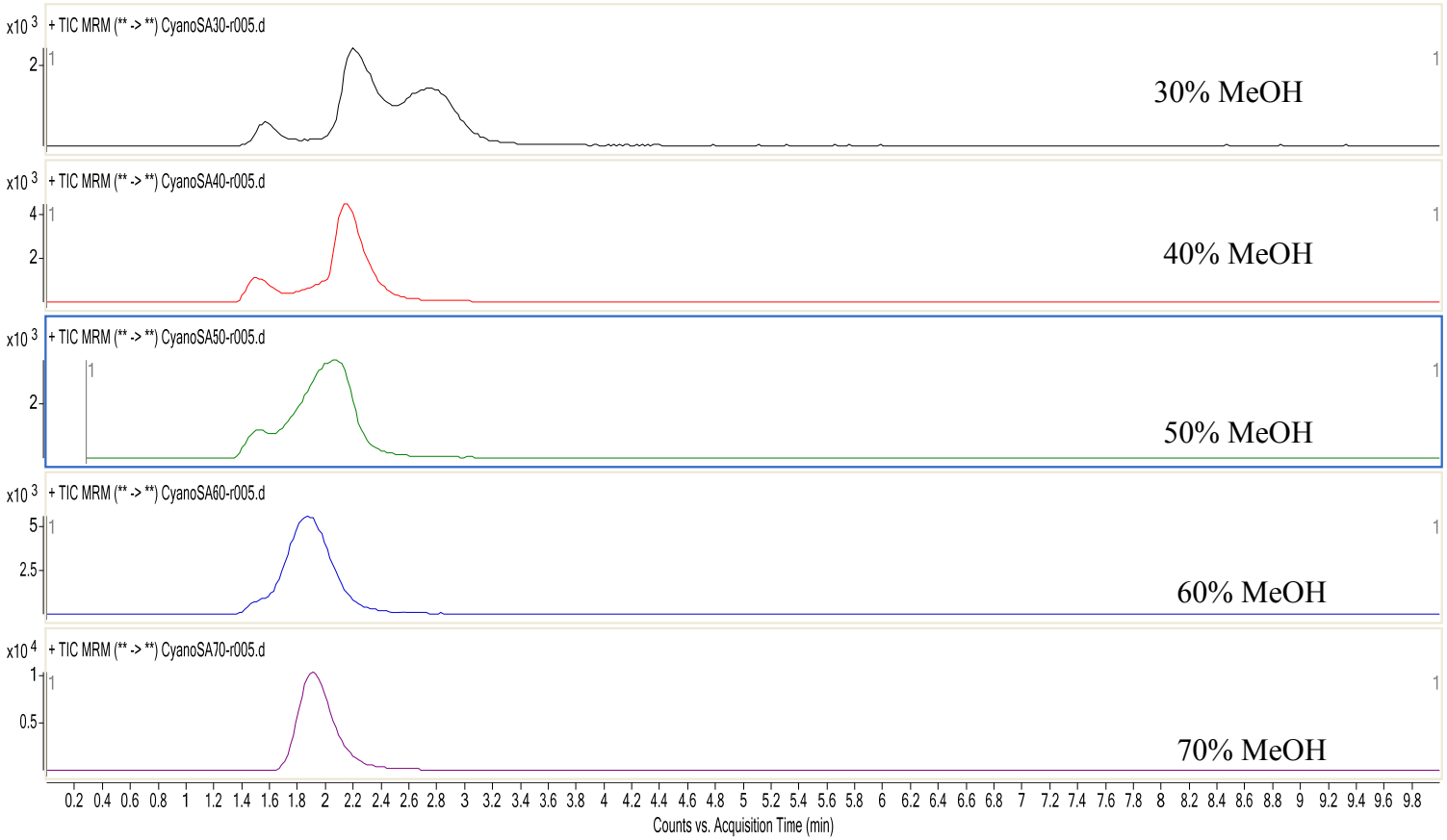


Figure 4.10: Comparison of the impact of different % MeOH mobile phase compositions on peak shape and retention for Scop and Atr using an Agilent Zorbax SB-CN column

It was decided to carry out further method development with regards to column selection. Structural properties of the analyte molecule play an important role in its retention characteristics. In general, an analyte with a larger hydrophobic surface area (C-H, C-C, and generally non-polar atomic bonds, such as S-S and others) results in a longer retention time because it increases the molecule's non-polar surface area, which is non-interacting with the water structure. On the other hand, polar groups, such as -OH, $-NH_2$, COO^- or $-NH_3^+$ reduce retention as they are well solvated with water molecules (Dong, 2006). Bearing this in mind and the structures of Scop and Atr, it was decided to investigate C18, C8 and Phenyl phases in order to find one capable of separating the two drugs.

Zorbax RX-C18 column

An Agilent Zorbax RX-C18 (narrow bore 150 x 2.1 mm, 5 μ) column was firstly investigated under the same chromatographic conditions as for the cyano column above. As discussed, with reversed phase stationary phases, retention time is longer for molecules which are less polar, while polar molecules elute more readily. By adding more water to the mobile phase the retention time was increased; thereby making the affinity of the hydrophobic analyte for the hydrophobic stationary phase stronger relative to the now more hydrophilic mobile phase (Kazakevich *et al.,* 2006). Figure 4.11 displays results using this column while varying the % MeOH between 30 and 70%. Buffer used was as before, 5 mM ammonium formate, pH 4. Using the same flowrate of 0.2 mL/min, 20 μL of a Scop and Atr 10 ng/mL mixed standard was injected. This column was unable to achieve separation between the two drugs.

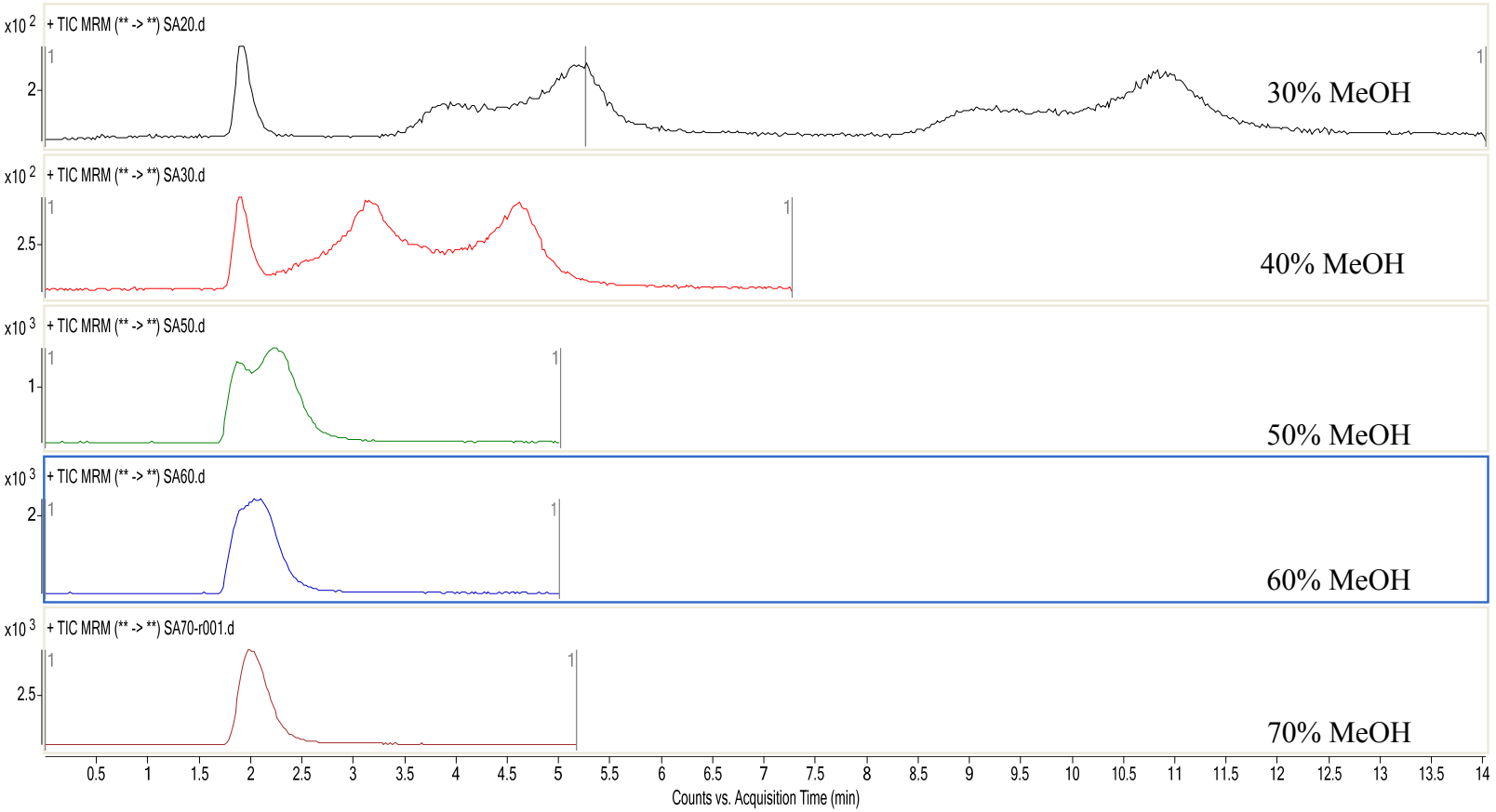


Figure 4.11: Comparison of the impact of different % MeOH mobile phase compositions on peak shape and retention for Scop and Atr using an Agilent Zorbax RX-C18 column

Zorbax Eclipse Rapid Resolution column

The second C18 column investigated was an Agilent Zorbax Eclipse Rapid Resolution (50 x 2.1 mm, 3.5 μm) column. Figure 4.12 displays results using this column while varying the % MeOH between 30 and 70%. Buffer used was as before, 5 mM ammonium formate, pH 4. Using the same flowrate of 0.2 mL/min, 20 μL of a Scop and Atr 10 ng/mL mixed standard was injected. This column was also unable to achieve separation between the two drugs.

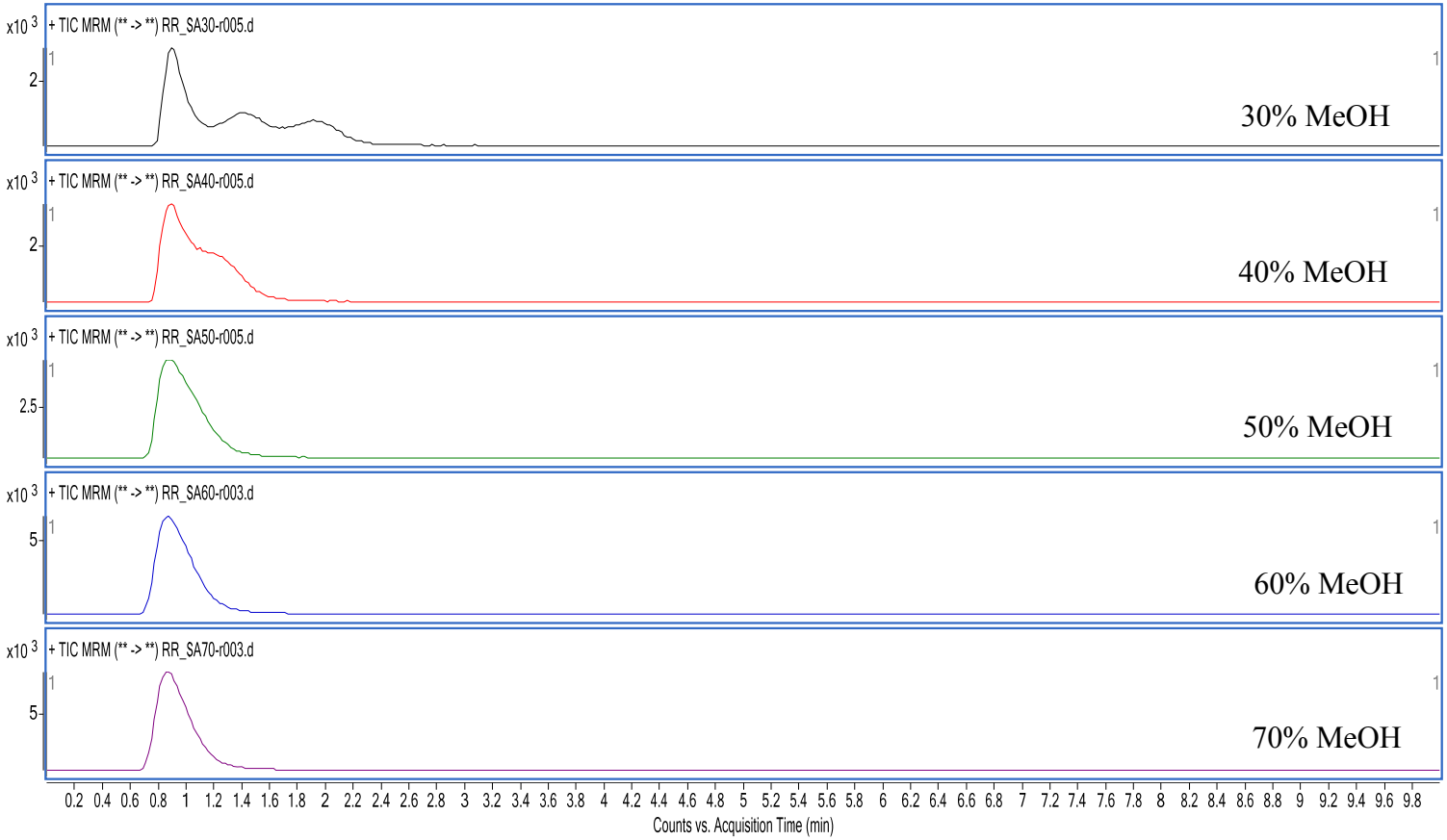


Figure 4.12: Comparison of the impact of different % MeOH mobile phase compositions on peak shape and retention for Scop and Atr using an Agilent Zorbax Eclipse Rapid Resolution column

Waters Nova-Pak C18 column

The third C18 column investigated was a Waters Nova-Pak C18 (150 × 3.9 mm, 4 μm). Due to the dimensions of the column, it was decided a higher flowrate than 0.2 mL/min was required. Preliminary work was carried out using 0.75 mL/min. Areas focused on for the optimisation of the chromatography of both drugs – Scop and Atr - included mobile phase composition, buffer concentration and pH. Different compositions of MeOH were firstly examined, see Figure 4.13. A flowrate of 0.6 mL/min and the same 20 μL of a Scop and Atr 10ng/mL standard was injected as with the previous column investigated. Ammonium formate (pH 4, adjusted with 1 M formic acid; 5 mM) - MeOH (10:90, v/v) was chosen for the mobile phase as separation of the drugs was achieved with a relatively short runtime.

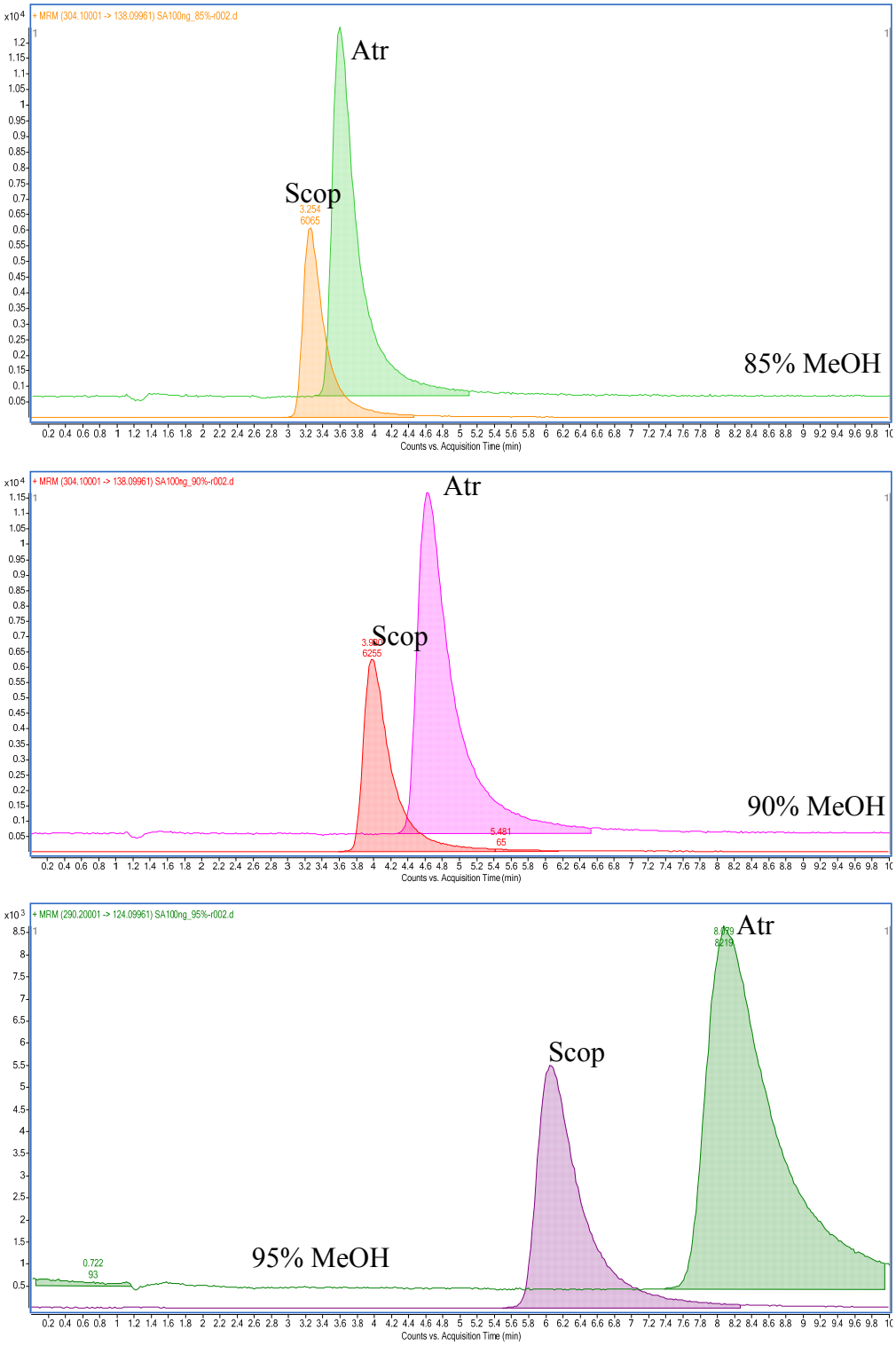


Figure 4.13: Comparison of the impact of different % MeOH mobile phase compositions on peak shape and retention for Scop and Atr using a Waters Nova-Pak C18 column

As discussed in Chapter 1, the selectivity or separation factor of a chromatographic system is a measure of the spacing between two given peaks. Represented by α, it describes how effectively a chromatographic system can separate two compounds (A and B) on the column (Weston *et al.,* 1997). See Eqn. 4.1. α is primarily a function of the packing material but can be manipulated to some extent by varying the mobile phase and/or temperature.

$$\alpha = k'_B / k'_A \qquad \text{Eqn. 4.1}$$

where *k'* (the retention/capacity factor) is defined as per Eqn. 4.2.

$$k'_A = t_R - t_M / t_M \qquad \text{Eqn. 4.2}$$

When calculating the selectivity factor, species A elutes faster than species B and so the selectivity factor is always greater than one. The value for α can range from 1 (when the retention times of the two compounds are identical), to infinity, (if the first component elutes in the void volume). If α is approaching 1, then regardless of efficiency or length of time on column, there will be no separation (Weston *et al.,* 1997). α was mathematically calculated for Scop and Atr using different chromatographic conditions.

Although 95% MeOH gave higher peak area, the peaks were broader and analysis time long, see Figure 4.13, and so 90% MeOH was chosen as the optimal MP as it offered the best results for both drug peaks with an acceptable α value of 1.33 as seen in Table 4.5**.**

% MeOH MP composition	t_R Scop	k' Scop	t_R Atr	k' Atr	α
85	3.3	1.71	3.8	2.18	1.27
90	3.9	2.32	4.9	3.08	1.33
95	6.3	4.25	8.4	6.0	1.41

Table 4.5: Comparison of different % MeOH mobile phase compositions on separation factor for Scop and Atr using a Waters Nova-Pak C18 column

As previously discussed, an important MP factor is the influence of the pH since this can change the hydrophobicity of the analyte. A buffering agent was hence required in order to control the pH. As ammonium formate and formic acid are routinely used in mass spectrometry, it was employed for this work. In order to further optimise the separation, different mobile phase buffer concentrations and pHs were looked at. Ammonium formate (pH 5 adjusted with 1 M formic acid) - MeOH (10:90, v/v) at 2, 5, 10 and 20 mM ammonium formate buffer concentrations were examined. See Figure 4.14. A 1% formic acid - MeOH (10:90, v/v) mobile phase was also investigated, however, from Figure 4.15 it can be seen the results obtained were not satisfactory. Separation between the peaks was poor and the peak area for Scop was low.

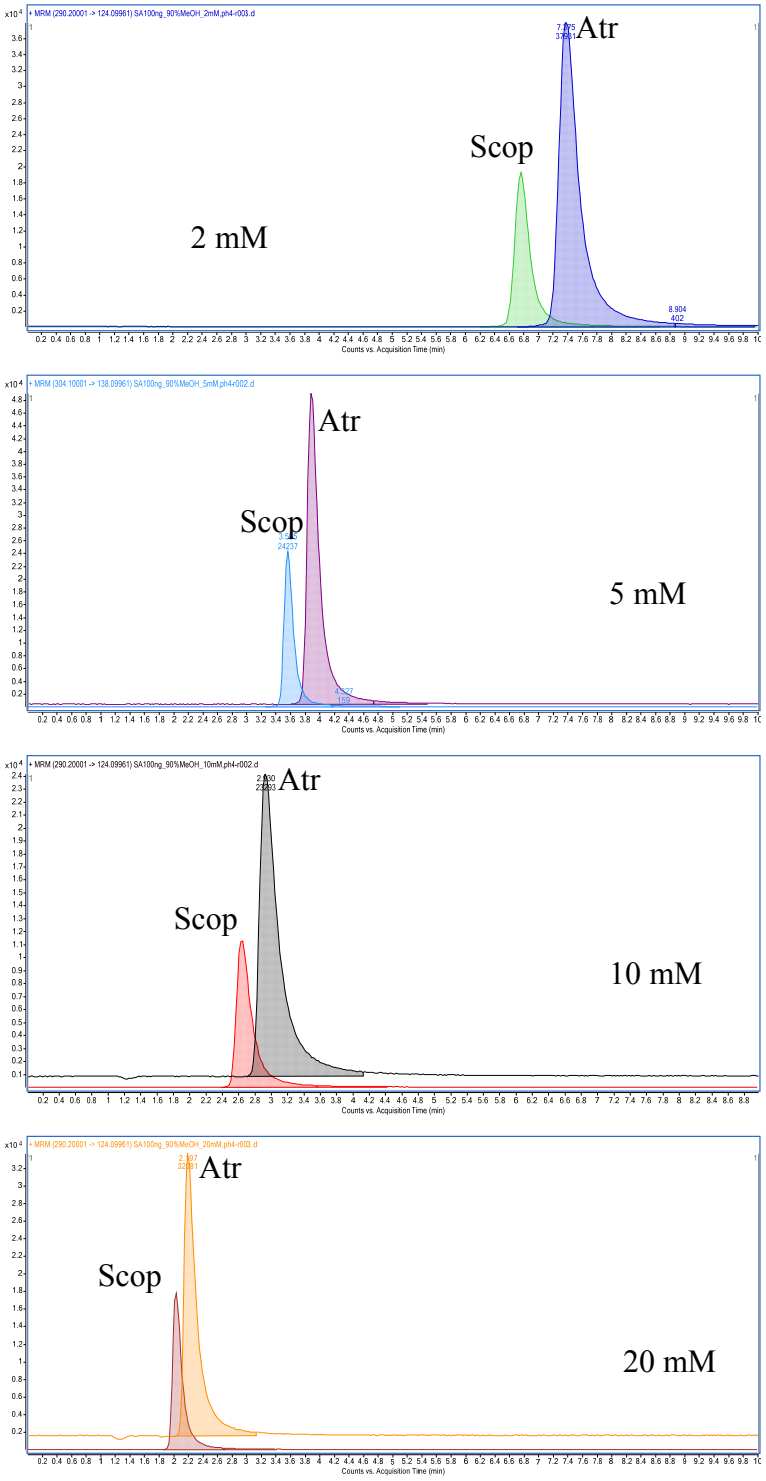


Figure 4.14: Comparison of the impact of different ammonium formate concentrations (pH 5) on peak shape and retention for Scop and Atr using a Waters Nova-Pak C18 column

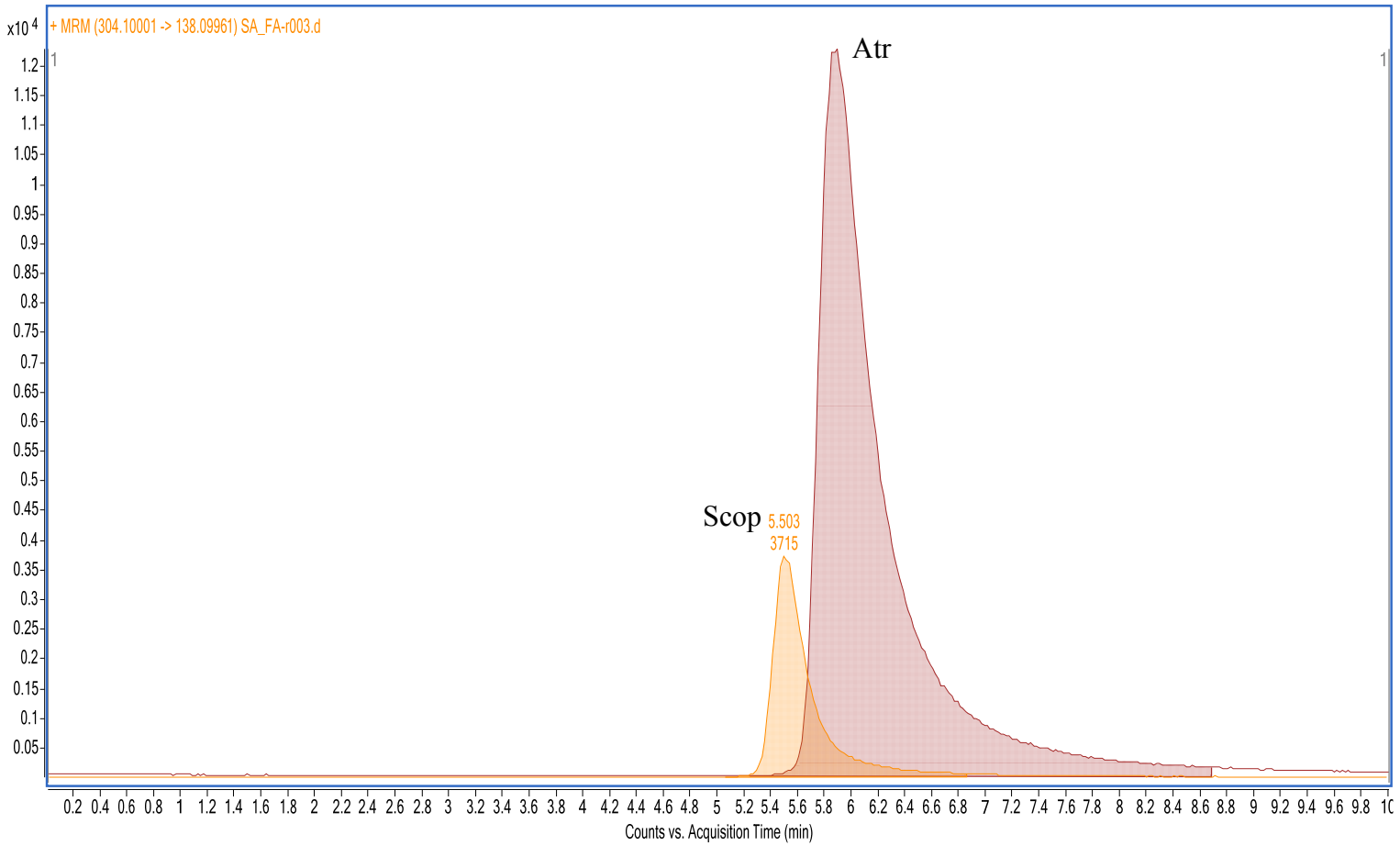


Figure 4.15: Chromatogram of Scop and Atr using 0.1% formic acid as mobile phase using a Waters Nova-Pak C18 column

It was decided to choose 5 mM ammonium formate as buffer for the mobile phase. Even though the peak area was slightly lower for both drugs in comparison to 2 mM, the retention times of the drugs are much shorter and Table 4.6 proves sufficient selectivity was achieved.

Ammonium formate conc. (mM)	**t_R Scop**	**k' Scop**	**t_R Atr**	**k' Atr**	**α**
2	6.8	4.7	7.4	5.1	1.1
5	3.6	2.0	4.0	2.3	1.2
10	2.7	1.3	2.9	1.4	1.2
20	2.1	0.8	2.2	0.8	1.1

Table 4.6: Comparison of different ammonium formate concentrations (pH 5) on retention factor for Scop and Atr using a Waters Nova-Pak C18 column

Using the concentration of 5 mM ammonium formate, different pHs were examined (3, 4 and 5). pH adjustment was achieved using formic acid. From Figure 4.16 it can be seen that pH 4 offered the best compromise between good peak shape and adequate separation. Also, the highest peak area values were achieved using pH 4. Again sufficient selectivity was achieved as seen in Table 4.7. Selectivity achieved using pH 3 and 5 was better than at pH 4, however, band broadening was evident at these pHs.

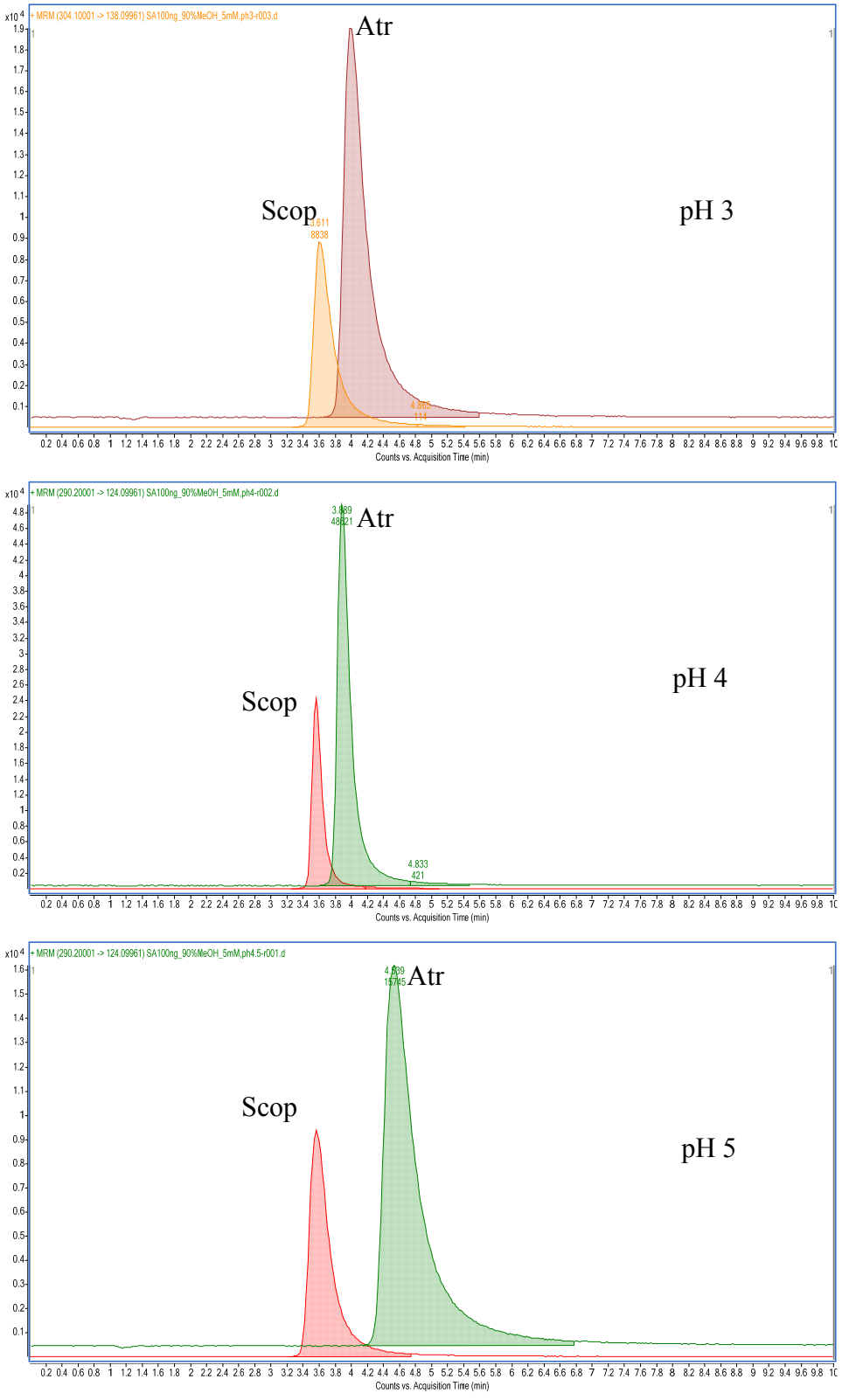


Figure 4.16: Comparison of the impact of different mobile phase pHs using 5 mM ammonium formate on peak shape and retention for Scop and Atr using a Waters Nova-Pak C18 column

Mobile phase pH	t_R Scop	k' Scop	t_R Atr	k' Atr	α
3	3.6	2.01	4.3	2.54	1.23
4	3.6	2.00	3.9	2.25	1.13
5	3.7	2.08	4.5	2.75	1.32

Table 4. 7: Comparison of different mobile phase pHs using 5 mM ammonium formate on retention factor for Scop and Atr using a Waters Nova-Pak C18 column

Column temperature was also varied in order to try and improve separation between the drugs. Temperatures of 20, 30 and 40 ºC were investigated. The change in temperature did not adversely affect the peak areas of the drugs and only a small shift in retention time was observed. Hence it was decided to maintain temperature at 20 ºC as it is a more convenient, stable and achievable temperature to maintain.

Four mobile phase flow rates from 0.5 to 0.75 mL/min were tested. The results are shown below in Figure 4.17 where it was evident, as might be expected, that the slowest flowrate gave the longest retention time and most tailing while it was noted that the slowest flowrate also yielded the highest peak areas. So a compromise of 0.6 mL/min was chosen as there was less peak tailing evident and the peak area obtained was higher than at the faster flowrates.

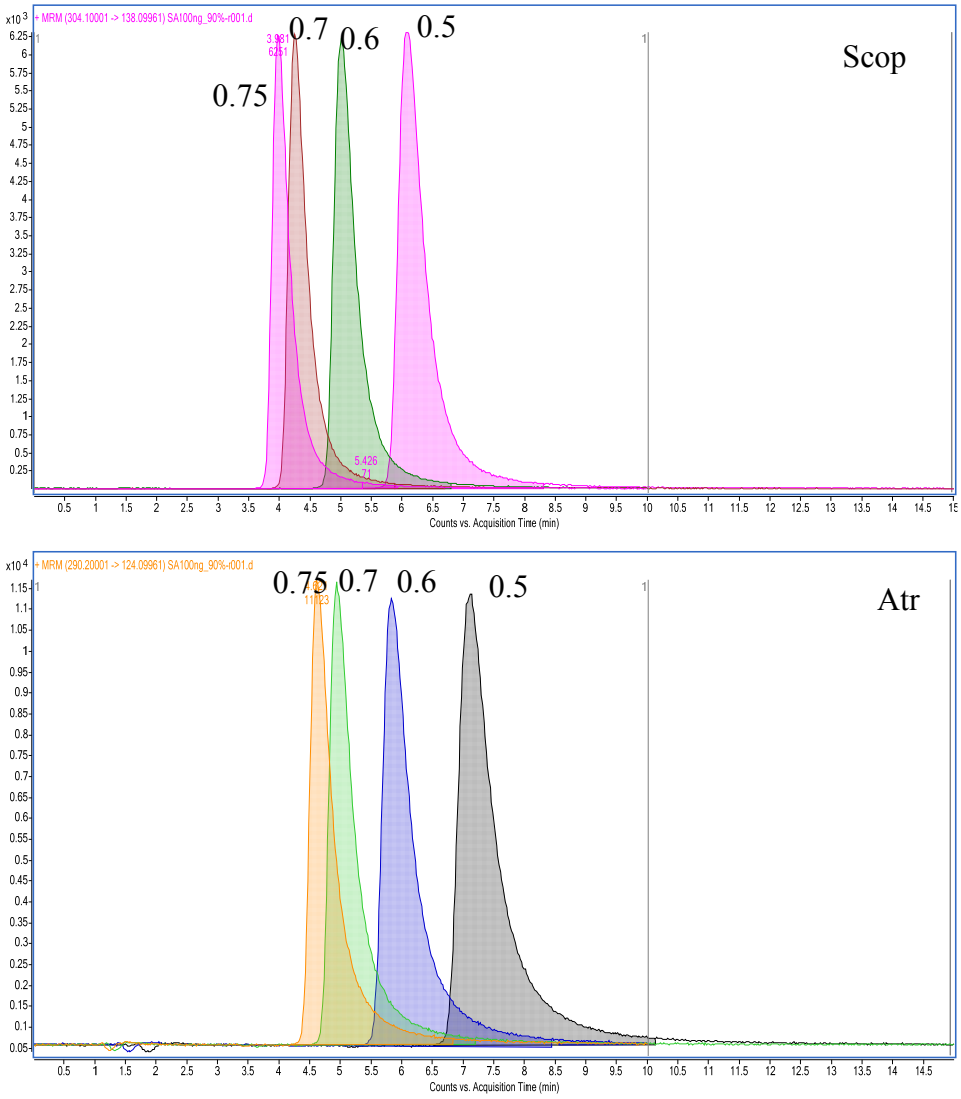


Figure 4.17: Comparison of the impact of different flowrates on peak shape and retention for Scop and Atr using a Waters Nova-Pak C18 column

A general downfall of the results obtained is the peak tailing associated with the Atr peak in particular. There are many sources of peak-tailing, ranging from column problems to chemistry problems to instrument problems. The most common reasons for peak tailing are extra-column band broadening, deterioration of the packed bed, and interaction of the analytes with active sites on the packing.

Also, the separation between both Scop and Atr, although borderline acceptable in terms of the α achieved, was not ideal. All areas of the chromatographic procedure were investigated in order to further improve this separation but to no avail.

Under the final Waters Nova-Pak C18 column (150 × 3.9 mm, 4 µm) chromatographic conditions a 20 µL injection volume was used. As it is a 4 µm particle size column, a higher injection volume is not recommended as it would lead to sample overloading and cause peak fronting. A mixture of ammonium formate (pH 4 adjusted with 1 M formic acid; 5 mM) - MeOH (10:90, v/v) was used as mobile phase, at a flow rate of 0.6 mL/min. The complete chromatographic run time of each sample was 10 min, which separated Scop and Atr from each other with retention times 5.1 and 6.1 min, respectively. See Figure 4.18. The sensitivity achieved for the drugs prepared in mobile phase was 100 pg/mL.

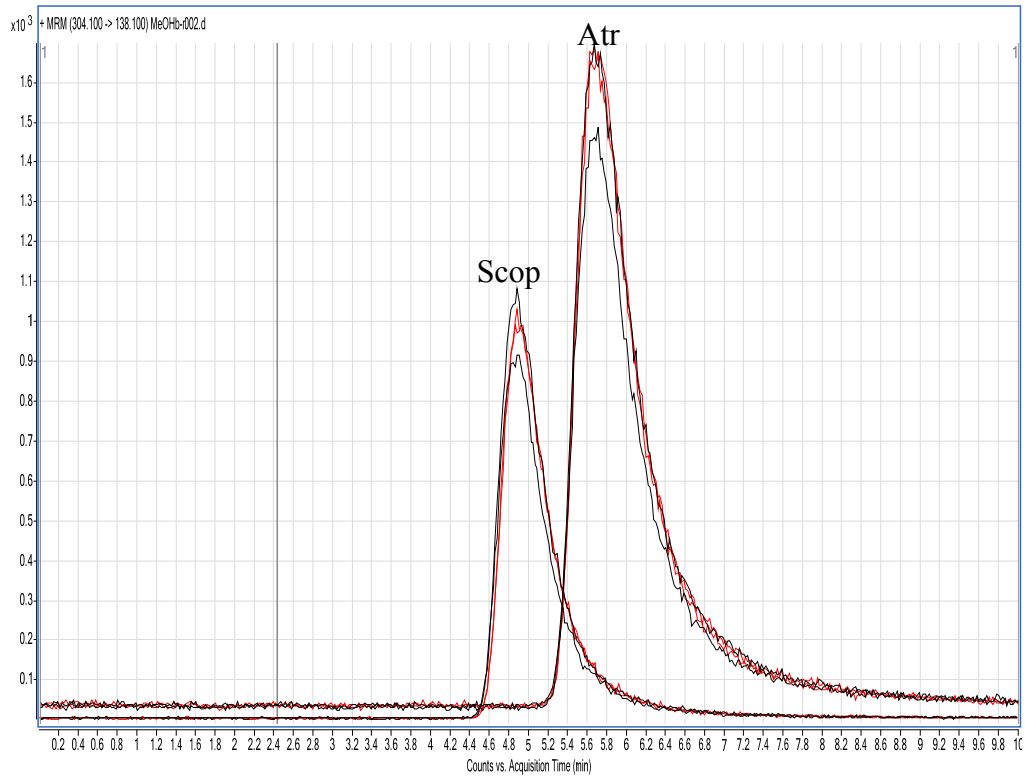


Figure 4.18: Chromatogram of Scop and Atr standard prepared in MP (10 ng/mL) using a Waters Nova-Pak C18 column

SPE development for use with Waters Nova-Pak C18 column

Solid phase extraction (SPE) was employed for the sample preparation step as the literature shows it to be successful and superior for the extraction of Scop and Atr, yielding good recoveries of the drug from serum. Five SPE cartridges

were investigated - Oasis HLB, MAX, WAX, MCX and WCX (all 1cc, 30 mg cartridges). These are each described briefly below:

Oasis HLB contains a polymeric water-wettable reversed-phase sorbent. This copolymer, with hydrophilic-lipophilic balance can retain both non-polar and polar compounds (Waters, 2010).

Oasis MCX; Mixed-Mode Cation eXchange and Reversed-Phase Sorbent is recommended for the extraction of basic compounds. It is synthesised from the reversed-phase Oasis HLB polymer and hence features two retention mechanisms; cation exchange and reversed-phase. Oasis MAX; Mixed-Mode Anion eXchange and Reversed-Phase Sorbent is for the extraction of acidic compounds. It too features two retention mechanisms; anion exchange and reversed-phase. Both MCX and MAX are water wettable, polymeric sorbents, stable from pH 0 to 14.

Oasis WCX; Weak Cation eXchange is for the extraction of strong bases and features cation exchange and reversed phase retention mechanisms. Oasis WAX; Weak Anion eXchange is for the extraction of strong acids. Again, its retention is mixed-mode, both ion exchange and reversed phase (Waters, 2010).

Table 4.8 outlines the SPE conditions used for the SPE Oasis WCX, WAX, MAX, WCX and HLB (all 1cc, 30 mg) cartridges. Four replicates for the blank serum and four replicates for the spiked serum were used on all 5 sorbents.

Cartridge	MCX	WAX	MAX	WCX	HLB
Condition:	1 mL MeOH	1 mL MeOH	1 mL MeOH	1 mL MeOH	1 mL MeOH
Equil:	1 mL H_2O	1 mL H_2O	1 mL H_2O	1 mL H_2O	1 mL H_2O
Load:	1 mL spiked serum / 1 mL blank serum	1 mL spiked serum / 1 mL blank serum	1 mL spiked serum / 1 mL blank serum	1 mL spiked serum / 1 mL blank serum	1 mL spiked serum / 1 mL blank serum
Wash :	1 mL 2% formic acid in H_2O	1 mL 2% formic acid in H_2O	1 mL 5% ammonium hydroxide in H_2O	1 mL 5% ammonium hydroxide in H_2O	1 mL 5% MeOH in H_2O
Eluate 1:	2 x 0.25mL MeOH	2 x 0.25mL MeOH	2 x 0.25mL MeOH	2 x 0.25mL MeOH	2 * 0.25mL MeOH
Eluate 2:	2 x 0.25 mL 5% ammonium hydroxide in MeOH	2 x 0.25 mL 5% ammonium hydroxide in MeOH	2 x 0.25mL 2% formic acid in MeOH	2 x 0.25mL 2% formic acid in MeOH	

Table 4.8: SPE conditions used for MCX, WAX, MAX, WCX and HLB

For all four ion exchange sorbents, the spiked serum eluate 1's were diluted with 0.5 mL MeOH and the blank serum eluate 1's diluted with 0.5mL recovery standard (100 pg/mL). For MCX, WAX, the spiked serum eluate 2 was diluted with 0.5 mL 2% formic acid in MeOH and the blank serum eluate was diluted with 0.5 mL Recovery standard in 2% formic acid in MeOH (100 pg/mL). For MAX, WCX, the spiked serum eluate 2 was diluted with 0.5 mL MeOH and the blank serum eluate 2 was diluted with 0.5 mL recovery standard (100 pg/mL).

The reconstituted sample was transferred to an autosampler vial and 20 µL was injected into the LC-MS system for analysis. See Tables 4.9, 4.10, 4.11 and 4.12 for results.

SPE cartridge type	**Scop recovery (%) ± % RSD**	**Atr recovery (%) ± % RSD**
	n = 4	**n = 4**
HLB	89.1 ± 1.1	72.0 ± 1.0
MAX	85.2 ± 1.4	102.8 ± 1.9
MCX	0.4 ± 0.3	1.1 ± 9.1
WAX	4.6 ± 4.3	26.4 ± 2.7
WCX	131.8 ± 3.9	57.7 ± 1.9

Table 4.9: Comparison of SPE approaches loading serum diluted 1:1 with 4% phosphoric acid

SPE cartridge type	Scop recovery (%) ± % RSD n = 4	Atr recovery (%) ± % RSD n = 4
HLB	89.0 ± 0.1	79.2 ± 0.5
MAX	80.4 ± 1.5	73.9 ± 0.3
MCX	0.0 ± 0.0	0.0 ± 0.0
WAX	3.5 ± 5.7	17.2 ± 1.2
WCX	53.3 ± 0.2	11.8 ± 10.1

Table 4.10: Comparison of SPE approaches loading serum diluted 1:1 with 4% ammonium hydroxide

SPE cartridge type	Scop recovery (%) ± % RSD n = 4	Atr recovery (%) ± % RSD n = 4
MAX	0.6 ± 5.0	1.3 ± 1.5
MCX	30.7 ± 0.3	59.5 ± 1.2
WAX	5.1 ± 0.9	26.4 ± 1.9
WCX	0.0 ± 0.0	8.5 ± 1.2

Table 4.11: Comparison of SPE approaches loading serum diluted 1:1 with 4% phosphoric acid (Eluate 2 results)

SPE cartridge type	Scop recovery (%) ± % RSD	Atr recovery (%) ± % RSD
	n = 4	n = 4
MAX	0.7 ± 0.9	1.1 ± 0.8
MCX	1.5 ± 0.5	13.6 ± 2.9
WAX	3.8 ± 2.6	17.0 ± 0.6
WCX	0.0 ± 0.0	0.3 ± 1.4

Table 4.12: Comparison of SPE approaches loading serum diluted 1:1 with 4% ammonium hydroxide (Eluate 2 results)

Phosphoric acid was used in the SPE load step; however, on MS analysis, high column back pressure and a high MS background noise were noted (even though the SPE protocol was recommended by Waters.) After analysis of test samples the column lost its ability to function due to extremely high background noise and back pressure and so was discarded. As discussed with the MP composition, phosphate buffers have dropped out of favor (Neue, 2002). Phosphates are not volatile, and - with time - clog the LC-MS interface. This results in significant downtime, and more importantly, in a large amount of work to clean up the interface again (Neue, 2002).

From these preliminary SPE results as per Tables 4.9 and 4.10, it can be seen best results in terms of recovery were achieved with the Oasis HLB cartridge. Hence, it was decided the SPE cartridge would be chosen for this work.

Replacing the column with a new one, phosphoric acid was eliminated from the extraction procedure in favour of formic acid for the load step. Similar recoveries were obtained for Scop using formic acid in the load step with the Oasis HLB but significant quantities of Atr eluted with the dead volume as can be seen in Figure 4.19. Hence quantitative results were unattainable.

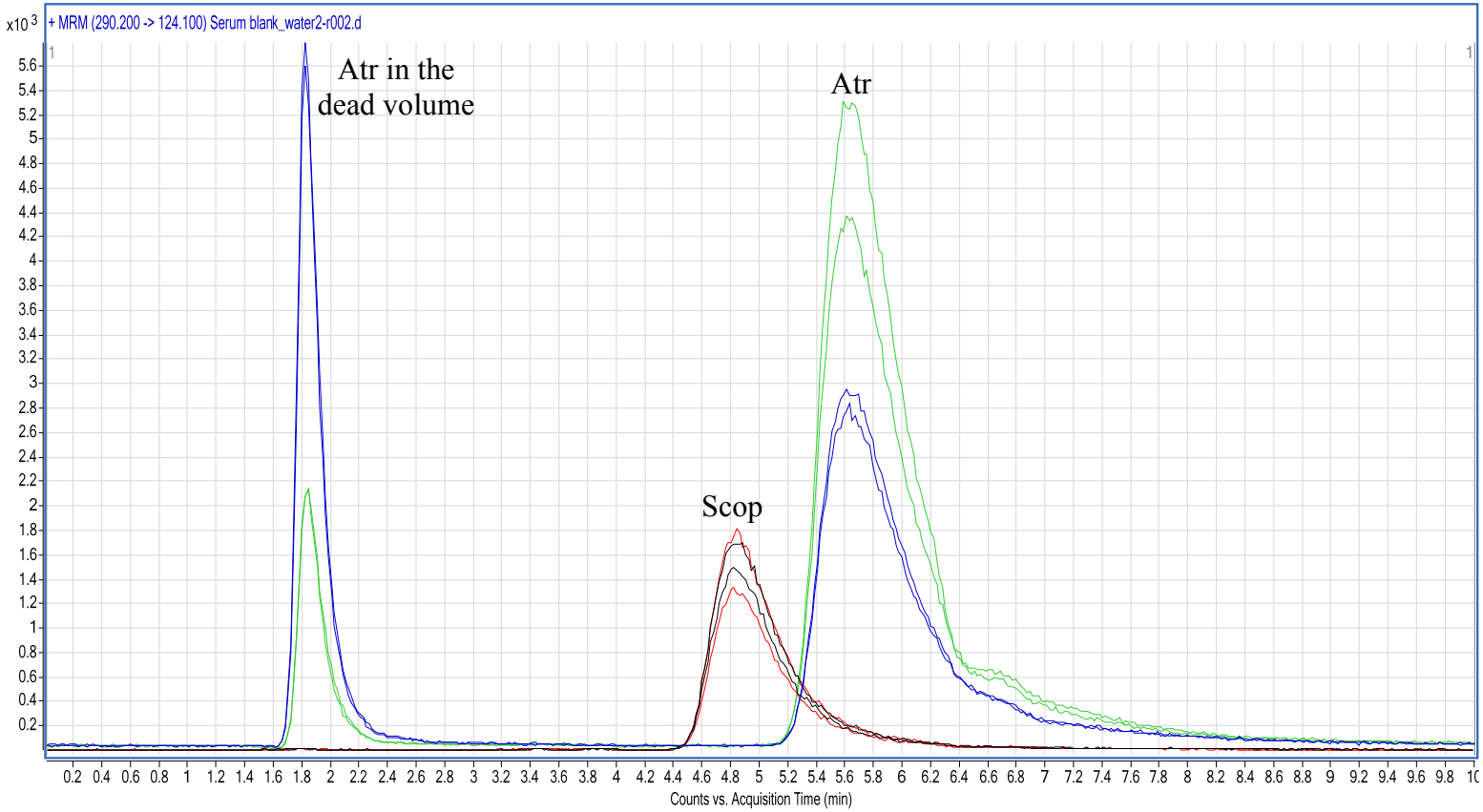


Figure 4.19: Chromatogram of an extracted 1ng/mL serum std loaded 1:1 with 1% formic acid using a Waters Nova-Pak C18 column

Green and Blue: Atr, Red and Black: Scop

Furthermore, upon investigating the sensitivity of the analytical protocol, an LOQ of 100 pg/mL in serum was obtained for Scop, not low enough for determination of the anticipated Scop serum concentrations post patch application. This column was hence abandoned as successful results would be impossible to attain even if 100% recovery of Scop was achieved via the extraction protocol along with no ion suppression.

Agilent Zorbax SB-C18 column

The fourth C18 column investigated was an Agilent Zorbax SB-C18 column (50 × 2.1 mm, 3.5 μm). Rapid Resolution columns reduce analysis time while maintaining resolution and have the advantage of substantial solvent savings. In comparison to the Waters Nova-Pak C18 (150 × 3.9 mm, 4 μm) column, the Agilent Zorbax SB-C18 column is much shorter, with a narrower internal diameter and smaller particle size. As a starting point, the optimised chromatographic conditions from the Waters Nova-Pak C18 column were used. Separation between Scop and Atr was achieved but in the injections of 20 and 10 μL, peak fronting was observed. See Figure 4.20. Broad peaks or fronting indicate sample overload. Fronting occurs when one or more of the compounds injected on the column exceeds the capacity of the liquid phase of the column. The thinner the liquid phase film, the less of each compound can be retained by the column (Neue, 2002). This includes both the injection volume and the concentration of each peak in the injection. As a result an injection volume of 20 μL and 10 μL was too large as a result of the column dimensions and so to begin with a smaller injection volume was required. Using a 5 μL injection the separation of the two drugs was achieved with a short runtime.

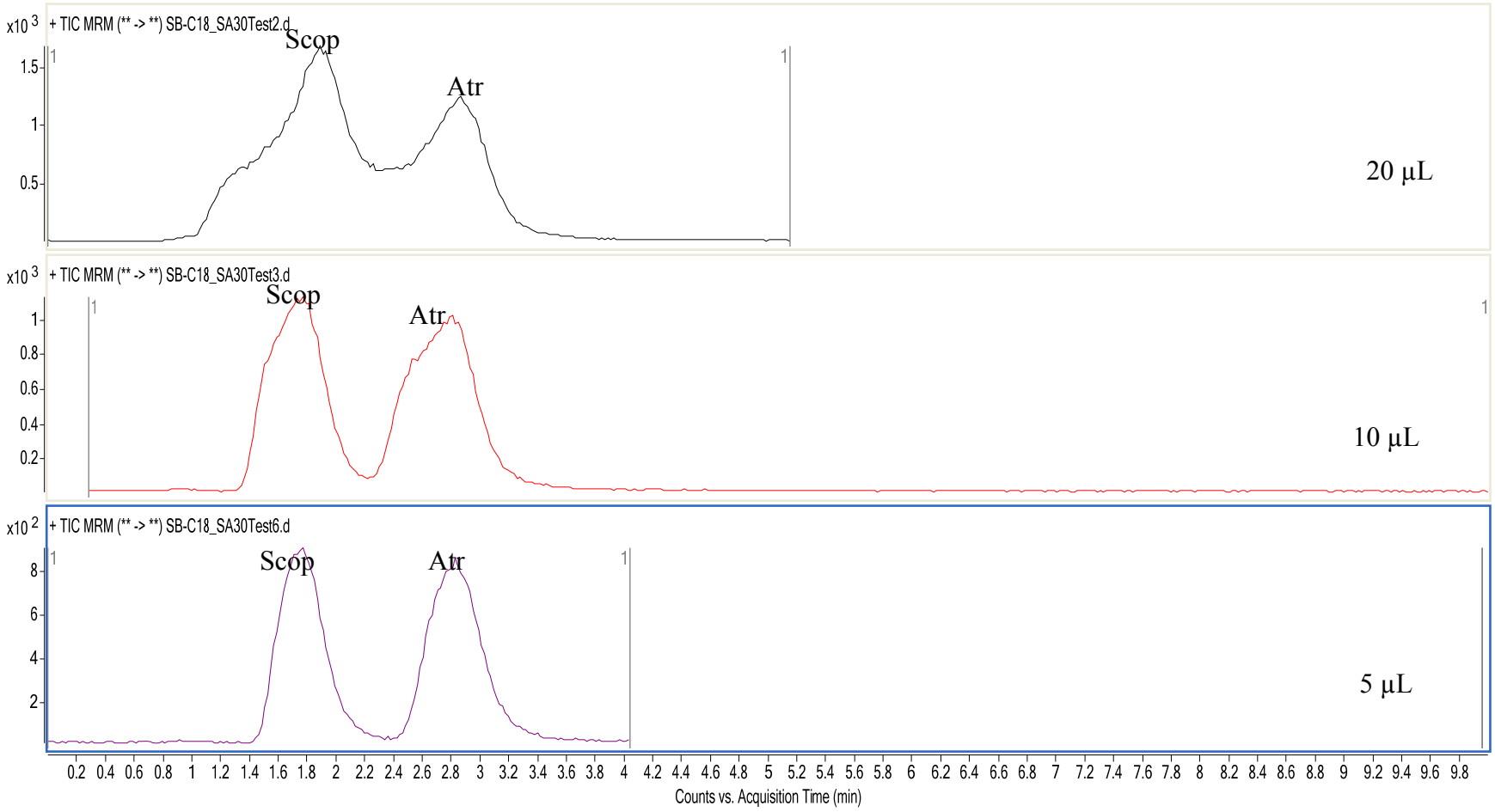


Figure 4.20: Comparison of the impact of different injection volumes on peak shape for Scop and Atr using an Agilent Zorbax SB-C18 column

Increasing the flow rate is the easiest way to decrease the analysis time. However, flow rate affects column efficiency, which in turn affects resolution and pressure. The flow rate for optimum efficiency is dependent on the particle size of the column packing material (Neue, 2002). The main disadvantage of increasing flow rate is higher pressure. Elevated pressures will shorten column lifetime as well as increase wear on the HPLC system. Increasing flow rate above the optimum will also reduce column efficiency and resolution. Decreasing the flow rate reduces system wear. However, it also increases analysis time. Due to the particle size of this column (3.5 µm), a flowrate of 0.6 mL/min was not possible as with the Waters Nova-Pak C18 column. A flowrate between 0.15 – 0.25 mL/min is recommended using the Zorbax SB-C18 column. See Figure 4.21 for a comparison of three different flowrates. 0.2 mL/min was chosen as optimal due to shortest analysis time and highest peak areas.

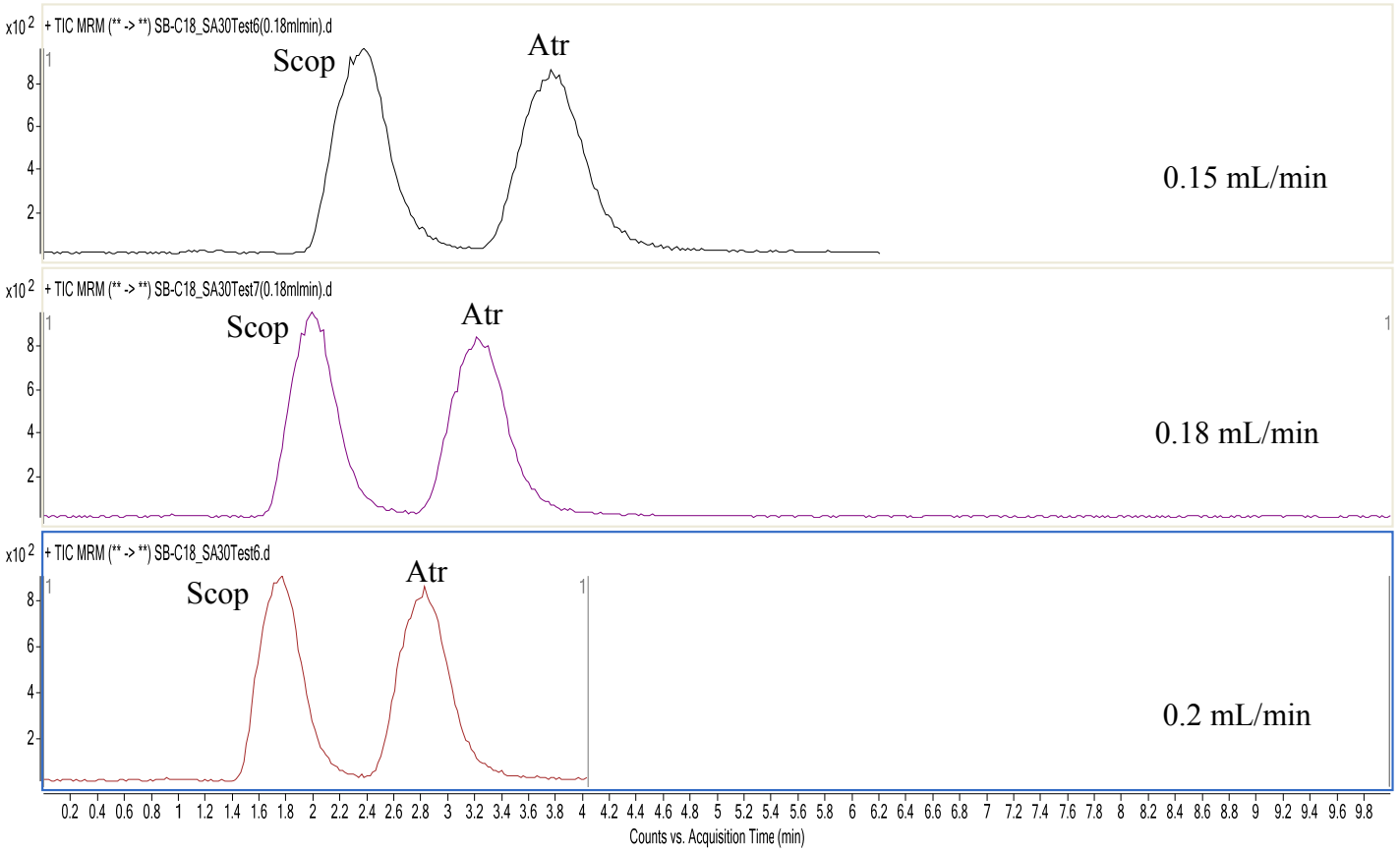


Figure 4.21: Comparison of the impact of different flowrates on peak shape and retention for Scop and Atr using an Agilent Zorbax SB-C18 column

Using this column at a flowrate of 0.2 mL/min, and injection volume 5 µL, the % MeOH played a significant role on the separation of the two drugs, see Figure 4.22. A decrease of % MeOH in the MP consisting of ammonium formate (pH 4, adjusted with 1 M formic acid, 5 mM) from 40 to 30% had a large impact on the separation of both drugs. The role of the organic modifier is to compete against the bonded phase molecule for attraction of the analyte molecules. Generally speaking, the higher the organic concentration the stronger the mobile phase and the shorter the retention (Jerkovich *et al.,* 2006). At 40% MeOH, it proved too strong and the two drugs co-eluted.

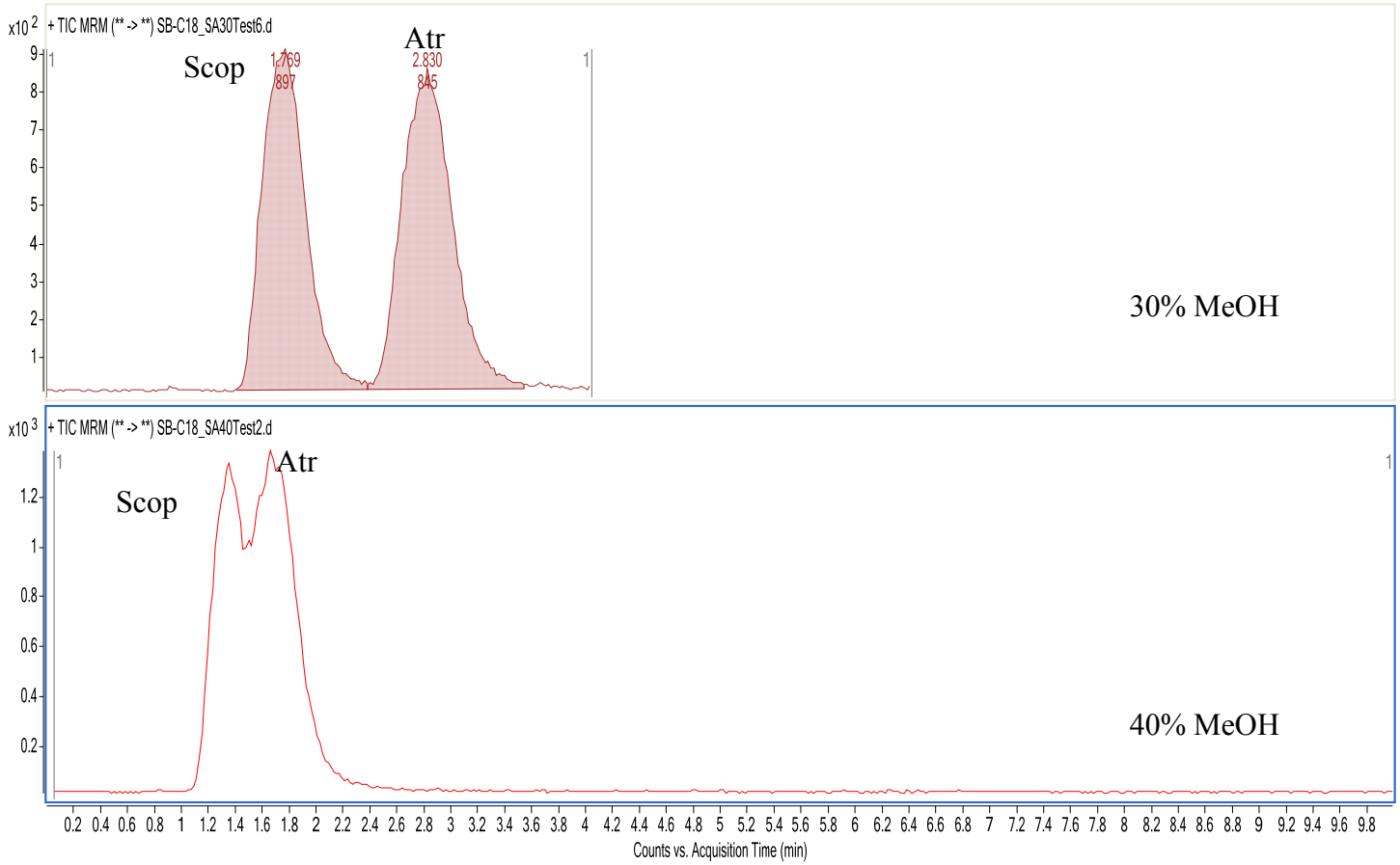


Figure 4.22: Comparison of the impact of different % MeOH mobile phase compositions on peak shape and retention for Scop and Atr using an Agilent Zorbax SB-C18 column

A buffering agent employing ammonium formate was again required in order to control the pH. In terms of concentration, 2 mM, 5mM and 10 mM ammonium formate buffers were investigated using ammonium formate (pH 4 adjusted with 1 M formic acid,) - MeOH (70:30, v/v). As the buffer concentration increased, the faster the analytes eluted. On comparing the peak areas (response) for Scop using these three buffers, it was concluded that the 2 mM buffer solution proved considerably better for the peak shape and area of the drug - see Figure 4.23.

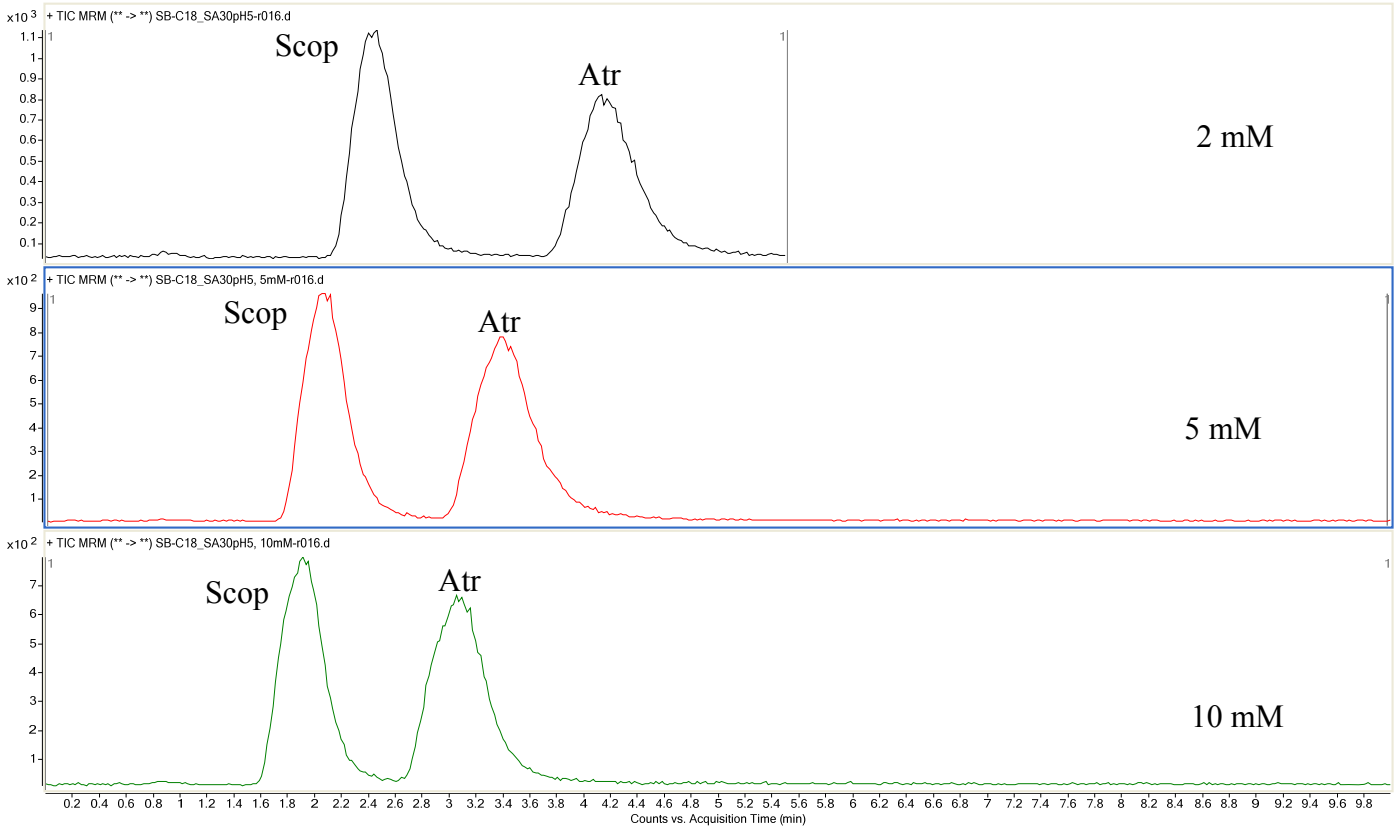


Figure 4.23: Comparison of the impact of different ammonium formate concentrations (pH 4) on peak shape and retention for Scop and Atr using an Agilent Zorbax SB-C18 column

Analytical mobile phases of differing pH values (3, 4 and 5) were tested using ammonium formate (2 mM) - MeOH (70:30, v/v). See Figure 4.24. The pH adjustment was achieved again by addition of formic acid. As mentioned, the pH of the mobile phase is a fundamental parameter in reversed phase HPLC that significantly affects the retention of ionisable analytes. (Neue, 2002). pH reflects the hydrogen ion concentration in solution. Adding acid (proton donor), increases the hydrogen ion concentration (lowers pH) of the solution. Changing the pH changes the degree of ionisation of molecules in solution. It thus affects their polarity, and as a consequence it changes their retention times in an HPLC separation. At low pH, the mobile phase protonates free silanols on the column and reduces peak tailing. At sufficiently low pH basic analytes are protonated and when ionised results in the analyte eluting more quickly as can be seen in Figure 4.24. The optimal pH for the LC-MS analysis was at pH 5 which resulted in good peak shape and highest peak area.

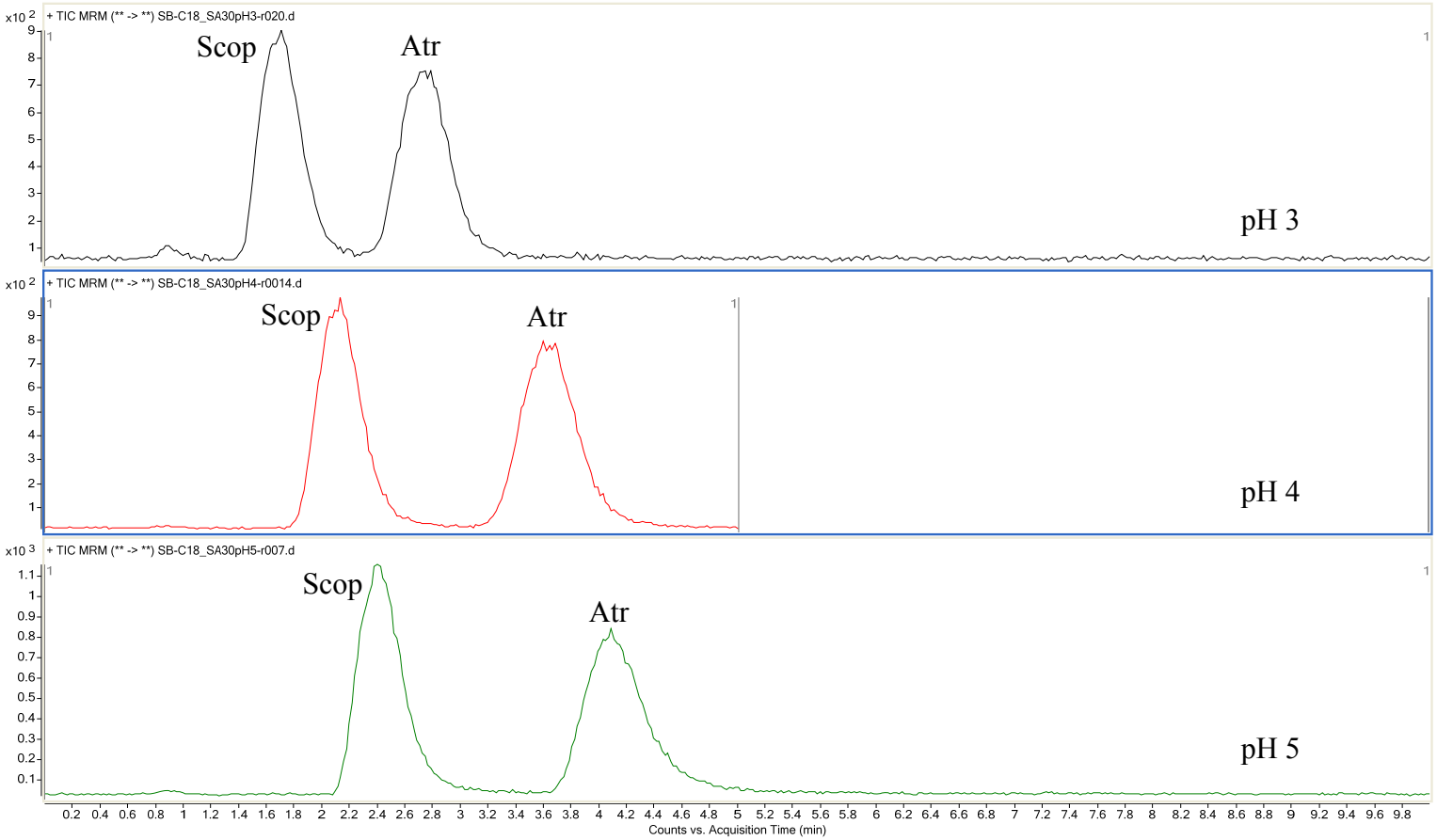


Figure 4.24: Comparison of the impact of different mobile phase pHs using 2 mM ammonium formate on peak shape and retention for Scop and Atr using an Agilent Zorbax SB-C18 column

Under the final chromatographic conditions using an Agilent Zorbax SB-C18 (50 × 2.1 mm, 3.5 µm) a 5 µL injection volume was used. A mixture of ammonium formate (pH 5 adjusted with 1 M formic acid; 2 mM) - MeOH (70:30, v/v) was used as mobile phase, at a flow rate of 0.2 mL/min. The complete chromatographic run time of each sample was 5 min, which separated Scop and Atr from each other with retention times 2.1 and 3.4 min, respectively. However, the sensitivity achieved for the drugs prepared in mobile phase was only 200 pg/mL, again likely to be too low for the demanding applications of our analyses.

SPE development for use with Agilent Zorbax SB-C18 column

Using the same SPE conditions as used with the Waters Nova-Pak C18 column it was noted problems were again occurring with regards to Atr, this time interfering Atr-mimicking endogenous peaks present in the serum blank extracted samples. See Figure 4.25.

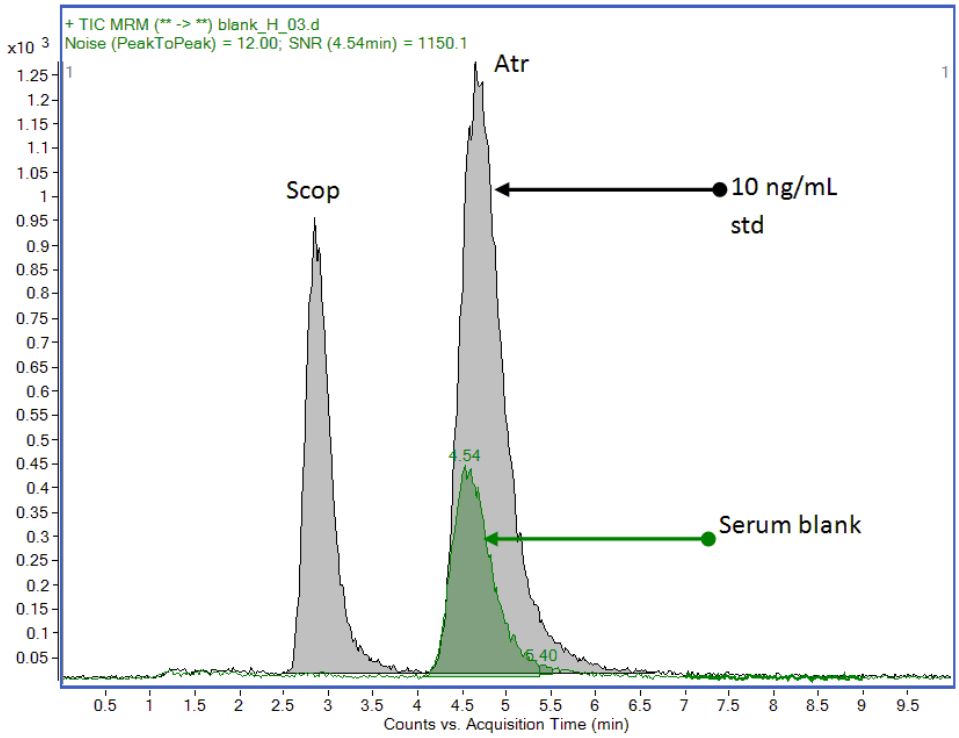


Figure 4.25: Overlay of a 10 ng/mL Scop and Atr standard in MP (grey) superimposed on extracted serum blank (green) using an Agilent Zorbax SB-C18 column

In the extracted serum blank a peak eluting at Atr's retention time was observed and showed significant intensity and signal/noise ratio. All potential sources of contamination were investigated. New Oasis HLB cartridges were ordered in along with all new reagents and chemicals. Also suspecting that the manifold could be a potential source of carryover, serum was extracted through the SPE cartridge without using the manifold. However, there was still a high level of Atr-mimicking contaminant detected.

Biological sample analysis is complicated because the samples contain macromolecules and other compounds such as proteins, endogenous and exogenous compounds. Materials in the extracted biological matrix can exist in much higher concentrations than the analyte. The aim of SPE is to remove as many of the unwanted compounds as possible without significant loss of the analytes of interest. Unwanted compounds can be present still in higher concentrations than the analytes of interest after the first clean-up. LC separation further separates analytes of interest from the unwanted compounds.

MS/MS offers a third stage of separation through selection of appropriate precursor and product ion pairs so that unwanted compounds are not registered by the detector. However, those unwanted and MS/MS unseen compounds present significant challenges, such as the Atr-mimicking peak in this work. In the LC/MS interface, unwanted compounds compete with analytes for ionisation and can cause inconsistent matrix effects that are detrimental to quantitative LC/MS/MS. If not separated from the analytes, some conjugated metabolites break down in the interface so that analyte concentration is overestimated.

Looking at Scop on its own it was noted a significant amount on ion suppression was occurring. See Figure 4.26. Displayed is serum blank extracted and reconstituted in 1 ng/mL standard of Scop (green) superimposed on the pure 1 ng/mL standard (blue) with the transition *m/z* 304 to 136 extracted out.

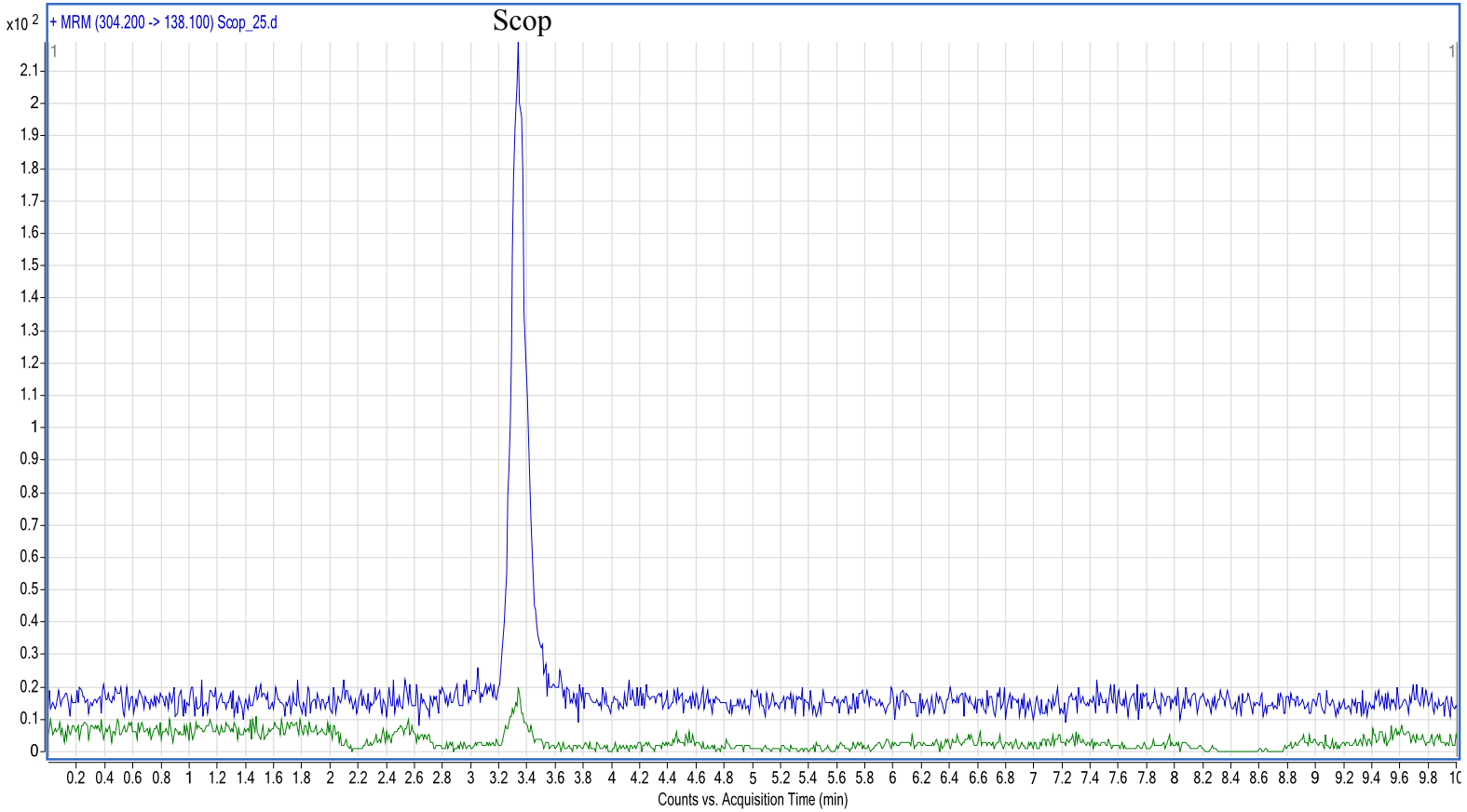


Figure 4.26: Serum blank extracted and reconstituted in 1 ng/mL standard of Scop (green) superimposed on the pure 1 ng/mL standard (blue) using an Agilent Zorbax SB-C18 column

This ion suppression is further proven by Figure 4.27. By overlaying the TIC scan of serum blank extracted with a MRM scan of a Scop pure standard it can be clearly seen Scop was eluting at the height of the endogenous material. As discussed in Chapter 1, the total ion current (TIC) chromatogram represents the summed intensity across the entire range of masses being detected at every point in the analysis.

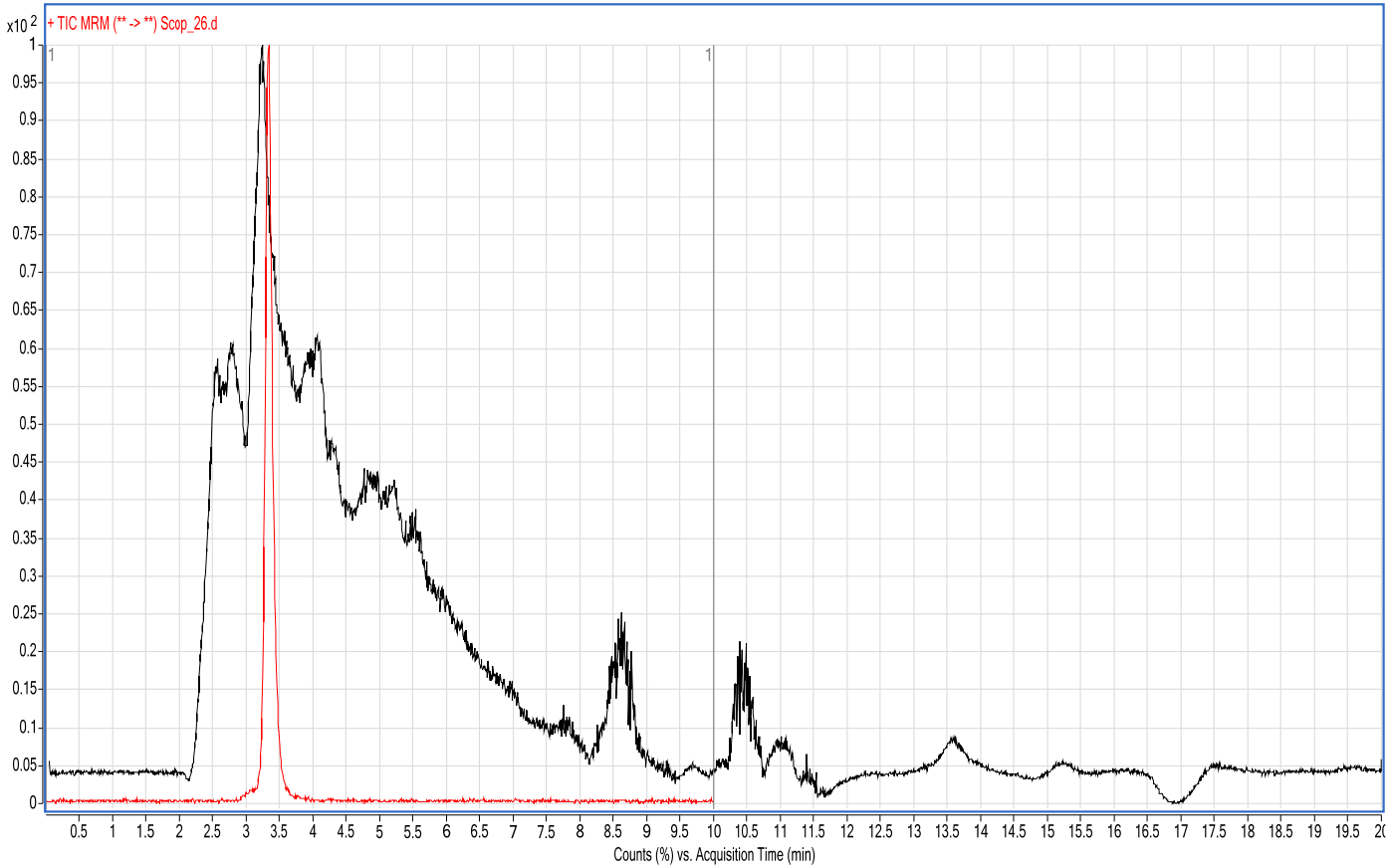


Figure 4.27: TIC scan of serum blank extracted (black) superimposed on a MRM scan of Scop pure standard (red) using an Agilent Zorbax SB-C18 column. Note: not to scale

Also in terms of sensitivity, the method was clearly not capable of reaching the desired LOQ. Figure 4.28 shows a serum blank extracted and reconstituted in MP superimposed on a 100 pg/mL serum standard extracted and reconstituted in MP with the transition *m/z* 304 to 138 extracted out.

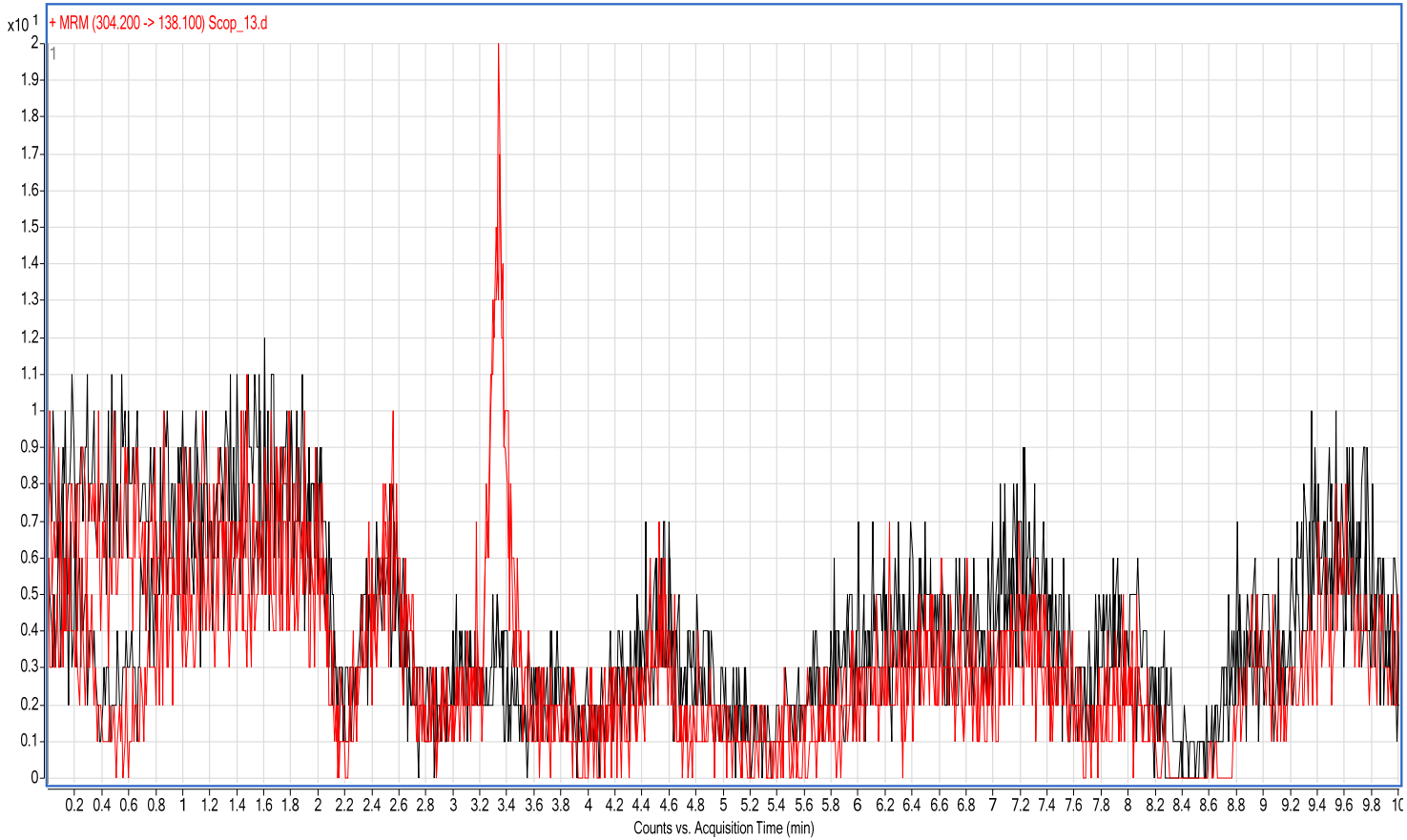


Figure 4.28: Chromatogram of an extracted serum blank superimposed on an extracted 100 pg/mL serum standard extracted using an Agilent Zorbax SB-C18 column

LLE development for use with Agilent Zorbax SB-C18 column

Due to unsuccessful attempts at optimising SPE conditions for Scop and Atr on two different columns, it was decided to investigate an alternative method of sample clean up. As discussed, there are several sample preparation techniques available for the pretreatment of biological samples. The simplest and fastest method for preparing samples is protein precipitation. Protein precipitation is most likely to cause ion suppression in ESI, since this method fails to sufficiently remove endogenous compounds such as lipids, phospholipids, fatty acids, etc. Co-elution of these compounds with the compound of interest affects the ESI droplet desolvation process. (Van Eeckhaut *et al.,* 2009). Although protein precipitation is quick and easy, it does not result in a very clean final extract and so was not investigated. Liquid–liquid extraction (LLE) is viewed by some researchers as an efficient means of sample preparation that is a cleaner option than protein precipitation (Chambers *et al.,* 2007); we therefore decided to investigate LLE as a potential extraction technique for the drugs in serum.

As discussed in Chapter 1, LLE provides sample clean-up whereby hydrophobic sample constituents are extracted from aqueous samples with a water-immiscible organic phase. Most LLE procedures are based on extraction of the target drugs into an organic solvent followed by solvent evaporation, reconstitution of residue, and injection into the HPLC system. Inorganic salts are normally poorly soluble in the organic solvents used for LLE, and consequently they principally remain in the aqueous sample phase. For serum samples, proteins may be of concern; however, they are almost insoluble in the organic solvents used for LLE and tend to precipitate. Although LLE provides excellent sample clean-up for salts and biological macromolecules a broad range of other compounds may be co-extracted during LLE.

LLE optimisation experiments for this work were carried out in a similar manner to a previously published LLE extraction procedure carried out in DCU (Roche *et al.,* 2009). Similar sample volumes and extraction techniques were used.

Scop is an uncharged tertiary amine. It has a weak basic character with a pKa of 7.6 and a reasonable lipid solubility (Renner *et al.,* 2005) which

allows for the transdermal absorption of the patch. Most alkaloids are poorly soluble in water but readily dissolve in organic solvents, such as diethyl ether and dichloromethane. With, acids, alkaloids form salts of various strengths. Those salts are usually soluble in water and alcohol and poorly soluble in most organic solvents. Exceptions include scopolamine hydrobromide, which is soluble in organic solvents.

To determine the optimum system for liquid–liquid extraction four water immiscible volatile organic solvents were tested: ethyl acetate; dichloromethane, *tert*-butyl methyl ether and chlorobutane. The optimised conditions for the Agilent Zorbax SB-C18 column (50 × 2.1 mm, 3.5 µm) were again used for LLE optimisation work.

200 µL of blank serum was added to the extraction tube, along with 100 µL of 1 M ammonium formate pH 8 buffer and 1.7 mL of extraction solvent. The extraction tubes were vortexed and mixed on a blood tube mixer for 15 min. The samples were centrifuged at 3200 × *g* for 5 min. 1.1 mL of solvent was transferred to conical bottomed glass LC autosampler vials. The vials were evaporated to dryness using a Genevac EZ-2 evaporator at ambient temperature, without light. The samples were reconstituted in 50 µL of MP with 20 µL injected automatically by the autosampler. 1 ng/mL spiked serum samples were also prepared and processed in the same manner as the above serum blanks samples. Results are shown in Figures 4.29, 4.30, 4.31 and 4.32.

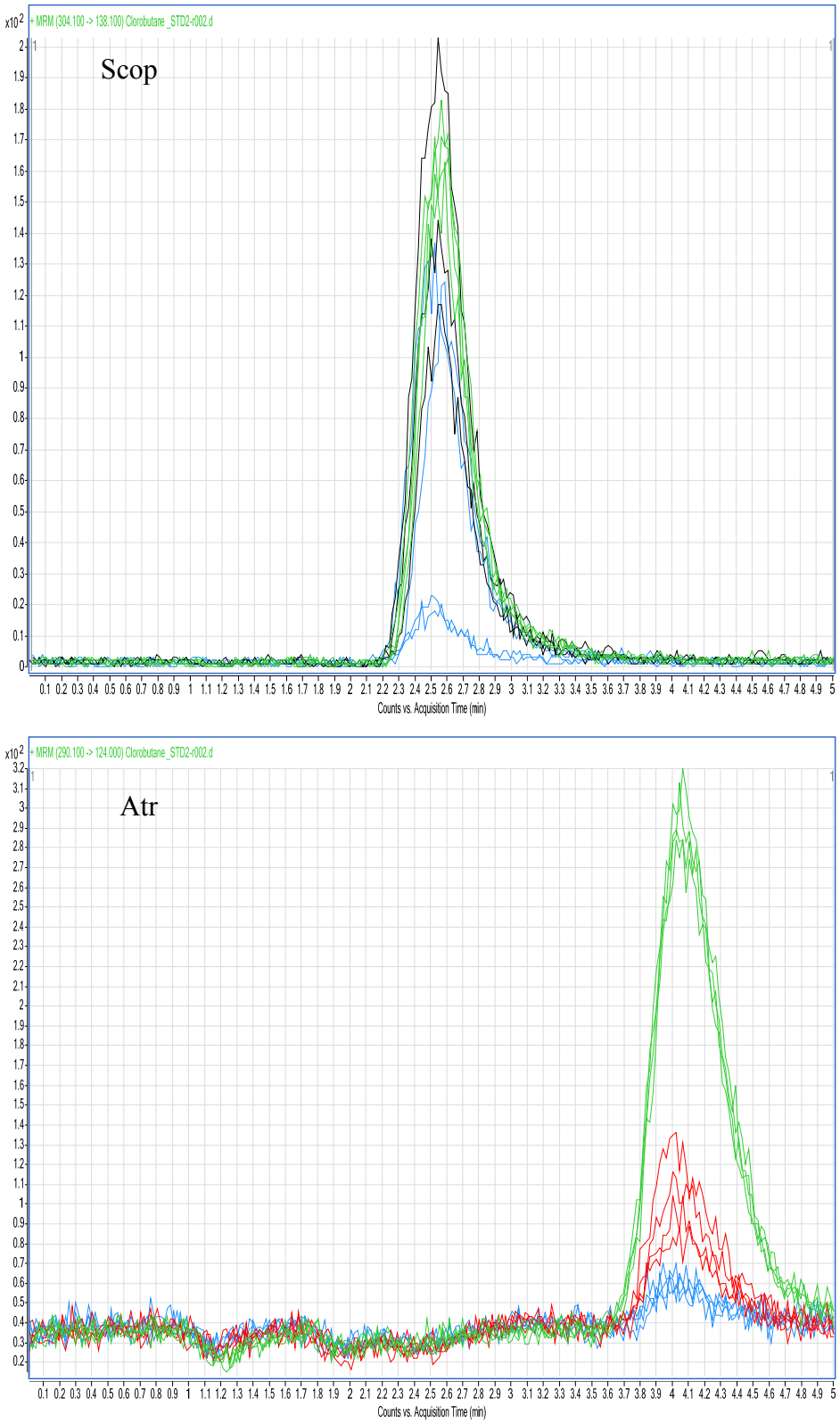


Figure 4.29: LC-MS chromatogram, in MRM mode of an extracted serum blank sample (blue) and an extracted spiked serum Scop standard (black) and Atr (red) superimposed on a pure std of both drugs in MP (green) using chlorobutane as extraction solvent

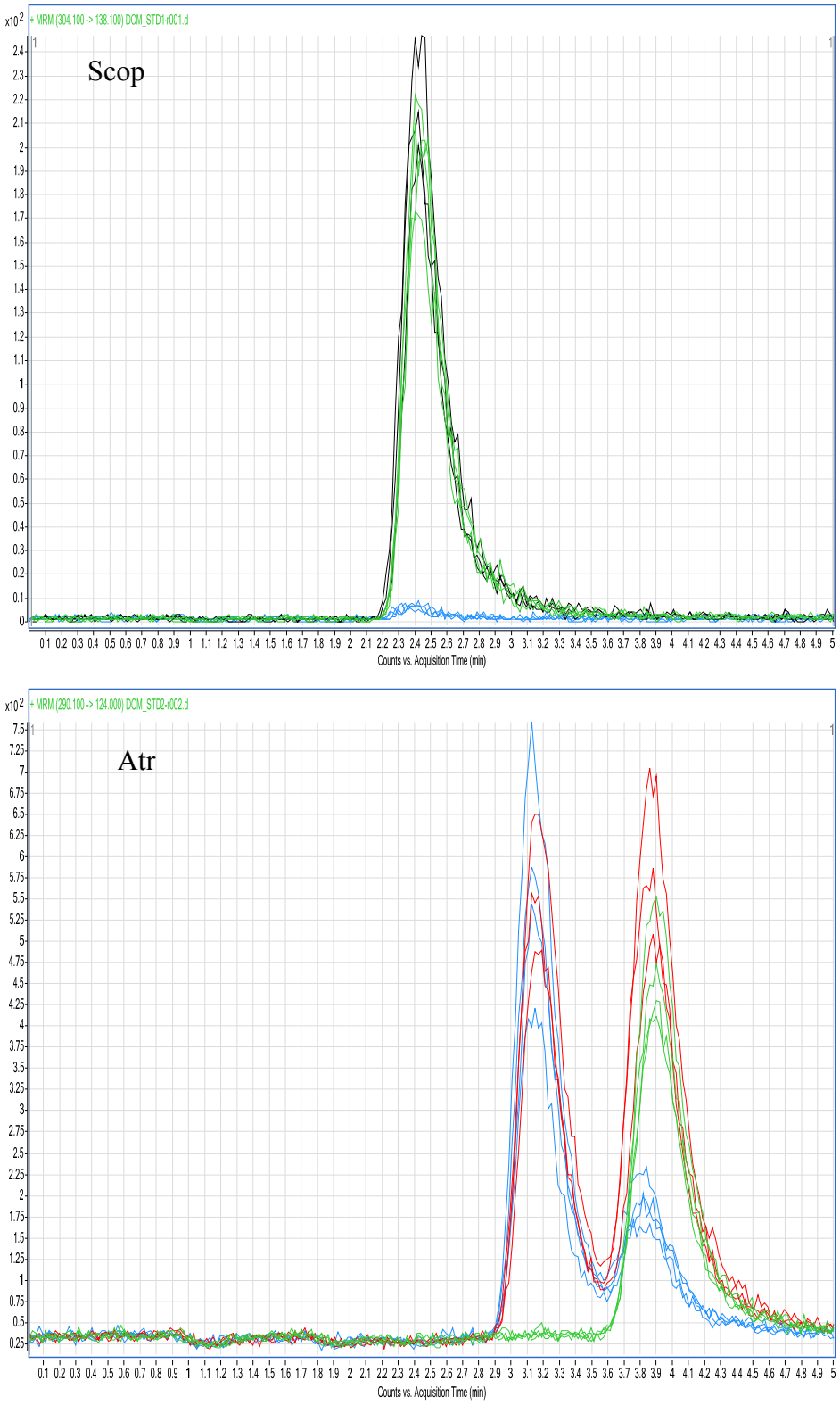


Figure 4.30: LC-MS chromatogram, in MRM mode of an extracted serum blank sample (blue) and an extracted spiked serum Scop standard (black) and Atr (red) superimposed on a pure std of both drugs in MP (green) using DCM as extraction solvent

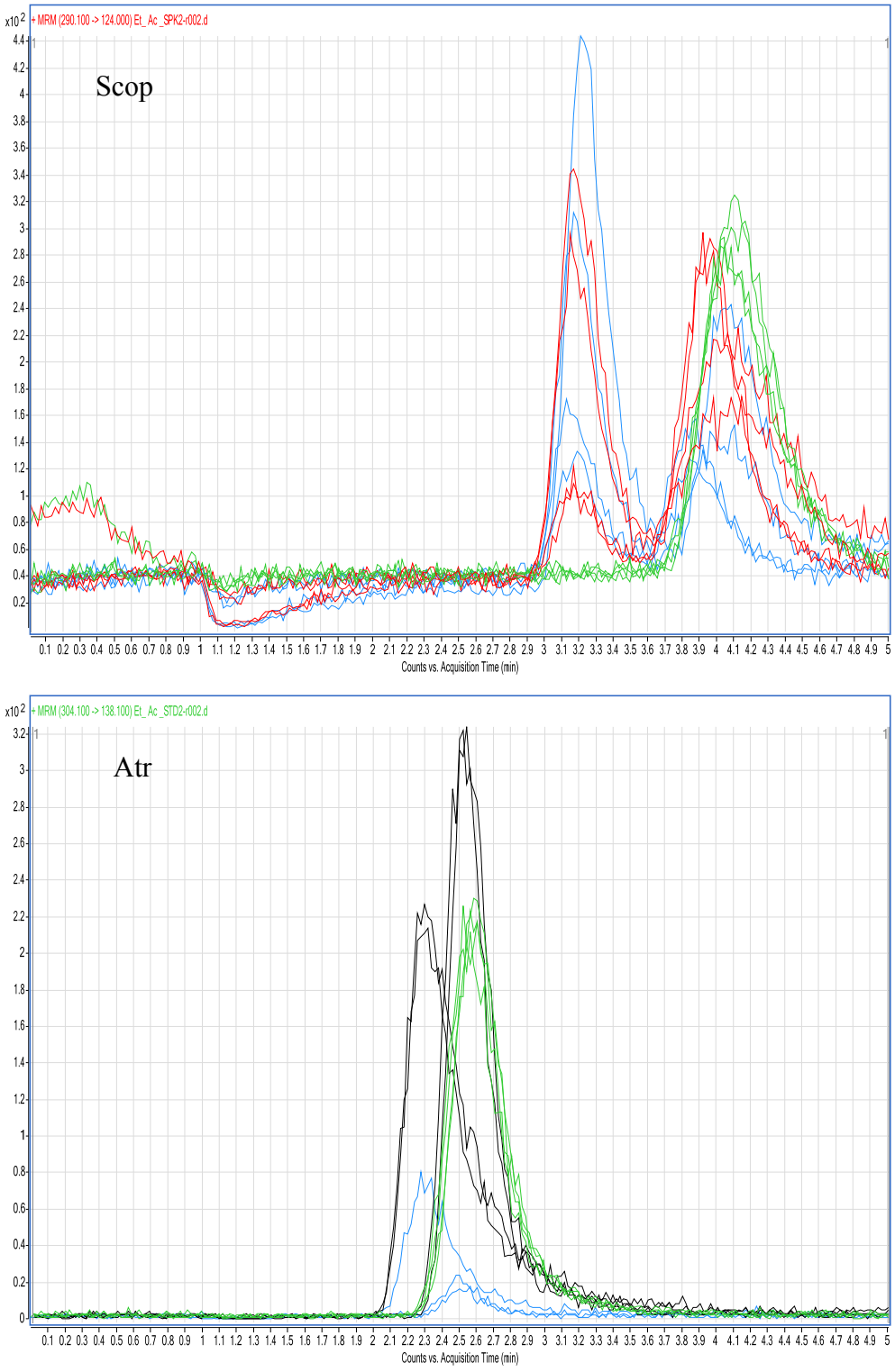


Figure 4.31: LC-MS chromatogram, in MRM mode of an extracted serum blank sample (blue) and an extracted spiked serum Scop standard (red) and Atr (black) superimposed on a pure std of both drugs in MP (green) using ethyl acetate as extraction solvent

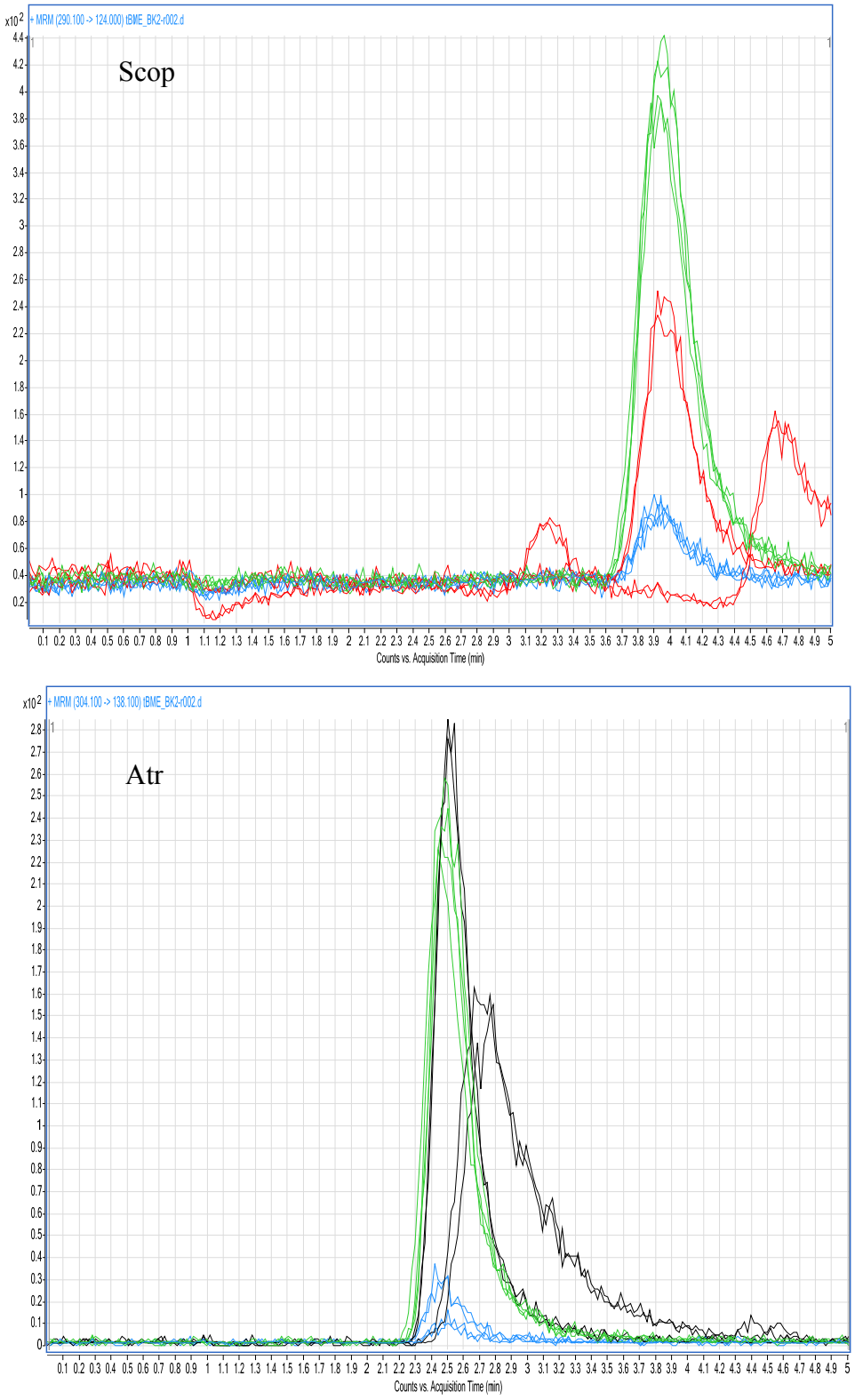


Figure 4.32: LC-MS chromatogram, in MRM mode of an extracted serum blank sample (blue) and an extracted spiked serum Scop standard (red) and Atr (black) superimposed on a pure std of both drugs in MP (green) using *tert*-butyl methyl ether as extraction solvent

Figure 4.33 shows a chromatogram of a pure standard of Scop and Atr in MP extracted through the LLE process. For the pure standards no endogenous peaks were observed indicating contaminating peaks were a result of the addition of serum to the extraction step. As already mentioned, blood contains an innumerable number of compounds that can interfere with analyte detection and may contribute to this Atr like peak eluting in the extracted blank serum samples.

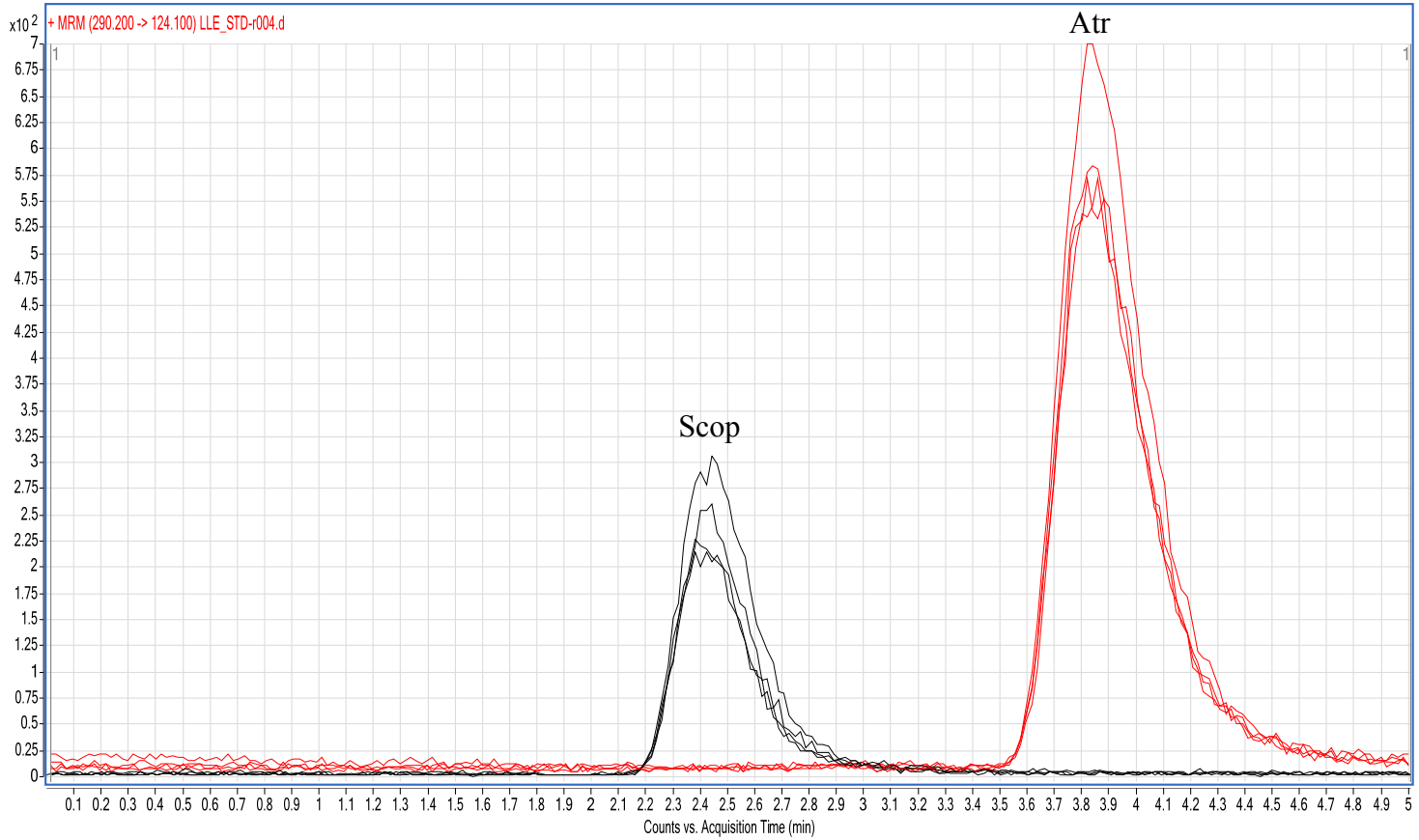


Figure 4.33: LC-MS chromatogram, in MRM mode, of Scop and Atr std in MP brought through the LLE extraction process (no serum) using an Agilent Zorbax SB-C18 column

From these results, contamination of the serum stock was suspected. A new batch of serum was ordered and brought through the same procedure as above. See Figure 4.34. An Atr like peak was again found in the new serum blanks extracted through LLE, however, for the new serum stock a single Atr like peak was observed in comparison to a split Atr like peak for the old serum stock.

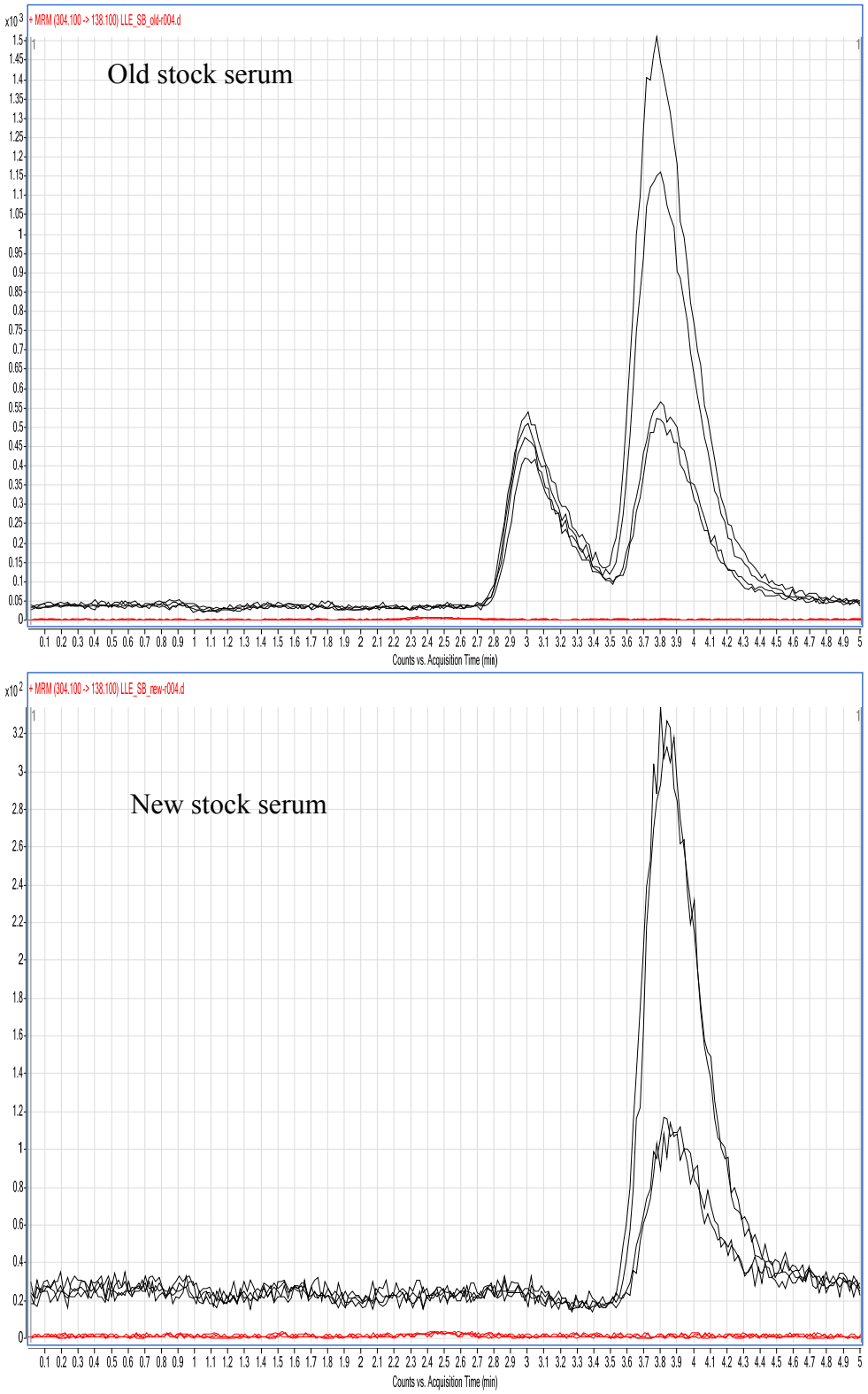


Figure 4.34: LC-MS chromatograms, in MRM mode comparing LLE extracted blank serum for old and new serum batches using an Agilent Zorbax SB-C18 column

Scop: red, Atr: black

The optimum pH of the extraction buffer was also investigated. Identical extractions were carried out using dichloromethane (DCM) as the solvent system and varying the pH of the extraction buffer. Due to its compatibility with mass spectrometry 1 M ammonium formate was selected as the aqueous buffer. The aqueous phase was tested across a pH range to determine an optimum in combination with DCM extraction solvent. As shown in Figure 4.35 the Atr like peak persisted at all pHs and made quantification impossible.

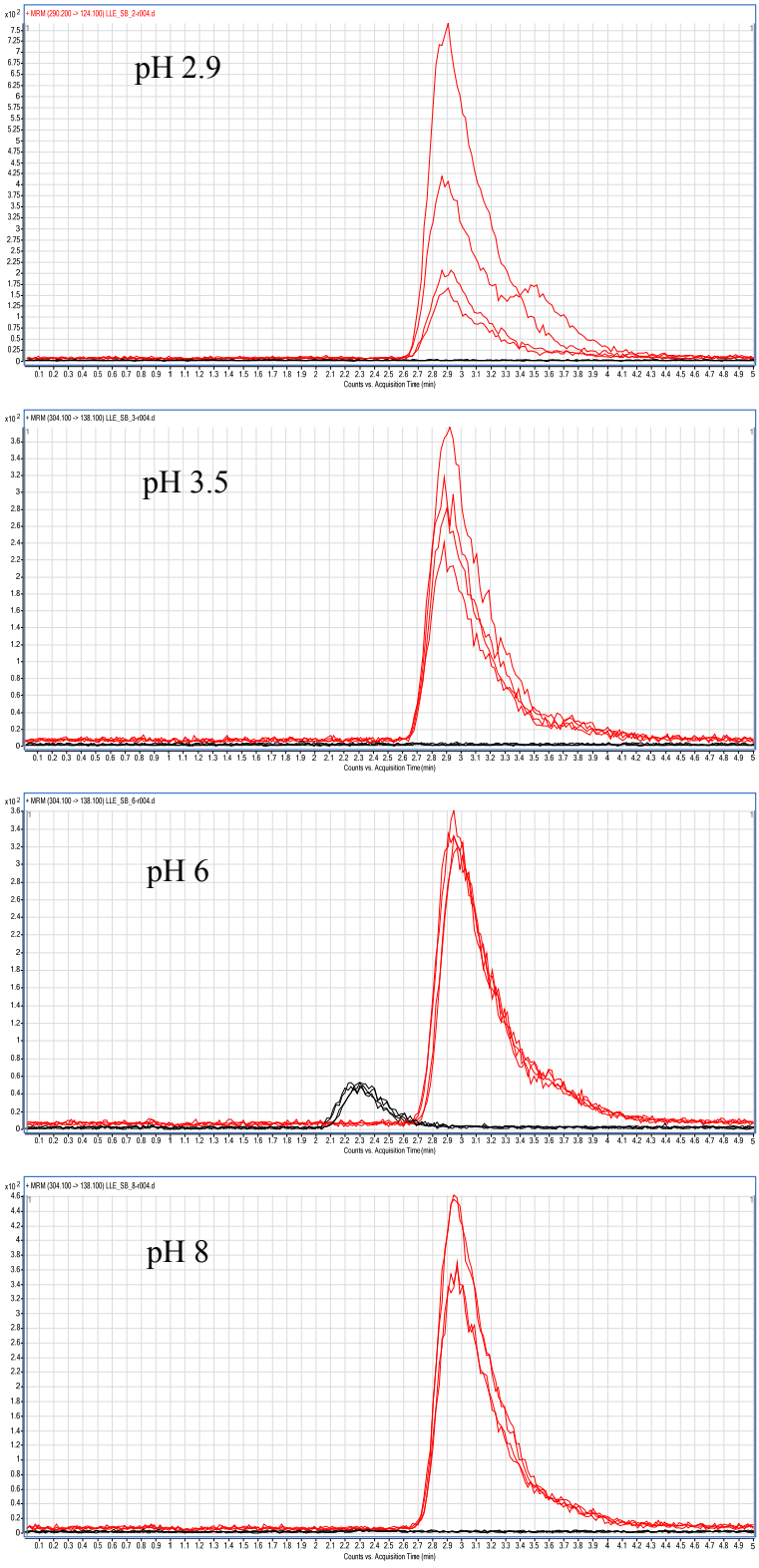


Figure 4.35: LC-MS chromatograms, in MRM mode, comparing the extraction of serum blanks at different pHs using an Agilent Zorbax SB-C18 column (Scop: black, Atr: Red)

The optimum extraction buffer pH for Scop was determined to be pH 8 as this gave greater recovery than the other pHs achieving 89%. Figure 4.36 shows overlaid chromatograms of both an extracted spiked serum standard and extracted blank serum sample at pH 8 and pH 3.5.

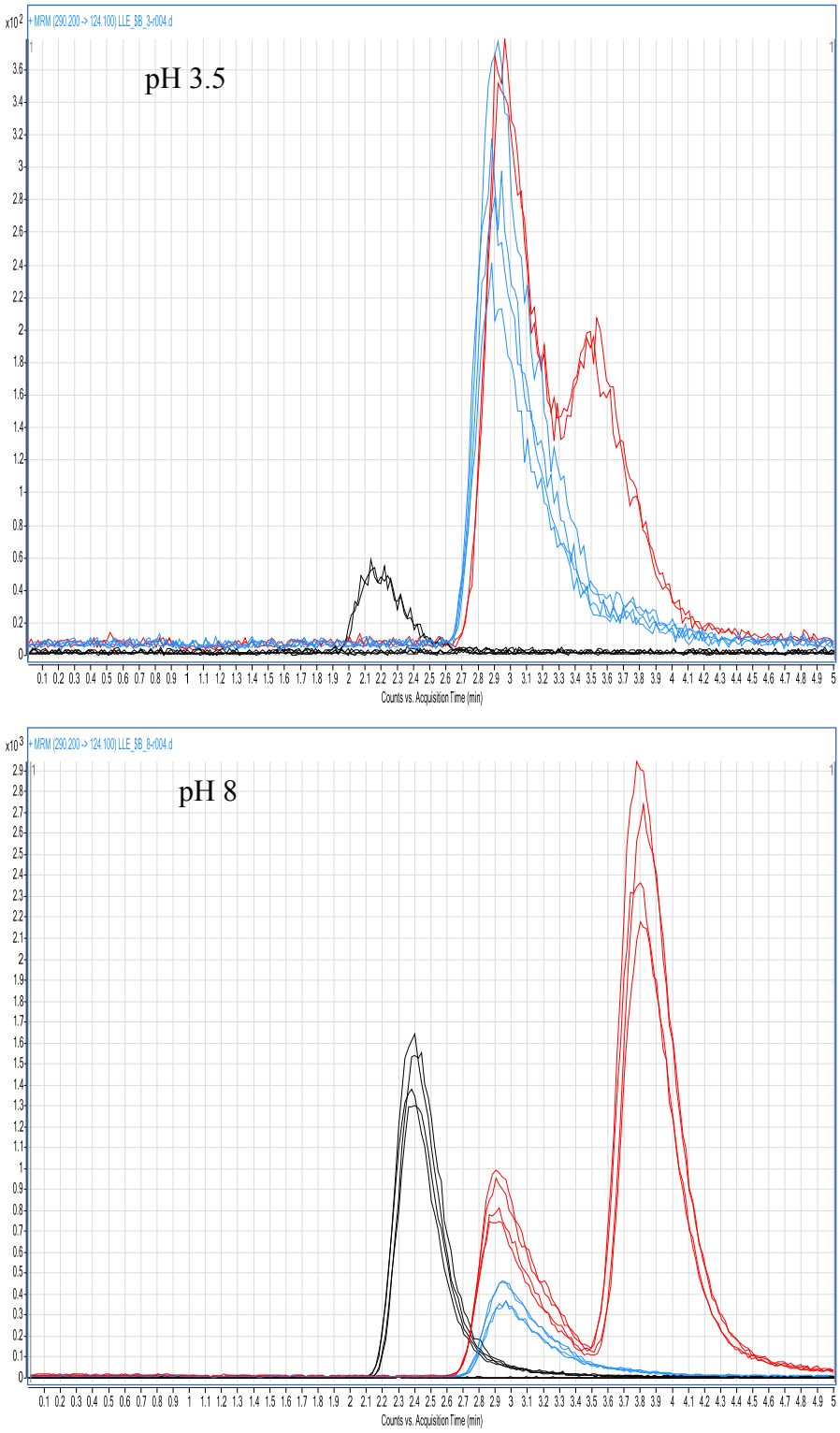


Figure 4.36: LC-MS chromatogram, in MRM mode, of extracted spiked serum standard superimposed on extracted blank serum sample using an Agilent Zorbax SB-C18 column.

Serum blank: blue, Scop black, Atr: red

Further LLE optimisation was not carried out as an LOD of only 200 pg/mL was achieved, see Figure 4.37, again not good enough in terms of sensitivity for Scop serum concentrations post patch application.

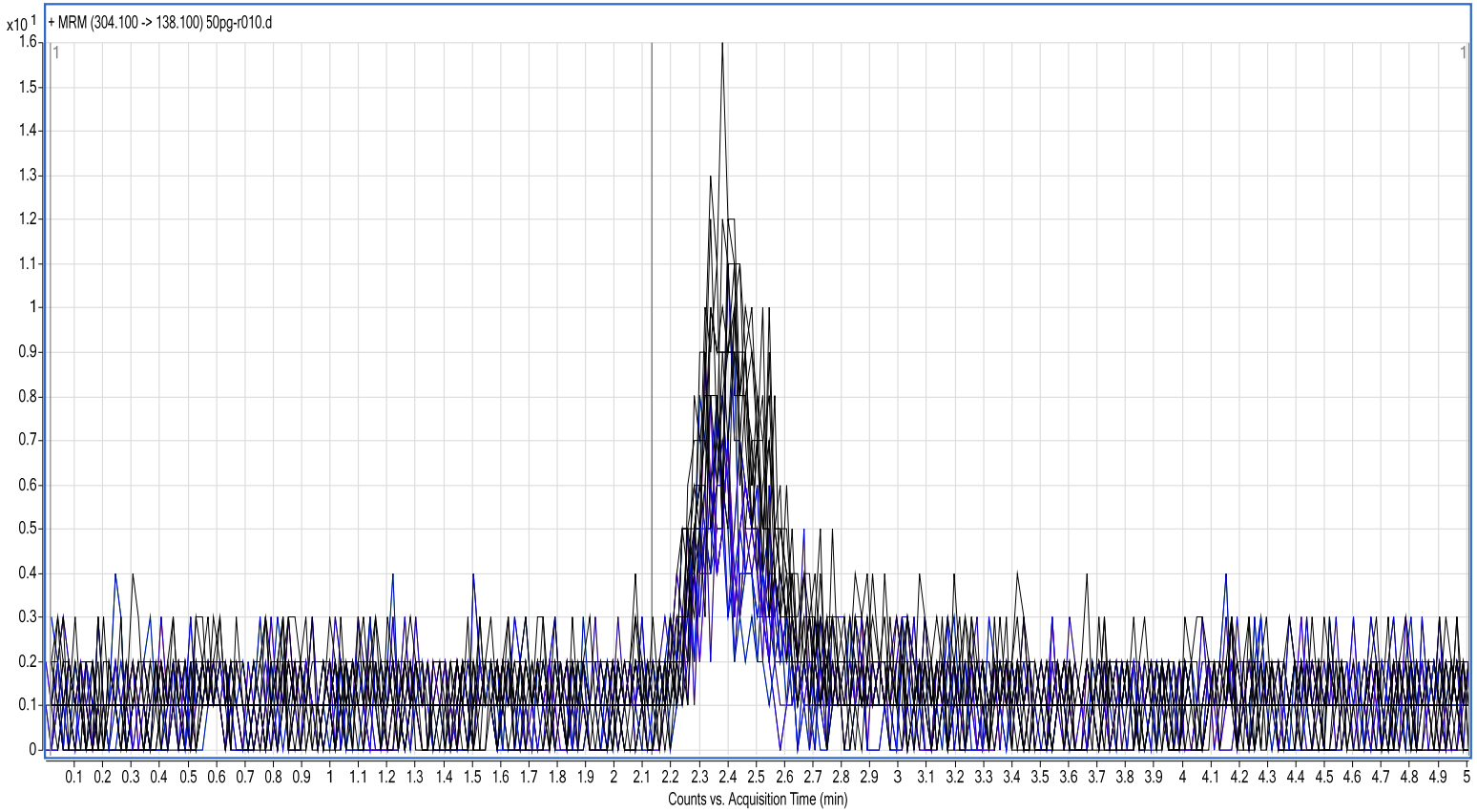


Figure 4.37: LC-MS chromatogram, in MRM mode of extracted spiked (200 pg/mL) serum standard (black) superimposed on extracted serum blank sample (blue) using an Agilent Zorbax SB-C18 column

Agilent Zorbax RX-C8 column

As C18 columns proved unsuccessful, it was decided to switch column chemistries from C18. Octylsilane (C8) is also a popular stationary phase in reversed-phase chromatography. It usually provides slightly less retention than the more popular C18 (Kazakevich *et al.,* 2006) and because of this, was too investigated. See Figure 4.38.

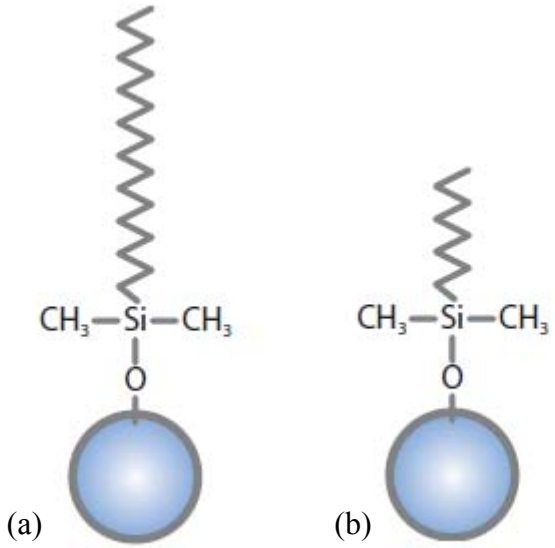


Figure 4.38: Reversed phase stationary phases (a) C18 and (b) C8

An Agilent Zorbax RX-C8 (narrow bore 150 x 2.1 mm, 5 µm) column was investigated. This column was also unable to achieve separation between the two drugs under the same chromatographic conditions. Figure 4.39 displays results using this column while varying the % MeOH between 40 and 70%. Buffer used was as before, 2 mM ammonium formate, pH 5. Using the same flowrate of 0.2 mL/min, 20 µL of a Scop and Atr 10 ng/mL mixed standard was injected.

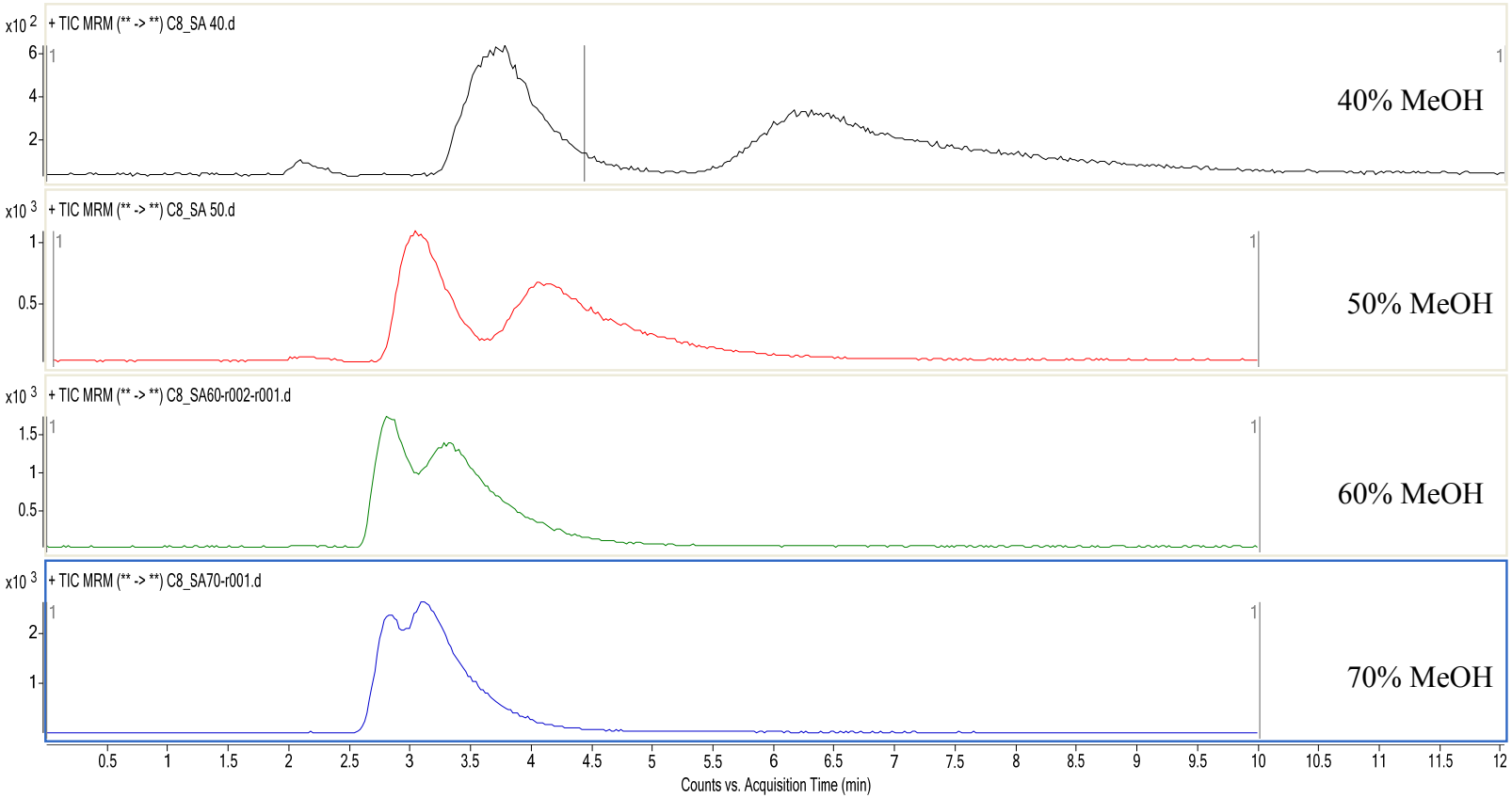


Figure 4.39: Comparison of the impact of different different % MeOH mobile phase compositions on peak shape and retention for Scop and Atr using an Agilent Zorbax RX-C8 column

Symmetry Shield RP8 column

A Symmetry Shield RP8 (75 x 4.6 mm, 3.5 µm) column was also investigated. This column was again unable to achieve separation between the two drugs under the same chromatographic conditions. Figure 4.40 displays results using this column at 40% MeOH in comparison to 40% MeOH using the above C8 column. Buffer used was as before, 2 mM ammonium formate, pH 5. Using the same flowrate of 0.2 mL/min, 20 µL of a Scop and Atr 10 ng/mL mixed standard was injected.

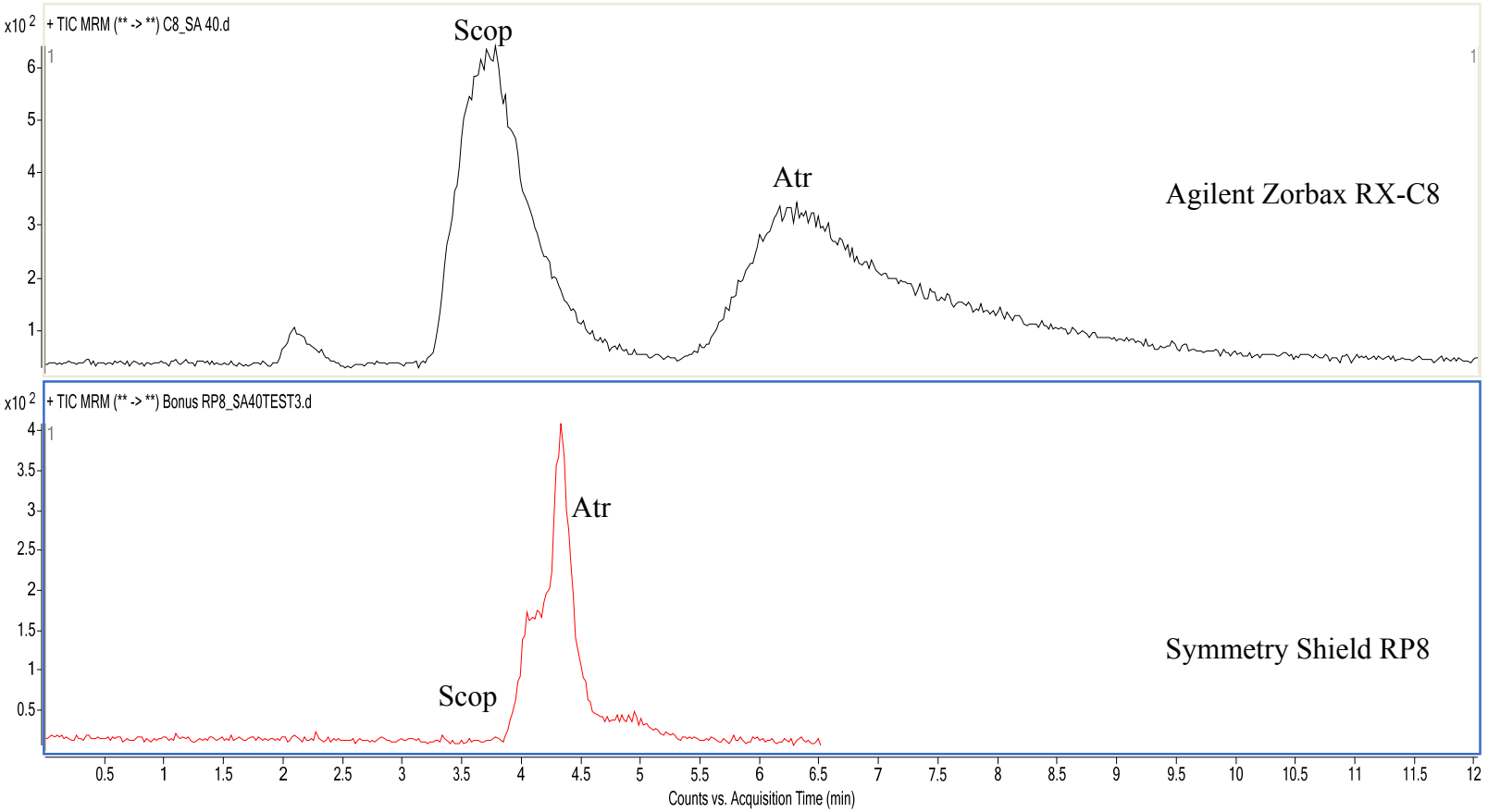


Figure 4.40: Comparison of Agilent Zorbax RX-C8 column to Symmetry Shield RP8 at 40% MeOH

Agilent Zorbax SB-Phenyl column

Following trials on a number of columns it was decided to investigate an Agilent Zorbax SB-Phenyl column (150 × 4.6 mm, 5 μm). Steenkamp *et al.,* 2004, reports the only other LC-MS method developed for the analysis of Scop from a biological matrix using a phenyl column. Their method was used to analyse viscera samples whereby Scop and Atr were detected in the stomach and its contents, which contained datura seeds. Steenkamp too evaluated various HPLC columns including reversed phase C18 in the method development but a Waters Xterra Phenyl HPLC column performed the best and so, was used by this group.

As phenyl columns offer unique selectivity from the alkyl phases and are generally less retentive than C8 or C18 phases (Kazakevich *et al.,* 2006) it was decided this phase would be a good alternative and worth investigating. See Figure 4.41.

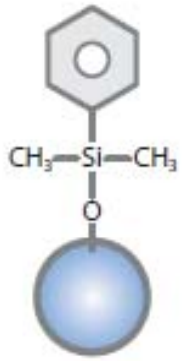


Figure 4.41: Reversed phase phenyl stationary phase

Figure 4.42 represents the preliminary results achieved using the Agilent Zorbax SB-Phenyl column (150 × 4.6 mm, 5 μm). The same flowrate of 0.2 mL/min and the same 20 μL of a Scop and Atr 10 ng/mL standard was injected as with the previous columns investigated. Separation between Scop and Atr was achieved but the retention times of the drugs, even under various % MeOH/ammonium formate compositions were too long with a runtime of 25 minutes required to elute the two drugs.

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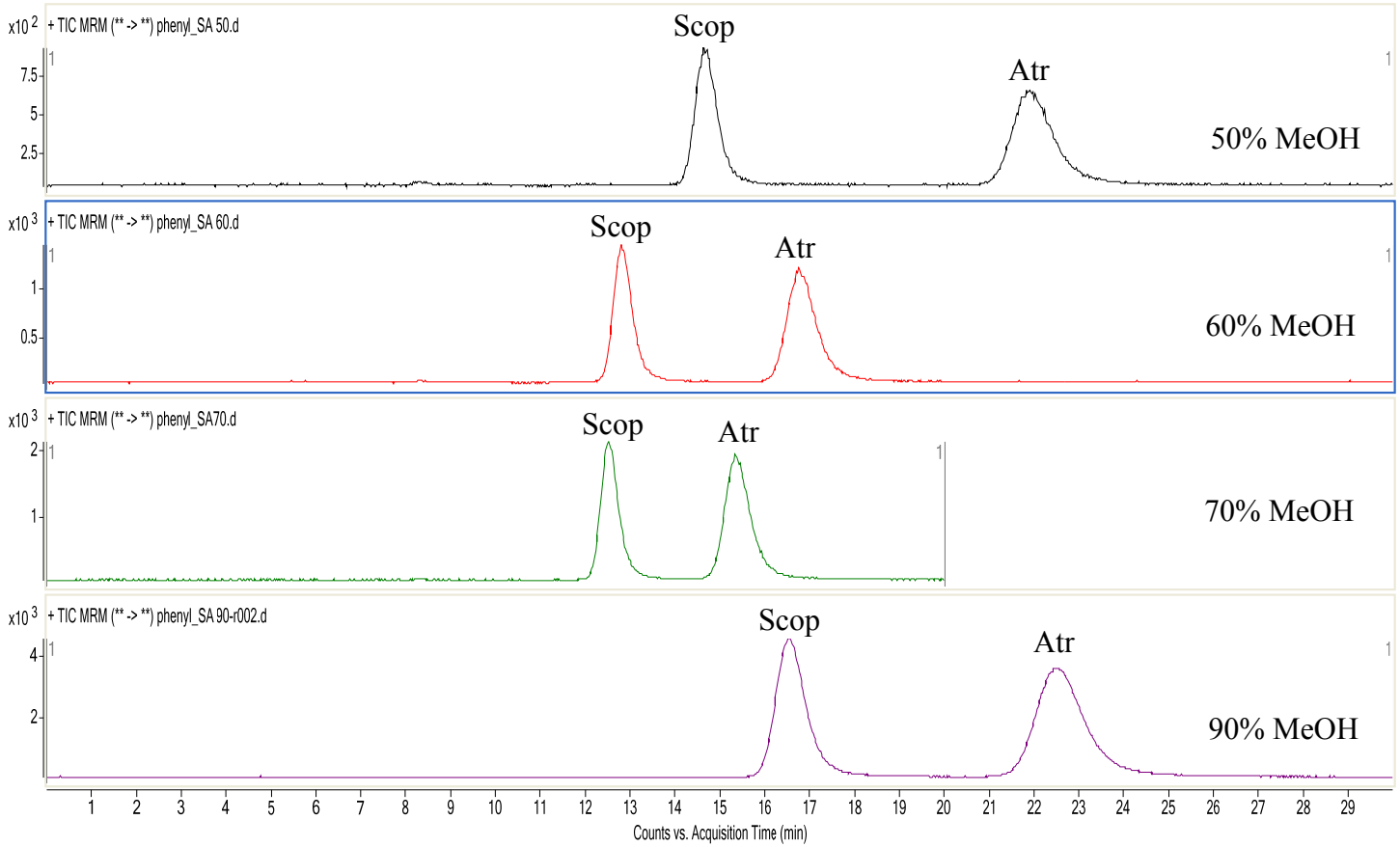

Figure 4.42: Comparison of the impact of different different % MeOH mobile phase compositions on peak shape and retention for Scop and Atr using an Agilent Zorbax SB-Phenyl column

As discussed, for quantitation of analytes in biological samples, the usual procedure is to add an internal standard in the calibration samples and real samples. An internal standard is generally incorporated to correct for sample and standard injection volume variations and errors. Since each sample and standard contains a constant internal standard amount, the analyte level (and its signal) should remain proportional to the internal standard level and the analyte concentration is therefore proportional to the ratio of the analyte to internal standard signals. Likewise, the sample and standard dilution volume can be compensated for by use of an internal standard. Proper selection of an internal standard also allows the internal standard to correct for extraction efficiency. The internal standard is typically chosen to be similar in structure, generally a structural analog to the analyte, so that they have similar partition coefficients in liquid extractions and extraction efficiencies in solid phase extraction (Grasso *et al.* 1998). However, use of Atr as an internal standard proved both complex and inadequate for this work.

The optimal internal standard is often regarded as a deuterated or carbon-14 analog of the analyte because they have identical chemical properties, resulting in identical extraction efficiencies and also identical reactivity for derivatisation. Since deuterated analogs chromatographically co-elute with the analyte, they can only be used with mass-selective detectors because mass spectrometers allow for the mass discrimination of the co-eluting compounds (Ohannesian *et al.,* Streeter 2002). As with more conventional internal standards, deuterated internal standards are used mainly to compensate for sample loss during clean up and correct the deviations of injection volumes and variability in detector response (Grasso *et al.* 1998). However, deuterated compounds are very expensive and are often not available. They must also be available in absolute purity since any non-deuterated material may confound the estimation of the target analyte. This can be a major limitation for the application of such standards in ultrasensitive determinations since any contamination by an undeuterated form of the analyte will lead to erroneous results.

It is recommended in cases where internal standards are not used for recovery correction, to use two to three replicate assays on a single sample to improve precision (Ohannesian *et al.,* Streeter 2002). Having concluded Atr

was extremely problematic in terms SPE quantification and getting a clear serum blank baseline it was decided not to proceed with analysis of this drug. Also, the high pKa value (10.2) for Atr posed some problems as most analytical columns cannot function at pH values higher than nine due to degradation of the silica support structure of the reversed phase analytical columns.

With these complexities in mind, it was decided to proceed without an internal standard and use three replicates per sample assayed for the remainder of the method. Fortunately, it was found that the results were sufficiently accurate and robust without the use of an internal standard.

Due to the wide bore nature of this column, the flow rate was increased from 0.2 mL/min to 0.6 mL/min in order to achieve faster analysis time. A flow rate reduced below optimum for the particle size can reduce efficiency and resolution due to increased band dispersion.

Figure 4.43 shows the effect of increasing the MP MeOH composition from 60-90% using ammonium formate (pH 5, adjusted with 1 M formic acid, 2 mM) and 0.6 mL/min flowrate again using the same 20 µL injection volume. Any decrease from 90% methanol had a massive impact on the sensitivity for Scop, with a decrease in both peak height and area observed.

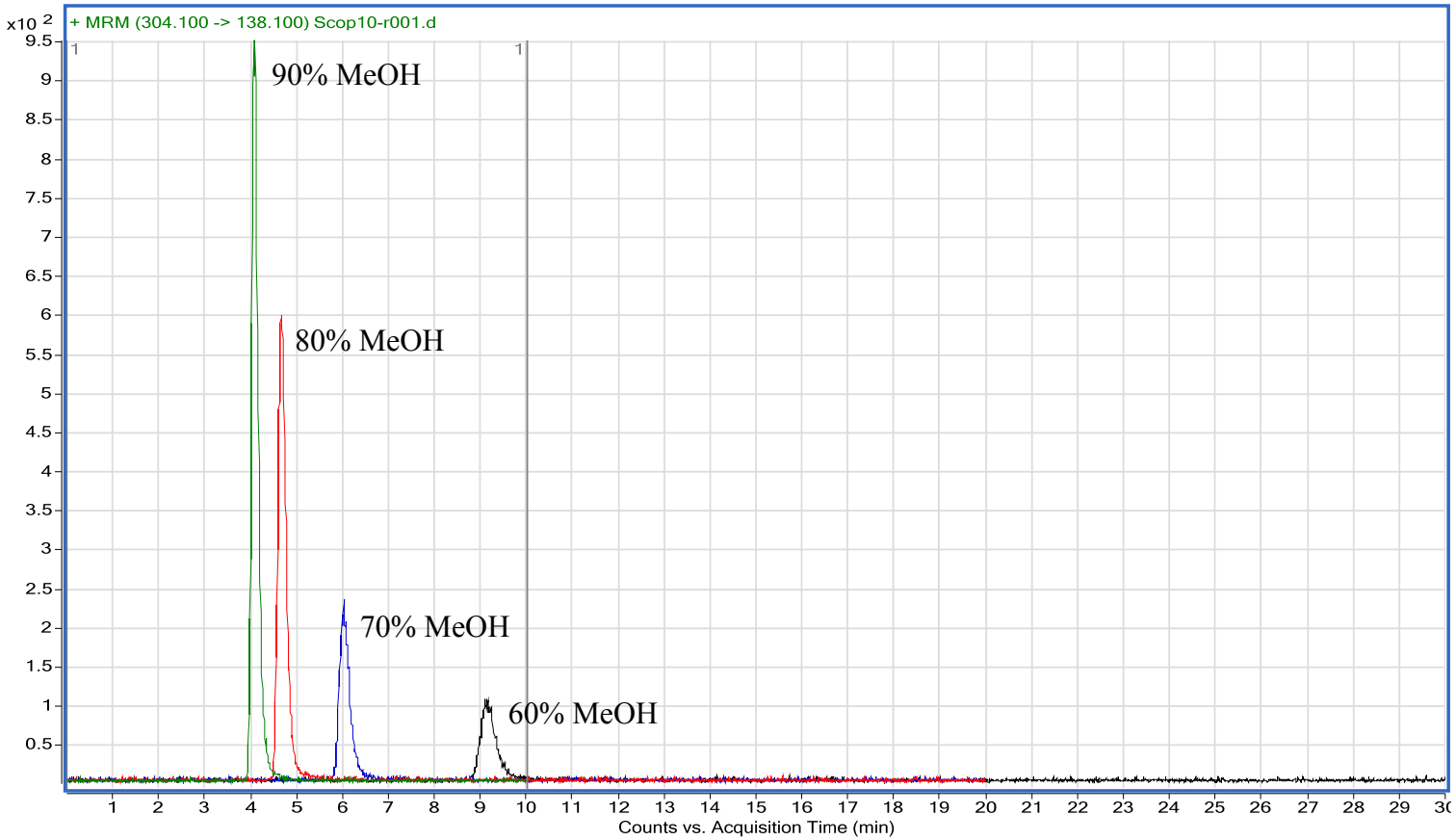

Figure 4.43: Comparison of the impact of different different % MeOH mobile phase compositions on peak shape and retention for Scop using an Agilent Zorbax SB-Phenyl column

In order to further optimise Scop's signal intensity, different mobile phase buffer concentrations and pHs were looked at. Using ammonium formate (pH 5 adjusted with 1 M formic acid) - MeOH (10:90, v/v), 2, 5, 10 and 20 mM ammonium formate buffer concentrations were examined. See Figure 4.44. It was decided to choose 10 mM ammonium formate as buffer for the mobile phase as it gave the best results in terms of area count in comparison to the others investigated.

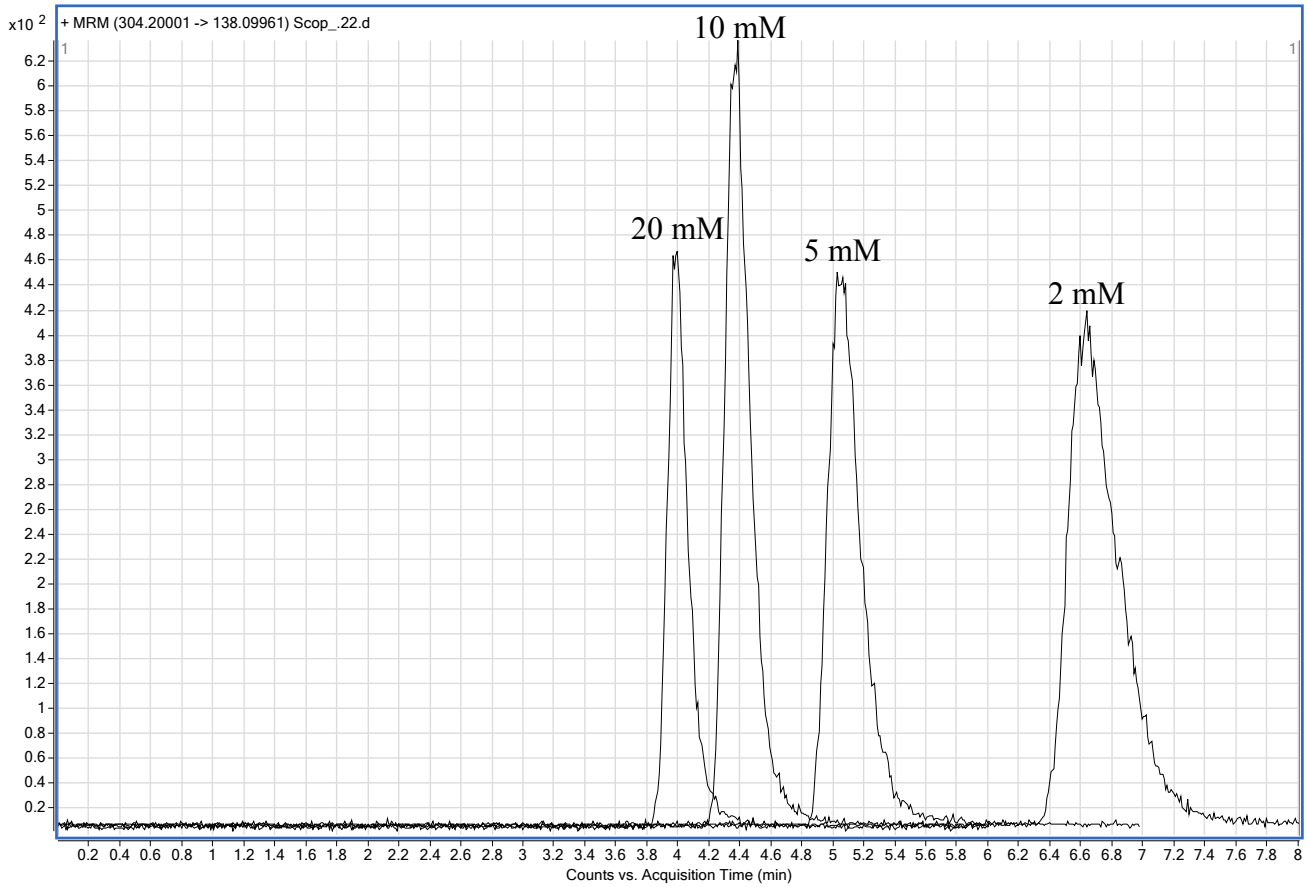

Figure 4.44: Comparison of the impact of different ammonium formate concentrations (pH 5) on peak shape and retention for Scop using an Agilent Zorbax SB-Phenyl column

As pH dictates the degree of ionisation of compounds that possess ionisable groups, the pH of the mobile phase influences both retention and ionisation efficiency in LC-MS. The pH of the mobile phase dictates whether analytes are positively or negatively charged, and the overall extent of charge. Using ammonium formate (10 mM) - MeOH (10:90, v/v), different pHs were then examined (4, 5, 7, 8 and 9). pH adjustment was achieved using formic acid and ammonium hydroxide. Although organic acids are the most common mobile phase additive for HPLC separations that employ MS detection, it may be necessary under certain circumstances to use more neutral conditions, either because the analytes are sensitive to acids or do not exhibit optimal resolution at low pH. When acids are not suitable, volatile salts, like ammonium formate or ammonium acetate, are the additives of choice. At higher pHs, basic compounds with pkas greater than 5 (such as Scop with a pKa value of 7.55-7.81 (Cherkaoui *et al.,* 1997)) become non-charged and the column retains them more. At pH > 5 the residual silanols on the silica surface become charged and this can lead to stronger interactions with basic compounds and increase retention. From Figure 4.45 it can be seen that high pH was needed to achieve greater retention of the basic compounds and pH 8 offered the best results in terms of peak area.

Steenkamp *et al*., 2004, also used basic chromatographic conditions in their approach to ensure the maximum retention of basic alkaloids. The phenyl column they used, however, could be employed at a pH > 9 and was found to be suitable for the chromatography of Atr. A gradient was employed starting with 90% water containing 10 mM ammonium acetate, the pH adjusted to 10.5 with ammonia. Due to the nature of the phenyl column used in our work, a pH this basic could not be employed and so was not investigated for further optimisation of Atr.

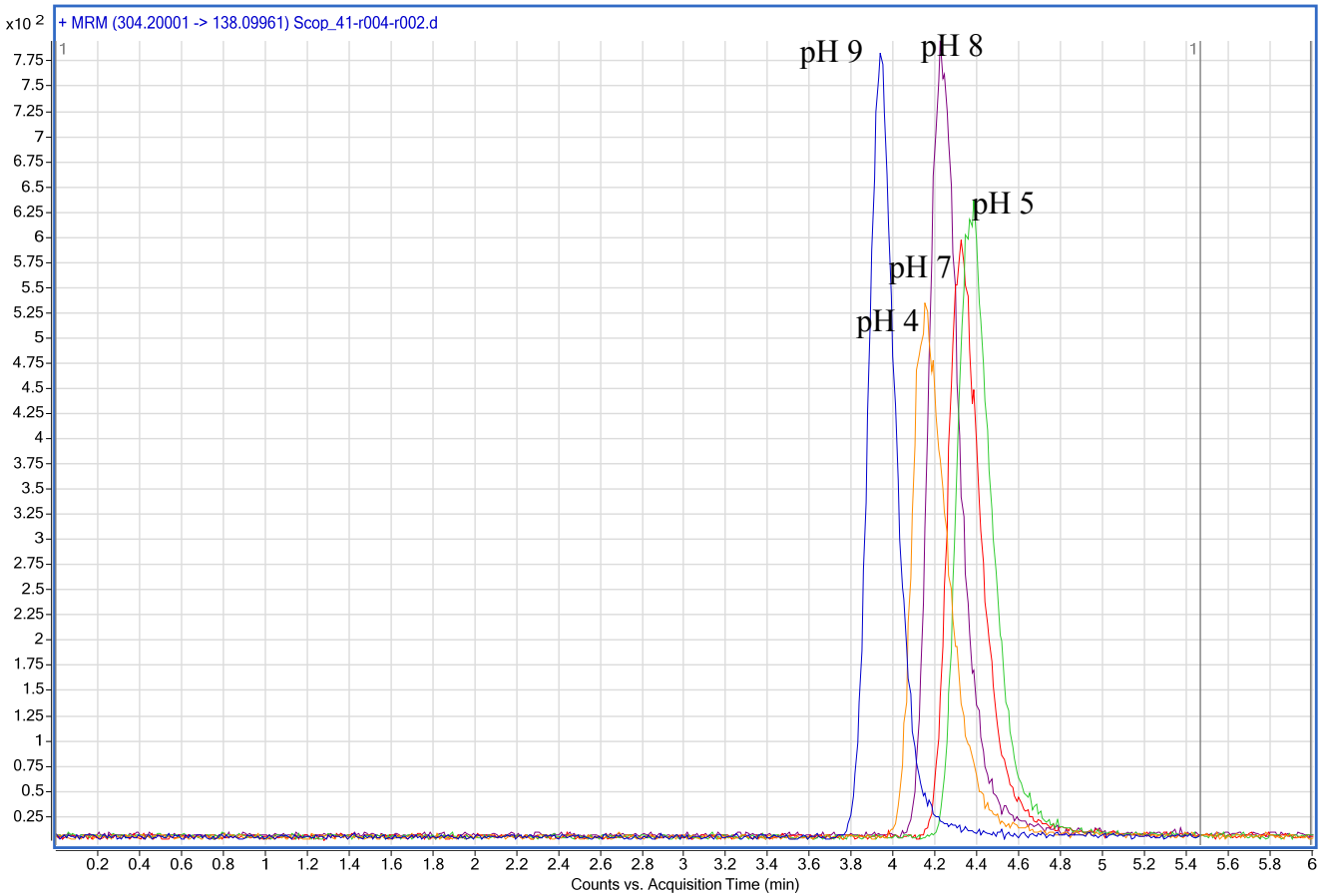

Figure 4.45: Comparison of the impact of different mobile phase pHs using 10 mM ammonium formate on peak shape and retention for Scop using an Agilent Zorbax SB-Phenyl column

Chromatographic separation was used for validation and analysis of the participant's serum samples was achieved using this Agilent Zorbax SB-Phenyl column (150 × 4.6 mm, 5 μm). A mixture of ammonium formate (pH 8 adjusted with ammonium hydroxide; 10 mM) - MeOH (10:90, v/v) was used as mobile phase, at a flow rate of 0.6 mL/min. The column temperature was maintained at 20 °C and the temperature of the autosampler was maintained 4 °C. The complete chromatographic run time of each sample was 10 min, which eluted Scop at 4.2 mins. Injection volume was 20 μL. The sensitivity achieved for the drugs prepared in mobile phase, 10 pg/mL was the best LOQ achieved out of all the columns investigated. As discussed, the solvent composition (organic/water ratio) is particularly important in the electrospray nebulisation and ionisation process, since it determines surface tension of the droplets formed and the vaporisation efficiency. As the surface tension of water is much higher than the surface tension of MeOH, the sensitivity is reduced when using a high % of aqueous mobile phases. This assay, however, required a high % of organic in the MP and may have been a contributing factor to the excellent LOQ achieved. It was hence decided to proceed with this column and re-optimise SPE conditions.

4.4.2.2 Extraction procedure

From the results obtained from the Waters Nova-Pak C18 column (150 × 3.9 mm, 4 μm) and SPE optimisation, it was determined the Oasis HLB cartridges gave the best results in terms of Scop SPE recovery in comparison to the four ion exchange sorbents. Also, and confirmed by the Agilent Zorbax SB-C18 column optimisation and SPE results, Atr did not give consistent results in terms of recovery, and some form of very similar contaminant was consistently found in SPE serum blanks in varying degrees. It was hence decided not to proceed with using Atr as internal standard with the Agilent Zorbax SB-Phenyl column. With the Agilent Zorbax SB-C18 column, ion suppression played a big problem in terms of Scop recovery. Further investigation was therefore required in order to obtain the cleanest results possible with the Agilent Zorbax SB-Phenyl column. It was decided to carry out an extensive study on the

retention of Scop to the Oasis HLB cartridge. Figure 4.46 displays the effect on varying the composition of MeOH in the wash step on the retention of Scop to the HLB cartridge. It was a crucial step in determining the optimum wash and also elution steps for Scop.

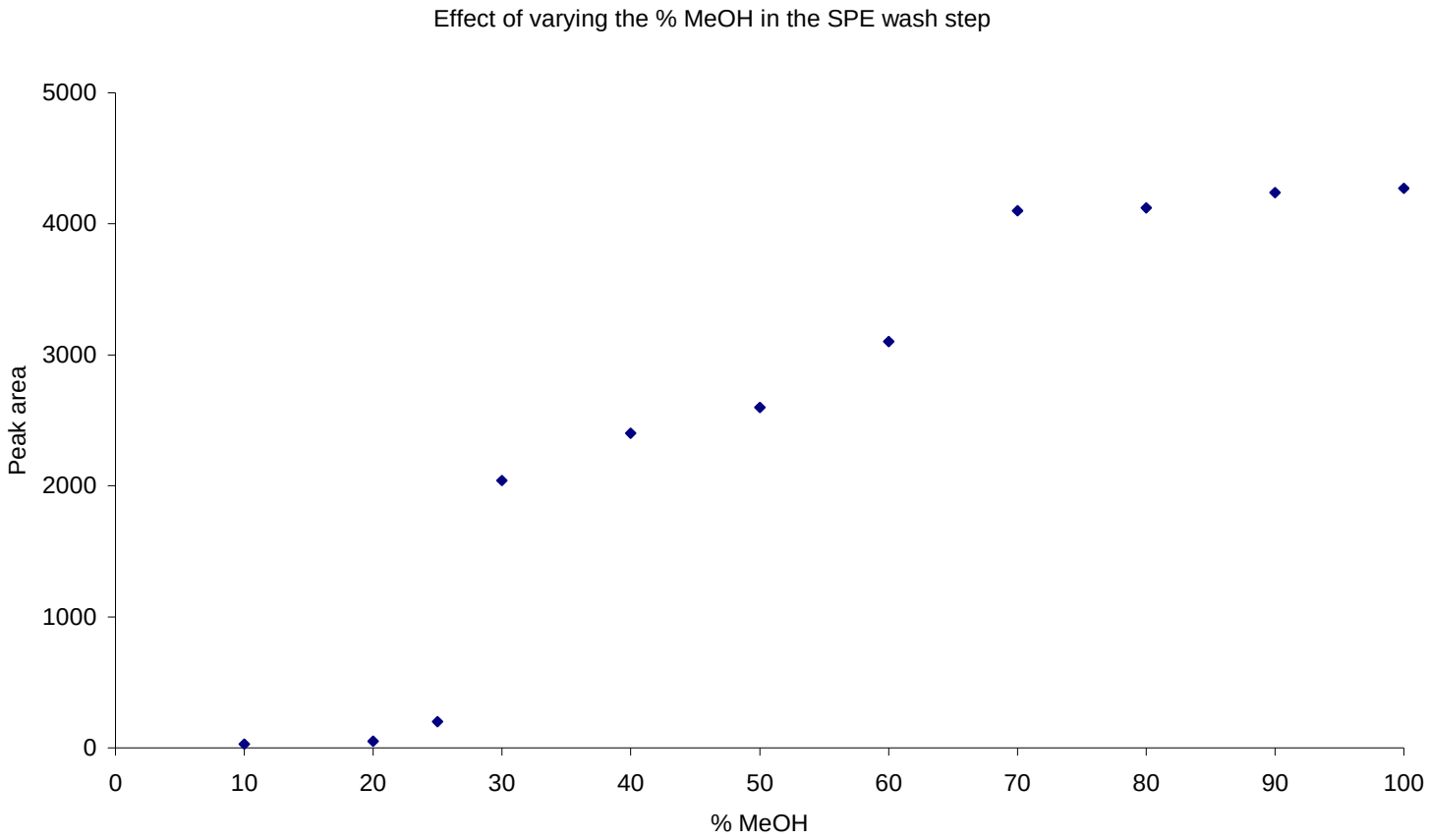

Figure 4.46: Comparison of different MeOH compositions in the SPE wash step on the retention of Scop

As discussed, the optimum wash solvent needed to be non-polar enough to elute as many interferences as possible, but not sufficiently non-polar to elute the analytes. This solvent was identified by washing the SPE cartridges starting with 100% water, then progressively increasing the proportion of MeOH present. Proportions of 10 to 100% MeOH in the wash solvent were evaluated. Each wash step was collected and analysed for the presence of the drugs. See Figure 4.46. There was a slight loss of recovery at 25% MeOH. The wash containing the highest percentage organic but no analytes was at 20% and provided the cleanest final extract.

From these results it can be seen, elution with 100% MeOH gave the best recovery for Scop in terms of achieving the highest peak area signal. However, on introducing serum to the elution step, eluting with 100% MeOH was shown to be detrimental to ion suppression results. As previously discussed, the SPE elution step requires use of the weakest elution solvent which has just enough hydrophobicity to give quantitative analyte elution. Eluting with 100% MeOH however, left behind on the sorbent the maximum number of contaminants as shown in Figure 4.47. A compromise using 75% MeOH proved satisfactory. With a slight decrease in recovery observed as seen Figure 4.46, a decrease in the number of contaminants eluting was evident as seen in Figure 4.47.

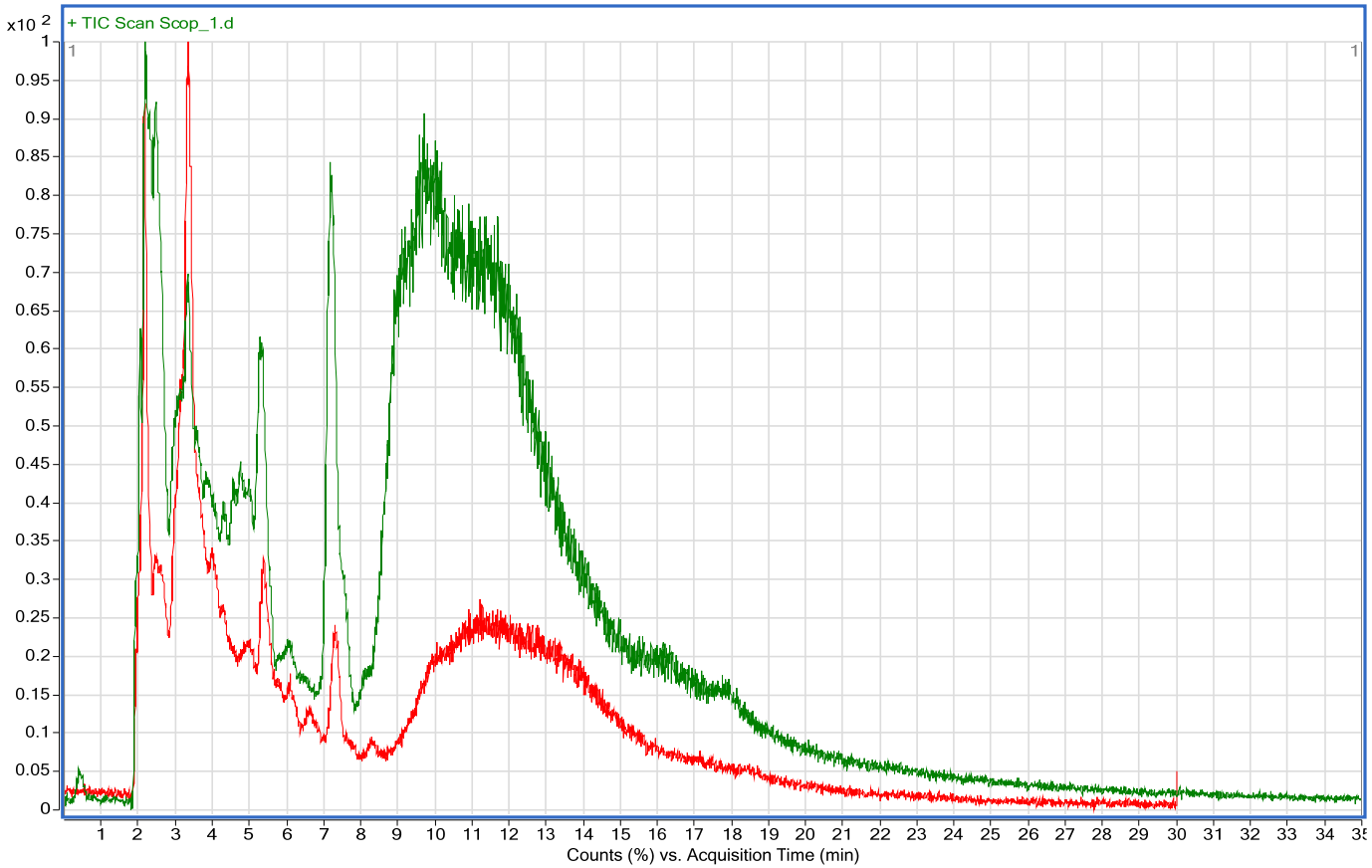

Figure 4.47: TIC scan showing differences between eluting with 100% MeOH (green) and 75% MeOH (red) using an Agilent Zorbax SB-Phenyl column

Figure 4.48 is a representation of where Scop eluted via MRM scan with transition *m/z* 304 to 138 extracted out in comparison to the TIC scan of serum blank extracted. It represents where Scop was eluting in relation to the endogenous material from the blank serum extracted.

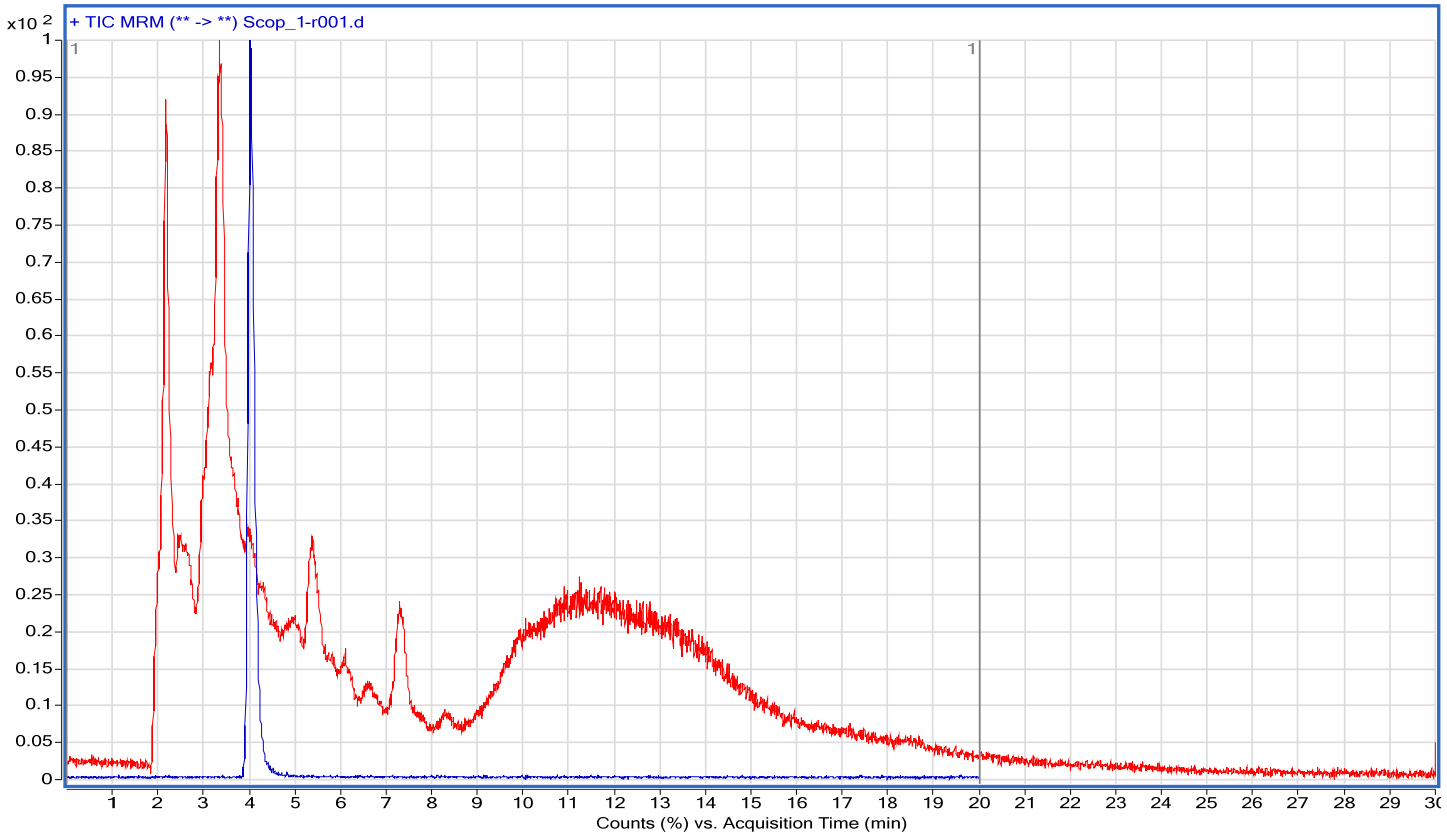

Figure 4.48: Chromatogram of TIC scan of spiked serum sample (red) superimposed on MRM scan of same Scop sample (blue) using an Agilent Zorbax SB-Phenyl column. Note: not to scale

Under the final optimised SPE conditions, Oasis HLB cartridges were conditioned with 1 mL MeOH prior to equilibration with 1 mL diluted buffer solution with water (1:10, v/v) used in load step (5 mM ammonium formate buffer; pH 8). 1 mL buffered serum (0.5 mL serum + 0.5 mL buffer (5 mM ammonium formate buffer, pH 8)), was loaded and subsequently washed with 2 mLs 20% MeOH. The sample was eluted with 0.5 mL 75% MeOH. The vials were evaporated to dryness using a Genevac EZ-2 evaporator at ambient temperature, without light for ~ 1.5 hours and reconstituted in 0.05 mL MeOH.

4.4.2.3 Mass spectrometric conditions

Mass spectrometry settings for Scop and Atr were optimized by injecting 250 ng/mL of drug, and by flow injection analysis (FIA) the precursor ion, optimum fragmentor voltage, optimum collision energy and product ion was determined. As discussed for MRM mode, the fragmentor voltage setting acts as a filter allowing the maximum number of precursor ions to enter the system in quadrupole one. In the collision cell a voltage is applied and the precursor ions are split into their products ions. This explosion occurs as the products ions smash in to the nitrogen ions already present with the energy from the voltage applied. These product ions continue to travel onwards to the third quadrupole. Here, accurate masses of the product ions are detected.

Each ion source/spray chamber's settings depend on the mobile phase composition, flow rate, and sample identity. The nebuliser pressure, drying gas flow, and drying gas temperature are dependent upon the mobile phase composition and flow rate. The settings used were set according to the MS operation manual for a mobile phase flowrate of 0.6 mL/min. Drying gas temperature was set to 375 ^{0}C, drying gas flow to 11 L/min and nebuliser pressure to 50 psi.

4.4.2.3.1 Precursor ion determination

From the autosampler, 10 µL of sample were injected into the mass spectrometer by FIA where a TIC scan was run in order to establish the predominant precursor ion. The range was set from *m/z* 200 to 400 for Scop and

from *m/z* 210 to 350 for Atr. These ranges were chosen as they bracketed the molecular weights of Scop and Atr. Once a prominent peak was seen at the expected *m/z* value, this was deemed to be the precursor ion; Scop *m/z* 304.1 and Atr *m/z* 290.2. See Figure 4.49.

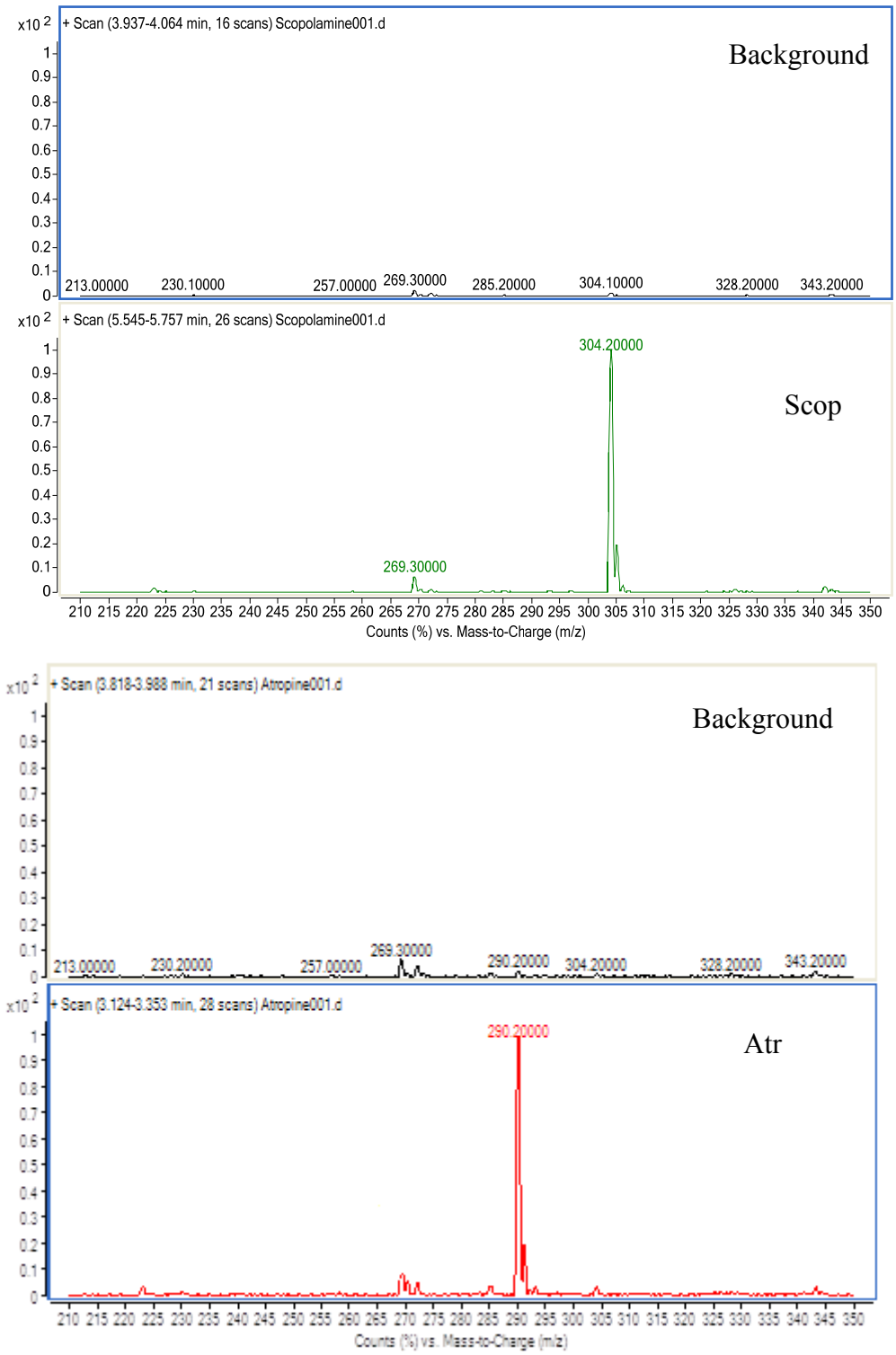

Figure 4.49: Comparison of background and Scop and background and Atr

4.4.2.3.2 Fragmentor voltage optimisation

Once the precursor ion had been established the next step was to determine the optimum fragmentation conditions for that precursor ion. The mass spectrometer was programmed to perform an MS selected ion monitoring (SIM) scan which detects only the selected precursor ion so that the optimum fragmentor voltage for that ion could be determined. These SIM scans were done in series where the fragmentor voltage was varied over a range of 0 to 160 V. Voltage increments of 20 V were used. 120 V achieved optimal results for Scop, with 140 V for Atr. See Table 4.13

Voltage (V)	**Scop**		**Atr**	
	Area	**Area %**	**Area**	**Area %**
0	374,077	28.0	75,207	27.6
	484,306	36.3	111,575	40.9
20	868,570	65.1	164,944	60.5
	792,053	59.4	161,351	59.2
40	961,364	72.1	177,633	65.1
	953,392	71.5	174,731	64.1
60	1,066,566	79.9	203,193	74.5
	1,049,472	78.7	196,597	72.1
80	1,154,427	86.5	211,708	77.6
	1,199,793	89.9	221,141	81.1
100	1,282,118	96.1	238,428	87.4
	1,286,714	96.4	241,916	88.7

120	1,334,297	100.0	252,353	92.5
	1,276,291	95.7	250,773	92.0
140	1,217,851	91.3	263,927	96.8
	963,963	72.2	272,707	100.0
160	940,971	70.5	272,675	100.0
	844,691	63.3	269,242	98.7

Table 4.13: Comparison of fragmentation voltages for Scop and Atr

4.4.2.3.3 Product ion determination

Having established the optimum fragmentor voltage for Scop and Atr, the next step involved carrying out a product ion scan in order to identify the product ions of Scop's and Atr's precursor ions. This was achieved by firstly isolating and then fragmenting the precursor ion with varying collision energy values ranging from 0 to 50, using increments of 10. The product ions were determined by analysing the fragments produced on collision.

For Scop, this resulted in four predominant product ions: *m/z* 110.1, *m/z* 121.1 *m/z* 138.1 and *m/z* 156.1. *M/z* 138 was the most abundant ion and so was used for quantification of Scop; the other 3 ions were monitored for qualitative purposes and hence ensuring specificity. According to Chen *et al*., 2005, the most abundant product ion at m/z 138.1 was formed by the loss of tropic acid ($C_9H_{10}O_3$, 166 Da) and the ion at m/z 156 was produced by the loss of $C_9H_8O_2$ (148 Da). See Figure 4.50. For Atr, this resulted in two predominant product ions: *m/z* 124.1 and *m/z* 93.1. *M/z* 93.1 was the most abundant ion and so was used for quantification of Atr.

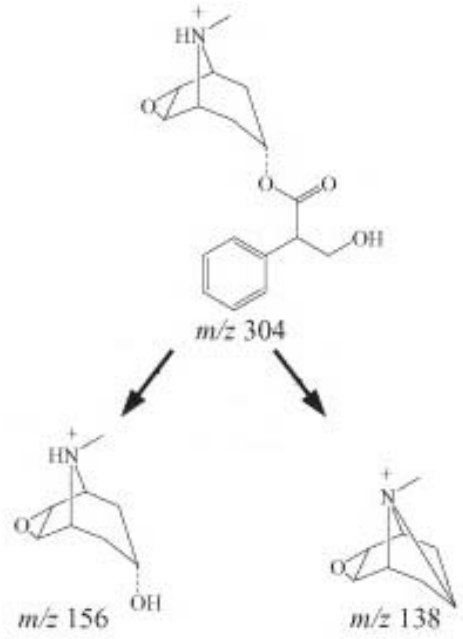

Figure 4.50: Fragmentation pathway of Scop

4.4.2.3.4 MRM transition optimisations

Repeated multiple reaction monitoring (MRM) scans which detected the MS/MS transitions were carried out in order to obtain the optimum collision energy for fragmentation of Scop and Atr to their product ions. Collision energy was raised in increments of 5 V in this case so that a more accurate collision energy could be established. The collision energy that gave the most intense peak for the chosen product ion was deemed to be the optimum value. See Table 4.14.

Compound	Product ion	Optimum collision energy (V)
Scop	110.1	30
	121.1	20
	138.1	20
	156.1	15
Atr	124.1	22.5
	93.1	35

Table 4.14: Collision energy results for product ions of Scop and Atr

The final MS conditions used in terms of precursor and product ions, collision energies and fragmentation voltages are shown in Table 4.15.

Compound	Precursor ion	Fragmentor voltage (V)	Product ion	Optimum collision energy
Scop	304.1	120	138	20
Atr	290.2	140	124.1	22.5

Table 4.15: Optimum positive mode ESI conditions for Scop and Atr

4.5 Method validation

A full validation in accordance with the FDA guidelines (FDA, 2001) was performed on the previously described method whereby chromatographic separation was achieved using an Agilent Zorbax SB-Phenyl (150 × 4.6 mm, 5 μm). A mixture of ammonium formate (pH 8 adjusted with ammonium hydroxide; 10 mM) - MeOH (10:90, v/v) was used as mobile phase, at a flow rate of 0.6 mL/min. The column temperature was maintained at 20 °C and the temperature of the autosampler was maintained 4 °C. The complete chromatographic run time of each sample was 10 min. Peaks were quantified using Agilent Masshunter Software.

The mass spectrometer was operated using an ESI source in the positive ion detection mode. The ionisation temperature was 350 °C, gas flow rate was 11 L/min and nebuliser pressure was 50 psi. Nitrogen was used as the ionisation source gas and ultrapure nitrogen as the collision cell gas. Analysis was performed in MRM mode with the following transitions: *m*/*z* 304.1 → *m*/*z* 138.1 for Scop, with a dwell time of 200 ms.

Extraction of Scop from serum was achieved using Oasis HLB cartridges which were conditioned with 1 mL MeOH prior to equilibration with 1 mL diluted buffer solution (5 mM ammonium formate buffer; pH 8) with water (1:10, v/v)) 1 mL buffered serum (0.5 mL serum + 0.5 mL buffer (5 mM ammonium formate buffer, pH 8)), was loaded and subsequently washed with 2 mLs 20% MeOH. The sample was eluted with 0.5 mL 75% MeOH. The vials were evaporated to dryness using a Genevac EZ-2 evaporator at ambient temperature, without light for ~ 1.5 hours and reconstituted in 0.05 mL MP.

The overall LC-MS method was validated for the following performance parameters- intra-day and inter-day precision, accuracy, sensitivity (LOD and LOQ), linearity and range, recovery and sample stability according to the guidelines described by the FDA (FDA, 2001).

4.5.1 Accuracy

In order to evaluate the accuracy of the method, five different concentrations of Scop (10, 20, 50, 100 and 200 pg/mL) were prepared in serum and analysed in triplicate on three consecutive days. The measured amounts were inserted into the equation of the calibration curves and treated as unknown concentrations. The calculated concentrations were compared with the nominal concentrations. Assay accuracy was expressed as % error i.e. [the absolute difference between calculated concentration and spiked concentration] / nominal concentration × 100. The accuracies obtained at the five concentrations examined were all acceptable with % error values < 15% - see Table 4.16. These are exceptionally good results given the very low concentration of Scop being detected.

4.5.2 Precision

The precision of Scop standards were evaluated by analysing five different concentrations of Scop (10, 20, 50, 100 and 200 pg/mL). The intra-day precision values were determined by processing each working standard concentration in sextuplicate on the same day and calculating the relative standard deviation (RSD) values. The inter-day precision values were determined by processing each working standard concentration in quadruplicate for four consecutive days and calculating the RSD values. % RSD was expressed as [deviation from the mean]/mean concentration × 100. Intra- and inter-assay precision RSD values were found to be less than 15% in all cases which, in accordance to FDA guidelines is the highest value accepted (FDA, 2001). See Table 4.16 for the summarised data. Again the results obtained, given the levels being detected are very good.

Compound	INTRA-DAY (n =4)			INTER-DAY (n = 3)		
	Calculated conc. (pg/mL)	Accuracy % Error	Precision % RSD	Calculated conc. (µg/mL)	Accuracy % Error	Precision % RSD
Scop	10.5	10.1	1.2	10.1	14.0	4.1
	20.3	-6.1	1.9	20.5	-4.3	4.2
	51.1	2.0	2.7	49.7	6.9	1.7
	99.1	1.4	7.2	100.0	-1.8	1.4
	200.9	4.3	2.1	199.9	-2.2	0.4

Table 4.16: A summary of the Intra-Day and Inter-Day precision and accuracy determinations

4.5.3 Sensitivity and selectivity

Both the limits of quantitation (LOQ) and detection (LOD) were determined for the assay. The LOQ was defined as the lowest concentration that produced a peak distinguishable from background noise with a minimum ratio of 10:1 (Shah *et al.,* 1992). LOD was defined as the lowest concentration that produced a peak distinguishable from background noise with a minimum ratio of 3:1. The limit of quantitation (LOQ) was an exceptional 10 pg/mL for Scop with a S/N of 16, the best sensitivity achieved to date for Scop extracted from biological matrix. See Figure 4.51. Extracted blank serum samples did not yield any endogenous peaks at the retention times of the drug compounds – see also Figure 4.51. The LOD was found to be 5 pg/mL with a S/N of 6. These values for LOD and LOQ enable pharmacokinetic monitoring of Scop serum samples. The signal-to-noise ratio was calculated by the Masshunter qualitative analysis software.

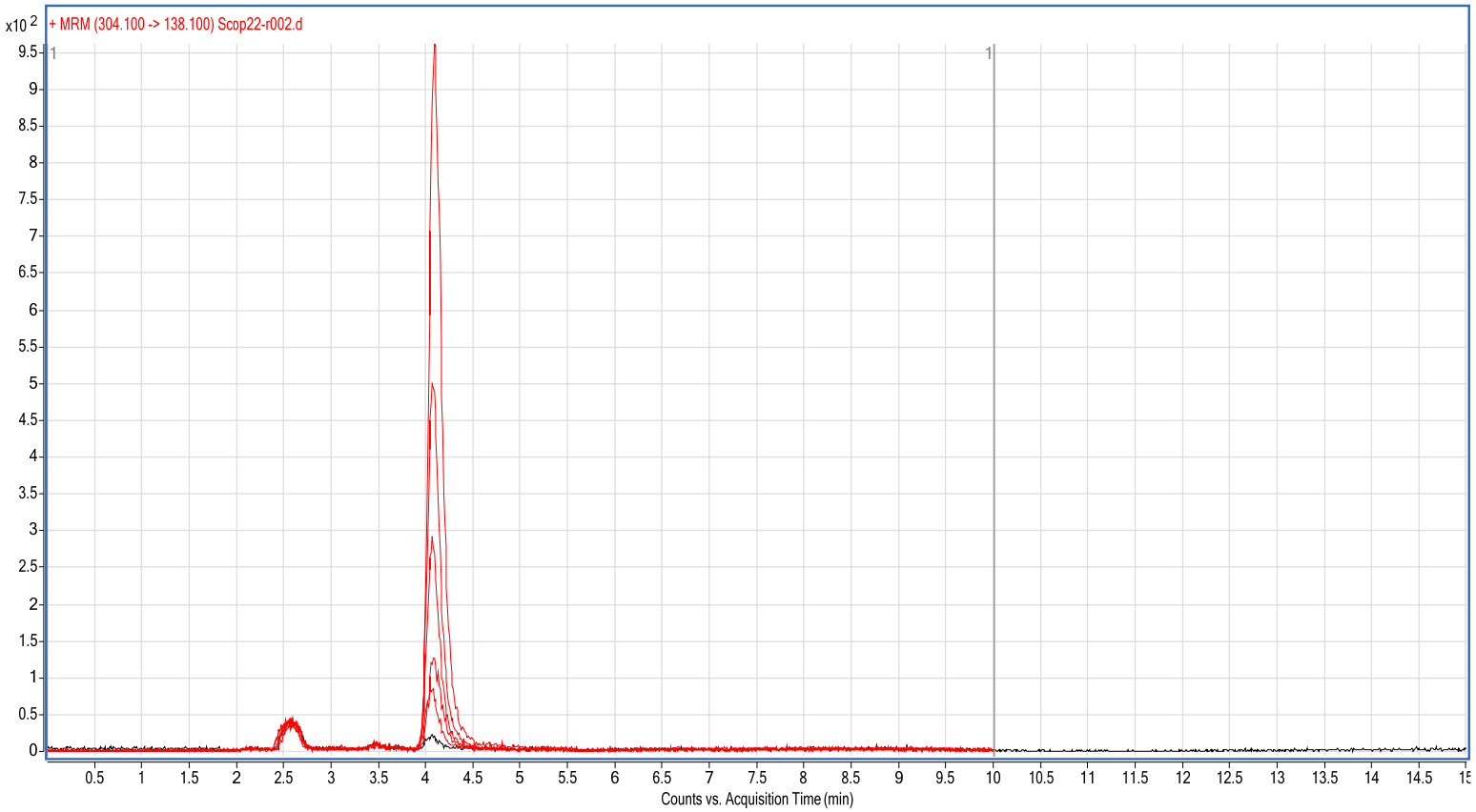

Figure 4.51: Chromatograms of extracted drug-free serum sample (black) superimposed on extracted serum spiked with 10, 20, 50, 100 and 200 pg/mL Scop (red) using an Agilent Zorbax SB-Phenyl column

Due to the high sensitivity of mass spectrometric detection and the low background when quantifying based on the molecular transitions of the ions in the collision cell, MRM gave excellent selectivity and the very low LOD and LOQs necessary to comfortably quantify Scop post patch (half or full) application.

4.5.4 Linearity and range

The calibration curves for Scop were linear over the range of 100–2000 pg/mL in mobile phase and recovery from serum was linear over the range 10–200 pg/mL, see Figure 4.52. The calibration curve was linear with a correlation coefficient (R^2) value of >0.999.

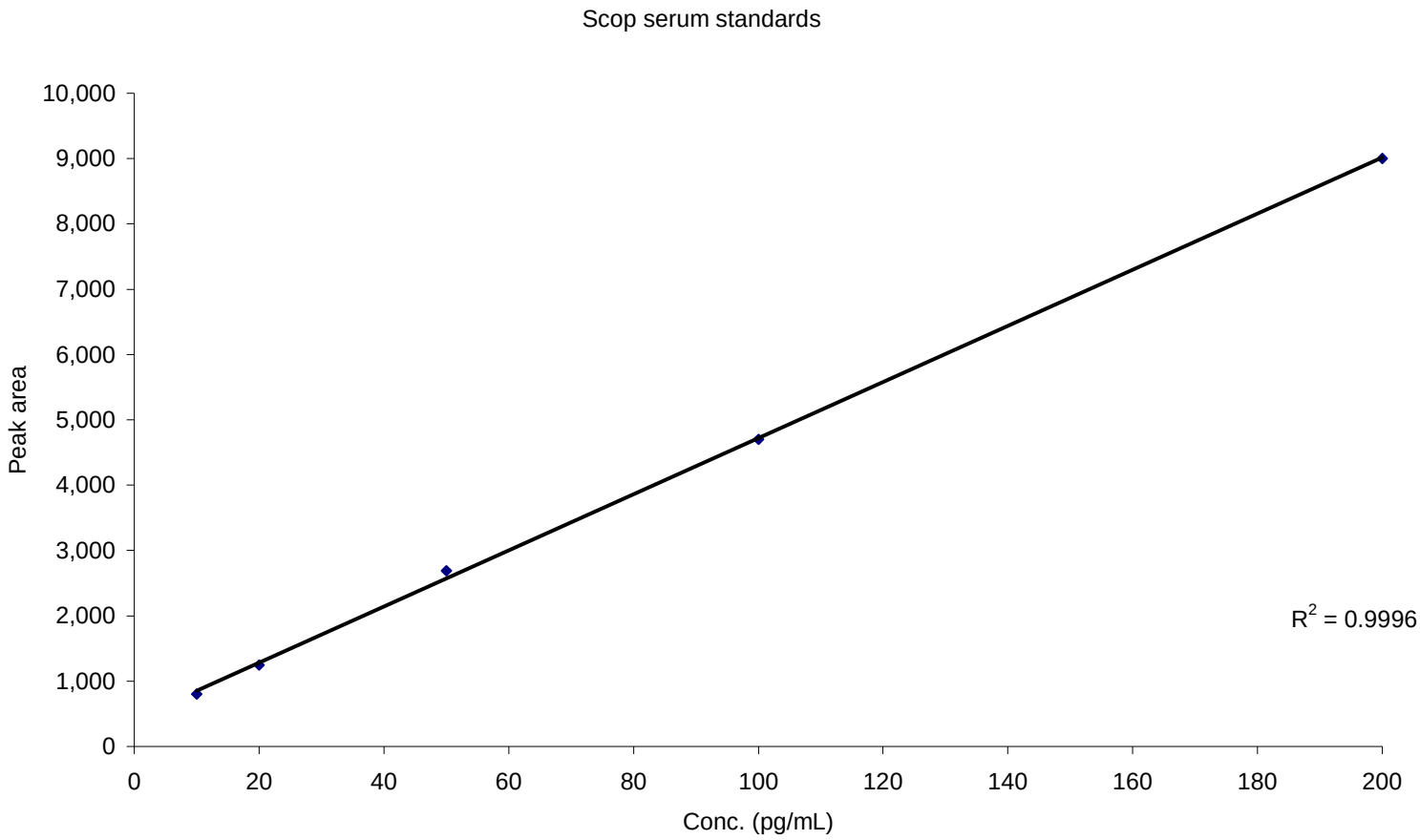

Figure 4.52: Linearity for Scop serum standards using an Agilent Zorbax SB-Phenyl column

4.5.5 Recovery

Varying concentrations of Scop (10, 100 and 200 pg/mL) were prepared in drug-free human serum and extracted using the SPE cartridges. The percentage of drug recovered from these serum samples was determined by comparing the calculated concentrations following extraction and HPLC assay with the calculated concentrations from post extracted spiked samples in MP of the same concentration after HPLC assay. Recoveries for serum samples spiked with 10, 100 and 200 pg/mL of Scop were ≥ 73% (Table 4.17).

There are very few LC-MS methods in the literature for the analysis of Scop from biological matrices. The SPE approaches exploited in the literature however, demonstrate poor Scop recovery or high sample volume required. Oertel *et al.*, 2002, developed a procedure for the extraction of Scop from serum employing Oasis HLB cartridges. A sample volume of 0.2 mL serum was required for the extraction but the recovery achieved was low at only 51%. Beyer *et al.*, 2007, used SPE for the extraction of Scop from plasma. A proportionately large sample volume was required (1 mL) and recovery ranged between 67 and 92% for Scop. In comparison, the recovery achieved for this assay was high at ≥ 73% and sample volume low at 0.5 mL serum required.

Conc. (pg/mL)	**Scop mean % recovery ± % RSD**
10	73.9 ± 1.6
100	76.1 ± 2.0
200	74.0 ± 1.1

Table 4.17: Recovery data for Scop in serum (n = 3)

4.5.6 Stability

To examine relevant variables around storage and long term stability we determined the impact of freeze–thaw cycles on the stability of the drugs in the presence of serum, fresh drug-free serum samples were spiked with 10, 100 and 200 pg/mL of Scop as shown in Table 4.18. Samples underwent three freeze-thaw (−20 °C to room temperature) cycles. The drug concentrations were then determined in triplicate on three separate occasions and compared to serum samples prepared to the same concentrations that were stored at −20 °C and only thawed once immediately prior to analysis. Finally, stock solution stability was evaluated by comparing the response obtained from standards prepared in mobile phase at three concentrations (100, 1000 and 2000 pg/mL) left at room temperature for 8 hours to freshly prepared stock solutions.

Compound	Conc. (pg/mL) ± % RSD	Cycle 1 % recovery ± % RSD	Cycle 2 % recovery ± % RSD	Cycle 3 % recovery ± % RSD
Scop	10	99.7 ± 2.1	98.1 ± 0.5	96.0 ± 3.7
	100	100.7 ± 3.0	100.8 ± 1.8	96.8 ± 2.0
	200	99.7 ± 1.9	99.5 ± 4.0	95.7 ± 0.9

Table 4.18: Summary of freeze-thaw stability findings for Scop (n = 3)

The data indicated that three freeze–thaw cycles had little significant impact on the Scop concentrations measured, which means that samples could be repeatedly thawed and re-analysed up to three times if required. The stability of the three stock solutions prepared in mobile phase was also evaluated. Standards (100, 1000 and 2000 pg/mL) left for eight hours at room temperature showed no decline in detector response at the three concentrations analysed which allowed for daily preparation of daily solutions.

4.5.7 Matrix effects

The impact of matrix effects on the accuracy, precision and robustness of bioanalytical methods is of growing concern in the pharmaceutical industry. Biological matrices such as plasma, serum and urine have been known to cause ion suppression or enhancement effects in mass spectrometric experiments. These effects result from co-eluting matrix components that affect the ionisation of the target analyte. Endogenous phospholipids are present in high concentrations in biological matrices, such as plasma and serum and have been implicated in causing ion suppression or enhancement in LC/MS/MS analyses. Researchers have described this phenomenon as being due to the effect they have on desolvation of the LC effluent droplets in electrospray MS analysis or as a result of competition for excess charges on the droplet surface (Chambers *et al.,* 2007). The potential impact of ion suppression/enhancement on the quantification was assessed by comparison of the results obtained with standard dilutions of each drug in methanol (standard solutions) against standard dilutions which were added to the eluate of SPE serum blanks (post extracted spiked samples) which were then dried and reconstituted in MP. Data indicated ~12% ion suppression, which according to FDA guidelines is acceptable. See Figure 4.53. This ion suppression could have been reduced further by either increasing the proportion of MeOH in the SPE wash step or by decreasing the proportion of MeOH in the elution step, both which would have been detrimental to the recovery of Scop as outlined previously. Matrix effects may also be reduced by simply injecting smaller volumes or diluting the sample. However, these solutions clearly influence the sensitivity of the method and were therefore not appropriate for this work. Fortunately, according to FDA guidelines (FDA, 2001), the value obtained for the % ion suppression is acceptable.

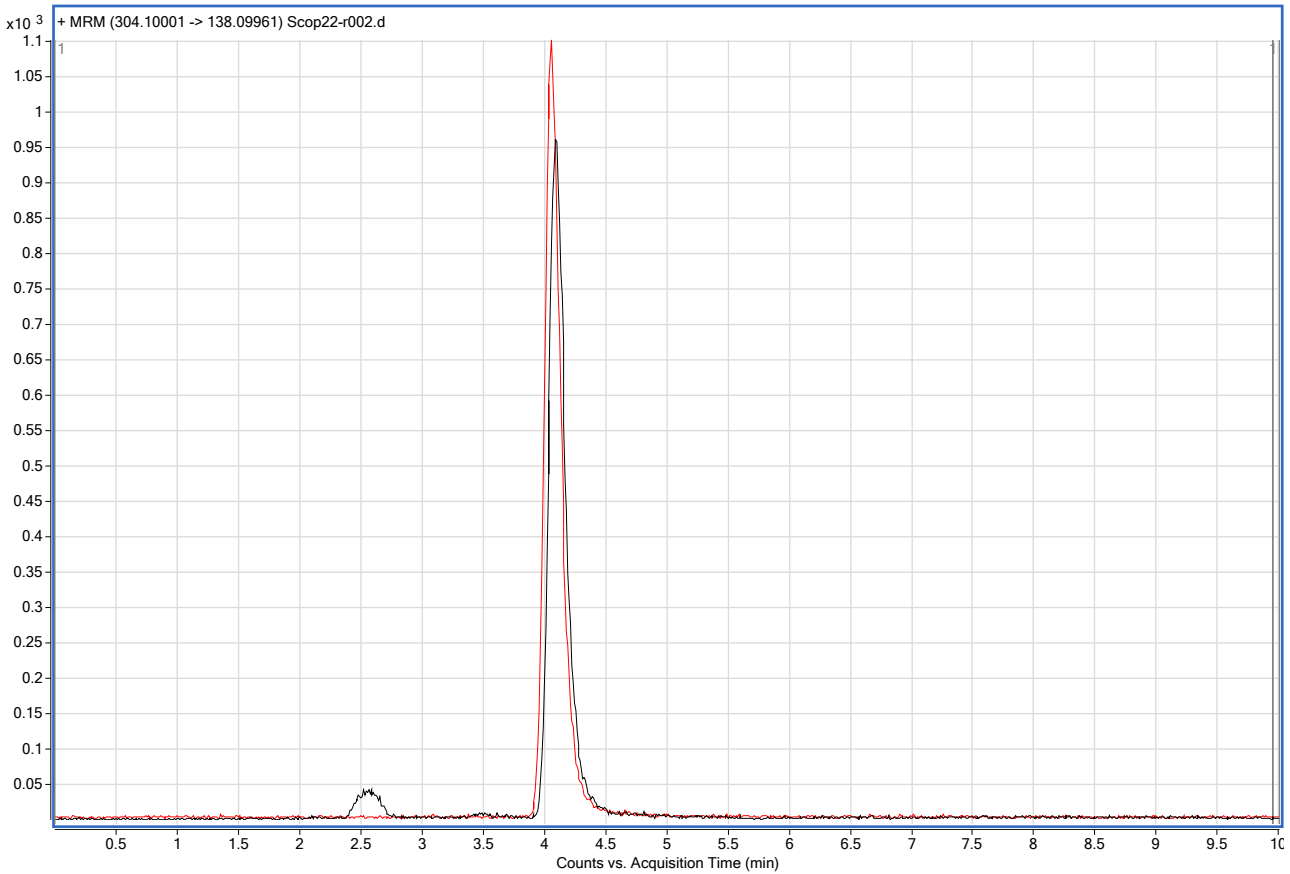

Figure 4.53: Chromatogram displaying ion suppression results. Red: 1ng/ml Scop in MeOH, Black: serum blank reconstituted in the same 1ng/ml Scop standard in MeOH

4.5.8 Patient sample results

Blood samples analysed were obtained from a participant of the pilot study discussed in Chapter 5. The participant was administered both the full and half Scop patch on different occasions. Samples were drawn at nine different time points (0, 3, 6, 7, 8, 9, 24, 48 and 72 hrs) over a 72 hour period. Presented in Figure 4.54 are the pharmacokinetic profiles generated from patient A's serum samples following the full and half transdermal Scop patch applications.

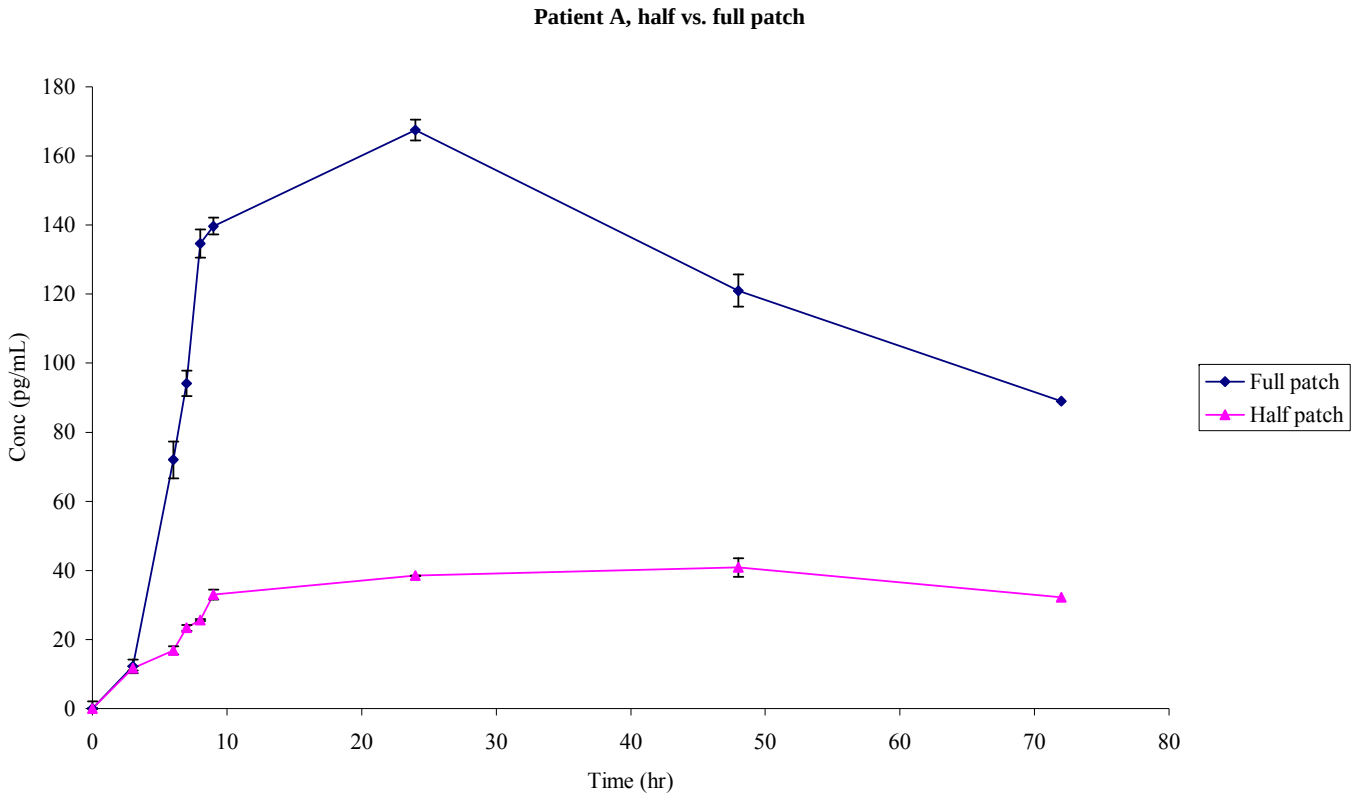

Figure 4.54: Comparison of pharmacokinetic profiles generated from patient A's serum samples following half and full transdermal Scop patch applications

Scop was not detected in serum of the patient pre administration of the patch at t=0. On application of the full transderm Scop patch, Scop partitioned from the drug saturated adhesive layer into the skin but absorption of Scop into the systemic circulation was somewhat slow with effective drug concentrations achieved 6-8 hrs post patch application. Maximum serum concentrations (C_{max}) of 167.5 ± 2.5 pg/mL of the alkaloid were reached at a T_{max}, (the time taken for the drug to reach its highest concentration in the blood stream) of about 24 hrs. As the patch releases Scop continuously over a 72 hr period, constant high Scop serum levels were observed, with a slight decline in drug concentration observed over time reaching 89.0 ± 4.6 pg/mL at 72 hrs.

On application of the half transderm Scop patch, the absorption of Scop into the systemic circulation was again somewhat slow with effective drug concentrations achieved 6-8 hrs post patch application. Maximum serum concentrations (C_{max}) of 40.8 ± 0.1 pg/mL were reached at a T_{max}, of about 48 hrs. High Scop serum levels were observed from 9 hrs onward.

Results of all participants in the Scop pilot study are discussed in more detail in chapter 5.

4.6 Conclusions

As discussed, LC–MS is currently the most common technique employed for the detection and quantification of tropane alkaloids. From the scientific literature read, there is no fast, simple, isocratic method reported which achieves the sensitivity required for the analysis of Scop in serum post patch and half patch application.

Oertel *et al.* developed two LC-MS methods for the analysis of Scop and achieved the best sensitivity for Scop published to date giving an LOQ of 20 pg/mL for Scop in serum (Oertel *et al.,* 2001). Even though the retention times for the drugs was fast, the chromatographic separation involved a gradient programme which required a 10 min equilibration time between runs and the resulting assay demonstrated poor Scop recovery. Oertel *el al.* subsequently investigated increasing sample throughput in pharmacological studies by using dual-column liquid chromatography with tandem MS (Oertel

et al., 2002). Even though good sensitivity was achieved at 50 pg/mL, a gradient was required and two columns were needed which added complexity to the method.

Beyer *et al.,* 2009, used APCI and ESI approaches for Scop's detection. APCI resulted in a LOQ of 5 ng/mL and ESI 0.1 ng/mL. Although the LOQ using ESI was low, the method required 1 mL plasma to achieve this sensitivity. Scop eluted at 4.8 min but again a gradient programme was requited for the method.

Steenkamp *et al.,* 2004, developed an LC-UV-MS method which resulted in an LOD of 100 pg/mL for Scop but suffered from Scop having an extremely long retention time of 20.0 min.

Many methods for the determination of Scop suffer from having to use a gradient programme in order to achieve their fast runtimes and low sensitivity, however, the assay developed and validated for this work required an isocratic elution which resulted in exceptional sensitivity and a short runtime. The assay described exhibits the best sensitivity achieved to date for Scop extracted from biological matrix.

In conclusion, a fast, novel and extremely sensitive assay has been developed and validated for the determination of Scop in serum using SPE coupled to LC–MS/MS. The isocratic elution scheme gives a simple, robust and reproducible chromatographic method with an exceptional LOQ of 10 pg/mL for Scop. The assay described has been proven accurate and precise for the determination of Scop in serum with a recovery of > 73% obtained. The easy sample preparation and fast separation makes this assay highly suitable for pharmacokinetic studies and therapeutic drug monitoring.

This method has been applied to a study evaluating the effects of the transdermal Scop patch both uncompromised and cut in half. As the assay is capable of monitoring serum levels of the drug over time it will also be used in the NASA-based motion sickness countermeasures study in order to evaluate the effects of the transdermal Scop patch cut in half in preventing motion sickness in astronauts induced by off-vertical axis rotation (OVAR).

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Chapter 5

A pilot comparison of the human pharmacology of a half versus full transdermal scopolamine patch

5.1 Scope of research

The National Aeronautics and Space Administration (NASA) are currently conducting a project entitled 'Evaluation of countermeasures for motion sicknesses'. The purpose of this study is to evaluate the effects of the transdermal scopolamine (Scop) patch in preventing motion sickness in astronauts induced by off-vertical axis rotation (OVAR). The main question being asked is 'Would cutting a Scop transdermal patch in half reduce the side effects associated with the medication while still giving effective protection from motion sickness?'

It had been hypothesised that if a transdermal patch of Scop is halved and only one half then used by the astronaut, that it is just as effective as a full patch but with less undesirable side effects. We therefore developed a pilot study to examine circulating levels of Scop in five volunteers treated with the full patch and a half patch to evaluate this hypothesis and further to consider if the patch is compromised by cutting it in half.

Also the study aimed to compare the severity of side effects induced by the transdermal Scop patch both full and in half and correlate these to the levels of Scop measured in serum over a 72 hour period.

5.2 Introduction

The skin represents an extraordinarily evolved organ. Not only does it physically encapsulate the organism and provide a multifunctional interface between us and our surroundings, but it is perpetually engaged in the assembly of a highly efficient homeostatic barrier to the outward loss of water. In so doing, it furnishes a membrane that is equally adept at limiting molecular transport both from and into the body. Overcoming this barrier function then, for the purpose of transdermal drug delivery, has been a challenging task for the pharmaceutical industry (Naik, *et al.,* 2000). Historically, developments in transdermal drug delivery have been incremental, focusing on overcoming problems associated with the barrier properties of the skin, reducing skin irritation rates and improving the aesthetics associated with passive systems.

The efficacy of a transdermal system is primarily dependent upon the barrier properties of the skin of the targeted species (Villarino *et al.,* 2006). The release of a therapeutic agent from a formulation applied to the skin surface and its transport to the systemic circulation is a multistep process which involves:

(a) drug dissolution within and release from the formulation
(b) partitioning of the agent into the skin's outermost layer, the stratum corneum
(c) diffusion through the stratum corneum
(d) partitioning from the stratum corneum into the more aqueous epidermal layer
(e) diffusion through the epidermis and into the upper dermis
(f) uptake into the local capillary network of the dermis and, eventually, the systemic circulation. (Villarino *et al.,* 2006)

The benefits of transdermal delivery include, not only convenience for patients but also increased compliance. Transdermal therapeutic systems allow drugs to be delivered in a rate-controlled manner, avoiding first-pass metabolism and the fluctuating plasma concentrations which can be encountered with some oral medication formulations. In most cases, there are fewer side effects than there are with oral delivery of drugs (Musel *et al.,* 2006). Scop was the first drug marketed as a transdermal therapeutic system. Today, numerous drugs have been delivered successfully through transdermal patches: Scop, nitroglycerin, nicotine, clonidine, fentanyl, estradiol, testosterone, oxybutynin, and, recently, methylphenidate, selegiline, rivastigmine and rotigotine (Farahmand *et al.,* 2009).

As discussed in Chapter 4, Scop has been shown to be one of the most effective agents for prevention of motion sickness. However, due to the short half-life of the drug in plasma and dose-dependent adverse effects, the clinical use of scopolamine administered orally or parenterally is limited. See Table 5.1 for a more comprehensive general list of Scop side effects commonly found at different doses orally administered (Barratt *et al.,* 2008).

Scop dose	Effect
0.5 mg	slight slowing of cardiac heart rate some dryness of mouth inhibition of sweating
1 mg	definite dryness of mouth thirst acceleration of heart rate sometimes preceded by slowing mild dilation of pupils
2 mg	rapid heart rate, palpitation marked dryness of mouth dilated pupils some blurring of near vision
5 mg	all the above symptoms marked difficulty in speaking and swallowing restlessness and fatigue headache, dry hot skin difficulty in urination reduced intestinal peristalsis
10 mg and above	above symptoms more marked pulse rapid and weak iris practically obliterated vision very blurred skin flushed hot dry and scarlet ataxia, restlessness and excitement hallucinations and delirium, coma

Table 5.1: Scop dose and effect

To minimise the relatively high incidence of side effects, the transdermal dosage form was developed (Renner *et al.,* 2005). Even though transdermal Scop has been associated with a lower incidence of side effects than orally or parenterally administered Scop, adverse systemic effects have still been frequently reported (Nachum *et al.,* 2006).

Motion sickness is a common affliction of mankind, caused by a neurological "disagreement" between visual signals and those generated by the balance organ in the inner ear when a person is moving (Gilles, 2007). Motion sickness is very common among astronauts. Space motion sickness and related symptoms remain a major limiting factor in space operations where the phenomenon can be seriously debilitating. There are several different forms of treatment for motion sickness. NASA provide the Scopderm patch for astronaut's treatment of motion sickness. However, the Administration has found that some individuals suffer significant side effects from these patches. The most commonly cited negative effects by people using transdermal Scop is dryness of the mouth and drowsiness. Temporary blurring of vision and dilation (widening) of the pupils has also been reported.

5.2.1 Transdermal scopolamine system

There are various transdermal therapeutic systems available which all use the same methodology. Tradenames such as Transderm-Scōp, Transderm-V, and Scopoderm are currently available in the market. The transdermal Scop system is a film, 0.2 mm thick and 2.5 cm^2 in size, made up of four layers. Proceeding from the visible surface towards the surface attached to the skin, these layers are a backing layer of tan coloured, aluminised, polyester film; a drug reservoir of Scop, light mineral oil, and polyisobutylene; a microporous polypropylene membrane that controls the rate of delivery of Scop from the system to the surface of the skin; and an adhesive formulation of mineral oil, polyisobutylene and Scop. A protective peel strip of siliconised polyester, which covers the adhesive layer, is removed before the system is used. The inactive components, mineral oil (12.4 mg) and polyisobutylene (11.4 mg) are not released from the system (Novartis, 2006). See Figure 5.1.

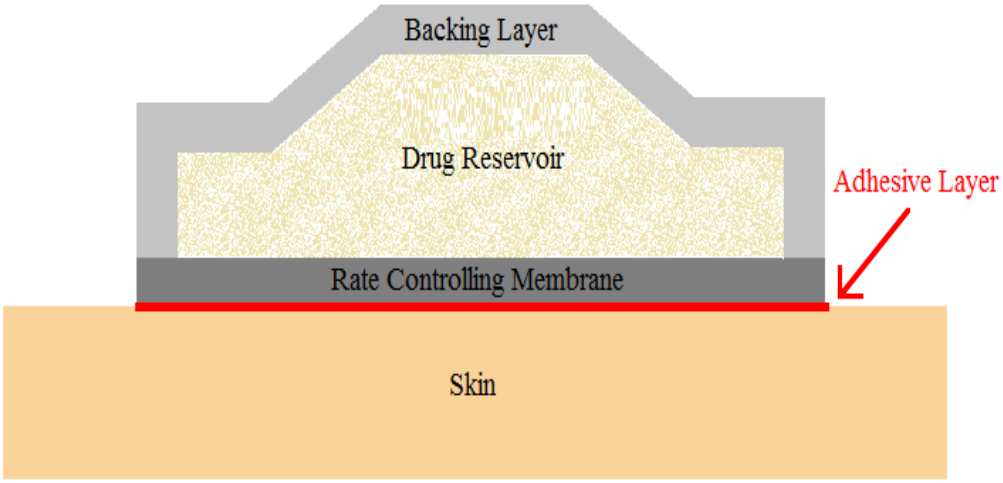

Figure 5.1: Diagrammatic representation of the cross section of the transdermal Scop patch

Each transdermal Scop patch contains a reservoir of 1.5 mg of Scop and is programmed to deliver *in vivo* approximately 1.0 mg of Scop at an approximately constant rate to the systemic circulation over three days (Renner *et al.*, 2005). Patients are instructed to place the patch behind the ear, as the permeability of the post auricular skin to Scop is 20 fold higher than other areas of the body (Farahmand *et al.*, 2009) for example the skin of the thigh (Streisand *et al.*, 1995).

Upon application, an initial priming dose (140 µg) of Scop is released from the adhesive layer to saturate the skin area and to accelerate the achievement of steady-state blood levels. The subsequent delivery of Scop to the blood is determined by the rate-controlling membrane and is designed to produce stable levels in the blood in a therapeutic range at a constant rate of approximately 5 µg/hr.

The literature indicates Scop serum levels of 50 pg/mL are generally accepted as affording prophylactic protection against motion sickness (Nachum *et al.*, 2001) These levels are attained 6 hours post patch application with a steady state of about 100 pg/mL achieved 8-12 hours after application (Nachum *et al.*, 2001).

5.2.2 Pharmacokinetics of scopolamine

Pharmacokinetics describes how the body affects a specific drug after administration The pharmacokinetics of a drug in the body is divided into several phases including absorption, distribution, metabolism and excretion. This is commonly referred to as the ADME scheme of a drug. Absorption is the process of a substance entering the blood circulation. Distribution is the dispersion or dissemination of substances throughout the fluids and tissues of the body. Metabolism is the irreversible transformation of parent compounds into daughter metabolites. Excretion (or elimination) is the elimination of the substances from the body. Pharmacokinetic properties of drugs may be affected by factors such as the site of administration and the dose of administered drug which may affect the absorption rate. The general ADME scheme for transdermally formulated Scop is as follows:

Absorption: As Scop is lipophilic, it is well absorbed percutaneously. Following application to the skin behind the ear, circulating blood levels are detected within four hours, with peak levels being obtained, on average, within 24 hours. The average plasma concentration produced is 87 pg/mL for free Scop and 354 pg/mL for total Scop (free and conjugates) (Novartis, 2006).

Distribution: The distribution of Scop is not well characterised. It crosses the placenta and the blood brain barrier and may be reversibly bound to plasma proteins.

Metabolism: Although not well characterised, Scop is extensively metabolised and conjugated with less than 5% of the total dose appearing unchanged in the urine. As Scop is an ester alkaloid, it is possible that small amounts are hydrolysed in the serum giving rise to tropic acid as well as scopine or scopoline. Scop glucuronides and sulfates are reported to be the main Scop metabolites in man (Stewart, 1979).

Elimination: The exact elimination pattern of Scop has not been determined. Following patch removal, plasma levels decline in a log linear fashion with an observed half life of 9.5 hours. Less than 10% of the total dose is excreted in the urine as parent and metabolites over 108 hours (Novartis, 2006).

5.2.3 Transdermal scopolamine patch application

The following instructions are given pertaining to patient preparation for Scop patch application. After washing the area behind the ear, it is wiped with a clean, dry tissue to ensure dryness. The transdermal Scop patch is removed from its protective pouch. To expose the adhesive surface of the patch, the clear plastic protective strip is peeled off and discarded. The adhesive side is placed against the skin. The patch is pressed firmly for 10-20 seconds, ensuring the edges adhered to the skin. After patch application behind the ear, hands are washed thoroughly in order to avoid contact with the exposed adhesive layer and prevent contamination of fingers with Scop. At the end of three days the patch is removed and thrown away.

5.2.4 Scopolamine pharmacokinetic studies

The literature contains limited information on transdermal Scop patch pharmacokinetics. Most studies on the transdermal Scop patch combine it with the oral dose to generate a more immediate effect. Also, the pharmacokinetic parameters and therapeutic efficacy of a half transdermal Scop patch have never been reported, not to mention a pharmacokinetic study which provides some initial correlates with side effects.

Nachum *et al.,* 2001, conducted a study entitled 'Scopolamine bioavailability in combined oral and transdermal delivery'. Their study evaluated the effects of combined transdermal and oral Scop administration on the pharmacokinetics of Scop in plasma, on cognitive and psychomotor performance, and on the incidence of adverse effects. Plasma levels of Scop high enough to prevent seasickness were obtained 0.5 to 2.5 hr after the oral administration of Scop as a supplement to the transdermal Scop patch. The combination was efficient in achieving therapeutic levels much faster than the patch alone, and in maintaining them for a prolonged period of time. These results resulted in participants using the combination of a transdermal Scop patch and a 0.3-mg tablet of Scop to prevent seasickness.

Nachum *et al*., 2006, also conducted a study entitled: Transdermal scopolamine for prevention of motion sickness: clinical pharmacokinetics and

therapeutic applications. The transdermal patch attained Scop concentrations of 50 pg/mL after 6 hours and a steady state of about 100 pg/mL was achieved 8-12 hours after application. Dry mouth occurred in about 50-60% of test subjects and drowsiness in up to 20%. A high % of allergic contact dermatitis was observed, affecting 10% of test subjects. No conventional trade name could be found in the paper which suggests that it was a developmental patch rather than one of the existing formulations.

5.2.4.1 Pharmacokinetics of non-patch scopolamine formulations

Putcha *et al.,* 1989, conducted a trial on the pharmacokinetics and bioavailability of Scop in six healthy male subjects receiving 0.4 mg of the drug by either oral or intravenous administration. Plasma and urine samples were analysed using a radioreceptor binding assay. Mean peak plasma concentrations were 2909.8 ± 240.9 pg/mL following IV administration and 528.6 ± 109.4 pg/mL following oral administration. Elimination half-life of the drug was 4.5 ± 1.7 hr. They concluded the variability in absorption and poor bioavailability of oral Scop achieved indicated that this route of administration may not be reliable or effective.

Putcha *et al.,* 1996, also investigated the bioavailability of intranasal Scop. The bioavailability of Scop in three dosage forms was compared in 12 healthy non-smoking male volunteers. Subjects received 0.4 mg doses of Scop bromide in intravenous (IV), intranasal (IN), or oral (PO) dosage forms on three occasions, with at least 2 weeks separating the doses. Plasma Scop concentrations were determined using a combined reverse-phase liquid chromatography radioreceptor binding assay. They concluded that IN Scop is an attractive alternative to IV administration, as indicated by rapid, reliable absorption, adequate bioavailability, and relatively low cost. They further concluded that measuring salivary flow rate could constitute a satisfactory, non-invasive means of evaluating the pharmacodynamics and absorption of anticholinergic drugs such as Scop.

5.3 Experimental

The LC-MS method developed and validated in Chapter 4 was ideal for studying the pharmacokinetics of the transdermal Scop patch in our study, firstly because of the complex nature of the blood matrix and secondly the need for high sensitivity to observe low concentration and long time point data. Standard curves were used for quantitation of the drug in serum samples. The samples represented different time points at which bloods were taken post patch application. Blank or t=0 samples taken before administration were important in determining background and ensuring data integrity with such a complex sample matrix.

All samples were analysed using the assay described in Chapter 4 which was validated for the determination of Scop in serum. Chromatographic separation was achieved using an Agilent Zorbax SB-Phenyl (150 × 4.6 mm, 5 µm). A mixture of ammonium formate (pH 8, adjusted with ammonium hydroxide, 10 mM), MeOH (10:90, v/v) was used as mobile phase, at a flow rate of 0.6 mL/min. The complete chromatographic run time of each sample was 10 min. Peaks were quantified using Agilent Masshunter Software.

The mass spectrometer was operated using an ESI source in the positive ion detection mode. The ionisation temperature was 350 °C, gas flow rate was 11 L/min and nebuliser pressure was 50 psi. Analysis was performed in MRM mode with the transition *m*/*z* 304.1 → *m*/*z* 138.1 for Scop; with a dwell time of 200 ms. Transitions and their optimal detector settings are listed in Table 5.2.

Compound	Precursor ion	Optimum fragmentor Voltage	Product ion	Optimum collision energy
Scop	304.1	120	110.1 121.1 138.1* 156.1	30 20 20 15

Table 5.2: Optimal fragmentor voltages and collision energy settings for Scop

* Quantifier ion

The quantifier ion is indicated in the table though all transitions were monitored with the qualifier ions adding specificity. Quantification was based on the integrated peak area as determined by the Masshunter quantification analysis software which quantitates the peak areas of the MRM transitions of each analyte.

5.3.1 Preparation of patient samples

Blood samples were obtained by informed consent in Dublin City University. Following application of a standard transdermal patch containing 1.5 mg of the drug, blood was drawn over 72 hours at different time points. The blood samples were centrifuged in a chilled centrifuge and the serum kept frozen at -80 ºC until analysis. A 500 µL aliquot of each patient sample was diluted 1:1 with a 5 mM ammonium formate buffer, pH 8 and loaded onto the SPE well. The sample was washed with 20% MeOH in water, eluted with 0.5 mL 75% MeOH, evaporated to dryness and reconstituted with 50 µL mobile phase. Samples were taken and prepared in duplicate.

5.3.2 Study Design

Following approval from the Dublin City University Research Ethics Committee, (See Appendix A) a study was carried out on five healthy volunteers, three males and two females aged between 25 and 28 years. As the patch is recommended for application over three days, it was decided to conduct blood sampling over this time in order to generate the pharmacokinetic profiles. As peak blood levels are obtained 6-9 hours post application, more frequent sampling was required around this time.

On week one of the study, the transdermal Scop full patch was applied to two of the patients and half patch to the remaining three patients and worn behind the ears over a period of three days. Blood samples were taken at the following time points after patch application; T=0 (baseline), 3, 6, 7, 8, 9, 24, 48 and 72 hours. An intravenous cannula was placed for the 6, 7, 8 and 9 hour timepoints. All blood samples were allowed to coagulate for 20 mins, and the resultant serum was placed into appropriate vials. Samples were frozen at -80

ºC until analysis by the LC-MS method developed for the project. A three week washout period in-between patch applications was employed, so complete elimination of Scop levels from the patient's blood was ensured.

For the second leg of the study, the same volunteers again applied the alternative transdermal Scop patch for the three days. Blood was again drawn at the same time points as above; time 0, 3, 6, 7, 8, 9, 24, 48, and 72 hrs. All blood samples were again allowed to coagulate for 20 mins, and the resultant serum will placed into appropriate vials. Samples were frozen at -80 ºC until analysis by the LC-MS method described in Chapter 4.

Pharmacokinetic parameters including the maximum serum concentrations of Scop in the blood stream (C_{max}) and the time taken for the drug to reach its highest concentration in the blood stream (T_{max}) were determined by visualisation of the graphed data. Also, an estimation of area under the concentration-time curve (AUC) was achieved using the trapezoidal method. AUC is the most reliable measure of a drug's bioavailability and is directly proportional to the total amount of unchanged drug that reaches systemic circulation.

5.4 Results and discussion

The trial was conducted with 5 healthy volunteers, 3 males and 2 females aged between 25 and 28 years. See Table 5.3 for further details on the 5 patients.

Patient	Sex (M/F)	Age (years)	Height (m)	Weight (kgs)
A	F	25	1.71	62
B	F	27	1.68	63
C	M	28	1.83	88
D	M	25	1.78	75
E	M	25	1.98	77

Table 5.3: Patient summary information

Adverse effects of the transdermal Scop patch were assessed throughout the 72 hour session by individual documentation of symptoms. The serum samples, processed from the blood samples were assayed for Scop concentration. The average Scop concentrations from the trial are plotted for the evaluation of the delivery profile over the 72 hours as outlined below.

5.4.1 Transdermal Scop full vs. half patch results and observations for Patient A

Presented in Figure 5.2 are the pharmacokinetic profiles generated from patient A's serum samples following the full and half transdermal Scop patch applications. Patient A was a 25 year old healthy female, of average height (1.71 m) and weight (62 kgs).

Upon application of the transdermal Scop patch, an initial priming dose of Scop is released from the adhesive layer to saturate the skin area. The subsequent delivery of Scop to the blood is determined by the rate-controlling membrane and is reported to deliver Scop at a constant rate of approximately 5 μg/hr.

Scop was not detected in serum of the patient pre administration of the patch at t=0. On application of the full transderm Scop patch, Scop partitioned from the drug saturated adhesive layer into the skin but absorption of Scop into the systemic circulation was somewhat slow with effective drug concentrations achieved 6-8 hrs post patch application. Maximum serum concentrations (C_{max}) of 167.4 ± 1.4 pg/mL of the alkaloid were reached at a T_{max}, (the time taken for the drug to reach its highest concentration in the blood stream) of about 24 hrs. As the patch releases Scop continuously over a 72 hr period, constant high Scop serum levels were observed, with a slight decline in drug concentration observed over time reaching 89.0 ± 5.2 pg/mL at 72 hrs.

From the literature it is reported circulating blood levels are detected within four hours, with peak levels obtained, on average, within 24 hours. The average plasma concentration produced is 87 pg/mL for free Scop (Novartis, 2006). The results obtained following application of the full transdermal Scop patch are in agreement with regards to timing, however, a concentration of 89.0

± 5.2 pg/mL is only reached after 72 hrs with much higher levels observed between 8 and 48 hrs.

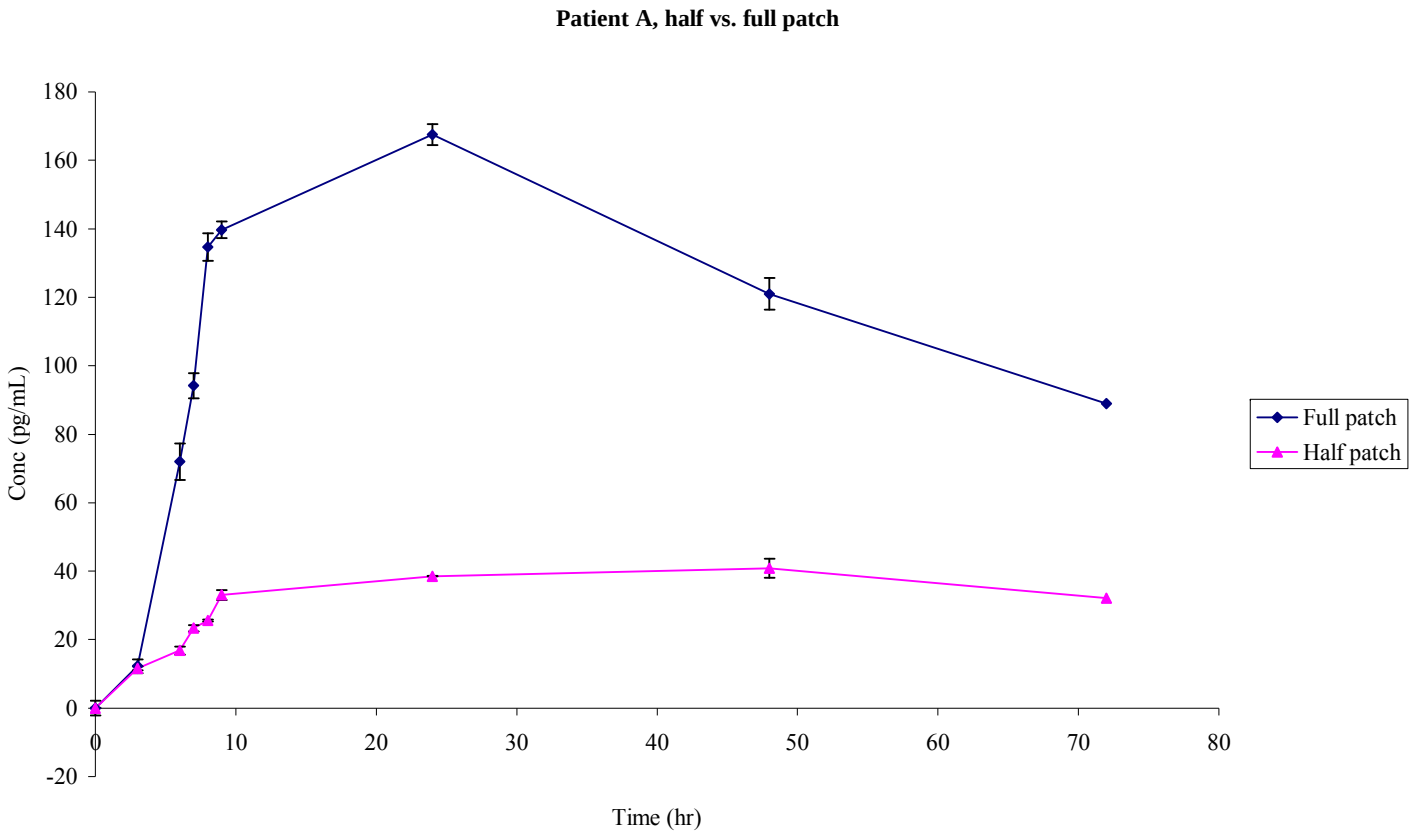

Figure 5.2: Comparison of pharmacokinetic profiles generated from patient A's serum samples following half and full transdermal Scop patch applications

Several methods exist for measuring the area under the concentration-time curve (AUC). For this work, a simple numeric estimation of area was achieved using the trapezoidal method. An advantage of this method is that it requires only a simple extension of a table of experimental data. Other methods involve either greater numeric complexity or fitting of an equation to the observations and then calculating the area by integrating the fitted equation (Tozer *et al.*, 2006).

For Patient A, Figure 5.2 is a plot of the concentration against time post drug administration. If a perpendicular line is drawn from the concentration at 3 hrs (12.2 pg/mL) down to the time-axis, then the area bounded between zero time and 3 hrs is a trapezoid with an area given the product of the average concentration time interval. The average concentration is obtained by adding the concentrations at the beginning and end of the time interval and dividing by 2. Since, in the first interval, the respective concentrations are 0 and 12.2 pg/mL and the time interval is 3, it follows that:

$$AUC_{0\text{-}3} = \frac{(0 + 12.2)}{2} \text{ pg/mL} \quad \text{x} \quad 3 \text{ hr}$$

The total AUC over all times is then simply expressed as the sum of the individual areas. See Table 5.4.

Patient A -Full patch				
Time (hr)	**Conc. (pg/mL)**	**Time interval (hr)**	**Average conc. (pg/mL)**	**AUC (pg-hr/mL)**
0	0.0	0	/	/
3	12.2	3	6.1	18.4
6	72.0	3	42.1	126.4
7	94.2	1	83.1	83.1
8	134.6	1	114.4	114.4
9	139.7	1	137.2	137.2
24	167.5	15	153.6	2303.8
48	121.0	24	144.2	3461.9
72	89.0	24	105.0	2520.2
			Total AUC $_{0\text{-}72}$:	**8765.3**

Table 5.4: AUC determination for Patient A full Scop patch administration

On application of the half transderm Scop patch, the absorption of Scop into the systemic circulation was again somewhat slow with effective drug concentrations achieved 6-8 hrs post patch application. Maximum serum concentrations (C_{max}) of 40.8 ± 0.3 pg/mL were reached at a T_{max}, of about 48 hrs. High Scop serum levels were observed from 9 hrs onward. See Table 5.5 for determination of AUC for Patient A post half Scop patch administration.

Patient A - Half patch				
Time (hr)	**Conc. (pg/mL)**	**Time interval (hr)**	**Average conc. (pg/mL)**	**AUC (pg-hr/mL)**
0	0.0	0	/	/
3	11.6	3	5.8	17.5
6	16.8	3	14.2	42.7
7	23.3	1	20.1	20.1
8	25.6	1	24.5	24.5
9	33.0	1	29.3	29.3
24	38.5	15	35.8	536.4
48	40.8	24	39.7	952.0
72	32.2	24	36.5	875.9
			Total AUC $_{0\text{-}72}$:	**2498.3**

Table 5.5: AUC determination for Patient A half Scop patch administration

See Table 5.6 for a comparison of the pharmacokinetic parameters determined post full and half Scop patch for Patient A.

Scop dose	**C_{max} (pg/mL) ± % RSD**	**T_{max} (hr)**	**AUC $_{0\text{-}72}$ (pg-hr/mL)**
Full patch	167.4 ± 1.4	24	8765.3
Half patch	40.8 ± 0. 3	48	2498.3

.

Table 5.6: Comparison of pharmacokinetic data following half and full transdermal Scop patch applications for Patient A

5.4.1.1 Transdermal Scop full vs. half patch side effects for Patient A

Patient A experienced severe dry mouth and blurred vision (particularly from 12 to 60 hours) following the full transdermal Scop patch, whereby day to day operational performance was seriously hindered. As can be seen in Figure 5.2, correlation of these severe adverse effects to higher than expected Scop levels over the aforementioned time period is observed. Other adverse effects observed by the full transdermal Scop patch on patient A included drowsiness and mild itching/redness on application site. These responses resolved on cessation of the Scop patch.

On application of the half transdermal Scop patch, to the same subject, the only adverse effect experienced by patient A was mild dry mouth from 9 to 72 hrs. This again correlates to max Scop levels observed in the serum samples over the time range mentioned, see Figure 5.2.

5.4.2 Transdermal Scop full vs. half patch results and observations for Patient B

Presented in Figure 5.3 are the pharmacokinetic profiles generated from patient B's serum samples following the full and half transdermal Scop patch applications. Patient B was a 27 year old healthy female was average in height (1.68 m) and weight (63 kgs).

Scop was not detected in serum of the patient pre administration of the patch at t=0. On application of the full transderm Scop patch, absorption of Scop into the systemic circulation was again somewhat slow with effective drug concentrations achieved 6-8 hrs post patch administration. A maximum serum concentration (C_{max}) of 139.5 ± 0.7 pg/mL was reached at a T_{max} of about 9 hrs. Post C_{max}, constant high Scop serum levels ranging from that at 24 hrs (94.5 ± 6.4 pg/mL) to a slight decline at 72 hrs (75.2 ± 3.5 pg/mL) were observed. The results obtained following application of the full transdermal Scop patch for Patient B are in agreement with levels reported in the literature. See Table 5.7 for determination of AUC for Patient B post full Scop patch administration.

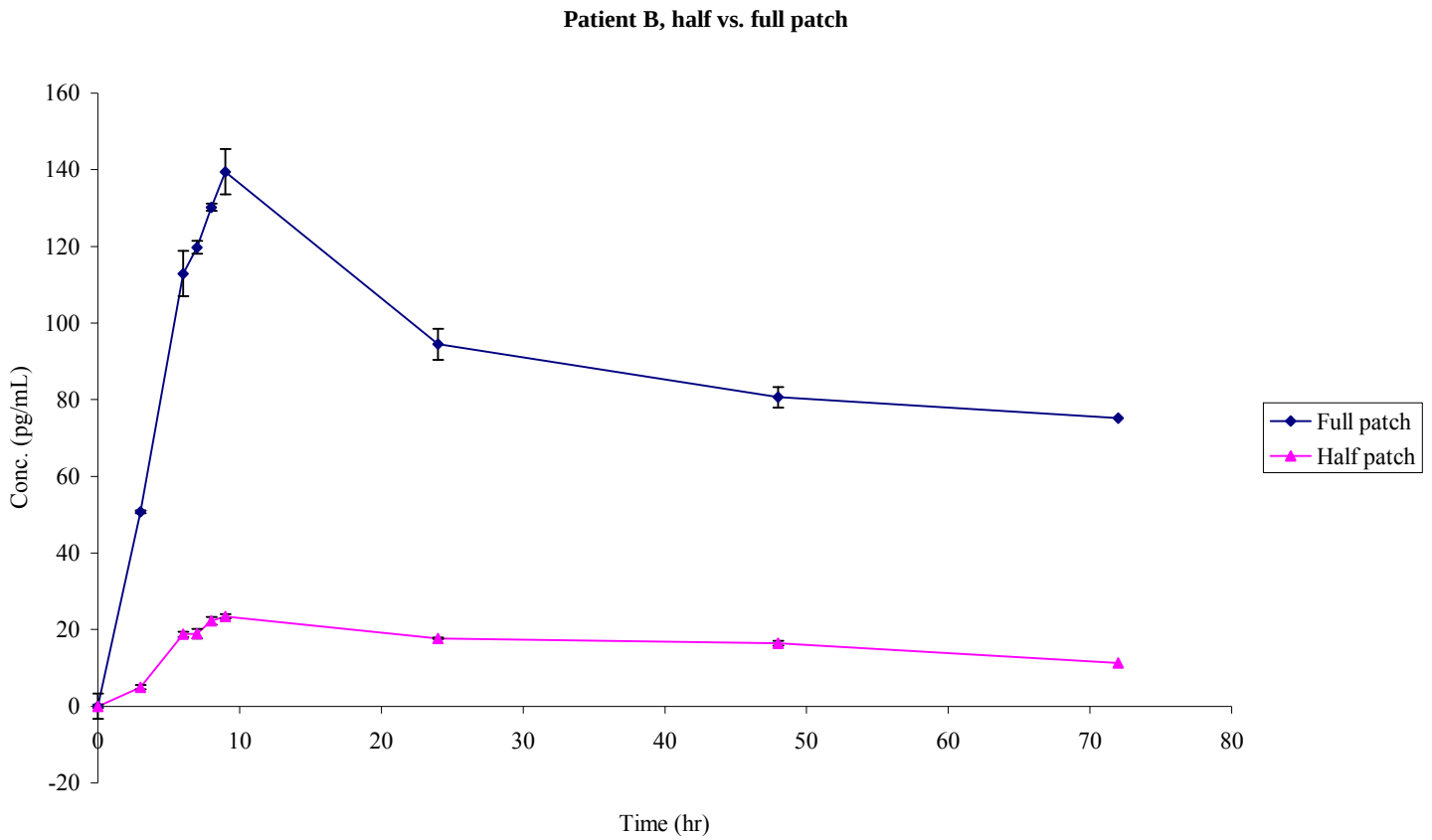

Figure 5.3: Comparison of pharmacokinetic profiles generated from patient B's serum samples following half and full transdermal Scop patch applications

Patient B -Full patch				
Time (hr)	**Conc. (pg/mL)**	**Time interval (hr)**	**Average conc. (pg/mL)**	**AUC (pg-hr/mL)**
0	0.0	0	/	/
3	50.7	3	25.3	76.0
6	112.9	3	81.8	245.4
7	119.7	1	116.3	116.3
8	130.2	1	125.0	125.0
9	139.5	1	134.8	134.8
24	94.5	15	117.0	1754.7
48	80.6	24	87.6	2101.4
72	75.2	24	77.9	1869.6
			Total AUC $_{0\text{-}72}$:	**6423.2**

Table 5.7: AUC determination for Patient B full Scop patch administration

On application of the half transderm Scop patch, the absorption of Scop into the systemic circulation was again somewhat slow with effective drug concentrations achieved 6-8 hrs post patch application. Maximum serum concentrations (C_{max}) of 23.46 ± 4.4 pg/mL were reached at a T_{max}, of about 9 hrs. Scop serum levels showed a slight decline in concentration from 9 hrs onward reaching 11.36 ± 5.9 pg/mL come 72 hrs. See Table 5.8 for determination of AUC for Patient B post half Scop patch administration.

Patient B - Half patch				
Time (hr)	**Conc. (pg/mL)**	**Time interval (hr)**	**Average conc. (pg/mL)**	**AUC (pg-hr/mL)**
0	0.0	0	/	/
3	5.0	3	2.5	7.5
6	18.7	3	11.9	35.6
7	19.0	1	18.8	18.8
8	22.3	1	20.6	20.6
9	23.5	1	22.9	22.9
24	17.7	15	20.6	308.8
48	16.4	24	17.1	409.8
72	11.4	24	13.9	333.6
			Total AUC_{0-72}:	**1157.7**

Table 5.8: AUC determination for Patient B half Scop patch administration

See Table 5.9 for a comparison of the pharmacokinetic parameters determined post full and half Scop patch for Patient B.

Scop dose	**C_{max} (pg/mL) ± % RSD**	**T_{max} (hr)**	**AUC_{0-72} (pg-hr/mL)**
Full patch	139.5 ± 0.97	9	6423.2
Half patch	23.5 ± 4.4	9	1157.7

Table 5.9: Comparison of pharmacokinetic data following half and full transdermal Scop patch applications for Patient B

5.4.2.1 Transdermal Scop full vs. half patch side effects for Patient B

Patient B experienced moderate dry mouth and slight blurred vision following the full transdermal Scop patch, with day-to-day operational performance fortunately unhindered. The most notable adverse effect for patient B was transient impairment of ocular accommodation, the result of circulating Scop blood levels or possibly finger-to-eye Scop contamination during application of the patch. This impairment of ocular accommodation has been reported before and it was thought to be due to finger to eye contamination (Nachum *et al.,* 2006). Other effects observed by the full transdermal Scop patch on patient B included some drowsiness. These responses became normal on cessation of the Scop patch.

On application of the half transdermal Scop patch, no adverse side effects were experienced by patient B This correlates to the low Scop serum concentrations observes post half patch application.

5.4.3 Transdermal Scop full vs. half patch results and observations for Patient C

Presented in Figure 5.4 are the pharmacokinetic profiles generated from patient C's serum samples following the full and half transdermal Scop patch applications. Patient C was a 28 year old healthy male, of height 1.83 m and weight 88 kgs.

Scop was not detected in serum of the patient pre administration of the patch at t=0. On application of the full transderm Scop patch, absorption of Scop into the systemic circulation was quite fast with effective drug concentrations achieved 3-6 hrs post patch administration. A maximum serum concentration (C_{max}) of 252.3 ± 1.5 pg/mL of the alkaloid was reached at a T_{max} of just 7 hrs. This is extremely high comparatively speaking with the other patients. Post C_{max}, a rapid decline of the drug serum level was observed reaching just 46.0 ± 0.7 pg/mL at 72 hrs and this again was unlike the response from the other patients. The results obtained following application of the full transdermal Scop patch for Patient C are not in agreement with that published in the literature. An initial extremely high concentration of Scop followed by a

rapid decrease of the drug concentration was observed and is not typical behaviour of the transdermal Scop patch. See Table 5.10 for determination of AUC for Patient C post full Scop patch administration.

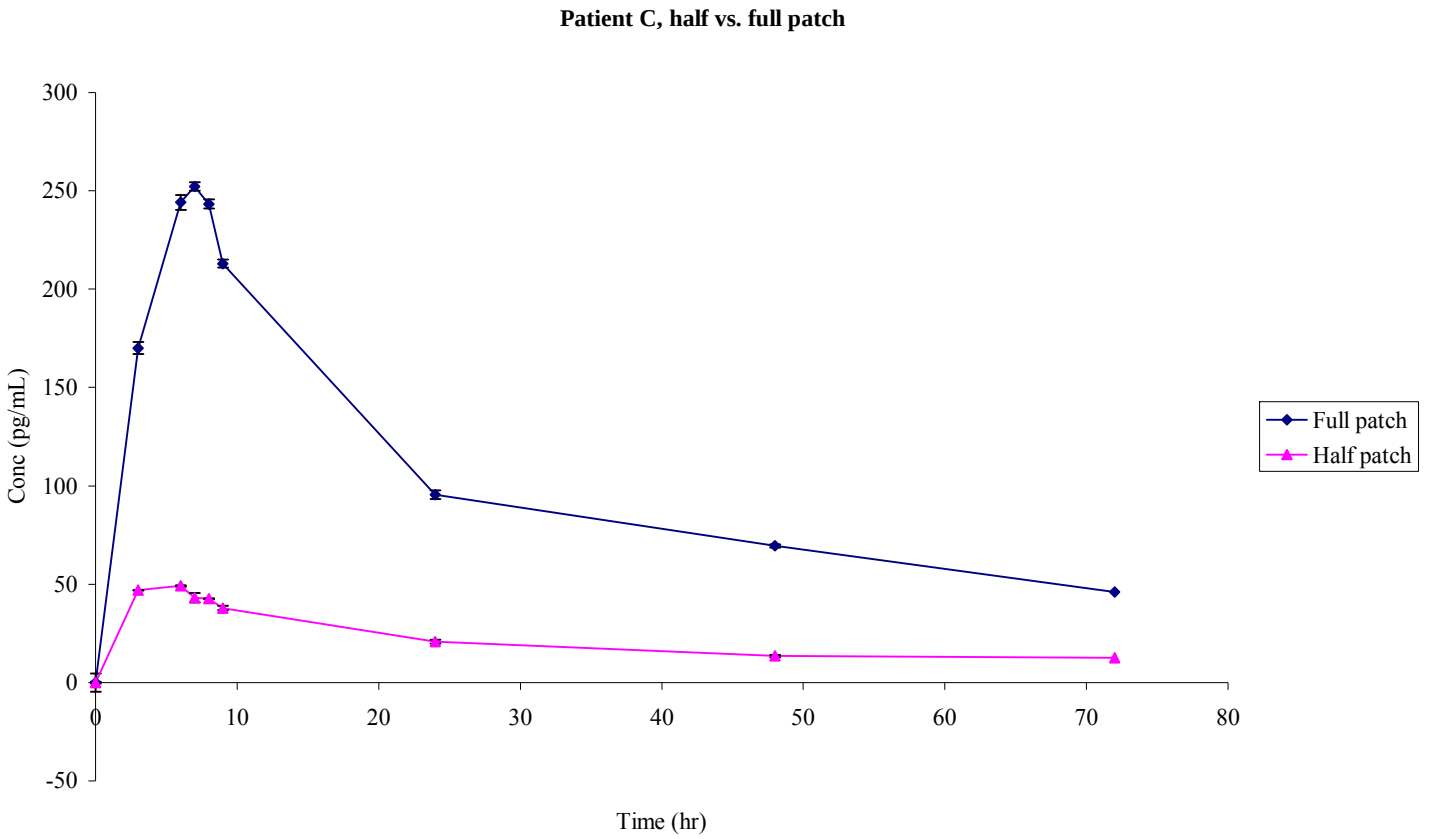

Figure 5.4: Comparison of pharmacokinetic profiles generated from patient C's serum samples following half and full transdermal Scop patch applications

Patient C -Full patch				
Time (hr)	**Conc. (pg/mL)**	**Time interval (hr)**	**Average conc. (pg/mL)**	**AUC (pg-hr/mL)**
0	0.0	0	/	/
3	170.1	3	85.1	255.2
6	244.2	3	207.1	621.4
7	252.2	1	248.2	248.2
8	243.3	1	247.8	247.8
9	213.0	1	228.2	228.2
24	95.6	15	154.3	2314.7
48	69.5	24	82.5	1981.0
72	46.0	24	57.7	1385.4
			Total AUC $_{0\text{-}72}$:	**7281.9**

Table 5.10: AUC determination for Patient C full Scop patch administration

On application of the half transderm Scop patch, the absorption of Scop into the systemic circulation was again somewhat fast with effective drug concentrations achieved 3 hrs post patch application. Maximum serum concentrations (C_{max}) of 49.10 ± 0.2 pg/mL were reached at a T_{max} of just 6 hrs. Scop serum levels showed a again a steady decline concentration from 6 hrs onward reaching 12.6 ± 4.8 pg/mL by 72 hrs. See Table 5.11 for determination of AUC for Patient C post half Scop patch administration.

Patient C - Half patch				
Time (hr)	**Conc. (pg/mL)**	**Time interval (hr)**	**Average conc. (pg/mL)**	**Area (pg-hr/mL)**
0	0.0	0	/	/
3	46.9	3	23.5	70.4
6	49.1	3	48.0	144.0
7	43.2	1	46.1	46.1
8	42.7	1	42.9	42.9
9	37.9	1	40.3	40.3
24	20.8	15	29.4	440.6
48	13.4	24	17.1	411.1
72	12.6	24	13.0	312.9
			Total AUC_{0-72}:	**1508.3**

Table 5.11: AUC determination for Patient C half Scop patch administration

See Table 5.12 for a comparison of the pharmacokinetic parameters determined post full and half Scop patch for Patient C.

Scop dose	**C_{max} (pg/mL) ± % RSD**	**T_{max} (hr)**	**AUC_{0-72} (pg-hr/mL)**
Full patch	252.25 ± 1.5	7	7281.9
Half patch	49.10 ± 0.2	6	1508.3

Table 5.12: Comparison of pharmacokinetic data following half and full transdermal Scop patch applications for Patient C

5.4.3.1 Transdermal Scop full vs. half patch side effects for Patient C

Patient C experienced moderate to severe dry mouth, dizziness, mild nausea and drowsiness following the full transdermal Scop patch, with day to day operational performance hindered. As with patient B, transient impairment of ocular accommodation was again noted. These responses became normal on cessation of the Scop patch. In comparison to all other patients, the most side effects were noted with patient C. This again correlates well to the unusually high concentrations of Scop determined in the serum samples analysed post patch application.

On application of the half transdermal Scop patch, no adverse side effects were experienced by patient C. This correlates to the low Scop serum concentrations observes post half patch application.

5.4.4 Transdermal Scop full vs. half patch results and observations for Patient D

Presented in Figure 5.5 are the pharmacokinetic profiles generated from patient D's serum samples following the full and half transdermal Scop patch applications. Patient D was a 25 year old healthy male of average height (1.78 m) and weight (75 kgs).

Scop was not detected in serum of the patient pre administration of the patch at t=0. On application of the full transderm Scop patch, absorption of Scop into the systemic circulation was somewhat slow with effective drug concentrations achieved 6-8 hrs post patch administration. A maximum serum concentration (C_{max}) of 96.7 ± 2.6 pg/mL of the alkaloid was reached at a T_{max} of 9 hrs. Post C_{max}, constant high Scop serum levels ranging from that at 24 hrs (73.8 ± 0.8 pg/mL) to a slight decline at 72 hrs (61.6 ± 2.9 pg/mL) were observed. The results obtained following application of the full transdermal Scop patch for Patient D are in agreement with published results. See Table 5.13 for determination of AUC for Patient D post full Scop patch administration.

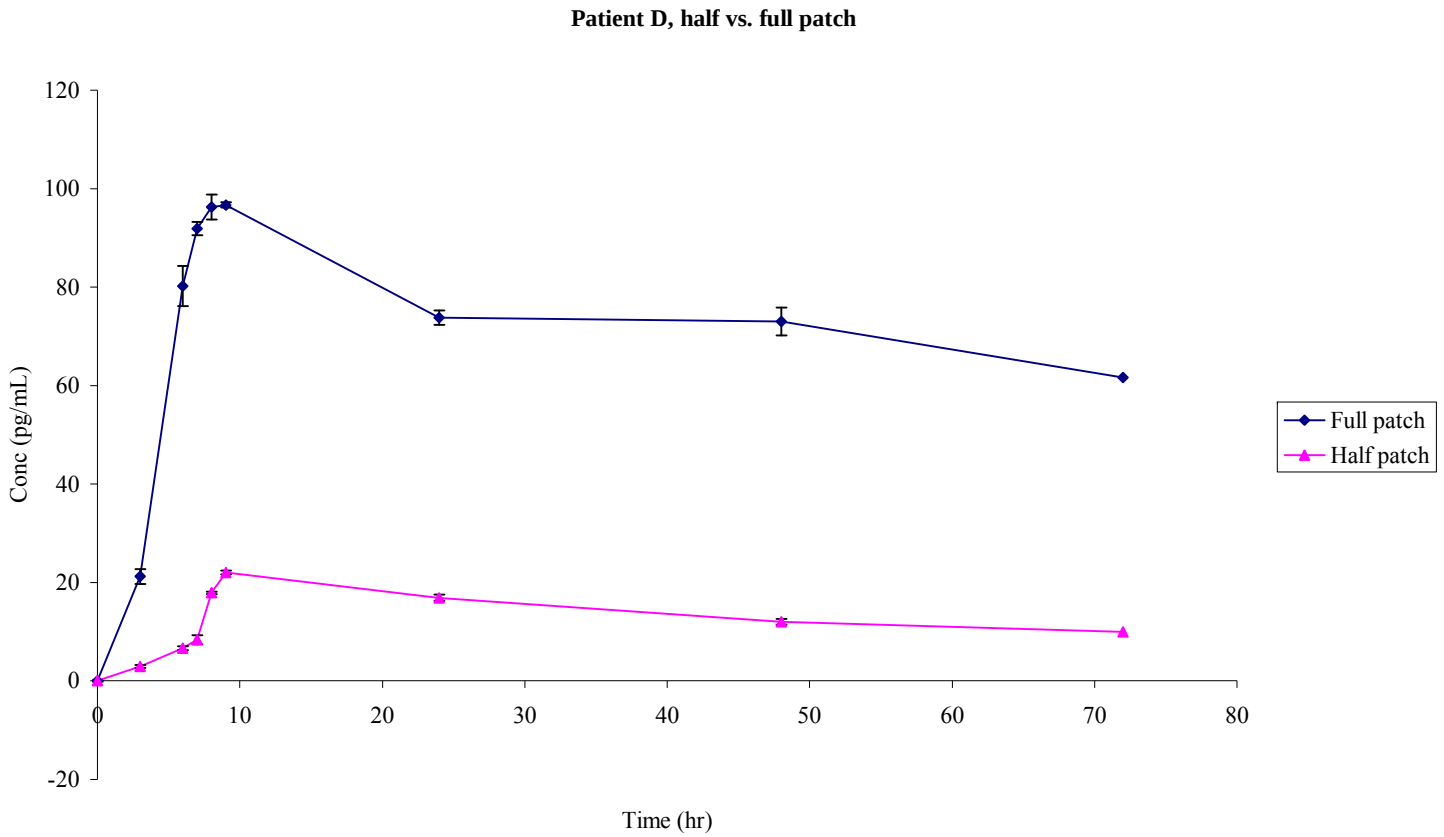

Figure 5.5: Comparison of pharmacokinetic profiles generated from patient D's serum samples following half and full transdermal Scop patch applications

Patient D -Full patch				
Time (hr)	**Conc. (pg/mL)**	**Time interval (hr)**	**Average conc. (pg/mL)**	**AUC (pg-hr/mL)**
0	0.0	0	/	/
3	21.2	3	10.6	31.9
6	80.2	3	50.7	152.2
7	91.9	1	86.0	86.0
8	96.3	1	94.1	94.1
9	96.7	1	96.5	96.5
24	73.8	15	85.2	1278.5
48	73.0	24	73.4	1761.6
72	61.6	24	67.3	1615.4
			Total AUC_{0-72}:	**5116.2**

Table 5.13: AUC determination for Patient D half Scop patch administration

On application of the half transderm Scop patch, the absorption of Scop into the systemic circulation was again somewhat slow with effective drug concentrations achieved 8 hrs post patch application. Maximum serum concentrations (C_{max}) of 22.0 ± 1.2 pg/mL were reached at a T_{max} of 9 hrs. Scop serum levels showed a again a steady decline concentration from 9 hrs onward reaching 10.0 ± 5.6 pg/mL at 72 hrs. See Table 5.14 for determination of AUC for Patient D post half Scop patch administration.

Patient D - Half patch				
Time (hr)	**Conc. (pg/mL)**	**Time interval (hr)**	**Average conc. (pg/mL)**	**AUC (pg-hr/mL)**
0	0.0	0	/	/
3	3.0	3	1.5	4.5
6	6.7	3	4.8	14.5
7	8.4	1	7.5	7.5
8	17.9	1	13.1	13.1
9	22.0	1	19.9	19.9
24	16.9	15	19.4	291.6
48	12.0	24	14.4	346.7
72	10.0	24	11.0	264.0
			Total AUC_{0-72}:	**961.9**

Table 5.14: AUC determination for Patient D half Scop patch administration

See Table 5.15 for a comparison of the pharmacokinetic parameters determined post full and half Scop patch for Patient D.

Scop dose	**C_{max} (pg/mL) ± % RSD**	**T_{max} (hr)**	**AUC_{0-72} (pg-hr/mL)**
Full patch	96.7 ± 2.6	9	5116.2
Half patch	22.0 ± 1.4	9	961.9

.

Table 5.15: Comparison of pharmacokinetic data following half and full transdermal Scop patch applications for Patient D

5.4.4.1 Transdermal Scop full vs. half patch side effects for Patient D

Patient D experienced moderate dry mouth and slight blurred vision following the full transdermal Scop patch, whereby day to day operational performance was unhindered. No other notable side effects were noted. These responses became normal on cessation of the Scop patch. Scop serum concentrations determined for Patient D were relatively low and so the side effects are fitting with the results.

On application of the half transdermal Scop patch, no adverse side effects were experienced by patient D. This correlates to the low Scop serum concentrations observes post half patch application.

5.4.5 Transdermal Scop full patch results and observations for Patient E

Presented in Figure 5.6 is the pharmacokinetic profile generated from patient E's serum samples following the full transdermal Scop patch application. Patient E was a 25 year old healthy male of height 1.98 m and weight 77 kgs .

Scop was not detected in serum of the patient pre administration of the patch at t=0. On application of the full transderm Scop patch, absorption of Scop into the systemic circulation was slow with effective drug concentrations achieved only after 24 hrs post patch administration. A maximum serum concentration (C_{max}) of 56.5 ± 0.1 pg/mL of the alkaloid was reached after this time. Post C_{max}, Scop serum levels ranging from that at 24 hrs to a slight decline come 72 hrs (54.9 ± 0.9 pg/mL) were observed. Unlike the other patients, no Scop was detected in the 3 hr time point post patch application for patient E.

The results obtained following application of the full transdermal Scop patch are for Patient E are much lower in concentration than those published with a C_{max} of only 56.5 ± 0.1 pg/mL obtained after 24 hours and maintained thereafter. See Table 5.16 for determination of AUC for Patient E post full Scop patch administration.

Due to unforeseen circumstances unrelated to the study, Patient E was unable to continue with the trial and so results for only the full transderm Scop patch were obtained.

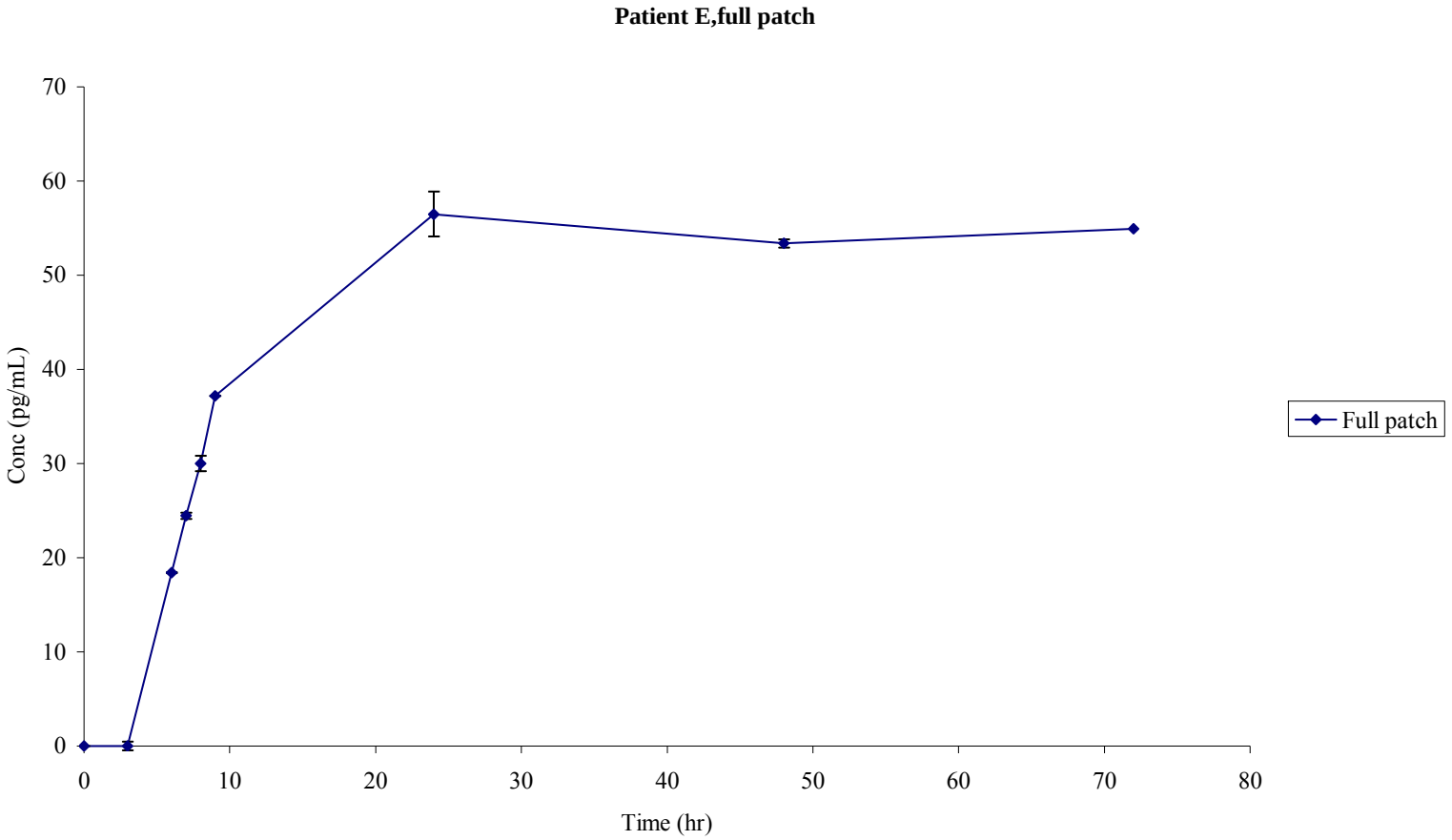

Figure 5.6: Pharmacokinetic profile generated from patient E's serum samples following full transdermal Scop patch application

Patient E -Full patch				
Time (hr)	**Conc. (pg/mL)**	**Time interval (hr)**	**Average conc. (pg/mL)**	**Area (pg-hr/mL)**
0	0.0	0	/	/
3	0.0	3	0.0	0.0
6	18.4	3	9.2	27.6
7	24.4	1	21.4	21.4
8	30.0	1	27.2	27.2
9	37.2	1	33.6	33.6
24	56.5	15	46.8	702.5
48	53.4	24	54.9	1318.2
72	54.9	24	54.2	1299.6
			Total AUC $_{0\text{-}72}$:	**3430.1**

Table 16: AUC determination for Patient E full Scop patch administration

See Table 5.17 for a summary of the pharmacokinetic parameters determined post full transdermal Scop patch for Patient E.

Scop dose	**C_{max} (pg/mL) ± % RSD**	**T_{max} (hr)**	**AUC $_{0\text{-}72}$ (pg-hr/mL)**
Full patch	56.5 ± 0.1	24	3430.1

.

Table 5.17: Pharmacokinetic data following full transdermal Scop patch application for Patient E

5.4.5.1 Transdermal Scop full patch side effects for Patient E

It is interesting to note Patient E experienced zero side effects following the full transdermal Scop patch application. This correlates well to the Scop serum concentrations calculated which were lower than in the other patients. Patient E was considerably taller (at 1.98 m) than all other patients participating in the trial, which could have impacted on the low concentrations obtained due to dilution of the circulating blood concentration of Scop.

5.4.6 Comparative analysis of all patient's results following full and half transdermal Scop patch applications

Following the full transdermal Scop patch application, serum concentrations of the drug determined indicate large interindividual variations between the patients. Scop levels in all five patients, up to 72 hrs post drug administration, are presented in Figure 5.7. Considerable differences were found between the five patients in particular the first 24 hrs post patch application. Thereafter, serum levels of Scop did not differ greatly between the patients.

Following the half transdermal Scop patch application, serum concentrations of the drug determined also indicated some interindividual variations between the patients. Scop levels in all four patients, up to 72 hrs post drug administration, are presented in Figure 5.8. Similar profiles to that of the corresponding full patch were observed, with again, significant differences found in the first 24 hrs post patch application. Thereafter, serum levels of Scop did not differ significantly between the patients.

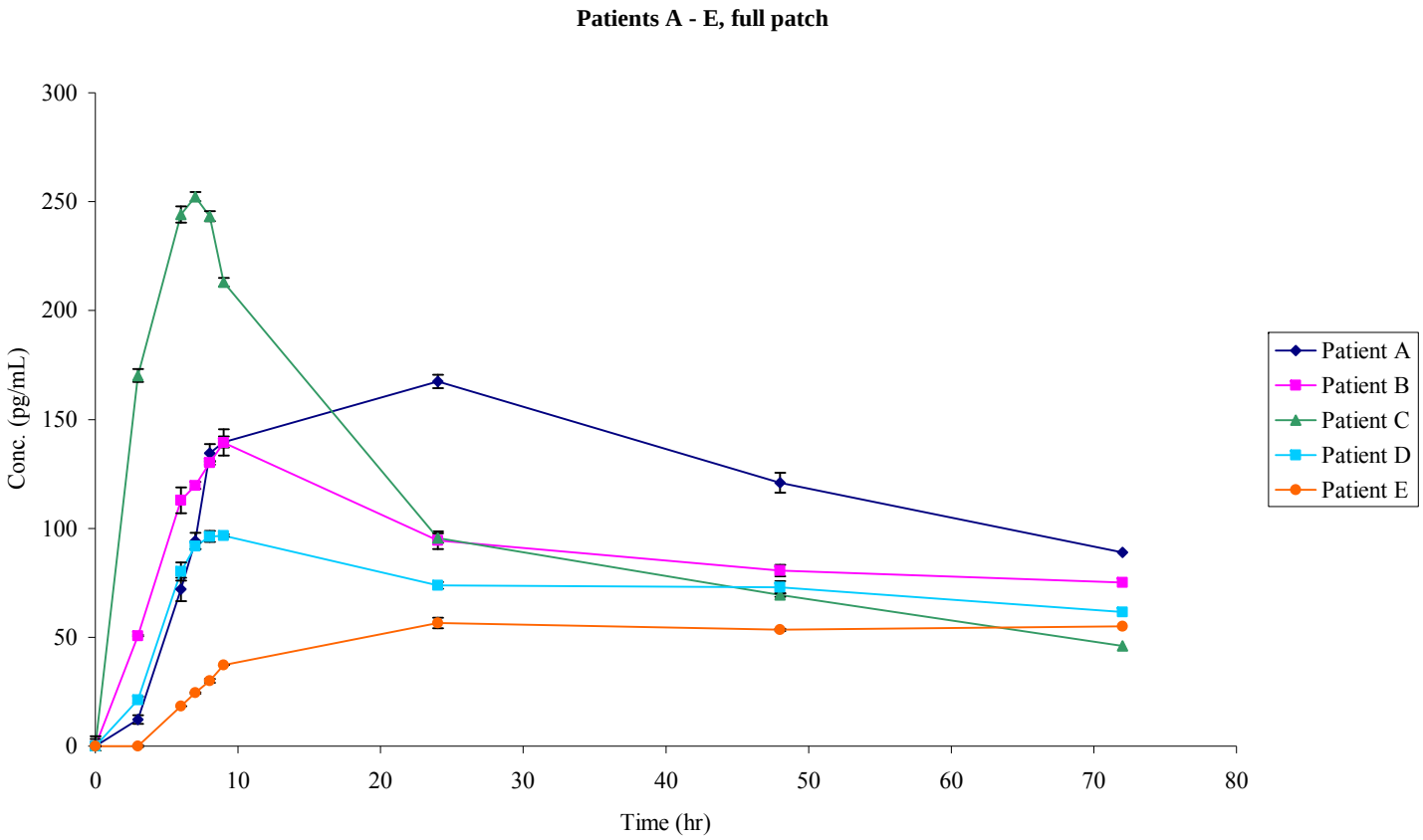

Figure 5.7: Comparison of pharmacokinetic profiles generated from Patient's A-E serum samples following full transdermal Scop patch application

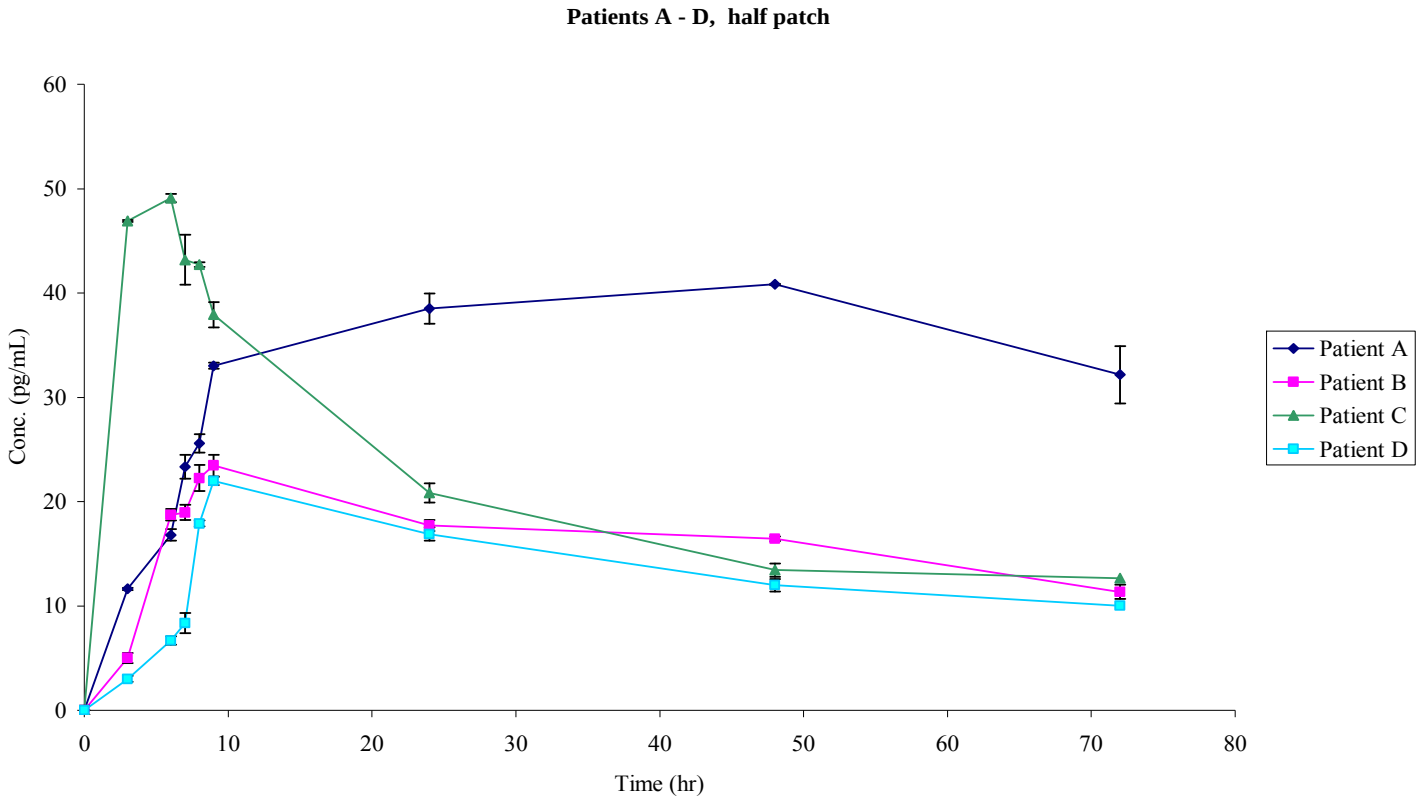

Figure 5.8: Comparison of pharmacokinetic profiles generated from Patient's A-D serum samples following half transdermal Scop patch application

As every person's skin absorbs the drug at a different rate, interindividual variations in the results obtained were expected. Besides the genetic aspects of variations between individuals in absorption and metabolism of drugs, ethnicity, age, gender, body weight, compliance, general health and skin condition are among the main subject parameters affecting the serum concentration profile of transdermally delivered drugs.

Although there are significant differences in the general appearance of skin and the distribution of hair follicles between males and females, there is no convincing evidence to suggest major differences in barrier function. In general, bioavailability and protein binding do not appear to be significantly affected by gender {{488 Ethel,Tur 1997}}.

Variation in results obtained affecting the serum concentration profile of the Scop transderm patch could be attributed to patient body weight and height, differences in transdermal penetration, absorption and metabolism of Scop or variation in serum assay/sampling. Table 5.18 and 5.19 presents a summary of the pharmacokinetic parameters determined for all patients.

Patient	**Full patch**		**Half patch**	
	C_{max} (pg/mL) ± % RSD	**T_{max} (hr)**	**C_{max} (pg/mL) ± % RSD**	**T_{max} (hr)**
A	167.5 ± 1.5	24	40.8 ± 0.2	48
B	139.5 ± 0.7	9	23.5 ± 4.4	9
C	252.3 ± 1.5	7	49.1 ± 0.2	6
D	96.7 ± 2.6	9	22.0 ± 1.2	9
E	56.5 ± 0.0	24	n/a	n/a

Table 5.18: Summary of Patient's C_{max} and T_{max} following both full and half transdermal Scop patch applications

Patient	Full patch AUC $_{0\text{-}72}$ (pg-hr/mL)	Half patch AUC $_{0\text{-}72}$ (pg-hr/mL)	% AUC present in half patch in comparison to full patch
A	8765.3	2498.3	28.5
B	6423.2	1157.7	18.0
C	7281.9	1508.3	20.7
D	5116.2	691.9	13.5
E	3430.1	n/a	n/a

Table 5.19: Summary of patients AUC data following both full and half transdermal Scop patch applications

From these results it can be seen the levels of Scop present in the half transdermal Scop patch vary, with the highest % obtained at ~ 28%. This indicates the patch cut in half is not delivering half the drug into the blood stream. The variability of the results also indicates the half patch is inconsistent in its delivery. Figure 5.9 represents a visual comparison of the AUC data following both full and half transdermal Scop patch applications.

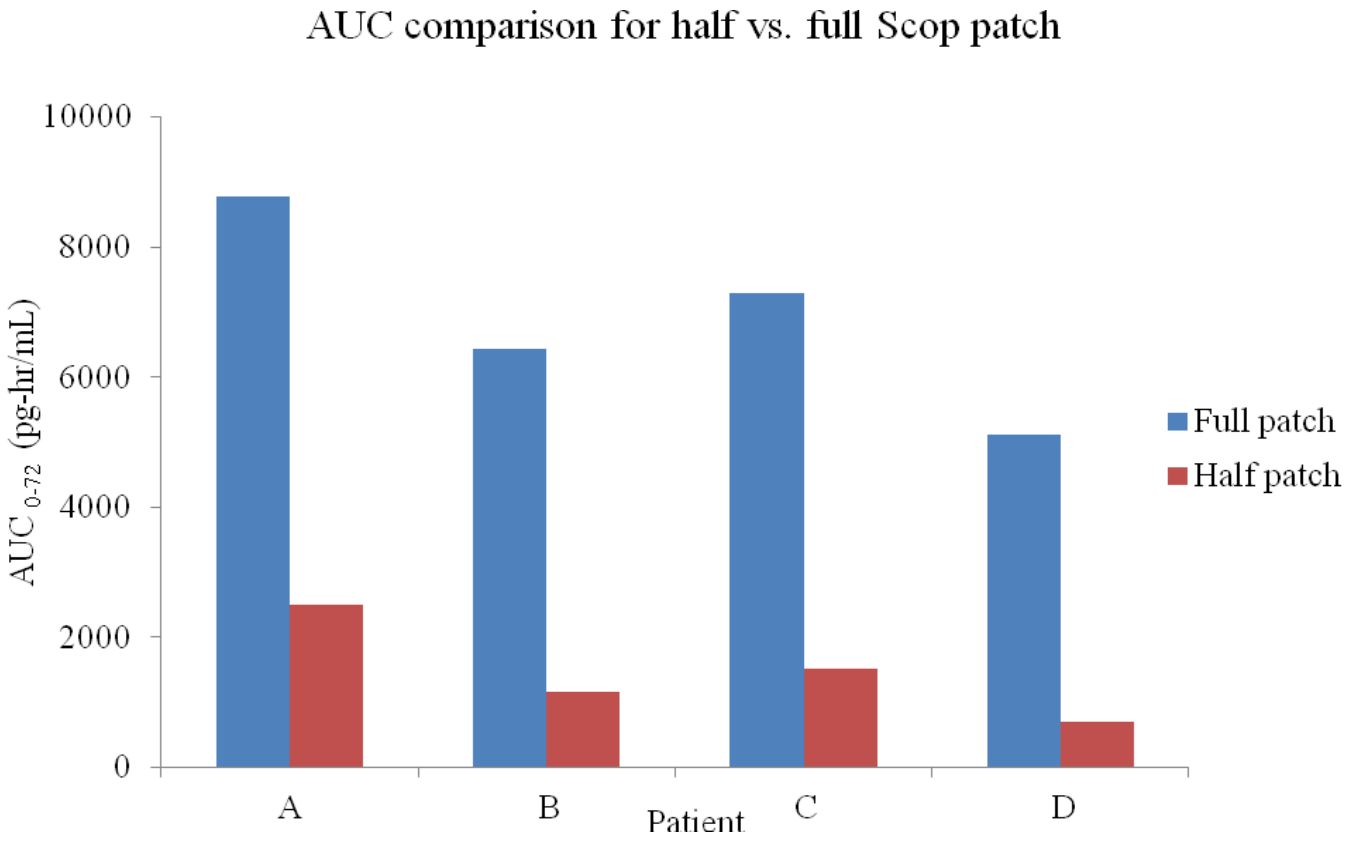

Figure 5.9: Comparison of AUC data following both full and half transdermal Scop patch applications

Subjective complaints of adverse effects differed significantly between the patients particularly following the full transdermal Scop patch application. Fortunately no patient was required to terminate the experiment due to the adverse effects experienced. Patient E, however, terminated the trial having completed the full transdermal Scop patch application due to unforeseen circumstances unrelated to the trial.

The unfavourable effects observed by the full transdermal Scop patch included dry mouth, dilated pupils, blurred vision, drowsiness, dizziness and mild itching/redness on application site. These responses returned to normal on cessation of the Scop patch. Table 5.20 presents a summary of all patient's symptoms and correlating C_{max} following the full transdermal Scop patch application and Table 5.21 following the half transdermal Scop patch application

Patient	C_{max} (pg/mL) ± % RSD	Dry mouth	Dilated pupils	Blurred vision	Dizziness	Nausea	Drowsiness	Itching on application site
A	167.5 ± 1.5	Y	N	Y	N	N	Y	Y
B	139.5 ± 0.6	Y	Y	Y	N	N	N	N
C	252.3 ± 1.5	Y	Y	N	Y	Y	Y	N
D	96.7 ± 2.6	Y	N	Y	N	N	N	Y
E	56.5 ± 0.1	N	N	N	N	N	N	N

Table 5.20: Patient's C_{max} and correlating side effects following full transdermal Scop patch application

Patient	C_{max} (pg/mL) ± % RSD	Dry mouth	Dilated pupils	Blurred vision	Dizziness	Nausea	Drowsiness	Itching on application site
A	40.8 ± 0.25	Y	N	N	N	N	N	N
B	23.5 ± 4.3	N	N	N	N	N	N	N
C	49.10 ± 0.2	N	N	N	N	N	N	N
D	22.0 ± 1.4	Y	N	N	N	N	N	N

Table 5.21: Patient's C_{max} and correlating side effects following half transdermal Scop patch application

Patients A and C were more adversely effected following the full transdermal Scop patch in comparison to the other patients. From Table 5.18, it can be seen the highest C_{max} values are associated with these two patients. Patient A experienced severe dry mouth and blurred vision whereby day-to-day operational performance was seriously hindered. Drowsiness and mild itching/redness on application site were also noted. Patient C experienced moderate to severe dry mouth, dizziness, mild nausea and drowsiness and transient impairment of ocular accommodation with day–to-day operational performance also hindered. Patient B, with the third highest C_{max} also experienced transient impairment of ocular accommodation. Blurred vision and dry mouth were also reported. Patient D, with a much lower C_{max} than Patients A-C, experienced some mild dry mouth and blurred vision and mild itching/redness on application site. On the extreme to Patients A and C is Patient E who experienced zero side effects following the same application. C_{max} for Patient E was the lowest obtained of all patients participating in the trail. The Scop serum concentrations calculated were much lower than those of the other patients and so, this correlated well with Patient E experiencing no adverse side effects.

In the comparative trial using the transdermal Scop half patch, hardly any side effects were experienced amongst the patients with just a minimal incidence of dryness of the mouth reported by Patient A.

5.5 Conclusions

The ‘Evaluation of countermeasures for motion sicknesses’, project currently being conducted in NASA, proposes the idea that cutting a transdermal Scop patch in half reduces the side effects associated with the medication while still having effective protection from motion sickness.

In Chapter 4 we developed an analytical protocol capable of supporting such a hypothesis and in this chapter a pilot study was instigated in order to examine circulating levels of Scop in patients treated with both the full and half transdermal Scop patch applications. Following both full and half transdermal

patch application, five subjects underwent subsequent blood collection and observation for each.

The study also aimed to compare the severity of side effects induced by the transdermal Scop patch both full and in half in correlation to the levels of Scop measured in serum over a 72 hour period The study concluded that by cutting the transdermal Scop patch in half, Scop levels are far less than half to those determined for the full Scop patch. Also, considerable differences in side effects between the single-dose and half-dose treatments of the transdermal Scop patch applications were observed. Cutting the patch seems to have had a disproportionate impact on delivery. As Scop is a reservoir patch whereby release is controlled by the polymer film next to the skin, cutting it in half could potentially have caused dose dumping of Scop from the reservoir on application. Furthermore, cutting it in half might have accelerated the dehydration of the medicine soaked reservoir or disrupted the rate-limiting membrane delivering the medication.

The results attained indicate that by cutting the patch in half, the levels of Scop detected are in fact substantially less than half those of the full dose, and so the integrity of the drug delivery system and adhesiveness of the patch is compromised.

Using the transdermal Scop full patch, severe side effects were observed which hindered operational performance in some of the patients. In the comparative trial using the transdermal Scop half patch, hardly any side effects were experienced amongst the patients. The literature indicates Scop serum levels of 50 pg/mL are generally accepted as affording prophylactic protection against motion sickness (Nachum *et al.,* 2001). However, the levels attained using the transdermal Scop patch cut in half are lower than this level. To date, there is no indication in the literature that a lower dose transdermal Scop patch is available. Due to the severe adverse effects reported using the full transdermal Scop patch, manufacture of a transdermal Scop patch with a lower level of Scop in the a drug reservoir of the patch is highly recommended.

The NASA-based motion sickness countermeasures study proposes the idea that cutting a transdermal Scop patch in half reduces the side effects associated with the medication while still having effective protection from motion sickness. In this study, it has been demonstrated that cutting the patch

in half does significantly reduce the side effects associated with the transdermal Scop patch, although the levels associated with this modification of the patch are not sufficient to reach the target threshold for suppressing motion sickness. It is, however, highly likely that the levels found would have some therapeutic effect and might reduce the severity of motion sickness symptoms or give greater resilience to these symptoms without affording full protection but also without the marked side effects which can afflict some individuals.

Continuation of the NASA based study should firstly evaluate if the low levels of Scop associated with cutting the transdermal Scop patch half actually prevent motion sickness in astronauts induced by off-vertical axis rotation (OVAR). If the levels are indeed too low to afford prophylactic protection against motion sickness, further investigation may warrant the manufacture a transdermal Scop patch possessing a lower level of Scop in the patch drug reservoir than the transdermal Scop formulations currently available.

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Overall conclusions and future work

One of the most difficult tasks faced by the analytical chemist is the clean-up and subsequent analysis of biological fluids. Bioanalytical methods developed for pharmacological and pharmacokinetic applications are even more challenging to develop and validate. This thesis achieved the development of novel methods for two very important and strategic investigations and their publication will aid the study of these drugs in human blood.

Chapter 2: Simultaneous determination of efavirenz, rifampicin and its main metabolite desacetyl rifampicin levels in human plasma

The first project involved the development and validation of an analytical protocol enabling the extraction, separation and determination of Efv and Rif and its metabolite, dRif, from human plasma. This was achieved using solid phase extraction and LC-UV analysis. Most challenging was the fact that Rif (and its metabolite, dRif) and Efv exhibit very different physiochemical properties, and so, developing this assay proved extremely challenging. The LC-UV method developed offers excellent sensitivity for each of the drugs, allows for the determination of Rif's metabolite which had not been done previously in the presence of Efv, and the sample volume required is low. The easy sample preparation and fast separation makes this assay highly suitable for pharmacokinetic studies and therapeutic drug monitoring in patients with HIV only, TB only or both diseases being treated with Efv and Rif.

This validated protocol was required for the SPhEAR project whereby the effects of Efv medication on the pharmacokinetics of oral Rif in the treatment of TB in HIV infected patients is currently being investigated. The fast, low cost and relatively easy UV-based assay developed can accurately detect the drug concentrations within a clinically relevant concentration range which renders it broadly applicable, especially in third world countries and in resource-limited settings where the burden of disease exists. Using standard LC-UV equipment, it has the advantage of being cost effective and easy to use.

Drug levels were measurable and significant differences in circulating Rif levels were found among the patients. Further work would involve completing analysis on all the SPhEAR study samples once all participating patients have undergone treatment and subsequent blood draw.

To summarise:

- A novel, sensitive isocratic high performance liquid chromatography assay employing solid phase extraction for the simultaneous determination of Rif, Efv and dRif from plasma was developed and validated.

- Various attractive features of the developed method include low volumes of plasma required for analysis, simple and fast extraction procedure and cost effective chromatographic equipment. This makes the method very rapid and economical, especially when a large number of samples are to be handled.

- With an LOQ of 100 ng/mL, Rif has a usefully short retention time of 3.1 min and a recovery of 94% from plasma. Similar to the LOQ for Rif, dRif's LOQ was again 100 ng/mL with a retention time of 2.75 min and a recovery of 97% from plasma achieved.

- There are limited reported methods for the simultaneous determination of dRif and Rif, the most successful achieving a sensitivity of 250 ng/mL. Our method is the best to date for the simultaneous determination of both drugs.

- Our method is capable of determining Rif's metabolite dRif. In the presence of Efv, this has never been investigated. This was important in order to determine the extent of Rif's metabolism to dRif in the presence of Efv.

- For Efv, there are numerous methods reported with LOQs of just 10 ng/mL but all suffer from either extremely long elution times, high sample volumes or both. With an LOQ of 100 ng/mL, and a sample volume of just 0.3 mL plasma required, Efv has a short retention time

of 6.1 mins which was very challenging in the presence of both dRif and Rif. A recovery of 70 % from plasma was obtained.

- There is only one reported LC-UV method for the simultaneous determination of Rif and Efv. It exhibits comparable sensitivity with the method we outlined (LOD 50 ng/mL) but requires gradient elution and hence suffers from a relatively long analysis time of 22 mins. The authors also did not measure Rif's main metabolite, dRif.

- Given the sensitivity of the method developed and the use of a SPE procedure delivering clean samples which had low background and excellent recoveries, this method was validated and used to quantitate the drug levels of patients participating in the SPhEAR study.

- The drugs can be detected within a clinically relevant concentration range using standard chromatography equipment employing UV detection which is both simple to use and cost effective (using low price equipment which is cheap to maintain and run).

- Adoption of such methodology is easily achievable in areas where there are high incidences of HIV infections, but limited laboratory budgets, such as sub-Saharan Africa, Eastern Europe and Asia.

Chapter 3: LC-MS determination of efavirenz and rifampicin levels in human plasma

It was decided to further investigate the methodology in order to achieve greater sensitivity for the assay. This was achieved using LC-MS analysis. The developed assay is the first capable of determining Rif and Efv simultaneously from a biological matrix using this technique. The sensitivity achieved for Rif in plasma is far superior to what is available in the scientific literature and for Efv the sensitivity achieved was comparable. Given more time, a decrease in the plasma sample volume would have been investigated. There would be

major advantages of using finger prick assays for blood rather than taking mLs using standard blood collection tubes which would allow for more routine analysis.

Following a full validation, this method would be capable of detecting extremely low levels of both drugs simultaneously in aqueous samples It would be interesting to apply the method to biological samples. It would be advantageous to modify the assay in order to quantify cellular and tissue levels of the drugs.

To summarise:

- There are currently no reported methods in the literature for the simultaneous analysis of Rif and Efv using LC-MS. The LC-UV method described in Chapter 2 was transferred to MS detection using similar chromatographic parameters as for the UV method.

- The sensitivity achieved was excellent with an LOQ of just 1 ng/mL for Rif in plasma. It is far superior to what is available in the scientific literature which reveals the lowest LOQ for serum spiked with Rif using MS to be 50 ng/mL and in plasma 100 ng/mL.

- An LOQ of 10 ng/mL was obtained for Efv prepared in plasma. Again this is an excellent result in comparison with what has previously been reported in the literature which reveals the lowest LOQ obtained for Efv was 10 ng/mL in plasma.

- The assay described in this chapter is the first capable of achieving such low levels of Rif and Efv from a biological matrix. Furthermore, the fast and easy sample treatment by SPE, as well as the short runtime of the assay allows for rapid sample processing and analysis.

Chapter 4: Development of a sensitive assay to extract, separate and determine scopolamine (anti-motion sickness medication) in human serum

The third analytical protocol developed and validated was for the determination of Scop in serum. This was achieved using solid-phase extraction coupled to LC-MS. This developed and validated protocol was required to support a project entitled 'Evaluation of countermeasures for motion sicknesses' being carried out currently in NASA. The purpose of this study is to evaluate the effects of the transdermal Scop patch in preventing motion sickness in astronauts induced by off-vertical axis rotation. It had been hypothesised that cutting a scopolamine transdermal patch in half will reduce the side effects associated with the medication while still providing effective protection from motion sickness.

LC-MS/MS remains one of the most useful tools available for bioanalysis and success required understanding the underlying principles of both chromatography and MS. The main challenge met here was developing a fast, isocratic method capable of determining the extremely low concentrations of scopolamine in serum post patch and half patch application. As the circulating level of Scop, especially when only half a patch is applied, is very low, both the extraction and the analytical methods were pushed to their very limits in order to reliably and reproducibly measure the levels of drug encountered. The isocratic elution scheme gives a simple, robust and reproducible chromatographic method with exceptional sensitivity for Scop. The easy sample preparation and fast separation makes this assay highly suitable for pharmacokinetic studies and therapeutic drug monitoring.

To summarise:

- A fast, novel and extremely sensitive assay has been developed and validated for the determination of Scop in serum using SPE coupled to LC–MS/MS.

- From the literature, there is no simple, isocratic method reported which achieves the sensitivity required for the analysis of Scop in serum post patch and half patch application. Methods suffer from having to use a gradient programme or a large sample volume in order to achieve their fast runtimes and low sensitivity.

- The isocratic elution scheme developed gives a simple, robust and reproducible chromatographic method with a short runtime and an exceptional LOQ of 10 pg/mL for Scop. Our experimental approach has yielded superior levels of sensitivity than those already in the literature for Scop extracted from biological matrix.

- The SPE approaches exploited in the literature demonstrate poor Scop recovery or high sample volume required. The assay described has been proven accurate and precise for the determination of Scop in serum with a recovery of 73% obtained.

- This method has been applied to a NASA bases study evaluating the effects of the transdermal Scop patch both uncompromised and cut in half.

Chapter 5: A pilot comparison of the human pharmacology of a half versus full transdermal scopolamine patch

In order to evaluate the NASA based countermeasures for motion sicknesses study hypothesis, we developed a pilot study to examine circulating levels of Scop in volunteers treated with the full patch and a half patch. It was investigated if the patch is compromised by cutting it in half and also to compare the severity of side effects induced by the transdermal Scop patch both full and in half and correlate these to the levels of Scop measured in serum over a 72 hour period. The results attained indicate that by cutting the patch in half, the levels of Scop detected are in fact substantially less than half those of the full dose, and so the integrity of the drug delivery system and adhesiveness of the patch was somewhat compromised. Also, there was a

considerable decrease in adverse side effects observed with the half transdermal Scop patch application in comparison to the full patch.

Continuation of the NASA-based study should firstly evaluate if the low levels of Scop associated with cutting the transdermal Scop patch in half actually prevent motion sickness in astronauts induced by off-vertical axis rotation (OVAR). If the levels are indeed too low to afford prophylactic protection against motion sickness, further investigation may warrant the manufacture of a transdermal Scop patch possessing a lower level of Scop in the patch drug reservoir than the transdermal Scop formulations currently available.

To summarise:

- The literature contains limited information on transdermal Scop patch pharmacokinetics. Most studies on the transdermal Scop patch combine it with the oral dose to generate a more immediate effect.

- The pharmacokinetic parameters and therapeutic efficacy of a half transdermal Scop patch have never been reported, not to mention a pharmacokinetic study.

- We developed a pilot study to examine circulating levels of Scop in five volunteers treated with the full patch and a half patch to evaluate the hypothesis that cutting a transdermal Scop patch in half reduces the side effects associated with the medication while still having effective protection from motion sickness.

- The LC-MS method developed and validated for Scop in serum was ideal for studying the pharmacokinetics of the transdermal Scop patch in our study due to the need for high sensitivity to observe low concentration and long time point data.

- The study compared the severity of side effects induced by the transdermal Scop patch both full and in half and correlated these to the levels of Scop measured in serum over a 72 hour period. Considerable differences between the single-dose and half-dose treatments of the transdermal Scop patch applications were observed.

- The results attained indicate that by cutting the patch in half, the levels of Scop detected are in fact substantially less than half those of the full dose, and so the integrity of the drug delivery system and adhesiveness of the patch is compromised.

To conclude, chromatographic methodologies for the analysis of different therapeutic drugs in biological matrices under challenging conditions representative of the extremes of real world analytical problems were developed. This work encountered and overcame problems associated with interferences from sample matrices, decomposition products, signal perturbations and extremely low levels of drug and metabolites. Successful method development was accomplished by means of careful assessment of these matrix effects and judicial use of the sample preparation methods coupled with suitable chromatography and detection.

Appendices

Appendix A: Ethics review document approved by DCU Research Ethics Committee

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Dublin City University
RESEARCH ETHICS COMMITTEE

APPLICATION FOR APPROVAL OF A PROJECT
INVOLVING HUMAN PARTICIPANTS

Application No. *(office use only)* DCUREC/2010/ ☐☐☐

Period of Approval *(office use only)* ...../...../..... to
...../...../....

This application form is to be used by researchers seeking ethics approval for individual projects and studies. The **signed original and an electronic copy** of your completed application must be submitted to the DCU Research Ethics Committee.

NB - The hard copy must be signed by the PI. The electronic copy should consist of <u>one file only</u>, which incorporates all supplementary documentation. The completed application must be proofread and spellchecked before submission to the REC. All sections of the application form should be completed. Applications which do not adhere to these requirements will not be accepted for review and will be returned directly to the applicant.

Applications must be completed on the form; answers in the form of attachments will not be accepted, except where indicated. No handwritten applications will be accepted. **Research must <u>not</u> commence until written approval has been received from the Research Ethics Committee.**

PROJECT TITLE **Validation of a method for the analysis of Scopolamine (anti-motion sickness medication) in human serum for the NASA space research programme**

PRINCIPAL INVESTIGATOR(S) **Dr. Gillian McMahon, Deirdre Fox**

Please confirm that **all** supplementary information is included in your application (in both signed original and electronic copy). If questionnaire or interview questions are submitted in draft form, a copy of the final documentation must be submitted for final approval when available.

	INCLUDED		**NOT APPLICABLE**
Bibliography	☒		☐
Recruitment advertisement	☒		☐
Plain language statement/Information Statement	☒		☐
Informed Consent form	☒		☐
Evidence of external approvals related to the research	☐		☒
Questionnaire	☐ draft	☐ final	☒
Interview Schedule	☐ draft	☐ final	☒
Debriefing material	☐		☒
Other	☐		☒

Please note:

1. Any amendments to the original approved proposal must receive prior REC approval.

2. As a condition of approval investigators are required to document and report immediately to the Secretary of the Research Ethics Committee any adverse events, any issues which might negatively impact on the conduct of the research and/or any complaint from a participant relating to their participation in the study

Please submit the **signed original, plus the electronic copy** of your completed application to:
Ms. Fiona Brennan, Research Officer, Office of the Vice-President for Research
(fiona.brennan@dcu.ie, Ph. 01-7007816)

1.1 PRINCIPAL INVESTIGATOR(S): *The named Principal Investigator is the person with primary responsibility for the research project. Doctoral researchers and Research Masters or their supervisors may be listed as Principal Investigators, depending on the conventions of the discipline and on the individual case. It should be made clear, in subsequent sections of this application, who is carrying out the research procedures. In the case of Taught Masters and undergraduate student projects the supervisors are Principal Investigators.*

2.0 PROJECT OUTLINE: *Provide a brief outline of the project, aims, methods, duration, funding, profile of participants and proposed interaction with them. This description must be in everyday language that is free from jargon. Please explain any technical terms or discipline-specific phrases.*

2.1 LAY DESCRIPTION: *Provide a brief outline of the project, including what participants will be required to do. This description must be in everyday language which is free from jargon. Please explain any technical terms or discipline-specific phrases. (No more than 300 words).*

2.2 AIMS OF AND JUSTIFICATION FOR THE RESEARCH: *State the aims and significance of the project (approx. 400 words). Where relevant, state the specific hypothesis to be tested. Also please provide a brief description of current research, a justification as to why this research should proceed and an explanation of any expected benefits to the community.* ***NB – all references cited should be listed in an attached bibliography.***

2.3 PROPOSED METHOD: *Provide an outline of the proposed method, including details of data collection techniques, tasks participants will be asked to do, the estimated time commitment involved, and how data will be analysed. If the project includes any procedure which is beyond already established and accepted techniques please include a description of it. (No more than 400 words.)*

2.4 PARTICIPANT PROFILE: *Provide number, age range and source of participants. Please provide a justification of your proposed sample size. Please provide a justification for selecting a specific gender.*

2.5 MEANS BY WHICH PARTICIPANTS ARE TO BE RECRUITED: *Please provide specific details as to how you will be recruiting participants. How will people be told you are doing this research? How will they be approached and asked if they are willing to participate? If you are mailing to or phoning people, please explain how you have obtained their names and contact details. This information will need to be included in the plain language statement. If a recruitment advertisement is to be used, please ensure you attach a copy to this application.*

3.3 POTENTIAL RISKS TO PARTICIPANTS AND RISK MANAGEMENT PROCEDURES*: Identify, as far as possible, all potential risks to participants (physical, psychological, social, legal or economic etc.), associated with the proposed research. Please explain what risk management procedures will be put in place.*

3.6 ADVERSE/UNEXPECTED OUTCOMES: *Please describe what measures you have in place in the event that there are any unexpected outcomes or adverse effects to participants arising from involvement in the project.*

3.7 MONITORING: *Please explain how you propose to monitor the conduct of the project (especially where several people are involved in recruiting or interviewing, administering procedures) to ensure that it conforms with the procedures set out in this application. In the case of student projects please give details of how the supervisor(s) will monitor the conduct of the project.*

3.8 SUPPORT FOR PARTICIPANTS: *Depending on risks to participants you may need to consider having additional support for participants during/after the study. Consider whether your project would require additional*

support, e.g., external counselling available to participants. Please advise what support will be available.

4.0 INVESTIGATORS' QUALIFICATIONS, EXPERIENCE AND SKILLS: *List the academic qualifications and outline the experience and skills relevant to this project that the researchers and any supporting staff have in carrying out the research and in dealing with any emergencies, unexpected outcomes, or contingencies that may arise.*

5.2 HOW WILL THE ANONYMITY OF THE PARTICIPANTS BE RESPECTED? *Please bear in mind that where the sample size is very small, it may be impossible to guarantee anonymity/confidentiality of participant identity. Participants involved in such projects need to be advised of this limitation.*

5.3 LEGAL LIMITATIONS TO DATA CONFIDENTIALITY: *Participants need to be aware that confidentiality of information provided can only be protected within the limitations of the law - i.e., it is possible for data to be subject to subpoena, freedom of information claim or mandated reporting by some professions. Depending on the research proposal you may need to specifically state these limitations.*

6.0 DATA/SAMPLE STORAGE, SECURITY AND DISPOSAL: *For the purpose of this section, "Data" includes that in a raw or processed state (e.g. interview audiotape, transcript or analysis). "Samples" include body fluids or tissue samples.*

8.0 PLAIN LANGUAGE STATEMENT: *Written information in plain language that you will be providing to participants, outlining the phases and nature of their involvement in the project and inviting their participation. Please note that the language used must reflect the participant age group and corresponding comprehension level.*

9.0 INFORMED CONSENT FORM: *This is a very important document that should be addressed by participants to researchers, requiring participants to indicate their consent to specific statements, and give their signature.*

FOR FURTHER INFORMATION AND NOTES ON THE DEVELOPMENT OF PLAIN LANGUAGE STATEMENTS AND INFORMED CONSENT FORMS, PLEASE CONSULT THE DCU REC WEBSITE: WWW.DCU.IE/RESEARCH/ETHICS

1. ADMINISTRATIVE DETAILS

THIS PROJECT IS: *(tick as many as apply)*

☒ Research Project ☐ Funded Consultancy

☐ Practical Class ☐ Clinical Trial

☐ Student Research Project *(please give details)*

☐ Research Masters ☐ Taught Masters

☒ PhD ☐ Undergraduate

☐ Other - *Please Describe:*

Project Start Date: **April 2010**

Project End date: **June 2010**

1.1 INVESTIGATOR CONTACT DETAILS *(see Guidelines)*

PRINCIPAL INVESTIGATOR(S):

TITLE	*SURNAME*	*FIRST NAME*	*PHONE*	*FAX*	*EMAIL*
Dr.	McMahon	Gillian	01 700 8351	n/a	gillian.mcmahon@dcu.ie
Ms	Fox	Deirdre	01 700 8351	n/a	deirdre.fox4@mail.dcu.ie

OTHER INVESTIGATORS:

TITL	*SURNAME*	*FIRST NAME*	*PHONE*	*FAX*	*EMAIL*

E					
Dr.	Levine	Lanfang	(321) 861-2931	n/a	lanfang.h.levine@nasa.gov

FACULTY/DEPARTMENT/SCHOOL/ CENTRE: School of Chemical Sciences

(NB – if Nursing, please note all students including PhD's must attach the letter from the Nursing Ethics Advisory Committee to this application)

1.2 WILL THE RESEARCH BE UNDERTAKEN ON-SITE AT DUBLIN CITY UNIVERSITY?

☒ YES ☐ NO *(If NO, give details of off-campus location.)*

1.3 IS THIS PROTOCOL BEING SUBMITTED TO ANOTHER ETHICS COMMITTEE, OR HAS IT BEEN PREVIOUSLY SUBMITTED TO AN ETHICS COMMITTEE?*)*

☐ YES ☒ NO *(If YES, please provide details and copies of approval(s) received etc.)*

DECLARATION BY INVESTIGATORS

The information contained herein is, to the best of my knowledge and belief, accurate. I have read the University's current research ethics guidelines, and

accept responsibility for the conduct of the procedures set out in the attached application in accordance with the guidelines, the University's policy on Conflict of Interest and any other condition laid down by the Dublin City University Research Ethics Committee or its Sub-Committees. I have attempted to identify all risks related to the research that may arise in conducting this research and acknowledge my obligations and the rights of the participants.

If there any affiliation or financial interest for researcher(s) in this research or its outcomes or any other circumstances which might represent a perceived, potential or actual conflict of interest this should be declared in accordance with Dublin City University policy on Conflicts of Interest.

I and my co-investigators or supporting staff have the appropriate qualifications, experience and facilities to conduct the research set out in the attached application and to deal with any emergencies and contingencies related to the research that may arise.

Signature(s):

Principal investigator(s): _____

Print name(s) in block letters: _____

Date:_____

2. PROJECT OUTLINE

2.1 LAY DESCRIPTION *(see Guidelines)*

Scopolamine is one of the substances used by NASA to combat motion sickness in space. The Scopolamine patch, an over the counter medication, is the most common form of the drug in the US. However, undesirable side effects have been reported. It had been hypothesised at NASA that if a transdermal patch of scopolamine is halved and then used by the astronaut, that it is just as effective as a full patch but with less undesirable side effects. In NASA, I developed and optimised a method for the determination of scopolamine in blood in order to evaluate if the levels of the half patch are indeed half of the Scopolamine level in full patch. However, the method was only semi-validated due to time restraints. On return to DCU, I was able to transfer the assay to a similar instrument here so that I could complete the work I had initially set out to do. Healthy volunteers will be asked to wear the full patch for 3 days orthe patch cut in half for 3 days with blood drawn at different time points which will subsequently be assayed for scopolamine concentration.

2.2 AIMS OF AND JUSTIFICATION FOR THE RESEARCH *(see Guidelines)*

This research is collaboration with NASA, Kennedy Space Centre, Florida where Dr. Daniel Woodard is conducting a project entitled ‘Evaluation of countermeasures for motion sicknesses’. The purpose of this study is to evaluate the effects of the transdermal scopolamine patch in preventing motion sickness in astronauts induced by off-vertical axis rotation (OVAR). The main question being asked is ‘Would cutting a scopolamine transdermal patch in half reduce the side effects associated with the medication while still having effective protection from motion sickness?’

By the oral route, scopolamine is effective within 0.5 h for a period of 6 h. Because of its short half-life in plasma and dose-dependent adverse effects,

the clinical use of scopolamine administered orally or parenterally is limited. To minimize the relatively high incidence of side effects, the transdermal dosage form has been developed. The Transderm Scop (transdermal scopolamine) system is a circular flat patch designed for continuous release of scopolamine following application to an area of intact skin on the head, behind the ear. Each system contains 1.5 mg of scopolamine base. The recommended dosage is a single patch applied at least 6-8 hours before the anti-motion sickness effect is required. After 72 hours, the patch should be removed and a new one applied behind the opposite ear. Therapeutic Scopolamine plasma levels are obtained very slowly, 6 to 8 h post patch application.

The literature contains limited information on Scopolamine patch pharmacokinetic information. Most studies on the Scopolamine transdermal patch combine it with the oral dose so as protection from motion sickness is effective immediately. Also, the Scopolamine patch cut in half to prevent motion sickness has never been reported, not to mention a pharmacokinetic study on it. The levels of Scopolamine post patch application are quite low at ~100ng/mL, and with the patch cut in half, levels would be lower again. This study would conclude if the integrity of the patch is compromised by cutting it in half. Methods previously published are not sensitive enough to achieve this goal.

Developed and optimised in NASA, Florida, is a novel reversed phase High Performance Liquid Chromatography assay with Mass Spectrometry detection and solid phase extraction for the determination of scopolamine. However, it was not fully validated due to time constraints. Following a technology transfer of the method here in DCU, completion of the validation is now planned. Real patient samples will be required for the validation section and also a pharmacokinetic study on the patch cut in half could be performed given approval from the REC as it has never been done before. The LC-MS method I’ve developed is extremely sensitive (Limit of Quantitation 10pg/mL) and achieving the anticipated levels of Scopolamine in blood post patch application and post half patch application should be well within the capability of the method.

Some studies have been conducted:

Lakshmi Putcha *et al.* [1] conducted a trial on the pharmacokinetics and bioavailability of scopolamine in six healthy male subjects receiving 0.4 mg of the drug by either oral or intravenous administration.

Lakshmi Putcha *et al.* [2] compared the bioavailability of scopolamine in three dosage forms in 12 healthy non-smoking male volunteers. Subjects received 0.4-mg doses of scopolamine bromide in intravenous (IV), intranasal (IN), or oral (PO) dosage forms on three occasions, with at least 2 weeks separating the doses. Saliva volume and flow rate and percent suppression of control flow rate were determined from each sample. Absorption after IN and PO scopolamine administration was rapid; plasma concentrations [1680 (IN) and 164 pg/mL (PO)] peaked within 1 h of dosing [0.37 (IN) and 0.78 h (PO)], respectively. IN and IV scopolamine suppressed salivary flow rate to similar extents (95% and 99.7%), respectively. Times to reach maximum effect were 1.05 and 0.27 h after IN and IV dosage, respectively.

Nachum *et al.* [3] headed a study entitled: Transdermal scopolamine for prevention of motion sickness: clinical pharmacokinetics and therapeutic applications. The protective plasma concentration of scopolamine is estimated to be 50 pg/mL. TTS-S attains that concentration after 6 hours; a steady state of about 100 pg/mL is achieved 8-12 hours after application. Dry mouth occurred in about 50-60% of subjects, drowsiness in up to 20%, and allergic contact dermatitis in 10%.

Nachum *et al.* [4] also conducted a study entitled : Scopolamine bioavailability in combined oral and transdermal delivery which investigated the pharmacokinetics of scopolamine after patch application in combination with oral tablets, 0.6 mg, 0.3 mg, or placebo.Subjects were 25 healthy, male naval-crew volunteers, , aged 18 to 20 years, randomly divided into three groups: group 1 (n = 9), TTS-S patch + 0.6 mg of scopolamine per os (p.o.); group 2 (n = 8), TTS-S patch + 0.3 mg of scopolamine p.o.; and group 3 (n =

8), TTS-S patch + placebo tablet. Subjects were drug free for at least 72 h before the study. They were required to refrain from strenuous physical activity during the study.Blood samples were collected before treatment and 0.5, 1, 1.5, 2.5, 3.5, 6, 8, and 22 h post-treatment, and were analysed for scopolamine levels.

2.3 PROPOSED METHOD *(see Guidelines)*

It has been agreed with Dr. Donal O Gorman in the School of Health and Human Performance that blood sampling will be conducted by experienced personnel in the School of Health and Human Performance.

As the patch is recommended for application over 3 days, it was decided to conduct blood sampling over this time in order to generate the pharmacokinetic profile. Peak blood levels are obtained 6-8 hours post application, hence more frequent sampling is required around this time.

The study will be conducted over 2 weeks. On week 1 (Monday, 9am) the Scopolamine transdermal full patch will be randomly applied to 10 of the participants and half patch to the remaining 10 participants and worn over a period of 3 days. Blood will be drawn at different time points; time 0, 3, 6, 7, 8, 9, 24, 48, and 72 hrs.

5 days will be left in-between the patch applications to ensure complete elimination of Scopolamine levels from the participant’s blood.

On week 2 (Tuesday, 9am), the same volunteers will again apply the Scopolamine transdermal patch (either half or full, depending on what was previously worn) for a period of 3 days. Blood will be drawn at the same time points as above; time 0, 3, 6, 7, 8, 9, 24, 48, and 72 hrs.

Healthy participants are required to wear the patch continuously over the three days and not to subject themselves to any strenuous activity. As there are so

many time point samples required participants are asked to eat and drink as normal. Participants are asked to note any side effects observed.

All blood samples will be allowed to coagulate for 20 mins, and the resultant serum will be placed into appropriate vials. Samples will be frozen at -20ºC until analysis by the LC-MS method developed for the project..

2.4 PARTICIPANT PROFILE *(see Guidelines)*

A total of 20 healthy adult participants aged 18-60 years of age will be recruited for this study. As this is a validation study, the sample number was based on existing studies that have been completed. The following studies, included in the reference list, studied between 6-25 participants.

Lakshmi Putcha *et al.* [2] used 12 participants for their trial while Nachum *et al.* [4] used 25 and Lakshmi Putcha *et al.* [1] used 6.

2.5 MEANS BY WHICH PARTICIPANTS ARE TO BE RECRUITED *(see Guidelines)*

Participants will be informed of this study through a circulating email to DCU staff and students.

Email:
Apologies to whom this does not concern.

The School of Chemical Sciences is currently undertaking a study to validate the measurement of Scopolamine in blood. Scopolamine comes in the form of an over-the-counter patch that prevents motion sickness. The aim of our study is to determine if half the patch would result in an adequate circulating level of Scopolamine. This research is in collaboration with NASA, Kennedy Space Centre, Florida. We are looking for volunteers to conduct two trials separated by one week. In one trial you will be asked to wear the Scopolamine patch for 3 days and the other trial is identical but only half the patch is worn. A blood

sample to determine the level of Scopolamine in circulation will be taken at a number of time points over each of the 3-day trials (0, 3, 6, 7, 8, 9, 24, 48, and 72 hrs post patch application).

If you would like to hear more about this study, please contact Deirdre Fox at deirdre.fox4@mail.dcu.ie or 01-7008351

2.6 PLEASE EXPLAIN WHEN, HOW, WHERE, AND TO WHOM RESULTS WILL BE DISSEMINATED, INCLUDING WHETHER PARTICIPANTS WILL BE PROVIDED WITH ANY INFORMATION AS TO THE FINDINGS OR OUTCOMES OF THE PROJECT?

All participants will be given a report of their results, and also the results of the group averages. Results will be compiled as part of a PhD thesis and will hopefully be presented at conferences and published in the scientific literature.

2.7 OTHER APPROVALS REQUIRED *Has permission to gain access to another location, organisation etc. been obtained? Copies of letters of approval to be provided when available.*

☐ YES ☒ NO ☐ NOT APPLICABLE

(If YES, please specify from whom and attach a copy. If NO, please explain when this will be obtained.)

2.8 HAS A SIMILAR PROPOSAL BEEN PREVIOUSLY APPROVED BY THE REC?

☐ YES ☒ NO

(If YES, please state both the REC Application Number and Project Title)

3. RISK AND RISK MANAGEMENT

3.1 ARE THE RISKS TO SUBJECTS AND/OR RESEARCHERS ASSOCIATED WITH YOUR PROJECT GREATER THAN THOSE ENCOUNTERED IN EVERYDAY LIFE?

☐ YES ☒ NO *If YES, this proposal will be subject to full REC review*

If NO, this proposal may be processed by expedited administrative review

3.2 DOES THE RESEARCH INVOLVE:

	YES	NO
• use of a questionnaire? (attach copy)?	☐	☒
• interviews (attach interview questions)?	☐	☒
• observation of participants without their knowledge?	☐	☒
• participant observation (provide details in section 2)?	☐	☒
• audio- or video-taping interviewees or events?	☐	☒
• access to personal and/or confidential data (including student, patient or client data) without the participant's specific consent?	☐	☒
• administration of any stimuli, tasks, investigations or procedures which may be experienced by participants as physically or mentally painful, stressful or unpleasant during or after the research process?	☐	☒
• performance of any acts which might diminish the self-esteem of participants or cause them to experience embarrassment, regret or depression?	☐	☒

• investigation of participants involved in illegal activities?	☐	☐
• procedures that involve deception of participants?	☐	☐
• administration of any substance or agent?	☒	☐
• use of non-treatment of placebo control conditions?	☐	☐
• collection of body tissues or fluid samples?	☒	☐
• collection and/or testing of DNA samples?	☐	☒
• participation in a clinical trial?	☐	☒
• administration of ionising radiation to participants?	☐	☒

3.3 POTENTIAL RISKS TO PARTICIPANTS AND RISK MANAGEMENT PROCEDURES *(see Guidelines)*

Scopolamine transdermal patch is an over the counter medication, and as the levels of the drug being released into the drug stream is low, side effects should be minimal. If side effects occur the patch can be removed at any time.

The following are some of the side effects that are known to be associated with this medicine. Just because a side effect is stated here does not mean that all people using this medicine will experience that or any side effect.

> Dry mouth, Drowsiness, Blurred vision, Irritation of skin at patch application site, Irritation of the eyelids, Dizziness, Dilated pupils, Restlessness, Confusion, Hallucinations, Difficulty passing urine, Headache, nausea, vomiting and disturbance of balance after removing the patch.

As some people are prone to drowsiness and dizziness while taking the patch, participants are not recommended to drive while taking the patch (half and full).

3.4 ARE THERE LIKELY TO BE ANY BENEFITS (DIRECT OR INDIRECT) TO PARTICIPANTS FROM THIS RESEARCH?

☐ YES ☒ NO *(If YES, provide details.)*

3.5 ARE THERE ANY SPECIFIC RISKS TO RESEARCHERS? *(e.g. risk of infection or where research is undertaken at an off-campus location)*

☐ YES ☒ NO *(If YES, please describe.)*

3.6 ADVERSE/UNEXPECTED OUTCOMES *(see Guidelines)*

If side effects occur, which will be minimal, patch can be removed at any time.

3.7 MONITORING *(see Guidelines)*

Regular check in's with personnel drawing blood, contact with participants (phone and email) with updates regarding timings of blood draws etc)

3.8 SUPPORT FOR PARTICIPANTS *(see Guidelines)*

Additional support for the subjects will not be required for this trial.

3.9 DO YOU PROPOSE TO OFFER PAYMENTS OR INCENTIVES TO PARTICIPANTS?

☐ YES ☒ NO *(If YES, please provide further details.)*

4. INVESTIGATORS' QUALIFICATIONS, EXPERIENCE AND SKILLS *(Approx. 200 words – see Guidelines)*

Dr. Gillian McMahon:

Role in proposed project: Overseeing the project and acting as a direct supervisor

- BSc in Analytical Science from Dublin City University, Ireland, 1992.
- PhD, The Royal College of Surgeons in Ireland/DCU, 1998.
- Postdoctoral research fellow in The National Centre for Sensor Research (NCSR) at Dublin City University
- Group research manager for the Chemical Sensors Group in the NCSR
- Currently: lecturer in the School of Chemical Sciences.

Deirdre Fox:

Role in proposed project: Responsible for doing analysis of blood samples

- BSc Analytical Chemistry with Quality Assurance, Cork Institute of Technology (CIT). 2006
- Currently: Third year of PhD entitled 'The evaluation of novel phases and approaches to selectively extract and analyse different drugs in human blood and other biological matrices'.

Dr. Lanfang Levine

Role in proposed project: Involved in the initial development of the methodology and is providing collaborative support for this project

- Ph.D. Agricultural Science, Chemistry of Forest Products (1990), Nagoya University, Nagoya, Japan

- M.S. Organic Chemistry (1985), Guangzhou Institute of Chemistry, Academia Sinica, Guangzhou, China
- B.S Chemical Engineering (1982), The South China Institute of Technology, Guangzhou, China
- Currently: Senior Research Chemist & Analytical Chemistry Discipline Coordinator in Dynamac Corporation Kennedy Space Centre, Florida.

5. CONFIDENTIALITY/ANONYMITY

5.1 WILL THE IDENTITY OF THE PARTICIPANTS BE PROTECTED?

☒ YES ☐ NO *(If NO, please explain)*

IF YOU ANSWERED YES TO 5.1, PLEASE ANSWER THE FOLLOWING QUESTIONS:

5.2 HOW WILL THE ANONYMITY OF THE PARTICIPANTS BE RESPECTED? *(see Guidelines)*

All participants will be assigned a random identification number by me. Their name will not be associated with any blood tubes or documentation thereafter. All information will be stored in a password protected computer and all written documentation will be held within a locked filing cabinet.

5.3 LEGAL LIMITATIONS TO DATA CONFIDENTIALITY: *(Have you included appropriate information in the plain language statement and consent form? See Guidelines)*

☒ YES ☐ NO *(If NO, please advise how participants will be advised.)*

6 DATA/SAMPLE STORAGE, SECURITY AND DISPOSAL *(see Guidelines)*

6.1 HOW WILL THE DATA/SAMPLES BE STORED? *(The REC recommends that all data be stored on campus)*

Stored at DCU ☒

Stored at another site ☐ **(***Please explain where and for what purpose)*

6.2 WHO WILL HAVE ACCESS TO DATA/SAMPLES?

Access by named researchers only ☒

Access by people other than named researcher(s) ☐ **(***Please explain who and for what purpose)*

Other *:* ☐ **(***Please explain)*

6.3 IF DATA/SAMPLES ARE TO BE DISPOSED OF, PLEASE EXPLAIN <u>HOW</u>, <u>WHEN</u> AND <u>BY WHOM</u> THIS WILL BE DONE?

I will be responsible for disposal of bio hazardous waste post analysis.

FUNDING

7.1 HOW IS THIS WORK BEING FUNDED?

IRCSET

7.2 PROJECT GRANT NUMBER (If relevant and/or known)

Not known

7.3 DOES THE PROJECT REQUIRE APPROVAL BEFORE CONSIDERATION FOR FUNDING BY A GRANTING BODY?

☐ YES ☒ NO

7.4 HOW WILL PARTICIPANTS BE INFORMED OF THE SOURCE OF THE FUNDING?

In plain language statement and Consent form

7.5 DO ANY OF THE RESEARCHERS, SUPERVISORS OR FUNDERS OF THIS PROJECT HAVE A PERSONAL, FINANCIAL OR COMMERCIAL INTEREST IN ITS OUTCOME THAT MIGHT COMPROMISE THE INDEPENDENCE AND INTEGRITY OF THE RESEARCH, OR BIAS THE CONDUCT OR RESULTS OF THE RESEARCH, OR UNDULY DELAY OR OTHERWISE AFFECT THEIR PUBLICATION?

YES ☒ NO *(If Yes, please specify how this conflict of interest will be addressed.)*

8. PLAIN LANGUAGE STATEMENT *(Approx. 400 words – see Guidelines)*

Research Study title: Validation of a method for the analysis of Scopolamine (anti-motion sickness medication) in human serum for the NASA space research programme

Principal Investigators: Dr. Gillian McMahon (gillian.mcmahon@dcu.ie) Deirdre Fox (Deirdre.fox4@mail.dcu.ie)

Department: School of Chemical Sciences, Dublin City University, Dublin 9

Purpose: The purpose of this study is to evaluate whether cutting a Scopolamine patch in half is delivering half of the anticipated Scopolamine or if the patch is compromised by cutting it in half.

Scopolamine is one of the substances used by NASA to combat motion sickness in space. The Scopolamine patch, an over the counter medication, is the most common form of the drug in the US. However, undesirable side effects have been reported. It had been hypothesised at NASA that if a transdermal patch of scopolamine is halved and then used by the astronaut, that it is just as successful as a full patch but that the side effects are much reduced. In NASA, developed and optimised is a method for the determination of scopolamine in order to evaluate if the levels of the half patch are indeed half of the Scopolamine level in full patch. However, the method was only semi-validated due to time restraints. The method developed in NASA was transferred to a similar instrument here so that completion of the work could be achieved.

The Scopolamine patch is a circular flat patch designed for continuous release of scopolamine over a period of 3 days, following application to the skin behind the ear. The main side effects reported while taking the patch are dry mouth, drowsiness and dizziness.

By conducting a study over a two week period, you will be asked to take either the full/ half patch on the first week, leaving 5 days before taking the full/half patch the following week. Blood draw times are 0, 3, 6, 7, 8, 9, 24, 48, and 72 hrs post patch application.

Post analysis of the blood, it will be concluded whether cutting the patch in half is delivering half amounts of Scopolamine in comparison to the full patch, into the blood or if the patch is indeed compromised by cutting it in half. A pharmacokinetic study will be carried out on the patch; both full and cut in half as it has never been done before.

This PhD research project is funded by IRCSET.

You will be assigned a random identification number by me. Your name will not be associated with any blood tubes or documentation thereafter. All information will be stored in a password protected computer and all written documentation will be held within a locked filing cabinet.

You may withdraw from the research study at any time point. There will be no penalty for withdrawing before all time point samples have been drawn. Confidentiality of information provided can only be protected within the limitations of the law - i.e., it is possible for data to be subject to subpoena, freedom of information claim or mandated reporting by some professions.

If you have concerns about this study and wish to contact an independent person, please contact: The Secretary, Dublin City University Research Ethics Committee, c/o Office of the Vice-President for Research, Dublin City University, Dublin 9. Tel 01-7008000

9. INFORMED CONSENT FORM *(Approx. 300 words – see Guidelines)*

Research Study title: Validation of a method for the analysis of Scopolamine (anti-motion sickness medication) in human serum for the NASA space research programme

Principal Investigators: Dr. Gillian McMahon, Deirdre Fox, Dr. Lanfang Levine

Department: School of Chemical Sciences, Dublin City University, Dublin 9

Purpose of Research: In NASA, Kennedy Space Centre, Florida, Dr. Daniel Woodard is conducting a project entitled 'Evaluation of countermeasures for motion sicknesses'. The purpose of this study is to evaluate the effects of the transdermal scopolamine patch in preventing motion sickness in astronauts induced by off-vertical axis rotation (OVAR). The main question being asked is 'Would cutting a scopolamine transdermal patch in half reduce the side effects associated with the medication while still having effective protection from motion sickness?' My role is to determine if the Scopolamine patch is compromised by cutting it in half. This study would also provide sufficient samples for pharmacokinetic profiling of the Scopolamine, both full and cut in half. This PhD research project is funded by IRCSET.

I understand I will be asked to take the full Scopolamine patch, applied behind my ear, for 3 days, give blood at 9 different time points (0, 3, 6, 7, 8, 9, 24, 48, and 72 hrs post patch application) and repeat this entire process 5 days later with the Scopolamine patch cut in half.

I understand associated side effects with taking the Scopolamine patch are:

Dry mouth, Drowsiness, Blurred vision, Irritation of skin at patch application site, Irritation of the eyelids, Dizziness, Dilated pupils, Restlessness, Confusion,

Hallucinations, Difficulty passing urine, Headache, nausea, vomiting and disturbance of balance after removing the patch.

As some people are prone to drowsiness while taking the patch, I have been advised not to drive for the duration of days taking the patch.

Please complete the following: (Circle yes or no for each question)

I have read the plain language statement?	Yes/No
I understand the information provided?	Yes/No
I have had an opportunity to ask questions and discuss this study?	Yes/No
I have received satisfactory answers to all my questions?	Yes/No

I understand I will be assigned a random identification number for this study and that my name will not be associated with any blood tubes or documentation associated.

I understand that I may withdraw from the research study at any time point. There will be no penalty for withdrawing before all time course samples have been drawn.

I have read and understand the information in this form. My questions and concerns have been answered by the researchers, and I have a copy of this consent form. Therefore I consent to take part in this research project

Participants Signature: _____

Name in Block Capitals: _____

Witness: _____

Date: _____

Bibliography

[1] L. Putcha, N.M. CintrÃ³n, J. Tsui, J.M. Vanderploeg, W.G. Kramer, Pharm. Res. 6 (1989) 481-485.

[2] L. Putcha, K.J. Tietze, D.W.A. Bourne, C.M. Parise, R.P. Hunter, N.M. Cintrón, J. Pharm. Sci. 85 (1996) 899-902, doi: 10.1021/js950327b.

[3] http://www.ncbi.nlm.nih.gov/pubmed/16719539

[4] http://www.ncbi.nlm.nih.gov/pubmed/11123371

Appendix B: Blood sampling protocol for scopolamine pilot study

Standard template for ethical justification for blood sampling associated with human studies conducted within DCU.

Version 1 September 2006

Completion instructions:

This document is intended to prompt responses to a number of standard questions which generally need to be answered to justify the sampling of blood associated with human studies.

The document is not meant to be an exhaustive exploration of the justification for such sampling and in specific situations, additional information may be required/ requested.

Answers are expected to be brief but should also be informative. See a sample completed form at the end.

Queries should be directed to the Secretary of the Research Ethics Committee in the OVPR office.

1) Briefly explain why blood sampling is required

The transdermal patch releases Scopolamine into the blood stream, my research involves evaluating the patch, both un compromised and cut in half.

2) Outline the analytes, components or general applications to be investigated in subject blood (now and any future studies)

Scopolamine from transdermal patch

3) Are any alternatives available to substitute the venous sampling of blood? yes/no.

Yes – saliva and urine. However this study is in order to complete validation using serum blood samples.

4) Will sampling require cannulation or direct vein puncture? yes/no

5) Outline the minimum volume of original subject blood (i.e. not serum or plasma) required to measure the required components.

3 mL

6) Are steps being taken in the protocol to minimise the volume of blood samples being taken? yes/no

Smallest blood collection tube

7) Are steps included to minimise the number of blood samples/vein puncture being taken? yes/no

Cannula inserted for first 6 time points (between 0 and 9 hours post patch application)

8) Anticipated sampling methodology

Volume of blood to be taken per sample	3 mL
Maximum number of samples to be taken per “sitting”	1
Maximum number of samples taken per day	6
Maximum number of samples to be taken over the course of the full study (if long duration study indicate the amount taken in an active 1 month period)	9 per trial
Maximum anticipate number of vein puncture episodes	4 per trial
Total volume of blood that will be taken from subject.	27 mL per trial

9) I certify that:-

- all persons sampling blood in this study are certified to do so through the school/unit where this work is being conducted
- that all those manipulating the resultant samples are fully trained in the safe practice of handling blood
- all persons handling this blood have received appropriate information according to current vaccination policy.

Signature of Study PI Date

An original signed copy must accompany electronic submissions. Alternatively, a PDF or other scanned version with a signature may be submitted

Appendix C: Publications

FOX, D., O'CONNOR, R., MALLON, P. and MCMAHON, G., 2011. Simultaneous determination of efavirenz, rifampicin and its metabolite desacetyl rifampicin levels in human plasma. *Journal of pharmaceutical and biomedical analysis*, **56**(4), pp. 785-791.

Appendix D: Oral and poster presentations

The work carried out for the duration of this thesis was presented in both poster form and oral presentations at various scientific conferences and meetings:

Oral Presentations

An oral presentation was presented to Kennedy Space Centre personnel on 13th Apr, 2009. The presentation was held at the Kennedy Space Centre, Florida.
Title: Development of an analytical protocol to extract, separate and determine the anti-motion sickness drug scopolamine in human serum
Authors: Fox D, Levine L, McMahon G.

An oral presentation was presented at the FAS closing ceremony on 15th Apr, 2009. The ceremony was held at the Kennedy Space Centre, Florida.
Title: Development of an analytical protocol to extract, separate and determine the anti-motion sickness drug scopolamine in human serum
Authors: Fox D, Levine L, McMahon G.

An oral presentation was presented on 04th April, 2010 to Dr. Patrick Mallon and all coordinating staff involved in the SPhEAR project. The presentation was held at Mater Misericordiae University Hospital, Dublin.
Title: Development of an analytical protocol to extract, separate and determine rifampicin (anti-TB medication) and efavirenz (anti- HIV medication) in blood samples of co- infected patients for SPhEAR programme
Authors: Fox D, O'Connor R, Mallon P, McMahon G

An oral presentation was presented at the Irish Universities Chemistry Research Colloquium (IUCRC) on 02nd July, 2010. The Colloquium was held at Queens University, Belfast.
Title: Development of an analytical protocol to extract, separate and determine efavirenz (anti-HIV medication) and rifampicin (anti-TB medication) in co-infected patients
Authors: Fox D, O'Connor R, Mallon P, McMahon G

Poster Presentations

A poster was presented at the *HTC, ExTech 10* conference held in Bruges, Belgium on Jan 28th to Feb 1st, 2008.
The poster title was: "Development of an analytical protocol to extract, separate and determine rifampicin (anti-TB medication) and efavirenz (anti-HIV medication) in co-infected patients.
Authors: Fox D, O'Connor R, Mallon P, McMahon G.

A poster was presented at the *Infectious Diseases Society of Ireland (IDSI), Inaugural Annual Scientific Meeting*, Dublin 2 on 11-13th June, 2008.
Title: Development of an analytical protocol to extract, separate and determine rifampicin (anti-TB medication) and efavirenz (anti-HIV medication) in co-infected patients.
Authors: Fox D, O'Connor R, Mallon P, McMahon G.

A poster was presented at the *Analytical Research Forum* (ARF) on 21-23rd July, 2008. The ARF was held at the University of Hull, UK.
Title: Development of an analytical protocol to extract, separate and determine rifampicin (anti-TB medication) and efavirenz (anti-HIV medication) in co-infected patients.
Authors: Fox D, O'Connor R, Mallon P, McMahon G.

A poster was presented at the *Analytical Research Forum* (ARF) on 21-23rd July, 2009. The ARF was held at the University of Kent, UK.

Title: Development of an analytical protocol to extract, separate and determine the anti-motion sickness drug scopolamine in human serum
Authors: Fox D, Levine L, McMahon G.

A poster was presented at the International Symposium on Chromatography (ISC) on 13- 16th Sept, 2010. The ISC was held at the Valencia Conference Centre, Spain.
Title: Development of a low cost, analytical process to extract, separate and determine efavirenz and rifampicin plasma concentrations in HIV/TB co-infected patients
Authors: Fox D, O'Connor R, Mallon P, McMahon G.

A poster was presented at the tenth International Congress on Drug Therapy in HIV infection 07-11th Nov, 2010. The HIV10 conference was held in Glasgow, UK.
Title: Development of a low cost, analytical process to extract, separate and determine efavirenz and rifampicin plasma concentrations in HIV/TB co-infected patients
Authors: Fox D, O'Connor R, Mallon P, McMahon G.

Scientific conferences prove a great way to network with fellow scientists and to explore what research is being conducted in similar fields of work.

Appendix E: Training received

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

- In house LC-UV training was given by my supervisor Dr. McMahon in Oct 2007 at Dublin City University. This was conducted in order to familiarise myself with the basic running and operation of the LC-UV instrument and also the Chemstation software.

- I was trained on LC-MS instrumentation in Kennedy Space Centre, Oct 2008, by my mentor Dr. Levine. This training was designed to show the basic running, operation and maintenance of LC-MS.

- A course was attended in the National Centre for Sensor Research, Dublin City University where the basic principles of operation of a MS were explained and a demonstration performed on MS operation. This two-day course was given by Dr. Maurice Burke.

- While at the HTC, Ex-Tech 10 (Jan 28th until Feb1st) conference in Brugge, Belgium I attended a short course entitled 'Modern Sample Preparation'. I found the course extremely helpful and beneficial as it was particularly relevant to the analytical research I was working on throughout my project.

- Official training on LC-MS instrumentation in the National Institute for Cellular Biotechnology was given by Dr. Sandra Roche in Oct 2009. This training gave me a good understanding of the operation and maintenance of the LC-MS used for this thesis.

Appendix F: Relevant experience gained

In Dublin City University, research students are obligated to partake in demonstrating duties. These duties involved overseeing a number of undergraduate students conducting practical experiments in scientific laboratories.

During my time in Dublin City University I have already taught:

- First year Science students between Oct and Mar 2007-2008. This was a lab based practical course on the basic techniques used in Chemistry. I had to supervise 12 students, each week with a different experiment. I had to grade weekly reports.

- Third year Analytical Science students between Oct and Dec 2009. This was a lab based practical course in the analysis of organic and inorganic species. I was supervising 2 experiments: High Performance Liquid Chromatography and Spectroscopy. I had to grade weekly reports submitted by the students.

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