Application of Solid Phase Microextraction for Quantitative Bioanalysis and Toxicokinetics: An Integrated Microsampling and Microanalysis Technique

By

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Dedication

To my loving family … my parents (Aras Ahmad and Barween Shakir) and my sister (Veyan Ahmad). Your love and support shines through everything I achieve. This thesis would not have been possible without you.

Abstract

Over the past decade, the growing field of microsampling has changed the way bioanalysis and preclinical studies are conducted. A variety of microsampling techniques have been adopted by the pharmaceutical industry and embedded into preclinical workflows. A technique known as solid phase microextraction (SPME) offers a distinctive advantage of measuring free drug concentrations within living organisms without the need for blood withdrawal. Despite its promise and potential advantages, SPME has not been extensively explored for preclinical use within the pharmaceutical industry. In this research, the application of SPME for quantitative bioanalysis and toxicokinetics was investigated for the first time within a pharmaceutical setting. This was performed through parallel *in vitro* and *in vivo* experiments.

Initially three test compounds were selected (metoprolol, propranolol and diclofenac) and LC-MS/MS methods were validated for all three. These were employed throughout the project to support quantitative analysis during the SPME *in vitro* and *in vivo* evaluation. SPME fibre blood exposure profiles and desorption profiles were constructed for the three tool compounds and parameters such as the impact of hematocrit levels, the effect of blood flow rate and on-fibre stability were investigated *in vitro*. SPME was then implemented *in vivo*. Practicalities of inserting the SPME fibre into the veins of animals was assessed using anesthetised rats and fibre blood exposure times were also determined during this first *in vivo* experiment.

Since SPME measures free drug concentrations, its potential benefits as a tool to determine protein binding values of drugs were examined and compared to a gold standard approach for protein binding experiments known as rapid equilibrium dialysis (RED). The three tool analytes were studied as they cover a range of plasma protein binding levels (~ 30 - 99%) at three different physiologically relevant concentrations (10, 100 and 500 ng/mL). This was followed by an *in vivo* experiment to identify whether SPME measures free drug concentrations in conscious rats. *In vivo* SPME samples were compared with whole blood samples withdrawn from the same rats and analysed using the RED device. A full toxicology study was subsequently conducted in conscious rats for seven days to mimic a typical preclinical rodent study. SPME was compared with conventional caudal venipuncture whole blood sampling for generating toxicokinetic data. The impact and biocompatibility of SPME was studied through pathological endpoints and using an Irwin behavioural study.

It was demonstrated that it may take up to 3 h for an analyte to reach equilibrium between the sample matrix and the SPME coating. This is not viable for *in vivo* applications due to ethical reasons and therefore pre-equilibrium conditions are more suited. Analyte desorption time of the SPME fibre was achieved between 15- 30 min. Levels of blood hematocrit had no impact on analyte response while blood flow rates may have an effect on analyte response and concentration. On-fibre stability was established for all three tool analytes for up to six weeks.

It was found that consistent results were obtained by SPME when measuring protein binding values of all three analytes across three concentrations. The percentage difference between protein binding values determined by SPME and RED was within recommended limits for bioanalysis (<15 %) across all analytes and concentrations. The time required to obtain plasma protein values using SPME was considerably quicker than by using the RED device (1 h compared to 8 h). It was demonstrated that SPME provides a compelling alternative platform for the efficient generation of high quality plasma protein binding values.

Pre-equilibrium conditions illustrated that using 2 min fibre exposure to systemic circulation was sufficient to produce reliable quantitative analysis. However, it was noted that current C18 fibre coatings did not detect metoprolol metabolite which exhibits a polar moiety. Mixed phase fibre coatings are required for metabolic analysis. The potential capacity of SPME to generate meaningful toxicokinetic data of free drug concentrations was shown. Biocompatibility of SPME was established by comparing pathological endpoints observed between SPME sampled and control rat groups.

Finally, a novel approach was described for quantitative bioanalysis by direct SPME-MS. SPME was coupled to a mass spectrometer to enable direct elution of analytes from the SPME fibre onto the MS. This was characterised with two test analytes, metoprolol and propranolol, spiked into control rat blood. The data indicated the significance of this approach to enable rapid, selective and highly sensitive (10 ng/mL lower limit of quantification) qualitative and quantitative chemical analysis.

Overall this research demonstrated that SPME could potentially provide a compelling alternative microsampling platform for preclinical studies.

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List of Abbreviations

AIDS	Immune Deficiency Syndrome
ALB	Albumin
ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
ANP	Atrial Natriuretic Peptide
APTT	Activated Partial Thromboplasin Time
AST	Aspartate Aminotransferase
AUC	Area under the Curve
BAS	Basophils
BCS	Biopharmaceutics Classification System
BEH	Ethylene Bridged Hybrid
CA	Calcium
CHOL	Cholesterol
CM	Capillary Microsampling
CREA	Creatinine
Crl:WI(Han)	Charles River : Wistar Han
CV	Caudal Venipuncture
CW-TPR	Carbowax-templated resin
CYP	Cytochrome
DAPCI	Desorption Atmospheric Pressure Chemical Ionization
DART	Direct Analysis in Real Time
DBS	Dried Blood Spots
DESI	Desorption Electrospray Ionization
DMF	Di-Methyl -Formamide
DMPK	Drug Metabolism and Pharmacokinetics
EBF	European Bioanalytical Forum

ECG	Electrocardiogram
EDS	Energy Dispersive Spectrometry
EDTA	Ethylene-Diamine-Tetraacetic Acid
EMA	European Medicines Agency
EOS	Eosinophils
ES	Electrospray
FDA	Food and Drug Administration
FIB	Fibrinogen Content
FOB	Functional Observation Battery
FTIH	First Time in Human
GC	Gas Chromatography
GCP	Good Clinical Practice
GLDH	Glutamate Dehydrogenase
GLP	Good Laboratory Practice
GLU	Glucose
GSK	GlaxoSmithKline
HB	Haemoglobin
HFBA	Heptafluorobutyric Acid
HPLC	High Performance Liquid chromatography
HRT	High Reticulocyte
HTC	Hematocrit
ICP	Inductively Coupled Plasma
IND	Investigational New Drug
IPA	Isopropyl Alcohol
IS	Internal Standard
ITS	Integrated Telemetry Systems
IUPAC	International Union of Pure and Applied Chemistry

IV	Intravenous
KRB	Krebs-Ringer Biocarbonate
LC	Liquid Chromatography
LLE	Liquid-Liquid Extraction
LLQ	Lower Limit of Quantification
LRT	Low Reticulocyte
LUC	Leucocyte Count
LYM	Lymphocytes
MALDI	Matrix Assisted Laser Desorption
MCH	Mean Cell Haemoglobin
MCHC	Mean Cell Haemoglobin Concentration
MCV	Mean Cell Volume
MON	Monocytes
MRM	Multiple Reaction Monitoring
MRT	Medium Reticulocyte
MS	Mass Spectrometry
MSD	Meso Scale Discovery
NC3Rs	National Centre for Replacement, Refinement and Reduction of Animals in Research
NEU	Neutrophils
NOAEL	No Observed Adverse Effect
NQ	Not Quantifiable
NSAID	Non-Steroidal Anti-inflammatory Drugs
PA	Polyacrylate
PAN	Polyacrilonitrile
PD	Pharmacodynamics
PDMS	Poly-Di-Methyl-Siloxane

PEEK	Polyetheretherketone
PEG	Polyethylene Glycol
PHOS	Inorganic Phosphorus
PK	Pharmacokinetics
PLT	Platelet Count
PP	Protein Precipitation
PPB	Plasma Protein Binding
PPY	Polypyrrole
PT	Prothrombin Time
QC	Quality Control
QToF	Quadrupole Time- of-Flight
RBC	Red Blood Cells
RBCR	Red Blood Cell Count
RDWR	Red Blood Cell Distribution Width
RETA	Reticulocytes
RED	Rapid Equilibrium Dialysis
SIL	Stable Isotope Labelled
SPE	Solid Phase Extraction
SPME	Solid Phase Micro-Extraction
TBIL	Total Bilirubin
TK	Toxicokinetics
TRIG	Triglycerides
UPLC	Ultra Performance Liquid Chromatography
UREA	Urea
VAMs	Volumetric Absorptive Microsampling
WB	Whole Blood
WBC	White Blood Cell Count

Chapter 1

Introduction

1.1 Modern Drug Discovery and Development Processes

The roots of the modern era of drug discovery and development lie back in the 19th century. Although human civilisation has been experimenting and consuming drugs for many centuries, it is only in the past hundred years that the foundation was laid for the systematic, industrial research and development of drugs and medicines. The industrialization of pharmaceutical development has revolutionised medical therapy in terms of identifying new drug targets, synthesising active drug candidates and ensuring drug safety and efficacy for human use [1].

Today, the task of developing new drugs is a lengthy process taking 10-15 years for a drug to progress from the research lab to the patient (Figure -1.1-). The average cost to identify and develop each drug that reaches the market is estimated to be between 800 million to 1 billion dollars[1]. This number includes the cost of unsuccessful NMEs (New Molecular Entities) that do not reach the market. Around 97% of preclinical candidates fail prior to reaching phase I trials[2]. In the early 1990's this lower rate of success could be accounted for by common causes such as physiochemical properties and bioavailability, but by the year 2000, these had been reduced and the main issues became safety and lack of efficacy during both pre-clinical and clinical phases[2]. Attrition is further contributed to the complexity of diseases, enhanced standard of care and the high expectations and demands from regulatory authorities. Testing for potential liabilities includes focusing on various drug metabolism and pharmacokinetic characteristics and paying particular attention to toxicity of major organ systems. A recent review of reasons for drug attrition identified cardiovascular toxicity and hepatotoxicity as the major contributors to drug attrition prior to clinical studies[3]. The current focus in the pharmaceutical industry is to exclude undesired compounds at the early stages of the discovery and development process rather than project closures at clinical phases which have a detrimental impact on resource.

The drug discovery and development process for small molecules can be divided into three major steps; discovery, preclinical development and clinical development. Early stages of drug discovery begin with understanding the disease or the clinical condition. Usually an

unmet clinical need is determined, and this is followed by target identification and choosing a biochemical mechanism which is putatively amenable to an interaction with a drug molecule. A target is a biological entity such as a gene or a protein, an intracellular enzyme or an extracellular receptor that has an affinity to bind to a drug i.e. a "druggable" moiety[4]. Once identified, the target is validated and assays are developed to enable characterisation of novel compounds. Such assays need to have a robust signal change with a measurable activity indicating compound potency. Transgenic animals and knockout studies form an attractive validation tool where gene manipulation is used to generate a range of phenotypic endpoints[1]. Designer mice are created with a targeted gene mutation to determine the *in vivo* function of various genes. Characteristics of mutant animals are identified in comparison to normal controls[4]. While transgenic and knockout models offer important scientific platforms to define the potential action of drugs, the cost of generating such unique species is an expensive process and therefore it is crucial to determine as much information as possible from these genetically engineered animals. The approach has always suffered from the strict regulations on the availability of blood volumes, terminal blood samples are usually collected to study various parameters[5,6].

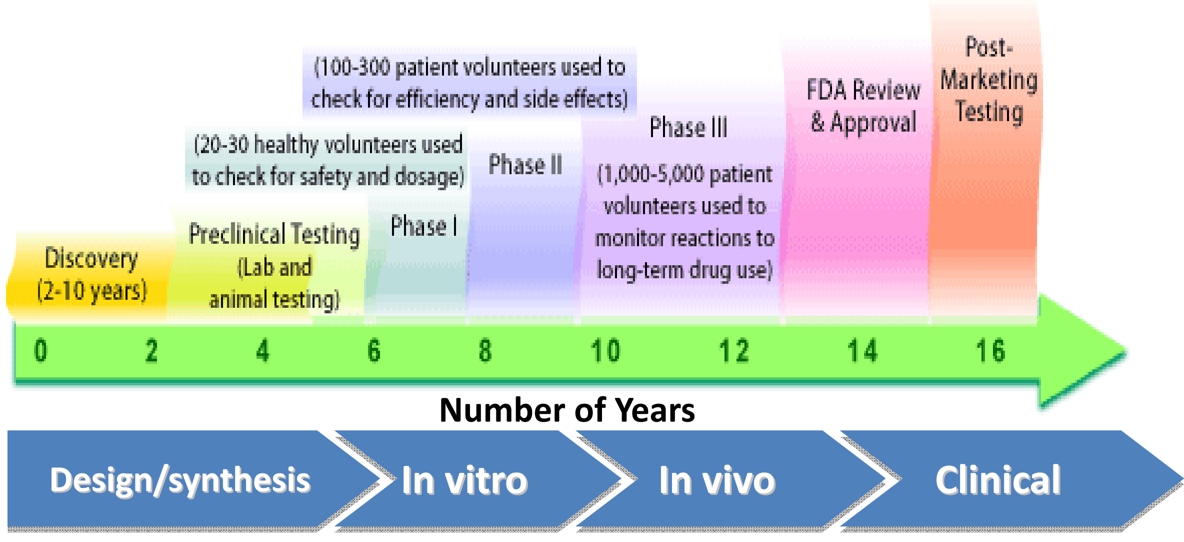


Figure -1.1- The drug discovery and development process [7]

Lead identification is the next stage of drug discovery where a large collection of molecules, being a chemical library of either small molecules or larger biologicals, undergo high throughput screening and a lead is selected based on the physiochemical characteristics,

binding properties to the putative target and the ability to elicit a biological response[4]. Thermodynamic evaluation of the mechanisms of interaction and the subsequent functional effects are assessed to give a better understanding of the mode of action of the active compound.

Lead optimisation is the final phase in the drug discovery process. The aim of this is to modify the lead structure to improve deficiencies in the pharmacodynamic (PD), pharmacokinetic (PK) or biopharmaceutical behaviour of the drug. Structure-activity relationship (SAR) and structure-kinetics relationships are evaluated to enhance various characteristics such as solubility, permeability, bioavailability and clearance[8]. In general, molecules are assessed using mechanistic *in vitro* screens to understand potential genotoxic liabilities, such as the Ames test which employs bacteria to identify the mutagenic potential of the chemical entity and its carcinogenic tendencies. Neurotoxicity behaviour is evaluated using *in vivo* models such as the Irwin test and metabolic profiling is carried out using repeat dosing PK. Other PK/PD studies are designed to test dose linearity and maximum tolerated dose[9].

Surviving molecules which meet the challenges of preliminary pharmacology and toxicology testing often become suitable for drug candidate selection. Once a lead candidate is identified and declared as a preclinical candidate, it enters preclinical development, a stage which encompasses studies that link laboratory drug discovery with clinical trials in human subjects.

Discovery work continues to find potential back-up molecules from the same series or other follow-up series which exhibit similar properties. This strategy ensures a flow of the pipeline in case candidate leads fail during preclinical development or clinical phases.

Following evaluation of candidates in preclinical animal models, an investigational new drug (IND) application is filed to the regulators such as the Food and Drug Administration (FDA) to ensure that the preclinical package supports the plan for phase I clinical trials[4].

Research in human subjects adheres to the principles of good clinical practice (GCP), regulations for the conduct of clinical trials, and compliance with these regulations is monitored by the FDA and the European Medicines Agency (EMA) (for conduct of trials in Europe). Clinical trials are categorized into four distinct phases I-IV investigating the safety and efficacy of the drugs by measuring certain outcomes in the participants at each stage of the trials. Phase I is the initial safety trial also known as first time in human study (FTIH) to establish a tolerated dose range in healthy volunteers using a single and multiple dose

escalation intervention. Phase II clinical trials are larger studies which include 100-300 subjects to explore efficacy and safety in selected populations of patients. The objective of this is to demonstrate proof of concept and show evidence of disease prevention or treatment in the target population[10].

Phase III trials are pivotal studies performed to support drug registration and licence to market. Bioequivalence studies comparing the bioavailability of the final manufactured product to existing treatments are monitored in large scale (1000-3000 patients). Long term safety and potential side effects are usually examined at this stage; the new product is required by the national regulatory authorities to demonstrate significant advantage over other approved treatments to progress into the market. Following drug approval, phase IV is usually conducted to gather information on the drug effects in various populations and side-effects associated with certain populations or with long-term use[10].

1.2 Toxicity Testing, Pharmacokinetics and Toxicokinetics

Rigorous testing is carried out to refine chemical and biological characterisation during the early stages of the development process but safety and toxicity are determined as part of the preclinical package to support clinical studies. Rodent and non-rodent models are used to delineate the PK profiles and toxicokinetic (TK) parameters prior to the conduct of human clinical studies[11].

Preclinical PK studies are usually conducted during early phases of drug discovery, the major emphasis of such studies being to obtain information on the absorption, distribution, metabolism and excretion (ADME) of the drug and to compare it to other candidate molecules. PK evaluation typically comprises of one day studies using suitable doses to provide preliminary data for pharmacologic or therapeutic events and hence, give an appreciation of the drug's PK-PD relationship.

In contrast, the discipline of TK is executed during preclinical development phases. Regulatory guidelines specify the need for pharmacology and toxicology information as part of the IND application submitted by sponsors to illustrate that the product is reasonably safe prior to initial use in humans. This section includes a description of the mechanism of drug

action in animals and also requires an integrated summary of the toxicologic effects of the drugs i.e. the adverse effects in animals and *in vitro*[12].

The International Conference of Harmonization (ICH) has also noted "the need for TK data and the extent of exposure assessment in individual toxicity studies"[11]. TK evaluation forms an integral part of the pharmaceutical safety assessment; it is defined as the generation of PK data either as an integral part in the conduct of non-clinical toxicity studies or in specially designed supportive studies. TK evaluation provides a detailed description of the systemic exposure and its relationship to toxicologic dose levels and the time course of the toxicity study [13]. Such information defines the potential of a compound to cause adverse effects and help in assessing the disposition and accumulation capacity upon repeated dosing [14].

The generation of exposure data in the animal species and its relationship to the administered dose is an FDA requirement and is well defined in the FDA guidelines for industry "...exposure to the parent substance and its major metabolites should be similar to or greater than that achieved in humans when such information is available"[12]. "Carefully designed and conducted *in vivo* studies allow evaluation of parent substance and metabolites and can enable estimation of safety margins"[13].

Selection of suitable toxicological species and dosage forms, as well as evaluation of between sex effects and inter-animal variability needs to be established and these data are used to set limits for clinical exposure and to calculate safety margins. Depending on the therapeutic indication of the drug being developed, a ratio of systemic exposure between animals and humans may be required to help predict the first clinical dose and provide a 'comfort zone' in assessment of risk prior to conducting clinical trials [16].

Various animal toxicity studies including single dose studies, repeat dose toxicity studies, safety pharmacology, reproductive, genotoxicity and carcinogenicity studies are typically performed and supported by TK measurements [12]. Table -1.1- shows a selection of the common toxicity studies performed in mice, rats and dogs. Other species such as mini-pigs, rabbits and monkeys are also utilised depending on compounds and indications.

TK measurements of the test drug and where appropriate metabolites, are usually made at appropriate time points during the course of individual studies. These measurements usually consist of plasma, whole blood or serum concentrations, where common parameters such as area under the concentration vs time curve (AUC) and maximum concentration (C_{max}) are

most commonly used to calculate exposure data. The AUC i.e. the area under a concentration versus time curve is a measure of the total drug exposure while C_{max} indicates the maximum concentration of compound observed in the matrix of interest. Other parameters such as clearance (the volume of fluid from which the drug is completely removed per unit time) and half-life (the time it takes for the concentration of compound to decrease by half) are also important indicators that are sometimes measured as part of TK studies. TK is generally integrated within toxicity studies, however such data can be generated in either all, or a representative subset of animals. For example, satellite groups may be used in the case of smaller rodents. Satellite animals are additional animals dosed as per protocol, but not subjected to toxicological and pathological observations and tests. Instead, they are used exclusively for the evaluation of PK characteristics of the test compound in blood, tissue or other body fluids[17]. The number of satellite animals commonly used is around 50 - 60% of the number of animals used in each main toxicity study[17].

This approach is necessary due to strict regulations on the availability of blood volumes; UK Home Office guidelines allow only 10% of the circulating blood volume to be sampled. Each sample size usually consists of 0.25 - 0.50 mL day^{-1} in rodents and up to 2 mL day^{-1} in non rodents[13]. These are taken at a series of time points after dose administration, typically from shortly after dosing up to 24 hours. The matrix for determining drug concentration could be blood, plasma or serum, however plasma samples have traditionally been used for generation of TK data due to the difficulties associated with handling whole blood [18]. This requires relatively large volumes (up to 500 μL) of blood in order to produce the required volume of plasma for analysis.

In rodent studies, 500 μL represents a relatively large volume of blood, the removal of which could cause anaemia or other secondary effects such as bone marrow and haematological changes which could confound interpretation of primary drug biological effects[14]. For this reason, blood samples are often taken from satellite animals which are employed for TK analysis only. The major drawbacks of this design are the large number of animals required to provide adequate information on both toxicology and PK data, and the inability to correlate toxicological effects with drug concentrations observed in the same animal, because pathology or functional effects are measured in the main study animals, while drug exposure is measured in TK satellite animals [19].

Table -1.1- Examples of toxicity study types performed during drug development

Species	Study Type	Non-GLP or GLP*
Mouse	7 Day Dose Range Finding	Non-GLP
	14 Day Repeat dose	GLP
	3 Months Repeat Dose	GLP
Rat	7 Day Candidate Selection	Non-GLP
	7 Day Dose Range Finding	Non-GLP
	Single Intravenous (IV) Dose	Non-GLP
	1 Month Repeat Dose	GLP
	4 Day IV study	GLP
	3 Months Repeat Dose	GLP
	6 Months Repeat Dose	GLP
	2 Years Carcinogenicity	GLP
Dog	Single Escalating Dose (Maximum Tolerated Dose)	Non-GLP
	7 Day Dose Range Finding	Non-GLP
	1 Month Repeat Dose	GLP
	3 Months Repeat Dose	GLP
	6 Months Repeat Dose	GLP
	2 Years Carcinogenicity	GLP

*GLP = Good Laboratory Practice is a set of principles intended to assure the quality and integrity of non-clinical laboratory studies regulated by government agencies. Non-GLP studies do not require the entire rigor of GLP studies.

1.3 3Rs and Microsampling

Although the use of *in vivo* experiments is essential to understand the fundamental mechanisms underpinning biological behaviour during drug development in the industry, particular attention must be paid to animal welfare. Strict regulations govern the use of animals in research to ensure that animals are only used when necessary. Russell and Burch proposed a 3Rs concept in 1959 for ethical principles; the replacement, refinement and reduction of animals in research[15]. Replacement refers to techniques that can avoid or replace the use of animals such as computer modelling and use of animal cell lines. Refinement involves the use of scientific techniques that decrease levels of potential pain, suffering or distress caused to the animal with a view to ultimately improve animal well-being [21].

Reduction, on the other hand, is defined as obtaining equivalent amounts of information using fewer animals, or generating more data from the same number of animals[13].

The idea gained significant momentum during the 1980s when governments, academia and industry became more involved. However, it was only when a UK government sponsored scientific organisation named the National Centre for Replacement, Refinement and Reduction of Animals in Research (NC3Rs) was established in 2004 to support animal research in the UK through application of the 3Rs principals, that the 3Rs developed a widespread interest and had a growing recognition of benefits. Since then, the NC3Rs has collaborated with the pharmaceutical industry, being one of the major users of animals in research, to identify and develop opportunities to minimize animal use and apply a range of 3Rs programmes to pharmaceutical strategies[15].

This has resulted in the development of new ways that allow for the use of animal in research to be replaced, reduced and refined without compromising the drug development process, regulatory requirements, or human safety.

One strategy that has been a focus point for this partnership is the use of microsampling techniques to assess drug levels in biological matrices. Microsampling refers to the collection of very small sample volumes, typically less than 50 µL[16] for the determination of drug concentrations and other biological entities such as biomarkers. The sample volume required for analysis is dictated by the analytical method, which is developed based on the expected level of exposure and the required lower limit of quantification. Highly sensitive methods are required to achieve lower limits of quantification (in the pg/mL to ng/mL range) to measure very low levels of the substance. Such methods have often involved the use of large sample volumes. However, advances in bioanalytical technology, specifically the sensitivity of detection techniques (e.g. mass spectrometry) have enabled the use of a decreased sample volume without a detrimental impact on the analytical range.

The method of sampling from a living organism, together with the method of bioanalysis form important pillars to obtaining reproducible and good quality data. For this reason, approaches that interfere minimally with the investigated organism are highly desirable.

Microsampling techniques for blood and plasma analysis is a paradigm shift for bioanalysis and a leap forward for toxicology and so it has recently been adopted by many

pharmaceutical companies. A recent survey demonstrated that almost 81% of 33 European based pharmaceutical companies are using microsampling techniques in their PK studies[17].

Improvements in both ethical and scientific aspects including of study design have been associated with the use of microsampling techniques. Small sample volumes allow serial/repeat sampling from the same animal, eliminating the need to use composite bleeds where timepoints are collected from several animals to obtain a profile over the full time course. In rodent TK studies, microsampling strategies facilitate the removal of satellite groups as shown in Table -1.2-.

Blood sampling can occur directly from the main study rodents without impacting clinical parameters or toxicological endpoints[18]. This not only helps to reduce animal usage in toxicology studies, it also enhances the quality of data generated by providing a direct correlation between drug exposure and toxicity observed in the same animal which in turn will enhance the reliability of the results and eliminates inter-subject variability[17]. Taking smaller volumes of blood avoids the need to pre-warm animals which is routinely performed in conventional analysis to encourage blood flow and collection of the necessary large blood volumes to generate plasma samples[19,20]. Microsampling consequently offers refinement and decreases levels of animal distress which causes fewer disturbances to critical physiological parameters such as heart and respiratory rates. Another significant benefit of microsampling is enabling juvenile toxicokinetic and paediatric studies where small blood volume is crucial as well as clinically facilitating studies to be performed in developing countries[21]. Analysis of rare matrices such as tears and other translucent fluids is another area where microsampling is of great interest. Furthermore from a business perspective such techniques provide potential advantages to cost reductions in terms of reduced compound and resource (dosing, handling and care) requirements, easier methods of sample shipping and storage as well as notable cost saving in the number of animals[22].

Table -1.2- Potential for reduction in animal numbers if study designs use microsampling techniques

Study Type	Conventional design with satellite animals	Microsampling design	Animal reduction
Dose Range Finding –rat	3M + 3F per group, plus 3M + 3F per group (dose level) for TK sampling	3M + 3F per group	50%
	Typical numbers = 48	Typical numbers = 24	
One month GLP toxicity study - rat	10M + 10F per group plus 3M + 3F per group for TK sampling	10M + 10F per group	23%
	Typical numbers = 104	Typical numbers = 80	
Three month GLP toxicity study mouse	10M + 10F per group plus 6M + 6F per group for TK sampling at beginning and end of study	10M + 10F per group	50%
	Typical numbers = 158	Typical numbers = 80	

Study design comparison between conventional studies and microsampling studies for rats during drug development. The number of male (M) and female (F) main study animals and satellite animals are shown. The reduction in animal use ranges from 23% to 50% depending on the numbers of satellite animals used and this differs between organisations and studies[17].

1.4 Types of Microsampling Techniques

Over the past few years, the benefits of microsampling in both nonclinical and clinical environments have been recognized throughout the pharmaceutical industry. In response, a number of microsampling techniques have recently been established and employed in the drug development process including dried blood spots (DBS), capillary microsampling (CM), plasma separation capillary (PSC) and volumetric absorptive microsampling (VAMs).

The main focus for microsampling techniques has been within the non-GLP arena as shown in Figure -1.2-. Due to the accumulation of evidence that microsampling can increase the amount of nonclinical safety information available[17], improve validity of those data by linking toxic effects to drug exposure in individual animals and that microsampling represents the most significant opportunity to reduce animal use in toxicology studies, more and more companies across the pharmaceutical industry have started to adopt such techniques. For this reason, the number of miscrosampling techniques has increased over the

past number of years which is starting to shape the future of regulatory within safety assessment and bioanalysis.

Although there is now a widespread use of microsampling in discovery and early pre-clinical studies, the diffusion of this extensive use has been limited in regulated studies[21]. Despite the encouraging feedback received from regulators for the use of microsampling in GLP studies, there still is a general perception in the industry that regulatory acceptance issues could still be a barrier to adoption. Employing new technologies in regulated studies requires scientists to file an educational component to build and support the innovation. This is usually established through collaborations and by building a working relationship between the vendors, scientists and regulators with a view to showcase the benefits of a technique if established data confirms that the benefit to risk ratio is favourable[23].

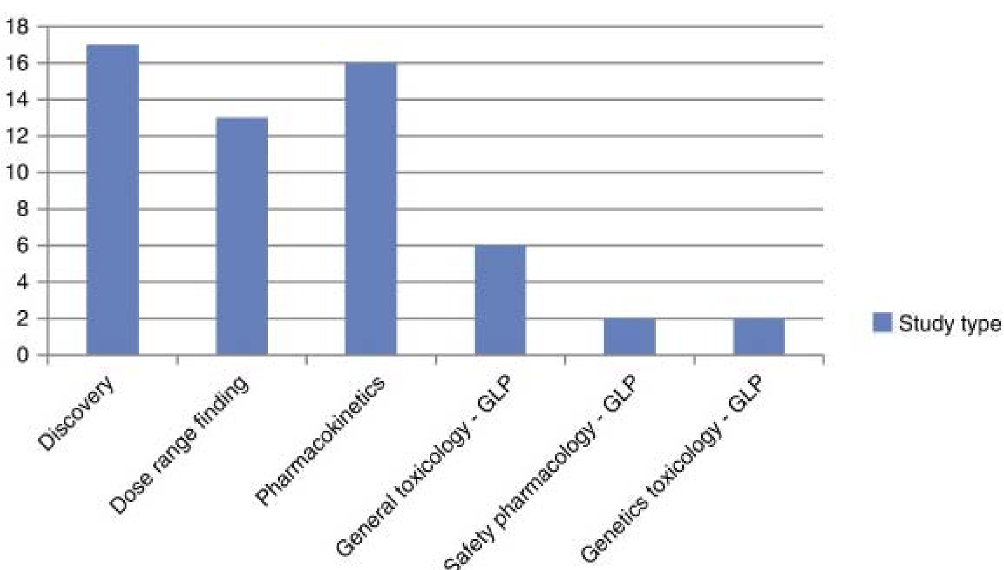


Figure -1.2- The number of companies out of 22 pharmaceutical companies, employing microsampling in non-Good Laboratory Practice (GLP) (discovery, dose range finding and pharmacokinetics) and GLP (general toxicology, safety pharmacology and genetic toxicology) studies[17].

1.4.1 Dried Blood Spot (DBS) Analysis

Dried blood spot analysis has been known for more than five decades, originally used for newborn screening and subsequently re-introduced in 2008 for bioanalytical support of drug development[24]. DBS provided a major breakthrough in the bioanalytical field, used by various pharmaceutical companies to support pre-clinical toxicokinetic studies and pharmacokinetic clinical phase I, II and III studies [24]. The technique relies on utilising filter paper or adsorbent cards onto which small volumes of blood (10 µL -20 µL) from animals or humans are spotted. Cards are air dried, stored and subsequently shipped to the analytical site. Circular discs are punched out of the DBS and the analyte is extracted using appropriate solvents (Figure -1.3-). DBS conveys many microsampling benefits particularly when coupled with sensitive and selective HPLC-MS/MS techniques [25].

Figure -1.3- Dried Blood Spots[25], image on the left showing sampled blood being applied onto a DBS card and image on the right showing punching of blood spot for analytical determination.

GlaxoSmithKline (GSK) and many companies consequently chose to utilize DBS sampling as the technique of choice for the evaluation of TK and PK for all new oral small molecule drugs selected as drug development candidates. A large body of high quality research was published and presented supporting the use of this technique[26-29].

Despite its many proven advantages, DBS has been challenged by regulators for its hematocrit effect limitations[30,31]. Hematocrit is a measure of the volume of blood occupied by red blood cells. Blood samples from various preclinical animals as well as clinical human

samples have a range of hematocrit levels[32]. This variability causes changes in blood viscosity which in turn leads to differences in blood diffusion on the card used for DBS sampling. This has a direct impact on the size of blood spot formed and will therefore affect the accuracy of analytical results [32]. This led to a period of reassessment of the implementation of DBS in the quantitative arena[33]. For this reason the quest for new microsampling techniques providing practical alternatives to DBS and maintaining the benefits of the 3Rs in animal use began[30,32].

1.4.2 Volumetric Absorptive Microsampling (VAMs)

With a view to overcoming the hematocrit issues associated with DBS, various approaches have been employed for a potential path forward for DBS. Most of these solutions rely on spotting an accurate volume of blood onto the sample collection card and subsequently sampling the entire spot; unfortunately, an aspect which current technologies could not readily support at the point of collection. For this reason a novel dried blood sampler, termed volumetric absorptive microsampler and marketed as “Mitra” has been designed and recently tested as described by Denniff *et al*[28] for collection of dried blood samples.

The VAMs sampler consists of an absorbent tip attached to a plastic handle as shown in (Figure -1.4-), the polymeric tip wicks up a fixed volume of blood (approximately 10 µL) upon exposure to wet blood, regardless of the blood hematocrit. In order to avoid overfilling, the tip of the sampler is not completely submerged into blood and the device is held at an angle to allow the tip to touch the surface of the blood pool. The wet tip is then left to dry for approximately 2 hours and is removed for desorption with an appropriate solvent[29].

This technique has the potential to displace DBS since it retains the microsampling advantages without the issues associated with haematocrit however it is notable that these benefits may not necessarily translate into the bioanalytical laboratory[34]. This is due to current lack of automation in addition to the complexity of method development and validation of the technique.

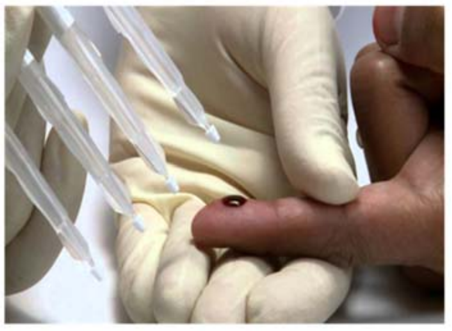
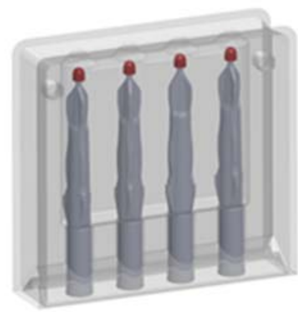



Figure -1.4- Volumetric Absorptive Microsampling[28]. Image on the left showing blood specimen collection and image on the right showing the clamshell packaging of the device to ensure sample protection.

1.4.3 Blood Capillary Microsampling (CM)

Reverting back to the use of wet sampling with reduced blood volumes rather than a dried matrix has received much recent attention within the pharmaceutical industry. A technique consisting of capillary microsampling allows the collection of a predefined volume of blood (< 35 μL) in a microcapillary[35]. A glass capillary coated with EDTA is filled end to end by capillary action (Figure 1.5); the filled capillary is placed inside a sample tube where the sample is washed out by an appropriate extraction solvent[30]. The diluted sample is then processed using conventional extraction techniques to measure drug concentrations. The bioanalytical method for CM samples is very similar to the validation of traditional large-volume methods. Multiple studies have been reported on the application of capillary samples used for analysis of samples from rodents, all of which indicate the robustness of the technique[30,31]. CM maintains the benefits of microsampling in addition to other important qualities such as stabilization of unstable compounds through fast sample collection and immediate addition of extraction solvent.

The drawback however is the difficulty of blood handling and the higher demand for bioanalytical methods to reach lower limits of quantification for lower systemic exposures, for which such low volumes of blood pose a hurdle. This technique also requires frozen sample shipment and storage.

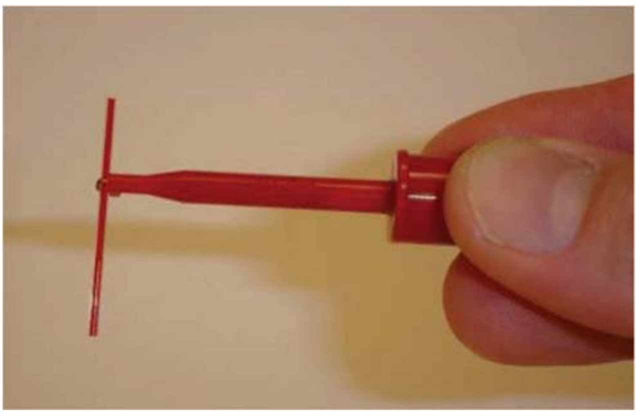


Figure -1.5- The exact blood volume collected in an EDTA-coated glass capillary micropipette, handled with a capillary holder[35].

1.4.4 Plasma Separation Capillary

Although blood is an acceptable matrix for exposure determination, plasma remains the matrix of choice among pharmacokineticists. This is due to the fact that unbound fractions of drug are usually determined in plasma, though conversions from blood to plasma can always be obtained through blood/plasma ratios established *in vitro*[36]. Also, plasma is considered easier to store and analyse.

A relatively new microsampling technique for wet plasma has been investigated and implemented in preclinical settings within the pharmaceutical industry[37]. The technique involves microcapillary tubes specifically designed to separate plasma from whole blood. Each EDTA treated capillary contains a thixotropic gel located in the middle of the empty tube with one end fitted with a self-sealing plug which swells to prevent "blow out" when centrifuging. Microvolumes of whole blood (< 75 µL) are withdrawn through capillary force and upon centrifugation, the gel provides a stable barrier between erythrocytes microvolumes of plasma (< 35 µL) based on density. Aliquots of plasma are dispensed with a prototype device (Drummond Scientific Co., Inc.; PA, USA) inserted into the capillary tube by pushing the plug end to eject the plasma out (Figure -1.6-). The recovered plasma is then stored frozen until it is received at the bioanalytical laboratory[33].

Figure -1.6- Novel capillary tube in carious process steps (A) empty capillary tube, (B) Blood filled capillary tubes, (C) centrifuged capillary tubes showing plasma separation and (D) Capillary tube filled fitted into a Micronic tube for centrifugation[17].

Following several successful implementations of the technique described by Jonsson and co-workers[38] as well as Bowen *et al.*[33], the utility of this technique for regulated preclinical studies and clinical applications is currently under investigation across several pharmaceutical companies.

Despite the notable shift towards using smaller volumes with recently established microsampling techniques, bioanalysts have not been able to completely move away from, or avoid using blood withdrawal. The quest for finding a "magic bullet" that can address both sampling and sample preparation issues has led to the development of a novel technique that has recently been applied to the field of *in vivo* bioanalysis. The technique moves 3Rs innovations a step further and allows for analyte extraction without the need for a defined sample volume, it also combines sampling, sample preparation and extraction in one step. This exciting concept is known as Solid Phase Microextraction (SPME)[39].

1.5 Solid Phase Micro-Extraction

Solid Phase Micro-Extraction, first established in the early 1990s by Pawliszyn at the University of Waterloo/Canada is a novel and effective sampling and extraction technique. It was introduced to fill several gaps in the current analytical approaches by addressing numerous issues, such as the need to reduce the volume of sample used and the size of typical extraction instrumentation to facilitate rapid and suitable laboratory and direct on-site analysis [40].

The technique is based on the use of a small amount of extracting phase dispersed on a solid support which is exposed to the sample matrix for a defined period of time [41]. An equilibrium process takes place in which the analyte partitions between the SPME coating and the sample matrix. The amount of analyte extracted by SPME is directly proportional to the concentration of analyte present in the sample matrix [41].

SPME is often mistakenly considered another form of solid-phase extraction (SPE) or micro-SPE[41], however there are significant differences between the two methods. SPE utilizes a large sorbent bed and a relatively large sample volume in which analytes are exhaustively extracted from the sample matrix. A selective desorption approach is employed to wash away any unwanted endogenous analytes, leaving the analyte of interest retained on the stationary phase. An additional step is required to remove the analyte of interest from the sorbent by using an appropriate eluent[41].

SPME, on the other hand, is a non-exhaustive approach i.e. the technique does not extract the total amount of analyte in the sample. Analytes are extracted with minimal disturbance to the system using a very small extracting phase relative to the sample volume. The selectivity of the coating phase and partitioning equilibria of the analyte are important factors for SPME. Both features facilitate selective extraction of the desired analyte and provide efficient on-site clean up without the need for intermediate steps[41].

In addition, the miniature size and the geometric configuration of the SPME device prohibits adhesion of macromolecules onto the extraction particles and limits access of unwanted substances present in the sample matrix. This is significantly different to the nature of SPE instrumentation where a large volume of sorbent is used with a void volume which may aid retention of non-adsorbed matrix components and in turn impact efficiency of sample clean up[39].

1.5.1 Theory of Solid Phase Microextraction

SPME makes use of equilibrium extraction. In one approach, a partitioning equilibrium between the coating/stationary phase and the sample matrix is reached, while a second method uses short pre-equilibrium extraction times and the amount of analyte extracted is related to exposure time if agitation of the system is kept constant. Quantification is then relatively calculated based on timed accumulation of the analyte in the coating[42].

The first approach relies on the fact that maximal extraction is achieved when equilibrium is reached between the extracting phase and the sample matrix. Using the law of mass conservation, this equilibrium can be described by Equation 1.1[43].

$$C_0 V_s = C_s^{\infty} V_s + C_f^{\infty} V_f \qquad \text{Equation 1.1}$$

Where C_0 is the initial concentration of analyte in the sample, C_f^{∞} is the equilibrium concentration in the fibre coating, C_s^{∞} is the equilibrium concentration in the sample, V_s and V_f are the volume of sample and the fibre coating, respectively.

K_{fs} is the distribution coefficient of the analyte between the fibre coating and the sample matrix. It can be described by the following equation;

$$K_{fs} = \frac{C_f^{\infty}}{C_s^{\infty}} \qquad \text{Equation 1.2}$$

Equations 1.1 and 1.2 can be combined and rearranged as follows,

$$C_f^{\infty} = C_0 \left(\frac{K_{fs} V_s}{K_{fs} V_f + V_s} \right) \qquad \text{Equation 1.3}$$

The above can then be used to calculate the number of moles of analyte (n) extracted by the SPME fibre as shown in Equation 1.4 below,

$$n = C_f^{\infty} V_f = C_0 \left(\frac{K_{fs} V_s V_f}{K_{fs} V_f + V_s} \right) \qquad \text{Equation 1.4}$$

The amount of analyte extracted (n) is directly proportional to the concentration of the analyte in the sample (C_0). When the volume of the sample is greater than the capacity of the fibre, Equation 1.4 can be expressed as,

$$n = K_{fs} V_f C_0 \qquad \text{given that} \quad V_s \gg K_{fs} V_f \qquad \text{Equation 1.5}$$

Equation 1.5 indicates that analyte extraction is independent of sample volume when the fibre is exposed to a sample volume larger than the coating capacity. This means that SPME can be directly exposed to various matrices without the need to collect a defined sample volume, eliminating the sampling step facilitates direct exposure to ambient air, rivers, flowing blood, etc. This simplified equation further illustrates that the amount of analyte extracted is directly proportional to the initial concentration of the sample[43].

For solid sorbent coatings, the number of active sites or the total surface area available for analyte adsorption is proportional to the coating volume of the sorbent. However, high analyte concentrations can cause saturation of active sites, resulting in non-linear extraction performance. Furthermore, the presence of an endogenous analyte at a high concentration can cause competitive adsorption of interfering analyte and displacement of the target analyte[44].

1.5.2 Environmental Applications of Solid Phase Microextraction

SPME has been used in several different disciplines including environmental applications. To date, the majority of analytical techniques used to detect environmental materials are themselves key contributors to pollution and act as a source of significant contamination[41]. SPME has offered simple on-site analytical procedures to detect trace levels of matter from various environmental matrices such as soil, water and sediments[45,46]. Numerous procedures have been described for the use of SPME to measure volatile organic compounds produced by biogenic sources such as phytoplankton and macroalgae in the marine environment and the implications of identifying such contaminants on the marine ecosystem [46].

Extraction and purification of pesticide residues such as atrazine, clomazone and pendimethalin from highly complex media have been enabled by employing headspace-SPME without the need to consume large solvent volumes as it is the case with traditional solid phase extraction methods. Trace amounts have been pre-concentrated prior to detection using this selective and efficient technique[47]. SPME use has facilitated the provision of critical soil characterisation data in Turkey allowing the establishment of sanitary landfill sites with sufficient rehabilitation to reduce current risks of contaminants[45]. The bioavailability of highly toxic hydrophobic insecticides such as pyrethroids have been determined using SPME, quantitative analysis of bifenthrin, cyfluthrin, and fenpropathrin were performed using disposable polydimethylsiloxane (PDMS) SPME fibres inserted into

sediments at many locations in California (USA) to detect freely dissolved insecticides that pose a threat to aquatic organisms [48]. Identification of compounds responsible for critical odour components of dairy manure has been possible using headspace-SPME[49]. Such sensory characterisation has a significant impact on livestock operations associated with emissions of odour, gases and particulate matter [49].

Lately, new SPME devices have been designed for sampling live fish for pharmaceutical residues without the use of terminal procedures; the device is directly used on living fish without the need for anaesthesia[50]. This has enabled detection of trace compounds to which wild fish can be exposed from municipal wastewater which can have major implication on consumer's health.

Overall SPME coupled to High Performance Liquid Chromatography (HPLC), Gas Chromatography (GC) and Inductively Coupled Plasma Mass Spectrometry (ICP-MS) and more recently to HPLC-MS has been utilised to measure and verify various volatile and non-volatile environmental emissions.

1.5.3 Food and Fragrance Applications of Solid Phase Microextraction

In addition to environmental applications, SPME has been employed intensively in food analysis to evaluate nutritional content, impurities, additives and toxic contaminants. Headspace SPME-GC analysis of volatile compounds from 65 hybrid citrus fruits such as mandarin and clementine has allowed the identification of profound flavours and off-flavours, in turn permitting the production of hybridised fruits with enhanced taste and quality [51]. SPME analysis of aroma- active compounds from milk samples has shown that milk from starved cows was of a better flavour quality compared to milk from grass fed cows that had tainted off- flavour. SPME provided a simple and reliable analytical technique to enable characterization of milk flavours [52]. A number of fungicides and insecticides such as pyrimethanil, procymidone and pirimicarb have been extracted and pre-concentrated from tomato samples using (PDMS) SPME fibres to analyse pesticide content and verify their long term toxicity effects[53].

To date SPME has been applied to various aspects of food analysis including determination of flavour compounds in soya sauce, monitoring contaminants causing organoleptic defects

of wine [54], identifying food mutagens such as heterocyclic amines in cooked meat products and quantifying volatile compounds in dry fermented sausages.

This rapid and selective technique has also found its way into the fragrance industry where SPME has been used to analyse floral scents where volatile compounds emitted from flowers were collected on PDMS fibres and analysed by GC-MS [55]. Extraction of perfume compounds from shampoo (aqueous dispersion) and subsequent quantitative analysis have been reported using SPME, thus providing a fast efficient method for quantification with decreased matrix effects [56].

1.5.4 Forensic and Military Applications of Solid Phase Microextraction

SPME has become a useful tool in forensic laboratories, where it has been utilised to analyse various antidepressant and narcotic compounds in human plasma such as mirtazapine, citalopram, paroxetine and amphetamines, drugs that often cause fatal poising due to overdose [40]. Highly sensitive SPME assays have been developed to enable extraction and detection of fentanyl from human plasma, a very potent sedative drug used in surgical analgesia but commonly misused with a small dose known to cause sudden death [57].

Non-invasive SPME has also been used as a sampling device to identify volatile emanations from human skin to clarify fingerprint characteristics of human odours that could provide valuable and important biomarker information used to identify criminals and diagnose diseases [58].

In addition, SPME methods have been utilised for detecting organophosphorus compounds of nerve agents present in military arsenals [59]. SPME has enabled analysis of explosives used for military purposes, industries, mining and agricultural activities. The chemical nature of explosives can cause serious health hazards due to their carcinogenic and toxic character, therefore detection of trace level concentrations is vital and of great importance in forensic applications. SPME coupled to HPLC has provided reliable and accurate analysis of such chemicals allowing effective pre-concentration of explosives and efficient separation[60].

1.5.5 Preclinical and Clinical Applications of Solid Phase Microextraction

SPME has recently found its way into the field of *in vivo* analysis in which devices have been inserted into a variety of preclinical species such as mice, rats and dogs to quantify and monitor drug concentrations. The first *in vivo* study of determining drug concentrations in beagle dogs was reported in 2003, where full PK profiles of benzodiazepines were obtained directly from the peripheral vein without the need to withdraw any blood [61]. Since then SPME has been implemented in various animal PK studies, with several interfaces such as indwelling catheters and adapters designed and developed to enable and simplify the insertion of the device into animals, or small rodents [62] [63].

The technique has been further developed for clinical use where *in vitro* SPME has been applied to extract and quantify a diverse range of drugs from urine, plasma and blood samples such as use of direct immersion SPME method developed for the determination of polynuclear aromatic hydrocarbons in human blood [64] and determination of non-steroidal anti-inflammatory drugs (NSAIDs) using SPME coupled to liquid chromatography [65].

1.5.6 Solid Phase Microextraction for Bioanalysis in the Pharmaceutical Industry

Despite the diverse applications of this novel technique, SPME has not been extensively utilised within a pharmaceutical industry setting for the quantitative bioanalysis of drugs, metabolites and biomarkers.

A promising application of SPME, which distinguishes this method from other extraction and sample preparation techniques, is its applicability to *in vivo* and on-site sampling. SPME combines sampling, sample preparation and extraction in one step. It gives the results reflecting the real condition at the time of sampling, particularly important in the case of drugs and metabolites, which are characterized by low *in vitro* stability or fast turnover. The miniaturization of the sampling device dimensions (D = 45 μm, L = 1.5 cm coating phase) (Figure-1.7-), allows sampling from small animals, such as rodents, enabling the full PK profile of drugs to be obtained [66].

The first SPME device consisted of an optical fibre covered with a layer of polymeric material. This was eventually developed into a metal wire or blade coated with a

biocompatible polymer. The biocompatible polymer has been adapted and miniaturized to be housed inside a hypodermic needle which can be inserted directly into the animal and the fibre is then subsequently retracted into the needle for protection and transport (Figure -1.7-).

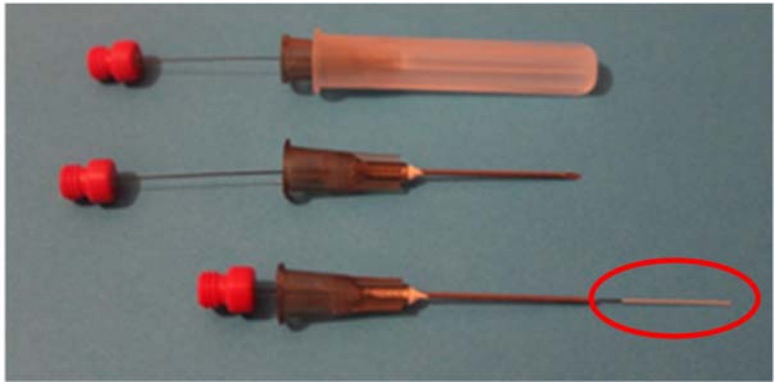


Figure -1.7- SPME Fibre housed inside a hypodermic needle. The coated phase is outlined in red[67].

Once the fibre is exposed to the sample matrix, the transport of analytes from the matrix to the coating begins immediately. SPME extraction is considered to be complete when the analyte concentration has reached distribution equilibrium between the sample matrix and the fibre coating[43]. The main advantage of direct *in vivo* SPME lies in the fact that it does not require any blood withdrawal, unlike almost all of the conventional methods where blood withdrawal is necessary to extract and quantify drug concentrations. For this reason, SPME promises the ethical and cost benefits of microsampling offering a positive future for the industry. At the same time, the technique provides numerous advantages to the analyst. The SPME device permits simultaneous sampling and sample preparation directly within the living organism of interest. Hence, reducing the overall number of sample processing steps. It eliminates the need for aliquoting sub-samples, centrifuging, freezing and defrosting of the sample which ultimately will provide increased speed and improved efficiency. The thin layer of biocompatible polymer of the SPME fibre prevents adhesion of large molecules such as proteins and phospholipids[41] providing effective sample clean-up, which in turn can reduce possible interferences and eliminates matrix effects. Ultimately, SPME could reduce overall analysis complexity and costs in terms of fewer animals and simplified procedures.

1.6 Aims and Objectives

The use of microsampling within the pharmaceutical industry has evolved over the last 10 years, leading to increasing use of small sample volumes for quantitative bioanalysis. In the quest for identifying new low volume sampling devices, significant attention has been drawn to SPME as it has all the attributes to be a new and exciting microsampling tool for the pharmaceutical industry.

This technique offers the potential for measuring free drug concentrations within living organisms without the need for blood withdrawal. Despite its promise and advantages, SPME has not been extensively explored or standardized for use within the pharmaceutical industry that deals with numerous samples at various stages of drug development. In this research, the feasibility of utilizing SPME as a microsampling technique within the pharmaceutical industry was investigated. The aim of this project was therefore to perform parallel *in vitro* and *in vivo* evaluation of the factors affecting SPME's use as a bio-microanalytical method, and to assess its potential applications and benefits for performing preclinical studies. The introduction detailed an overview of the fundamentals of microsampling techniques along with an insight to SPME and its current status and applications. The objectives of the project were thus as follows.

- Select appropriate test compounds based on factors that may impact SPME extraction, such as drug binding properties and develop LC-MS/MS methods to enable quantification of the chosen compounds.
- Use *in vitro* experiments to establish the essential parameters that may impact the extraction of small molecules by SPME. These factors include blood exposure profiles, desorption time profiles, the impact of hematocrit, the effect of blood flow rate and on fibre stability.
- Evaluate the use of SPME as a tool for measuring plasma protein binding values of drugs. Compare its use with a gold standard technique that is currently employed as a routine platform for assessing protein binding values.
- Investigate the *in vivo* application of SPME using anesthetised rats followed by live rodents, to understand the viability of the technique for *in vivo* use and to determine free concentrations from live animals.
- Design a toxicology study to determine analyte toxicokinetic profiles using SPME and perform a full tolerability assessment of the impact of SPME on animal stress

levels as well as clinical pathology endpoints. This will require comparison of SPME with a conventional sampling technique to identify the compatibility of SPME for preclinical studies.

- Investigate the feasibility of introducing a sample directly from the SPME fibre into the mass spectrometer to enable direct SPME-MS analysis without the need for off-line extraction and chromatographic separation. This will require building a SPME MS-inlet and testing the technique using *in vitro* samples to determine whether analytes can be desorbed off the fibre directly into the mass spectrometer.

Chapter 2

Selection of Test Compounds and Validation of Bioanalytical Methods

2.1 Introduction

An important aspect of the progression of drug discovery and development is the accurate quantification of drugs and endogenous components in biological samples. Bioanalysis is one of the few disciplines which is required throughout the entire drug discovery and development process. Bioanalytical methods are the keys to accurate toxicokinetic and/or pharmacokinetic assessment of drug candidates in support of regional or worldwide regulatory submissions[68]. The requirements for assay validation are clearly set-out in guidance documents issued by regulatory agencies[12,69]. Bioanalysis in support of drug discovery is not routinely operated to regulatory guidance, or to the exacting standards of GLP and Good Clinical Practice (GCP), as the data is used for internal decision making by the innovator company and is unlikely to be included in any regulatory submission[4]. Hence, suitably validated generic methods are often used for these studies. However, at the drug development stage, rigorous bioanalytical method validation is required to support GLP toxicology and clinical studies[12,70]. Bioanalytical method validation is performed to demonstrate whether a particular method is fit for purpose when determining an analyte concentration within a specific biological matrix, such as blood, serum, plasma, urine, or saliva. This includes assessment of drug selectivity, specificity, sensitivity, linearity, accuracy and precision, short term and long term stability, dilution integrity and carryover[68].

The validation criteria and guidance were developed by regulators with a focus on the integrity of data derived from later stage bioavailability/bioequivalence studies[12]. Since then, the guidance has been universally implemented across all clinical and toxicological study types, regardless of the study type and what such data is being used for. This results in a significant resource investment for drug development programmes. However, there is a current consensus by bioanalysts that a tailored version of the validation criteria should be developed and implemented for PK studies in man and animals[71].

Recently, an alternative validation approach has been adopted within the pharmaceutical industry. This has been known as the tiered or scientific validation approach[72,73]. The concept revolves around selecting the appropriate experiments that are scientifically required to adequately define a procedure to support a given endpoint. The idea was initiated for two main reasons; the first being the demand to deliver drugs faster and in a more cost effective manner to patients and, second, the emerging new applications in support of biological and chemical portfolio which require a different bioanalytical method development concept[74]. There is a growing gap between the scientific relevance of the bioanalytical guidelines and their applicability to the broadening variety of studies requiring quantitative bioanalytical support. Current guidelines for regulated bioanalysis are based on late stage clinical studies but from a scientific point of view, these studies are performed in later stages when a lot of analyte knowledge has already been established and accumulated throughout the drug's life in development. While some study types do not require these regulatory aspects, bioanalytical laboratories invest resource and cost to try and meet these requirements without a real scientific need or merit. Some examples include performing full validations for tissue and urine samples, biomarkers or the use of regulated bioanalysis standards in novel technologies which does not reflect the scientific relevance for such validations. For these reasons, assay appropriate scientific validation has been introduced to establish method validation specific for each study need[72]. Embracing new technologies such as microsampling is a prime example where a scientific validation approach can be applied to enable progress more rapidly using fit for purpose bioanalytical methods.

For this reason, the bioanalytical methods utilized for SPME evaluation in this research will be scientifically validated methods that will assess linearity, precision and accuracy and selectivity. Other parameters such as matrix effects and recovery will not be assessed during method validation because the internal standard response will be monitored during the validation and sample analysis. Assessment of dilutions will be performed within study runs if needed.

2.1.1 Sample Preparation and the Use of Internal Standards

Sample preparation is the first step encountered in bioanalysis and is often the most critical in terms of extracting the required analyte from a complex matrix. Various matrices have different challenges and each analyte has its own unique characteristics, both of these aspects dictate the type of extraction approach utilised during method development and validation[75].

A biological sample consists of proteins, salts, lipids, acids and numerous other endogenous components from which the analyte of interest is retrieved. Relatively low concentrations of the target analyte make the task of removing unwanted constituents even more difficult. For this reason, sample clean-up is an essential element of bioanalysis[68].

Historically, a small number of extraction techniques have dominated the world of sample clean-up for small molecules; these include protein precipitation (PP), solid phase extraction (SPE) and liquid-liquid extractions (LLE)[76]. Since then many other innovative techniques have emerged and been applied in bioanalysis such as DBS[26] sampling, but protein precipitation remains one of the simplest and most widely used sample preparation technique and is considered by many as the gold standard technique for measurement of total drug concentration of small molecules in bioanalysis, due to its simplicity, costs and ready compatibility with LC-MS/MS analysis [68,77]. It is a process that achieves analyte separation from proteins by conversion of soluble proteins to an insoluble state either through addition of organic solvents such as acetonitrile or methanol, or with a change of pH i.e. acidification which leads to formation of insoluble salts resulting in protein precipitation[78]. Another effective procedure involves salting out the proteins using agents such as zinc chloride. The underlying mechanism of precipitation involves alteration to the solvation potential of the sample i.e. precipitation is achieved by lowering the solubility of the solute by using a suitable reagent[77].

The reagent usually contains an internal standard (IS). The main purpose of the IS is to improve the accuracy and precision of quantitation as well as ensuring the robustness of bioanalytical methods[79,80]. The IS generally consists of either a structural analogue of the analyte or preferably a stable isotope labelled compound (SIL) where selected atoms are replaced with ^{13}C, ^{15}N or ^{2}H. It should have similar physiochemical properties as the analyte and behave in a comparable manner to the analyte during sample extraction, chromatography and detection.

A known and an equal amount of IS is typically added to all samples in a batch and the analyte/IS response ratios are used for quantification. The internal standard acts as the analyte "shadow", it tracks the analyte performance through extraction, separation and detection. Any changes in the IS response can indicate instrumental issues or no changes in the IS response may indicate problems associated with the actual analyte or sample such as sample loss through adsorption, evaporation or transfer[80].

The bioanalytical methods employed throughout this project were validated in rat whole blood with protein precipitation rather than SPME extraction. This was because biocompatible SPME fibres were prototypes and in short supply and so not enough fibres were available to explore method linearity, precision and accuracy of test compounds with enough SPME fibres remaining to perform further *in vitro* and *in vivo* SPME experiments. A compromise was therefore made to use protein precipitation for bioanalytical method validation and these methods were subsequently utilized to quantify samples extracted by SPME throughout the project. However, a limited number of SPME fibres were still employed during the method validation to investigate which test compounds could be readily bound and desorbed off the SPME fibres.

2.1.2 Use of LC-MS/MS

Since its commercial introduction in the 1980s, liquid chromatography-mass spectrometry (LC-MS) or primarily, tandem mass spectrometry (LC-MS/MS) has rapidly become the standard separation and detection instrument in any well-equipped bioanalytical laboratory[81]. LC-MS combines the physiochemical separation capabilities of liquid chromatography (LC) with the mass separation/detection capabilities of mass spectrometry (MS). Liquid chromatography or high performance liquid chromatography (HPLC) is a technique whereby pumps are utilized to pass pressurized liquid known as the mobile phase containing the sample mixture through a column filled with solid particles (stationary phase) leading to the separation of the sample components. Present day liquid chromatography employs ultra-high pressure systems (UPLC) enabling analyte separation using columns packed with small particles approximately (1.7 – 1.8 μm) to reach high performance and withstand mobile phase pressures up to 1000 bar, thus providing better resolution, sensitivity and speed.

Combining this with mass spectrometry provides a powerful tool for quantifying the concentration of active drug and/or its metabolite. MS is a detection technique based on sample ionization and separation of analytes according to their mass/charge ratio. The instrument consists of three components; an ion source that converts sample molecules into ions, a mass analyzer that separates the ions by their masses and a detector that measures the abundance of each ion present[82].

Since the 1980s, liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) has been used extensively in pharmaceutical laboratories for small molecule bioanalysis, and is favoured for its high throughput, sensitivity and accuracy. LC-MS/MS also offers excellent selectivity, being able to distinguish and quantify highly homologous isoforms, even at low levels, with accuracy and precision over a wide linear dynamic range.

2.1.3 Rationale for Selection of Test Compounds

A number of test compounds must be identified prior to investigating the suitability of SPME as a microsampling technique within the pharmaceutical industry. The set of selected small molecule compounds must be chosen based on their physiochemical properties and their appropriateness for SPME extractions. The selection criteria must take into consideration the application of SPME to determine free unbound drug concentrations which suggests that plasma protein binding is a vital property that should be accounted for. Such factors will impact adsorption and equilibration times. Other aspects including practicalities associated with the scope of work, log P values, size (molecular weight), cost and availability of compounds and their SILs as well as physiologically relevant concentrations should be acknowledged. Overall, a set of small molecules that mimics typical NCE properties are required to evaluate SPME.

A range of compounds with varying chemical and biological characteristics should be screened and tested to confirm their potential suitability for SPME investigative work.

2.1.4 Aims and Objectives

The aims and objectives of this chapter are to select suitable compounds to evaluate the SPME technique *in vitro* and *in vivo* and also to develop suitable LC-MS/MS bioanalytical methods to support these investigations throughout this research project.

2.2 Experimental

2.2.1 Chemicals and Materials

Amitriptyline, caffeine, chloroquine, diclofenac, diclofenac $^{13}C_6$ sodium salt 4.5-hydrate, fluoxetine, metoprolol tartrate, midazolam, naproxen, propranolol hydrochloride were purchased from Sigma Aldrich (Dorset, UK), metoprolol-d_7 and propranolol-d_7 were acquired from Toronto Research Chemicals (Ontario, Canada). BioSPME silica probes consisting of a titanium wire coated with a biocompatible C18 extraction phase, housed inside hypodermic needle (medical grade, stainless steel, 22 gauge outer tubes) were supplied by Supelco (Bellefonte, PA, USA); each fibre has a thickness of 45 µm and 15 mm length of coating. Control rat blood and control rat plasma containing K2-EDTA to prevent coagulation was obtained from B&K Universal (Grimston, Hull, UK). Control rat blood was stored at +4ºC and used within 48 h of collection. Dimethylformamide (DMF) and formic acid (reagent grade ≥ 95%) were purchased from Sigma-Aldrich (Dorset, UK). Methanol, acetonitrile, propanol and water were of HPLC gradient grade and obtained from Fischer Scientific Ltd (Loughborough, UK).

2.2.2 Preparation of Standard Stocks, Working Solutions and Test Samples

Primary stock solutions for each test compound and internal standard (IS) were prepared in DMF (1 mg/mL). Serial dilutions of each analyte's stock solution were performed in acetonitrile/water (1:1, v/v) to give working standard concentrations of 1, 10 and 100 µg/mL. Internal standard working solutions were prepared from the primary stock solution to give a final concentration of 100 ng/mL in acetonitrile.

Test samples for all compounds were prepared at 10 ng/mL and 500 ng/mL in control rat whole blood by spiking a suitable volume of the working solution. Non-matrix volumes used to spike the samples were < 5% of the total sample volume[12]. These were then extracted using SPME to determine their extraction suitability and to enable the selection of the final set of test compounds.

Subsequent to the selection of the final set of compounds (metoprolol, propranolol and diclofenac), calibration standards and quality control (QCs) samples were prepared for all three compounds to establish linearity and to test assay precision and accuracy. A

concentration range of 10 – 500 ng/mL was used. QCs were prepared using an appropriate spiking scheme to give nominal concentrations of 10, 30, 200, 400 and 500 ng/mL.

Selectivity was assessed using total blanks (control samples with no drug) and blank containing IS samples from 6 different batches of rat whole blood (B&K Universal, Grimston, Hull, UK).

2.2.3 Extraction of Validation Samples

A set of test compounds were examined for compatibility with the SPME coating phase (C18). SPME extraction was performed by conditioning the fibres for 15 min in methanol followed by 15 min in water contained within 1.4 micronic tubes (Micronic^{TM}, Aston, USA). Fibres were then exposed to the spiked test samples in rat blood for 1 h. Analytes were desorbed off the SPME fibres through 15 min exposure to 200 µL of 100% acetonitrile. Fibre conditioning, extraction and desorption were performed with 500 rpm agitation using a compact laboratory shaker (MS 3 Digital, IKA). Agitation of the sample was applied in an attempt to mimic the existence of a ‘stirred’ medium which would surround the fibres analogous to intravenous blood flow in a living organism. Extracts were then analysed using a generic LC-MS/MS method developed as detailed in Section 2.2.4 below.

Whole blood samples (calibration standards (n =8) and QCs (n = 6)) assessing linearity, assay precision and accuracy, were extracted using protein precipitation. This was performed by taking a 25 µL aliquot of standard or QC samples into clean 1.4 micronic tubes (Micronic^{TM}, Aston, USA). 200 µL of internal standard working solution was added to all samples while 200 µL of 100% acetonitrile was added to double blanks. Tubes were capped, vortex mixed and then centrifuged for approximately 10 min at 3000 g (5810R, Eppendorf, Germany). Following centrifugation, the supernatant was transferred into clean micronic tubes and injected into the LC-MS/MS system.

2.2.4 LC-MS/MS Method Development

A generic LC method that could be applied for all three chosen compounds (metoprolol, propranolol and diclofenac) was developed.

Chromatographic separation was achieved using an Acquity UPLC system (Waters, MA, USA) equipped with a sample manager, sample organizer, a binary solvent manager and column oven. Analytes were separated using an Acquity C18 BEH column 50 x 2.1 mm i.d., 1.7 µm particle-size (Waters, MA, USA). This was kept at 50ºC and a gradient elution was applied employing the mobile phases, deionised water containing 0.1% formic acid (mobile phase A) and 100% acetonitrile (mobile phase B). Following sample injection (4 µL), the mobile phase was held at 95% A for 0.5 min followed by rapid gradient to 10% A at 1.10 min. The composition was kept at an isocratic period to 1.30 min and was ramped to 95% A at 1.50 min and finally held at the same composition to 2.00 min, re-equilibrating the column prior to the next cycle. The flow rate was 0.8 mL/min and HPLC effluent was diverted to waste for the first 0.5 min of chromatographic run time using a divert switching valve (Kinesis, USA).

MS detection occurred using an API-5000 tandem quadrupole mass spectrometer (AB Sciex, USA) equipped with a heated electrospray ionisation. The tuning parameters of the MS were optimized by continuous infusion of 25 ng/mL of each compound and its IS flowing at a 0.5 mL/min by means of an external infusion pump directly connected to the mass spectrometer. A full scan was conducted in both positive and negative ion mode, separately, to identify the most suitable ion mode for detection using a scan range of 50 – 400 Da and scan speed of 10 Da/s. The ionization of the analytes was carried out using ESI in positive mode. The ion spray source temperature was set at 500 ºC and an ion spray voltage of 5500 V. The analysis was performed using multiple reaction monitoring (MRM) mode using instrument settings as described in Table -2.1-. All gases used were nitrogen, a dwell time of 100 ms was employed for ion monitoring and unit resolution was applied to both Q1 and Q3.

Table -2.1- Summary of MS/MS parameters for the analysis of test compounds

Analyte	Q1 Mass (amu)	Q3 Mass (amu)	Declustering Potential (V)	Entrance Potential (V)	Collision Energy (V)	Cell Exit Potential (V)
Metoprolol	268.3	116.2	78	10	26.4	13
Metoprolol-d_7	275.3	191.0	78	10	26.4	13
Propranolol	260.0	183.0	125	12	28	20
Propranolol-d_7	267.0	183.0	125	12	28	20
Diclofenac	296.0	214.0	93	12	49	30
Diclofenac-$^{13}C_6$	302.0	220.0	93	12	49	30

LC-MS/MS data were acquired and processed using the proprietary software application Analyst™ (Version 1.6.1 Applied Biosystems/MDS Sciex, Canada). Calibration plots (two calibration lines per run) of analyte/internal standard peak area ratio versus analyte concentration were constructed and a Linear - Weighted 1/(x*x) regression applied to the data. The choice of weighting was based upon the sum of % residual errors. Concentrations of analytes in QC samples (n = 6 per concentration) were determined from the appropriate calibration line, and used to calculate the bias and precision of the method.

To ensure that the LC-MS/MS system performance (which can vary from day to day, depending on a number of factors such as maintenance) remained consistent, system suitability test (SST) samples utilized as reference mixture were injected prior to study runs. These consisted of the tool compounds (metoprolol, propranolol and diclofenac) spiked in 50/50 acetonitrile/water at 10 ng/mL. When SST fell outside acceptable limits i.e. when low sensitivity or poor chromatography were observed, action was taken to identify the cause and once rectified the system was used for data acquisition.

2.3 Results and Discussion

2.3.1 Selection of Test Compounds

In order to select an appropriate set of compounds to investigate the applicability of the SPME technique, a range of small drug molecules (shown in Table -2.2-) were screened for physiochemical properties, protein binding values and their chromatography characteristics. A generic LC separation method (detailed in Section 2.2.4) was developed to facilitate the screening and testing of the compounds.

Selection of suitable compounds largely depended on their protein binding characteristics; however other factors such as commercial availability, compatibility with the *in vivo* SPME phase (C18) and applicability of the generic chromatography were taken into consideration when choosing appropriate compounds. All compounds were extracted with SPME fibres and analysed using LC-MS/MS. The compatibility of the selected compounds with the SPME phase was a crucial aspect due to the fact that the C18 coating phase was the only available biocompatible phase. Generic extraction conditions were applied for SPME, since it is not possible to change any *in vivo* conditions such as pH levels or apply any other sample treatment when using SPME for in vivo extraction directly from the veins of living organisms. Below are two selected examples of the rationale for rejecting some compounds. Naproxen and chloroquine were rejected based on compatibility with SPME phase and chromatography issues, respectively.

Naproxen was extracted at two concentrations using the *in vivo* SPME fibres. The analyte response at 10 ng/mL was approximately the same as 500 ng/mL as shown in Figures -2.1- and -2.2-

Table -2.2- A range of compounds showing a selection of varied physiochemical properties and protein binding characteristics[83]

Name	Average Molecular Weight (g/mol)	Log P	Plasma Protein Binding (% Bound)	pK_a (Strongest Acidic)	pK_a (Strongest Basic)
Caffeine	194.2	-0.24	30	-	-0.92
Chloroquine	319.9	5.28	45	-	10.32
Clotrimazole	344.8	5.48	90	-	6.62
Diclofenac	296.2	4.98	99	4.00	-2.10
Fluoxetine	309.3	4.09	87	-	9.80
Metoprolol	267.4	1.88	30	14.09	9.67
Midazolam	325.7	3.89	97	-	6.57
Naproxen	230.6	3.29	1	4.19	-4.80
Propranolol	259.4	3.03	90	14.09	9.67
Simvastatin	418.6	4.51	95	14.91	-2.80

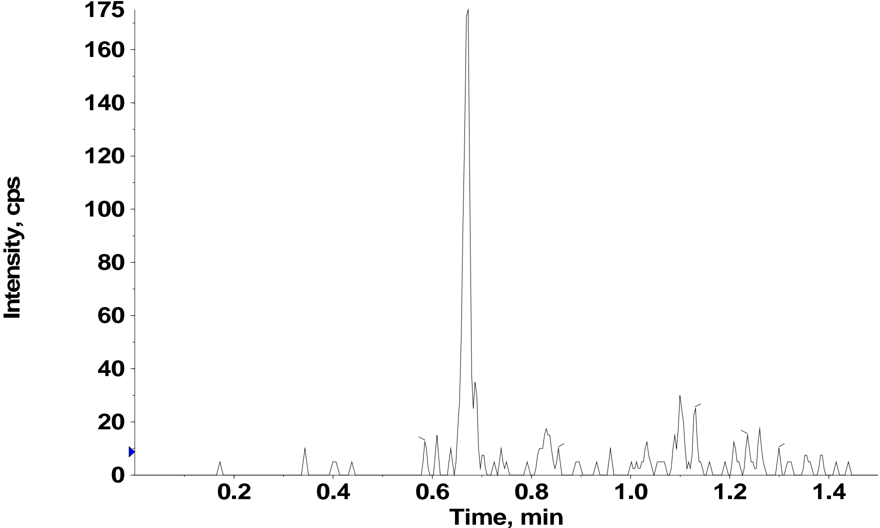


Figure -2.1- LC-MS/MS chromatogram of naproxen at 10 ng/mL extracted from rat blood by biocompatible C18 SPME.

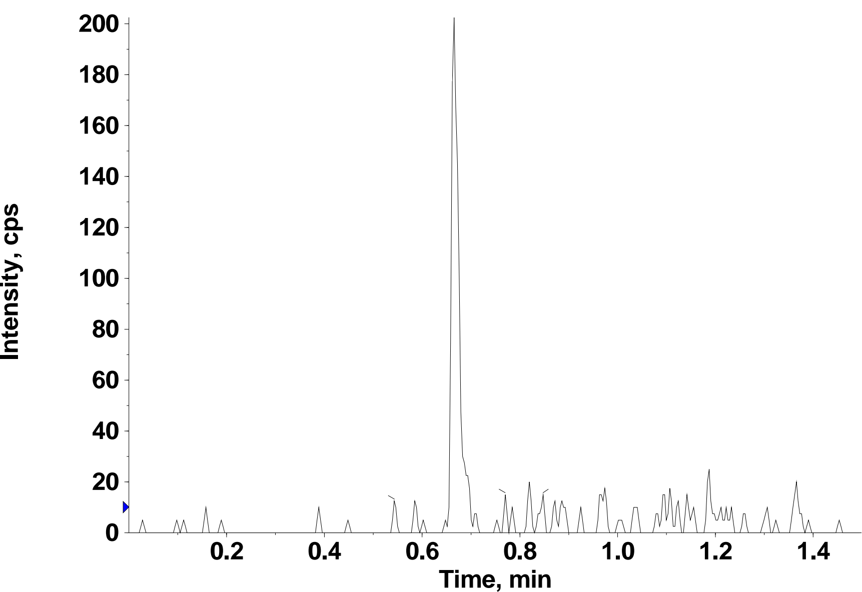


Figure -2.2- LC-MS/MS chromatogram of naproxen at 500 ng/mL extracted from rat blood by biocompatible C18 SPME.

Despite the 50 fold difference in concentration between the 10 and 500 ng/mL samples, the analyte response for naproxen was approximately the same. Initially this was deemed as an experimental error and the procedure was repeated for both concentrations. The same results were obtained upon re-extraction and re-analysis. In order to determine whether these results were related to the SPME extraction, the same spiked whole blood samples were extracted using protein precipitation. The results as expected were linear and analyte response at 500 ng/mL was approximately 50 fold higher than the 10 ng/mL sample.

This suggested a problem with the SPME extraction. Naproxen is an acidic molecule and the SPME C18 phase is likely to extract un-dissociated/neutral species of analytes and therefore the extraction efficiency is largely impacted if the appropriate phase is not utilised during the extraction. For this reason, considering sample pH adjustment was executed to test whether this will improve extraction efficiency of naproxen. Figure -2.3- shows the analyte response of naproxen at 500 ng/mL extracted by SPME, following pH adjustment of the whole blood sample to pH 3. This confirmed that low pH values improve the extraction of acidic compounds. This enhanced extraction was also observed by Aresta *et al*.[65] when human urine samples were pH adjusted *in vitro* to increase extraction efficiency of naproxen.

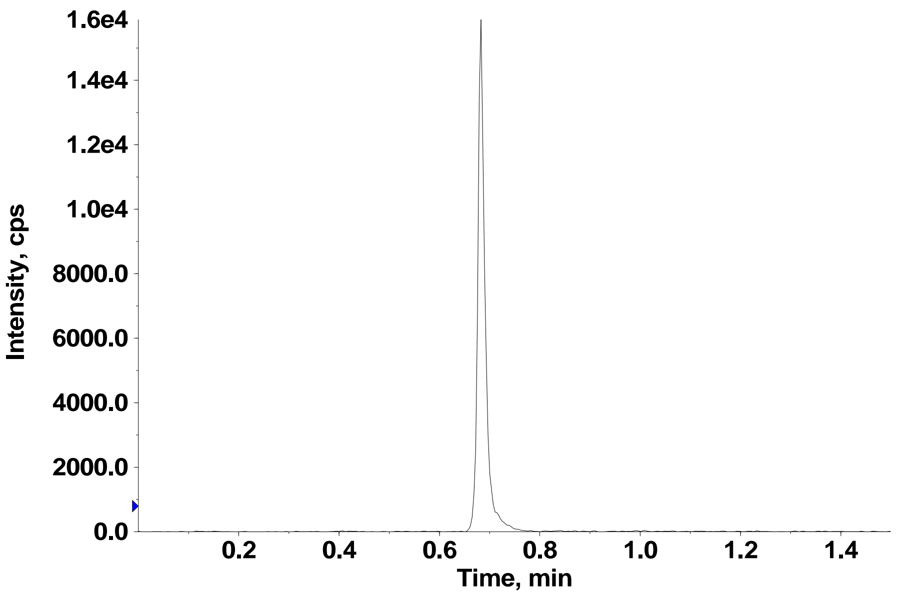


Figure -2.3- LC-MS/MS chromatogram of naproxen at 500 ng/mL extracted from rat blood by biocompatible C18 SPME following sample pH adjustment to pH 3.

The adjustment of pH is a viable approach for *in vitro* samples, however this is not feasible for *in vivo* extraction directly from a living organism's veins. Other SPME phases, suitable for polar molecules were required. For this reason, naproxen was one of the compounds that were not selected for the test compounds within this research.

Chloroquine is another example of one of the compounds that was excluded from the chosen test compounds. The extraction efficiency of chloroquine using SPME behaved in a proportional manner, where the analyte response at 500 ng/mL of chloroquine was approximately 50 fold higher than the 10 ng/mL analyte response. However, the chromatographic separation for chloroquine involved developing a separate method to the generic LC-MS/MS method. Figure -2.4- shows a representative chromatogram of chloroquine when the extracted sample was analysed using the generic LC-MS/MS method.

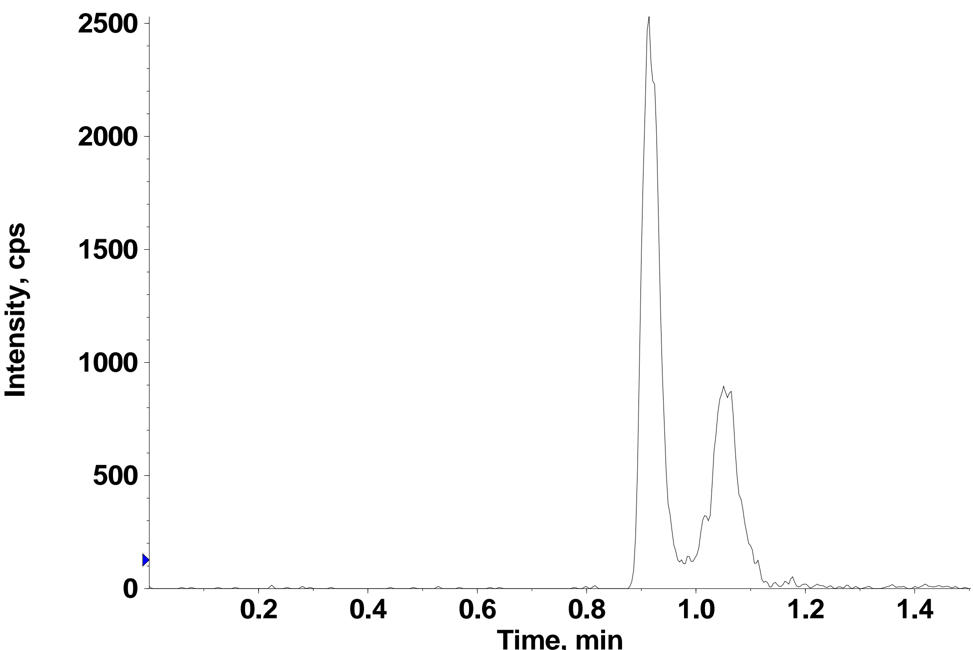


Figure -2.4- LC-MS/MS chromatogram of chloroquine at 10 ng/mL extracted from rat blood by biocompatible C18 SPME and analysed using a generic LC-MS/MS method.

As shown above, using the generic LC-MS/MS method resulted in a double peak for chloroquine. For this reason, the chromatographic separation for chloroquine was re-evaluated and achieved using reverse phase and ion pair chromatography. Figure -2.5- illustrates a chloroquine chromatogram subsequent to re-development of the LC method.

Heptafluorobutyric acid (HFBA), 0.1%, an ion-pairing reagent, was used for mobile phase (A) and 100% acetonitrile for mobile phase (B) eluting at an isocratic composition A:B 63/37 (v/v). A Thermo Hypersil Gold column, 50 x 3mm, i.d. 5 µm, kept at 40°C was used and the flow rate maintained at 0.8 mL/min with a sample injection volume of 5 µL.

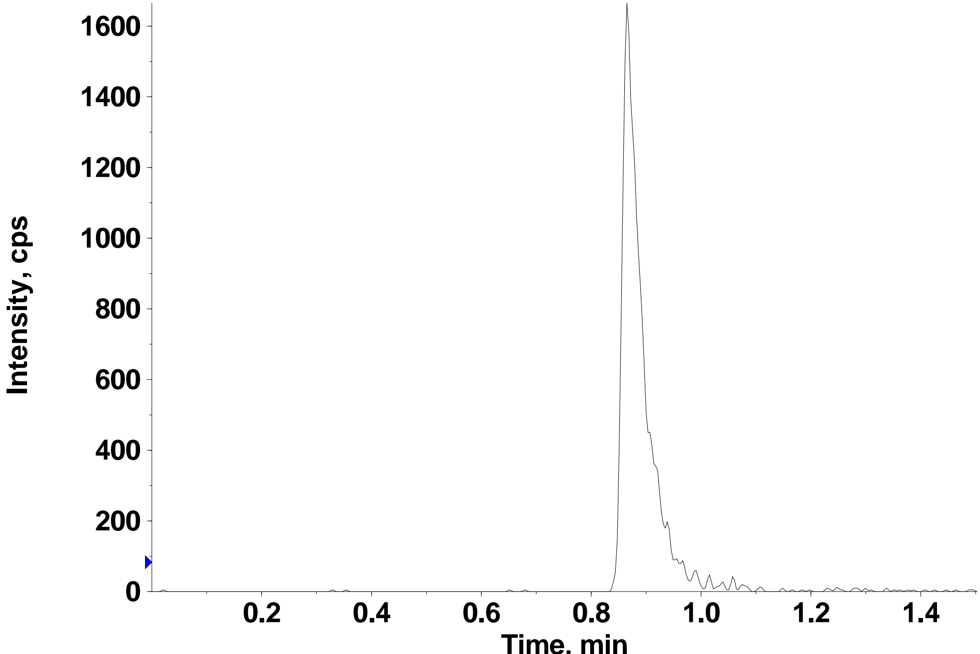


Figure -2.5- LC-MS/MS chromatogram of chloroquine at 10 ng/mL extracted from rat blood by biocompatible C18 SPME and analysed using a modified LC-MS/MS method.

In order to simplify the process, choosing compounds that require different separation and detection methods was not practical. Therefore, such compounds including chloroquine and naproxen were eliminated from the selection of the test compounds. Other compounds such as midazolam were eliminated due to limited commercial availability, toxicity and the legally controlled status.

The final selected set of compounds consisted of metoprolol, propranolol and diclofenac as shown in Table -2.3-. These drug molecules cover a range of protein binding values (approx

30%, 90% and 99%) and were all compatible with the C18 biocompatible fibre phase as well as the generic LC separation method. Log P values of these three compounds range from 1.88 to 4.98. This is considered a typical range for NCEs displaying moderate hydrophobicity values. Such Log P values enable drug delivery to the site of action and facilitate biological activity as a consequence of drug absorption through various body compartments. Log P values of less than -2 and greater than 5 can lead to solubility issues and compromise drug penetration as well as drug absorption within the living organism[84,85].

Table -2.3- Chemical structures, molecular weights and protein binding values for the selected test compounds[83].

Compound	Structure	Average Molecular Weight (g/mol)	Plasma Protein Binding (% Bound)
Metoprolol	OH, H N, CH_3, CH_3, H_3CO, O	267.4	30
Propranolol	CH_3, O, NH, CH_3, OH	259.4	90
Diclofenac	Cl, NH, Cl, OH, O	296.2	99

2.3.2 Method Validation

A scientific validation approach was followed to develop validated methods for all three compounds. Precision (% CV) and bias were assessed using whole blood protein precipitation followed by LC-MS/MS analysis. Protein precipitation was utilized due to the limited number of SPME fibres which were prototypes and in short supply. This is scientifically justifiable as those methods were validated using a conventional reliable extraction technique (protein precipitation). The selectivity of the method was established by the analysis of blank (control whole blood sample extracted using acetonitrile containing IS) and double blank samples (control whole blood sample extracted using 100% acetonitrile) of control rat whole blood from 6 individual animals.

HPLC-MS/MS chromatograms of the blanks and validation samples were visually examined and compared for chromatographic integrity and potential interferences. Representative chromatograms of a double blank sample and samples at the lower limit of quantification (LLQ) are shown in Figure -2.6- to Figure 2.8 for metoprolol, propranolol and diclofenac, respectively. No unacceptable interferences at the retention times of each analyte and its internal standard were observed and the level of signal to noise ratio at the LLQ was greater than 10:1 for all three analytes.

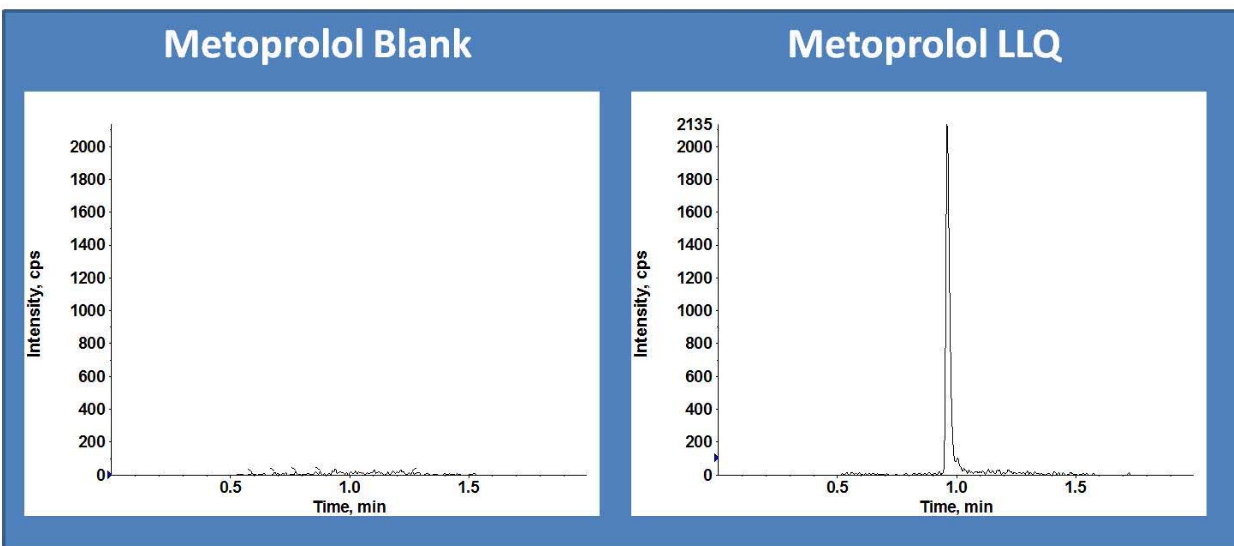


Figure -2.6- Representative chromatograms of a blank rat whole blood sample and an LLQ sample of metoprolol at 10 ng/mL.

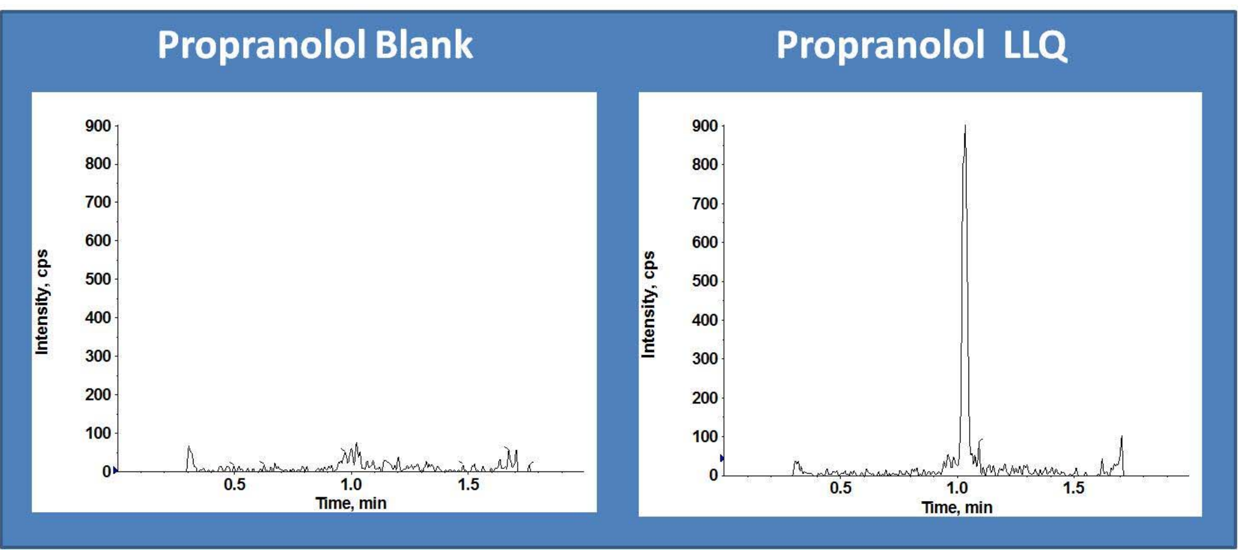


Figure -2.7- Representative chromatograms of a blank rat whole blood sample and an LLQ sample of propranolol at 10 ng/mL.

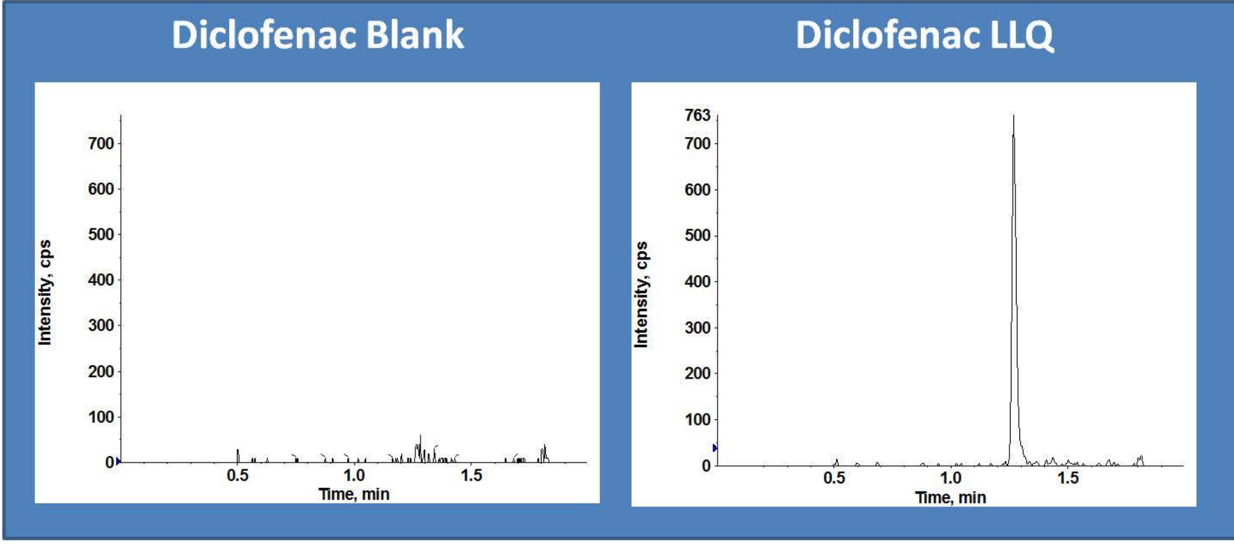


Figure -2.8- Representative chromatograms of a blank rat whole blood sample and an LLQ sample of diclofenac at 10 ng/mL.

Linear responses in the analyte/internal standard peak area ratios were observed for all three analytes when calibration standards were assessed as part of method validation. The correlation coefficients obtained using linear - weighted 1/(x*x) regression were 0.9967, 0.9976 and 0.9986 for metoprolol, propranolol and diclofenac, as shown in Figures -2.9-, -2.10- and -2.11-, respectively. The weighted regression is typically used to maximize the estimation efficiency of each parameter[86]. This is performed by treating all of the data points equally. Since a calibration curve contains data over several orders of magnitude, the effect of the high-end point of the calibration curve changes the mean value of the response (the dependant variable value of Y) and dominates the calculation of the slope. For this reason, a weighting regression is utilised to give emphasis to data points at the lower end of the curve and give a better fit for the data set[86].

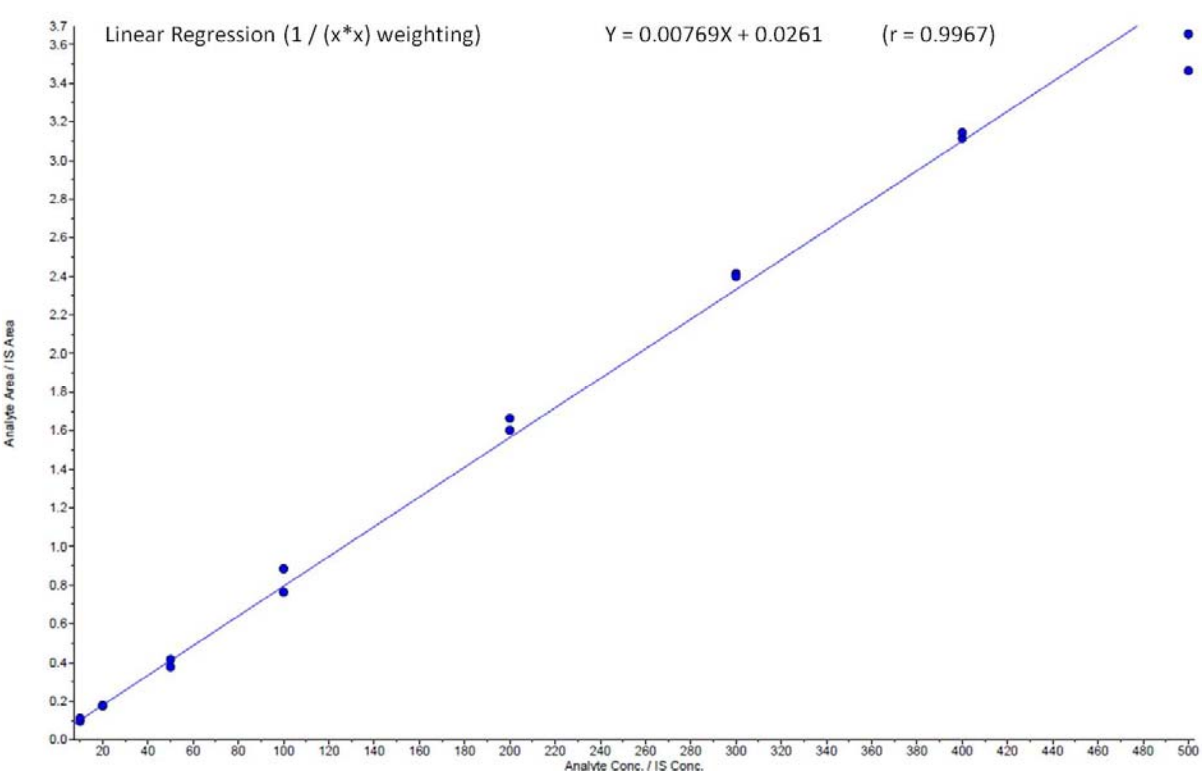


Figure -2.9- Calibration plot (range 10 – 500 ng/mL) for metoprolol extracted from rat whole blood using protein precipitation followed by LC-MS/MS, n= 2 at each concentration level.

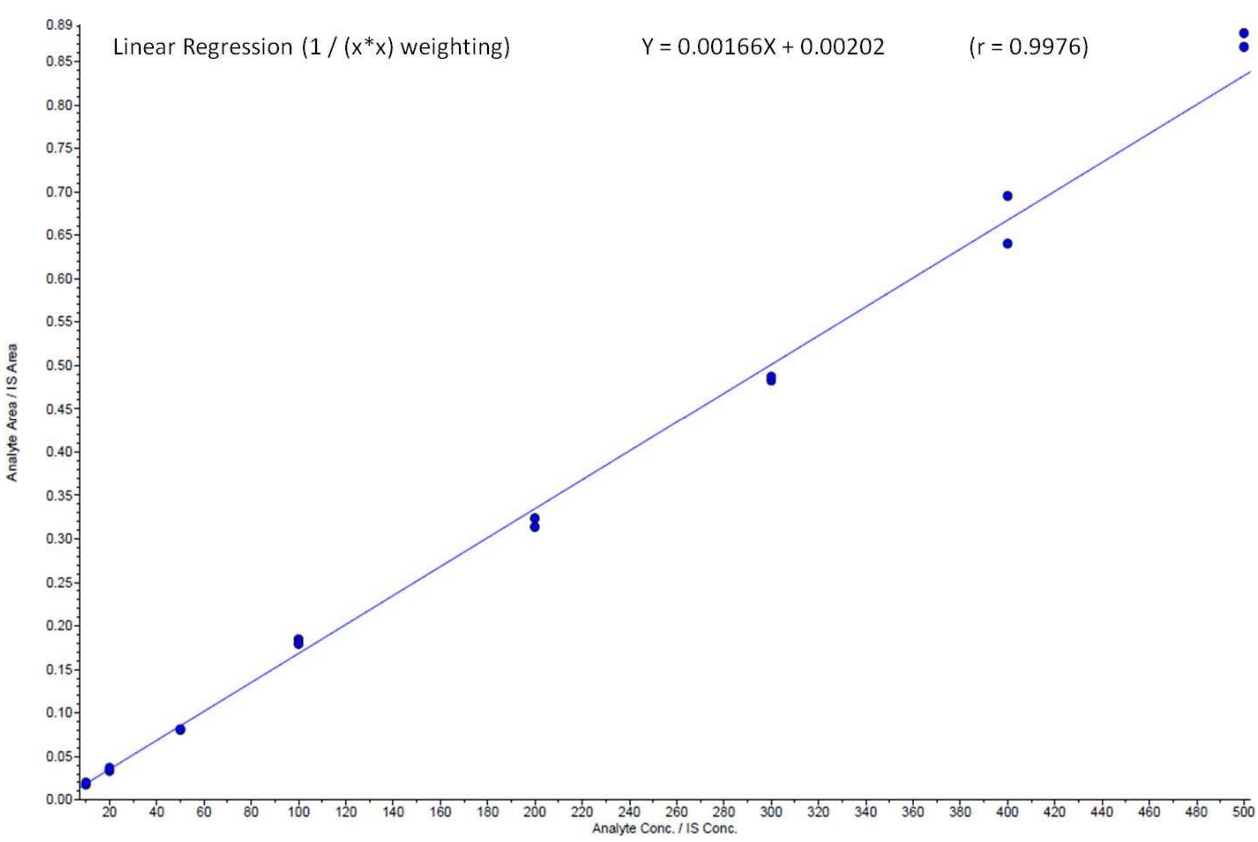


Figure -2.10- Calibration plot (range 10 – 500 ng/mL) for propranolol extracted from rat whole blood using protein precipitation followed by LC-MS/MS, n= 2 at each concentration level.

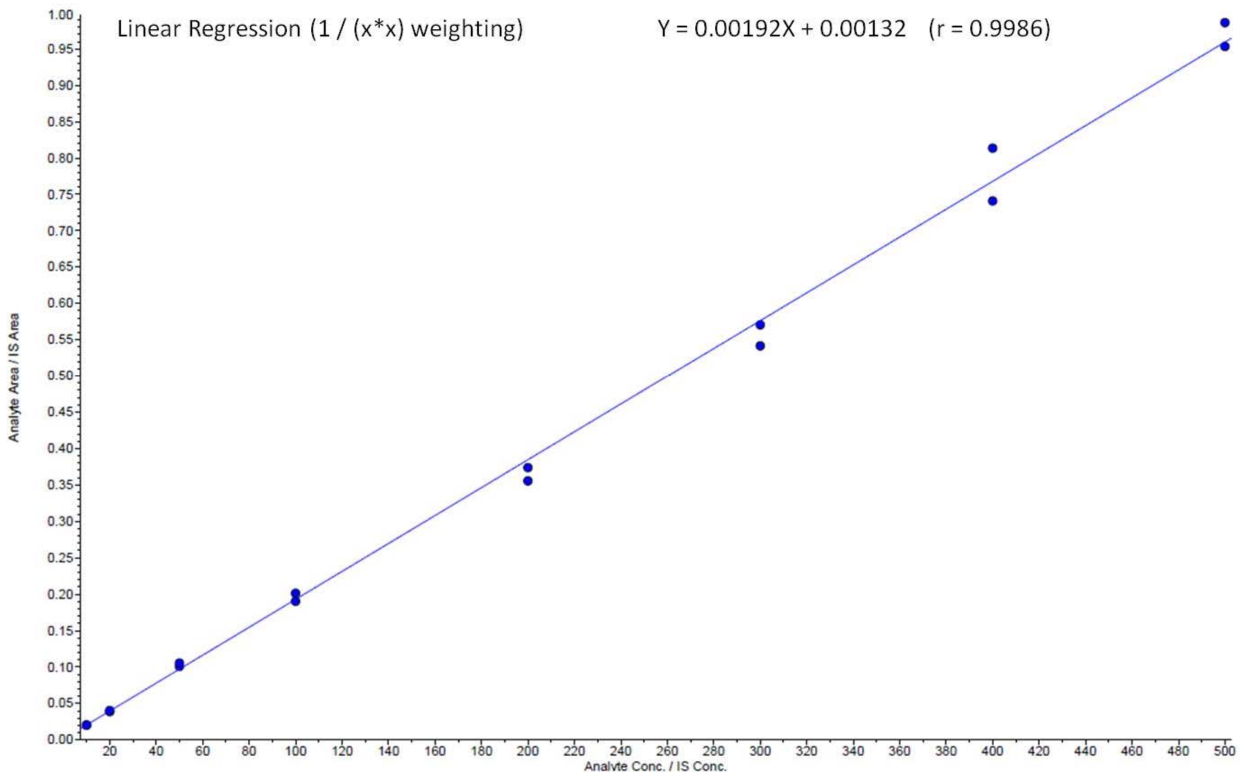


Figure -2.11- Calibration plot (range 10 – 500 ng/mL) for diclofenac extracted from rat whole blood using protein precipitation followed by LC-MS/MS, n= 2 at each concentration level.

The accuracy (% bias) and precision (% CV) calculated as shown below, of each method was evaluated using the quality control samples analysed against the calibration standards.

$$\% \, CV = \left\lfloor \frac{Standard \ Deviation}{Mean} \right\rfloor x \ 100 \qquad \text{Equation 2.1}$$

At all quality control concentrations examined, the accuracy and precision values were within 15% as shown in Tables -2.4-, -2.5- and -2.6-.

Table -2.4- Bias, precision (% CV) and individual validation sample concentration data for metoprolol extracted from rat whole blood

Nominal Concentration	QC 10 10 ng/mL	QC 30 30 ng/mL	QC 200 200 ng/mL	QC 400 400 ng/mL	QC 500 500 ng/mL
	10.8	32.6	237.8	394.3	498.9
	9.7	33.5	218.0	408.8	448.9
	11.8	33.3	212.8	407.2	504.8
	9.2	34.1	224.0	391.3	496.8
	12.8	29.4	232.3	396.7	464.7
	11.5	30.8	227.5	459.7	520.2
Mean	11.0	32.3	225.4	409.7	489.0
Standard Deviation	1.3	1.8	9.2	25.5	26.8
Precision (%)	12.1	5.6	4.1	6.2	5.5
Bias (%)	9.7	7.6	12.8	2.4	-2.2
N	6	6	6	6	6

Table -2.5- Bias, precision (% CV) and individual validation sample concentration data for propranolol extracted from rat whole blood

Nominal Concentration	QC 10 10 ng/mL	QC 30 30 ng/mL	QC 200 200 ng/mL	QC 400 400 ng/mL	QC 500 500 ng/mL
	9.5	31.0	190.3	404.2	559.7
	10.8	30.2	197.0	558.7	525.5
	10.2	32.7	209.0	431.4	499.5
	10.7	32.2	207.8	398.5	560.0
	10.0	27.0	214.4	408.9	538.2
	11.0	29.4	212.2	432.9	490.9
Mean	10.3	30.4	205.1	439.1	528.9
Standard Deviation	0.56	2.0	9.4	60.3	29.4
Precision (%)	5.4	6.7	4.6	13.7	5.6
Bias (%)	3.4	1.3	2.6	9.8	5.8
N	6	6	6	6	6

Table -2.6- Bias, precision (%CV) and individual validation sample concentration data for diclofenac extracted from rat whole blood

Nominal Concentration	QC 10 10 ng/mL	QC 30 30 ng/mL	QC 200 200 ng/mL	QC 400 400 ng/mL	QC 500 500 ng/mL
	10.5	31.6	191.6	406.5	552.2
	10.8	30.6	189.0	430.8	522.8
	11.0	30.5	194.4	438.7	509.3
	9.8	33.1	200.6	444.1	532.4
	10.0	31.5	198.1	417.0	503.0
	10.6	29.7	192.6	433.6	497.1
Mean	10.4	31.2	194.4	428.5	519.5
Standard Deviation	0.45	1.2	4.3	14.1	20.6
Precision (%)	4.3	3.8	2.2	3.3	4.0
Bias (%)	4.3	3.9	-2.8	7.1	3.9
N	6	6	6	6	6

The accuracy of an analytical method describes the closeness of mean test results obtained by the method to the true value (concentration) of the analyte. Accuracy is determined by replicate analysis of samples containing known amounts of the analyte. A minimum of three concentrations in the range of expected concentrations is recommended by the FDA[12]. According to the FDA guidance for GLP studies[12], the mean value should be within 15% of the actual value except at LLQ, where it should not deviate by more than 20%. However, for non-GLP studies 20% accuracy is generally accepted. The accuracy for all three analytes was within 15% of the actual value at each concentration.

The overall generic LC separation method and MS detection parameters were accurate and precise for all three analytes. This instils confidence in the LC-MS/MS part of the analysis when exploring the validity of the SPME technique and enables easier workflow throughout this research. Subsequent *in vitro* and *in vivo* experiments throughout this research contain calibration standards and quality control samples prepared using SPME fibres to suitably monitor the performance of the analytical method for experimental data acquisition.

2.4 Conclusion

Selecting an appropriate set of tool compounds to evaluate a technology remains a challenging aspect for the pharmaceutical industry. The variety of NCE moieties that pass the drug discovery and development process is wide, such molecules have diverse physiochemical properties and multiple pharmacological characteristics. Covering the whole range of possible compounds when evaluating a technology is impossible. Also regardless of the number of compounds used to evaluate a technique, a specific method validation for every analyte will have to be executed prior to any study conduct. Therefore, selecting a narrow range with moderately variable properties that are relevant to the technique is sufficient to give a better understanding of the technique. The main rationale for choosing metoprolol, propranolol and diclofenac for the evaluation of SPME was the protein binding characteristics of the three compounds which covers a suitable range from high to low binding values. Other important aspects included log P values, compatibility with the C18 SPME phase, commercial availability, cost and applicability to generic LC-MS/MS analysis.

A scientific validation approach was followed and methods were validated for all three analytes. The overall bias and precision (%CV) of the validation runs were within 15% of nominal concentrations for all three analytes. These methods were both selective and sensitive and will be used throughout this research.

Chapter 3

In vitro Evaluation of SPME Fibres; Considerations of Parameters Impacting Bioanalytical Method Development

3.1 Introduction

3.1.1 SPME Method Development

The success of *in vivo* SPME sampling and extraction, depends significantly on the *in vitro* validation of the SPME method and the optimisation of extraction characteristics required to achieve adequate sensitivity, accuracy and precision. All of which are essential to have sufficient confidence in the data which in turn will be used to make appropriate preclinical and clinical decisions that can ultimately affect patients.

Several factors are considered to be fundamental for developing a high quality SPME method including; appropriate selection of fibre coating, evaluation of extraction time, choice of agitation mode, selection of efficient desorption solvents and assessment of various other parameters such as on fibre stability, hematocrit effect and inter-fibre variability. *In vivo* experimental conditions need to be optimised and reproduced *in vitro*, for this reason *in vitro* SPME method development must be conducted prior to any *in vivo* SPME application.

3.1.2 Fibre Coating

The main element that determines the success of SPME as an extraction technique is the coating of the fibre[87]. The coating material is primarily responsible for the extraction of analytes. It is therefore crucial to have a range of fibre coatings that can extract a series of analytes with a wide range of characteristics including a variety of molecular weights and polarities. The extent of extraction is directly dependent on the affinity of the analyte towards the coating phase versus the sample matrix, which is defined by the magnitude of the distribution constant K_{fs}. Coatings are designed to have high K_{fs} values to improve analytical sensitivity[87].

Most of the currently available SPME fibres have been designed for GC applications with a very limited number of fibre coatings developed for HPLC use. Desorption of the analyte from the extraction phase entails the use of organic solvents as desorption solvents which are compatible with commonly used HPLC solvents. However, utilising organic solvents can cause swelling of the fibre coatings and eventually leading to breakage and stripping off the coated layer[42]. Four coatings were initially developed for HPLC use; polydimethylsiloxane, polydimethylsiloxane/divinylbenzene, polyacrylate and Carbowax-templated resin (CW-TPR), some of which still lack the required properties of durability, inter-fibre reproducibility and good extraction efficiency[41]. However, a new series of fibres have been developed for *in vivo* SPME applications of small molecules; these consist of polymeric octadecyl (C18) bonded silica particles embedded into a biocompatible binder. The binder is composed of a non-swelling polymer which is resistant to fouling upon exposure to biological matrices; its biocompatibility feature refers to the non-toxic effect or the absence of adverse effects when the material is inserted into a living organism[42]. Furthermore, the biocompatible polymer prevents adhesion of macromolecules such as proteins, complex carbohydrates and lipids on its surface but permits the movement of analytes and metabolites through the binder to interact with the C18 particles[88]. This in turn facilitates efficient sample clean up and enables reduced levels of matrix interference.

Two types of coatings determine the mechanism of extraction; these are classified as either absorbent or adsorbent types (Figure -3.1-). Absorbent fibre coatings such as the C18 coated fibres consist of viscous fluid like polymers, cross linked to the fibre core, the mechanism of analyte interaction depends exclusively on the thickness of the coating, where analytes migrate through the layers and are captured as they enter the deeper levels of the coating[89].

The adsorbent coating is composed of solid particles suspended into a liquid polymer where extraction of analyte is dependent on interactions between the analyte and the solid particles, the nature of interaction depends on the polarity of the analyte and its ability to form hydrogen bonding, pi-pi bonding or contribute to van der Waals interactions.

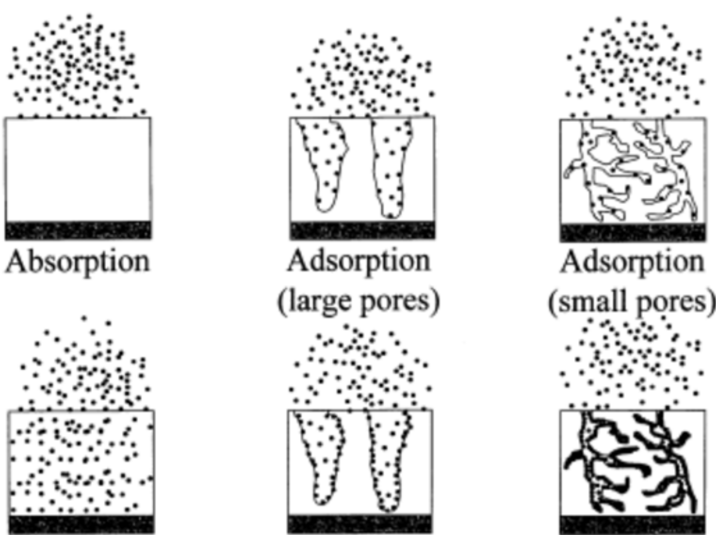


Figure -3.1- Schematic representation of absorptive versus adsorptive extraction[89].

3.1.3 Fibre Capacity and the Effect of Analyte Properties

Fibre capacity is determined by the thickness and the size of coating particles. The thicker the coating, the larger the number of active sites available for analytes to bind and interact with. Smaller coating particles such as 2-3 µm have higher capacity relative to larger 7 µm coating particles. This is due to the greater total surface area supplied by smaller particles[90].

The typical particle size of adsorbents used for the *in vivo* SPME fibres is 3-5 µm. Fibre capacity is enhanced by applying multiple coats to produce the required thickness (45 µm)[42]. However, it is worth noting that the overall dimensions of the device should be small enough to be inserted into a living organism to ensure minimal tissue damage and short extraction times. Miniaturization of the device has two fundamental implications, one of which is the smaller amount of extracted analyte which may compromise analytical sensitivity and linear dynamic range. Second is the need for coatings with higher distribution constants (K_{fs}) to improve the compromised analytical sensitivity[62]. Coating fused silica involves pulling the fibre through a special coating applicator numerous times to obtain the necessary thickness. Controlling the speed with which the fibre is pulled through the applicator and producing fibres with similar lengths and coated portions (typically 1 cm long) per sample per batch of fibres are important factors that play a vital role in the durability and reproducibility of fibres[42]. Acquiring similar results when multiple fibres are used is an essential feature for

bioanalytical applications since, accuracy and precision of data should be within pre-defined limited acceptance criteria, as outlined by regulatory guidelines[12].

The extent of analyte retention on the fibre is dependent on several factors, including the polarity of the coating phase, the molecular weight of the analyte and its ionisation state. Polar coating materials offer selectivity for adsorption of hydrophilic analytes, while non-polar phases with alkyl functional groups provide affinity for lipophilic analytes i.e. typically mid to non-polar substances. Most SPME fibres have bipolar properties to some degree, meaning that they are capable of extracting both slightly polar and non-polar analytes.

Two commonly used polar fibre coatings have been reported including polyethylene glycol (PEG) phase and polyacrylate (PA) phase, both of which have demonstrated desirable selectivity towards extraction of polar analytes. Although both coating types are commercially available and perform very well for SPME-GC applications, their characteristics have not yet been extensively tested for LC suitability or developed for *in vivo* applications[41].

The molecular weight and size of the analyte governs the dynamic movement of the analyte in and out of the coating phase. Smaller analytes migrate rapidly in and out of the coating layers and therefore display poor retention characteristics but shorter equilibration times due to the fast movement, while larger analytes move through the coating in a slower manner gradually reaching deeper layers of the coating material but taking longer to reach equilibrium[42]. On the other hand, smaller sized molecules may occupy more surface area and bind tightly to the stationary phase as they can penetrate into the cavities that cannot be accessed by larger molecules.

3.1.4 Extraction and Equilibration Time

Extraction time is the amount of time in which the extraction phase i.e. the coated portion of the fibre, is in contact with the sample matrix. SPME is an equilibrium extraction technique, it requires long enough period of time for the concentration of analyte to reach a state of equilibrium between the coating phase and the sample medium[91].

Equilibration time is governed by the time needed for sufficient mass of analyte to reach the surface of the extraction phase in addition to the time required for the analyte to distribute within that phase. Both of these factors depend on the rate of extraction and fibre capacity[92].

Fibre capacity denotes the thickness of the coating phase; it takes longer to reach equilibrium with a thicker coating compared to a thin coating. It is therefore essential for the fibre coating to be sufficiently thin to permit for faster equilibration times but thick enough to give adequate assay sensitivity.

Ideally, SPME extraction should be performed at equilibrium conditions where the maximum amount of analyte is extracted by a given extraction phase and further increase in exposure time does not result in additional amounts of extracted analyte. This facilitates reduced data variation and improved method reproducibility. However the time needed to reach complete equilibrium can sometimes be inconveniently long, Lord *et al* [61] assessed the PK profile of diazepam and its metabolites in beagle dogs using 30 min extraction time which was considered adequate time to establish equilibrium between the SPME fibre and circulating blood. Such long sampling and extraction time are not compatible with ethical animal use and can adversely affect temporal resolution especially in PK studies where drug concentrations can rapidly change during early timepoints. For this reason, extraction time should be shorter than the time difference between two sampling timepoints but should also be sufficient to provide good sensitivity and reproducibility.

One approach to improve temporal resolution of sampling is to employ pre-equilibrium extraction, the use of shorter fibre exposure time. However, if this approach is followed, it is vital to control extraction conditions and to carefully manage fibre exposure time to guarantee accurate and precise results. This is particularly important for the utility of external *in vitro* calibration curves, which may become challenging since the same experimental parameters such as matrix composition and pH need to be reproduced *in vitro*. Nevertheless, alternative calibration routes have been reported in the literature[93] to minimize possible variations during pre-equilibrium extraction. Such strategies are based on kinetic calibration, where fibre coatings are preloaded with an appropriate standard, either a stable isotopically labelled form of the analyte, or an analogue of the compound with similar mass transfer kinetics to the parent analyte. The fundamentals of the kinetic calibration method is built on the relationship between absorption of the analyte from the sample matrix onto the SPME fibre and the desorption of preloaded standards from the fibre into the sample matrix[94]. The preloaded calibrant is desorbed during the extraction process and therefore the concentration of the extracted analyte is determined by establishing the desorption of the preloaded standards from the fibre.

The concentration of the analyte can be calculated using the following equation;

$$C_0 = \frac{nq_0}{q_0 - Q} \; x \; \frac{1}{K_{fs}V_f} \qquad \text{Equation 3.1}$$

where C_0 is the initial concentration of analyte, n is the amount of analyte extracted, Q is the amount of standard remaining in the extraction phase after exposure of the extraction phase to the sample matrix for the sampling time, V_f is the volume of the fibre, K_{fs} is the fibre coating/sample distribution coefficient of the analyte, and q_0 is the amount of standard that is preloaded onto the extraction phase[41,95]. This technique has been successfully applied for the extraction of organic contaminants of field sediments[96].

The main advantage of this technique is that it accounts for potential variability caused by any potential disturbance to the exact length of sampling time, the uniformity of agitation in the system and sample composition. But a major drawback is the ethical and technical impact of desorbing the preloaded calibrant into the living organism as well as the additional cost and time required to preload the fibres. Another potential problem with pre-loaded calibrants is the possible effect of fibre heterogeneity which may impact pre-loading and subsequent desorption. Furthermore, the product $K_{fs}V_f$ must be accurately determined *in vitro* using equilibrium extraction in the matrix of interest prior to performing the pre-equilibrium kinetic calibration[97].

Alternative calibration methods such as standard addition procedures have been proposed and implemented for fast pre-equilibrium sampling[63,94]. However, utilising such techniques is less suitable for high throughput *in vivo* studies within the pharmaceutical industry due to the time, cost and labour intensive demand for such procedures. Overall, selecting an appropriate exposure time is a compromise between reproducibility, assay sensitivity and time resolution, so it has to be optimized for each analyte during method development using an extraction-time profile. The extraction can be interrupted and the fibre analysed prior to equilibrium as long as the same conditions are applied to both *ex vivo* calibration standards and *in vivo* samples. However, to obtain reproducible data, constant agitation conditions and careful timing of the extraction are necessary[93].

3.1.5 Sample Volume

Most conventional sample preparation techniques require the utility of increased sample volume to enhance method sensitivity. On the contrary, the amount of analyte extracted with SPME fibres increases with sample volume up to a point, after which method sensitivity is unaffected by further increase in sample size[41]. When sample volume is greater than $K_{fs}V_f$ (the product of analyte distribution constant and fibre coating volume) the number of moles of analyte extracted is independent of sample volume[98]. *In vivo* sampling involves a very large sample volume such as that of the circulatory system and the amount of analyte extracted is considered to be negligible, therefore it is independent of sample volume. During *in vitro* experiments, the sample volume is mainly governed by the size of the vial containing the sample. Given that the vial dimensions are appropriate to retain a sample volume that could submerge the entire SPME fibre, sufficient amount of analyte will be extracted even from very small sample volumes. This eliminates the need to collect a defined sample volume[66].

3.1.6 Agitation Method

Sample agitation *in vitro* aids mass transport between the sample and the coating phase, this in turn leads to shorter extraction times required to achieve equilibrium. Under perfect agitation conditions the time required to reach equilibrium is determined only by the fibre geometry and the diffusion of analyte in the sample[97]. The rate of extraction is impacted by the ability of the analyte to diffuse through the boundary layer of the sample matrix. The boundary layer is the term used to describe a hydrodynamic phenomenon where the liquid phase moves slowly passed a solid surface[99]. Intermolecular forces cause the solvent to stagnate near a surface, in this case the fibre coating, and so the only way a molecule can reach the coated phase is by diffusion across the boundary layer[89]. The size of the boundary layer is determined by both the matrix and the rate of sample agitation. Essentially the boundary layer (Figure 3.2) is thicker in a viscous medium, and in a medium that is poorly agitated. Agitation increases the molecular mobility of the solvent molecules (i.e. thinner boundary) and also causes turbulence in the sample matrix (the 'pocket' of solvent that has been depleted of analyte at the boundary layer is replaced more efficiently). This results in improved mass transport of the analyte to the fibre surface[89].

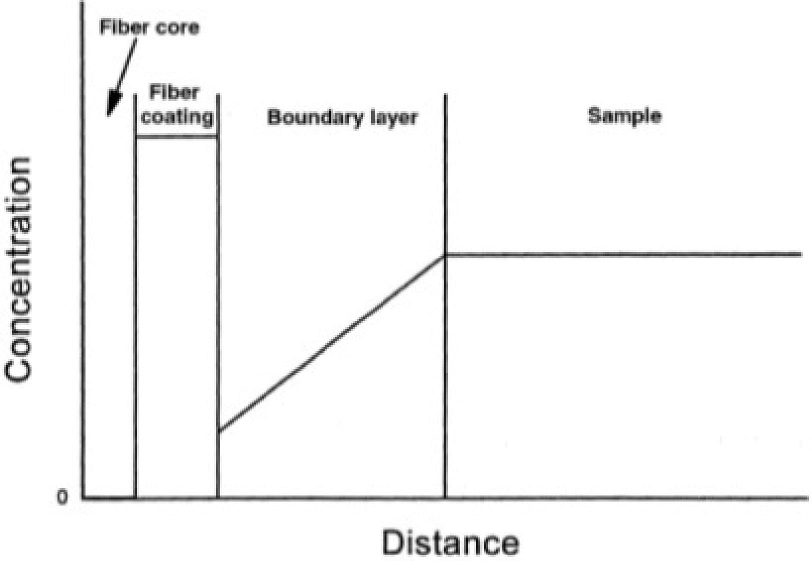


Figure-3.2- The boundary layer model of SPME sample matrix[89]

Several agitation techniques could be utilized based on the mode of application, these include; orbital shaking, needle vibration, sonication and magnetic stirring. They all have their advantages and disadvantages but some are more appropriate than others depending on the application. For example, orbital shaking using plate mixers is more suitable for multiple fibres, applying uniform agitation to all samples. On the other hand, magnetic stirring although is simple to use without the need for sophisticated equipment, the magnetic stir bar must be small enough to be placed inside the sample vial and may cause unwanted heating, interference or introduce contamination into the sample vial. While needle vibration is ideal for very small sample volumes, but may exert substantial stress on the fibre coating and the needle as a whole[100].

Overall improved agitation efficiency is needed to reduce the thickness of the boundary layer and to increase mass transfer rate which ultimately leads to shorter equilibration time.

3.1.7 Desorption Conditions

Desorption is the process of releasing the extracted analyte from the coated solid phase of the fibre. It is therefore essential to optimise desorption conditions to enhance extraction efficiency and maximise method sensitivity. Desorption procedures are generally more complicated for HPLC applications compared with SPME-GC. The kinetics of desorption process in liquid phase are significantly slower than in gas-phase, this is due to the fact that in gas-phase, analytes are desorbed off at high temperatures where diffusion coefficients are higher leading to faster desorption rates[101]. Therefore the process in liquid phase involves optimisation of several factors including; type of desorption solvent, volume of desorption solvent, desorption time and assessment of potential carryover[102].

Selection of suitable desorption solvents with appropriate elution strength is crucial to achieve adequate removal of analyte from the coating phase. Different mixtures with varying proportions of LC-MS compatible solvents such as acetonitrile, methanol and water, are typically utilized depending on the combination of analytes being extracted and the solid phase coating from which analytes are being desorbed off. Adjusting the pH of the chosen solvent composition may also increase desorption efficiency, depending on the type of the molecule and the nature of the coated solid phase[103].

Desorption can be performed in different ways including; i) manual desorption, ii) off-line desorption and iii) direct desorption.

Manual desorption involves using a HPLC interface (a desorption chamber similar to a conventional HPLC injection system) where a static or dynamic mode can be applied. In dynamic mode, a continuous flow of mobile phase is passed through the interface containing the SPME fibre to desorb off the analyte. However a large internal desorption volume may cause peak broadening and therefore interface volume maybe reduced to improve peak shape, but desorption efficiency is compromised and all of the analyte may not completely get desorbed in a reasonable amount of time[41].

Alternatively, static desorption has been utilized where the interface is pre-filled with a defined volume of solvent and the fibre is exposed to this solvent for a length of time, subsequently the solvent containing the extracted analyte is diverted onto the HPLC system. The main disadvantage of this approach is the high risk of potential carryover due to

incomplete desorption, however this can be reduced by employing a wash step to clean the interface between analytes or to use disposable/single use fibres.

Offline desorption is similar to static desorption, but the extracted analyte is desorbed into sample vials containing an appropriate solvent offline, without direct desorption into the HPLC system. The amount of solvent selected for desorption should be small enough to aid method sensitivity, but also sufficient to enable complete immersion of the coated phase. Desorbed analytes may then be injected using HPLC autosamplers into the LC-MS/MS system from sample vials or 96 well plates. The main drawback is the need to desorb the analytes into relatively large solvent volumes (> 200 μL) to submerge the entire coated fibre and ultimately facilitate efficient desorption process, which could lead to loss of sensitivity. One way to overcome this problem and improve sensitivity is to evaporate the extract to dryness immediately after desorption using a stream of nitrogen gas in a commercial plate dryer[97]. The sample can then be reconstituted with a small volume of HPLC compatible solvent, followed by injection into the LC-MS/MS. The shape and dimensions of the chosen vial and the use of various inserts placed inside vials or 96 well plates may also aid the use of small volumes.

The internal standard can be added in either, or both of the desorption and the reconstitution solvent to control any pipetting and injection variability. The primary advantage of offline desorption is the ability to perform desorption of multiple samples at the same time with or without agitation. Use of agitation may speed the desorption process. Automated systems such as Concept 96 robotic station[104] which allows for parallel desorption of up to 96 samples providing high throughput could be employed.

Desorption time is determined as part of method validation, as reported in the literature[88,97]. It is appropriate to test between 5 - 30 min desorption time period with agitation, depending on the coating type. Practical limitations maybe encountered with less than 5 min desorption and inadequate throughput if more than 30 min is applied.

Evaluation of carryover on the probe is sometimes necessary. This needs to be investigated when the same fibre is used *in vitro* multiple times, to determine the amount of analyte that may remain on the fibre after desorption. This could be assessed by performing multiple desorption steps using the same solvent following the first extraction. The efficiency of desorption is determined through a series of repeated re-desorption of the same fibre to ensure that no further analyte is eluted off the fibre, the amount of compound observed in

subsequent washes should be within an acceptable level of the original desorbed material to determine whether additional steps should be taken to reduce the carryover. This issue is not applicable to this research as single-use disposable fibres will be used throughout this project.

In recent years, direct desorption off the fibre into the mass spectrometer has also been explored where various configurations of SPME have been directly coupled to mass spectrometry and desorption is performed online from the fibre into the detector[105,106]. This aspect is further discussed in Chapter 7.

3.1.8 On-Fibre Stability

The chemical stability of a compound is often a concern for bioanalysis. A valid analysis cannot be performed if losses or (gains) of analyte occur during sample collection, transport, storage and analysis. Many drugs are stable and often do not require further treatment other than confirmation of stability for the maximum storage time at specified conditions. Nevertheless, some analytes may exhibit instability, this could be biological, chemical, thermal or photo degradation, which needs to be addressed prior to sample collection. In some cases, certain sample carriers or sample preparation techniques may stimulate instability and so it is vital to perform pre-study stability evaluation as part of the bioanalytical method development to cover the expected sample handling and storage conditions during the conduct of the study as well as shipment and any subsequent length of storage[68]. Several key documents including regulatory guidelines and publications have highlighted the significance of stability assessment and have given specific recommendations on how to establish analyte stability[11,107].

For *in vivo* SPME extractions, it is anticipated that the analyte is extracted onto the fibre at the clinical site or animal laboratory and is then transported to the bioanalytical site for analyte quantification. As analysis is not usually performed directly after sample collection, it is essential to investigate whether analyte stability on-fibre is maintained over the relevant storage period. It is also important to identify whether the SPME coating deteriorates over the same period of storage time to understand impact on desorption.

On a separate matter, prodrug compounds are often designed to be rapidly converted to the active molecule *in vivo*. However, in some cases prodrug quantification is necessary but may prove to be difficult to stabilise and inhibit conversion into the active in the collected sample.

In such cases, SPME may provide a potential advantage over other sample preparation and extraction techniques, as SPME could be utilized to determine the real time concentration of prodrugs directly *in vivo* without the need to stabilise the collected sample.

3.1.9 Hematocrit Effect

Blood can be divided into two major components; plasma and cellular constituents including red and white blood cells. The hematocrit is a measure of the proportion of blood that is composed of red blood cells which is expressed as a percentage of the cellular blood volume compared to the total blood volume, Equation 3.2[108]. This percentage could have a minimum value of 0% and a maximum value of 100%, there are no definitive values in animals or humans for normal hematocrit, as the levels vary with age, sex and health status Table -3.1-[32].

$$Hct = \left(\frac{V_{RBC}}{V_{Blood}}\right) x\ 100 \qquad \text{Equation 3.2}$$

Where Hct is the level of hematocrit, V_{RBC} is the volume of red blood cells and V_{blood} is the volume of blood.

Furthermore, certain medical conditions such as anemia and polycythemia could result in extreme hematocrit values. Changes in hematocrit lead to changes in the viscosity of the blood where a low hematocrit has a lower viscosity relative to higher hematocrit blood, this is because red blood cells are large (6-8 µm) and so a higher proportion will increase the viscosity of blood[109]. This disparity could have a potential effect on the performance of SPME extractions as it has previously been demonstrated that it has a major impact on dried blood spot analysis[32]. SPME is a technique that does not require blood withdrawal, blood absorption or spotting. So control of blood volume is not a necessity if the SPME probe is totally immersed but the viscosity of blood may affect the kinetics of extraction[41]. This could prove to be true if blood used for preparation of *in vitro* SPME calibration standards is of a notably different hematocrit level to the *in vivo* blood sample, which could cause assay bias and may ultimately lead to misleading bioanalytical data. For this reason, it is important to

investigate the effects the hematocrit level may impose on analyte response when employing SPME extractions.

Table -3.1- Typical human hematocrit levels[32]

Age	Hematocrit Levels (%)
Birth	42 – 64
Less than 1 month	31 – 67
1 month – 2 years	28 – 55
2 – 12 years	34 – 45
12 – 18 years, female	36 – 46
12 – 18 years, male	37 – 49
Adult female	36 – 44
Adult male	41 – 50

3.1.10 pH and Temperature

Typical commercially available SPME coatings (C18) currently being evaluated throughout this research are likely to extract analytes in their undissociated/neutral state at physiological pH due to the non-polar nature of the C18 coating[41]. For this reason, extraction efficiency could be optimised by conversion of analytes into their neutral forms by making pH adjustment to the matrix. Therefore, a low pH value will improve the extraction of acidic compounds and a high pH will enhance extraction efficiency for basic compounds. For example naproxen which is acidic has an enhanced extraction efficiency observed at pH 3[65].

For *in vitro* SPME extraction, the matrix is commonly modified to favour extraction by increasing the affinity or partition coefficient of the analyte for the extraction phase. However, this could adversely affect the quality of the coating and damage the fibre if extreme pH levels are utilised.

On the other hand, it is not possible for *in vivo* extractions to perform pH optimisation as it is not viable to change the pH within a living organism without causing death or harm. For this

reason, pH adjustment of the sample is not an option for *in vivo* sampling and since *in vitro* calibration and quality control samples should mimic the *in vivo* samples, pH optimisation is unnecessary during method development of *in vivo* applications.

Temperature modification also plays a vital role for SPME extractions, increasing the temperature may have an impact on reduction of the equilibration time. However, temperature alteration is also not feasible *in vivo* therefore it is not required during *in vitro* method development for *in vivo* application. Although the temperature of the blood used to prepare calibration standards and quality control samples (*in vitro*) should match physiological temperature. In which case, the blood will require warming to approximately 37°C prior to spiking *in vitro* standards and QCs. Post-spiked standards and QCs should also be left at this temperature for a set time to match study samples.

3.1.11 Effect of Anticoagulant

Anticoagulants are used to prevent blood coagulation at the point of sample collection by conventional venepuncture. Keeping the sample in liquid form helps with downstream manipulation, such as taking sub-aliquots for extraction and analysis. Blood collection of samples and control matrices for bioanalytical analysis are typically performed using sample tubes containing an anticoagulant[110]. The choice of anticoagulant for bioanalysis is sometimes based on what has historically been used within an animal facility. Selection of the right anticoagulant is vital for some clinical chemistry applications. For example, measuring the sodium content of blood requires an anticoagulant that does not contain a sodium counter ion.

The most commonly used anticoagulants for bioanalysis are ethylenediaminetetra acetic acid (EDTA) and potassium oxalate. These anticoagulants exist as salt forms with different cations (counter ions), for example potassium K1 and K2 EDTA[110]. This aspect is not considered crucial for bioanalytical applications, recently, the European Bioanalytical Forum (EBF) collected validation data to examine the impact of anticoagulant counter ion change and showed no effect on the precision and accuracy of more than 40 different LC-MS/MS bioassays[111].

Considerations of the impact of anticoagulants on SPME applications must be highlighted since there is a discrepancy between *in vivo* samples and *in vitro* samples. *In vivo* SPME

samples are directly exposed to the systemic circulation without the need for an anticoagulant. However, these would be quantified against *in vitro* samples which are exposed to spiked calibration standards and quality control samples prepared in whole blood containing an anticoagulant. Thus, to offer a realistic comparison between the two, blood collected without anticoagulation must be utilised alongside blood collected into tubes containing anticoagulant. The issue with this approach is that blood collected without anticoagulation readily clots at room temperature and it would be difficult to maintain matrix homogeneity while exposing SPME fibres to the matrix for a pre-defined time period.

This could potentially be a limitation of the SPME technique, however previous work with other microsampling techniques has shown no impact of anticoagulation on bioanalytical data. For example, the viability of using volumetric absorptive microsampling (VAMs) devices without pre-treatment with anticoagulants was investigated by Miao *et al*[112]. No difference was found between samples with and without the anticoagulant. A similar study was conducted by Mastronardi *et al*[113] assessing the levels of glycated haemoglobin (HbA1.) The study utilized DBS cards where blood collection was performed directly from a finger prick onto a DBS card without the use of an anticoagulant versus whole blood extraction using blood collected into EDTA tubes. The results from both types of sample collection were comparable without significant differences observed.

Although the use of an anticoagulant agent remains a potential risk/ limitation of the SPME technique, the above examples clearly demonstrate that there is a low risk of the anticoagulant on bioanalytical data.

3.1.12 Aims and Objectives

The aims and objectives of this chapter are to describe and assess the *in vitro* experiments typically conducted during the method development of SPME assays. Prior to *in vivo* study start, essential parameters such as extraction time profiles, desorption time profiles and on-fibre stability must be examined to build an *in vitro* protocol that can be applied to the *in vivo* study. This will be performed by establishing *in vitro* equilibrium extraction and desorption profiles of the three test compounds (metoprolol, propranolol and diclofenac), followed by on-fibre stability investigations for all three analytes at ambient temperature for up to six weeks. Other parameters such as characterization of the physical properties of SPME fibres will be identified using scanning electron microscope, the impact of hematocrit levels on SPME extractions will be assessed and the effect of blood flow rate on SPME extractions will also be examined.

3.2 Experimental

3.2.1 Chemicals and Materials

Metoprolol tartrate, propranolol hydrochloride, diclofenac sodium salt and diclofenac $^{13}C_6$ sodium salt 4.5-hydrate were purchased from Sigma-Aldrich (Dorset, UK); metoprolol-d_7 and propranolol-d_7 were acquired from Toronto Research Chemicals (Ontario, Canada). BioSPME silica probes consisting of a titanium wire coated with a biocompatible C18 extraction phase, housed inside hypodermic needle (medical grade, stainless steel, 22 gauge outer tubes) were supplied by Supelco (Bellefonte, PA, USA); each fibre has a thickness of 45 µm and 15 mm length of coating, particle size approximately 5 µm. Control rat blood stored at +4ºC, used within 48 h of collection, and control rat plasma containing K2-EDTA to prevent coagulation were obtained from B&K Universal (Grimston, Hull, UK). Control fresh bull blood containing heparin was obtained from a local abattoir (Leeches, Royston, UK). All animal studies were ethically reviewed and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals. Phosphate buffered saline tablets, dimethylformamide (DMF) and formic acid (reagent grade ≥ 95%) were purchased from Sigma-Aldrich (Dorset, UK). Methanol, acetonitrile, propanol and water were of HPLC gradient grade and obtained from Fischer Scientific Ltd (Loughborough, UK).

3.2.2 Sample Preparation for Scanning Electron Microscope (SEM) Analysis

Physical characterisation of BioSPME fibres was performed using scanning electron microscope (field emission SEM, ZEISS SUPRA, Germany). SEM images were acquired by a skilled SEM expert Dr. Nathalie Fa (Product Development/GSK) using an acceleration voltage of 3kV and a magnification range of 100 – 20,000 X, images acquired using SEM, SmartPI software, Germany.

Five sets of fibres (n=3) were prepared for SEM examination; Set 1 = blank fibres, Set 2 = fibres exposed to 200 µL of fresh control rat blood containing K2-EDTA for 30 min, Set 3 = fibres stored for two days at ambient temperature subsequent to rat blood exposure, Set 4 = fibres washed with 200 µL of deionised water following exposure to rat blood and Set 5 = fibres desorbed with 200 µL of 100% acetonitrile (15 min desorption period) subsequent to

blood exposure. In addition, a small section of one of the blank fibre surface was intentionally removed using a scalpel to identify the coating thickness.

Each fibre was cut using a blade cutter, into 10 mm piece, mounted using carbon conductive tape (Agar Scientific, UK) on individual SEM specimen target (ZEISS SUPRA, Germany) and then sputtered with a platinum coat using a sputtering device (Quorum Technologies, UK) and analysed on the SEM.

3.2.3 Preparation of Standard Stocks, Working Solutions and Test Samples

Primary stock solutions for each test compound (metoprolol, propranolol and diclofenac) and their stable label isotopes utilised as internal standards (IS) were prepared in DMF (1 mg/mL). Serial dilutions of each analyte's stock solution were performed in acetonitrile/water (1:1, v/v) to give working standard concentrations of 1, 10 and 100 µg/mL. Internal standard working solutions for each analyte were prepared from the primary stock solution to give a final concentration of 100 ng/mL in acetonitrile.

Analytical test samples were prepared fresh on the day of analysis by spiking an appropriate volume of the working standard solutions into fresh control rat blood containing EDTA, stored at + 4°C and used within 48 h of blood collection. The solvent used to spike into the blood matrix did not exceed 5% of the total volume. Three concentrations 10, 100 and 500 ng/mL were prepared for each test analyte.

3.2.4 Extraction and Desorption Time Profile

SPME samples were preconditioned by immersing the fibres into 200 µL of methanol, followed by 200 µL water for a period of 15 min in each solvent. This step is necessary to wet the C18 chains of the coated phase and ultimately facilitate optimal extraction efficiency. An extraction time profile was constructed for each test compound by exposing n = 6 fibres, per timepoint per concentration, to 200 µL blood aliquot of the spiked test samples (Concentration 10, 100 and 500 ng/mL). Seven extraction time points were studied (0.5, 1, 2, 3, 5, 10 and 30 min) at ambient temperature and samples were extracted at each time point by removing the SPME probes and rinsing them briefly for 30 s with 200 µL purified water to ensure removal of sample residue adhered to the outside of the coating. Subsequently the

fibres were placed in 200 µL of desorption solvent, 100% acetonitrile containing 100 ng/mL of internal standard, for 15 min. Extracted samples were then analysed by LC-MS/MS. In all cases; preconditioning, extraction and desorption were performed under constant orbital agitation of 500 rpm using a compact laboratory shaker (MS 3 Digital, IKA). All fibres were directed through the needle into a 96 deep well plate with a frame to ensure that the entire extraction phase (coated region) was immersed in the sample (Figure -3.3-).

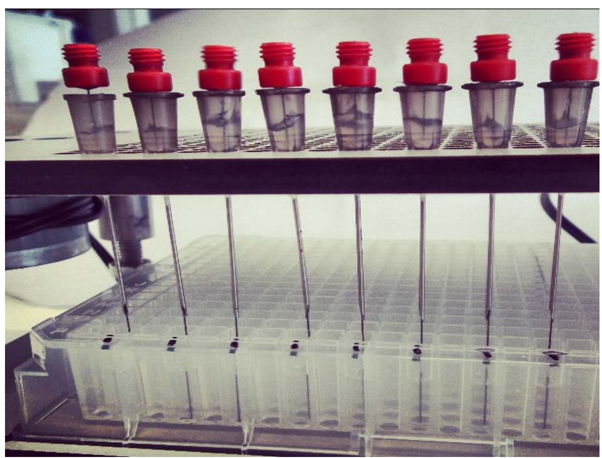


Figure -3.3- SPME fibres exposed to methanol for preconditioning of the coated C18 phase.

To evaluate the desorption time profile, the above procedure for the blood extraction was repeated, keeping the fibres exposed to the blood sample for 30 min. But three desorption timepoints were investigated. Analytes were desorbed off the fibres (n=6) after being exposed to the desorption solvent for 15, 30 and 60 min.

3.2.5 Optimisation of Desorption Solvent

In order to assess the efficiency of desorption solvent, four different solvents and solvent mixtures were investigated; 100% acetonitrile, 100% methanol, 70/30 acetonitrile/water (v/v) and 70/30 acetonitrile/water (v/v) containing 0.1% formic acid. The impact of the differences in organic composition and pH of desorption solvent were investigated to determine the effect on the measured analyte response. SPME samples were prepared by extracting n =3

fibres exposed to rat blood containing K2-EDTA, spiked with 10 and 500 ng/mL of each test compound. The extraction procedure was performed at 30 min and desorption at 15 min under constant orbital agitation of 500 rpm. All fibres were preconditioned with methanol and water for 15 min in each solvent and were rinsed with water for 30 s following extraction. The desorbed extracts were subsequently injected onto the LC-MS/MS.

3.2.6 The Effect of Hematocrit Level on Assay Bias

The impact of variable hematocrit levels on SPME extraction was investigated using metoprolol as the test analyte. Rat blood samples with a range of nominal hematocrit values 20 - 80% were prepared. Control rat blood was centrifuged (5810R, Eppendorf, Germany) at 1500 g for 20 min to produce a layer of plasma separated from blood cells. Prior to centrifugation, the hematocrit level of the control rat blood was determined (50%). Six additional blood samples were produced with the following hematocrit levels; 20, 30, 40, 50, 60, 70 and 80% by either removing appropriate volumes of plasma from centrifuged blood, or adding plasma to uncentrifuged blood, followed by gentle mixing to avoid blood cell lysis. The hematocrit value for each blood sample was confirmed by centrifugation on a haematospin (1300, Hawksley, UK) using 1.15 x 75 mm micro hematocrit tubes (Brand, Germany) (Figure -3.4-), followed by observing hematocrit levels on a micro-hematocrit reader (Hawksley, England).

Figure -3.4- Hematospin containing micro-hematocrit tubes for measuring blood hematocrit levels.

Each blood sample was spiked with an appropriate volume of metoprolol working solution (< 5% non-matrix solvent) to give a final target concentration of 100 ng/mL. SPME samples (n = 3) were prepared by preconditioning fibres with methanol and water, 15 min in each solvent and exposure to blood samples for 30 min, followed by desorption with 100% acetonitrile containing 100 ng/mL of internal standard. The whole process was performed under constant orbital agitation of 500 rpm. Extracts were then analysed by LC-MS/MS.

3.2.7 Sample Preparation for Stability Analysis

On-fibre compound stability was assessed for all three test analytes (metoprolol, propranolol and diclofenac). Three concentration levels were examined (10, 100 and 500 ng/mL) and n = 6 fibres were extracted at each concentration and time point. Three sets of fibres (n = 6 per set) were conditioned and exposed to spiked rat blood for 30 min and washed with water for 30 s post extraction. One set of fibres were desorbed at T_0 straight after sampling, with 200 µL of 100% acetonitrile containing 100 ng/mL internal standard. The other two sets were stored post extraction at ambient temperature, one set was subsequently desorbed following storage for two weeks and the last batch were desorbed after six weeks. All extracts were analysed by LC-MS/MS on the same desorption days. Analyte response following fibre storage was compared to analyte response at T_0. Instrument performance was evaluated using system suitability solutions injected onto the LC-MS/MS on each day of analysis to verify that system performance was reproducible and unchanged during this time. System suitability solution is a test mix containing metoprolol, propranolol and diclofenac at 10 ng/mL prepared in bulk at the start of the stability investigation and injected on analysis days to assess system performance.

3.2.8 Preparation of Bull's Blood for *ex vivo* Circulatory Simulation

Approximately 2 L of fresh bull's blood was obtained from a local abattoir in 4 plastic bottles each containing approximately 6,000 units of heparin dissolved in 2 mL of Ringer's saline. The bottles were rotated to ensure their interior surfaces were washed by the heparin solution. Red blood cells (RBCs) were prepared as detailed below;

Spin 1: The blood was taken and divided between 4 centrifuge pots and spun at approximately 3000 g for 15 min in a refrigerated centrifuge at +4 ºC. The layer containing

white blood cells and platelets (buffy coat) in addition to the plasma supernatant were then removed by aspiration.

Spin 2: The remaining RBCs were diluted to approximately double their volume with Ringer's saline solution containing approximately 10,000 units/L heparin and centrifuged as described above.

Spin 3: The supernatant was again removed and the above process repeated using un-heparinised Ringer's solution.

Prior to storage, approximately 120 mg of glucose (Merck, AR), 1 mL of penicillin streptomycin solution (10000 IU/mL-10000 uG/mL, Gibco BRL) dissolved in 2 mL of Ringer's saline was added per approximate 100 mL of RBC pellet. Further additions of this solution were made every five days during storage. Prepared RBCs were stored at 0-5 ^{o}C and discarded after a maximum of 14 days.

On each experimental day, the required volume of washed bovine RBCs were split into 50 mL centrifuge tubes (4 x 20 mL) and diluted approximately 1:1 with Krebs-Ringer Biocarbonate (KRB) (K4002, Sigma Aldrich, UK). The cells were centrifuged at 3000 g for 10 min and the supernatant removed and discarded. This process was repeated, and following removal of the supernatant for the RBC pellets were pooled.

The hematocrit count of the final RBC pellet was determined as (95%). This was used to calculate the volume of pellet required to obtain a hematocrit level of 50-52% (mimicking expected rodent hematocrit level) in the final perfusate (total volume 150 mL).

Approximately 4.5 g of bovine serum albumin (A2153, Sigma Aldrich, UK) was dissolved in 50 mL KRB. To this solution the calculated volume of RBC pellet was added, and further KRB added until a final volume of 150 mL was obtained. Finally, 150 mg of glucose were added to this solution.

The hematocrit level was then measured using hematocrit tubes and a hematospin centrifuge to ensure that 50% hematocrit was prepared.

3.2.9 *ex vivo* Circulatory Simulation for Effect of Blood Flow Rate

In order to achieve relevant *in vitro* experimental conditions that partially mimic the dynamic *in vivo* extraction conditions and aid assessment of the effect of blood flow rate on SPME, a simulated circulatory blood system was employed. Figure -3.5- shows a photo of the flow system model of the systemic circulation. A peristaltic pump (Watson Marlow, 505S) was

utilized to function as an artificial heart, pumping bull's blood containing heparin, from a 150 mL reservoir of matrix. Rat blood was replaced with bull blood due the large volumes needed for the *ex vivo* simulations. A range of Tygon® silicone tubing (Saint-Gobain Performance Plastics, France) were used to simulate the circulatory system. Bull blood was pumped from the reservoir up through the tubing into a heat exchanger to maintain the temperature of the blood at approximately 36-37 °C and then entered a distilling column shaped flask acting as an artificial lung where blood is oxygenated from exposure to air through vents located on either sides of the flask. The blood then passed through flexible plastic tubing mounted on a holder to enable sampling and needle piercing of the SPME probe (Figure 3.6-). A manual pressure regulator was also employed to provide pressure control. The pH of the blood was measured at the onset and at the end of the experiment. This remained the same (approximately 7.0) throughout the entire procedure. Appropriate volumes of each analyte working solution were added to the matrix reservoir to give three concentrations (10, 100, 500 ng/mL). Three blood flow rates were studied (20, 30 and 75 ml/min) at each concentration level and n = 6 fibres were analysed at each flow rate and analyte concentration. SPME fibres were conditioned by immersing the fibres into 200 µL of methanol followed by 200 µL of water and then inserted inside the simulated vein for 30 min. Desorption occurred using 200 µL of 100% acetonitrile containing 100 ng/mL of analyte IS. The above process was repeated for each test compound. The entire system was washed with purified water, flushed with PBS and tubing replaced prior to changing to another analyte.

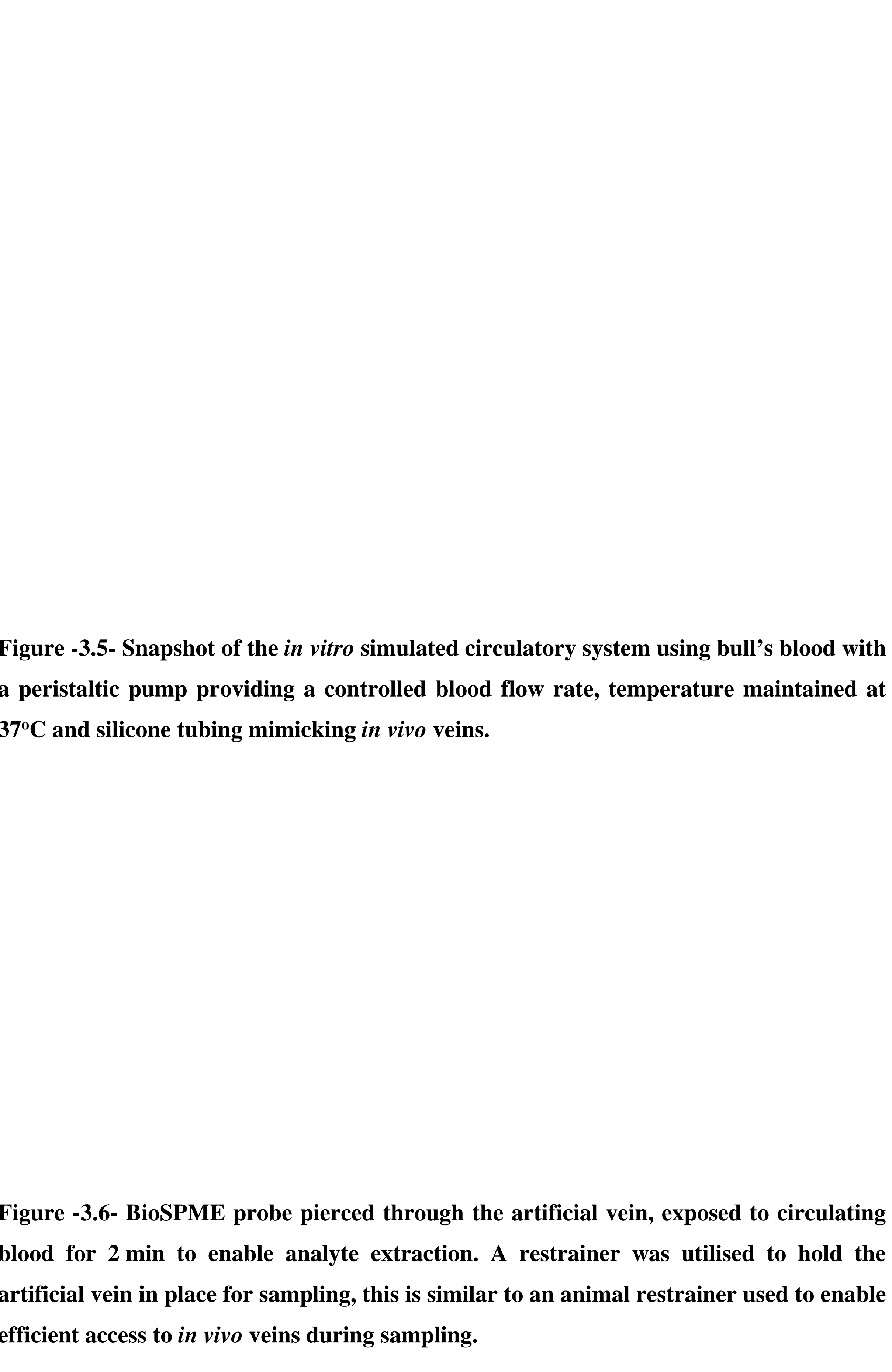


Figure -3.5- Snapshot of the *in vitro* simulated circulatory system using bull's blood with a peristaltic pump providing a controlled blood flow rate, temperature maintained at 37°C and silicone tubing mimicking *in vivo* veins.

Figure -3.6- BioSPME probe pierced through the artificial vein, exposed to circulating blood for 2 min to enable analyte extraction. A restrainer was utilised to hold the artificial vein in place for sampling, this is similar to an animal restrainer used to enable efficient access to *in vivo* veins during sampling.

3.2.10 LC-MS/MS Analysis

LC-MS/MS analysis was performed using the same methodology described in Chapter 2, Section 2.2.4.

3.3 Results and Discussion

3.3.1 Physical Characterisation of SPME Fibres

The surface of the SPME fibres were examined using scanning electron microscope with a view to characterise the physical properties of the fibres at various stages of the SPME process. Figure-3.7- shows the structure of the coated phase, which consists of silica beads with C18 particles bonded to the surface of a stainless steel wire with the particles bonded to each other by use of polyethylene glycol (PEG) glue. The fibres appear to have a smooth thin coated surface when viewed with the naked eye, however SEM images in Figure -3.7- of blank fibres suggest that the coating contains unfilled parts, "holes" scattered between the C18 silica beads.

Figure-3.7- image C and D show a cracked fragment and a cavity in the blank fibres (size ~8µm) suggesting inconsistencies with the coating process. This non uniform coating is expected to be an important contributing factor to inter-fibre variability, which ultimately leads to irregular extraction efficiency and reduction in reproducibility. Vuckovic *et al*[42] investigated the inter-fibre reproducibility of biocompatible C18 fibres using model drugs and the results showed that reproducibility was ≤11% RSD for n = 10 fibres. Musteata *et al*[114] also assessed inter-fibre reproducibility of custom made biocompatible fibres where RSD values were ≤10%. While Lord *et al*[61] utilised polypyrrole coated probes that has approximately 30% inter-fibre variability and Schubert *et al*[115] reported 47 – 52% RSD values for the extraction of linezolid. Such values are considered to be undesirably high for regulated bioanalytical applications, where total analytical variability is required to be within ±15%[12]. However, this aspect is currently being explored by the vendors (Supelco/Millipore Merck) in response to feedback from this research. They are currently looking to further improve inter-fibre reproducibility through production of highly homogenous fibres and better quality control procedures.

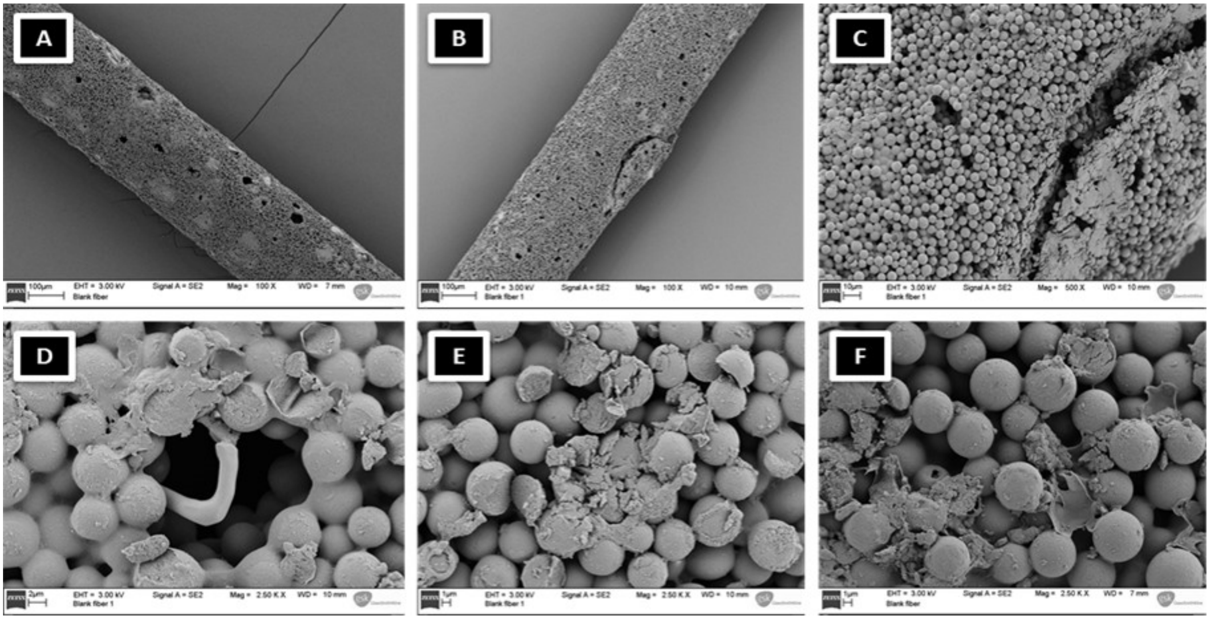


Figure -3.7- SEM images of blank SPME fibres coated with 5 µm C18- bonded silica particles. (A = blank fibre, Mag. = 100 X), (B = cracked blank fibre, Mag. = 100 X), (C = cracked blank fibre, Mag. = 500 X), (D = hole within fibre, Mag. = 2.5 K X), (E = fibre silica particles, Mag. = 2.5 K X), (F = fibre silica particles, Mag. = 2.5 K X).

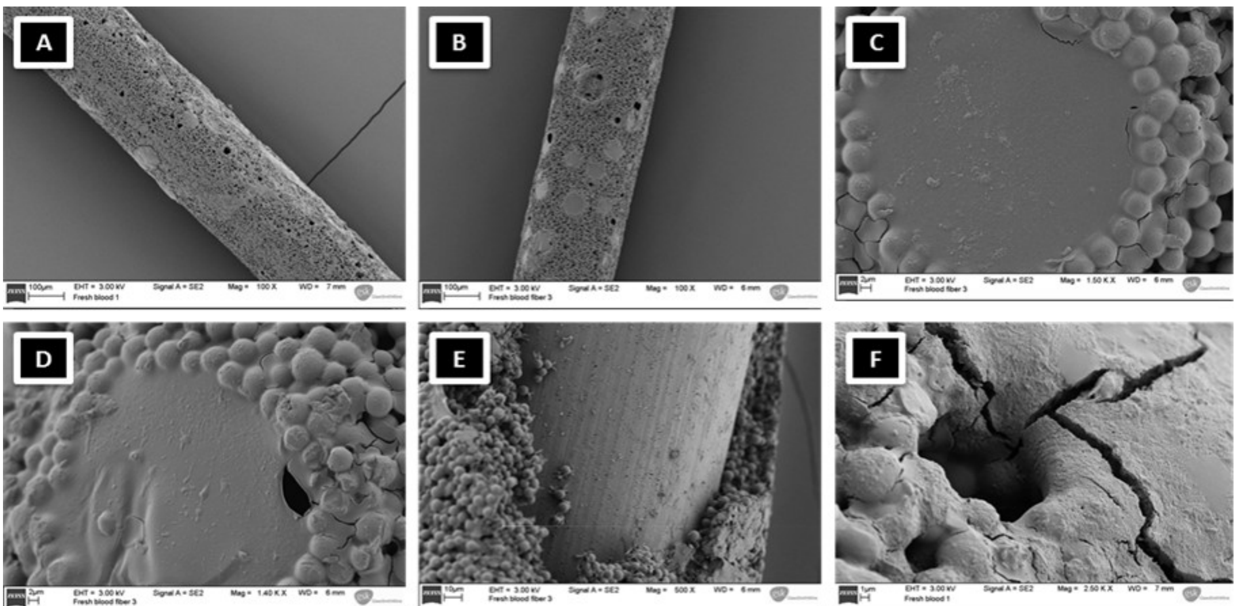


Figure -3.8- SEM images of SPME fibres exposed to fresh rat blood for 30 min without wash or desorption steps. (A = fibre exposed to blood, Mag. = 100 X), (B = fibre exposed to blood, Mag. = 100 X), (C = hole within fibre, Mag. = 1.5 K X), (D = hole within fibre, Mag. = 1.4 K X), (E = stripped fibre coating, Mag. = 600 X), (F = hole within fibre, Mag. = 2.5 K X).

The size of each C18 silica bead is approximately 5 µm as shown in Figure-3.7- image D, E and F. This is consistent with the manufacturer's specification for the biocompatible fibres[43]. Figure -3.8- illustrates fibres subsequent to blood exposure. Images A-D show a thin layer covering some of the empty spaces or the holes previously detected on the blank fibres. This could be a sheet of fat or matrix components that adhere to the fibre during blood exposure. Other techniques such as energy dispersive spectrometry (EDS)[116] or matrix assisted laser desorption (MALDI)[117] could be utilised to identify the chemical nature of these components and confirm whether its matrix related or if its material leaching from the fibre itself. These techniques are suitable for surface imaging of biological and chemical materials to identify the individual components[117].

Although the size of a red blood cell (~7-8 µm) allows it to fit into these holes, the way the SEM samples are prepared involves the use of vacuum followed by platinum coating which potentially may cause intact cells to collapse. For this reason, it is anticipated that this thin sheet of material is not composed of intact blood cells, but could be debris of red blood cells.

Figure -3.8- image E shows a deliberately stripped section of a fibre revealing the number of C18 layers (~ 6 to 7) coated onto the fibre which in turn provides an overview of the fibre thickness (~ 6 to 7 equating to approximately 40 µm fibre thickness) as well as the number of active sites. Similar findings were observed by Vuckovic *et al*[42] where 40 µm fibre thickness was shown upon removal of a portion of the fibre coating.

Figure -3.9- shows stored fibres subsequent to blood exposure for 30 min. Although in practice, fibres are washed prior to storage but in this instance fibres were stored immediately after blood exposure to observe the impact if blood residues were left on fibre and not completely washed off after extraction. Images A-F demonstrate dried blood forming a crusty hard layer on the fibre following storage at ambient temperature for 24 h. Figure-3.10- shows fibres washed with water for 30 s following blood exposure, image A illustrates a mucus looking substance attached to the fibre after the wash step.

Figure-3.11- shows fibre images post desorption which clearly demonstrates the recovery of smooth, well defined C18 particles without any smears or uneven surfaces. Although most of the thin sheets or layers covering the hollow spaces have been distorted, their presence is still apparent which indicates that exposure to an organic solvent during the desorption step had no impact on this. The impact of this is unknown, whether it could contribute to the variability of extraction results, further investigations are required to understand the nature of these thin sheets and their effect. This could be performed using MALDI[117] and or EDS[118] as described earlier.

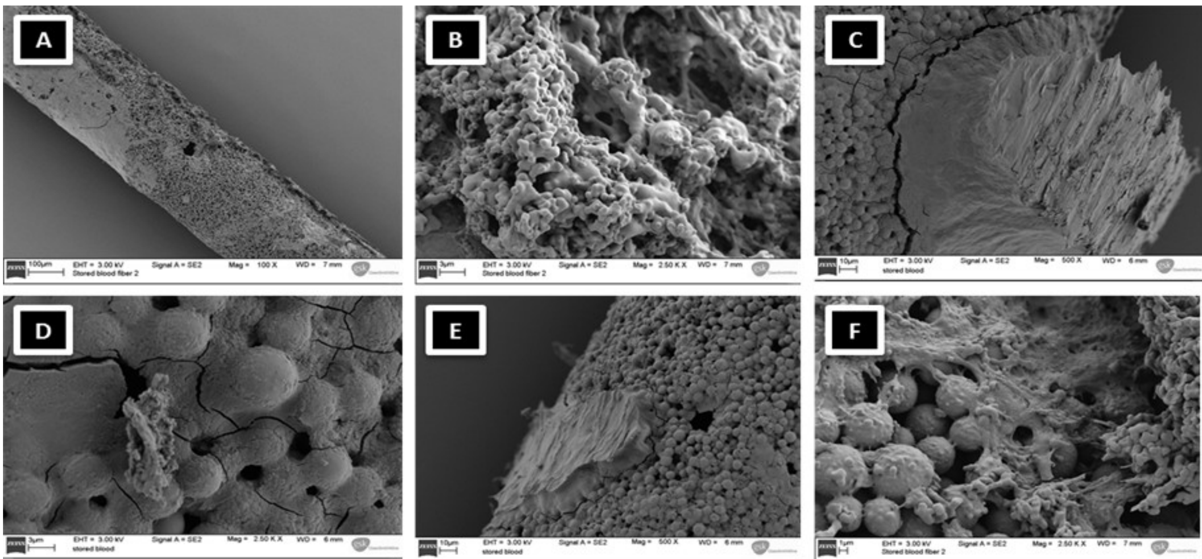


Figure -3.9- SEM images of SPME fibres stored for 24 hours at ambient temperature following exposure to control rat blood for 30 min. (A = fibre exposed to blood and stored, Mag. = 100 X), (B = fibre silica particles with blood following storage, Mag. = 2.5 K X), (C = dried blood residue on fibre upon storage, Mag. = 500 X), (D = silica particles of fibre covered with blood upon storage, Mag. = 2.5 K X), (E = silica particles of fibre covered with blood upon storage, Mag. = 500 X), (F = silica particles of fibre covered with blood upon storage, Mag. = 2.5 K X).

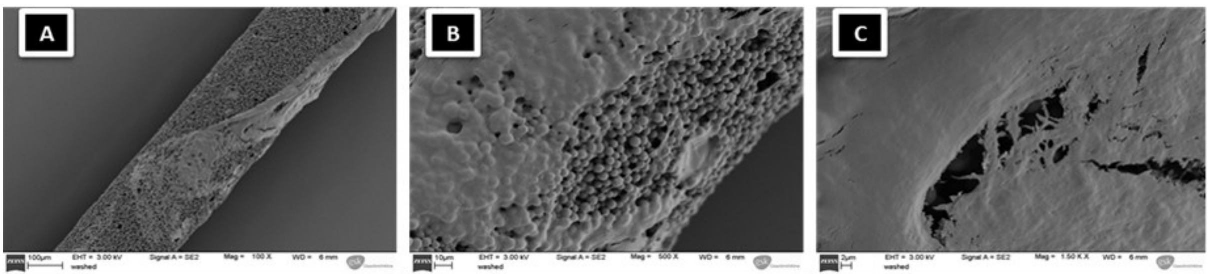


Figure -3.10- SEM images of SPME fibres after the wash step (rinsing with water for 30 s) subsequent to blood exposure for 30 min and prior to desorption. (A = fibre subsequent to wash, Mag. = 100 X), (B = fibre silica particles subsequent to wash, Mag. = 500 X), (C = hole within fibre subsequent to wash, Mag. = 1.5 K X).

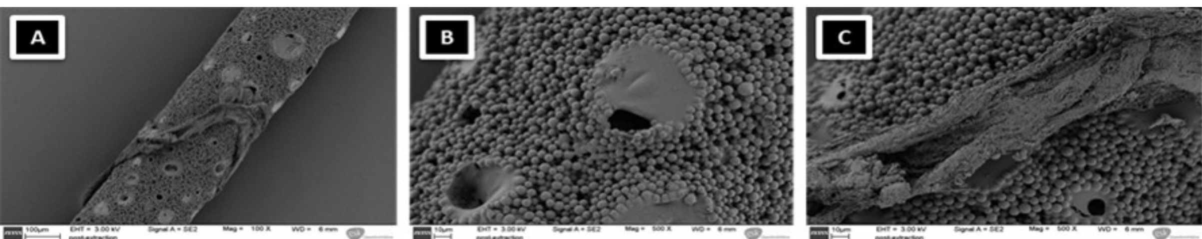


Figure -3.11- SEM images of SPME fibres subsequent to a desorption step, post analyte extraction. (A = fibre following desorption, Mag. = 100 X), (B = fibre silica particles following desorption, Mag. = 500 X), (C = fibre silica particles following desorption, Mag. = 500 X).

3.3.2 Extraction Time Profile

In order to study the equilibration time profile to establish the time required for the SPME C18 fibres to reach equilibrium, *in vitro* extraction time-course experiments were conducted in rat blood. An optimum equilibrium or pre-equilibrium time for *in vivo* applications must be determined as part of the *in vitro* method development.

Prior to this, it was necessary to assess the suitability of the instruments used to establish the extraction time profiles. For this reason, an experiment was performed to investigate the linearity of the mass spectrometer response. This was conducted using spiked calibration standards in PBS for all three analytes (metoprolol, propranolol and diclofenac). These samples were extracted using protein precipitation. PBS was utilised rather than blood or plasma to decouple the outcome from any matrix effects. Also protein precipitation extraction was used to minimize the impact of the extraction technique i.e. to isolate any potential variability contributions that could be added if SPME was used.

Figure -3.12- shows that the response of the mass spectrometer is linear for all analytes across a large dynamic concentration range (1- 1000 ng/mL) with the following regression coefficients 0.993, 0.999 and 0.998 for metoprolol, propranolol and diclofenac respectively. This confirms the results of the validated methods developed in Chapter 2 for all three analytes. This also indicates that the detector response will be linear for all three analytes at the concentrations that will be investigated throughout this research. So, if any non-linearity is observed for adsorption or desorption profiles, it has now been confirmed that it is not due to the detector.

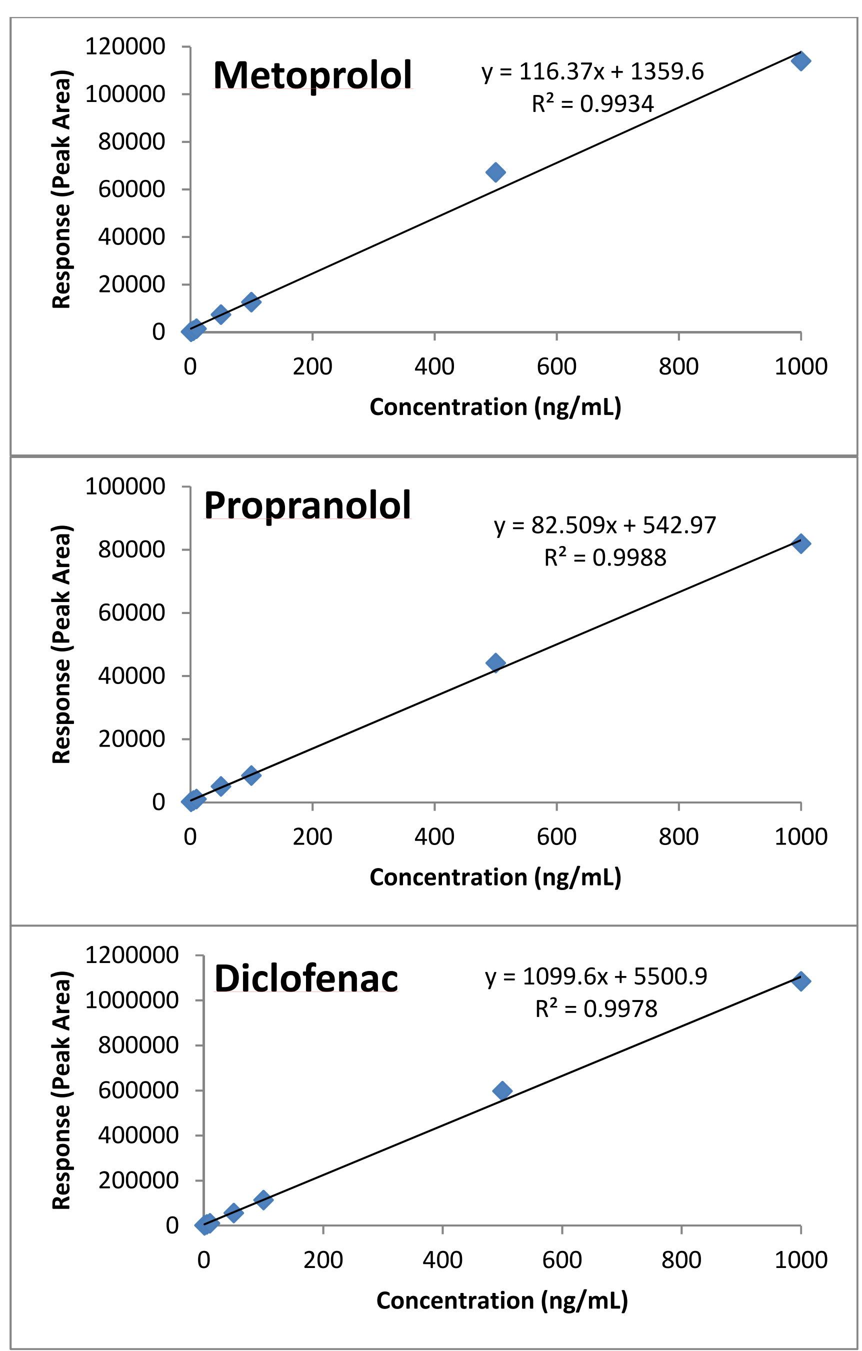


Figure -3.12- Response versus concentration calibration curve for metoprolol, propranolol and diclofenac at a range of 1-1000 ng/mL, analytes were spiked into PBS and extracted using protein precipitation followed by LC-MS/MS analysis.

Figures -3.13-, -3.14-and -3.15- illustrate an extraction time profile for metoprolol, propranolol and difclofenac respectively, at three different concentration levels (10, 100 and 500 ng/mL) achieved by measuring the analyte response as a function of time. The data were normalized by dividing the analyte response (peak area) by the actual spiked concentration.

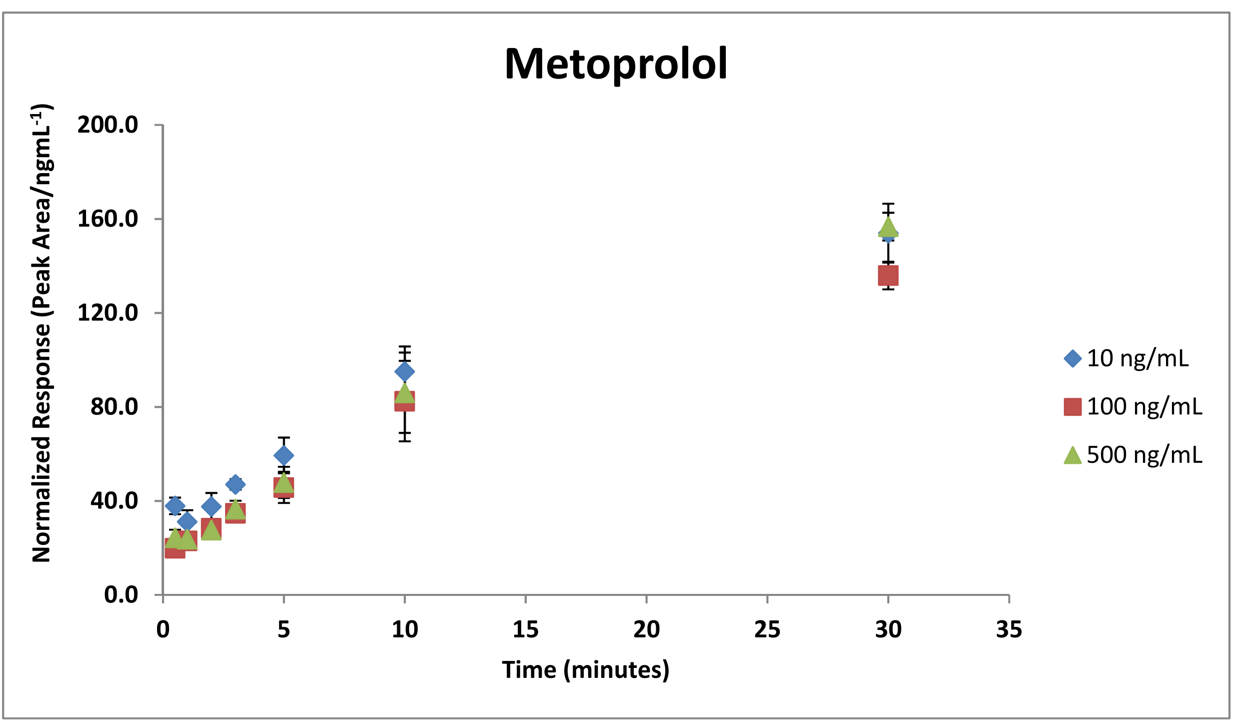


Figure -3.13- Normalized response versus extraction time profile of metoprolol extracted from rat blood spiked at 10, 100 and 500 ng/mL. Data represents mean analyte peak area for n = 6 fibres at each time point, results were normalized by dividing the response (peak area) by actual spiked concentrations. Error bars constructed based on standard deviations (1 SD used).

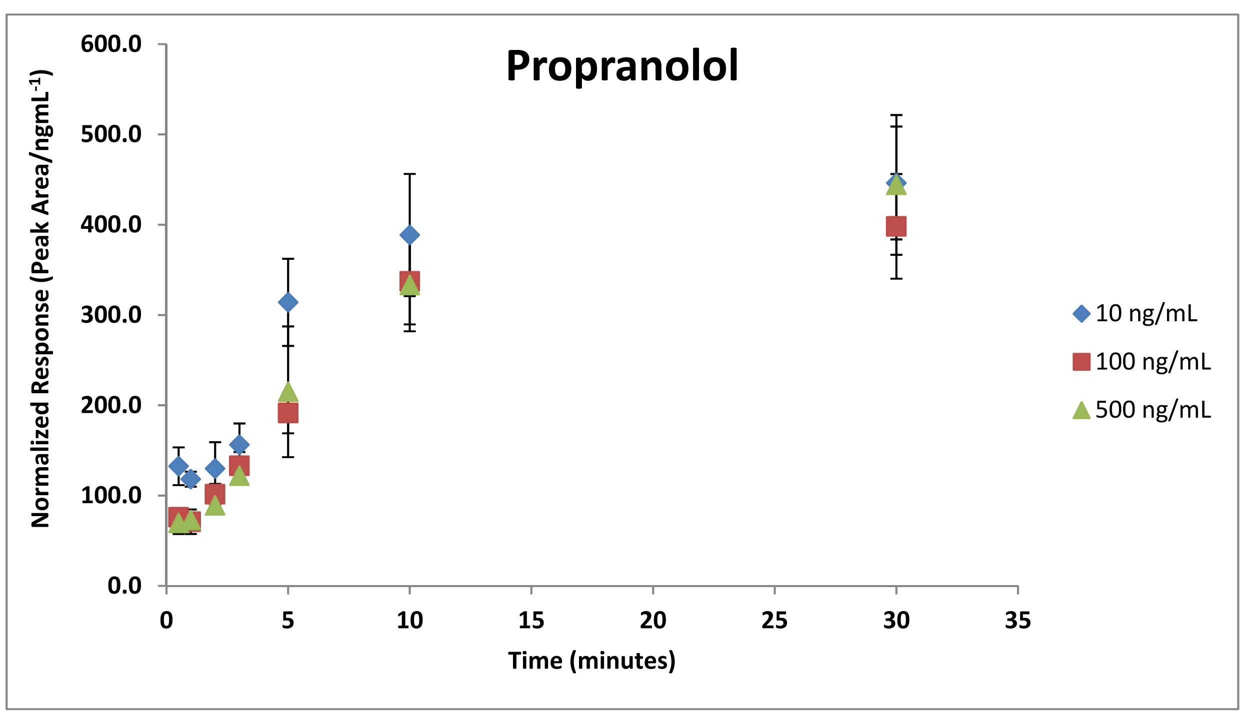


Figure -3.14- Normalized response versus extraction time profile of propranolol extracted from rat blood spiked at 10, 100 and 500 ng/mL. Data represents mean analyte peak area for n = 6 fibres at each time point, results were normalized by dividing the response (peak area) by actual spiked concentrations. Error bars constructed based on standard deviations (1 SD used).

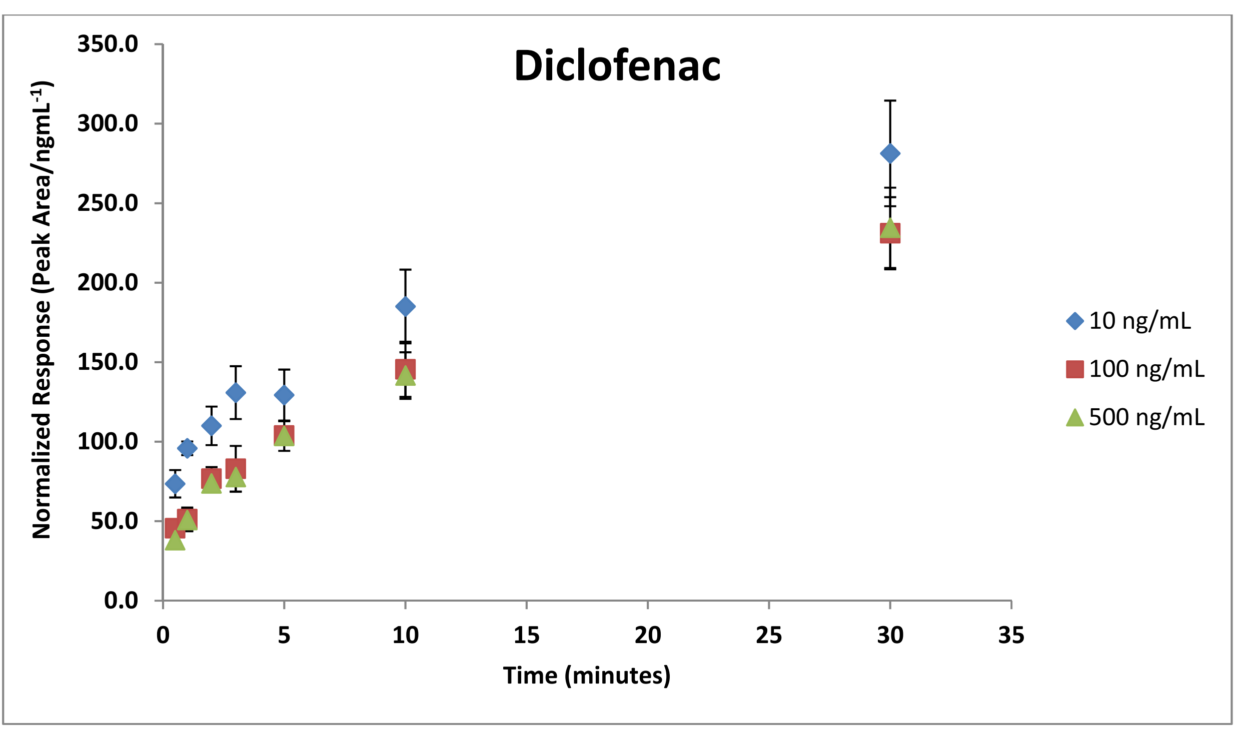


Figure -3.15- Normalized response versus extraction time profile of diclofenac extracted from rat blood spiked at 10, 100 and 500 ng/mL. Data represents mean analyte peak area for n = 6 fibres at each time point, results were normalized by dividing the response (peak area) by actual spiked concentrations. Error bars constructed based on standard deviations (1 SD used).

The initial data points for the different concentrations of the metoprolol extraction time profile (Figure -3.13-) do not overlay, despite them being within the linear region of the response versus concentration from the detector. There is a difference between the low concentration (10 ng/mL) data compared with the mid and high concentrations at the early time points, with a greater response being observed at lower concentrations. The profiles then follow the same pattern and flatten out at the same position which indicates that they are following similar dynamics. Similar profiles were observed for propranolol and diclofenac. However, the normalized profile for the low concentration (10 ng/mL) for diclofenac is consistently higher than the mid and high profiles at all time points. This discrepancy could be due to the quality of the SPME fibres, where the inter-fibre variability could be more apparent at the lower concentration which impacts the mean of the response for n = 6 fibres, making the low concentration point appear to be higher than the data points for the mid and high concentrations.

It is noted that in all cases analyte response increased with time, however the complete profile was not accomplished and equilibrium was not reached by 30 min. The amount of analyte extracted at equilibrium is the largest analyte amount that can be extracted from a given sample by a given SPME fibre, and further increase in time does not contribute to additional increase in the extracted amount of analyte[97]. Thus, the equilibration time can sometimes be inconveniently long because of the slow analyte diffusion rates. The physiochemical properties of the analytes as well as other factors such as the boundary layer may also significantly contribute to the time required to reach equilibrium.

Similar results were reported by Lord *et al*[61] when assessing the time-extraction profiles of benzodiazepines. Extraction equilibrium was reached after 60 min, however pre-equilibrium conditions were used and 5 min sample exposure was employed. Aresta *et al*[65] also showed extraction time profiles for naproxen using carbowax coated fibres, equilibrium was not reached by 60 min. While shorter equilibration times (15 – 20 min) were shown by Boussahel *et al*[119] for pesticide extractions from water using PDMS/GC fibres. In all cases such equilibration times are not suitable for *in vivo* PK/TK applications.

Due to the fact that equilibrium in this study was not reached by 30 min for all three analytes, a follow up experiment was conducted for one of the analytes (metoprolol). It was decided that only one analyte would be further examined due to the limited number of the prototype SPME fibres available. Metoprolol was chosen because this analyte was to be used in further studies within this research. An extended extraction time was used to investigate the time at which equilibrium is achieved. This experiment was performed by quantifying the recovered concentrations of metoprolol at each time point rather than using the obtained response to gain a better understanding of recovery throughout the equilibrium profile. Quantification was achieved using calibration standards prepared in the desorption solvent (acetonitrile) spiked with metoprolol at a range of 1 – 1000 ng/mL.

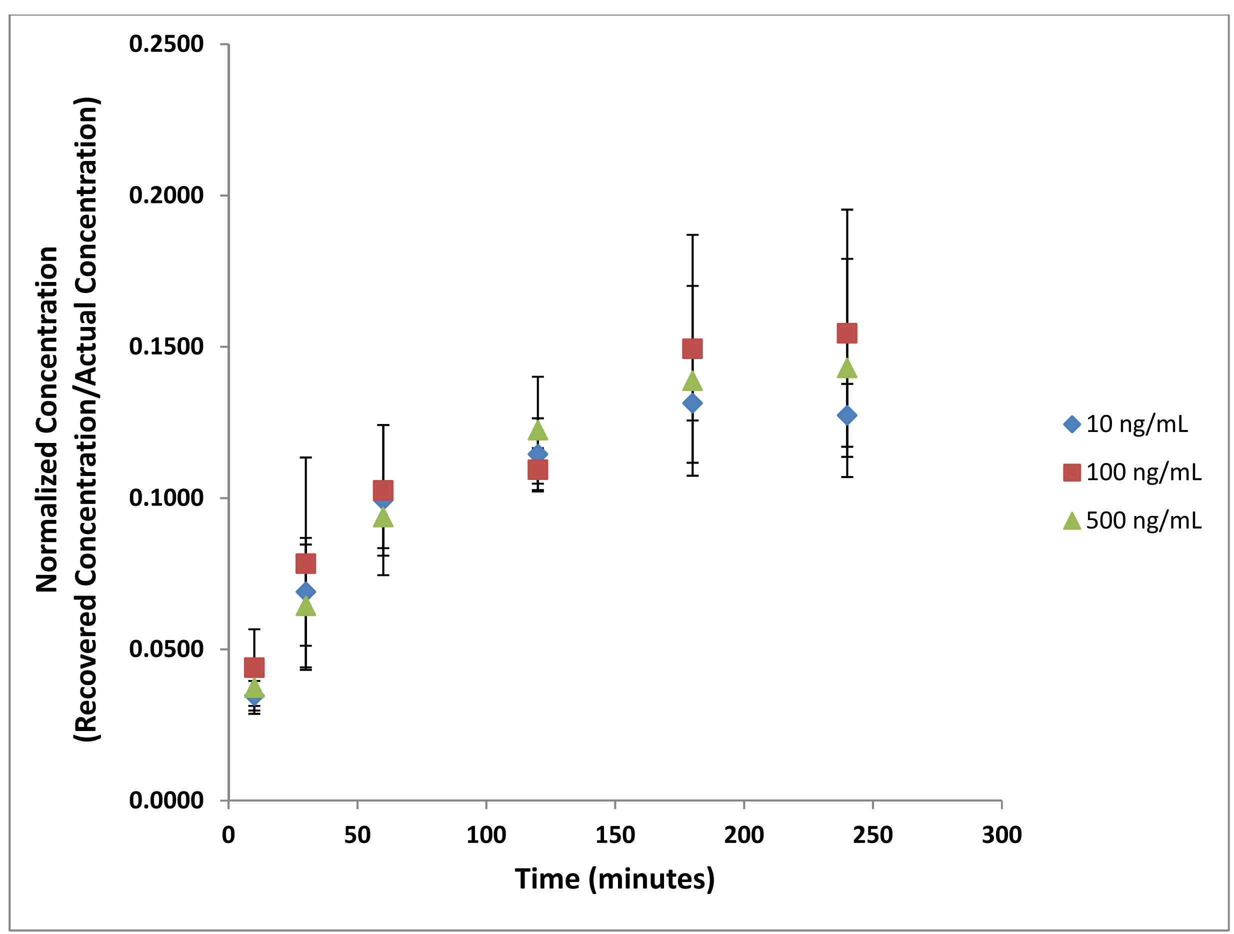


Figure -3.16- Combined extraction-time profile of metoprolol where recovered concentrations were normalised by dividing them by the actual spiked concentrations (mean ± SD, of n = 6 fibre determinations) (1 SD used).

The quantitative extraction-time profile for metoprolol displayed in Figure -3.16- shows that the curve started to flatten out indicating that equilibrium was achieved after 180 min (3 h) exposure to blood. It also shows that concentration is proportional to analyte response (peak area) when comparing Figure -3.13- to -3.16-. This further confirms that equilibrium was not reached after 30 min as shown previously in Figure -3.13-. Each profile needed additional time for equilibrium, this could also be the case for propranolol and diclofenac.

However, such long equilibration times provide insufficient temporal resolution to construct an accurate PK profile when applied *in vivo*. This is particularly true for early PK time points, where it is sometimes necessary to take multiple samples within a very short period of time

such as 5 min, 15 min, 25 min and 1 h after dosing. Also the practicality of leaving the probe within a living organism for this length of time is of a major concern in addition to ethical considerations.

The time required to reach equilibrium is independent of sample concentration, this is evident in Figures -3.13-, -3.14- and -3.15- and -3.16- and has been previously established in various studies published in literature[41]. The ratio of the amount of analyte extracted at each timepoint for different concentrations remained constant throughout the extraction profile within acceptable experimental error (± 15% as outlined by bioanalytical FDA guidelines[12]). For example, the ratio between the amount of metoprolol extracted at 10 min for the low and mid concentration is 1.27, this ratio at 30 min is 1.14 and at 4 h is 1.21 as shown in Table -3.2-. This trend was observed for all three concentrations throughout the time profile. This indicates that pre-equilibrium conditions and shorter extraction times could be utilized as long as all *in vitro* calibration standards and quality control samples are exposed to the matrix for the same length of time as *in vivo* sampling time. As previously mentioned, equilibrium time is affected by coating thickness, agitation conditions and analyte distribution constants. The long equilibration times observed for the fibres could be attributed to the thickness of the coated phase which is approximately 45 µm, thicker fibres requires longer periods of time for the analyte to diffuse through the particles layers and therefore result in longer equilibration times[41]. Reduction in the particle size of the extraction phase may allow for thinner coated fibres without impacting the number of active sites available for extraction and therefore decreasing the length of time required for equilibrium.

The mechanics of agitation and fluid dynamics could also impact the equilibration process although only minor changes to equilibration times were observed by Lord *et al*[97] when maximum orbital sample agitation was compared to no agitation.

Table -3.2- Recovered concentration ratio of metoprolol at each time point (mean ± SD, n = 6) (1 SD used).

Time (minutes)	Recovered Concentration Ratio		
	100 ngmL^{-1}/10 ngmL^{-1}	**500 ngmL^{-1}/100 ngmL^{-1}**	**500 ngmL^{-1}/10 ngmL^{-1}**
10	**1.27** ± 0.035	**0.849** ± 0.044	**1.08** ± 0.037
30	**1.14** ± 0.069	**0.822** ± 0.078	**0.932** ± 0.064
60	**1.03** ± 0.099	**0.914** ± 0.103	**0.944** ± 0.094
120	**0.96** ± 0.115	**1.120** ± 0.109	**1.07** ± 0.122
180	**1.14** ± 0.131	**0.929** ± 0.149	**1.06** ± 0.139
240	**1.21** ± 0.127	**0.926** ± 0.154	**1.12** ± 0.143

Several experimental limitations should also be considered when constructing *in vitro* extraction-time profiles during analyte method development; these include the potential change in blood consistency over time where some blood congealing effects were observed at the later timepoints, adhesion of matrix components to the outer parts of the probe i.e. the needle area due to long blood exposure periods which may require longer washing steps prior to desorption and finally the practicalities of executing such experiments within a working day.

3.3.3 Desorption Time Profile and Optimisation of Desorption Solvent

Desorption, the final step in the SPME extraction process plays a vital role in maximizing method sensitivity. Increasing desorption efficiency by optimising the length of desorption time and selecting appropriate desorption solvents is a crucial element of *in vitro* method development. Figures -3.17, -3.18- and -3.19- show desorption time profiles for metoprolol, propranolol and diclofenac respectively. Desorption was performed using 100% acetonitrile containing IS.

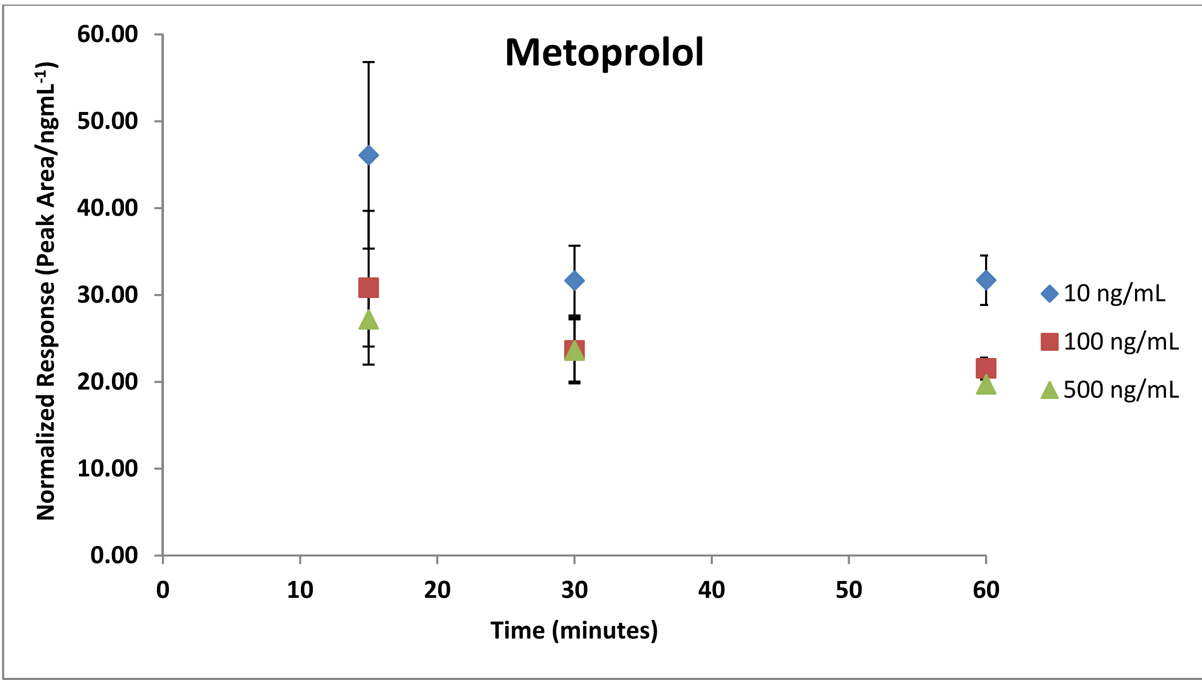


Figure -3.17- Normalized response versus desorption time profile of metoprolol extracted from rat blood spiked at 10, 100 and 500 ng/mL. Metoprolol desorbed using 100% acetonitrile containing IS. Data represents the mean analyte peak area for n= 6 fibres at each timepoint. Data were normalized to spiked concentrations and error bars constructed based on standard deviation (1 SD used).

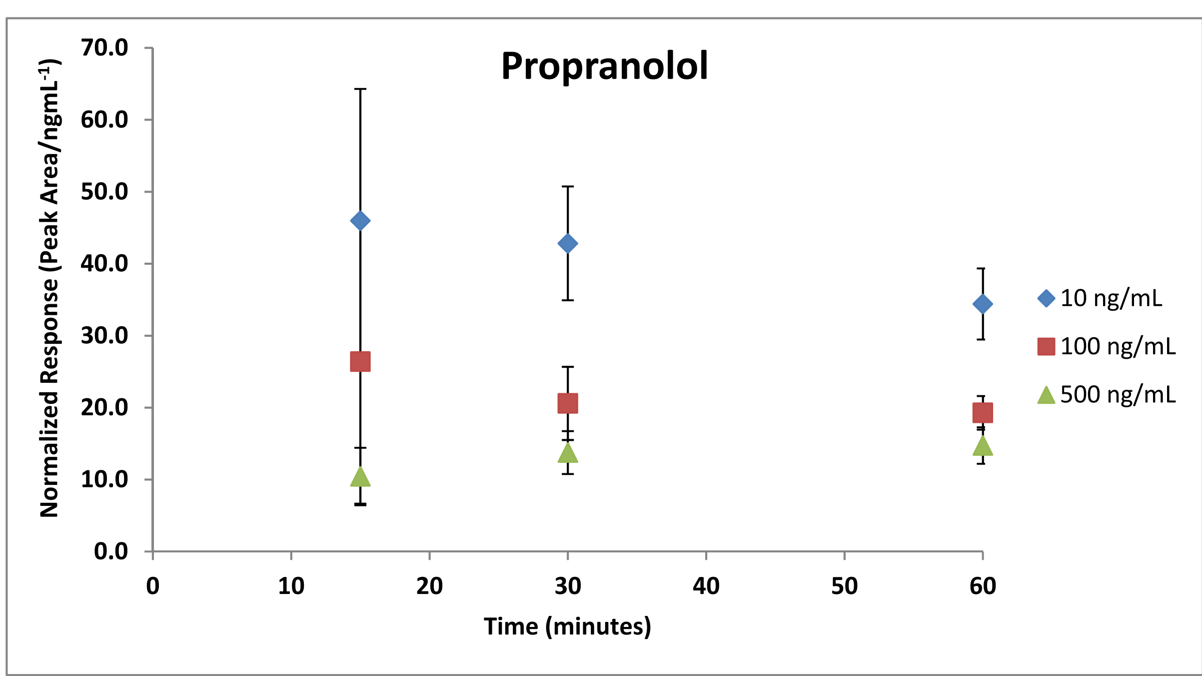


Figure -3.18- Normalized response versus desorption time profile of propranolol extracted from rat blood spiked at 10, 100 and 500 ng/mL. Propranolol desorbed using 100% acetonitrile containing IS. Data represents the mean analyte peak area for n= 6 fibres at each timepoint. Data were normalized to spiked concentrations and error bars constructed based on standard deviation (1 SD used).

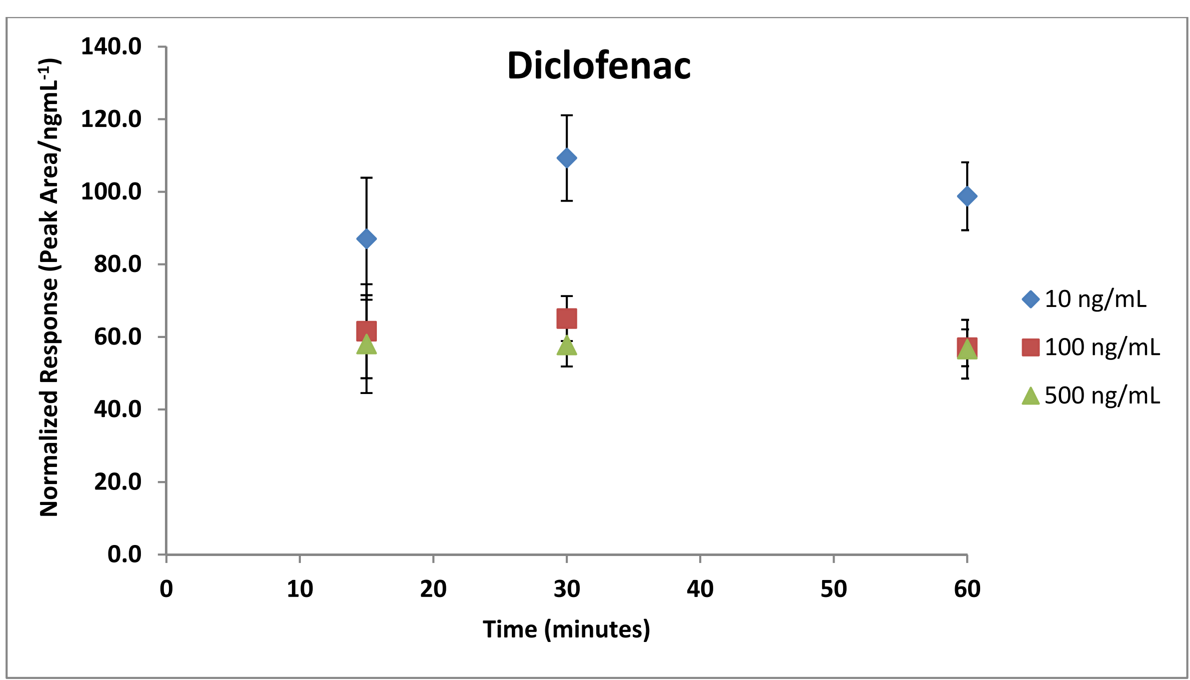


Figure -3.19- Normalized response versus desorption time profile of diclofenac extracted from rat blood spiked at 10, 100 and 500 ng/mL. Diclofenac desorbed using 100% acetonitrile containing IS. Data represents the mean analyte peak area for n= 6 fibres at each timepoint. Data were normalized to spiked concentrations and error bars constructed based on standard deviation (1 SD used).

All desorption profiles are relatively flat suggesting that complete desorption was achieved after 15 minutes for all analytes across three different concentrations. Concentration dependency was also observed with desorption profiles of all three analytes, where normalized response of the lower concentration (10 ng/mL) was higher than the mid and high concentrations. This could be due to higher recovery at lower concentrations, potentially due to solvent saturation at higher concentrations or detector saturation. However, the latter is unlikely because the response for all anlytes is in the linear range of the detector as demonstrated earlier in Figure-3.12-.

An interesting trend was observed throughout the majority of the profiles where variability decreased with time. This could potentially be due to longer agitation periods which seems to improve the variability. Or it could be due to the fact that the analyte is desorbed off the fibre from different areas, starting with the surface followed by the inner coated particles and therefore different desorption pathways and kinetics may lead to larger variability.

Another potential reason could be that complete desorption equilibrium is established with longer desorption periods, but some of the desorbed analyte might be re-adsorbed onto the SPME fibre. Finally, a portion of the analyte could be sticking to the vial (desorption tube) giving better consistency.

The desorption profiles shown above were flat between 15-60 min, therefore the time required for maximal desorption could be selected between 15-60 min. Desorption time periods of < 5 min do not provide practical advantages and those > 30 min are disadvantageous for throughput. Current bioanalytical extraction or clean up techniques prior to analysis range in complexity from very quick and simple protein precipitation methods to more complex liquid-liquid extraction and SPE methods[77]. While microsampling techniques such as dried blood spots (DBS) require additional steps such as sample drying on the DBS card (typical recommended time is 2 h), punching out the DBS disk or whole spot followed by solvent extraction using a suitable shaker for 2 h[120]. Thus, the time taken for the analyst to complete the extraction process is variable depending on the extraction technique. This implies that a SPME desorption time of approximately 30 min can offer operational advantages to the analyst. Particularly, when sample freezing and defrosting, aliquoting and centrifuging steps are all removed and the only step subsequent to sample collection is the desorption stage within the bioanalytical laboratory. This step could be further improved with decreased manual intervention and introduction of laboratory SPME automation techniques.

It is also important to select a desorption solution and volume that optimize both desorption efficiency and the degree of pre-concentration (small volume) while maintaining adequate chromatographic peak shape through matching of the solvent composition to that of the mobile phase as much as possible[87].

Different desorption solvents were also evaluated to understand the impact on analyte recovery. The choice of solvents varied from 100% organic such as acetonitrile to others containing aqueous and finally an acidified composition. This is to understand whether the affinity of the test compounds for each solvent is different and in turn result in higher or lower analyte recovery. Figures -3.20-, -3.21- and -3.22- illustrate the normalized peak area values for two concentration levels (10 and 500 ng/mL) obtained after desorption with various solvent compositions. It was noted that desorption efficiency was almost halved for propranolol desorbed with methanol compared with acetonitrile at the high concentration (500 ng/mL), this effect was less apparent with metoprolol. Propranolol is less hydrophilic

than metoprolol[121], therefore the affinity of each analyte to various solvents is different depending on the chemical structure of the analyte and its physiochemical properties.

Diclofenac on the other hand, showed no difference in response at 500 ng/mL across all desorption solvents and only a minimal effect was observed at the lower concentration 10 ng/mL where addition of aqueous composition enhanced the performance of diclofenac desorption. A non-linear or an un-proportional trend was observed between both concentration levels (10 and 500 ng/mL) for both propranolol and diclofenac which may impact the accuracy (recovery) of the drug concentrations when using SPME. For this reason, this must be assessed during method validation and the impact, if any, on the overall recovery should be determined.

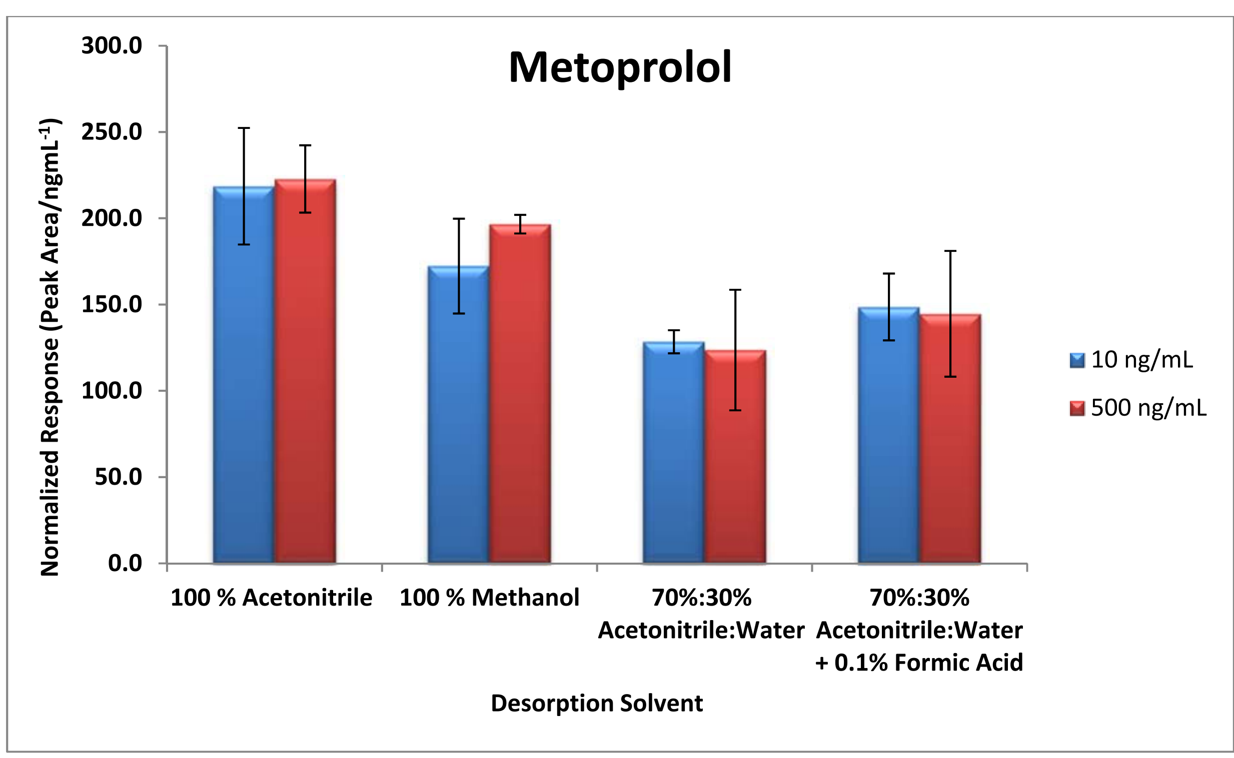


Figure -3.20- Optimization of desorption solvent for metoprolol extracted from rat blood (mean ±SD, n = 3) spiked at 10 and 500 ng/mL, desorption exposure was performed for 15 min using agitation of 500 rpm. Data was normalized to spiked concentrations and error bars constructed based on standard deviation (1 SD used).

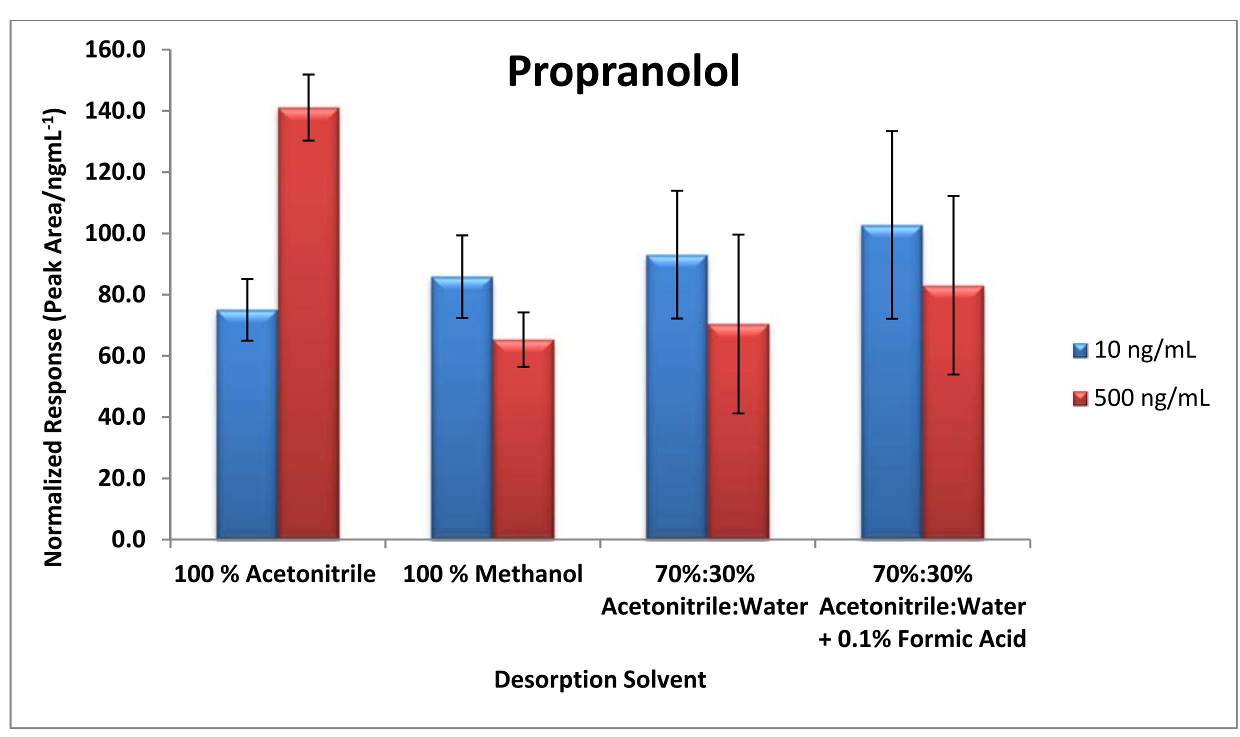


Figure -3.21- Optimization of desorption solvent for propranolol extracted from rat blood (mean ±SD, n = 3) spiked at 10 and 500 ng/mL, desorption exposure was performed for 15 min using agitation of 500 rpm. Data was normalized to spiked concentrations and error bars constructed based on standard deviation (1 SD used).

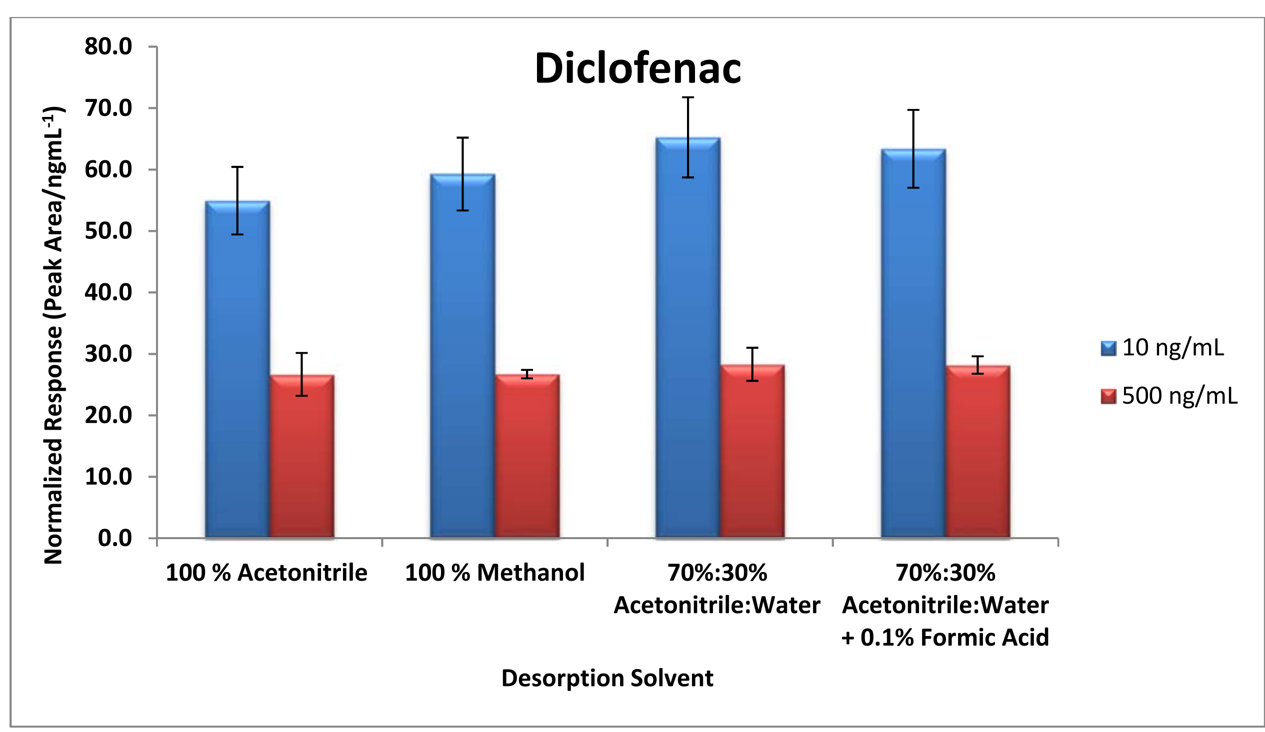


Figure -3.22- Optimization of desorption solvent for diclofenac extracted from rat blood (mean ±SD, n = 3) spiked at 10 and 500 ng/mL, desorption exposure was performed for 15 min using agitation of 500 rpm. Data was normalized to spiked concentrations and error bars constructed based on standard deviation (1 SD used).

In general, the results indicate that acetonitrile was superior in performance for the majority of the targeted compounds and for all concentrations. This correlates with the supplier's (Supelco/Sigma Aldrich) recommendation for desorption of C18 SPME fibres[42] and is therefore a good default eluent solvent. However, if additional sensitivity is required, then other solvents may need to be explored.

The practice of identifying an optimum solvent for greater extraction efficiency has previously been adopted and applied during the method development of other microsampling techniques such as DBS[24,122]. The choice of extraction solvent is dependent on the nature of the analyte in terms of its physiochemical properties and its relative affinity for the stationary and solvent phases. However generic solvents that may be applicable for a range of analytes are generally preferable for ease of use and flexible workflow, particularly if sufficient assay

sensitivity at low enough reproducibility is obtained. In the case of SPME, acetonitrile would be the starting point as a generic desorption solvent.

3.3.4 The Impact of Hematocrit Level on SPME Extraction

The effect of blood hematocrit level on analyte response was studied for the SPME extraction of metoprolol from rat blood samples with varying hematocrit levels (20 - 80%). Only one analyte was investigated (metoprolol) due to the limited number of prototype *in vivo* SPME available. Figure -3.23- shows that no consistent correlation was observed between changing hematocrit level and analyte response, suggesting that there is no evidence of hematocrit impact on SPME extraction. Results for the 60% hematocrit level were not reportable due to instrumental failure which prevented sample injection. The data obtained were then normalised to the difference in response from the response acquired for the original hematocrit level of rat blood prior to centrifugation (i.e. nominal HCT value for rat blood = 50%) as shown in Figure -3.24-. The difference in response across the hematocrit range is < 25% with no particular trends noted.

The amount of bias that is considered acceptable by international regulatory agencies for a quantitative bioanalytical method to be valid is ±15%[107], Figure -3.24- shows that the percentage difference in response is < 15% from nominal over the range of 20 - 70% hematocrit levels which is within the acceptance criteria as stated above. Also, the range of HCT covered is more than the range that is likely to be encountered within a preclinical study.

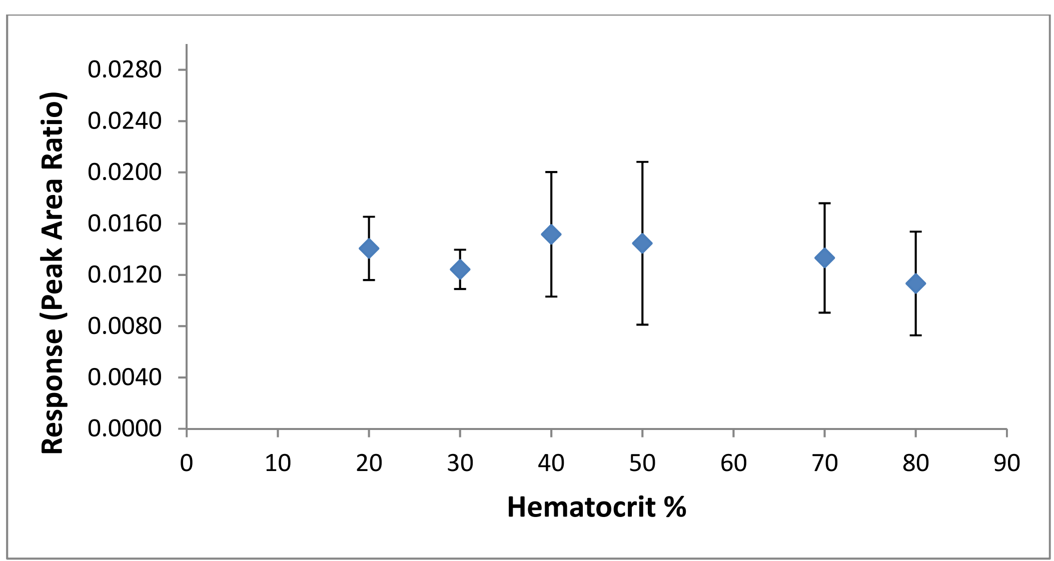


Figure -3.23- Relationship between rat blood hematocrit and analyte response for metoprolol (100 ng/mL). Each data point is the average analyte response of n = 6 fibres. Error bars set at standard deviation (1 SD used).

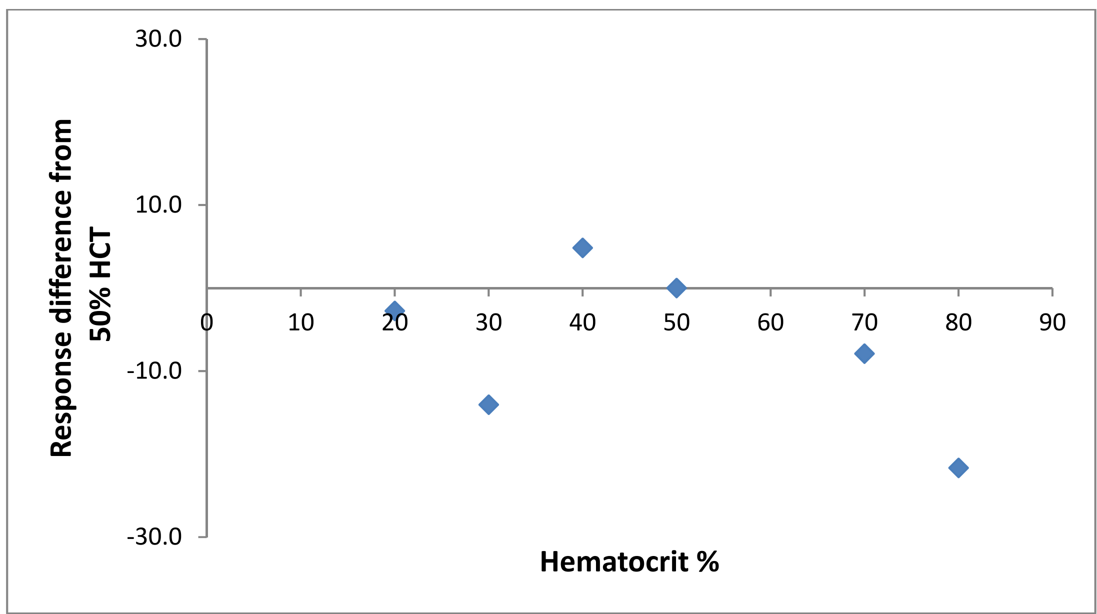


Figure -3.24- Metoprolol mean response normalized to 50% hematocrit level.

Studies have shown that an increase in red cell hematocrit leads to an increase in relative whole blood viscosity[123]. Also diffusion coefficients are inversely related to viscosity where the rate of analyte diffusion decreases with increased viscosity and therefore an increase in the equilibration time is expected for more viscous matrix. This could explain the low analyte response observed for 80% hematocrit sample where a longer equilibration time may have been required to achieve a satisfactory response equivalent to that of a nominal hematocrit sample.

Overall the level of hematocrit for individual animals or humans is unlikely to change over a short period of time under healthy conditions, i.e. over the course of pharmacokinetic studies, however the potential for inter-individual hematocrit variability remains a risk. Nevertheless, this means that SPME has the potential to provide a fit for purpose microsampling technique that may overcome issues associated with the quantification of samples with varying hematocrit levels and offer benefits over previously established problems with the dried blood spot technology[124,125].

3.3.5 On-Fibre Stability

A stability experiment was performed to investigate on-fibre compound stability upon storage at ambient temperature. Three anaytes (metoprolol, propranolol and diclofenac) were extracted from spiked rat blood onto SPME fibres and stored at ambient temperature for two and six weeks. Stability was assessed at three different concentrations (10,100 and 500 ng/mL) by comparing the mean response (peak area ratio) after storage for two (T2 wks) and six weeks (T6 wks) with the original analyte response extracted fresh at time zero (T0).

Table -3.3- On-Fibre Stability Data for Metoprolol

Nominal Concentration (ng/mL)	10			100			500		
Extraction Time*	**T0**	**T2**	**T6**	**T0**	**T2**	**T6**	**T0**	**T2**	**T6**
Mean (n=6) Peak Area Ratio (Analyte/IS)	0.00859	0.00931	0.00911	0.0793	0.0781	0.0792	0.319	0.316	0.319
SD	0.0020	0.00252	0.00263	0.0111	0.0127	0.0120	0.0619	0.0661	0.0612
CV (%)	24.3	27.8	28.9	14.8	15.4	16.2	19.1	21.0	19.2
Difference from T0 (%)		**8.6**	**7.0**		**-1.5**	**-0.1**		**-0.9**	**-0.1**

* T0 = Extraction at Time 0

T2 = Extraction after storage at ambient temperature for two weeks

T6 = Extraction after storage at ambient temperature for six weeks

Table -3.4- On-Fibre Stability Data for Propranolol

Nominal Concentration (ng/mL)	10			100			500		
Extraction Time*	**T0**	**T2**	**T6**	**T0**	**T2**	**T6**	**T0**	**T2**	**T6**
Mean (n=6) Peak Area Ratio (Analyte/IS)	0.00553	0.00521	0.00624	0.0463	0.0429	0.0465	0.218	0.203	0.226
SD	0.000712	0.000934	0.000723	0.00621	0.00446	0.00514	0.0152	0.01353	0.0145
CV (%)	13.9	17.3	12.5	14.0	10.3	11.0	6.9	6.4	6.2
Difference from T0 (%)		**-4.9**	**12.7**		**-7.3**	**0.4**		**-6.9**	**3.7**

* T0 = Extraction at Time 0

T2 = Extraction after storage at ambient temperature for two weeks

T6 = Extraction after storage at ambient temperature for six weeks

Table -3.5- On-Fibre Stability Data for Diclofenac

Nominal Concentration (ng/mL)	10			100			500		
Extraction Time*	**T0**	**T2**	**T6**	**T0**	**T2**	**T6**	**T0**	**T2**	**T6**
Mean (n=6) Peak Area Ratio (Analyte/IS)	0.000224	0.000316	0.000321	0.00233	0.00243	0.00235	0.00936	0.00901	0.00914
SD	0.000812	0.000767	0.000783	0.000321	0.000332	0.000414	0.00245	0.00222	0.00241
CV (%)	28.6	22.2	24.8	14.3	13.7	20.5	25.9	24.4	26.4
Difference from T0 (%)		**10.7**	**12.7**		**5.1**	**0.8**		**-2.9**	**-1.4**

* T0 = Extraction at Time 0

T2 = Extraction after storage at ambient temperature for two weeks

T6 = Extraction after storage at ambient temperature for six weeks

The results in Tables -3.3-, -3.4- and -3.5- show that there was no significant difference (<15%) in analyte response between samples extracted fresh and after on-fibre storage for two and six weeks across all concentrations for all three analytes. This in turn indicates that all three analytes are stable on-fibre for at least six weeks stored at ambient temperature.

Depending on the location of the in-life study and the site responsible for bioanalysis, the distance between the two could range from 0.5 to 10,000 miles and several weeks or even months could pass before samples are analyzed, therefore ensuring compound stability on-fibre is vital during method development.

The data presented in this study illustrate the possibility of storing fibres with extracted analytes at ambient temperature. This is an important aspect for SPME as the technique could be applied to studies in remote areas where refrigerating and centrifugation are not commonly available. The ability to ship samples at ambient temperature offers the potential for considerable cost savings compared with shipment of frozen wet samples[126].

Furthermore, extreme temperature and humidity storage levels should also be investigated prior to sample collection if knowledge of such extreme environmental conditions is known and that samples will be subjected to long distance transfer due to the location of sampling and bioanalytical sites.

3.3.6 The Impact of Blood Flow Rate on SPME Extraction

Changes in body temperature have a direct impact on blood flow rates, for this reason conventional sampling techniques such as venipuncture require rodents to be warmed by placing them on a warm plate or exposing them to warm water prior to taking a sample in order to dilate the blood vessel and increase blood flow rate. Raman *et al*[127] has established that there is a linear relationship between body temperature and blood flow rate. Tail blood flow rate in rodents rises as a linear function of body temperature. This is because at low body temperature, blood flows primarily in central veins of the tail, while at higher body temperature, blood flows in peripheral tail veins[127]. Also the rate of blood flow is varied between species, such physiological parameters cannot be controlled when performing *in vivo* sampling and so it is vital to understand the potential effect of these factors on SPME extraction[127]. In this study, the impact of changes in blood flow rates on analyte response was investigated through a simulated *in vitro* circulatory system. Three analytes (metoprolol, propranolol and diclofenac) were evaluated at three different concentrations (10, 100 and 500 ng/mL) and three different blood flow rates (20, 30 and 75 ml/min). The choice of blood flow rates was a compromise between interspecies hepatic blood flow variation for human, dog and rat (~ 20, 30, 75 mL/min respectively)[128] and the capacity of the peristaltic pump utilised to drive the blood through the *in vitro* set up.

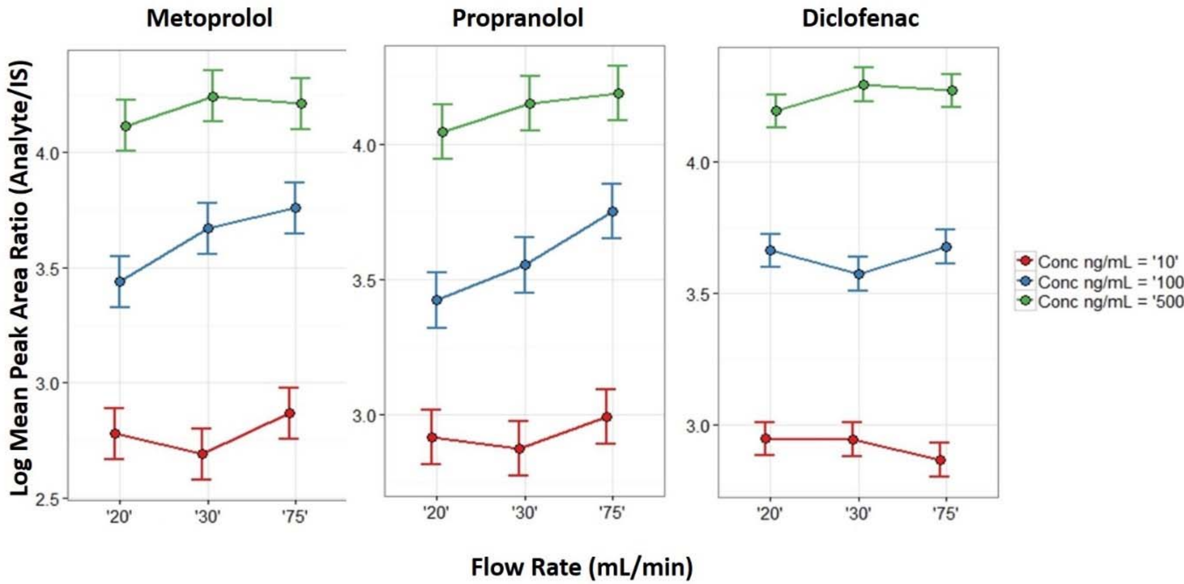


Figure -3.25- The effect of blood flow rate on analyte response. Plots of the log mean peak area ratio of analyte/IS (n = 6) with 95% confidence intervals versus blood flow rate for metoprolol, propranolol and diclofenac at 10, 100 and 500 ng/mL. Plots generated using InVivoStat, version 3.0.

Table -3.6- ANOVA table calculated for the effect of blood flow rate on analyte response using a type III model fit.

Analysis of variance (ANOVA) table			
Analyte	**Degrees of freedom**	**F-value**	**p-value**
Metoprolol	2	4.12	0.0227
Propranolol	2	5.62	0.0066
Diclofenac	2	1.44	0.2487

The data in Figure -3.25- show that in general analyte response (log mean n= 6) increased with increasing blood flow rates for metoprolol and propranolol while no consistent trend was observed for diclofenac. These observations were further confirmed by the results of the statistical analysis shown in Table -3.6-, where a type III model was utilized which assesses the effect of the blood flow rate and the effect of concentration as well as the interaction between both parameters. The data for analyte response were statistically different for metoprolol and propranolol (p values were < 0.05, 0.0227 and 0.0066 for metoprolol and propranolol respectively) while no statistical differences were shown for diclofenac (p value > 0.05, 0.2487).

It is also notable that the change in analyte response at higher blood flow rate was more apparent at the lower concentrations (10 and 100 ng/mL) for metoprolol and propranolol than at the higher concentration (500 ng/mL). This could be due to the fact that mass transfer of the analyte via diffusion increases with increased agitation levels i.e. higher blood flow rates cause faster diffusion which in turn decreases the equilibration time and hence leads to higher analyte response. Nevertheless, the amount of analyte extracted could potentially be the maximum amount of drug extracted at equilibrium, this means that at higher flow rates, shorter equilibration times are anticipated and so equilibrium is reached faster which affects the amount of extracted drug within the 2 min probe exposure period. This concept correlates with results that have previously been reported by Es-haghi *et al*[129]. Despite the apparent impact of blood flow rate for metoprolol and propranolol, it should be noted that blood flow rates within a specific species will not drastically change over time[127,130] as shown in this experiment therefore it is highly unlikely that such parameter will have an impact on PK/TK data within one species.

Inter-species differences have been previously encountered in other microsampling techniques such as DBS. The spot size and appearance of a DBS sample has been shown to be variable between different species where rabbit blood spot size appears to be larger and

forms bigger halo around the spot compared to other species[120,131]. However, as all unknown samples are quantified against calibration standards and QCs prepared with control matrix of the same species then such issue becomes practically insignificant with no impact on the quality of the data produced.

Although the extraction of n = 6 fibres was performed simultaneously for each blood flow rate at each concentration with accurate timing of 2 min exposure to the flowing system, experimental limitations may have impacted the precision (%CV) of the results. This is shown by the poor % CV values that exceeded 30% in some cases as displayed in Table -3.7.

Table -3.7- % CV of analyte response at different blood flow rates (% CV of n = 6)

	Concentration (ng/mL)	**10**			**100**			**500**		
	Flow Rate (ml/min)	**75**	**30**	**20**	**75**	**30**	**20**	**75**	**30**	**20**
Analyte Response % CV	**Metoprolol**	23.8	18.1	13.5	30.3	12.7	36.3	31.7	23.9	23.2
	Propranolol	13.3	5.6	11.4	7.5	18.5	29.2	29.5	19.0	24.6
	Diclofenac	18.2	13.1	8.7	15.9	25.9	13.4	19.6	16.4	22.8

Contributions to overall experimental variance include the positioning of each fibre with respect to the central plug of blood flow, the proximity of the fibre to the walls of the artificial vein, the length of fibre or how far each fibre is actually exposed to the flow at each occasion as well as accuracy of timing in addition to any evaporative or adsorption losses during washing of the fibre or throughout the entire extraction process. This experiment has highlighted the potential for increased inter-fibre variability which could be encountered due to *in vivo* sampling, such variability is associated with the process of handling fibres during *in vivo* applications compared with *in vitro* implementation where most parameters are controlled. For this reason, improved quantitative data could be achieved by enhancing the quality of the manufactured product i.e. by reducing the intrinsic variability of BioSPME fibres down to very low percentages. This will then permit for further variability which maybe encountered during *in vivo* sampling as well as instrumental variability of bioanalytical LC-MS/MS analysis or other quantification techniques.

3.4 Conclusion

An *in vitro* evaluation of the critical parameters that play a major role in the development of a suitable SPME method for the extraction of small molecules was performed. The effect of some of these parameters highlighted some of the potential issues that may impact the application of SPME *in vivo.*

The results of the analyte extraction time profiles, illustrated that analyte adsorption onto the SPME fibre is dependent on matrix exposure time. It may take up to or more than 3 h for the analyte concentration to reach equilibrium between the fibre and the sample matrix. This length of time is not viable for *in vivo* applications. For this reason, pre-equilibrium conditions with short fibre exposure time (1-2 min) will have to be used during *in vivo* applications. Therefore, calibration standards and quality control samples need to be exposed to the matrix for the same length of time as the *in vivo* fibre exposure.

On the other hand, it was noted that desorption time has very little influence on analyte recovery. However, data variability decreased with time which indicates that 15-60 min of analyte desorption is a compromise between throughput and variability.

Moreover, 100% acetonitrile efficiently desorbed both metoprolol and propranolol, while addition of water enhanced the desorption of diclofenac. This indicates that optimisation of desorption solvent is necessary during *in vitro* method development.

The level of blood hematocrit was found to have no impact on analyte response following SPME extraction, while blood flow rate may have an effect on analyte response and concentration.

On-fibre analyte stability for all three analytes was established for six weeks. This indicated the suitability of the SPME technique to be utilized in remote areas where access to centrifuges and freezers is limited.

Overall, this study showed the various parameters that should be investigated and evaluated *in vitro* prior to *in vivo* SPME application. The results from this chapter indicate that pre-equilibrium time should be used to measure metoprolol concentrations during the *in vivo* TK study investigation in later chapters. While desorption of metoprolol could be performed within 15 – 60 min and that 100% acetonitrile is the most efficient desorption solvent for metoprolol.

Chapter 4

SPME for Assessment of Plasma Protein Binding, a Complementary Technique to Rapid Equilibrium Dialysis

4.1 Introduction

Administered drugs can partition between the red blood cell and plasma components of circulating blood. Historically, in drug concentration assays, the use of plasma has been preferred as a matrix over blood. This is due to perceptions concerning its ease of handling, ability to be frozen and thawed successfully for sample storage and shipment, potential for increased assay sensitivity and the reduction of matrix interference[24]. Plasma contains proteins, small molecules and inorganic ions. Almost 60% of the plasma protein is serum albumin, while 3% is α-1-acidglycoprotein and the remainder is immunoglobulin[132]. Within the plasma fraction, drug molecules can be found either non-covalently bound to plasma proteins (mainly to serum albumin and acidglycoprotein), termed plasma protein binding (PPB), or be found free (unbound) and can diffuse through biological membranes or bind to receptors[132].

According to the well-established free drug hypothesis, only the free drug concentration is distributed to the site of action where it effects biological activity leading to efficacy and toxicity[36]. Hence, accurate determination of this parameter is essential for therapeutic drug monitoring, specifically for drugs with a narrow therapeutic window i.e. for drugs that have a small difference between therapeutic and toxic doses, such as digoxin and lithium[133]. The importance of determining the unbound drug concentration for monitoring various classes of drugs has been previously highlighted[134].

The current approach in drug discovery is to use *in vitro* experiments to determine PPB values to ultimately help establishing structural drug designs and aid selection of candidates for further *in vivo* experiments[135]. The extent of protein binding depends on several aspects including the physiochemical properties of the compound, the concentration of the drug and *in vivo* protein content. Interspecies difference in PPB and variability between individuals

specifically in disease states such as renal, liver and thyroid disease can also significantly alter the binding characteristics of a drug[136].

For this reason, several regulatory authorities recommend the determination of PPB prior to clinical trials to support the assessment of drug-drug interactions[6]. Therefore, PPB is considered to be a crucial parameter in ongoing drug development projects and in drug discovery studies[137].

Despite the importance of this parameter, due to reasons of convenience and precedence, the majority of bioanalytical assay techniques in current use measure the total (free and bound) drug concentration, rather than the potentially more relevant concentration of free drug [132]. The sole use of total drug levels might be misleading and may not reflect the true significance of the relationship between clinical pharmacokinetics (PK) and pharmacodynamics (PD) of a drug[36]. Although in some cases, total concentrations of drugs from PK studies are related back to free using PPB values determined *in vitro* during early discovery experiments[138,139].

The most widely used *in vitro* methodologies for determining the plasma protein binding of drugs include equilibrium dialysis, rapid equilibrium dialysis (RED), ultrafiltration and ultracentrifugation. Each technique displays a variety of advantages and disadvantages in terms of speed, data quality and complexity. Comparative evaluations of each method have been reported in the literature[140-142].

Several analytical challenges are known to be associated with some of these techniques. For example, ultrafiltration is a quick and easy method, where a special exclusion filter is used to filter the analyte from a matrix. However, the analyte may bind to the filter and cause disturbance to the equilibrium which in turn will impact the quality of the data[11].

Ultracentrifugation, on the other hand, requires the use of a powerful centrifuge (up to 250,000 g) along with lengthy centrifugation periods (approximately 16 hours) to separate the binding matrix from the drug which impose cost and time problems [12].

Perhaps the most frequently used method in the pharmaceutical industry is equilibrium dialysis which has been considered the “gold standard” for protein binding assessments[143]. This technique involves the use of two containers, one with the matrix sample and one with a suitable buffer such as phosphate buffered saline (PBS), separated by a membrane (Figure -4.1-). The free drug concentration is determined when equilibrium is reached between the two vessels[11]. Equilibrium dialysis avoids the dependence on nonspecific binding of the

analyte and the large plasma volumes required for the other procedures mentioned above. Even if some compounds may bind non-specifically, they will not interfere with the equilibrium because binding will be very minimal.

On the other hand, equilibrium dialysis is considered to be time consuming and labour intensive therefore the use of more recently developed technique, the RED device, has become the dominant method for protein binding analysis[143].

The RED device consists of a Teflon base plate which holds up to 48 disposable dialysis cells. Each cell is composed of two chambers separated by a dialysis membrane with a high membrane surface area to volume ratio. Plasma containing the analyte is placed in one of the chambers and buffer, such as phosphate buffered saline, is added to the other side and incubated for a period of time (typically > 6 hours). Subsequent to equalizing each matrix with blank plasma or PBS, aliquots from both sides are analysed with an appropriate quantification method e.g. LC-MS/MS[143]. Results are simply expressed as the ratio in concentration between both sides of the membrane. Although higher assay throughput is achieved compared to routine equilibrium dialysis, which is an advantage for rapid screening in drug discovery and large numbers of clinical samples in drug development, the length of equilibration time remains a major drawback for this technique[14].

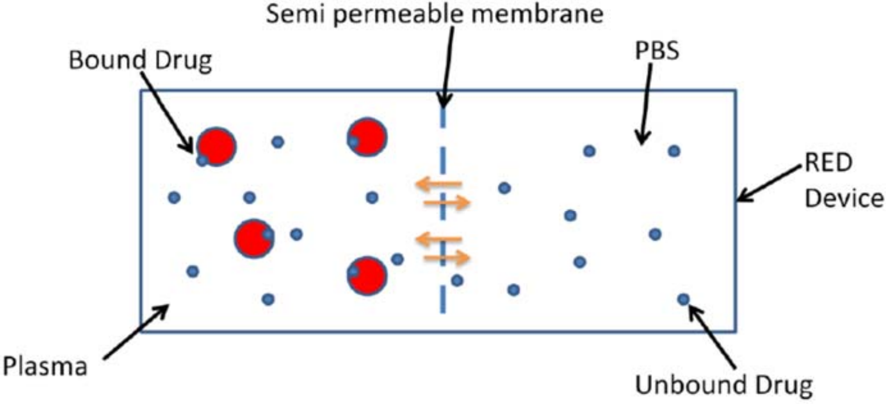


Figure -4.1- Basic principles of equilibrium dialysis, drug in plasma on one side (left) and PBS on the other (right). This is incubated at 37°C to reach equilibrium with constant shaking.

Recent advances in SPME have opened up new possibilities for addressing some of the challenges encountered when measuring plasma protein binding values[39,144]. SPME, as mentioned in earlier chapters, is an equilibrium process in which the analyte partitions between the SPME coating and the sample matrix (Figure 4.2). The amount of analyte extracted by SPME is directly proportional to the concentration of unbound analyte present in the sample matrix[89]. The non-exhaustive nature of SPME, represents an important benefit, since typically only a small portion of the analyte is removed from the matrix which does not cause any interference with the equilibrium[41].

Analyte extraction from the matrix is independent of sample volume when the fibre is exposed to a sample volume larger than the coating capacity ($V_{sample} >> V_{fibre}$). This means that SPME can be directly exposed to various matrices without the need to collect a defined sample volume as long as sample volume is large enough to immerse the whole fibre coating[63].

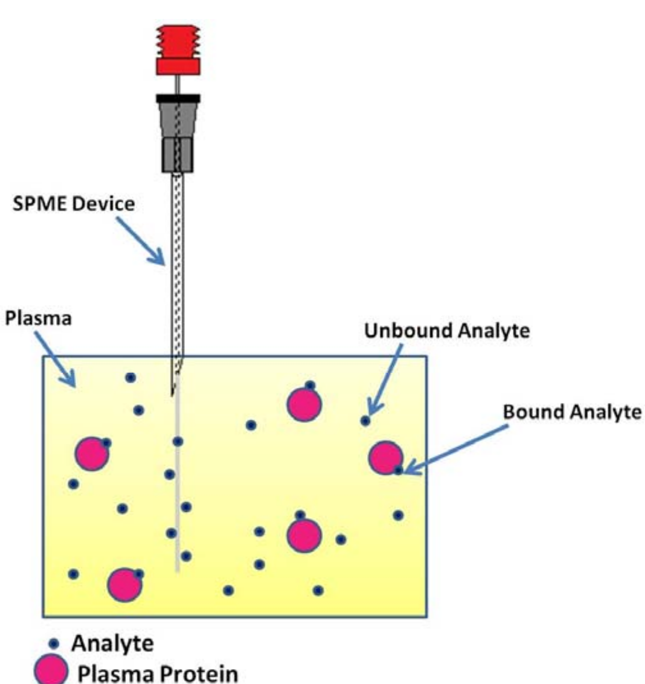


Figure -4.2- SPME for the measurement of unbound drug concentrations in plasma.

The SPME approach, therefore, may also be used to determine PPB values *in vitro* to characterise the distribution of small molecules with respect to the plasma compartment. SPME has the potential to offer a simple approach to accurately estimate protein binding affinities in early drug discovery, or during drug development. It may overcome several limitations of current methodologies by providing short experimental time and ease of use in addition to accuracy and reliability.

The determination of PPB by SPME is based on establishing the free concentration of drug in plasma in the presence of proteins which is then compared with total drug concentration measured by SPME in the absence of proteins[21]. In practical terms, this can be performed by spiking a known concentration of analyte into plasma and the same is repeated in a suitable buffer such as PBS. The plasma compartment will contain the free concentration of drug in the presence of proteins while the buffer compartment will act as a surrogate for the total drug concentration in the absence of proteins. Analyte concentrations from both compartments can be extracted by SPME and compared to determine the PPB of the drug. The percentage of drug binding to plasma proteins is simply calculated from the total and free concentrations of the drug as shown below;

$$PPB = \frac{C_{total} - C_{free\,plasma}}{C_{total}} \; x\; 100\% \qquad \text{Equation -4.1-}$$

This technique can be relatively fast and offers the possibility of automation as well as the ability to directly study complex sample matrices including whole blood and tissue.

4.1.1 Aims and Objectives

The aims and objectives of this chapter are to investigate the use of SPME as a tool for *in vitro* determination of plasma protein binding. This will be performed by comparing SPME to the RED device, the current golden standard method for PPB measurements. Three test compounds (metoprolol, propranolol and diclofenac) will be used as they are representative small molecule drugs that cover a range of binding values (30-99%) in rat plasma. Three concentrations will be assessed for each drug across a physiologically relevant range (10 - 500 ng/mL) using the validated bioanalytical methods.

4.2 Experimental

4.2.1 Chemicals and Materials

Metoprolol tartrate, propranolol hydrochloride, diclofenac sodium salt and diclofenac $^{13}C_6$ sodium salt 4.5-hydrate were purchased from Sigma-Aldrich (Dorset, UK); metoprolol-d_7 and propranolol-d_7 were acquired from Toronto Research Chemicals (Ontario, Canada). BioSPME silica probes consisting of a titanium wire coated with a biocompatible C18 extraction phase, housed inside hypodermic needle (medical grade, stainless steel, 22 gauge outer tubes) were supplied by Supelco (Bellefonte, PA, USA); each fibre has a thickness of 45 µm and 15 mm length of coating. Control rat plasma containing K2-EDTA to prevent coagulation was obtained from B&K Universal (Grimston, Hull, UK). RED device was purchased from ThermoFisher Scientific (UK). Phosphate buffered saline (PBS) tablets, dimethylformamide (DMF) and formic acid (reagent grade ≥ 95%) were purchased from Sigma-Aldrich (Dorset, UK). Methanol, acetonitrile, propranolol and water were of HPLC gradient grade and obtained from Fischer Scientific Ltd (Loughborough, UK).

4.2.2 Preparation of Standard Stocks, Working Solutions and Test Samples

Primary stock solutions for each test compound (metoprolol, propranolol and diclofenac) and their stable label isotopes utilised as internal standards (IS) were prepared in DMF (1 mg/mL). Serial dilutions of each analyte's stock solution were performed in acetonitrile/water (1:1, v/v) to give working standard concentrations of 1, 10 and 100 µg/mL. Internal standard working solutions for each analyte were prepared from the primary stock solution to give a final concentration of 100 ng/mL in acetonitrile.

4.2.3 SPME Procedure for Analysis of Plasma Protein Binding

PBS solution was prepared by dissolving one PBS tablet into 200 mL of deionised water (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride, pH 7.4).

SPME fibres were preconditioned with methanol followed by water for 15 min in each solvent. Appropriate volumes of analyte working solutions were spiked into fresh rat plasma

and into PBS at target concentrations of 10, 100 and 500 ng/mL. Non-matrix volumes used to spike the samples were < 5% of the total sample volume[12,69]. Spiked rat plasma was gently mixed on a roller mixer (Progen Scientific, UK) for 15 min at 37ºC.

One set of SPME fibres (n = 6) was immersed into 200 µL aliquots of spiked plasma and a second set was placed into 200 µL aliquots of spiked PBS for each target concentration. SPME extraction was conducted following 30 min incubation at 37ºC by removing the fibres from the samples, rinsing them with water for 30 s and desorbing them in 200 µL of 100% acetonitrile containing 100 ng/mL of the appropriate internal standard for 15 min. All extracts were subsequently analysed by LC-MS/MS. The entire SPME extraction procedure was performed with constant orbital agitation at 500 rpm using a compact laboratory shaker (MS 3 Digital, IKA). The percentage of binding to plasma proteins was calculated from the total and free analyte response as follows;

$$\text{PPB \%} = \left(\frac{Analyte{:}IS\ Peak\ Area\ Ratio_{\ PBS} - Analyte{:}IS\ Peak\ Area\ Ratio_{\ Plasma}}{Analyte{:}IS\ Peak\ Area\ Ratio_{\ PBS}} \right) \ x \ 100$$

Equation -4.2-

4.2.4 RED Procedure for Analysis of Plasma Protein Binding

A single-use RED plate preloaded with 48 equilibrium dialysis membrane inserts was used and 300 µL aliquots (n = 6) of spiked rat plasma in addition to 300 µL aliquots (n = 6) of control blank plasma (same plasma batch as the spiked plasma) were placed into the sample chambers of the RED device. This was dialysed against 500 µL aliquot (n = 6) of PBS added into the buffer chambers. The RED unit was covered with self-adhesive plate seal and incubated at 37ºC on a flatbed orbital shaker (MS 3 Digital, IKA) set at 300 rpm for approximately 6 h. Following equilibration, dialysis was stopped and 25 µL aliquots were taken from each compartment, placed into 1.4 mL matrix tubes (Micronics, Platinastraat, Netherlands), and an equal volume of dialysed blank plasma was added to the PBS aliquot and 25 µL of dialysed PBS was added to the spiked plasma compartment aliquot to ensure matrix matching of samples prior to extraction and analysis.

Samples were extracted by protein precipitation through addition of 200 µL of 100% acetonitrile containing 100ng/mL of internal standard. All tubes were vortex mixed for 5 min

and centrifuged (5810R, Eppendorf, Germany) at 3000 g for 10 min. The supernatant was transferred into clean tubes and injected onto the LC-MS/MS. Analyte binding calculation for the RED approach was performed as shown below;

$$PPB\ \% = \left(\frac{Analyte{:}IS\ Peak\ Area\ Ratio_{\ Plasma} - Analyte{:}IS\ Peak\ Area\ Ratio_{\ PBS}}{Analyte{:}IS\ Peak\ Area\ Ratio_{\ Plasma}} \right)\ x\ 100$$

Equation -4.3-

4.2.5 LC-MS/MS Analysis

LC-MS/MS analysis was performed using the same methodology described in Chapter 2, Section 2.2.4.

4.3 Results and Discussion

SPME fibres have been reported for the measurement of unbound, circulating plasma concentrations of xenobiotic compounds during *in vivo* experiments[61,92,145]. Due to the fact that SPME can measure free drug concentrations, and its (comparative) ease of use, this raised the interesting prospect of SPME's application for a rapid throughput method for routine plasma protein binding determination. To demonstrate the utility of SPME fibres for measuring free drug concentration from biological matrices, an *in vitro* experiment was conducted to compare the amount of drug extracted from protein free matrix (PBS) with the amount of drug determined from rat plasma (high protein content matrix). The percentage of bound drug concentration extracted was then calculated and compared with values obtained using a conventional technique, the RED device, routinely utilised for establishing plasma drug protein binding. The results in Table -4.1- display the calculated protein binding values for metoprolol, propranolol and diclofenac across a range of concentrations (10, 100 and 500 ng/mL) using SPME and RED.

Table -4.1- Comparison of protein binding values for metoprolol, propranolol and diclofenac across a concentration range of 10-500 ng/mL obtained using rapid equilibrium dialysis (RED) and SPME. Data represent mean ± SD, n = 6 determinations.

Analyte Concentration (ng/mL)	RED %PPB*	SPME %PPB*	% Difference between both techniques**
Metoprolol / Literature values for %PPB = ~30%[146]			
10	34.3 ± 0.336	31.8 ± 0.784	7.3 ± 0.027
100	33.6 ± 0.415	31.6 ± 0.562	6.0 ± 0.022
500	31.5 ± 0.180	31.8 ± 1.09	-1.0 ± 0.035
Propranolol / Literature values for %PPB = ~90%[143]			
10	89.3 ± 0.0742	91.4 ± 1.01	-2.4 ± 0.011
100	90.0 ± 0.0816	91.0 ± 0.735	-1.1 ± 0.008
500	87.6 ± 0.0504	77.4 ± 0.961	11.6 ± 0.012
Diclofenac / Literature values for %PPB = ~99%[147]			
10	98.7 ± 0.0589	98.5 ± 0.941	0.203 ± 0.010
100	99.4 ± 0.0367	99.5 ± 0.857	-0.100 ± 0.009
500	99.4 ± 0.0363	99.1 ± 0.721	0.302 ± 0.007

*Errors were based on standard deviation and calculated using error propagation methodologies.

$$** \ \% \ \text{Difference} = \frac{\%PPB_{RED} - \%PPB_{SPME}}{\%PPB_{RED}} \ x \ 100$$

The calculated bound percentage by SPME correlated well with bound values determined by the RED device, which indicates that SPME did indeed measure the free circulating drug concentration within a complex biological matrix. It was found that consistent results were obtained by SPME for each analyte across all three concentrations with ≤15% difference[12] between concentrations.

The percentage difference between the two techniques, SPME and RED was < 15% across all analytes and concentrations. In the case of diclofenac, the magnitude of the difference

between RED and SPME was <1%. All results also correlated well with average protein binding values quoted in the literature for each compound[143,146,147]. The small differences between the values obtained in this study and protein binding values previously published in literature[148,149] can be explained by inter-animal variations in plasma protein content or due to typical analytical experimental errors.

The SD of SPME was greater than the SD from the RED assay which suggests that the variability of the SPME assay is higher than the RED although not high enough to impact the reliability of the data. This could be due to the quality of the fibres used and the inter-fibre variability associated with it[41,87] which was also discussed in Chapter 3, Section 3.3.1. Another reason for this variability could be due to the pre-equilibrium conditions which were applied during this study. As it was highlighted in Chapter 3, Section 3.3.2, the time required for each compound to reach equilibrium could potentially be > 3h. This in turn has an adverse effect on high throughput determination of plasma protein binding of drug molecules. For this reason, pre-equilibrium conditions were applied during this study and 30 min exposure time was chosen to equilibrate the SPME fibres with the sample matrix. This was deemed an appropriate length of time for *in vitro* applications and a good compromise between throughput and data reproducibility[41]. The use of pre-equilibrium SPME conditions has no impact on free concentration measurements or protein binding determination as long as all samples are extracted using the same length of exposure time as illustrated by the data shown in Table -4.1-.

A paired t-test was conducted to compare the PPB values obtained using RED for all three analytes with PPB values measured using SPME. There was no significant difference in the values for RED and SPME, $p > 0.05$. The test was performed on $n = 18$ samples (i.e. three concentrations X six replicates) for each drug by SPME versus RED. This suggests that data obtained using SPME is equivalent to the data obtained using the RED device and therefore a suitable alternative method allowing more rapid analytical throughput.

A two-way analysis of variance was also performed to understand the influence of two independent variables, namely the concentration of analyte and the effect of the analytical technique on the PPB values. The analyte concentration included three levels (10, 100 and 500 ng/mL) and analytical techniques consisted of the RED and SPME. Neither effect was statistically significant at the 0.05 significance level. The effect of analyte concentration yielded $F = 1.02$, $p > 0.05$, indicating that the effect of concentration was not significant. The

impact of the analytical technique yielded $F = 2.89$, $p > 0.05$, indicating that there is no significant difference between both analytical techniques.

The data obtained in this study clearly demonstrate that SPME measured only the unbound drug fractions for a range of different compounds and this is in agreement with several previous reports of SPME use [91,150,151]. The technique uses an extraction phase that adsorbs analytes and prevents adhesion of large molecules and, therefore, provides a simple approach for the measurement of free drug concentration which is a key parameter for the interpretation of compound bioavailability and its PD action. The depletion of the free fraction of drug from the matrix is negligible with SPME, such that it does not have an effect on the equilibrium between the bound and free fraction of the analyte within the matrix[41]. Although, negligible extraction may lead to smaller drug amounts available for analysis and therefore lead to detection problems, such issues are now resolved by highly sensitive MS instrumentation that permit successful detection of low analyte concentrations.

An important aspect for consideration is the thickness of the coating on the SPME fibres. This determines the capacity of the fibres which in turn impacts the concentrations at which the protein binding is measured. For higher sample concentrations, a thicker coating would be required, the thicker the coating the larger is the number of adsorption sites available for analytes to bind and interact with.

Overall, the experimental findings of the current study indicate that SPME is an approach that could be utilised *in vitro* for determining the percentage PPB of a compound in a biological matrix. SPME allowed monitoring of PPB values for analytes with a range of binding affinities which can be classified as low, medium and highly bound compounds (30 - 99% bound)[36].

Compared to the RED device, SPME offers several advantages for use in protein binding measurements including; short analysis time of less than 1 h for SPME compared with greater than 6 h for RED; the ability to study complex matrices such as blood directly without the need for dilutions or subsequent extractions; the elimination of the need for protein precipitation with the RED device which ultimately minimises concerns associated with matrix interference. In addition to the above, SPME provides a relatively easy sampling and extraction procedure compared to other conventional PPB assays. No sample aliquoting or centrifugation is required, and extracted samples can be stored on fibre as shown in Chapter 3, Section 3.3.5 allowing for desorption later if analyte stability is established.

SPME could potentially be automated using several stations equipped with agitators where fibres could be conditioned, exposed to the sample matrix and analytes desorbed off using robotic handlers which ultimately will facilitate high sample throughput with minimal labour. The ultimate applicability of SPME for direct *in vivo* determination of the unbound concentration of drug is also a promising aspect, which has been supported by the *in vitro* findings of the current work.

The use of SPME as a tool to determine PPB values enables simplified workflow and higher throughput when compared to other PPB techniques and their challenging issues such as RED, ultracentrifugation and ultrafiltration as mentioned in Section 4.1. The SPME technique is not commonly used for this purpose because it has not been presented to the pharmaceutical industry as a PPB tool. Limited knowledge/publications are currently available about using SPME for PPB measurements. Commercial availability and promotion of the technique for PPB is another hindrance, although SPME devices can be tailored according to the researcher's needs, the commercial availability of ready-to-use devices for PPB experiments is still limited and not widely published. However, refocusing commercialisation of SPME for routine analysis of PPB would likely increase interest in the technology as a tool for such *in vitro* experiments.

4.4 Conclusion

The impact of measuring the degree of protein binding is high when trying to understand the relationship between the PK and PD of drugs. Although RED has dominated this field, the potential for the use of a complementary technique such as SPME with a direct comparison to RED has not been studied previously.

This investigation demonstrated the use of SPME for the measurement of PPB *in vitro* and highlighted its favourable comparison to existing techniques. The data obtained using SPME clearly showed that this approach provided accurate estimates of PPB values across a range of bound drug levels (30 – 99 %) at a several physiologically relevant concentrations. Compared to RED, SPME offered a number of benefits including simplicity as well as short equilibration and analysis time, where the overall procedure for SPME was completed within 1 h compared to 6-8 h using RED. Importantly, the ability of SPME to determine the unbound drug concentrations in plasma (of relevance for direct *in vivo* microsampling) has been shown in this work using the industrial-standard, comparative RED technique. SPME also offers the future possibility of automation which will consequently enhance throughput and increase the number of samples processed.

In this chapter a pre-equilibrium measurement was developed suitable for the high throughput determination of plasma protein binding of drug molecules.

Chapter 5

Bridging the Gap Between *in vitro* and *in vivo* SPME: Investigation into the Use of SPME for Detection of Exogenous Analytes in Rats

5.1 Introduction

5.1.1 Direct *in vivo* Insertion of SPME

The development of biocompatible coating materials was a major breakthrough for SPME. It has opened opportunities for the use of SPME as an *in vivo* tool facilitating direct extraction from complex biological matrices[42]. The term "biocompatibility" as described by the International Union of Pure and Applied Chemistry (IUPAC) refers to the characteristics of a material that enables it to be in contact with a living organism without triggering an adverse effect[13]. A material could be considered biocompatible if the sum of adverse hormonal and cellular reactions occurring during exposure to the material is lower than that or equivalent to a reference material[41]. This feature permits direct immersion into biological matrices, both *ex vivo* and *in vivo*.

Biocompatible SPME coatings use a biocompatible binder "glue" to immobilize coated particles[114]. However, one of the major shortcomings of SPME is the small choice of commercially available stationary phases; in fact the choice is limited to silica particles coated with octadecylsilane particles (C_{18}), polyacrilonitrile (PAN), polyethylene glycol (PEG), polyacrylate (PA) and polypyrrole (PPY), all of which are polymers that are deemed biocompatible[42]. Of these coatings, the C_{18} phase is the only coating which has currently been assembled within a hypodermic needle and is commercially available for direct *in vivo* sampling[42].

The use of the *in vivo* SPME device for rodent studies has previously been limited to implementation only in conjunction with an interface. In-dwelling catheters and adapters connected to syringes that allow fibre penetration, have been employed as interfaces for *in*

vivo SPME rodent studies[63]. This is due to the large dimensions of earlier *in vivo* devices prior to the hypodermic needle assembly, which prohibited direct SPME probe insertion into the veins of rodents[91]. Reports in literature have shown usage of various custom-made sampling interfaces that enable application of *in vivo* SPME in rodent studies[92]. To date direct immersion of the SPME probe into the vasculature has mostly been demonstrated in large animals such as beagle dogs[61], however miniaturization of the sampling device has allowed for the availability of a range of needle gauges (21 – 23 G) suitable for direct sampling from different size animals including rodents.

Blood vessels in a rat's tail are 0.15 to 0.5 mm in diameter as approximate dimensions measured by Staszyk *et al*[152], while recent biocompatible SPME probes have a diameter of approximately 45-50 µm i.e. small enough to penetrate inside the veins of a rat's tail without completely obstructing them. The lateral tail vein is usually accessed for sampling approximately one-third along the length of the tail from the tail tip, moving towards the base of the tail for multiple samples. Blood samples are only usually taken from the base of the tail if no vein is visible elsewhere. Taking the first sample/s from the proximal end of the tail can result in a perivascular clot and inflammation that significantly reduces blood flow to the distal portion of the vessel[16]. It therefore important to assess the occurrence of adverse events such as perivascular clotting when inserting the SPME into the veins of an organism.

The first *in vivo* investigation within this research project will involve preliminary determination of the feasibility of inserting the SPME probe into anesthetised rats and capturing whether *in vivo* SPME is viable for use within a rat's tail vein without the use of an interface. The investigation will also monitor the length of time required for fibre exposure to blood in order to produce reliable results.

One of the most common methods of reducing animal suffering while testing or investigating a new invasive sampling technique is the use of anaesthesia. This is to reduce pain if the procedure is new and if the mechanism of action is unknown. It is also useful to enable initial handling of the device without dealing with any signs of distress or other unexpected animal response.

In small research animals such as rodents, induction with volatile anaesthetic agents is usually fast and smooth, and is easily achieved by placing the animal in a plastic chamber, to which the volatile agent is delivered. The first anaesthetic to be used in this fashion was ether which is highly irritating to the respiratory tract as well as inducing signs of distress. When

more modern volatile agents (isoflurane, sevoflurane) are used for rodents, induction is usually performed without struggling or obvious signs of excitement[153]. Anaesthetic agents such as isoflurane are known to reduce cardiac output and slow down blood flow to body organs[154]; this in turn has a direct impact on conventional sampling techniques where blood withdrawal becomes difficult when studies are performed under general anaesthesia. However, SPME sampling can be used in surgeries and other studies that require anaesthetised animals as no blood withdrawal is needed. For example, Lord *et al*[97] assessed the PK profile of linezolid in anaesthetised pigs after a single IV dose using *in vivo* SPME. The length of extraction was 5 min and their results illustrated the potential for rapid free drug determination within surgical or intensive care situations.

5.1.2 Potential of SPME to Measure Free Drug Concentrations *in vivo*

The importance of measuring free drug concentrations lies in the fact that this parameter provides basis for accurate determination of *in vivo* drug efficacy[137]. For example, under certain conditions the equilibrium between bound and free drugs can be disrupted leading to elevated free concentration levels which is significantly higher than predicted levels calculated *in vitro* using total drug concentrations. For instance, elevated free phenytoin concentrations have been reported in patients suffering from acquired immune deficiency syndrome (AIDS)[36]. This in turn can have major consequences on patient safety and may lead to fatal cases.

Despite its importance, free drug monitoring is not a routine procedure in clinical laboratories due to technical difficulties and lack of established reference ranges for free drugs[36]. Numerous techniques that are applicable for direct *in vivo* analysis of free drug concentrations have been widely available. These include arrays of sensors, microdialysis, microfluidics and nanotechnology[88]. But most of these systems remain complicated and bulkier than a simple miniature *in vivo* device, in many cases, external optics, pumps and detectors are required to operate the devices[155]. Regardless of some distinctive advantages, some of these techniques such as nanomaterials have significant cytotoxicity and require major modifications prior to *in vivo* applications[156]. Biocompatible SPME was initially developed to address these issues and provide a simple *in vivo* procedure that can monitor free drug concentration within a living organism. Due to its microextraction nature, SPME

removes a minimal amount of analyte from the system under investigation without disturbing the normal balance of *in vivo* chemical components, whereas large volumes of blood would be needed to assess the free concentration with traditional techniques such as rapid equilibrium dialysis[143].

Studies described in Chapter 4 confirmed the capability of SPME in measuring free drug concentrations *in vitro.* The feasibility of using SPME for protein binding experiments was also demonstrated. In order to confirm that these findings are translated to *in vivo* measurements, an *in vivo* study was conducted in this chapter. SPME sampling in conscious rats was performed to determine *in vivo* free drug concentration. This was compared with data obtained using conventional blood withdrawal and *in vitro* rapid equilibrium dialysis. This examined the feasibility of SPME for direct *in vivo* assessment of drug concentrations.

5.1.3 Aims and Objectives

The aims and objectives of this chapter were to perform two *in vivo* studies. The first study was carried out in anesthetised rats to achieve the following;

- Utilise the SPME fibre in anesthetised rats for the first time in this research without employing adapters and interfaces such as catheters.
- Determine the duration of fibre exposure during the period before sample equilibration has been reached (i.e. pre-equilibrium) to systemic circulation while considering ethical limitations.
- Qualitative preliminary assessment of the practicality of using the prototype device and its applicability for implementation in a toxicology study.

The second *in vivo* study was performed in conscious rats. This was to demonstrate the use of biocompatible SPME fibres for the measurement of free drug concentrations *in vivo* and to compare the results with a conventional protein binding assay (the RED device). This aimed to determine whether the data obtained in Chapter 4 for *in vitro* SPME could be translated to an *in vivo* microsampling approach.

5.2 Experimental

5.2.1 Chemicals and Materials

Metoprolol tartrate was obtained from Sigma Aldrich (Dorset, UK). The sources of all other chemicals and materials were as described in Chapter 2, Section 2.2.1.

5.2.2 Procedure for *in vivo* SPME Study in Anesthetised Rats

All animal studies were ethically reviewed and carried out in accordance with the Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals.

The design of the first *in vivo* study involved the use of two sampling techniques; SPME and conventional caudal venipuncture (CV) in anesthetised male rats. The purpose of this study was to identify a suitable pre-equilibrium length of time for the SPME fibre exposure within the rat's tail. In order to achieve this, drug extraction was performed at steady state drug concentration. This was to ensure that all fibres were exposed to the same *in vivo* drug concentration but for different periods of time. Conventional CV samples were also taken to compare the data generated with the data obtained using SPME sampling.

Four male rates (Wistar Han) were obtained from Charles River Labs (St. Constant, PQ, Canada) weighing 250 to 300 g. The rats were acclimatised for a week prior to experimental start date. They were kept in plastic solid bottom cages and fed 5CR4 rodent diet (Purina Mills International) along with filtered mains water (Veolia Water plc).

Animals were anesthetised with isoflurane/O2 and were maintained under anaesthesia throughout the duration of the experiment. Subsequent to being anaesthetised, all four rats were placed on their back and their body temperature was maintained by use of heat mats with thermostat.

Rats were surgically prepared with a jugular vein cannula two days prior to experimental start date. This was performed to facilitate IV drug (metoprolol) administration. An angiocath (SAI, Infusion Technologies, UK) was placed in the right lateral tail vein to facilitate conventional blood sampling. A dose of 5 mg/kg (0.2 mg/mL) of metoprolol tartrate was

applied at an IV rate of 3 mL/h/kg through the jugular vein. The duration of infusion was approximately 4 h. These parameters were determined using the *in silico* modelling described in Section 5.2.3 to give an appropriate steady state concentration.

Prior to the *in vivo* sampling, prototype biocompatible SPME probes housed within 22-gauge hypodermic needle and consisting of biocompatible C_{18} coating (45 µm thickness, 5 µm particle size, Supelco, Bellefonte, PA, USA), were preconditioned with methanol followed by water for 15 min.

In vivo sampling was performed after metoprolol concentration in blood had reached steady state. This was achieved using continuous intravenous infusion (IV) of metoprolol solution. Four fibre exposure times were applied. This experiment was performed to determine the required length of fibre exposure to circulating blood within a living organism. At each sampling point, the SPME fibre was exposed to the systemic circulation for a defined period of time. The chosen durations of pre-equilibrium SPME exposure to systemic circulation were (30 s, 60 s, 90 s and 120 s) as shown in Table -5.1-. The latter times were chosen based on ethical considerations for leaving the fibre within a live animal. A whole blood sample was also withdrawn at each occasion to compare with the SPME concentrations.

Table -5.1- SPME sampling schedule in anesthetised rats

Animal Number	Sample 1	Sample 2	Sample 3	Sample 4
Rat 1	30 s	120 s	90 s	60 s
Rat 2	60 s	30 s	120 s	90 s
Rat 3	90 s	60 s	30 s	120 s
Rat 4	120 s	90 s	60 s	30 s

Desorption of metoprolol from SPME probes was achieved using 200 µL of desorption solvent containing internal standard (IS) i.e. acetonitrile containing 100 ng/mL of metoprolol-d_7. Desorption was performed for 15 min under orbital agitation of 500 rpm. The resulting extracts were then analysed by LC-MS/MS.

Whole blood samples (100 µL) were withdrawn into microtainer tubes containing K_3EDTA as the anticoagulant (BUNZIL Healthcare, UK). The whole blood samples were withdrawn at the same time as each SPME sampling occasion in order to obtain a realist comparison of both techniques. A 22-gauge angiocath (venous catheter) was utilized for the CV procedure to facilitate whole blood withdrawal.

Whole blood samples were extracted using protein precipitation where an aliquot of 25 µL was precipitated using 200 µL of IS solution (acetonitrile containing 100 ng/mL of metoprolol-d_7). The supernatant was removed from the pellet into clean 1.4 micronic tubes (Micronic™, Aston, USA) and analysed by LC-MS/MS.

SPME samples were quantified against calibration standards and QCs prepared in rat blood and extracted by the SPME procedure described above. Whole blood samples were quantified against calibration lines and QCs extracted by protein precipitation.

5.2.3 *In silico* Modelling of Metoprolol Steady State Concentration

In order to estimate the length of time required for continuous IV infusion to achieve a steady state concentration of metoprolol between 100 – 200 ng/mL, published metoprolol data from Yoon *et al*[157] was utilised to model and predict the required experimental parameters.

The mean plasma concentrations after 1 min IV infusion of metoprolol at 1 mg/kg from Yoon *et al*[157] was entered into a TK software (Phoenix 32, version 6.0, Pharsight Corporation, CA, USA) to perform the PK modelling and simulation. Initially a 2-compartment IV bolus model was employed to generate the initial parameters. These were subsequently utilized in a 2-compartment IV infusion model from which the infusion rate (3 mL/h/kg) and the duration of the infusion (4 h) were determined.

5.2.4 Post Mortem Tail Dissection

Animals were killed by terminal anaesthesia where pentabarbitone was administered through the jugular cannula. Subsequent to death confirmation, SPME fibres were inserted into the tail vein and dissection of the tail was performed. The procedure involved careful lateral cutting of the tail around the SPME probe to examine the penetration of the fibre into the

vein. This assessment was performed to identify whether the fibre was inserted correctly into the vein of the tail. The intention of this experiment was for qualitative necropsy only i.e. making observations with the naked eye to ensure that the fibre was inserted into the vein.

5.2.5 *In vivo* Measurement of Protein Binding Values by SPME and RED

A second study in conscious rats was designed to confirm that SPME measures free drug concentrations *in vivo*. Similar to the *in vitro* study in Chapter 4, two techniques, SPME and RED, were utilised and compared.

The study design involved use of six male rats (Wistar Han, Charles River Labs, St. Constant, PQ, Canada) weighing 250 to 300 g. The rats were acclimatised for a week prior to experimental start date. They were kept in plastic solid bottom cages and fed 5CR4 rodent diet (Purina Mills International) along with filtered mains water (Veolia Water plc).

Similar to the anesthetised rat study design, rats were surgically prepared two days prior to experimental start date to insert a jugular vein cannula. This is to facilitate tethered IV infusion in live rats. Subsequent to two days recovery period, the rats were tethered to the infusion device through a rodent jacket that allows IV infusion with free rat movement within the cage while being connected to an infusion pump.

A dose of 5 mg/kg (0.2 mg/mL) of metoprolol was applied at an IV rate of 3 mL/h/kg through the jugular vein for 4 h. Each rat was sampled twice as shown in Table -5.2-, once using preconditioned SPME probes and the second sample was a whole blood withdrawal (200 µL) using a conventional butterfly needle (caudal venepuncture). SPME fibres were preconditioned using methanol followed by water for 15 min. Whole blood samples were collected into microtainer tubes containing K_3EDTA as the anticoagulant (BUNZIL Healthcare, UK). Metoprolol was desorbed off the SPME fibres using 200 µL of IS solution (acetonitrile containing 100 ng/mL of metoprolol-d_7).

Whole blood samples were split into two (100 µL aliquots) as shown in Figure -5.1-. One sample was extracted by protein precipitation using 25 µL of whole blood sample and 200 µL of acetonitrile containing 100 ng/mL of metoprolol-d_7. This was centrifuged at 3000 g for 10 min (5810R, Eppendorf, Germany). The supernatant was removed and injected onto the LC-MS/MS. This was performed to determine the total drug concentration. The second

whole blood aliquot was analysed using the RED device to determine the protein binding values of metoprolol in each rat. The procedure used for the RED analysis was as described in Chapter 4, Section 4.2.3. however, plasma was replaced with whole blood samples.

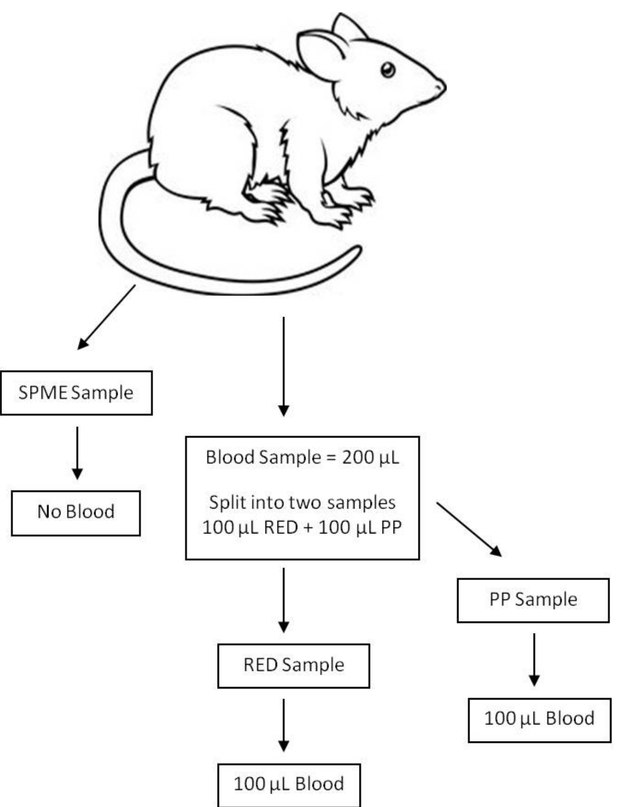


Figure -5.1- Illustration of the type of samples taken from each rat and the fate of each sample. PP samples are whole blood samples analysed by protein precipitation (PP) and RED samples are samples that were analysed by the rapid equilibrium dialysis device followed by PP extraction.

Table -5.2- Order of sampling and sampling timepoints for six conscious rats dosed with metoprolol using continuous IV infusion

Order of sampling	Animal Number and Sampling Time*						
	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 6
Sample 1	SPME 4.75 h	CV 4.83 h	SPME 4.92 h	CV 5.00 h	SPME 5.083 h	CV 5.16 h	SPME 5.25 h
Sample 2	CV 5.33 h	SPME 5.41 h	CV 5.50 h	SPME 5.58 h	CV 5.66 h	SPME 5.75 h	CV 5.83 h

* Sampling time after dose start

5.2.6 LC-MS/MS Analysis

LC-MS/MS analysis was performed using the validated methodology described in Chapter 2, Section 2.2.4.

Analysis of metoprolol biotransformation was performed using a quadrupole time- of-flight mass spectrometer (QToF) coupled to an Acquity UPLC system (both Waters, Ltd, UK). External mass calibration of the mass spectrometer was performed over m/z range of 50 -1000 using a solution of NaCsI (purchased from Waters Ltd.). The instrument was tuned to give the best possible performance using a 10 µg/mL solution of metoprolol in 50% (v/v) acetonitrile in water, infused into a solvent flow of 0.15 mL/min 1:1 0.1% (v/v) formic acid in water: acetonitrile, using the integrated syringe pump. An external lock mass (1 µg/mL leucine enkephalin in 50% (v/v) acetonitrile in water containing 0.1% (v/v) formic acid, m/z 556.2771) was infused into the reference sprayer of the MS source at a flow rate of 20 µL/min using a dedicated HPLC pump (Shimadzu, Manchester, UK) during each analyses, for automatic mass correction.

5.3 Results and Discussion

5.3.1 *In vivo* SPME Study in Anesthetised Rats

The *in vivo* exposure time trends of SPME fibres were evaluated using four rats. Results from Chapter 3, Section 3.3.1 showed that extraction equilibrium was not reached even after 30 min of fibre exposure to spiked samples *in vitro*. However, it can be assumed that as long as calibration standards and QCs prepared *in vitro*[97], mimic the length of *in vivo* exposure, then resultant concentrations of unknown samples should give reliable data and reflect *in vivo* concentrations.

In order to test this, a study in anesthetised rats was performed and a number of exposure timepoints were explored. The exposure times were limited by ethical constrains of the duration the fibre could be left in the tail vein without causing too much distress to the live animal. For this reason, exposure times of (30 s, 60 s, 90 s and 120 s) were examined. Parallel whole blood samples were withdrawn using conventional caudal venepuncture sampling to compare with the SPME extraction data.

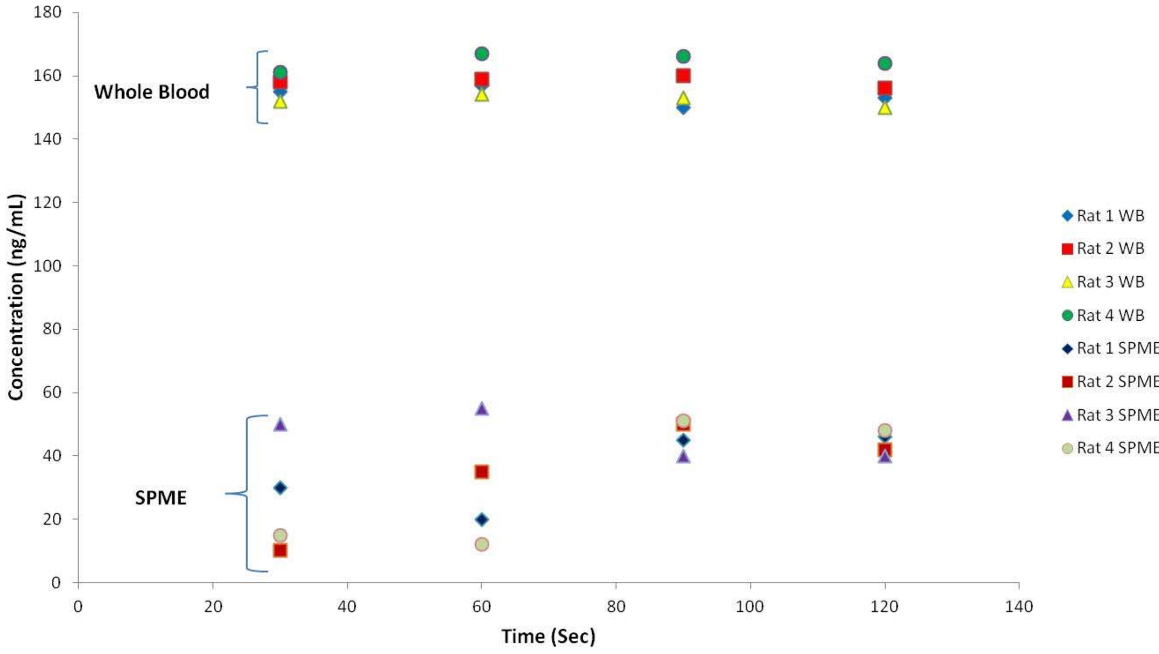


Figure -5.2- Extracted metoprolol at steady state concentration in anesthetised rats. *In vivo* exposure time profiles were investigated for SPME (free) concentrations and were compared to whole blood (total) concentration using protein precipitation extraction.

Figure -5.2- shows extracted metoprolol concentrations using *in vivo* SPME versus whole blood concentrations extracted by protein precipitation. The SPME time exposure profile *in vivo* showed that 30 s and 60 s fibre exposure resulted in variable concentrations between the four rats with an approximate 5-fold difference between the lowest and highest concentrations observed for the same sampling timepoint. This variability was reduced for the 90 s exposure timepoint and was further decreased for the 120 s exposure timepoint. These data seem to indicate that concentration consistency increased with length of fibre exposure time to the circulatory system. This finding is expected as SPME is an equilibrium extraction method; therefore, extraction time is the limiting step for the SPME procedure. Selection of the optimum extraction time is a critical step to obtain reliable results, particularly for pre-equilibrium extraction approach. Equilibrium extraction provides the highest sensitivity and reliability but in most *in vivo* SPME applications, pre-equilibrium conditions are used[97], since equilibrium extraction times tend to be long and impractical for *in vivo* use.

However, in the case of pre-equilibrium extraction, the longer the extraction times and the less steep the extraction profile versus time curve slope, the smaller the relative errors that occur. When the chosen extraction time is in the steep area of the curve, a small error in timing may cause much higher relative errors in analyte adsorption as it is the case with the 30 s and 60 s exposure times. Pre-equilibrium SPME offers distinct advantages due to its better temporal resolution and short sampling intervals which allow for monitoring of analytes within highly dynamic systems with rapid analyte concentration changes. Previous research has shown that extraction time for certain compounds can be as short as 30 s whilst providing fully reproducible and quantitative results[158]. Lord *et al*[97] assessed the pharmacokinetic profile of linezolid in anesthetised pigs after a single IV dosage using a 5 min extraction time. This length of time (5 min) could still pose a potential problem, if the PK profile for the analyte in an IV study is rapidly changing with time.

Overall, based on the data presented above, both equilibrium and pre-equilibrium extractions need precise and perfectly repeatable timing which should be determined as part of the bioanalytical method, although for the latter method, timing is more critical.

5.3.2 Biotransformation of Metoprolol

Upon processing the SPME and the whole blood data, two peaks with the same MRM (multiple reaction monitoring) transition as metoprolol were observed as shown in Figure -5.3-. The major peak was identified as metoprolol, its retention time corresponded to the same retention time as the metoprolol internal standard. The "unidentified peak" eluted earlier than the metoprolol peak on a reversed phase LC system, which indicated that it was a more polar component. This unidentified peak was only observed in the whole blood sample extracts while the SPME extracts only contained the peak corresponding to the parent (metoprolol).

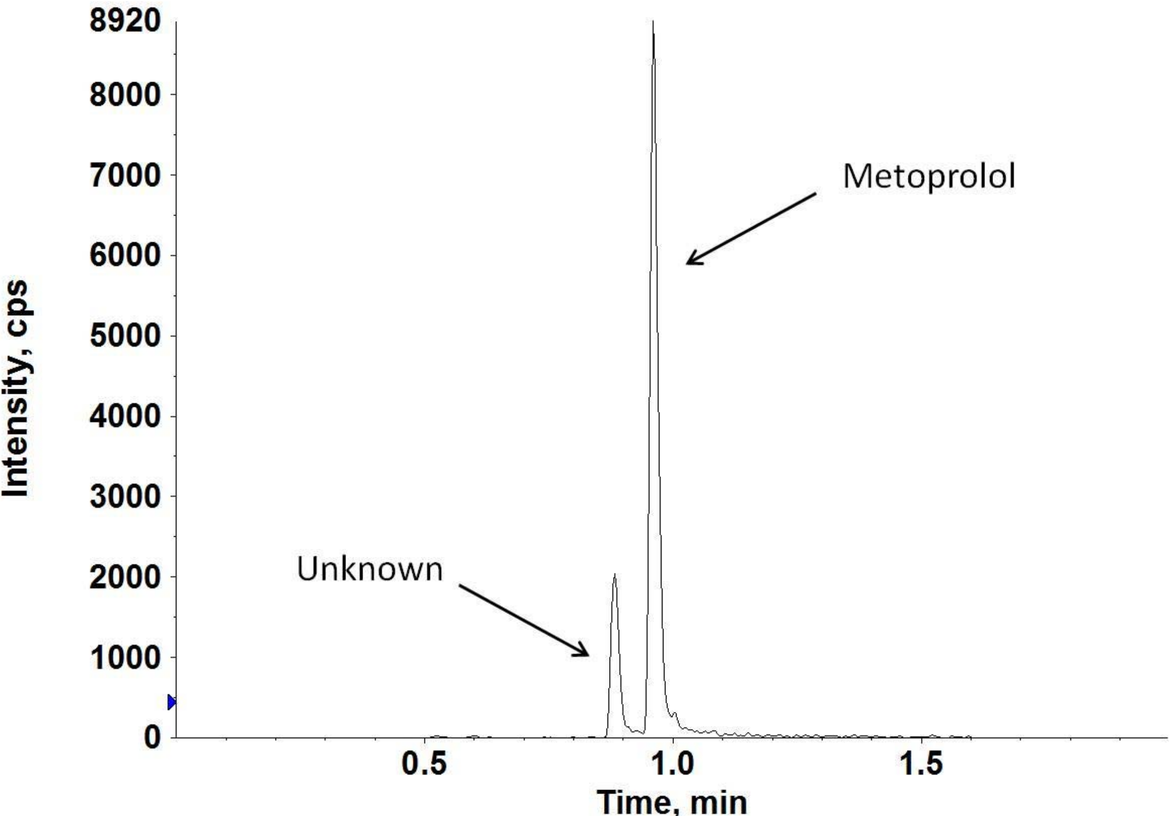


Figure -5.3- Representative LC-MS/MS chromatogram of a whole blood sample taken at steady state post continuous IV infusion of metoprolol for 4 h in anesthetised rats. Whole blood samples extracted using protein precipitation.

In order to investigate the origin and identify the moiety of the unidentified peak, residual whole blood samples from all four rats were pooled to create a large enough sample for the investigation. A 50 µL aliquot was precipitated with 200 µL of acetonitrile containing 100 ng/mL of metoprolol-d_7 and centrifuged (5810R, Eppendorf, Germany) at 3000 g for 10 min. The resultant supernatant was removed and analysed using a QToF. The rationale for using this instrument lies behind its capability to detect and resolve compounds with improved mass accuracy. This enables collection of data that can distinguish drug metabolites from most if not all isobaric endogenous components and that can determine elemental compositions of metabolite ions and their fragments. The high mass measurement accuracy allows exact mass measurement of small molecules. The QToF is a hybrid quadrupole time-of-flight mass spectrometer with MS/MS capacity, the quadrupole acts as an ion guide in MS mode and as a mass filter when utilised in MS/MS mode. A reflectron time-of-flight analyser is positioned orthogonally to the quadrupole to serve as a mass resolving tool. The high resolving power of this instrument allowed for elemental composition analysis of the pooled sample. A full scan of the pooled extracted sample was performed to determine the accurate mass of metoprolol and the unidentified (unknown) peak. The accurate mass corresponded to molecular formulae differing by C, 4 x H and O indicating a loss of CH_4 and a gain of O as shown in Figure -5.4-.

	Accurate Mass detected using QToF (amu)	Corresponding Molecular Formula
Metoprolol	268.1922	C15H26NO3
Unknown	268.1536	C14H22NO4

Figure -5.4- The detected accurate mass for metoprolol and the unknown peak when analysed using the QToF.

A standard stock solution of metoprolol was diluted to 100 ng/mL and analysed along with metoprolol-d_7 by MS^n to determine the fragmentation pattern which in turn would enable elucidation of the individual fragments. These were compared to the MS^n fragments of the unknown peak to determine the location of biotransformation on the metoprolol molecule as shown in Figure -5.5-.

Figure -5.5- MS^n fragments of the unknown peak to elucidate the biotransformation.

Figure -5.6- Biotransformation of metoprolol in rat.

The retention time, accurate mass and fragmentation data indicated that the biotransformation was O-demethylation and further oxidation to a carboxylic acid as shown in Figure -5.6-. These findings correlate well with published literature regarding the metabolism pathway of metoprolol[159]. Metoprolol is primarily metabolised by cytochromes (CYP2D6) and (CYP3A4) where α-hydroxylation and O-demethylation of metoprolol occurs to produce inactive metabolites[160].

The unknown peak corresponds to a metoprolol metabolite which was only observed in the whole blood extract and was not detected in the SPME extract. This suggests that the selectivity of the C_{18} coated SPME fibres was limited to adsorption of metoprolol only without its metabolite. Although a C_{18} column was still utilized for chromatographic separation of the whole blood sample, this was combined with gradient elution which in turn enabled separation between the two peaks (unknown and metoprolol). As discussed above, the metabolite is of a more polar moiety than the parent. Therefore, the type of SPME phase utilised in this experiment did not exhibit the appropriate coating properties to detect the metabolite. The type of phase applied determines the polarity of the SPME coating. Polarity can provide selectivity by enhancing the affinity of the coating for polar analytes compared to a non-polar fibre coating.

The lack of commercially available mixed phase *in vivo* SPME coatings for more polar analytes is a current drawback of the SPME technology. The choice of coating phases particularly for *in vivo* applications is very limited which poses a challenge for the extraction of polar components such as metabolites. Drugs entering the body undergo biotransformation, some leading to active metabolites that may bind to the therapeutic target receptors or interact with other targets causing adverse effects. For this reason, the FDA guidance for metabolites in safety testing[161] recommends metabolic profiling of drugs during different stages of development using *in vitro* and *in vivo* methods. Generally, active metabolites as well as ones that have been identified *in vitro* using human hepatocytes are considered for exposure and safety assessments. In such cases, sensitive and selective analytical techniques are required to detect and characterize metabolites derived from the parent drug. Although SPME has been successfully applied in numerous metabolomic studies i.e. the analysis of metabolites or metabolite fingerprinting[151], the SPME probes employed in those studies were custom-made or specifically designed for each study. For example, Vuckovic, D. *et al*[42] utilized mixed-mode coatings, polar-enhanced polystyrene − divinylbenzene, and phenylboronic acid custom made coatings for the extraction of hydrophilic and hyrophobic metabolites at physiological conditions.

The use of SPME for metabolic profiling and metabolomics in general could potentially transform the way metabolites are currently captured and quantified. Measuring unstable metabolites or even unstable prodrugs has always been a complicated task for bioanalysts. *In vitro* concentrations of unstable components do not always match the real *in vivo* concentrations. In some cases, the time between withdrawing a sample and treating it with a stabilizer is enough to degrade an unstable moiety. For this reason, capturing unstable metabolites or prodrugs within a living organism using suitable SPME probes can lead to real-time drug monitoring i.e. reflect true in-life drug concentrations. This can have a substantial impact on current quantification methods for unstable metabolites and prodrugs. The small amount of analyte required for SPME analysis permits detection of metabolites circulating at low concentrations. SPME may offer a good representation of the true metabolome at the time of sampling and therefore act as a powerful tool for metabolomic analysis[62,151]. But this capability is currently limited by the chemistry of the coating and the commercially available SPME products. Increased coverage of hydrophilic molecules will only be achieved when a diverse range of *in vivo* coating phases become commercially available.

5.3.3 Post Mortem Tail Dissection

Necropsy is considered as an important step in research, especially when evaluating new sampling techniques. In this study, the insertion of SPME fibre into the rat's tail vein was followed by tail dissection. This was performed to identify the positioning of the fibre within the vein and possibilities of vein puncture if the fibre is advanced into the vein with increased handling force.

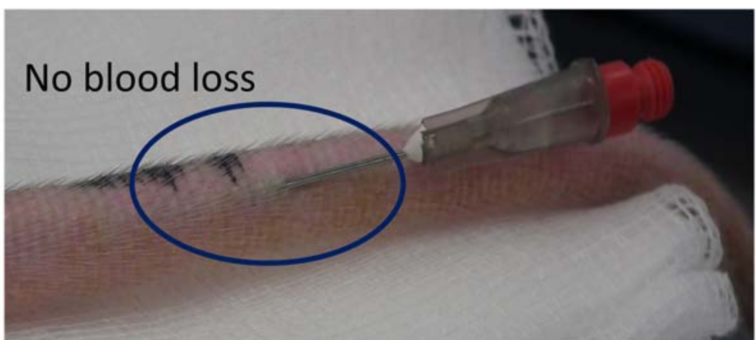


Figure -5.7- SPME fibre fully extended into a Wistar Han rat tail vein.

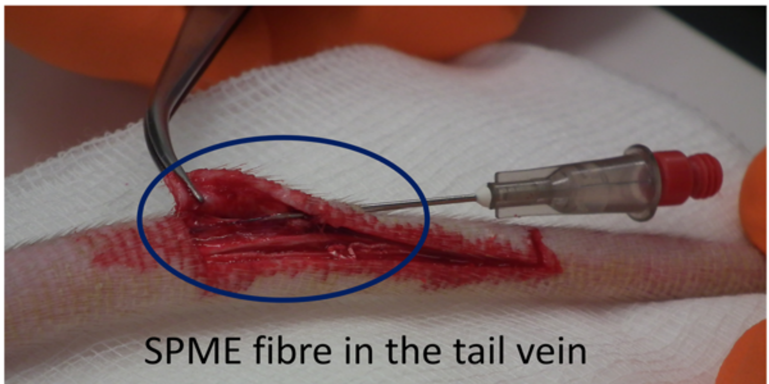


Figure -5.8- Necropsy of Wistar Han rat tail vein illustrating the SPME insertion into the vein.

In addition to the fact that SPME sampling does not require blood withdrawal, Figure -5.7- illustrates that there was no blood loss during the insertion of the device into the living organism. Figure -5.8- demonstrates the fibre penetration inside the vein. The laboratory animal technician and study vet both confirmed that the necropsy further revealed that the fibre had been advanced into the tail vein without causing any damage to the vein. The laboratory animal technicians stated that resistance was exerted in the reverse direction to the fibre when the device was pushed into the vein vigorously. This meant that vein puncture was not possible as the fibre is too flexible and thin to cause any damage to the vein.

Preliminary assessments of SPME probe insertion into the living organism showed that the device was easy to handle. Despite the fact that the current *in vivo* SPME probe is a prototype device which requires modification and product development, animal technicians found the device simple to use within a busy laboratory setting. This was a satisfactory indicator that the device could potentially be applied and used for a toxicology study.

In comparison to other microsampling techniques, extensive training was not required for the animal technicians to utilise the SPME device. While one of the core concerns with the dried blood spot technology was training of staff to accurately spot the blood sample onto the card[162]. On the other hand, the SPME device was used in anesthetised rats in this instance so the ease of use could have been masked by the fact that a response was not observed upon insertion of the device due to the anesthesia effect. The ease of use requires full assessment when the technique is applied in a toxicology study of live animals. This is described in more detail in Chapter 6.

5.3.4 *In vivo* Measurement of Protein Binding Values by SPME and RED

Assessment of PPB of candidate drugs in each laboratory animal is not a common approach within preclinical drug development. Traditional and current ways of identifying bioavailability and relating total drug concentrations to free circulating drug concentrations have been through *in vitro* plasma protein binding measurements. Values determined *in vitro* are then applied to total drug concentrations measured *in vivo* to produce a calculated value.

In this study, the percentages of metoprolol binding to protein were measured individually for six conscious rats, by taking whole blood samples and analysing them using the RED device.

Total metoprolol concentrations were also measured by protein precipitation extraction. The two values were used to calculate the theoretical free concentration using Equation -5.1-.

$$Theoretical\ Free\ Conc.\ = Total\ WB\ Conc. determined\ by\ PP\ \ x\ \ Calculated\ \%\ Free$$

Equation -5.1-

This was compared with free metoprolol concentration (Table -5.3-) determined using SPME which was sampled at approximately the same timepoint as the whole blood sampling.

Table -5.3- Total and free metoprolol concentrations measured in live rats using RED and SPME

Animal Number	**Total WB Conc. determined by PP (ng/mL)**	**% Bound determined by RED**	**Calculated % Free By RED**	**Calculated Theoretical Free Conc. (ng/mL)**	**Actual free Conc. determined using SPME (ng/mL)**	**% Difference between SPME and Theoretical Conc.**
Rat 1	49.4	21.6	78.4	38.7	34.4	11.2
Rat 2	52.0	22.0	78.0	40.6	46.0	-13.4
Rat 3*	-	-	-	-	-	-
Rat 4	56.5	36.8	63.2	35.7	40.2	-12.5
Rat 5	85.3	21.1	78.9	67.3	61.4	8.72
Rat 6	83.1	10.1	89.9	74.7	73.6	1.38
Mean	**65.3**	**22.3**	**77.7**	**51.4**	**51.1**	
SD	**17.5**	**9.5**	**9.5**	**18.1**	**16.1**	

*Rat 3 chewed the connections of the infusion harness and escaped from the tethered IV jacket and therefore no data was collected for rat 3.

Table -5.3- shows the data collected for *in vivo* metoprolol concentrations using RED and SPME. The first observation was that protein binding values measured using the RED device have a large inter-animal range, varying between 10.1% - 36.8%. Although the overall mean correlates with reported literature values for metoprolol (20 - 30%)[146], the inter-animal variability was much larger than the observed variability for the *in vitro* RED data in Chapter 4, Section 4.3. The calculated theoretical *in vivo* free metoprolol concentration obtained using the RED device correlated well with the free concentrations determined using SPME. It was found that the percentage difference between the free drug concentrations obtained by the two techniques was less than ±15%. This is expected because the SPME fibre was not competing with the proteins for the drug since fibres only adsorb a small amount of drug which does not impact the blood/protein equilibrium. This indicates and further confirms that SPME measures *in vivo* free circulating drug concentration.

The overall results of this experiment showed that SPME can be used to determine free circulating drug concentrations in live animals. In the current drug discovery and development setting, knowledge of drug/protein binding property is determined during the early stages of the drug discovery[36]. This is used to extrapolate preclinical animal data to predict drug's efficacy and toxicity in human subjects. Although the amount of circulating free drug concentration is crucial to determine the drug's bioavailability, hepatic clearance and other parameters, the common practice in the pharmaceutical industry is to report total drug concentrations rather than free. This is mainly due to the fact that there is equilibrium between bound and free drugs, and concentration of free drug can be predicted from total drug concentrations. Also the limited range of analytical techniques for direct monitoring of free drug concentrations adds to the complexity of utilising this parameter in preclinical and clinical settings. However, under certain conditions such as disease states, if the equilibrium between bound and unbound drug is disturbed, the free drug concentration can become significantly higher than expected in which case a patient may experience drug toxicity even if the total drug concentration is within the therapeutic range. The converse is also true. In hyperalbuminaemia more drug will be bound to the plasma proteins than in a healthy patient and the amount of free drug would therefore be lower than expected i.e. below efficacious levels[163].

For this reason, it is essential in some cases to monitor the free concentration and SPME has the potential to be used *in vivo* for direct monitoring of free rather than total concentrations. Currently, microdialysis is recognised as the most established *in vivo* technique for measuring

free drug concentrations[164]. The technology is widely used for monitoring neurotransmitters in the brain and for the measurement of selected metabolites as a bed-side instrument. However, the technique still has limitations such as complicated initial set up of the sampling device and carryover issues. All other techniques require blood withdrawal followed by *in vitro* analysis of drug binding values to elucidate the free concentrations. Therefore, SPME offers a simple and compelling alternative to these techniques.

The current pharmaceutical environment as discussed above utilises total drug concentrations for preclinical and clinical studies as this has been the routine approach for many years. The mindset of the industry will have to change before a technique such as SPME becomes the routine technique for reporting drug exposure levels. But if the science behind SPME can be demonstrated, then there is every possibility of it being adopted in the future because of the stated advantages, although this is likely to take time and require support from regulators. Despite this, SPME can potentially be used immediately for certain study types such as PK discovery studies and preclinical non-GLP studies, where free drug concentrations and sample blood volumes play a major role.

5.4 Conclusion

The work reported in this chapter, showed the feasibility of transferring the SPME technique from *in vitro* to *in vivo* bioanalysis. It has been demonstrated that pre-equilibrium conditions can be applied *in vivo* to reduce the length of sampling time. In practice, the time required to reach equilibrium is usually too long and cannot be implemented for two reasons; ethical considerations of leaving the needle and fibre within the living animal for a long period of time which may cause animal distress and second, the rapid drug concentration change within a dynamic system. Essential parts of the drug PK profile could be missed if the length of sampling time is too long. However, the main disadvantage of employing pre-equilibrium conditions lies in the fact that time of extraction requires optimisation during the *in vitro* method development and that shorter sampling times may compromise analyte recovery and sensitivity. But such hurdles can be addressed by current highly sensitive detection techniques such as mass spectrometry.

The small blood vessel size of rodents meant that the use of interfaces was a necessity to allow *in vivo* SPME applications. This study showed that the new miniaturized SPME

devices were small enough to be inserted directly into rodent veins without the need for a catheter or other interfaces.

This was further confirmed by the necropsy which revealed ease of fibre penetration into the vein. This feature enables ease of use and flexible handling for animal technicians particularly within a busy preclinical study laboratory.

The information generated in this study presented for the first time a direct comparison between RED and SPME for monitoring free drug concentrations in individual animals. The difference between the two techniques was ±15%, however, the RED device involved blood withdrawal followed by *in vitro* analysis while SPME did not require blood withdrawal and it enabled sampling and extraction in one step. Despite the apparent advantages of SPME in monitoring free drug concentrations, there is currently only one phase (C18) which is housed within hypodermic needles and is commercially available for *in vivo* applications. This coating phase is a versatile phase but it does not cover a wide range of analyte polarities, for this reason metabolites that exhibit polar moieties cannot be extracted using this phase as was the case with the metoprolol metabolite. Product development issues and the need for mixed phase coatings to expand the portfolio of *in vivo* drug extraction will have to be addressed prior before the SPME technique could be accepted as a mainstream bioanalytical procedure.

Chapter 6

Comparison of SPME and Conventional Blood Sampling in a 7 Day Toxicity Study in Male Rats

6.1 Introduction

As previously discussed in Chapter 1, Section 1.2, the primary objective of toxicokinetics is to demonstrate the systemic exposure achieved in animals and its relationship to the drug dose level and the time course of a toxicity study[165]. This is then related to toxicological findings if any, and their relevance to clinical safety in human studies[165]. Thus, toxicokinetic measurements are normally integrated within toxicity studies using separate satellite groups with a view to enhance the value of the toxicological data generated.

Laboratory animals utilised within preclinical investigations including toxicity studies, require stable and defined physiological states so that response to the variable of interest (drug under evaluation) is not masked by external factors such as sampling procedures. Compromising animal wellbeing leads to unreliable data which may give false interpretations with reduced credibility of study outcomes[166]. One of the major hurdles for implementing microsampling techniques in regulatory studies is the removal of TK satellite animals and the potential consequences of using microsampling on functional, behavioural and clinical pathological endpoints in main study animals[17,167]. Any adverse impact of generating TK measurements from the main toxicity study animals can lead to regulators not accepting the data[17]. For this reason, systematic evaluation of toxicologically sensitive parameters including haematological and clinical chemistry changes is essential to understand the impact of subjecting animals to a new microsampling device. Changes between control and test groups are monitored to observe and assess the biological and the toxicological significance of any differences between the two groups[17].

Such assessments are also important in revealing the biocompatibility of the microsampling tool, specifically if it is invasive. In the case of SPME, investigating toxic and inflammatory reactions as well as monitoring other stress markers is necessary as the mechanism of the

device involves leaving the fibre in the vein of a living organism for a defined period of time, therefore the possibility of adverse reactions to the device is high.

In addition to assessing the impact of microsampling techniques on toxicological parameters, it is also essential to ensure that the TK data generated using microsampling devices is comparable to TK data obtained by conventional sampling. Therefore, using SPME in a typical preclinical study within a pharmaceutical setting and comparing this technique to a conventional sampling tool to generate TK data is vital to evaluate the applicability of the technique.

6.1.1 Tail Vein Sampling

To date, blood removal is one of the most common procedures used to address the needs of toxicokinetic, pharmacokinetic and toxicology studies[168]. The ideal method for blood sampling from laboratory rodents should have minimal physiological impact on the animal, removes the requirement for anaesthetics and finally enables fast and repeated sampling without causing stress to the animal or having an effect on any toxicological parameters[169].

Tail vein sampling is a popular technique suitable for rodent studies. It is quick and simple to perform. However, this method requires warming rodent tails by immersion into warm water or through whole animal warming using a hot box or an incubator. This is to enable blood vessel dilation and in turn allow collection of sufficient blood volume for analysis[170]. The rodent is usually placed in a restrainer which consists of a plastic tube and a 21 gauge butterfly-needle is inserted into the blood vessel and blood is collected directly into tubes or through a syringe or a capillary tube. To avoid bruising and damage to the tail, no more than eight samples should be taken over 24 h[15]. The recommended number of attempts is also minimal (no more than three needle sticks in any one attempt). Removal of blood can have a direct impact on the pathology and other endpoints of main study animals including haematological changes which may mask the potential effects of the test compound. For this reason, satellite groups have been previously employed to determine toxicokinetic parameters.

In vivo SPME has the potential to eliminate the need for satellite groups as no blood withdrawal is required. Furthermore, the device is housed within a 21 gauge needle, thereby

maintaining the same dimensions of conventional sampling tools. However, the number of needle sticks will remain the same as the conventional technique i.e. a needle stick is required per each PK/TK timepoint unless the current prototype SPME device can be further developed to encompass an indwelling cannula that can accommodate interchangeable fibres without the need for multiple needle sticks.

6.1.2 Clinical Pathology Measurements

The assessment of clinical pathology is a key tool for the evaluation of drug safety within a biological system and it is also a clear indicator of physiological changes as a consequence of exposure to foreign materials such as sampling tools[171]. The most common analysis includes clinical chemistry measurements looking at several enzymes that are used as biomarkers for cellular injury and organ toxicity/damage. For example elevated levels of alanine aminotransferase and glutamate dehydrogenase are regarded as indicators of hepatotoxicity whereas alterations in alkaline phosphatase activities are a sign of changes in food intake[172]. Cardiac dysfunction is observed through changes in atrial natriuretic peptide (ProANP) and tropinin levels. High influx of white blood cells is also a clear indicator of inflammation in response to damage caused by sampling procedures. Other core clinical chemistry tests involve determination of glucose, urea, total protein and other hepatocellular and hepatobiliary parameters[173]. These biomarkers act as important pointers to distinguish the direct and downstream effects of sampling techniques on animals.

Haematology examination is also performed concurrently with chemistry tests, the standard parameters that are clear pointers of haemorrhage and functional disturbances of the blood platelets are erythrocyte count, leucocyte count, haemoglobin levels, haematocrit as well as several others such as white blood cell count that provide insight to production of haematopoietic tissues and give indications of bone marrow toxicity[171].
In addition to the above, coagulation markers are also monitored to reveal vascular injuries and show signs of a haemostatic disturbance where an increased tendency to bleeding is suspected[171]. Also initiation of coagulation is an indicator of body response upon exposure to foreign materials i.e. a marker for biocompatibility[19]. These markers include prothrombin time, activated thromboplastin time and fibrinogen levels. The coagulation system which promotes the formation of blood clot during tissue damage starts by platelet aggregation

which is activated by thrombin, this is then followed by fibrinogen formation which stimulates platelet clumping to accommodate formation of the plug[172]. For this reason, the coagulation system plays an important role as a biomarker for changes associated with the vascular system.

Histopathology of certain organs is also one of the requirements for clinical pathology assessments, for example; the spleen is a site of red blood cell production and therefore is a clear indicator of disturbance to the red blood cell count while liver toxicity and lung damage can occur as a consequence of changes associated with stress[174]. Tail tissue is usually examined for inflammation, irritation and extent of vascular damage/bruises[14,175] to determine the impact of new sampling tools; hence terminal tissue samples of these organs are retained and microscopically examined.

6.1.3 Neurobehavioral Assessment

How an animal cope with a given situation and how it responds to internal and external factors is what defines an animal's wellbeing. The extent of animal wellbeing is not exclusive to physiological measures; it is a combination of physiological and behavioural indicators. Animal behaviour including eating and drinking patterns, playing, sleeping, grooming and posture serve as important markers that characterise the animal's health and its response to stress[16].

Any alterations from the regular state will trigger a protection mechanism where adaptive coping responses are activated to return the animal to its normal condition of what is known as "wellbeing"[176]. Abnormal responses may lead to distress, disability or death. A systematic observation of an animal's behaviour offers the possibility for determining the effects of drugs and exposure to surgical or sampling tools on the psychological and behavioural state of the animal. Samuel Irwin[177] developed an observational procedure which was further developed into a functional observation battery (FOB) that consists of a series of situations applied to the animal to determine the overall sensory, coordination and motor deficits. The procedure is designed to comprehensively assess activities related to stimulation of the central nerves system such as excitation, jumping and autonomic functions such as salivation, lachrymation and several other responses[177]. The duration of the test lasts between 10-15 minutes where visual and other sensory stimuli are applied to the animal to determine its

ability to detect and respond in a normal manner, after which rodents are returned to their cages[177,178].

The "Irwin neurobehavioral study" is suitable for identifying the suitability of SPME as a microsampling tool. The SPME device is considered to be a 'stressor' (an external factor which may cause pain), to which the animal will respond through behavioural and autonomic ways. Depending on the magnitude of the stressor, the animal's biological function maybe altered to either have a minimal effect on its wellbeing or have a greater impact that could result in distress[179]. Animal distress can adversely impact the quality of experimental results, data interpretation and may also affect the ethical conduct of the study[176]. If SPME is shown to be psychologically and toxicologically benign, its adoption in preclinical and future clinical studies may have the potential to evolve a new area of microsampling without having to withdraw any blood.

6.1.4 Aims and Objectives

The aims and objectives of this chapter are to assess the ethical and practical impact of using biocompatible SPME as a novel microsampling technique for repeat blood sampling without blood withdrawal, and to compare the use of SPME with a conventional tail blood sampling procedure (caudal venepuncture) to generate TK data. This will be performed through a 7 day repeat dose toxicity study in male rats. The study design will mimic a typical preclinical study used within the pharmaceutical industry, in terms of duration, dosage, animal numbers and analysis of various endpoints. In this study, SPME will be used for serial toxicokinetic sampling from conscious rats on day 1 and day 7 to replicate a standard toxicokinetic assessment. This will be conducted to demonstrate the quality of the TK data generated using SPME and whether it is comparable to conventional sampling. A full tolerability evaluation assessing stress levels and clinical pathology is to be conducted for all animals to establish the suitability of this novel microsampling technique. SPME sampling and conventional blood withdrawal sampling will also be compared to a control group to observe the impact of each sampling technique on the animals and to assess the toxicological significance of any differences between them.

6.2 Experimental

6.2.1 Test Material

Metoprolol tartrate obtained from Sigma Aldrich (Dorset, UK) was formulated as a solution using sterile water (6 mg/mL) and stored at approximately 2 -8 ºC, protected from light for 15 days.

6.2.2 Rationale for Test Material

Metoprolol is a selective beta 1 receptor blocker which is used to lower blood pressure in patients with high blood pressure, heart failure or angina. It has been chosen as a tool compound because it is a class I compound (high solubility and permeability) according to the Biopharmaceutics Classification System (BCS), with a wealth of published information (*in vitro* and *in vivo*)[146,148], defined pharmacokinetics (rapidly cleared) and pharmacodynamic effect (blood pressure). A previous study has been reported investigating the pharmacokinetics, pharmacodynamics and toxicity of metoprolol in Wistar albino rats at 60, 120 and 240 mg/kg/day for 28 days[180]. Based on this a dose of 60 mg/kg/day was selected as the identified no observed adverse effect (NOAEL) from Nandi *et al*[180] to provide a suitable concentration-time profile for the study duration. Metoprolol was to be administered once daily for 7 days by oral gavage to male Crl:WI(Han) rats to determine the toxicokinetics and systemic exposure of metoprolol at a target dose level of 60 mg/kg/day.

6.2.3 Animals and Housing

24 Male Wister (Han) rats were obtained from Charles River UK Ltd (Margate, Kent). Male rats were used to minimize data variability caused by sex differences. The approximate age of the rats at day 1 of dosing was 10 weeks. Animals were split into 4 groups as shown in Table -6.1-, each containing 6 rats. The number of animals per group was chosen based on the minimum recommended number of rodents used on 7 day and 1 month preclinical toxicology studies[165]. Plastic solid bottom cages were used for housing, each containing Aspen 4H bedding (Datesand Ltd). 3 animals were randomly housed within each cage (same treatment group) and were acclimatised for 6 days prior to day 1 of dosing. All animals were kept at an

approximate temperature of 19 -23ºC and a relative humidity range of 55 ± 10% on a 6 am to 6 pm light cycle. They were given 5CR4 rodent diet (Purina Mills International) and filtered mains water (Veolia Water plc). Chew sticks, nesting material and tunnels were provided for environmental enrichment.

Animals were randomly allocated to one of four treatment groups (each group is defined in Section 6.2.4) and were identified using microchips and permanent markers on the tail with numbers as shown in Table -6.1- below;

Table -6.1- Animal numbers within each group

Animals	Group Number			
	1	2	3	4
Males	001 002 003 004 005 006	007 008 009 010 011 012	013 014 015 016 017 018	019 020 021 022 023 024

6.2.4 Study Design

The design of the study consisted of 4 animal groups as shown in Table -6.2- below. The rationale for each group choice was based on the fact that the difference between Groups 1 and 2 would examine the impact of the SPME device on the animal. While the difference between Groups 2 and 3 would examine the impact of the test article (metoprolol) and finally the difference between Groups 3 and 4 would examine the impact of SPME compared to conventional sampling i.e. caudal venipuncture (CV).

Table -6.2- Study design and animal group numbers with sampling types and dosing schedule

Group Number	Group Name	TK Blood Sampling	Sampling Method	Dose* (mg/kg/day)	Dose Conc *(mg/mL)	Number of Males
1	Group 1	No	Control/None	Vehicle	0	6
2	Group 2	Yes	SPME	Vehicle	0	6
3	Group 3	Yes	SPME	60	6	6
4	Group 4	Yes	CV	60	6	6

6.2.5 Dosing Regimen and Sampling

Metoprolol was administered, once a day, orally at a dose volume of 10 mL/kg which is equivalent to 60 mg/Kg. Groups 1 and 2 were dosed 10 mL/kg of sterile water by oral gavage.

Toxicokinetic sampling was performed on days 1 and 7 at the following timepoints; 0.5, 1, 3, 5, 7 and 24 h after dosing. Groups 2 (vehicle) and 3 (dosed/metoprolol) were sampled using SPME by placing the SPME needle into the rat vein. Once the needle was inserted into the caudal vein, the fibre was projected inside the vein and exposed to the systemic circulation for a defined period of time (2 min). Prior to sampling, each fibre was pre-conditioned with methanol for 15 min followed by water for another 15 min.

Group 4 was sampled using caudal venipuncture (CV). This method involved using a standard 21 g cut-off butterfly needle inserted into the caudal vein. Approximately 150 µL of blood was collected into 0.5 mL microtainer tubes containing K_3EDTA as the anticoagulant (BUNZIL Healthcare, UK). Bleeding was staunched using light pressure and a swab or other absorbent material at the end of each collection. Subsequent bleeds required a fresh cut-off butterfly needle to be inserted into the caudal vein at each occasion. All blood samples were gently mixed on a roller mixer (Progen Scientific, UK).

Animals were sacrificed after the 24 h timpepoint on day 8 by exsanguination via the abdominal aorta under isoflurane anaesthesia. Confirmation of death was performed by cutting of the major blood vessel (cessation of circulation). All animal studies were ethically

reviewed (GSK POL-GSKF-403 POL_87182 (7.0)) and carried out in accordance with Animals (Scientific Procedures) Act 1986 and the GSK Policy on the Care, Welfare and Treatment of Animals.

6.2.6 Bioanalysis and Toxicokinetics

SPME samples collected from Group 3 were desorbed using acetonitrile containing metoprolol-d_7 as per the SPME procedure described in Chapter 3, Section 3.2.4. Whole blood samples collected from Group 4 were extracted (25 µL aliquots) using protein precipitation (full methodology for metoprolol whole blood analysis is detailed in Chapter 2, Sections 2.2.3 and 2.2.4). Concentrations of metoprolol from SPME samples (Group 3) and blood (Group 4) were determined by using the bioanalytical LC-MS/MS method validated for metoprolol (as detailed in Chapter 2). Two sets of calibration lines and QC samples were analysed, one was extracted using the SPME procedure described above and the second was extracted using whole blood protein precipitation as detailed in Chapter 2, Sections 2.2.3 and 2.2.4.

Toxicokinetic evaluation was performed using a non-compartmental pharmacokinetic analysis method (Phoenix 32, version 6.0, Pharsight Corporation, CA, USA). Systemic exposure to metoprolol was determined by calculating the area under the plasma concentration-time curve (AUC) from the start of dosing to the last quantifiable timepoint (AUC_{0-t}) using the linear-logarithmic trapezoidal rule. The maximum observed peak plasma concentration (C_{max}) and the time at which it was observed (T_{max}) were determined by inspection of the observed data.

6.2.7 Clinical Observations

All animals were thoroughly examined for clinical signs on day -3 prior to dosing and at least three times daily during treatment days by animal laboratory technicians. Body weights and food consumption were also recorded on day -3 pre-treatment and daily during treatment days.

6.2.8 Clinical Pathology Measurements

Necropsy was performed using samples taken on day 8 by puncture of the abdominal aorta under isoflurane anaesthesia (with food withheld overnight prior to necropsy). 0.8 mL of blood was collected into potassium EDTA tubes and analysed using standard ADVIA 2110 haematology methodology[181] (full protocol available from Siemens Healthcare Diagnostics) to determine the following haematology parameters:

Haematocrit (HCT), haemoglobin (HB), total red blood cell count (RBCR), mean cell volume (MCVR), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC), red blood cell distribution width (RDWR), reticulocytes (RETA), platelet count (PLT), white blood cell count (WBC), neutrophils (NEU), lymphocytes (LYM), monocytes (MON), eosinophils (EOS), basophils (BAS), low reticulocyte (LRT), medium reticulocyte (MRT), high reticulocyte (HRT), leucocyte count (LUC), platelet count (PLT).

Clinical chemistry parameters were measured using ADVIA 2120 chemistry methodology[181] (full protocol available from Siemens Healthcare Diagnostics), 2.0 mL of blood was collected into tubes containing lithium heparin to measure the following parameters:

Alanine aminotransferase (ALT), glutamate dehydrogenase (GLDH), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin (TBIL), glucose (GLU), albumin (ALB), total protein (TP), urea (UREA), creatinine (CREA), inorganic phosphorus (PHOS), calcium (CA), cholesterol (CHOL), triglycerides (TRIG), sodium (NA), potassium (K), chloride (CL).

Coagulation activity was monitored using 1.8 mL of blood collected into tubes containing 0.2 mL of 0.106 M trisodium citrate. A standard CA 1500 coagulation methodology (full protocol available from Siemens Healthcare Diagnostics) was employed to measure prothrombin time (PT), activated partial thromboplasin time (APTT) and fibrinogen content (FIB).

Cardiac output was investigated using standard Centaur CP methodology (full protocol available from Siemens Healthcare Diagnostics), 0.8 mL of blood was collected into Minicollect Z serum gel activator tubes to assess the level of troponin I (CTNI). A further 0.8 mL of blood was utilised to determine NT-pro-atrial natriuretic peptide (NT pro-ANP)

levels. An enzyme-linked immunosorbent assay on a meso scale discovery (MSD) platform was employed.

All tissues from all animals (Groups 1-4) were preserved in 10% buffered formalin and macroscopic examination of any abnormalities was performed. Specific tissues including the liver, lung, spleen and tail were histologically examined for all animals.

6.2.9 Neurobehavioral Assessment

Animals were observed by animal laboratory technicians according to a standardised observation battery which allows the assessment of both peripheral and central nervous systems activities (e.g. motor activity, behaviour, co-ordination, somatic sensory/motor reflex responses and autonomic responses such as piloerection, pupil size, lachrymation, and salivation, overt cardiovascular and gastrointestinal effects). Methods were adapted from those originally described by Irwin for detecting behavioural effects in mice[177] and subsequently modified as the neurologically based Functional Observational Battery (FOB) adapted for use in rats[182].

Animals were examined on day -1 i.e. pre-treatment and on days 1 and 7 between 2 and 4 hours after dosing. Each animal was subjected to a number of stimuli to assess the response. The true identification of each animal was blinded for the purposes of these examinations (microchipped). In addition, the order in which animals were presented to the examining technicians on each respective occasion was also randomised.

6.3 Results and Discussion

6.3.1 Bioanalysis and Toxicokinetics

The evaluation of pharmacokinetic profiles and examination of toxicokinetic parameters is a crucial part of all toxicology studies. The feasibility of PK/TK studies by *in vivo* SPME was investigated and compared with conventional blood sampling. Blood sampling by SPME measures the unbound free drug concentration (Group 3). This was also shown in Chapter 5, Section 5.3.4. Whereas total drug concentration was measured (Group 4) using conventional

caudal tail vein (CV) sampling followed by protein precipitation extraction. Metoprolol concentrations from Groups 3 and 4 are shown in Tables -6.3- and -6.4- respectively.

Table -6.3- Free concentrations of metoprolol extracted by SPME at each timepoint on days 1 and 7 following oral administration at a nominal dose of 60 mg/kg/day to male rats. Data reported to 3 significant figures.

Free Concentrations of Metoprolol Extracted by SPME (ng/mL)							
Period	Time (h)	Animal Number					
		13	14	15	16	17	18
Day 1	0.5	601	548	250	797	525	378
	1	394	333	197	321	228	587
	3	67.1	99.2	55.6	52.4	56.4	91.9
	5	4.97	14.5	4.26	5.57	8.75	9.57
	7	3.20	4.28	3.09	NQ	3.85	3.78
	24	NQ	NQ	NQ	NQ	NQ	NQ
Day 7	0.5	135	183	334	344	491	482
	1	198	140	239	216	294	703
	3	32.0	54.8	61.2	51.4	40.7	58.8
	5	NQ	14.2	4.03	5.49	5.55	3.07
	7	NQ	5.79	8.44	NQ	1.54	1.97
	24	NQ	NQ	NQ	NQ	NQ	NQ

NQ – Not Quantifiable

Table -6.4- Total whole blood concentrations of metoprolol by conventional CV sampling at each timepoint on days 1 and 7 following oral administration at a nominal dose of 60 mg/kg/day to male rats

Total Whole Blood Concentrations of Metoprolol (ng/mL)							
Period	Time (h)	Animal Number					
		19	20	21	22	23	24
Day 1	0.5	458	927	483	658	558	1418
	1	270	811	394	399	502	952
	3	60.7	279	108	40.4	73.3	195
	5	11.0	27.1	21.3	4.14	12.4	63.0
	7	4.56	6.63	9.15	NQ	7.31	15.5
	24	NQ	NQ	NQ	NQ	NQ	NQ
Day 7	0.5	636	734	676	1411	277	645
	1	334	418	500	946	299	749
	3	28.1	127	152	150	74.0	137
	5	NQ	13.0	23.3	30.5	13.9	30.5
	7	NQ	10.6	12.6	12.1	NQ	11.9
	24	NQ	NQ	NQ	NQ	NQ	NQ

NQ – Not Quantifiable

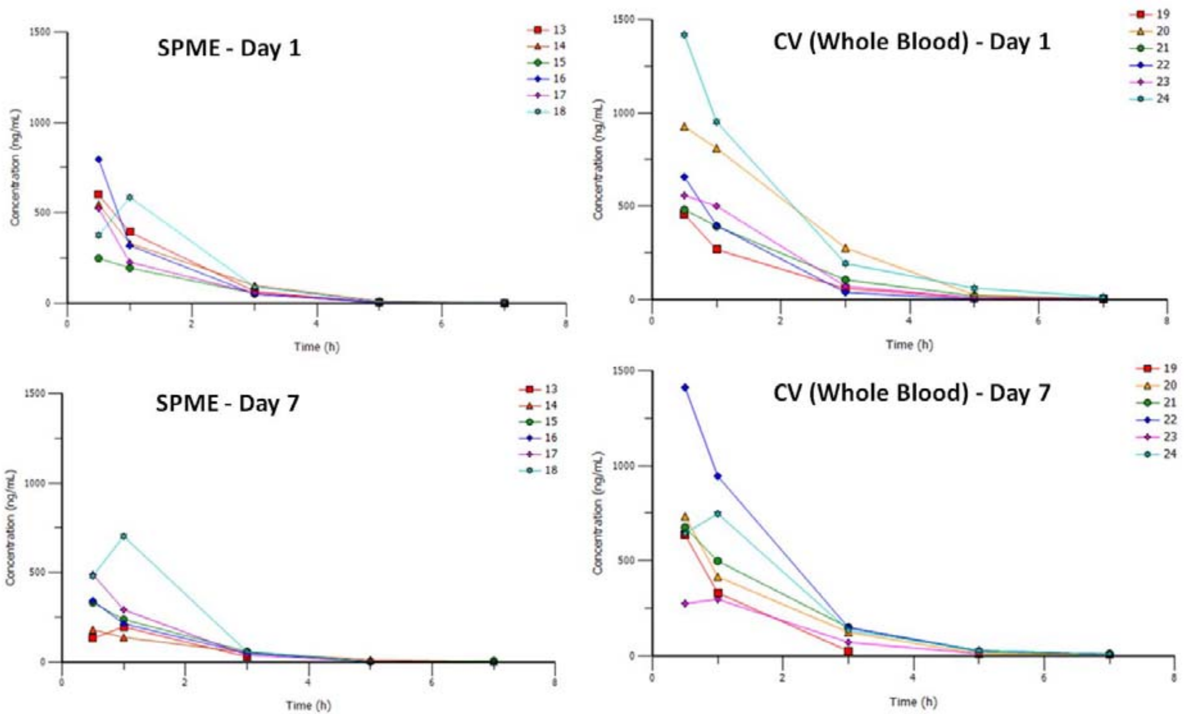


Figure -6.1- Individual concentration-time profiles of metoprolol sampled by SPME and CV on days 1 and 7 following oral administration at a nominal dose of 60 mg/kg/day to male rats (individual rodent numbers are indicated in the coloured key to each plot).

Following oral administration of metoprolol to male rats at 60 mg/kg/day, concentrations of metoprolol were quantifiable in blood up to 7 h after dosing from both blood sampling techniques. Similar concentration-time profiles were observed for metoprolol following both blood sampling techniques, using SPME and CV, for day 1 and day 7, respectively. However, Figure -6.1- shows a difference in the concentration levels between Groups 3 and 4. This is expected as both techniques are measuring different aspects, one is measuring unbound drug concentration (SPME/Group 3) and the other is measuring total drug concentration (CV/Group 4).

For this reason and in order to establish a like for like comparison between Groups 3 and 4, a correction factor consisting of the average protein binding value for metoprolol (22.3%) determined *in vivo* in Chapter 5, Section 5.3.4 was applied to adjust the free concentrations from SPME to give equivalent total concentrations as shown in Table -6.5- below.

Table -6.5- Concentrations of metoprolol extracted by SPME adjusted for protein binding fraction (22.3%), to give total concentration at each timepoint on days 1 and 7 following oral administration at a nominal dose of 60 mg/kg/day to male rats

Corrected Total Concentrations of Metoprolol Extracted by SPME (ng/mL)							
Period	Time (h)	Animal Number					
		13	14	15	16	17	18
Day 1	0.5	774	705	322	1026	676	486
	1	508	429	253	414	294	756
	3	86.4	128	71.5	67.5	72.7	118
	5	6.40	18.7	5.48	7.17	11.3	12.3
	7	4.12	5.51	3.98	NQ	4.96	4.87
	24	NQ	NQ	NQ	NQ	NQ	NQ
Day 7	0.5	174	236	430	443	632	620
	1	255	180	308	278	378	905
	3	41.2	70.5	78.8	66.2	52.4	75.6
	5	NQ	18.3	5.19	7.07	7.14	3.95
	7	NQ	7.45	10.9	NQ	1.98	2.54
	24	NQ	NQ	NQ	NQ	NQ	NQ

NQ – Not Quantifiable

Table -6.6-Calculated toxicokinetic parameters for groups 3 (free concentration/SPME), (corrected equivalent total concentration/SPME) and 4 (total concentration/CV)

Toxicokinetic Parameters:					
Parameter	Period		Male		
			Dose of Metoprolol (60 mg/kg/day)		
			Group Number		
			Group 3 (SPME) (Unbound Free)	Group 3 (SPME) (Corrected/ Equivalent Total)	Group 4 (CV) (Total Whole Blood)
AUC_{0-t} (ng.h/mL)	Day 1	**Mean**	**747**	**962**	**1230**
		Min	444	572	646
		Max	955	1230	2200
	Day 7	**Mean**	**552**	**710**	**1160**
		Min	300	386	607
		Max	978	1260	1990
C_{max} (ng/mL)	Day 1	**Mean**	**551**	**710**	**750**
		Min	250	322	458
		Max	797	1030	1420
	Day 7	**Mean**	**375**	**483**	**751**
		Min	183	236	299
		Max	703	905	1410
T_{max} (h)	Day 1	**Median**	**0.5**	**0.5**	**0.5**
		Min	0.5	0.5	0.5
		Max	1.0	1.0	0.5
	Day 7	**Median**	**0.5**	**0.5**	**0.5**
		Min	0.5	0.5	0.5
		Max	1.0	1.0	1.0

When comparing the original TK parameters of Group 3 with Group 4, a difference in TK parameters (as defined by mean AUC_{0-t} and C_{max}) was noted but as expected this is due to using free and total concentrations measured by SPME and CV, respectively. Group 4 (CV) appears to have higher systemic exposure to metoprolol when compared with Group 3 (SPME), but the difference is potentially consistent with the known protein binding. However, when the corrected TK parameters from Group 3 are compared with Group 4 values, a much more realistic picture was illustrated and similar values were observed for the parameters from both groups. T_{max} was observed at 0.5 and 1 h of dosing. Due to the fact that the SPME sampling and the conventional blood withdrawal sampling were conducted in two different groups of animals and due to inter-animal variability, it is inappropriate to employ statistical methods to compare the data obtained from SPME and whole blood analysis. However TK data generated from such small group sizes over many years in GSK laboratories for multiple NCEs suggest that TK data can vary by up to two-fold and still be considered comparable[24]. Hence, the TK parameters generated using SPME with the < 1.7 fold variability in data compared with data generated using the conventional sampling technique (CV) demonstrate that they are within the limits of physiological variability[24]. To conclude whether SPME and CV sampling generate equivalent TK data, several statistically powered studies would be required. This could potentially be generated over the years when SPME is applied to numerous TK studies as was the case with other techniques such as DBS.

Figure -6.2- shows the level of data variability between Groups 3 and 4 at each timepoint on days 1 and 7. The level of variability as illustrated by the box plots for Group 4 seems to be higher than Group 3. This suggests that the data generated using SPME is comparable to the data generated using CV sampling with similar or lower levels of variability. Figure -6.3- show clearly the comparison between the data of both groups. The range of AUC_{0-t} and C_{max} values from Group 3 (corrected) on day 1 correlated to the range of AUC_{0-t} and C_{max} values of Group 4. However, the TK parameters of Group 3 (corrected) seem to be lower on day 7 than the TK parameters for Group 4. This difference could be due to inter-animal variability as well as analytical extraction variability between the two bioanalytical techniques. Higher levels of variability were also observed for both AUC_{0-t} and C_{max} values within Group 4 when compared to variability within Group 3 (corrected) as shown in Figure-6.3-. This observation potentially suggests that the variability of the SPME sampling technique is lower than the standard conventional (CV) sampling approach.

Metoprolol exposure profiles AUC_{0-t} , C_{max} and T_{max} values generated using both SPME and CV sampling techniques compare favourably with published data by Nandi *et al*[180] and Yoon *et al*[157].

This study revealed the feasibility of obtaining six timepoints from each animal using the SPME procedure without the requirement for blood withdrawal. This in turn suggests that SPME could be utilized to generate TK data from main study animals without the need to use separate satellite groups. According to the Home Office Guidelines[183] for use of animals in research, the number of needle sticks permitted per timepoint can be up to 3 attempts and between 6 to 7 timepoints can be taken over the duration of 24 h[183,184]. This implies that there is scope for using SPME to take additional samples for either more TK timepoints or samples that can be used for other endpoints such as PD or biomarker investigations.

Overall, the data demonstrated that SPME is a technique that can be used to measure unbound free drug concentrations and in turn enable evaluation of toxicokinetic parameters. These findings are consistent with data obtained by Lord *et al*[61] who conducted the first *in vivo* SPME study in dogs within an academic set up. The study utilized polypyrrole custom made SPME fibres to determine diazepam and its metabolite concentration in dog. This chapter has built on this work and highlighted the suitability of *in vivo* SPME for PK/TK evaluation within pharmaceutical toxicology studies without the need for separate satellite groups.

Figure -6.2- Comparison between the corrected total concentrations of metoprolol determined by SPME for Group 3 and CV samples for Group 4 determined by CV sampling at each timepoint for days 1 and 7. Box plots consisting of the interquartile range with the median displayed by the bold line within each box. Outliers defined by the black dots represent the values which are outside the distribution range.

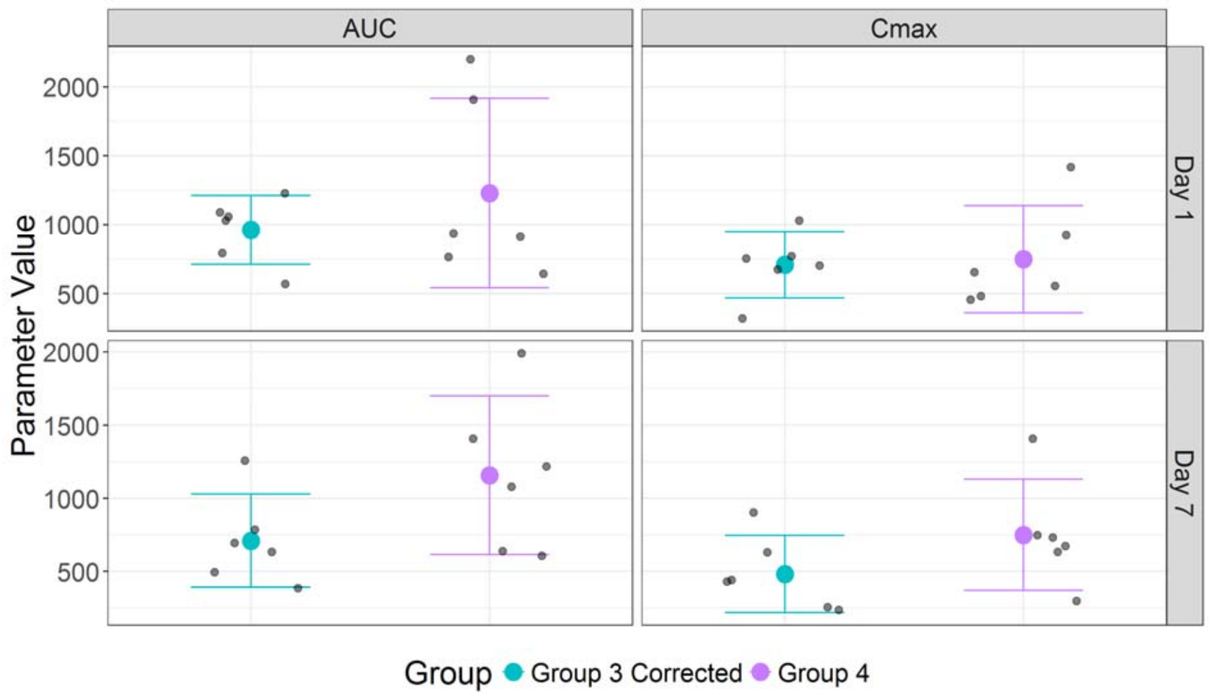


Figure -6.3- Summary TK data, mean and 95% confidence levels. Comparison of TK parameters between the corrected total concentrations determined by SPME for Group 3 and parameters for Group 4 determined by CV sampling for days 1 and 7.

6.3.2 Clinical Observations

There were no procedure related clinical observations noted. These include cage-side observations such as general health and mortality, no rats died throughout the duration of this study. There were no prominent changes in body weight or food consumption for any of the four groups. This indicated that both SPME and CV sampling had no adverse effects on the overall clinical wellbeing of the animals.

6.3.3 Clinical Pathology Measurements

Summary data of haematology parameters are presented in Figures -6.4- to -6.7-. Haematology samples from control male 6 (vehicle dosed/No sampling) and dosed male 13 and 18 (60 mg/kg/day – SPME) were clotted therefore they were considered not suitable for analysis.

Group 4 animals, given 60 mg/kg/day and sampled using CV showed a significant reduction in haemoglobin, haematocrit concentration and red blood cell count as shown in Figure -6.4-. This suggests red blood cell mass loss, reduced production or increased blood cell removal. An increased reticulocyte count and reticulocyte subpopulation was also demonstrated in Figure -6.5-, indicating a response from the bone marrow to blood loss. This compensatory increase in the low, medium and high reticulocytes of Group 4 is a clear indication of body response to compensate for the blood loss caused by the CV sampling procedure. All other parameters for all groups were within normal levels[185] and within the range of analytical variability. In contrast, Groups 2 and 3 which were SPME sampled showed similar haematology patterns to the control Group 1 i.e. all within normal levels[186], suggesting no effect of SPME procedure on haematology parameters.

The impact of losing approximately 15-20% of the total blood volume by CV sampling causes a substantial cholinergic release with intense arteriolar constriction. This is a known potential consequence of blood loss and is accompanied with metabolic acidosis due to anaerobic glycolysis and oxygen shortage[187]. Such changes may have undesirable effects on the outcomes of pre-clinical toxicology studies and may give false interpretations of the impact of drugs on animal's physiological and behavioural changes[188].

Although SPME has not been used in a toxicology study where the haematological impact has been investigated, similar studies in literature have shown that the effects of other microsampling techniques on haematological parameters were similar to the outcomes of this study[189]. For example Powles-Glover *et al*[20] showed that conventional sampling volumes showed a significant decrease in haemoglobin, haematocrit and red blood cell count compared with microsampled (6 x 32 µL) animals which showed a slight decrease in haemoglobin concentration relative to the control group. Even though other microsampling techniques have less impact on haematology parameters compared to conventional techniques, they still do have slight effects compared to SPME which showed no changes when compared to the control group.

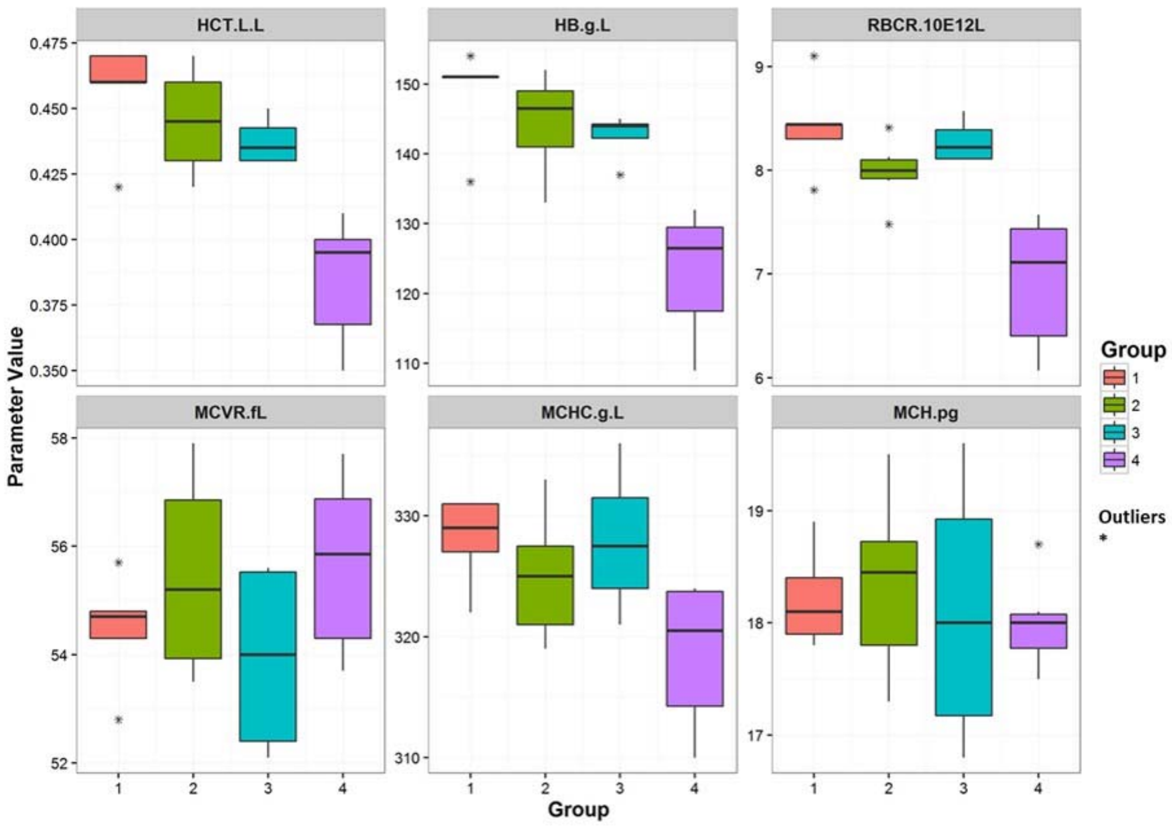


Figure -6.4- Haematology parameters comparing Groups 1-4. Box plots consisting of the interquartile range with the median displayed by the bold line within each box. Outliers define the values which are outside the distribution range. Group 1 (Control), Group 2 (Vehicle/SPME), Group 3 (Dosed/SPME) and Group 4 (Dosed/CV) Abbreviations described in Section 6.2.8.

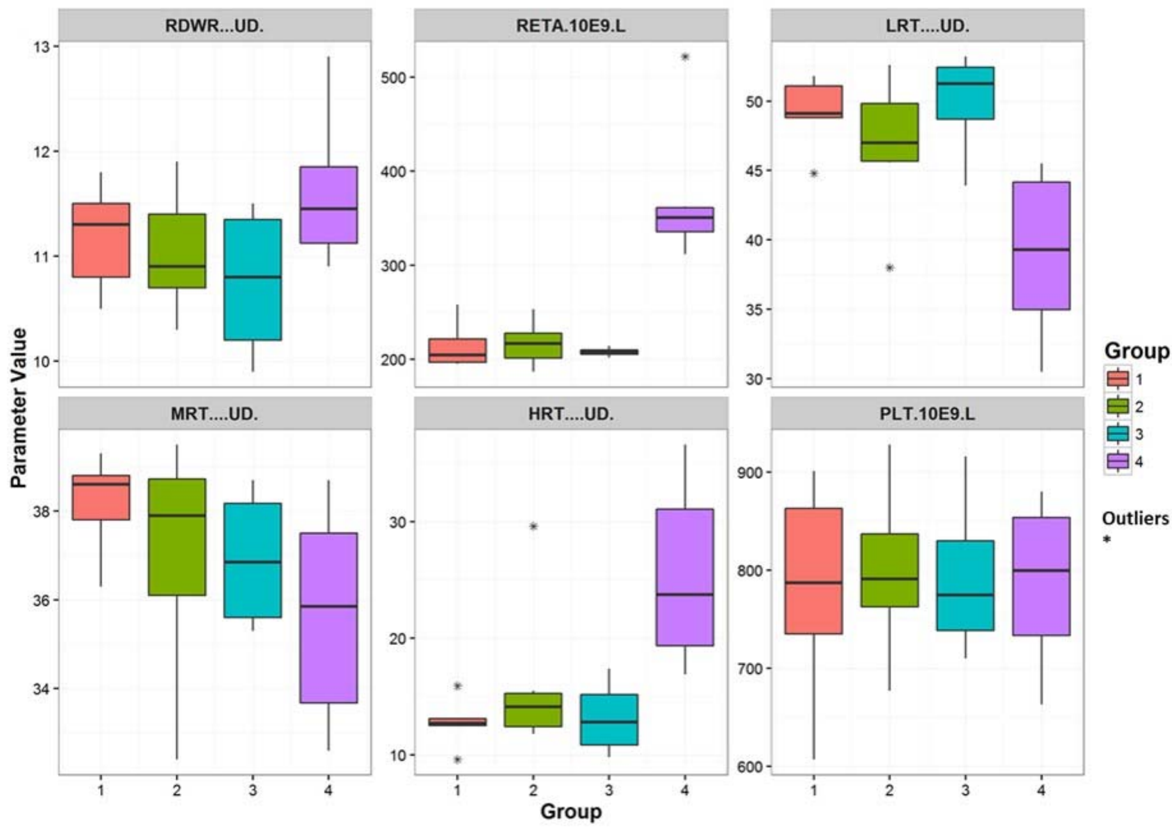


Figure -6.5- Haematology parameters comparing Groups 1-4. Box plots consisting of the interquartile range with the median displayed by the bold line within each box. Outliers define the values which are outside the distribution range. Group 1 (Control), Group 2 (Vehicle/SPME), Group 3 (Dosed/SPME) and Group 4 (Dosed/CV) Abbreviations described in Section 6.2.8.

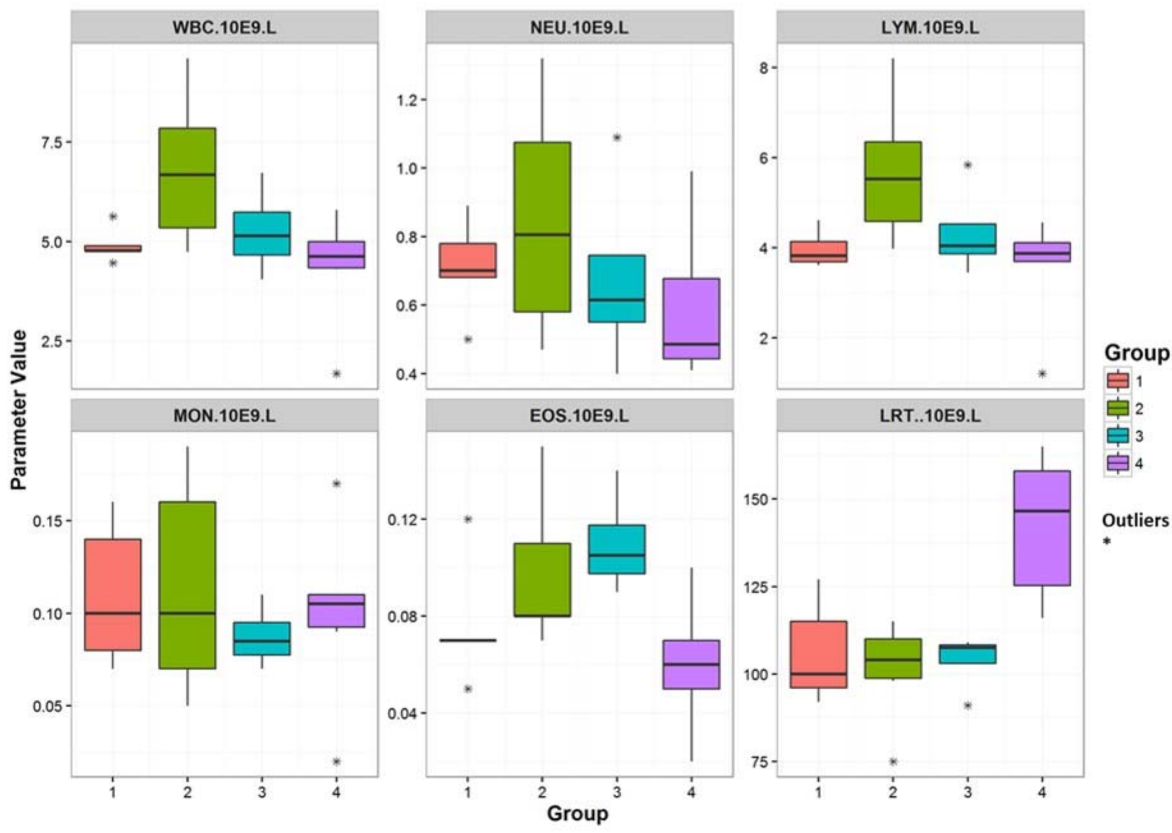


Figure -6.6- Haematology parameters comparing Groups 1-4. Box plots consisting of the interquartile range with the median displayed by the bold line within each box. Outliers define the values which are outside the distribution range. Group 1 (Control), Group 2 (Vehicle/SPME), Group 3 (Dosed/SPME) and Group 4 (Dosed/CV) Abbreviations described in Section 6.2.8.

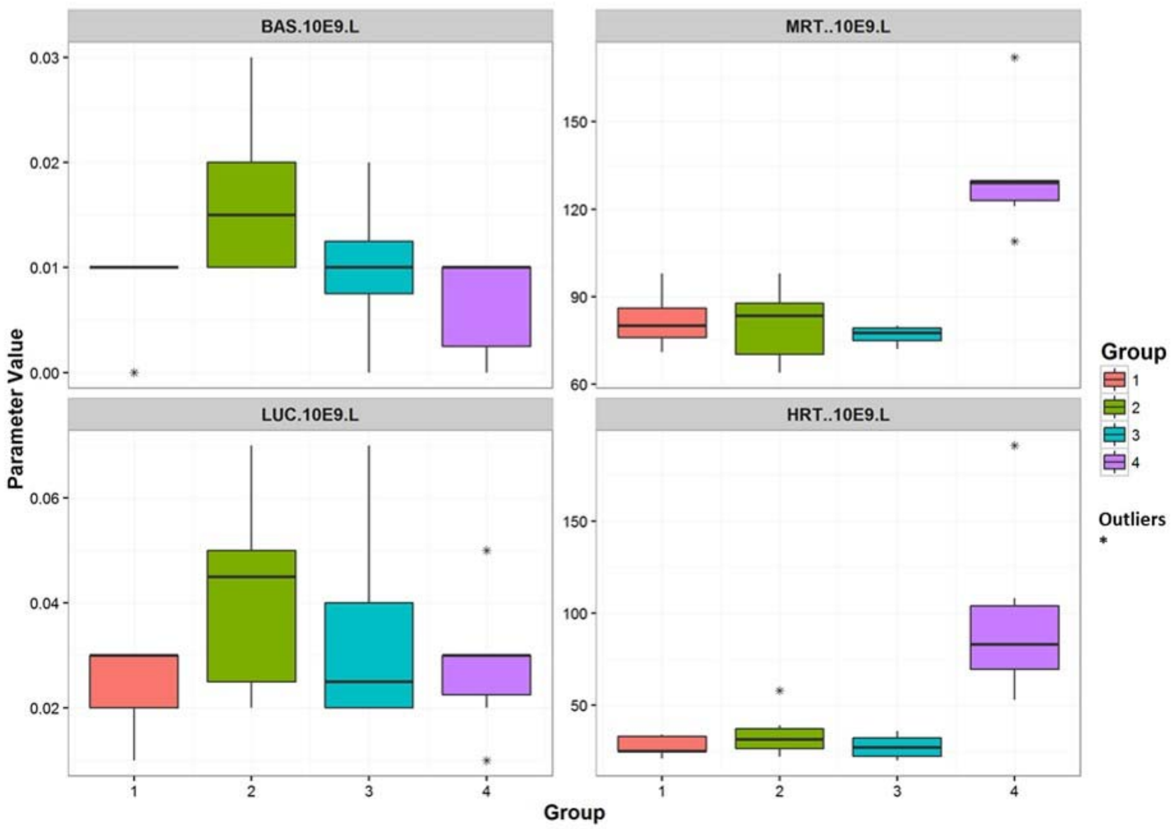


Figure -6.7- Haematology parameters comparing Groups 1-4. Box plots consisting of the interquartile range with the median displayed by the bold line within each box. Outliers define the values which are outside the distribution range. Group 1 (Control), Group 2 (Vehicle/SPME), Group 3 (Dosed/SPME) and Group 4 (Dosed/CV) Abbreviations described in Section 6.2.8.

The clinical chemistry and coagulation data as well as the cardiac output for all four groups were compared in Figures -6.8- to -6.11-. The significant changes and differences were summarized in Table -6.7-. All other parameters presented in Figures -6.8- to -6.11- measured were neither of significance nor differing from control values and are not discussed further. No differences were observed between control Group 1 and Group 2 however animal number 10 (vehicle/SPME) demonstrated a high plasma GLDH activity (40.1 U/L) and a high WBC, LYM count (9.60 and 8.20 x 10^9/L respectively). These values fell outside normal biological range[185,190]. This could be attributed to inter-animal variability as 1 out of 6

animals is not significant enough to be procedure related or associated with the test article because Group 2 were dosed vehicle only. R.Hall[171] commented on inter-animal variability and outliers which may be caused by genetic defects or other biological pathway interferences that may lead to such outliers.

The data outlined in Table -6.7-, showed that there were no differences in clinical chemistry data between Group 3 (SPME/dosed) and Group 1 (control) which is highlighted in grey to illustrate no difference in values. This further confirms that the SPME procedure had no impact on animals in terms of changes in clinical chemistry data. Whereas differences were observed between Group 4 (CV/dosed) and Group 1 (control) showing that conventional sampling did indeed affect clinical chemistry parameters.

Also, the difference between Groups 4 (dosed/CV) and 3 (dosed/SPME) shown in Table -6.7- is similar to the difference in parameters between Groups 4 (dosed/CV) and 1 (control/no sampling). This suggests that the SPME sampled group is behaving in a similar manner to the control/not sampled group i.e. no noteworthy changes were observed in the clinical chemistry data for the SPME sampled groups. These findings concur with similar findings by Niu *et al*[189] which reported no impact of capillary microsampling on biochemical endpoints.

In summary, the above observations showed the potential advantage of using SPME sampling to generate TK data from main study animals without impacting clinical chemistry or haematology endpoints during a toxicity study. Conversely, conventional sampling did have a clear impact on those parameters and may cause false interpretations of drug effects, which further confirms the reason for the current practice of using separate animal groups for TK analysis within the pharmaceutical industry when applying conventional sampling techniques.

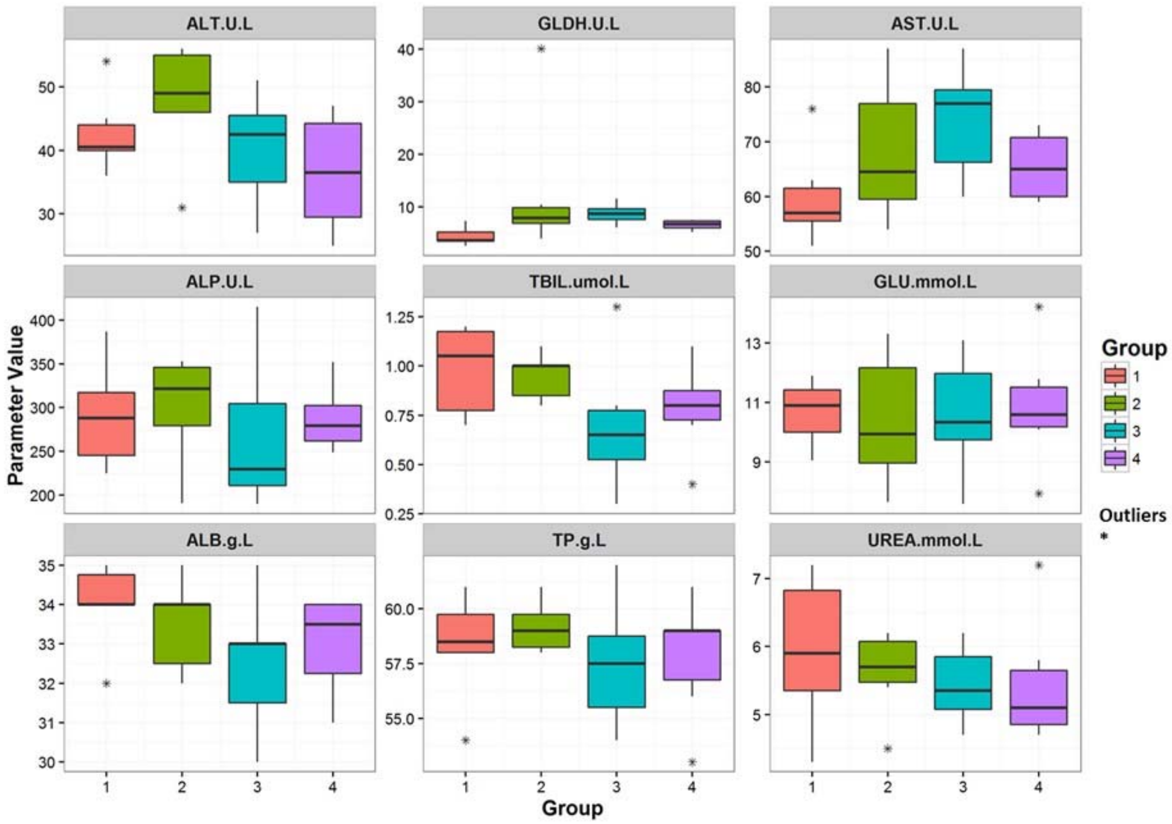


Figure -6.8- Clinical chemistry parameters comparing Groups 1-4. Box plots consisting of the interquartile range with the median displayed by the bold line within each box. Outliers define the values which are outside the distribution range. Group 1 (Control), Group 2 (Vehicle/SPME), Group 3 (Dosed/SPME) and Group 4 (Dosed/CV) Abbreviations described in Section 6.2.8.

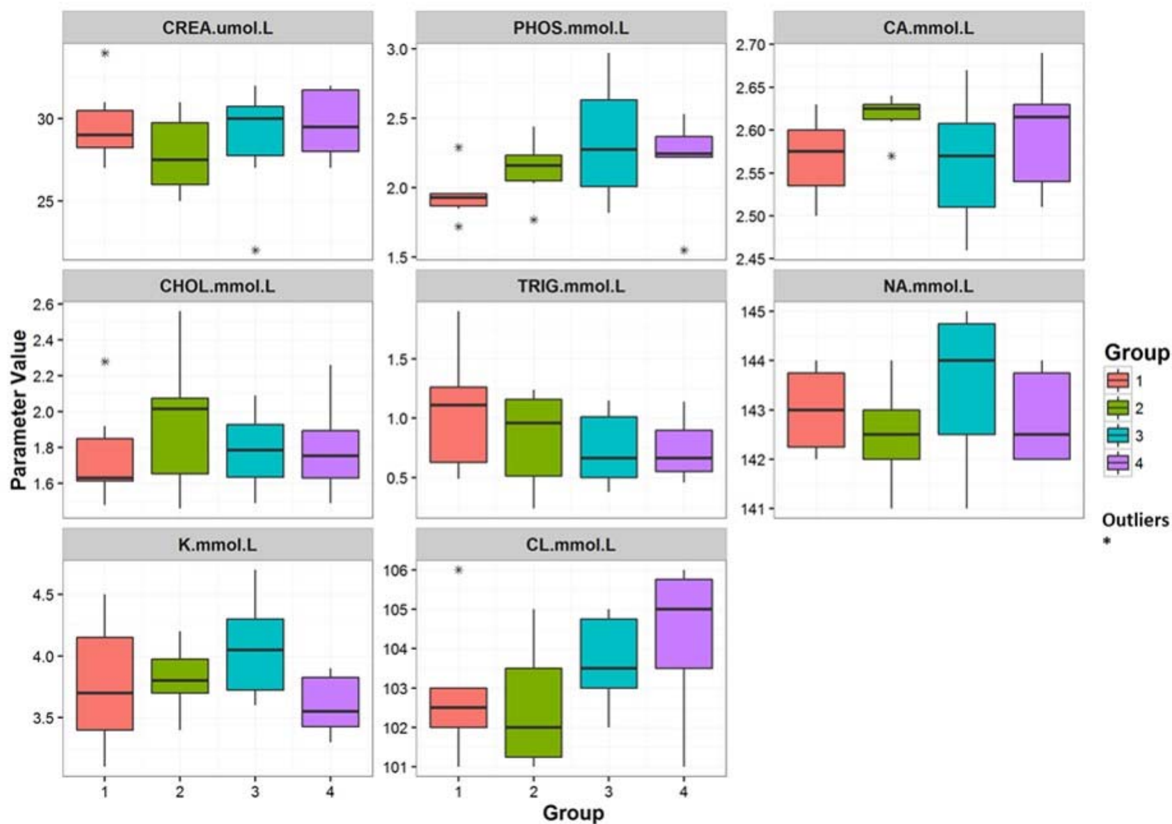


Figure -6.9- Clinical chemistry parameters comparing Groups 1-4. Box plots consisting of the interquartile range with the median displayed by the bold line within each box. Outliers define the values which are outside the distribution range. Group 1 (Control), Group 2 (Vehicle/SPME), Group 3 (Dosed/SPME) and Group 4 (Dosed/CV) Abbreviations described in Section 6.2.8.

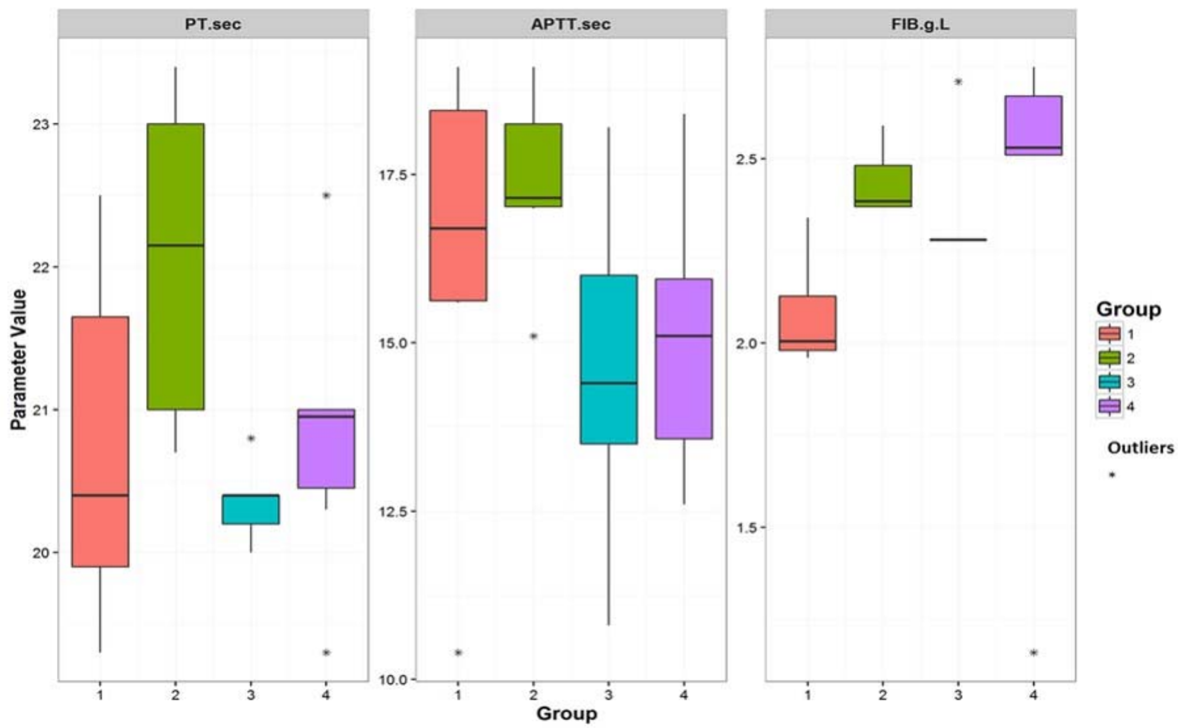


Figure -6.10- Coagulation parameters comparing Groups 1-4. Box plots consisting of the interquartile range with the median displayed by the bold line within each box. Outliers define the values which are outside the distribution range. Group 1 (Control), Group 2 (Vehicle/SPME), Group 3 (Dosed/SPME) and Group 4 (Dosed/CV) Abbreviations described in Section 6.2.8.

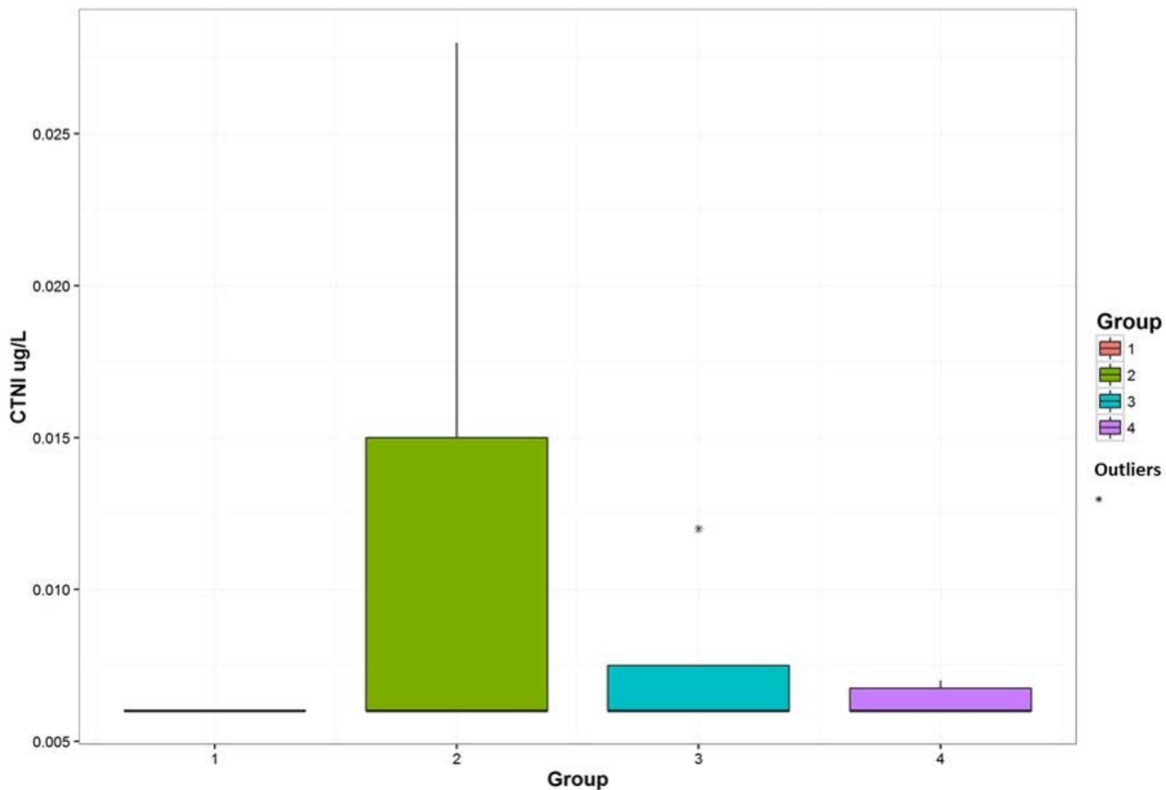


Figure -6.11- Cardiac output comparing Groups 1-4. Box plots consisting of the interquartile range with the median displayed by the bold line within each box. Outliers define the values which are outside the distribution range. Group 1 (Control), Group 2 (Vehicle/SPME), Group 3 (Dosed/SPME) and Group 4 (Dosed/CV) Abbreviations described in Section 6.2.8.

Table -6.7- Between group differences in haematology and clinical chemistry parameters. Abbreviations described in Section 6.2.8.

	Male Rats*			
Group	3		4	
Parameter	60 (SPME)		60 (CV)	
	[X] Group 1	[X] Group 2	[X] Group 3	[X] Group 1
ALB	•	**0.98 (2/6)**	•	•
PHOS	•	**1.09 (2/6)**	•	•
HCT	•	•	**0.89**	**0.85**
HB	•	•	**0.86**	**0.83**
MCHC	•	•	**0.97 (2/6)**	**0.97 (2/6)**
RETA	•	•	**1.79**	**1.73**
LRT#	•	•	**1.37 (4/6)**	**1.35 (4/6)**
MRT#	•	•	**1.72**	**1.60**
HRT#	•	•	**3.6**	**3.6**
RBCR	•	•	**0.84**	**0.82**
NEU	•	•	**0.87 (4/6)**	**0.83 (4/6)**

- No difference

*Differences were calculated by dividing the mean of each group by the mean of the opposing group and then observing the number of animals within each group which is outside the range of the control group for each parameter.

The haematology and clinical chemistry results supported the histology findings of the spleen. The spleen is the site of direct and indirect toxicity, a target for some carcinogens, and also a site for metastatic neoplasia and a primary site of extramedullary haematopoiesis[174]. In the spleen, mild or moderate extramedullary haematopoiesis was seen in all the animals in Groups 1, 2 and 3. Haematopoiesis refers to the formation of blood cellular components[174]. Animals given 60 mg/kg/day metoprolol and subjected to conventional caudal vein blood sampling suffered from severe extramedullary haematopoiesis. This change was associated with a reduction in haemoglobin, haematocrit concentration, red blood cell count and increased reticulocyte count and reticulocyte subpopulation. Figure -6.12- shows the histopathology of spleen samples from Groups 1,3 and 4. Minimal extramedullary haematopoiesis was seen in control animals of Group 1 that were not sampled by either technique. This is indicated by the purple spots of the images in Figure 6.12-; the first image shows a minimal number of purple spots corresponding to minimal production of blood cellular components in Group 1 rats. While the second image shows an increase in the number of purple spots signifying a moderate level of blood cell

production. The last image in Figure -6.12- shows a rise in the number of purple spots suggesting a high level of extramedullary haematopoiesis. The increased incidence and severity of the extramedullary haematopoiesis and the associated changes in haematological parameters with conventional sampling method was considered to be secondary to the blood loss with homeostatic compensation by increasing the level of blood cell production.

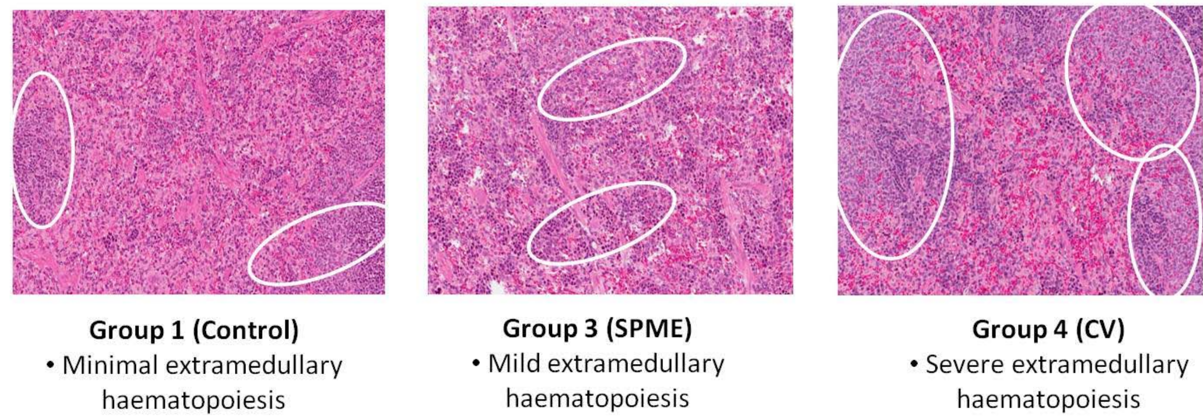


Figure -6.12- Representative images of the spleen histology slides from Groups 1, 3 and 4 showing varying levels of extramedullary haematopoiesis. The dark purple dots, marked with a white circle indicate areas of blood cell production (haematopoiesis).

Tail histology was performed by taking four sections from the top to the bottom of the tail for all animals as shown in Figure -6.13- to ensure full examination of the sampling sites. In the blood sampling sites in the tail, there were no differences in inflammatory reaction between the groups that were subjected to the conventional or the SPME sampling techniques. Minimal or mild inflammatory reaction, characterised by perivascular haemorrhage and/or inflammatory cell infiltrate and/or vascular wall necrosis, was seen in all the animals that were sampled by the conventional or the novel SPME technique. These changes were considered to be the consequence of soft tissue trauma caused by the venepuncture technique i.e. needle insertion into the blood vessel. No similar changes were seen in the animals of Group 1 that were not sampled. Figure -6.14- shows histology images which reveal differences between the injection sites in the tail from Groups 3 and 4 as well as an example

image of the control tail where no sampling occurred. Because of the relatively large amount of blood taken during conventional sampling, the wound needs to be larger and deeper to yield sufficient quantities of blood, hence the source of severe haemorrhage and inflammation that was observed for Group 4.

These histopathological findings highlight the impact of conventional blood withdrawal sampling procedures on sampling sites compared to the SPME technique. Although both of the SPME and CV procedures are invasive and cause inflammatory response upon needle insertion into the tail, it is apparent that blood withdrawal can cause additional damage to the vessels which was accompanied by severe haemorrhage compared to the no blood withdrawal method of SPME.

There were no noteworthy organ weight changes in all animals or any other histological findings in the liver or lung associated with either of the blood sampling techniques.

Figure -6.13- Representative histology slide (control male animal 1) showing the general tail sectioning for microscopic examination, lateral sectioning starting from the top (1) to the bottom (4) of the tail. This type of sectioning was applied to all animal groups.

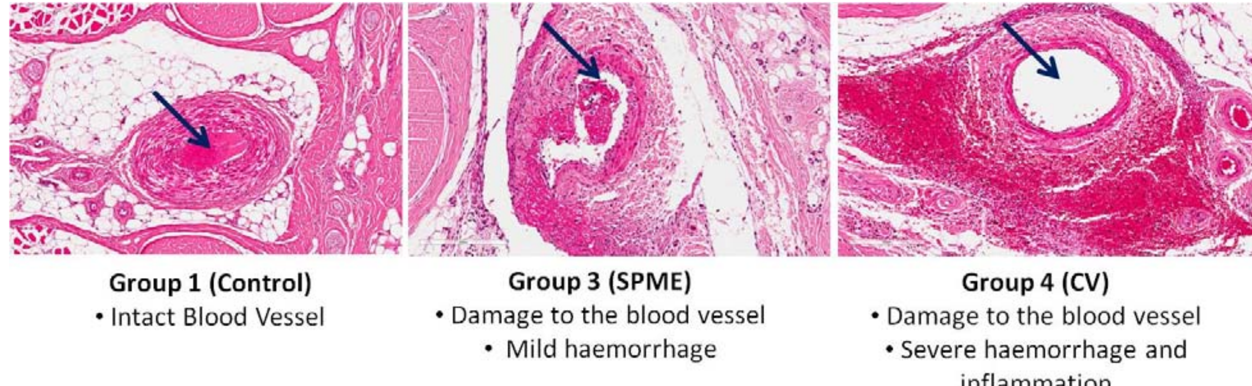


Figure -6.14- Examples of tail histology slides at the sampling site from Groups 3 and 4 showing the effect of sampling on the blood vessel. Group 1 was not sampled but tail histology was taken for comparison with sampled groups. Black arrows indicate the site of blood vessel and the red marks surrounding that area illustrate haemorrhage.

6.3.4 Neurobehavioral Assessment

The Irwin test which consists of a battery of general behavioural and psychological observations in the rodent has been utilized for many years to study the effect of NCEs on central nervous system activity and neurological function in rodents. This was later adopted to investigate the impact of new sampling procedures on the behavioural and psychological functions of rodents[191,192]. For this reason, this test was incorporated into the toxicity study to assess the impact of SPME and conventional sampling on neurobehavioral endpoints.

As part of the neurobehavioral observation assessment, aggression and irritability was noted in 2 out of 6 rats following treatment of 60 mg/kg metoprolol and sampled by the conventional caudal venepuncture on day 7. These observations could be attributed to the noise generated by the study conduct and not treatment or sampling related as this was not consistent across all sampled groups. All neurobehavioral data are shown in Tables -6.8- to -6.10-, there were no SPME related effects on the behaviour of the animals.

Occasional differences were noted in positional passivity and grooming in several animals including in the vehicle no sampling group (Group 1), these changes were not considered

treatment or procedure related as they observed in the control group. Rearing was reduced in all groups over time and is considered to be related to habituation to the open field arena. Physiological parameters such as urination and defecation were variable during all observations. Pupil diameter was not assessed due to a failure of the ophthalmoscope.

Although the above observations and the results of this test indicate that there were no procedure related changes and there were no behavioural or psychological differences between the conventional and the SPME sampling techniques, the Irwin test may not have been sensitive enough to assess the effects of sampling procedures. The Irwin observation test is commonly used to evaluate the effects of a new compound to determine potential neurotoxicity or impact of new drugs on a specific psychological function[193]. The relevance of this test to examine the effect of sampling procedures remains debatable. The interpretation of behavioural tests such as those conducted within the Irwin study, is rarely obvious. For example, grooming or body movements might fail to reflect pain or other sampling effects. Other tests such as the use of micro-sensors that can detect behavioural changes or video recordings that monitor all animal throughout the duration of the study may reveal better understanding of the impact of sampling on rodent behaviour[194].

Table -6.8- Effects on neurobehavioral functions of male rats on day -2 prior to study start

Observation		Day -2 (pre-treatment) Treatment Groups: Group 1 (Control)	Group 2 (Vehicle/SPME sampling)	Group 3 (Dosed/SPME sampling)	Group 4 (Dosed/CV Sampling)
Awareness	Arousal	● green	● green	● green	● green
	Finger Approach	● green	● green	● green	● green
	Head Touch	● green	● green	● green	● green
	Positional Passivity	● red	● red	● green	● green
	Visual Placing	● green	● green	● green	● green
	Catalepsy	● green	● green	● green	● green
Mood	Fear	● green	● green	● green	● green
	Grooming	● yellow	● green	● green	● green
	Aggressiveness/ Irritability	● green	● green	● green	● green
	Vocalisation	● green	● green	● green	● green
Motor Activity	Body Position	● green	● green	● green	● green
	Spontanous Locomotor Activity	● green	● green	● green	● green
	Rearing	● red	● red	● green	● red
	Ataxic Gait	● green	● green	● green	● green
Central Excitation	Twitches	● green	● green	● green	● green
	Convulsions	● green	● green	● green	● green
	Trembling	● green	● green	● green	● green
	Startle Response	● green	● green	● green	● green
	Sensitivity to pinching of the tail	● green	● green	● green	● green
Muscle Tone	Hypotonic Gait	● green	● green	● green	● green
	Grip Strength	● green	● green	● green	● green
	Body Tone	● green	● green	● green	● green
	Abdominal Tone	● green	● green	● green	● green
	Limb Tone	● green	● green	● green	● green
Reflexes	Corneal Reflex	● green	● green	● green	● green
	Pinna Reflex	● green	● green	● green	● green
	Hind Limb Reflex	● green	● green	● green	● green
	Righting Reflex	● green	● green	● green	● green
Autonomic Profile	Tail Position	● green	● green	● green	● green
	Piloerection	● green	● green	● green	● green
	Eyes Opening	● green	● green	● green	● green
	Exopthalmos	● green	● green	● green	● green
	Lachrymation	● green	● green	● green	● green
	Chromodacryorrhea	● green	● green	● green	● green
	Salivation	● green	● green	● green	● green
	Faeces Consistency	● green	● green	● green	● green
	Urine colour	● green	● green	● green	● green
	Death	● green	● green	● green	● green

● green **Within Normal Range**
● yellow **Moderate Activity**
● red **Extreme Activity**

Table -6.9- Effects on neurobehavioral functions of male rats on day 1 of study start

Observation		Day 1 – Treatment Groups: Group 1 (Control)	Group 2 (Vehicle/SPME sampling)	Group 3 (Dosed/SPME sampling)	Group 4 (Dosed/CV Sampling)
Awareness	Arousal	🟢	🟢	🟢	🟢
	Finger Approach	🟢	🟢	🟢	🟢
	Head Touch	🟢	🟢	🟢	🟢
	Positional Passivity	🔴	🔴	🟢	🔴
	Visual Placing	🟢	🟢	🟢	🟢
	Catalepsy	🟢	🟢	🟢	🟢
Mood	Fear	🟢	🟢	🟢	🟢
	Grooming	🟢	🟢	🟢	🟢
	Aggressiveness/ Irritability	🟢	🟢	🟢	🟢
	Vocalisation	🟢	🟢	🟡	🟢
Motor Activity	Body Position	🟢	🟢	🟢	🟢
	Spontanous Locomotor Activity	🟢	🟢	🟢	🟢
	Rearing	🔴	🟡	🟡	🟡
	Ataxic Gait	🟢	🟢	🟢	🟢
Central Excitation	Twitches	🟢	🟢	🟢	🟢
	Convulsions	🟢	🟢	🟢	🟢
	Trembling	🟢	🟢	🟢	🟢
	Startle Response	🟢	🟢	🟢	🟢
	Sensitivity to pinching of the tail	🟢	🟢	🟢	🟢
Muscle Tone	Hypotonic Gait	🟢	🟢	🟢	🟢
	Grip Strength	🟢	🟢	🟢	🟢
	Body Tone	🟢	🟢	🟢	🟢
	Abdominal Tone	🟢	🟢	🟢	🟢
	Limb Tone	🟢	🟢	🟢	🟢
Reflexes	Corneal Reflex	🟢	🟢	🟢	🟢
	Pinna Reflex	🟢	🟢	🟢	🟢
	Hind Limb Reflex	🟢	🟢	🟢	🟢
	Righting Reflex	🟢	🟢	🟢	🟢
Autonomic Profile	Tail Position	🟢	🟢	🟢	🟢
	Piloerection	🟢	🟢	🟢	🟢
	Eyes Opening	🟢	🟢	🟢	🟢
	Exopthalmos	🟢	🟢	🟢	🟢
	Lachrymation	🟢	🟢	🟢	🟢
	Chromodacryorrhea	🟢	🟢	🟢	🟢
	Salivation	🟢	🟢	🟢	🟢
	Faeces Consistency	🟢	🟢	🟢	🟢
	Urine colour	🟢	🟢	🟢	🟢
	Death	🟢	🟢	🟢	🟢

🟢 **Within Normal Range**
🟡 **Moderate Activity**
🔴 **Extreme Activity**

Table -6.10- Effects on neurobehavioral functions of male rats on day 7 of study start

Observation		Day 7 – Treatment Groups: Group 1 (Control)	Group 2 (Vehicle/SPME sampling)	Group 3 (Dosed/SPME sampling)	Group 4 (Dosed/CV Sampling)
Awareness	Arousal	🟢	🟢	🟢	🟢
	Finger Approach	🟢	🟢	🟢	🟢
	Head Touch	🟢	🟢	🟢	🟢
	Positional Passivity	🟢	🟢	🟢	🟢
	Visual Placing	🟢	🟢	🟢	🟢
	Catalepsy	🟢	🟢	🟢	🟢
Mood	Fear	🟢	🟢	🟢	🟢
	Grooming	🟢	🟡	🟢	🟢
	Aggressiveness/ Irritability	🟢	🟢	🟢	🔴
	Vocalisation	🟢	🟢	🟢	🟢
Motor Activity	Body Position	🟢	🟢	🟢	🟢
	Spontanous Locomotor Activity	🟢	🟢	🟢	🟢
	Rearing	🟡	🟡	🟡	🟡
	Ataxic Gait	🟢	🟢	🟢	🟢
Central Excitation	Twitches	🟢	🟢	🟢	🟢
	Convulsions	🟢	🟢	🟢	🟢
	Trembling	🟢	🟢	🟢	🟢
	Startle Response	🟢	🟢	🟢	🟢
	Sensitivity to pinching of the tail	🟢	🟢	🟢	🟢
Muscle Tone	Hypotonic Gait	🟢	🟢	🟢	🟢
	Grip Strength	🟢	🟢	🟢	🟢
	Body Tone	🟢	🟢	🟢	🟢
	Abdominal Tone	🟢	🟢	🟢	🟢
	Limb Tone	🟢	🟢	🟢	🟢
Reflexes	Corneal Reflex	🟢	🟢	🟢	🟢
	Pinna Reflex	🟢	🟢	🟢	🟢
	Hind Limb Reflex	🟢	🟢	🟢	🟢
	Righting Reflex	🟢	🟢	🟢	🟢
Autonomic Profile	Tail Position	🟢	🟢	🟢	🟢
	Piloerection	🟢	🟢	🟢	🟢
	Eyes Opening	🟢	🟢	🟢	🟢
	Exopthalmos	🟢	🟢	🟢	🟢
	Lachrymation	🟢	🟢	🟢	🟢
	Chromodacryorrhea	🟢	🟢	🟢	🟢
	Salivation	🟢	🟢	🟢	🟢
	Faeces Consistency	🟢	🟢	🟢	🟢
	Urine colour	🟢	🟢	🟢	🟢
	Death	🟢	🟢	🟢	🟢

🟢 **Within Normal Range**
🟡 **Moderate Activity**
🔴 **Extreme Activity**

6.3.5 Feedback from Laboratory Animal Technicians

Six laboratory animal technicians were trained on the use of SPME, some of whom were exposed to the technique prior to the start of the study and others received minimal training throughout the study conduct. SPME is a novel technique that has not been utilized within toxicology studies in the pharmaceutical industry. The use of the technique is different to the conventional procedures that the technicians had been using. Although the SPME needle is similar to a butterfly needle applied for CV sampling, advancing the actual SPME fibre into the vein of the living organism remains a task that requires training and skills. Despite this, minimal training was given during the study and all six technicians agreed that the prototype SPME device was simple to use and that the fibre was easy to insert and to adjust into the rat tail vein. Five out of the six technicians found no difference between using the SPME device compared with the conventional butterfly needle. Suggestions for improvements were given with regards to reducing the time of fibre exposure to the systemic circulation within the vein from 2 min to 1 min or less though leaving the fibre for 2 min did not impact the behavior of the animals during or after sampling. Other comments included no blood loss during SPME sampling compared with other microsampling and conventional techniques. Overall, positive feedback was received from all six technicians regarding the use of SPME, throughout the conduct of the study and during the after study review meeting. These comments agreed with previous positive feedback received from technicians throughout the conduct of the first *in vivo* study which was performed in Chapter 5.

6.4 Conclusion

A seven day toxicology study was conducted to evaluate the application of *in vivo* SPME sampling and extraction in rodents. The SPME technique was also compared to conventional sampling procedures within this study. This is the first study where *in vivo* SPME has been applied within a preclinical pharmaceutical setting; the technique has shown powerful potential for future use as a microsampling tool that could be applied in upcoming preclinical and clinical studies. The device proved its strength in terms of enabling repeat sampling without blood withdrawal and minimal blood loss, its biocompatibility was established with no impact on toxicology endpoints and no observed adverse effects. Meaningful PK profiles and TK parameters were obtained via a simplified sampling method (SPME) which enabled monitoring of free unbound drug concentration. No difference in inflammatory reaction at the blood sampling site (tail) was observed between the novel SPME microsampling and the conventional tail vein blood sampling technique. Whereas in the spleen, an observable difference in extramedullary haematopoiesis was seen in all the animals given 60 mg/kg/day metoprolol and subjected to conventional caudal vein blood sampling compared to SPME.

SPME was, therefore, demonstrated to be a promising microsampling technique that could be applied to toxicology studies to determine TK in the main study animals. It could also be applied in juvenile preclinical studies where rodents are too small to undergo blood withdrawal procedures without sacrificing a large number of neonates. Despite the design of the probe still being at a relatively early stage of development, the prototype device was easy to use as described by laboratory animal technicians. Further modification to the mechanics of the *in vivo* device will facilitate simpler workflow allowing more convenient SPME sampling.

Overall, this study showed that SPME is pathologically benign and that its adoption as a microsampling tool could be a beneficial, more viable and ethical form of blood sampling based on haematology, clinical chemistry and coagulation data in conjunction with procedure-related microscopic changes seen in the spleen and tail sampling sites and derived toxicokinetics.

Chapter 7

Direct Ambient SPME-MS for Quantitative Analysis of Drugs

7.1 Introduction

The quantitative analysis of complex samples consists of several steps, each of which is essential for obtaining accurate and informative results for the analyte of interest. Previous chapters have shown the suitability of SPME for bioanalytical analysis and for direct sampling from rodents in PK/TK studies as described in Chapter 6. Perhaps, the two key bottleneck procedures in bioanalysis are still sample clean-up and chromatographic separation[195], both of which are considered time consuming as well as labour and cost intensive. Ideally, techniques that can eliminate the laborious manual extraction procedures associated with sample clean-up and the analytical complexity of chromatography separation will significantly simplify the entire bioanalytical procedure. For this reason, enormous efforts have been made in the past to simplify these processes through various approaches including attempts to reduce extraction complexity by employing automated liquid handlers[196], minimise cost and increase throughput by utilizing high speed separation techniques and introducing online direct analysis[197].

The term "direct analysis" is used to describe techniques that eliminate the need for separation or enable direct ionization off the sample surface[197]. This means direct introduction of complex samples into the mass spectrometer at ambient temperature and atmospheric pressure, which provides a powerful tool for rapid analysis without sample preparation. To date, direct ambient mass spectrometry has been applied for monitoring a variety of different molecules including pharmaceuticals, explosives, forensic and environmental samples[198]. Detection of these analytes has been performed from a broad range of matrices including biological samples at physiologically relevant concentrations[199], forensic samples such as ink aging analysis[200], food applications such as lipid content in milk[201] and pesticides in fruits as well as many other environmental matrices such as pesticides in water[202] and hydrogen peroxide in ambient air[203].

The second contemporary generation of mass spectrometers hyphenated with direct desorption/ionization techniques emerged in 2004[198]. Since then desorption electrospray ionization (DESI) and direct analysis in real time (DART) have set the benchmarks for this generation of direct MS approaches. Subsequently, they have led to a remarkable stream of rapidly expanding variants to support the analysis of myriads of analytes[106,204]. DESI which was introduced by Cooks and co-workers[205] is an ambient ionization technique, in which a high velocity pneumatically assisted electrospray jet, is continuously directed toward the probed surface. The jet forms a thin solvent film on the sample where rapid analyte extraction occurs. Secondary droplets are formed by the incoming jet, from which gas phase ions are created and ejected into the MS inlet for downstream detection. DESI mimics ESI in terms of ionization pathways but, by being one of the major ambient MS techniques, it exhibits added analytical capabilities.

DART on the other hand, introduced by Laramee and Cody[206], involves generation of a direct current or radiofrequency upon exposure to flowing helium or nitrogen gas. This in turn creates a stream of ionized molecules that are directed toward the sample to promote desorption of analytes. The desorbed discharge is then directed to the MS inlet to enable detection.

While qualitative analysis has been demonstrated by DESI and DART, to date the quantitative performance of many of these techniques has not been sufficient to enable general adoption of the technology for routine quantitative applications such as bioanalysis. The consistency of various analytical parameters such as robustness, reproducibility and sensitivity has been the main issue associated with direct analytical techniques. For example many direct desorption techniques do not match the sensitivity on offer from other traditional routes that involve manual sample extraction followed by chromatography separation[197]. For some applications this is not an issue such as metabolic profiling, biomarker identification, monitoring of food additives and many other qualitative processes. However, for quantitative analysis with regulatory restrictions, such barriers will have to be overcome before direct analysis techniques can be used to support such bioanalytical studies.

Despite this, a number of publications have demonstrated acceptable quantification of drugs in biofluids and tissues with paper spray ionization[207]. Some of which have had low enough or adequate variability that has been sufficient to meet regulatory bioanalytical criteria. Paperspray ionization involves analyte transport by wicking in a porous material with a

macroscopically sharp point, and a high electric field is used to perform ionization[105]. Multiple versions of paper spray ionization sources have been developed and utilised for various applications such as quantification of small molecule pharmaceuticals form dried blood spot samples[105,208,209]. This has also led to the development of other substrate spray techniques such as "Nib-Spray-MS" where bamboo nibs were used as sample emitters and have been applied for rapid sampling and screening of saliva samples[210].

Direct desorption techniques in general are still at a relatively early stage of development but a number of these techniques show huge potential analytical gains that can enable efficiency and simpler bioanalytical workflows. However, the challenges described above clearly show that sensitivity and robustness will have to be addressed prior to routine implementation for bioanalytical applications. Table 7.1 summarises some of these techniques and lists their primary advantages and disadvantages.

Table -7.1- Summary of some currently available direct analysis techniques and their key advantages and disadvantages

Direct Analysis Technique*	Key Advantages*	Disadvantages*
- Desorption Electrospray Ionization (DESI) - Direct Analysis in Real Time (DART) - Desorption Atmospheric Pressure Chemical Ionization (DAPCI) - Paperspray - Nib-Spray - Easy Ambient Sonic Electrospray Ionization (EASI) - Desorption Corona Beam Ionization (DCBI)	- Minimal sample preparation. - Sample maintenance under ambient conditions outside the vacuum system. - Rapid with high throughout analysis. - Gentle ionization methods. - Simplified overall workflow.	- Analyte detection depends largely on the matrix such as tissue, fruits, which in some cases results in lack of quantification. - Difficulty of modifying or switching between existing mass spectrometers and ambient mass spectrometers. - Lack of automation in some cases which has a negative impact on productivity and throughput. - Potential problems with labile metabolites converting back to parent (acyl glucuronides, N oxides, sulphates). - Potential matrix effects, leading to lower sensitivity.

*[106,198,210-214]

7.1.1 Direct SPME-MS

As highlighted above, the potential advantages on offer from direct desorption techniques, such as simpler bioanalytical workflows are significant enough to reshape the future of bioanalysis as well as other fields, where analytical instrumentation is utilized for non-regulated applications. The recent surge in interest in ambient spectrometry and direct elution[215] instigated the idea of combining two disparate but powerful techniques, direct SPME with direct MS. This combination allows for the merger of microsampling benefits with direct detection without the involvement of chromatographic separation. Currently SPME analysis requires offline analyte desorption from the fibre into a suitable solvent followed by LC-MS/MS analysis. The proposed idea, similarly to paperspray, involves direct desorption and ionization of the analyte directly off the SPME fibre itself into the MS inlet.

Direct SPME-MS is a novel ionisation method that could allow for both qualitative and quantitative analysis of pharmaceutical drugs from *in vivo* and *ex vivo* systems with minimal sample usage, without the need for sample preparation or separation. The process in which SPME samples are collected, minimizes the impact of other matrix components which are associated with wet whole blood or plasma samples. This means enhanced sample clean-up is achieved with SPME, therefore, interference with quantitative analysis of analytes is limited. This in turn allows for enhanced direct analysis despite lack of separation (chromatography).

Subsequent to analyte extraction by the SPME coating, the fibre is mounted in close proximity to the orifice of the MS and a solvent is applied to the fibre. A high voltage is then employed to induce electrospray ionization at the tip of the fibre, enabling the quantitative analysis of the analyte.

SPME and paper spray have multiple similarities in terms of the design characteristics where the spraying tip consists of a sharp tip, the spray solvent transports the analytes using a high voltage to create a high electric field between the device and mass spectrometer inlet. This type of analysis can potentially be applied to a wide range of analytes *in vivo* and may offer a simple and effective approach to the quantitative determination of circulating analyte concentrations, particularly where sample volumes are limited (e.g. rodent and paediatric studies)[88], or in situations where conventional wet blood / plasma sample collection and processing may be difficult.

This novel technique (SPME-MS) has the compelling advantages of direct *in vivo* extraction, monitoring unbound drug concentrations with minimal blood removal and the simplicity and speed of obtaining immediate results by direct MS analysis[61]. It would offer a significant impact when applied in the drug discovery arena for early phase drug screening and even for therapeutic drug monitoring, where the level of regulatory restrictions is somewhat less complicated than regulated drug development studies. Direct SPME-MS is an attractive technique for any field that requires a quick, easy, simple and cheap analysis, for example point-of-care diagnostics and forensic applications[88]. In this case, user friendly devices are desirable that are simple enough for non-clinicians as well as clinicians to perform sampling with accuracy and where samples can be safely shipped without the need for freezing and refrigerating.

7.1.2 Potential Challenges

Eliminating the need for chromatographic separation is the ultimate advantage of direct desorption. However, it is also one of the challenges that must be overcome if it is intended to be introduced to the regulated bioanalytical arena. GLP drug development studies require a high level of confidence in the accuracy of the analytical data with tight statistical outcomes[12]. Removing liquid chromatography can result in decreased sensitivity due to ion suppression and also cause reduced selectivity and in turn could reduce bioanalytical performance[216]. Therefore, particular attention must be paid to achieving adequate sensitivity and selectivity across a range of representative NCEs when using direct desorption techniques.

Control of contamination and carryover is also essential to ensure confidence in the analytical results. The level of analyte contamination in blank control samples should not exceed 20% of the analyte response at the lower limit of quantification (LLQ) as stated in the bioanalytical regulatory guidance[12]. Carryover should also not be large enough to significantly bias subsequent samples.

Carryover and cross contamination can be avoided using integrated wash systems that can reduce the effect of any residual analyte levels. Current direct techniques[106,207,217] have not been identified to suffer from carryover issues. However, a better understanding will be

gained once direct desorption techniques become more widely applied within the pharmaceutical industry.

In addition to the above, the financial implications of modifying MS sources to integrate direct desorption technologies and the need to train personnel is subject to scrutiny. For a technique to be worth such efforts, the advantages have to be significant and for many bioanalysts to even consider a new technology to be a suitable alternative, it needs to be complementary to established techniques that are readily accepted and "work" even if the new technology offers many benefits, it needs to essentially demonstrate a simpler working process which in itself may pose a challenge.

7.1.3 Aims and Objectives

The aims and objectives are to design a novel SPME-MS direct desorption technique to eliminate the need for chromatographic separation and to demonstrate the first application of direct SPME-MS for the extraction and detection of NCEs from whole blood. This will be performed by building an interface to facilitate direct elution from SPME fibres into the MS. Instrumentation factors of importance to direct SPME-MS analysis will be identified and optimised to provide quantitative direct SPME-MS analysis. Components of this chapter have been used as the basis of a publication in Analytical Chemistry[67].

7.2 Experimental

7.2.1 Chemicals and Materials

Metoprolol tartrate and propranolol hydrochloride were purchased from Sigma Aldrich (Dorset, UK), metoprolol-d_7 and propranolol-d_7 were acquired from Toronto Research Chemicals (Ontario, Canada). SPME silica probes consisting of a titanium wire coated with a biocompatible C18 extraction phase, housed inside a hypodermic needle (medical grade, stainless steel, 22 gauge outer tubes) were supplied by Supelco (Bellefonte, PA, USA); each fibre has a thickness of 45 µm and 15 mm length of coating. Control rat blood (stored for 48 h at + 4ºC) containing K2-EDTA to prevent coagulation was obtained from B&K Universal (Grimston, Hull, UK). Methanol, acetonitrile and water were of HPLC gradient grade and obtained from Fischer Scientific Ltd (Loughborough, UK). Dimethylformamide (DMF) was purchased from Sigma Aldrich (Dorset, UK).

7.2.2 Preparation of Standard Stocks and Working Solutions

Primary stock solutions for each test compound and internal standard (IS) were prepared in DMF (1 mg/mL). Serial dilutions of each analyte's stock solution were performed in acetonitrile/water (1:1, v/v) to give working standard concentrations of 1, 10 and 100 µg/mL. IS working solutions for each analyte were prepared from the primary stock solution to give a final concentration of 100 ng/mL in acetonitrile.

7.2.3 Preparation of Test Samples

Analytical test samples were prepared freshly on the day of analysis by spiking an appropriate volume of the working standard solutions into fresh control rat blood containing EDTA. The solvent used to spike into the blood matrix did not exceed 5% of the total volume. A concentration range relevant for the physiological exposure of the drugs was utilised to give final concentrations of 10, 50, 200, 500, 800 and 1000 ng/ml for each of the analytes.

SPME extraction characteristics, including equilibration time profile for blood exposure were previously determined for both metoprolol and propranolol in Chapter 3. The SPME samples were prepared by preconditioning the fibres with methanol followed by water for a period of

15 min in each solvent. This step is necessary to wet the C18 chains of the coated phase and ultimately facilitate optimal extraction efficiency. The fibres were exposed to 200 µL aliquots of the test samples for a minimum of 2 min with 500 rpm agitation using a compact laboratory shaker (MS 3 Digital, IKA). Agitation of the sample was applied in an attempt to mimic the existence of a 'stirred' medium which would surround the fibres due to intravenous blood flow in a living organism. This assists the mass transport between the sample and the fibre coating and decreases the time needed to reach equilibrium. After extraction, the SPME probes were rinsed briefly for 30 s using purified water in order to remove any sample droplets adhered to the outside of the coating as a result of surface tension. In all cases (preconditioning and extraction), fibres were directed through the needle into a 96 deep well plate with a frame (Figure -7.1-) to ensure that the entire extraction phase (coated region) was immersed in the sample. To assess the accuracy and reproducibility of the method, three fibres were extracted and analysed at each concentration level. All samples were prepared on the same day of analysis. SPME fibres are currently disposable and designed for single use extractions for both *in-vivo* and *in vitro* applications.

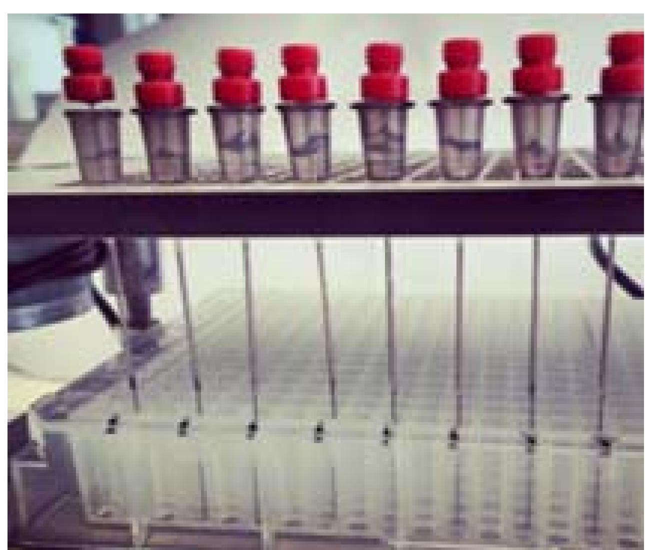


Figure -7.1- SPME fibres directed through a frame into a 96 deep well plate to ensure that the entire extraction phase is immersed into the sample.

7.2.4 Initial Direct SPME Ionization Prototype

In order to establish the feasibility of direct SPME-MS, an initial prototype was developed using a spent 100 µL glass CTC (Hamilton, UK) syringe. The syringe was employed as both, a desorption chamber with a known volume of solvent capacity (100 µL) and as a tool to facilitate ionization. The syringe metal needle section was removed and replaced with a modified coaxial Sciex ionizer nozzle (part number 016323.C, electrospray assembly). A test sample of metoprolol at 100 ng/mL was extracted from rat blood by SPME and the fibre was subsequently placed inside the CTC syringe, the rear end of the syringe was blanked off with a septum seal. Previously a radial side hole (1 mm) was diamond drilled into the centre of the glass syringe to fit a 10 µm PEEK tubing which was bonded liquid tight into the glass (Figure -7.2-). The PEEK tubing was connected to a syringe infusion pump. The syringe containing the SPME fibre was positioned in front of the mass spectrometer and acetonitrile was applied through the infusion pump with a high voltage maintained during desorption inside the syringe. A favourable MS response for metoprolol was observed which led to the second stage of spraying directly from SPME fibres.

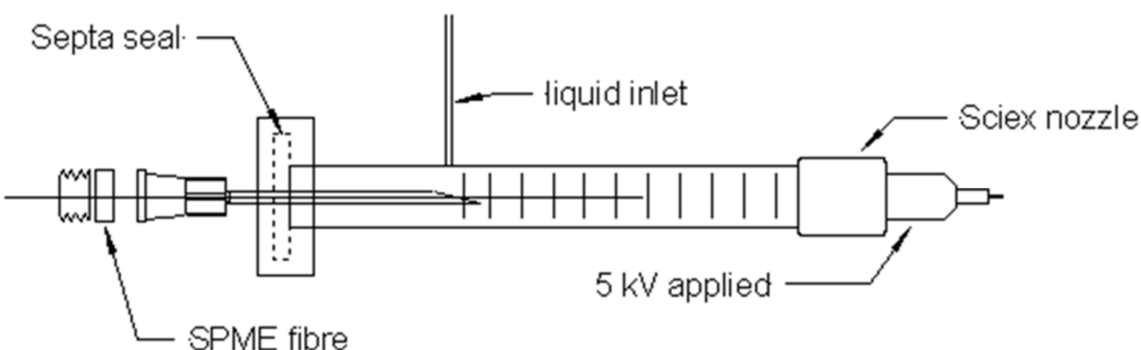


Figure -7.2- Schematic diagram of the initial experimental set up [67]. A modified CTC syringe acts as desorption chamber and a tool to facilitate ionization through the nozzle.

7.2.5 Direct SPME Ionization Set-up and Optimising Operating Conditions

For the final SPME ionization set-up, a source with a direct fibre inlet holder was designed to allow for precise introduction of the SPME needle and close proximity of the coated fibre to the MS inlet. A spray solvent (which also functions as the desorption solvent for the compounds adsorbed onto the SPME fibre) consisting of acetonitrile containing the isotopically labelled internal standard (100 ng/mL) was applied onto the entire coated fibre through a syringe infusion pump delivering solvent at a constant flow rate. PEEK tubing (10 µm) which was placed at the end of the SPME fibre was utilized to deliver the spray solvent. The voltage was then set to 5000 V and applied to the rear titanium wire of the SPME fibre as shown in Figure -7.3- and direct ionization was performed by simultaneously starting MS data acquisition. The application of a voltage to the SPME device (titanium wire) initiated the formation of a spray at the tip of the fibre and resulted in the immediate detection of analyte and internal standard ions. After 588 scans (5 min), the voltage was set to zero and the fibre was retracted. This length of time was chosen because the MS signal of the LLQ (10 ng/mL) started to deteriorate after 5 min. Therefore, 5 min was deemed appropriate for desorption and was used for all samples.

The operating conditions were systematically altered to examine the essential factors that may influence the quality of the MS signal achieved. These included parameters such as the distance between the SPME fibre tip and the MS inlet. This was varied from 1 to 10 cm to identify whether this will have an impact on the analysis. The flow rate of the spray solvent was also investigated by changing the flow rate at 5 µL/min intervals from a low rate of (5 µL/min) to high (30 µL/min). Carryover was assessed by analysing a control fibre which had been exposed to blank rat blood (drug free) following analysis of a HLQ sample (1000 ng/mL). This was utilised to ensure and assess the selectivity of the method as well as potential for carryover.

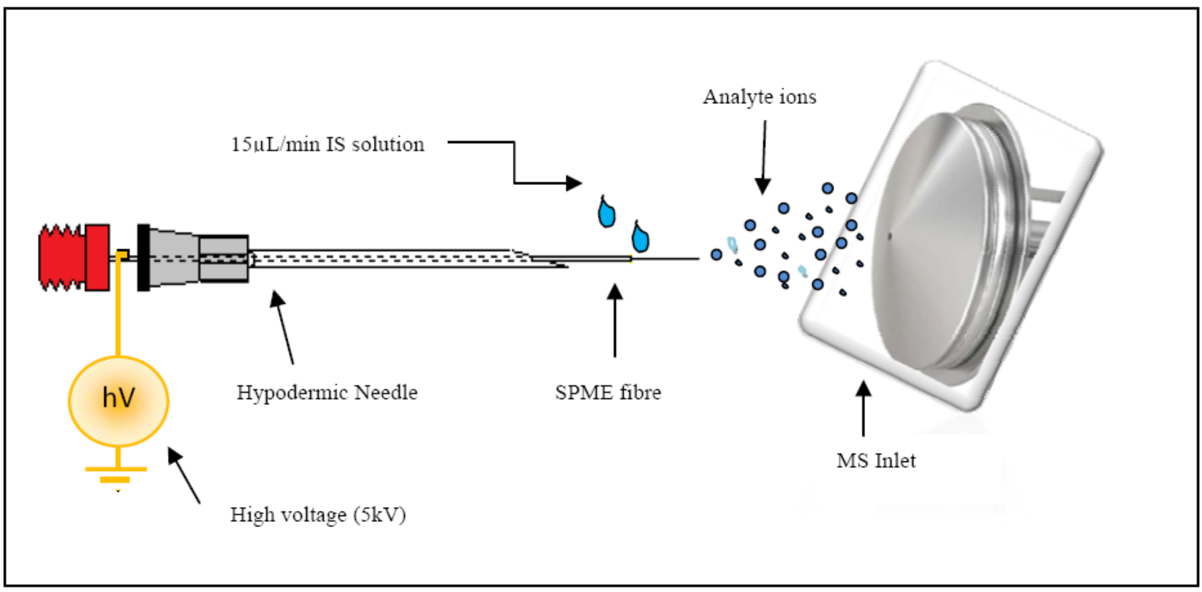


Figure -7.3- A schematic illustration of direct SPME-MS [67]. SPME fibre mounted on a 3D stage to allow movement towards the MS inlet. Spray solvent was applied through a 10 µm PEEK tubing connected to a syringe pump. Direct SPME-MS does not require sheath gas and is performed at ambient temperature.

7.2.6 Mass Spectrometry

All experiments were performed using a Sciex API3000 (Applied Biosystems/MDS Sciex, Canada). The instrument was operated in positive ion mode with a declustering potential and a focusing potential for both analytes of 60 V and 100 V respectively. The curtain gas (N_2) was set to 12 psi, scan rate was 5 s^{-1} and MS unit resolution was chosen. The analytes and their internal standards were detected in Q1 scan mode using the characteristic protonated molecular ions $[M+H]^+$ corresponding to the following: metoprolol: m/z 268, metoprolol-d_7: m/z 275, propranolol: m/z 260 and propranolol-d_7: m/z 267. Preliminary experiments were performed using full scan mode however for quantitative analysis, selected ion recordings were acquired using narrow scan ranges m/z 267-276 for metoprolol and m/z 259-268 for propranolol.

MS data were acquired and processed (integrated) using Analyst software (v1.4.2 Applied Biosystems/MDS Sciex, Canada).

7.3 Results and Discussion

7.3.1 Direct SPME Ionization

The desire to achieve direct ionization and removal of distinct separation systems, initiated the idea of exploring the possibility to directly ionize analytes from a SPME fibre. As detailed in Section 7.1, the main advantage of direct analysis is the elimination of liquid chromatographic separation. Such aspect will impose significant simplification of the current bioanalytical workflow which involves manual sample preparation (analyte extraction) as well as chromatographic separation.

This initial procedure of the first SPME-MS prototype was performed to determine proof of principle i.e. to establish whether a signal can directly be produced from a loaded fibre using a known desorption volume and established ion optics. The purpose of the above was to identify experimental and instrumental variables related to desorption and ionization with a view to understand practicalities such as accurate timing of sample desorption and ability to change the fibre without encountering carryover issues when using different concentrations and obtaining adequate inter-fibre reproducibility. This ensured that such limitations were understood prior to attempting direct spray from the fibre without the assistance of a CTC syringe. The outcome from this preliminary experiment showed that the basic instrumental requirements of SPME-MS is very similar to paper spray analysis[105], a spray solvent is required to facilitate desorption of analyte from the fibre and a high electric field to perform ionization. Pneumatic assistance was not required to transport the analyte: a spray solvent and a voltage are simply applied to the fibre, which is held in front of a mass spectrometer. A signal was generated as shown in Figure -7.4-.

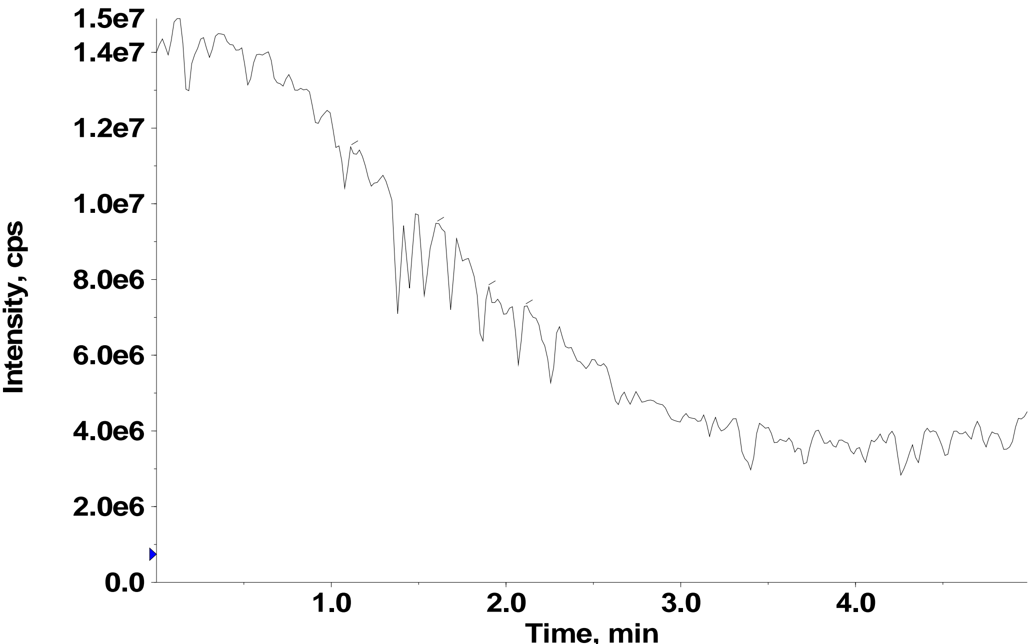


Figure -7.4- Direct SPME-MS analysis of metoprolol (10 ng/mL) extracted from fresh rat blood, response versus time chronogram illustrating electrospray ionisation upon voltage onset using the initial set up, SPME fibre within a CTC syringe.

Subsequent to the initial experiment, a modified source was designed to enable direct SPME-MS analysis. Figure -7.3- shows in schematic form the general experimental set up for direct SPME-MS. Stable electrospray ionization was observed upon voltage onset (Figure -7.5-).

The coated titanium SPME fibre is conductive and when the spray solvent is applied, the high electric field generated between the SPME tip and the MS inlet breaks the liquid to form a mist of charged fine droplets which subsequently desolvates to produce gaseous ions. In common with paper spray MS[207] and nib-spray MS[210], direct SPME-MS required the fibre to possess its characteristic sharp tip to establish the high electric field and a manually-blunted SPME tip caused loss of signal. It is believed that the coating and the bonding materials of the fibre function as insulators[41] therefore ensuring that the tip is exposed gives rise to a much more stable spray. Visual observation of the Taylor cone and examination of the mass spectra obtained for both metoprolol and propranolol samples suggested that a controlled spray producing protonated ions was achieved and that ESI mechanism may be responsible for the resultant ionization. The analysis of each sample was halted after 5 min (588 cycles)

i.e. a fixed desorption period was implemented for all samples in order to compare like-for-like. This desorption period was chosen because the signal stabilized by 2 min and remained approximately stable for more than 5 min as shown in Figure-7.5-.

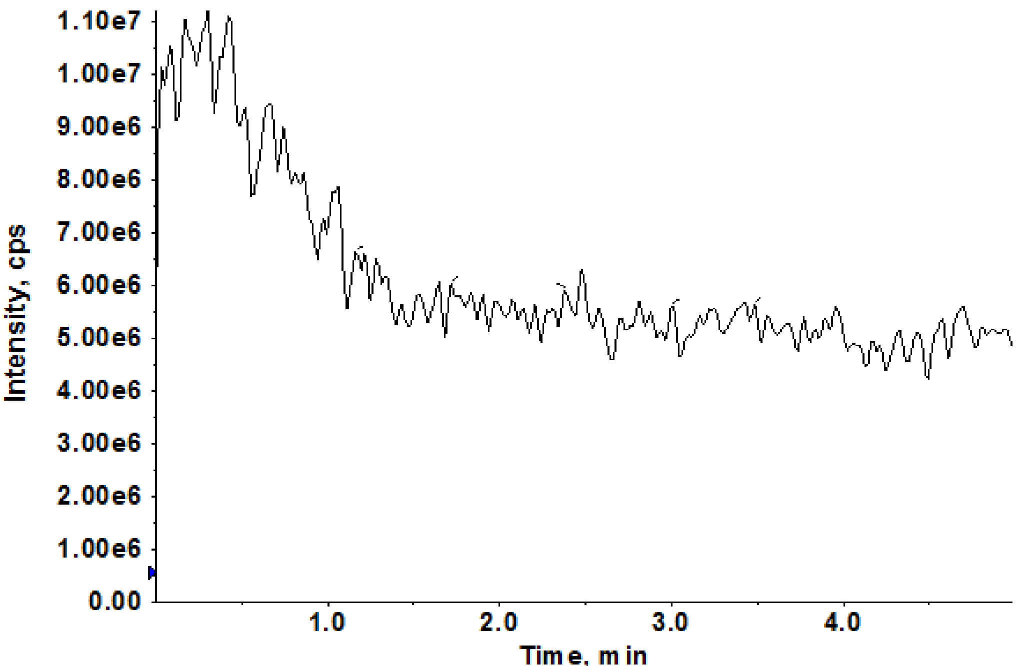


Figure -7.5- Direct SPME-MS analysis of metoprolol (10 ng/mL) extracted from fresh rat blood, response versus time chronogram illustrating electrospray ionisation upon voltage onset. Voltage switched off after 5 min[67].

Since no chromatographic separation is taking place, the solvent used to desorb the analyte from the SPME fibre is referred to as the "spray solvent" rather than the mobile phase. In this case, the C18 coating of the fibre is acting as the stationary phase and the spray solvent as the mobile phase eluting the analyte off the fibre. It is also important to note that this technique is generating a "MS response" of the total ion current rather than a chromatographic peak. For this reason, the height of the response produced i.e. the sum of the mass spectral intensity of the analyte over the full 5 min was treated in the same way as a chromatographic peak to elucidate sensitivity and precision data.

7.3.2 Optimising Operating Conditions

The spray efficiency was found to be dependent on several factors, one of which is the flow rate at which the spray solvent was applied; higher flow rates (> 30 µL/min) resulted in excess solvent forming larger droplets that ultimately compromised the shape and efficiency of the cone jet and in turn impacted the quality of spectrum obtained. A poor performance was also observed with lower solvent flow rates (< 10 µL/min) which led to a non-uniform flow over the tip and a correspondingly erratic, unstable signal for desorbing analyte. Thus a suitable range (10-30 µL/min) of spray solvent flow rate was identified and an optimum flow rate of (15 µL/min) was consequently utilised.

Another important aspect for effective ionization is the position of the fibre relative to the MS orifice. The distance of the fibre tip from the MS inlet plays a vital role, placing the tip too close to the inlet (< 3 cm) generated sparking and consequently produced low and variable responses (Figure -7.6-) while moving the fibre too far from the inlet (> 7 cm) caused loss of signal intensity and a change in the spray plume. For this reason, the XYZ configuration and positioning of the fibre was optimised to be within a distance of 3-5 cm from the MS using a 3D moving stage on which the SPME holder was mounted. Approximate alignment with the MS was also essential. Further investigations and execution of a full evaluation are required to transform this technique into a routine bioanalytical tool.

Carryover was minimal (< 20% of the LLQ peak area) i.e. no unacceptable interferences corresponding to metoprolol and propranolol ions were observed, representative spectra of a blank sample and test sample for both metoprolol and propranolol at 200 ng/mL are shown in Figure -7.7- and Figure -7.8-.

Subsequently optimised parameters enabled production of desired signals for direct SPME-MS without substantial carryover (< 20% of the LLQ signal) which led to subsequent quantitative experiments.

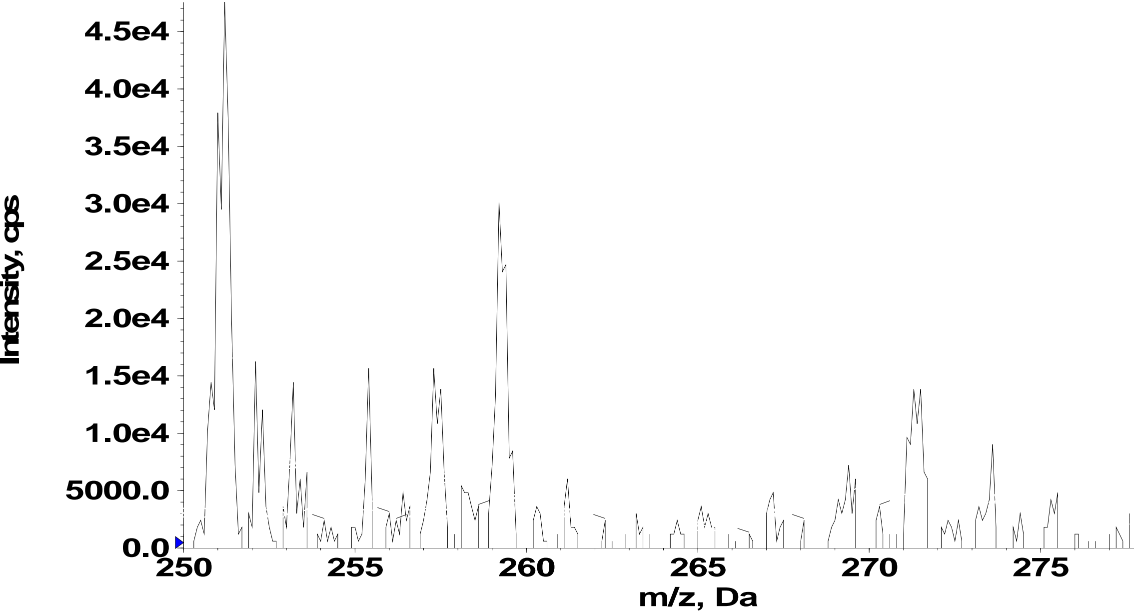
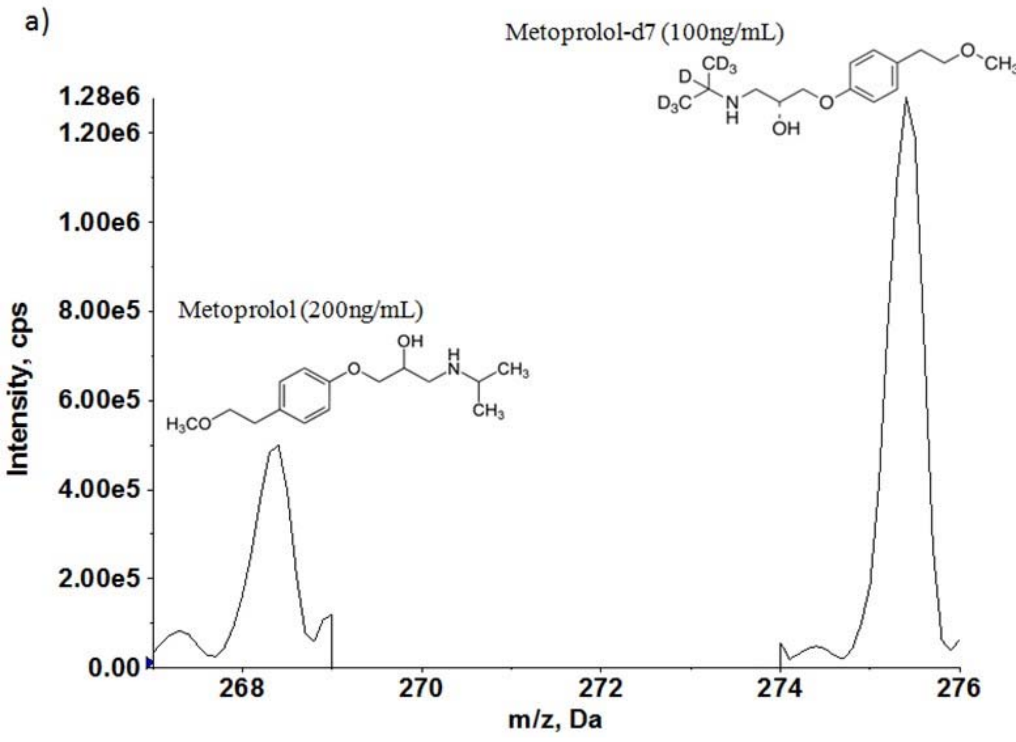


Figure -7.6- Example mass spectrum of metoprolol (200 ng/mL) when the fibre tip was place < 3 cm from the MS inlet. Variable response generated due to sparking.



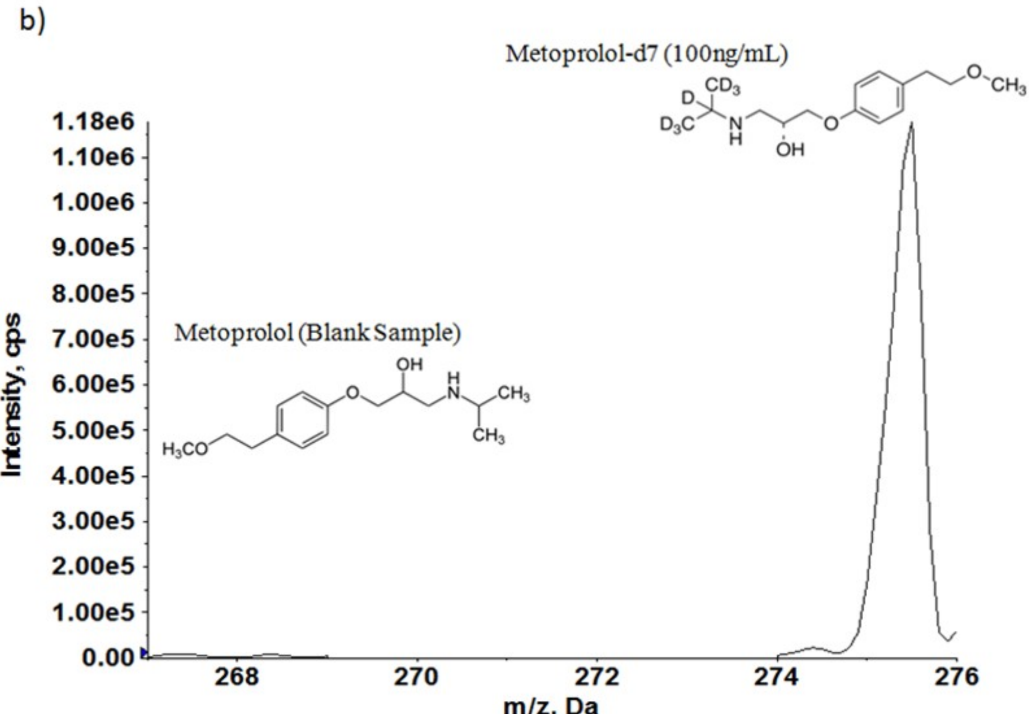
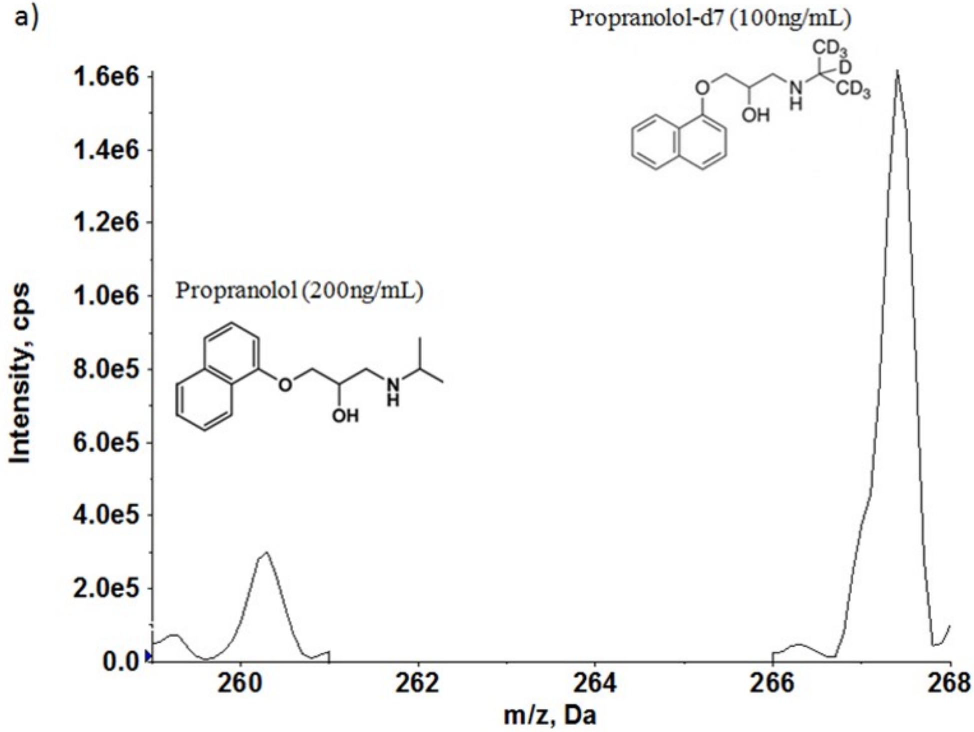


Figure -7.7- a) Mass spectrum of metoprolol (200 ng/mL) and its internal standard (100 ng/mL) analysed by direct SPME-MS. b) Mass spectrum of blank sample and metoprolol-d_7 (100 ng/mL) analysed by direct SPME-MS showing minimal carryover, blank sample analysed immediately after the highest concentration (1000 ng/mL)[67].



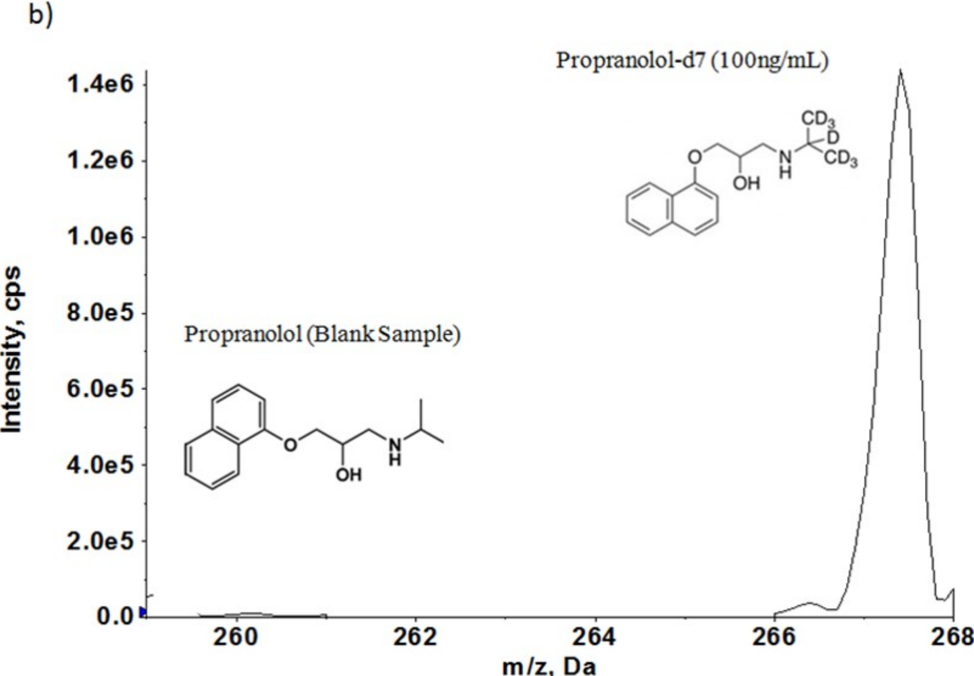


Figure -7.8- a) Mass spectrum of propranolol (200 ng/mL) and its internal standard (100 ng/mL) analysed by direct SPME-MS. b) Mass spectrum of blank sample and propranolol-d_7 (100 ng/mL) analysed by direct SPME-MS showing minimal carryover, blank sample analysed immediately after the highest concentration (1000 ng/mL)[67].

7.3.3 Analyte Quantification Using Direct SPME-MS

The analysis of a set of standards (range 10 – 1000 ng/mL) for both metoprolol and propranolol was performed (n = 3 for each concentration) and used as examples of the results typically obtainable for small molecule quantitation by direct SPME-MS. The results showed that analyte peak height/IS ratio increased in a proportional manner with increasing concentration levels for both metoprolol and propranolol test samples. Plots of analyte/IS peak height ratio versus the nominal concentration were constructed as shown in Figure-7.9- and Figure-7.10-. The signal of the lowest concentration analysed, for both metoprolol and propranolol was sensitive enough to detect a lower limit of quantification (LLQ) of 10 ng/mL. This performance was achieved through a Q1 scan of an API3000. The Q1 scan mode was utilised to identify any competing ions i.e. possible background ions that may have dominated charge exchange and therefore reduced the ion intensity of ions of interest. No competing ions were observed. In the future MS/MS with multiple reaction monitoring (MRM) scans will be acquired to mask any unwanted species and ultimately enhance performance. It is also anticipated that using superior instruments such as high resolution accurate mass spectrometry would lead to acquisition of greater selectivity and sensitivity.

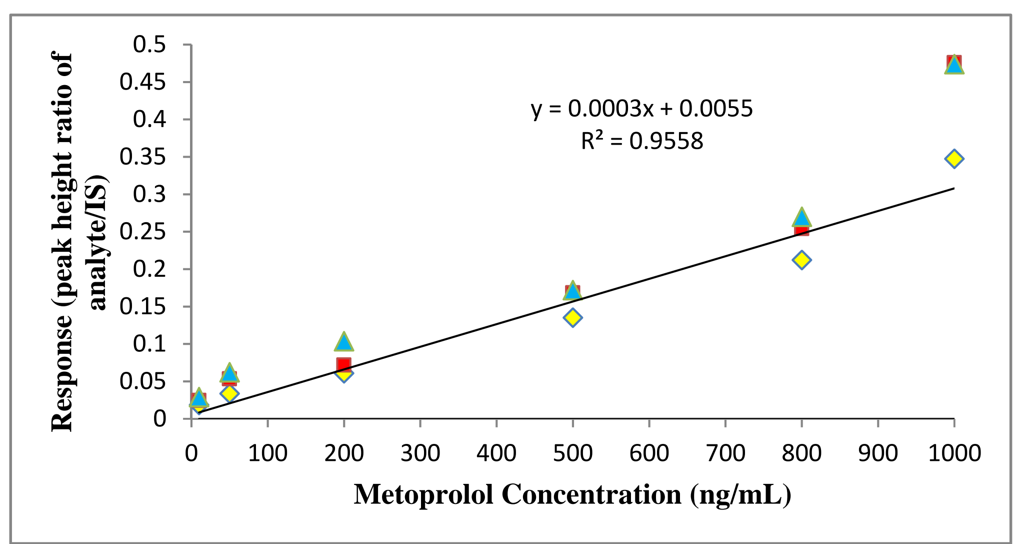


Figure -7.9- Quantitative analysis by direct SPME-MS of metoprolol. Data represents the standard curve (ratio of instrument response to that of internal standard versus analyte concentration) for n=3 replicates at each concentration[67].

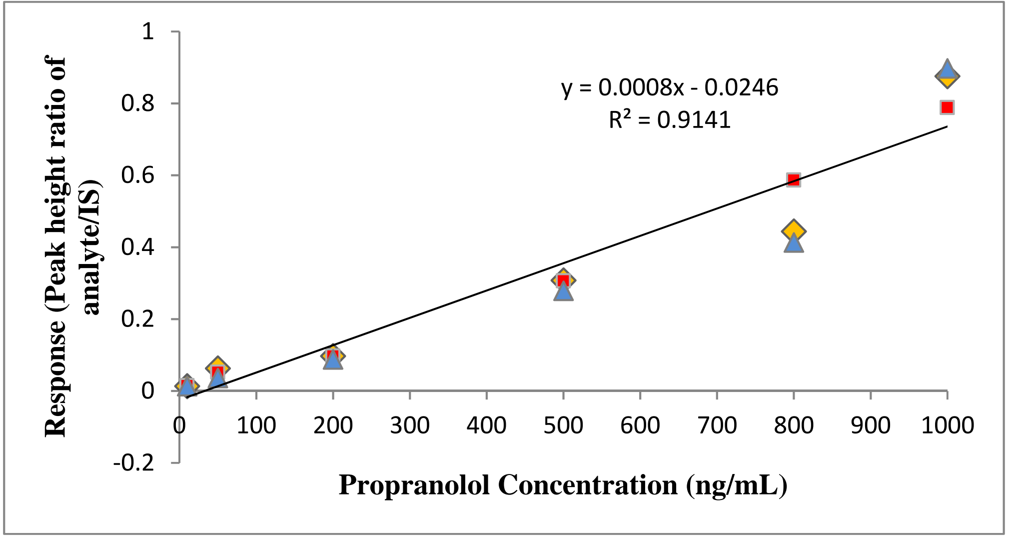


Figure -7.10- Quantitative analysis by direct SPME-MS of propranolol. Data represents the standard curve (ratio of instrument response to that of internal standard versus analyte concentration) for n=3 replicates at each concentration[67].

The initial results showed that good linearity and reproducibility were achieved in this proof of concept study up to 800 ng/mL but the trend was less linear at 1000 ng/mL. This may be attributed to the analyte being deeply embedded in the fibre and more difficult to desorb off the fibre at low concentrations while at higher concentrations the analyte is more widely distributed (including on the surface) leading to more facile desorption.

Precision (% CV) data generated from the replicate measurements (n=3) at each concentration were below 30% for both metoprolol and propranolol as shown in Table -7.2- and Table -7.3-. It should be noted that some of the variation observed could be attributed to the inter-fibre variability of the SPME probes that was observed in previous chapters (Chapters 3 and 4). At this stage of early development, such precision data demonstrated for two test analytes suggests that this technique was capable of generating acceptable reproducibility and if additional investigations prove successful, the precision could be further improved to meet current bioanalytical acceptance criteria (15-20%). Precision values obtained by direct SPME-MS compare favourably well and in some cases better than precision data obtained for other direct analysis techniques. For example < 30% has been achieved for paper spray analysis of citalopram in dried blood spots[105], while >30% has been shown for DART analysis of indomethacin[195] at 10 ng/mL from rat plasma and 33% for verapamil analysis[195] from rat plasma.

Table -7.2- Peak height ratio of metoprolol standards /IS analysed using direct SPME-MS

Conc. (ng/mL)		10	50	200	500	800	1000
Peak Height Ratio (Analyte/IS)	Sample 1	0.0184	0.0336	0.0608	0.1351	0.2121	0.3473
	Sample 2	0.0244	0.0533	0.0717	0.1682	0.2540	0.4756
	Sample 3	0.0288	0.0621	0.1035	0.1718	0.2699	0.4737
	Mean	0.0239	0.0497	0.0787	0.1584	0.2453	0.4322
	SD	0.0052	0.0146	0.0222	0.0202	0.0298	0.0735
	% CV	**21.9**	**29.4**	**28.2**	**12.8**	**12.2**	**17.0**

Table -7.3- Peak height ratio of propranolol standards /IS analysed using direct SPME-MS

Conc. (ng/mL)		10	50	200	500	800	1000
Peak Height Ratio (Analyte/IS)	Sample 1	0.0133	0.0627	0.0967	0.3073	0.4439	0.8754
	Sample 2	0.0146	0.0519	0.0971	0.3060	0.5864	0.7882
	Sample 3	0.0131	0.0360	0.0878	0.2784	0.4131	0.8974
	Mean	0.0137	0.0502	0.0939	0.2972	0.4811	0.8536
	SD	0.0008	0.0134	0.0053	0.0164	0.0924	0.0578
	% CV	**5.99**	**26.7**	**5.65**	**5.50**	**19.2**	**6.77**

Additional work is required to improve and build on the current findings with a view to achieving linear calibrations which will enable accurate quantification of unknown concentrations. Further tests using a number of compounds may improve understanding of the overall mechanism of direct ionization and help compare this technique to conventional analytical methods such as protein precipitation and solid phase extraction combined with liquid chromatographic separation.

SPME fibres can easily be introduced into a source of a triple quadrupole mass spectrometer without the need for complicated modifications. This in turn means compatibility with pre-existing resources as triple quadrupole mass spectrometers are currently the detection method of choice in many industrial bioanalytical labs. This poses a major advantage in terms of retaining high levels of sensitivity and selectivity using available mass spectrometers which are prevalent in quantitative and regulated bioanalysis.

Furthermore, the use of direct SPME-MS for structural elucidation on high resolution accurate mass-spectrometers is envisaged for future technical development with a view to identify metabolites in the same sample as the parent compound.
Also investigating various SPME coating phases for the extraction of molecules with a broad range of log P values will allow detection of unstable metabolites such as acyl glucuronides and N-oxides. The rapid acquisition of metabolism data will provide a better understanding of disease pathways and the discovery of new biomarkers.

A number of modifications to the current design of the direct source need to be developed, the present device, although very simple, is still manually controlled. Therefore, the ability to characterize and analyse large numbers of compounds and samples in a high sample throughput mode can only be achieved with a fully automated system. This involves incorporating a wash procedure in between analysis to flush the system and ensure elimination of any contamination or deposits of residuals. Manually operated direct desorption technique involves a setup consisting of the extraction/ionization device coupled to the MS inlet where each sample is manually uploaded onto this arrangement. Such setup might be adequate for investigative low sample throughput applications. However, for routine drug development and discovery studies, the requirement to analyse hundreds of study samples will necessitate reliable automation where samples can be racked up and left for analysis overnight without the need for human interaction[196].
This could be achieved by utilizing a robotic arm that can pick SPME fibres from a rack and transfer them to the MS inlet with accurate positioning in front of the orifice. Alternative methods could involve a rotary device where a set of SPME fibres can be uploaded (Figure -7.11-) into a rotary cylinder and each fibre would be presented at the MS orifice for the duration of the analysis. A new fibre is rotated into position subsequent to the extraction of the previous sample and a new set of fibres can be uploaded from an automated carousel-like magazine. Additional functionalities such as application of the internal standard i.e. the desorption solution, as well as visual recognition of samples should also be considered when designing SPME automated devices.

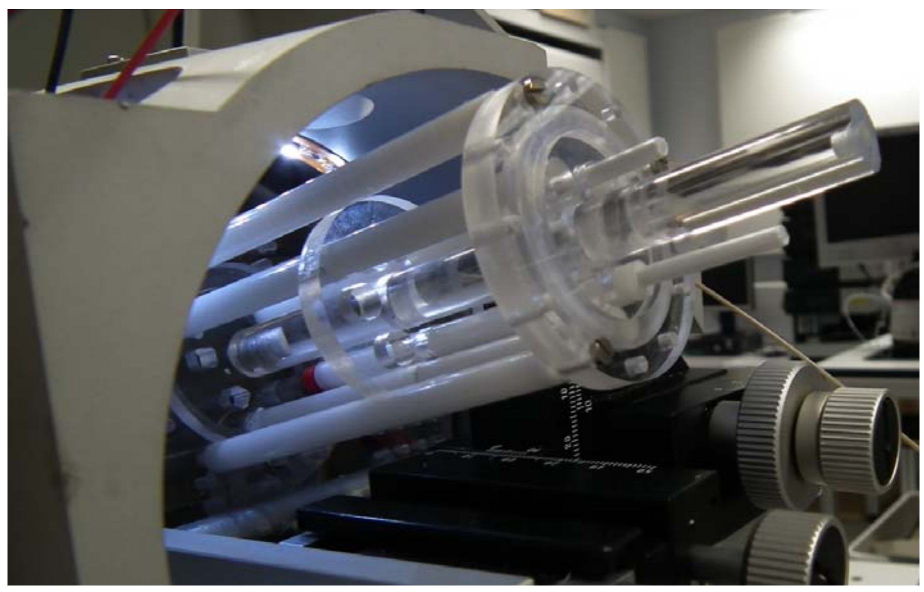


Figure -7.11- Rotary bespoke automation concept for direct SPME-MS, designed by Sheelan Ahmad and developed by Michael Tucker at GlaxoSmithKline.

Robust automation of SPME is a challenge that requires attention to enable direct desorption to be utilized for drug development studies. Automation errors should be minimal and comparable to errors seen with current sample injection systems in HPLC instruments. Error handling is essential if direct analysis techniques are to be deemed as alternatives for separation techniques. The automated direct analysis device must also be able to communicate and synchronize with the detector so that samples are reconciled against a sample sequence list that maybe submitted from the MS computer system. Although significant progress has been witnessed in SPME automation, currently there are no commercially available automation devices for any SPME direct analysis techniques.

Overall direct SPME-MS substantially simplifies MS analysis where no sample preparation or separation is required and data is generated at ambient temperature. This may considerably reduce time, costs and aid higher analytical throughput with minimal sample usage and no blood withdrawal. The fundamental drive and rationale for direct analysis is the potential removal of the sample preparation interface which will aid process simplification and enable time saving.

7.4 Conclusion

The intention of this work was to identify the possibility to perform direct ionization from a SPME fibre by applying voltage and desorption solvent. This chapter has presented the proof of concept for direct SPME-MS as a means to conduct quantitative analysis of small molecules directly from whole blood samples. The capability of direct SPME-MS analysis has been characterised with two test analytes, metoprolol and propranolol, spiked into control rat blood. The data indicated the significance of this approach to enable rapid, selective and highly sensitive (10 ng/mL lower limit of quantification) qualitative and quantitative chemical and biochemical analysis.

Analysis is carried out on the same SPME fibre that is used for extracting the analyte from the investigated medium or the living organism, thus immediate determination of unbound drug concentration could be achieved from biological samples. The approach combined the highly attractive features of two very powerful techniques; SPME and MS. The technique has wide ranging potential for future preclinical and clinical tests as well as therapeutic drug monitoring. Exploring the technique in more depth for both qualitative and quantitative use has the potential to open the door to further applications and transform the field of bioanalysis through combining the selectivity of SPME with the sensitivity of direct SPME-MS analysis.

Chapter 8

Research Summary and Future Directions

8.1 Research Summary

For many years analysts have been trying to identify techniques with better analytical sensitivity and tools that reduce multistep sample handling procedures. This has been recently accompanied by a shift towards using smaller sample volumes without impacting the quality of the bionanalytical data[21,218]. This is to cope with the demand for highly sensitive assays that require low LLOQ and to improve ethical considerations around the use of reduced animal numbers in research. As discussed in Chapter 1, a number of microsampling techniques have been applied over the last decade to address the above. Each with its own advantages and disadvantages (Chapter 1).

Meanwhile, a technique known as solid phase microextraction has been growing in popularity (Figure -8.1-) among scientists from several different disciplines due its unique properties. However, some of its distinctive characteristics such as analyte extraction without the need for blood withdrawal had not been fully explored to address bioanalytical and sampling issues within the pharmaceutical industry. *In vivo* SPME applications have been mostly confined to academic research laboratories rather than the pharmaceutical industry[42].

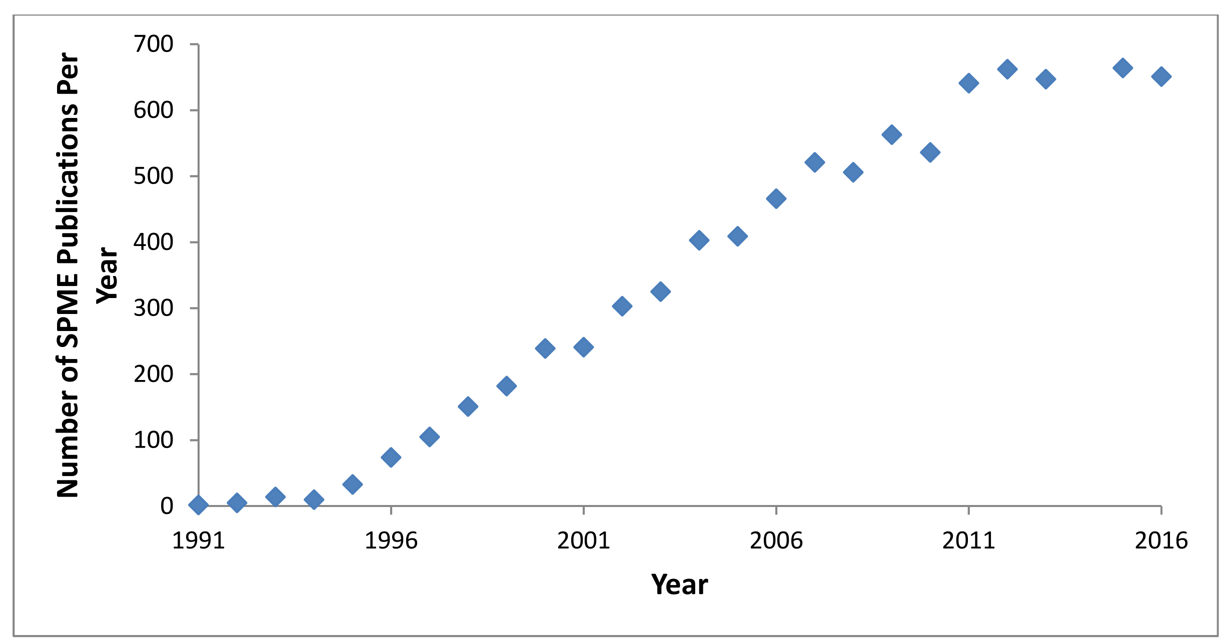


Figure -8.1- Number of SPME publications per year for the last 25 years that contain the term "SPME". Data collected using the Scopus search facility (Elsevier).

This research explored the feasibility of utilising SPME as a microsampling technique within the pharmaceutical industry with a view to evaluate the factors affecting SPME's use as a bio-microanalytical device for preclinical studies. Parallel *in vitro* and *in vivo* experiments were conducted to assess the technique and its application within the pharmaceutical industry.

At the start of the project, the *in vitro* investigations in Chapter 3 revealed that SPME is suitable for determining physiologically relevant analyte concentrations from blood without the need to lose any sample volume. This in turn meant that SPME can be used as a microsampling device in live animals without the requirement to withdraw any blood. However, the probe requires equilibration with the analyte within the sample matrix. But equilibration profiles determined in Chapter 3 showed that equilibrium is not reached prior to 3 h exposure. This has adverse effects on ethical, practical and scientific considerations for the technique. Leaving the SPME fibre within a living organism for long periods of time will cause animal distress and jeopardise data quality if earlier PK timepoints are missed. As such, shorter sampling times i.e. pre-equilibrium extraction was assessed in Chapter 5 (first *in vivo* study). This study was successful showing that 2 min extraction is sufficient providing that calibration standards and quality control samples are exposed to spiked samples for the same

length of time *in vitro*. Analyte desorption time profiles, on the other hand, showed that sufficient analyte recovery was achieved within 15 - 30 min of desorption exposure. This in turn greatly simplifies and shortens the analytical effort and time for the analyst, enabling simpler workflow.

One of the over-riding findings of this project has been that SPME extracts free analyte concentrations rather than total concentrations. This aspect was employed and assessed in Chapter 4 to use SPME as a tool to determine protein binding values *in vitro*. The technique was further explored and was compared to a gold standard protein binding technique known as the RED device. SPME was found to be quicker producing results within 1 h compared to 8 h with the RED device and generating comparable data with < 15% difference between the two techniques. SPME provided a compelling alternative platform for the efficient generation of high quality plasma protein binding values. This opens another door for useful applications of the SPME device within the pharmaceutical industry. The current approach across the industry is to identify protein binding values of candidate drugs during the early discovery work. However, this is typically performed *in vitro* using traditional techniques such as RED and ultracentrifugation that require time and resource. SPME can simplify this approach if utilized for protein binding experiments both *in vitro* and directly *in vivo* giving individual animal protein binding parameters.

One apparent barrier to wider applications of *in vivo* SPME which was discovered in Chapter 2 (method development of naproxen) and Chapter 5 (identification of metoprolol metabolite) is the need for mixed-phase fibre coatings. The current *in vivo* biocompatible SPME fibres which are commercially available are C18 coated. This type of coating is not suitable for polar/hydrophilic compounds. Despite the potential importance of SPME application in the metabolism and metabolomic fields, the accessibility to varied biocompatible phase/chemistry coating types is still limited. Working in collaboration with the SPME vendors (Supelco/Merck Millipore), a new line of biocompatible mixed-mode coatings are currently being produced and efforts are under way to evaluate them. However, it is important to mention that challenges in terms of SPME coating do remain. In particular, the thickness of the fibre coating determines the capacity of the device. Thicker coatings allow for wider dynamic concentration ranges but mean that longer equilibration periods are required, while linearity is lost at higher concentrations with thinner coatings due to limited capacity. For this reason, several ideas have been proposed to vendors/collaborators to improve coating qualities through smaller particle dimensions therefore increasing surface

area without compromising coating thickness. Another aspect that needs to be highlighted is the inter-fibre variability encountered throughout this project, high %CV values of inter-fibre variability during stability and blood flow rate experiments in Chapter 3 Sections 3.3.5 and 3.3.6 were observed. This has been fed back to the manufacturers and is currently being addressed through better quality control of fibre batch production.

The biocompatibility of SPME, the ability to construct PK/TK profiles and the feasibility of inserting the fibres directly into rat tails without the need for an interface device were demonstrated for the first time in this project. The full tolerability study in Chapter 6 assessing serial TK sampling and evaluation of SPME within a preclinical setting highlighted the fact that SPME provides a unique microsampling platform without the need to withdraw any sample. Biocompatibility was confirmed through clinical pathology endpoints as well as animal stress levels when subjected to the SPME fibres. This work illustrated the importance of *in vivo* SPME and no blood withdrawal to preclinical rodent studies. No blood removal enables serial or repeat sampling from the same animal without the need for extra satellite groups in addition to toxicology animal groups. This leads to improved data quality and reduced animal use which has a huge ethical impact and permits cost savings.

Another essential outcome of this project has been introducing the SPME technique to laboratory animal technicians. Previously, the industry has witnessed the implementation of a number of microsampling techniques including dried blood spots, capillary microsampling etc. and there has been initial difficulties and push back from staff regarding complex procedures where extensive training was required. Despite this, many animal technicians realise the microsampling benefits and so they are keen to try and implement new microsampling techniques. The feedback received from all six technicians that utilized the SPME device during the *in vivo* studies was very positive, complementing the ease of use and handling flexibility being comparative to the use of butterfly needles and other traditional sampling tools. Minimal training was required during study conduct; all staff used the device without training prior to *in vivo* study days. Nevertheless, it is vital to point out that fibre conditioning with methanol and water prior to sampling was performed and handled separately. If this was to be carried out by technicians at the animal laboratory, it would have added complexity, time and effort. This step is currently being reviewed by the manufacturers and may be eliminated by using smaller particle size that does not require surface activation.

The availability of such single-use SPME devices which are biocompatible is extremely important to make *in vivo* SPME technology stand out within the pharmaceutical industry. The potential applications of the technique both as a microsampling device and as a tool to measure unbound drug concentrations within the pharmaceutical industry is extremely wide.

Finally, this research has led to the development of an innovative direct ionization technique involving *in vivo* SPME. This enabled the analysis of analytes by spraying directly from SPME fibres straight into the mass spectrometer without the need for offline sample preparation and chromatographic separation. A direct SPME-MS source was designed and tested to present a proof of concept for a technique that provides rapid, selective and highly sensitive qualitative and quantitative chemical and biochemical analysis. This particular outcome of this project is relevant not only for the use of SPME in bioanalysis, but for the analysis of analytes in environmental, food, fragrance and forensic industries.

Overall, the work in this project has shown the potential for applying *in vivo* SPME in several different stages of the pharmaceutical industry. Based on the work and the data generated in this research, *in vivo* SPME can now be immediately utilised for non-GLP preclinical studies where the bioanalytical acceptance criteria is a little wider than those defined in the regulated guidance, 20 - 25% compared to 15%[12,71,73]. The next stage will be to implement the technique in regulated toxicology studies but prior to that, several aspects including inter-fibre reproducibility and simplified preconditioning steps as well as sample audit trail will have to be addressed.

8.2 Future Directions

The advantages on offer from the SPME technique could potentially be utilized at various stages of the drug discovery and development process. Starting with early discovery phases, SPME can be applied in 3D cell cultures to determine the ability of the drug to elicit a biological response inside an *in vitro* model where the cellular function is examined prior to full commitment in the *in vivo* system.

More importantly, SPME forms an attractive tool for knockout studies in which transgenic animals are used to understand mutagenesis and validate genetic variations. Such studies have been limited almost exclusively to mice models by virtue of the ease of genetic

manipulation and their close reflection of the human physiology. The generation of these unique species is an expensive process and therefore it is crucial to determine as much information as possible from these genetically engineered animals. The approach however, has always suffered from the strict regulations on the availability of blood volumes, but this will no longer be an issue with the direct, no blood withdrawal aspect of SPME. However, the needle residence time within the vein of a living organism may be an issue if this is not further reduced through manufacturing modifications to the design and particle size of coating phases.

The technique has been applied in the metabolomic area facilitating effective *in vivo* metabolite monitoring using custom-produced coating phases[97] and also for *in vitro* studies to monitor the metabolomics of disease pathways using relevant cell lines[219]. Multiple blood-free sampling aids the process of capturing unstable metabolites within the living organism, reflecting the actual metabolite component in real time i.e. a true snapshot of the metabolome[151]. The acquisition of rapid metabolism data known as "Metabolism quenching" will provide the pharmaceutical industry with information that can form the foundations of a comprehensive metabolomic database used for designing future personalised medications.

Biomarker monitoring is another area worth shedding some light on. SPME has been used for the detection of volatile compounds that act as indicators of various disorders. Recent publications have described the use of SPME -GC-MS coupled with nano-sensors for the successful identification of 42 volatile compounds[220]. These small molecules which correspond to lung-cancer biomarkers were detected using patient breath sampling. Tumour growth biomarkers and potential regulators of angiogenesis have also been captured by SPME[221]. If these yet un-commercialised prototype devices could be further developed into commercial tools with selective coatings specific for biomarkers, then SPME can add further prognostic values to the industry.

Analysis of peptide biomarkers and larger biopharmaceutical molecules such as antibodies and proteins is another area where SPME could potentially add value. The development of biotherapeutics is now an integral part of the pharmaceutical industry where, traditionally immunoassays have dominated the field of quantifying such molecules. However, with current advances in LC-MS/MS, it has been possible to accurately measure peptides and proteins with lower limits of quantification. So although it may not yet be so well established, it is believed that with intricate design of SPME fibres, it may prove feasible to coat fibres

with specific antibodies/immunosorbents to act as binding beds for antigens. This will enable a highly specific “lock and key” mechanism between the fibre and the target harvested from the complex biological matrix, all of which would take place within the living organism, avoiding the necessity to collect blood samples.
Potentially SPME applications could be further extended to tissue analysis. The device can penetrate organ tissues without causing much regional damage compared with microdialysis probes[164]. Solvent compatibility and the difficulty of coupling microdialysis with LC-MS remains unsolved[164]; therefore the use of SPME coupled with LC-MS will enable detection of low concentrations without the ion suppression associated with microdialysis. Measurements of drug levels in the brain of conscious free moving rodents have been performed without the requirement for organ removal[222]. This opens the door for quantitative PK and TK analysis of drugs with complete organ exposure or accumulation profiles without the need to take terminal samples for subsequent tissue homogenising and wet sample analysis.

The clinical suitability of SPME is also an important consideration. Although sample volume is not a major concern when dealing with the majority of human subjects, there are numerous cases where avoidance of blood withdrawal is essential, specifically in paediatric studies or blood coagulation disorders. To date, most human SPME applications have been clustered around breath and skin analysis. However, the quantification of analgesic drugs in human urine samples has also been reported[58,221]. A notable novel application anticipated for SPME within the clinical arena is the possibility of the technique to serve as a diagnostic tool in the operating theatre. Monitoring of blood drug concentrations during surgery is a critical element of many clinical procedures. An example that illustrates this significance is anaesthetic management of liver transplantation patients. The function of the hepatic system varies during liver transplantation surgeries; this has a direct impact on the metabolism of the combination of drugs used for general anaesthetics. SPME can provide a simple method to measure the concentration of parent drug and metabolites throughout the various stages of the transplant process specially if coupled with direct MS analysis. This in turn will enable dosage control of anaesthetics during surgical procedures.

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