Evaluation of paper spray mass spectrometry for direct analysis of small molecule drugs and hemoglobin constant spring peptide markers

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2016

Takyi-Williams, J. (2016). Evaluation of paper spray mass spectrometry for direct analysis of small molecule drugs and hemoglobin constant spring peptide markers. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/68927>

<https://doi.org/10.32657/10356/68927>

NANYANG
TECHNOLOGICAL
UNIVERSITY

**EVALUATION OF PAPER SPRAY MASS
SPECTROMETRY FOR DIRECT ANALYSIS OF SMALL
MOLECULE DRUGS AND HEMOGLOBIN CONSTANT
SPRING PEPTIDE MARKERS**

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SCHOOL OF BIOLOGICAL SCIENCES

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A thesis submitted to the Nanyang Technological University in partial fulfilment of
the requirement for the degree of Doctor of Philosophy

2016

Acknowledgement

I would like to express my utmost gratitude to my advisors Dr. Tang Kai, Dr. Liu Chuan Fa and Dr. Thomas Gong Haiqing. Dr. Tang Kai guided me in a brilliant and fatherly manner whilst impacting in me his enormous experience and valuable suggestions and ideas. Dr. Liu Chuan Fa provided me with immeasurable support and advice. He also gave me the opportunity to partake in drug discovery studies which afforded me with profound experience in preclinical studies. Dr. Thomas Gong Haiqing was my thesis advisory committee member and his lab fabricated the paper spray prototype which I used extensively in this thesis.

I also thank the following: Singapore Government Award (SINGA) for my study scholarship; Janssen, a division of Johnson & Johnson for the sponsorship of the paper spray research, especially Dr. Wang Yang and Dr. Wenying Jian for their helpful discussions; Thalassemia research center, Mahidol University, Thailand, for the provision of the samples for the biomarker discovery and validation study; Dr. Zhou Qingyu (University of South Florida, Tampa) for her valuable assistance and discussions in animal model experiments; Prof Andrew Tan for his permission to be my IACUC supervisor and Mr. Rinson Lai of Thermofischer for his technical assistance on LTQ-Orbitrap instrument.

I would like to convey my gratitude to my labmates and colleagues in NTU.

Lastly, I would like to thank my family for their love and support.

Abbreviations

Hb	Hemoglobin
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
DBS	Dried blood spot
DPS	Dried plasma spot
DSS	Dried sample spot
PCR	Polymerase chain reaction
FDA	U.S. Food and Drug Administration
S&S	Schleicher and Schuell
EDTA	Ethylene diamine tetra acetic acid
DMPK	Drug metabolism and pharmacokinetics
ADME	Absorption, Distribution, Metabolism and Elimination
LC-MS/MS	Liquid chromatography tandem mass spectrometry
PS	Paper Spray
PS-MS	Paper Spray Mass spectrometry
LC	Liquid Chromatography
UPLC	Ultra Performance Liquid Chromatography
HPLC-UV	High Performance Liquid Chromatography-Ultraviolet detection
Avg.	Average
MW	Molecular weight
AUC _{0-∞}	The area under the analyte plasma concentration-time curve
AUC	Area under the curve
TIC	Total ion chromatogram

SPE	Solid Phase Extraction
LLE	Liquid-Liquid Extraction
kV	kilovolt
CL	Total Clearance
Vd	Apparent volume of distribution
Hb CS	Hemoglobin Constant Spring
LLOQ	Lower limit of quantification
TK	Toxicokinetics
PK	Pharmacokinetics
PD	Pharmacodynamics
IV	Intravenous
ELISA	Enzyme Linked Immunosorbent Assay
CID	Collision induced dissociation
Hct	Hematocrit
TDM	Therapeutic Drug Monitoring
AMS	Ambient Mass Spectrometry
API	Atmospheric pressure ionization
Chr	Chromatography
SEM	Standard error of mean
IS	Internal standard
SIL	Stable isotope labeled
ESI	Electrospray ionization
SRM	Selective Reaction Monitoring (precursor to product transition)
$t_{1/2}$	Half-life (time)
m/z	Mass to charge ratio

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Abstract

A significant limitation in performing preclinical studies using small animals is the need for macrovolume blood sampling, a similar situation encountered with newborn or neonates screening. Dried blood spot (DBS) method which requires small sample volume has overcome this limitation. However, the required extensive sample pre-treatment prior to chromatographic separation and detection is a major bottleneck. High throughput analysis is a priority in preclinical drug discovery and clinical settings. Paper spray mass spectrometry which is ideally suited to direct DBS analysis without sample pre-treatment and chromatographic separation prior to mass analysis at ambient environment could provide the required throughput. At present there is no report on the application of paper spray mass spectrometry for preclinical studies or biomarker analysis. The studies described in this thesis were undertaken to evaluate the application of paper spray mass spectrometry for direct analysis of small molecule drugs and biomarker in dried matrix spot samples.

In the first part of the thesis, paper spray mass spectrometry (PS-MS) method was developed and validated. Potential analytical liabilities, such as carryover and ion suppression were systematically evaluated using structurally diverse test compounds. It was observed that ionization suppression was prevalent for the test compounds, but it could be effectively managed by using a stable isotope labeled internal standard (SIL-IS). Carryover was found to be minimal for some compounds and variable for others and could generally be overcome by cleaning the metal clip and inserting matrix blanks in-between samples. The results from the validation study indicated that the PS-MS assay was precise and accurate, obtaining a LLOQ of 1 and 5 ng/mL for sunitinib and benzethonium respectively in dried plasma spots (DPS). The test compound, sunitinib, was stable in all conditions studied and analyte extraction and detection sensitivity could be improved with sufficient off-line or on-line pre-moistening of the dried spot prior to PS-MS analysis after longer storage period. For quantitative bioanalysis, SIL-IS coated PCR tube sampler prepared by evaporation and concentration technique was used to sample microvolume (5 μ L) of plasma, serum and whole blood and the results compared to that of conventional pre-mix method. The tube sampler results were found to correlate well with those from a conventional laboratory pre-mixed procedure and comparable accuracy and precision were achieved. Hence, < 10 μ L blood could be sampled directly from small rodents during preclinical studies. Evaluation of the PS-MS assay to ascertain that it will provide comparable data to LC-MS/MS assay when used for *in vivo* bioanalytical analysis showed that PS-MS assay results correlate well with those from conventional LC-MS system, and comparable accuracy, precision, linearity and sensitivity (LLOQ of 1 ng/mL of sunitinib in DBS) were achieved with analysis speed of about 48-fold higher with sample volume of 5 μ L. In addition, the presence of stable

metabolite in the samples showed no impact on the parent quantitation for the test compound in PS-MS assay. Further application of the PS-MS assay to preclinical pharmacokinetic (PK) study found that, the assay was simple and rapid with overall analysis time of 1 min and a full plasma concentration vs. time profile of sunitinib could be obtained from a single mouse. The assay required small sample volume, thereby reducing the overall cost and the use of animals in the preclinical study. Therefore, PS-MS assay could be a potential valuable tool for high throughput *in vivo* screening of drug candidates.

In the second part of the thesis, DBS method was developed as an alternative sampling method for the analysis of biomarkers in DBS samples and further investigated the direct detection of biomarkers from DBS samples using paper spray method. Hemoglobin Constant Spring (Hb CS) was the selected disease model used for this study, which was among a group of inherited blood diseases that were previously studied in our lab and the potential peptide markers identified using peptidomic approach. A DBS prepared by spotting 10 μ L packed red cell on Schleicher & Schuell (S&S) 903 filter paper, dried for 4 h and punched-out spots of eight taken with subsequent solvent extraction and precipitation with 0.1 % formic acid in acetonitrile was sufficient to detect all Hb CS specific peptide markers using MALDI-MS and nanoLC-MS/MS analysis with comparable results to liquid packed red cell samples of 100 μ L. Stability results also showed that peptide markers were stable in the DBS at 4 °C and room temperature for a period up to 12 months and at 37 °C and 40 °C for 1 month period. Direct analysis of the peptide markers from DBS using PS-MS method proved unsuccessful probably due to the nature of the paper substrate used or low percentage of Hb CS specific peptide markers present in the peripheral blood. However, this sampling method can potentially be used to screen Hb CS disease in newborns, critically ill patients or individuals from remote/resource-limited areas or be coupled to the discovery and validation study.

Chapter 1

1.0 Introduction

1.1 Background

A significant limitation in performing preclinical studies using small animals is the need for macrovolume blood sampling, a similar situation encountered with newborn or neonates screening. In the early stage drug discovery studies, drug candidates are screened using small animal models. Plasma or serum from venous blood utilized to measure analyte concentration demands collection of high blood volume (100-500 μL) at each time point to obtain the sample volume sufficient for the analyte(s) concentration measurement. This makes repeated sampling from small animals impossible due to ethical and regulatory issues ¹. Therefore, in preclinical studies, composite sampling is mostly adopted, but increases the number of animals per study which tend to reduce the quality of data and simultaneously increases associated cost and the quantity of test items required for the preclinical discovery package ^{2, 3}. Similarly, during biomarker discovery and validation using proteomics or metabolomics approaches, larger blood volume ($> 100 \mu\text{L}$) is mostly required for the study making blood collection from neonates/newborn or sick patients almost impossible.

Microsampling coupled with dried blood spot (DBS) represents an alternative blood sampling method for the measurement of circulating analyte(s). Since its introduction, it has been demonstrated for the analyses of several analyte(s) in whole blood, plasma or serum spots, etc. in discovery ², preclinical development ³, and human sampling ⁴. This sampling technique involves microvolume blood collection and spotting on filter paper followed by drying. The DBS samples are then processed by an off-line solvent extraction method prior to chromatographic separation and mass spectrometry (MS) detection. Merits of DBS include use of small sample volume ($< 50 \mu\text{L}$), cost-effective process, analyte stability, improved safety, etc ⁵. However, one of the major limitations of

DBS technique is the required off-line extensive solvent extraction which is usually time-consuming and not suited for the current high throughput-demand environment. Though, further integration of the sample processing into the chromatographic step improved the throughput, the assay still required sample pre-treatment and chromatographic separation prior to MS analysis.

Therefore, direct analysis of DBS samples can provide the required high throughput analysis currently demanded in research or clinical settings ⁶. Paper spray ionization, an ambient ionization method, is most suited for direct MS analysis of DBS samples without sample preparation or chromatographic separation ⁶. It harnesses the many advantages of DBS and the paper substrate is primarily used for the ionization process. In literature, paper spray has been used as ambient ionization method for small molecule analysis in a range of applications including therapeutic drug monitoring ⁷, food safety ⁸, neonatal screening ⁹ and tissue analysis ¹⁰. Potential application of paper spray (PS) ionization in preclinical studies or detection of disease biomarkers is yet to be harnessed. Therefore, we are proposing to extend the application of PS to *in vivo* pharmacokinetic studies and biomarker detection. The first part of the thesis shall look into the evaluation of PS for preclinical study using commercially available small molecule drugs as model compounds. The significance of this research lies in the fact that, microvolume blood collection coupled with direct MS analysis of DBS can be achieved during preclinical studies, which will enable drastic reduction in quantity of test items used and overall cost of the study. It shall also provide high throughput analysis which is a priority in the screening of drug candidates or analogues in the early stages of preclinical studies.

The second part of the thesis shall explore the analysis of biomarkers in DBS and further evaluate the potential application of PS for direct analysis of these biomarkers in DBS using one of the inherited blood diseases, hemoglobin Constant Spring (Hb CS), as disease model. Successful detection of the biomarkers from microvolume DBS will be beneficial to the peptidomic research platform and also could extend its application to clinical settings for diagnostics, newborn screening or population screening in remote/resource-limited areas. Furthermore, the direct MS analysis of these Hb CS

biomarkers in DBS samples using PS method will provide the required high throughput analysis currently demanded in clinical/research settings.

1.2 Dried Blood Spot sampling technique

Traditionally, whole blood/plasma samples for clinical and/or toxicological analyses are generally obtained by venipuncture sampling technique. Ivar Bang, considered as the father of modern clinical microanalysis published the first report of blood spots on filter paper as a laboratory technique almost a century ago ¹¹. And in the 1960's, Dr. Robert Guthrie introduced DBS samples for wider use where he applied it for the screening of phenylketonuria in newborn population ¹². This blood sampling method has since been used for over 40 years for the detection of DNA, viral RNA, antibodies, hormones and biomarkers ^{13, 14}.

Since the introduction of DBS sampling method, it has exhibited many advantages over the conventional venipuncture blood collection method and has since become popular in the clinical and pharmaceutical communities over the last decades. The use of small blood volume (< 50 µL) is one of the major advantages of dried blood spot sampling. Thus, serial blood sampling from satellite animals is made possible in preclinical pharmacokinetics (PK) studies whilst, microvolume blood collection for population based screening programs especially newborn screening is also guaranteed. The method is less invasive and relatively painless ^{15, 16}. The blood sample collection can be easily done in non-hospital environment or by minimally trained technicians ^{17, 18}. It also enables the stability of most analytes in the blood due to the absence of endogenous enzymes in the dried state. Stability of analytes in DBS over weeks and longer periods at different temperatures has been highlighted in a number of publications ¹⁹. Moreover, the sampling technique simplifies storage and specimen shipment without the need for any special care from the point of collection to the analytical laboratories or clinics, thereby reducing the cost substantially ^{20, 21}. The infection risk of DBS samples is comparatively less due to the deactivation of the pathogenic agents and safety of transfer of blood onto the filter paper ^{17, 22}. In addition, these advantages of DBS sampling method make it

attractive for undertaking multisite or multinational research studies in areas where lack of venipuncture logistics, electricity and dry-ice shipping may hamper the research.

Although, DBS was utilized as the sampling method in newborn screening, therapeutic drug monitoring, preclinical studies, etc. the apparent disadvantages associated with this method limited its potential to replace conventional whole blood, serum or plasma collection. There was likely reduction in sensitivity due to the small sample volume used, particularly for analyte(s) at low circulating levels. This problem was compensated for by the introduction of highly sensitive new generation mass spectrometer or tandem MS (MS/MS) with the capability to reach low detection levels²³. Hence, many studies showing the coupling of DBS with liquid chromatography tandem mass spectrometry (LC-MS/MS) have been published recently for the measurement of therapeutic agents or biomarkers in research and clinical settings^{24, 25, 3, 26, 27}.

During discovery of potential drug candidates, DBS cannot be utilized in all situations, since DBS might not be a surrogate for unbound fraction of the drug. Therefore *in vitro* investigation on certain factors such as the magnitude of the blood-to-plasma concentration ratio of drug, drug clearance and kinetics of distribution within blood are carried out prior to the selection of DBS as the preferred matrix²⁸. However, in certain studies, dried plasma or serum spots are utilized to mimic the use of the gold standard matrix, though additional centrifugation may be required²⁹.

The stability of analytes in DBS is one of the major merits of this sampling technique. However, some enzyme labile compounds, especially prodrugs, are unstable during drying³⁰. Therefore, ways to incorporate enzyme inhibitors during sampling are essential but could be a challenge.

Finally, DBS samples are traditionally processed by an off-line solvent extraction procedure which is often time-consuming and not suited to the current high throughput environment⁵. The integration of the sample preparation step to the chromatographic separation as a single process improved the sample throughput; however, sample preparation and chromatographic separation prior to MS analysis is still required^{31, 32, 33}. Direct mass spectrometry analysis by ambient ionization techniques such as paper spray

ionization could potentially enable the required high throughput analysis in the applicable areas ⁵.

1.2.1 Application of dried blood spot in preclinical study

Over the years, DBS sampling technique has attracted the attention of the pharmaceutical industry for preclinical studies of drug metabolism and pharmacokinetics (DMPK) ³⁴. During the early stages of drug discovery studies, a large number of drug candidates or analogues are tested for ADME (absorption, distribution, metabolism and elimination) properties using small animal models. The priority at this stage of the discovery is to rapidly screen for analogues characteristics, rank-order them and eliminate the poor ones. High throughput analysis is therefore a priority at this stage of the discovery studies. The conventional analytical methods use plasma or serum from venous blood sampling as the gold standard matrix for quantitation. However, the sample volume requirement by these analytical methods demand blood sampling volume of >100 µL at each time point from a single animal, making serial sampling impossible from small animals such as mouse or rat due to the limited blood volume permissible to be taken from a single individual animal per day or specific period ¹. Thus, composite sampling is mostly adopted but increases the number of animals per study which tends to reduce the quality of data and simultaneously increases associated cost and the quantity of test items required for the preclinical development package ^{2,3}.

The introduction of DBS technique in early stage preclinical studies has overcome these aforementioned limitations and its application has soared over the last decade enabling serial blood sampling from single small animals whilst complying with the 3R's initiative which aims to reduce, refine and replace animal use in drug discovery studies ^{2, 35, 36}. Moreover, better quality of PK data is ensured due to the use of a single animal ²⁶. Also, the quantity of test items and the overall cost of study are drastically reduced. Therefore, several reports have demonstrated the development and application of DBS sampling technique for small molecule drug analysis in preclinical PK studies ^{3, 37, 38, 39}.

1.2.2 Blood concentration measurements for PK evaluation in preclinical study

Plasma measurements has been used for many years in obtaining concentration-time profile of compound, estimate PK parameters and provide assessment for drug safety and exposure. Total drug measurements from either plasma or DBS comprise the sum of bound and unbound drug; however, it is the unbound drug which is free in the plasma and able to exert pharmacological effect. Due to this assumption, plasma drug concentration measurement is preferred over blood in drug discovery and development ⁴⁰. As stipulated in regulatory guidelines, drug concentration measurements in plasma, serum or blood matrices could be done to represent its exposure ⁴¹. In view of this, DBS can be considered as an alternative viable matrix to measure drug concentration and obtain its PK parameters. However, as reported in literature, there can be a problem in PK interpretation when drug measurement from blood is used over that of plasma ⁴⁰. This problem is particularly prevalent for drugs with preferential distribution into plasma or red blood cells and in situations where the factors (protein binding and hematocrit) which govern the partitioning process are expected in the study population. Therefore, it is advisable to consider the outcome and appropriateness of adopting DBS sampling within the context of the study. However, *in vitro* experiments can be used to explore and address these factors prior to the study in order to evaluate the suitability of using blood to measure drug concentrations.

1.2.3 Application of dried blood spot in research/clinical settings

The various advantages of DBS sampling techniques have also attracted attention in the research /clinical settings for biomarker discovery or screening. Therefore, DBS has been used in population screening; for instance newborn gene screening in pediatric and neonates. Also, through the use of DBS method, sample collection from individuals in remote regions during population-based screening can be achieved. Application of DBS method in peptide-based approach for hemoglobinopathy screening has been reported. For instance, identification of fetal chains of hemoglobin (Hb) was made through the measurement of glycated HbF (GHbF) and acetylated glycated fetal Hb (AcHbF) in dried blood spots ⁴². In other reports, tryptic peptide markers of clinically relevant hemoglobin variants (Hb S, Hb C and Hb E) and β -thalassemia were generated from DBS samples prior to the use of tandem mass spectrometry ^{43, 44}. Population screening for β -thalassemia trait through the quantitative measurement of δ : β -globin peptide ratios as potential surrogate markers of HbA₂ in dried blood spots after tryptic digestion has been reported ⁴⁵. Moreover, ceruloplasmin-specific peptide markers from DBS samples through trypsin digestion for newborn screening of Wilson disease have also been published ⁴⁶.

Peptidome biomarker discovery relies on the hypothesis that changes in proteome as a result of disease or mutation may be determined by the changes in the peptidome. Hence, some recent studies demonstrated peptidomic markers' potential for detection of various diseases ^{47, 48, 49, 50} including type 1 diabetes ⁵¹, ovarian cancers ⁵², breast cancer ⁵³ and kidney disease ⁵⁴. In a previous study in our lab, peptidomic biomarker discovery for inherited blood diseases was conducted ⁵⁵. Peptides that originated from hemoglobin degradation due to protease activity or instability of hemoglobin were identified as potential markers. During the validation studies, peptides with specific mutation sites were considered as markers with ultimate specificity. Hemoglobin Constant Spring (Hb CS) was among these inherited blood diseases that were studied and the targeted peptide markers are shown in Figure 1.1.

1	MVLSPADKTNVKAAWGKVGAGHAGEYGAEALERMFLSFPTTKTYFPHFDLSHGSAQVKGHG	60
61	KKVADALTNAVAHVDDMPNALSALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTP	120
121	AVHASLDKFLASVSTVLTSKYR QAGASVAVPPARWASQRALLPSLHRPFLVFE	173
		[M+H]
		RPFLVFE - 907
		HRPFLVFE - 1044
		SLHRPFLVFE - 1244
		LPSLHRPFLVFE - 1454
		LLPSLHRPFLVFE - 1567
		ALLPSLHRPFLVFE - 1638
		SQRALLPSLHRPFLVFE - 2010

Figure 1.1: The alpha globin chain of Hb CS with a mutated stop codon at position 142. The peptides identified as markers with their molecular masses are listed, all originating from the unstable portion of the alpha globin chain.

A liquid packed red cell sample of 0.1 mL was used during the peptidomic discovery and validation studies. The use of DBS as alternative sampling method will greatly reduce the required blood volume and cost of shipment of the samples in the peptidomic research. It can also extend the potential application of Hb CS-specific peptide markers for diagnostic purposes in clinical settings.

1.3 Hemoglobin Constant Spring as a disease model for DBS studies

Hemoglobin Constant Spring is the disease model that will be used to study the application of dried blood spot sampling technique. It is a non-deletional α -thalassemia caused by the mutation of the $\alpha 2$ globin gene stop codon. Information on the Hb CS-specific peptide markers have been identified already in a previous study in our lab using liquid packed red cell.

1.3.1 Thalassemia

Thalassemia disorder is a heterogeneous group of anaemias characterized by gene mutation that causes absence or reduction in the synthesis of normal structure globin chains^{56, 57}. The classification of thalassemia disorder is based on the affected globin chains thus alpha- and beta-thalassemia. Each of these disorders causes the red cells to become hypochromic and microcytic in nature with varying degree of anaemia⁵⁸. Thalassemia is observed in southern part of Africa, Middle East, Mediterranean and Southeast Asia.

1.3.2 Alpha thalassemia

Alpha thalassemia (α -thalassemia) is used to describe all conditions which cause the reduction/absence of α -globin chains of hemoglobin. The normal hemoglobin contains two α -globin chains located on each chromosome 16. In the diseased state, there is reduction in the expression of the α -globin genes due to either deletional or non-deletional mechanism. Studies have shown that deletion in α -globin chains is the main cause of α -thalassemia disorder in the Southeast Asian populace^{59, 60}.

1.3.3 Hemoglobin Constant Spring

Hemoglobin Constant Spring is a non-deletional α -thalassemia caused by the mutation of the $\alpha 2$ globin gene stop codon (UAA > CAA) at 142nd position resulting in additional 31 amino acids to the normal α -globin sequence as shown in Figure 1.2. The stop codon mutation allows the mRNA to be translated until the next in-phase stop codon, thus generating elongated protein. There are six possible mutations at the 142nd codon able to generate elongated protein, of which five have been observed including Hb CS which is most prevalent among Southeast Asian population with gene frequency

varying between 1 to 8 per cent ⁵⁶. It is also distributed in Southern China and has also been found among individuals of Mediterranean and Middle East origin ⁶¹. The heterogenous Hb CS usually has around 1 % of Hb CS in their blood cells, comparatively lower than the expected 25 % ⁶². Moreover, when mRNA amount in erythroid precursor from bone marrow and matured erythroid are compared, there was significant decreased in the mRNA levels which was not observable ⁶³. These observations indicated instability in Hb CS mRNA, which may be due to the disruption in poly A tail structures ⁶⁴, as alpha globin 3'UTR regions are required for mRNA stability in erythroid cells ⁶⁵. The instability in Hb CS mRNA causes reduction in the rate of the α -globin chain synthesis ⁵⁶, ⁶⁶ in the mutant gene, thus lead to small amount of Hb CS in the peripheral blood making it difficult to detect, especially in heterozygote ⁶⁷.

Figure 1.2: Illustration of HbCS mutation and globin synthesis. Chain terminating mutant in which the normal chain-terminating codon (UAA) had mutated to a glutamine codon (CAA) by a single gene substitution resulting in additional 31 amino acids to the normal sequence.

The interaction of Hb CS with α -thalassaemia-1 leads to Hb H-CS disease which tends to be more severe than Hb H disease caused by triple α gene deletion in adults. The disease patients have lifelong history of haemolytic anaemia, susceptible to infection and

progressive enlargement of the spleen. Patients may also be at risk of portal vein thrombosis due to splenectomy requirements. Hence, patient may be more dependent on transfusion support than the deletion type of Hb H disease ⁶⁸ which may lead to iron overload in most patients due to increase in gastrointestinal tract. Moreover, there is a report on transfusion-dependent Hb H-CS disease or Hb H-CS hydrops fetalis ⁶⁹. However, heterozygote Hb CS is known to be clinically and haematologically normal. Therefore a diagnostic assay to determine Hb CS is essential for genetic counseling and prenatal diagnosis ⁶⁹.

1.4 Blood sampling and preparation of dried blood spots

The DBS sampling commences with the collection of blood from heel or finger punctures using sterile and disposable lancet devices designed to deliver a controlled, uniform puncture that stimulates sufficient capillary blood flow with minimum pain and bruising ^{70, 15}. The skin puncture depth recommended for adults, children and infants are 2.5 mm, 2.0 mm and 1.5 mm respectively ⁷¹. The blood sampling and collection stage is critical for newborn screening program since any errors encountered will ruin the entire program ⁷². Similarly, blood samples can be collected from the tail veins of small animals through incision.

Due to blood sampling through the skin puncture, the collected sample is a mixture of arteriole, capillary and venule blood and may be contaminated with interstitial and intracellular fluids depending on the pressure exerted during sampling ⁷³. The first blood drop is recommended to be wiped away whilst the subsequent blood drops are applied to the filter paper (Whatman 903) to soak through and completely fill it. Care is taken to ensure that the blood uniformly penetrates and saturate the filter paper to provide reliable results ⁷⁴. The indirect approach of DBS preparation involves the collection of blood into a glass capillary tube (containing anticoagulant) and then dispensed onto the filter paper using suction bulb ⁷⁵. In addition, blood can also be collected in anticoagulant containing eppendorf tubes and blood spotting done with pipette.

Proper drying of the blood spot specimen is paramount before storage or transport due to moisture and humidity effects. The blood spot specimen is air-dried for at least 3 h over an open non-absorbent surface at ambient temperature to prevent the effect of moisture and humidity which may cause induction of bacterial growth and also influence elution properties. The drying time depends on the filter paper used, temperature and humidity ⁷⁶. The drying of blood spot is carried out at the absence of direct sunlight and heat sources. Also, stacking of the dried blood spot specimens is avoided and not allowed to touch surfaces in the event of drying. The drying period is also critical for some analyte(s) which tend to degrade, hence, in such situations stabilizers are added to the blood samples prior to spotting.

After drying of the dried blood spots, the samples are stored in re-sealable plastic bags with desiccants and humidity indicator cards. For long term storage, the packed DBS samples are stored in a reliable lab-grade freezer to maintain the integrity of the analytes in the samples ¹⁵. Although the filter paper matrix appears to provide some degree of sample protection against degradation, repeated cycles of freezing and thawing is not advisable ^{77, 78, 79, 80}. Studies have shown that proper care of blood spot specimens can maintain the integrity of analyte(s) in storage for many weeks and years ^{81, 82, 83}. All collected DBS samples are considered as diagnostic specimens for shipment. The properly packed DBS is again kept in another secondary container such as bond envelope or cardboard box ⁸⁴, where it can be shipped either domestically or internationally without any special permit or package requirements and under any circumstance where permit is required for packaged DBS, issued permit is requested from the responsible authority. Factors considered during DBS method development include type of anticoagulant used, hematocrit and punching location, drying and storage conditions, sample volume and type of filter paper ^{16, 85}.

1.5 Processing of DBS samples

The sample preparation procedure for DBS comprises an off-line multi-step process prior to chromatographic separation and MS analysis. It begins with obtaining a punch of

specific diameter from the center of the filter using a micro-punch and cutting mat into a vial. The disc punched from the filter paper provides a volumetric measurement that is similar to the application of a pipette for liquid measurements ¹⁶. The DBS sample is either cut entirely into smaller pieces, ⁸⁶ pre-cut ⁸⁷ or perforated ⁸⁵. Usually, standard micro-punch devices with reproducible diameter are utilized.

Figure 1.3: The schematic diagram of the DBS processing⁸⁸.

The analyte in the punched disc is then extracted with an organic solvent (mostly methanol or acetonitrile) or a mixture of aqueous and organic solvents, usually containing internal standard (IS) ^{89, 90, 91} in case of quantitation. To ensure adequate recovery of analyte from DBS, extensive mixing, agitation or sonication may be required ³⁷. The extract is centrifuged to remove any residues and an aliquot of it subjected to appropriate analytical technique for analysis or the extract taking through further sample cleanup steps such as solid phase and liquid-liquid extraction ^{92, 93, 23} or desalting and concentration using Millipore C18 Ziptip prior to analysis.

1.6 Analytical methods for DBS analysis

DBS samples are compatible with a large number of bioanalytical methods, including chromatography, mass spectrometry, DNA and immunoassays.

1.6.1 Chromatography/mass spectrometry

For the past decades, DBS samples combined off-line or online with chromatography and (tandem) MS has been the major analytical tool for DBS analysis^{94, 95, 96, 97, 98, 99}. One of the disadvantages of DBS technique is the likelihood of the assay to suffer from sensitivity due to the use of microvolume sample of blood, plasma or serum; however, this problem has been compensated for by the use of highly sensitive MS or tandem MS (MS/MS) coupled to liquid chromatography (LC) as a separating method. Over the past two decades, the capabilities of this general method have geared towards the coupling of the DBS sample processing step to the LC system (online) or utilizing two-dimensional (2D) UHPLC^{100, 24, 25, 101, 102} to achieve high throughput. Contrary to the manual offline procedure, the recent improvement integrates the DBS sample processing to the LC-MS system into a single process that enables direct extraction and analysis. However, the desired throughput is not yet achieved on these recent systems due to the requirement for sample preparation and chromatographic separation.

1.6.2 Direct mass spectrometry analysis of DBS samples

Recent capabilities of mass spectrometric systems have enabled direct analysis of the DBS samples without sensitivity problems as mentioned above. Almost a decade ago, direct elution method was reported in literature. Abu-Rabie *et al.* applied thin layer chromatography-MS (TLC-MS) for direct analysis of drugs in DBS with or without chromatographic separation¹⁰³. Surface sampling probe methods were also introduced afterwards. One of them, liquid-extraction-based–sealing-surface -sampling probe (SSSP)

was applied for direct MS analysis of targeted drugs and metabolites in DBS ¹⁰⁴ whilst the second approach, Liquid microjunction-surface sampling probe (LMJ-SP) was used for direct MS analysis of hemoglobin variants in DBS ¹⁰⁵.

Alternatively, DBS samples can be directly analyzed by ambient ionization methods in the open environment without the need for sample pre-treatment and chromatographic separation prior to MS analysis ¹⁰⁶. When interfaced with tandem MS instruments, rapid identification and quantitation can be achieved in short total run time. Thus, these features of ambient ionization mass spectrometry make them particularly suitable for high throughput analysis, e.g. large scale screening and pharmacokinetics/toxicokinetics (PK/TK) studies ⁶. Direct analysis of DBS samples by ambient ionization methods was first demonstrated by Corso and co-workers where they employed atmospheric pressure thermal desorption chemical ionization (APTDCI)-MS/MS to directly desorb and ionize sterols (acylcarnitines) from DBS ¹⁰⁷. Desorption electrospray ionization (DESI) was also successfully used for direct quantitative analysis of xenobiotics in DBS without sample preparation and chromatographic separation by Wiseman *et al.* ¹⁰⁸. Paper spray ionization, particularly suited to direct analysis of DBS and dried spots of tissue homogenates was also introduced by Cooks and co-workers in 2010 and has since been demonstrated to be useful for fast, qualitative and quantitative analysis of complex mixtures ^{109, 110}. For instance, direct MS analysis of a wide range of compounds, from small molecules to large molecules in DBS and dried spots for therapeutic drug and substance abuse monitoring, food safety, neonatal screening and tissue analysis have been achieved ^{7, 8, 9, 10}. Summarized in Table 1.1 are the ambient ionization techniques which have been demonstrated for direct analyses of DBS so far, including paper spray ionization method which will be explained further in this thesis.

While the gold standard approach to DBS analysis still requires punch-out, analyte(s) extraction and analysis by LC-MS/MS, the direct desorption/ionization approach (e.g. paper spray) shows that the direct analyses of DBS samples is possible through the elimination of extensive sample preparation or cleanup and provide the avenue for the development of high throughput analyses (Figure 1.4).

Novel biomarker discovery is vital for elucidating disease states determination, therapeutic response monitoring and determination of prognosis ¹¹¹. Conventional LC-MS/MS has been the most reliable analytical tool in the investigation of biomarkers by approaches such as proteomics and metabolomics over the past decade ¹¹². Regarding the addition of DBS in the LC-MS/MS workflow, direct DBS analysis may be a novel means of supporting the different stages in the biomarker pipeline from discovery to the validation steps ¹¹³. A study by Thomas *et al.* demonstrated the direct desorption of 2 µl dried plasma spots into the MS instrument without pre-treatment or LC separation which enabled a high throughput fingerprinting of phospholipidome in atherosclerotic mice ¹¹⁴. Based on multiple MS-MS experiments and complex data mining, this strategy allowed sample classification between atherosclerotic and control mice and highlighted the discriminated species. Moreover, the feasibility of using paper spray ionization process for direct analysis of peptides and proteins in dried spots on filter paper has been demonstrated ^{109, 115}. However, in literature, there is no report on the application of ambient ionization methods in proteomics or peptidomics¹¹⁶, probably due to the low concentration levels in which these protein or peptide markers exist in the biofluid. Therefore, one of the study aims in this thesis includes the investigation into the direct analysis of Hb CS-peptide markers in dried blood spots.

Figure 1.4: Methods comparison for dried blood spots analysis by conventional LC-MS and ambient MS. AMS: ambient MS, DBS: dried blood spot ¹⁰⁶. (Adapted and modified from ref.)

Table 1.1: Ambient ionization techniques demonstrated for direct MS analysis of dried sample spot (DSS)

Ambient ionization techniques	Acronyms	Analyte(s) in DSS
Atmospheric pressure thermal desorption chemical ionization ¹¹⁷	APTDCI	Sterols ¹⁰⁷
Desorption electrospray ionization ¹¹⁸	DESI	Xenobiotics ¹⁰⁸
Paper spray ¹⁰⁹	PS	Therapeutic drugs ⁷ , illicit drugs ⁸ , sterols ⁹ , proteins and peptides ¹⁰⁹ , ¹¹⁵
Direct analysis in real time ¹¹⁹	DART	Xenobiotics ¹²⁰
Laser diode thermal desorption atmospheric pressure chemical ionization ¹²¹	LDTD- APCI	Xenobiotics ¹²²

1.7 Mass spectrometry

The first report on the use of mass spectrometer was demonstrated in the work of J.J Thompson ¹²³. Currently, mass spectrometry is one of the most sensitive analytical methods. It is used to measure the mass of a molecule. Prior to detection, the analyte in a condensed phase is converted to gaseous state with simultaneous ionization ¹²⁴. After ionization, the ions are transferred into the mass analyzer in a high vacuum environment where they are separated based on their mass to charge ratio (m/z), and their relative intensities displayed in the mass spectrum ¹²³. In addition, the ions can undergo further fragmentation for structural elucidation of a molecule or to determine transition ions to be used in multiple reaction monitoring (MRM) methods. Monoisotopic or nominal mass represent the mass of an ion in MS and it is estimated using the mass of the most predominant isotope ¹²³. As shown in Figure 1.5, a mass spectrometer consists of three basic components, namely, an ion source, a mass analyzer and an ion detector.

Figure 1.5: Block diagram of a mass spectrometer. The sample is first introduced into the ion source for ionization at the gaseous phase to produce ions. Secondly, the ions are transferred into the mass analyzer for separation and after sent to the detector which converts the ions into electrical signal and finally the mass spectrum is displayed on the computer ¹²⁵. (Adapted and modified from reference)

1.7.1 Separation and ionization

In the conventional analytical methods, sample preparation of complex samples and chromatographic separation are carried out prior to MS analysis. After chromatographic separation, ionization proceeds either inside the vacuum MS or at atmospheric pressure by different ionization techniques. Contrary to these ionization techniques, a new trend that enables direct atmospheric pressure ionization of samples without the need for sample preparation or chromatographic separation has been introduced. One of such methods is paper spray ionization which uses the principles of electrospray ionization and the process is explained below.

1.7.1.1 Atmospheric pressure ionization (API)

The main limiting factor which involved the use of a mass spectrometer as a detector was the process of introducing ionized sample into the high vacuum environment required for MS detection. Hence, the invention and utilization of ion sources of greater power and general applicability can be viewed as an essential aspect in MS development. This led to the development of several varieties of ‘soft’ atmospheric pressure ionization (API) methods including electrospray ionization (ESI)^{126, 127}, matrix assisted laser desorption ionization (MALDI)^{128, 129, 130} and atmospheric pressure chemical ionization (APCI)¹³¹. Further progresses in research to curtail the need for sample pre-treatment and chromatographic separation led to the introduction of ambient ionization techniques¹³². These methods operate in open air and enable direct ionization of ordinary/native samples without sample preparation or chromatographic separation.

1.7.1.1.1 Electrospray Ionization

Electrospray ionization method was the first of the API methods and has since been used for the past two decades. It is more suited to analysis of polar to moderately polar compounds¹²³. Due to its inherent soft ionization in the gas phase, they are mostly used for the analysis of large and fragile polar molecules. ESI enables the transfer of ionic species from solution into the gas phase in three step processes¹³³ which comprise dispersal of a fine spray of charge droplets, followed by solvent evaporation and ejection of ion from highly charged droplets as shown in Figure 1.6. ESI ionizes compounds through one of the following processes including formation of adduct in both positive and negative ion modes (e.g., $[M + H]^+$, $[M + Cl]^-$); direct release of intact cations and anions from salts; and deprotonation in the negative ion mode¹³⁴. The ionization principle of paper spray is similar to that of ESI.

Figure 1.6: Electrospray ionization mechanism¹³³. (Adapted and modified from reference)

Briefly, ESI is produced by applying a strong electric field under atmospheric pressure to a liquid flowing through a thin silica capillary which generates a fine mist of highly charged droplets after Taylor cone formation¹²³. The potential difference between the thin silica capillary and the counter-electrode directs these highly charged droplets towards the inlet of the mass spectrometer. The solvent contained in the highly charged

droplets evaporates through the aid of an elevated ESI-source temperature and/or stream of nitrogen drying gas leading to an increase of surface charge density and a decrease of the droplet radius¹³³. The decrease in the droplet radius continues until the Rayleigh limit is reached at which it is kinetically and energetically possible for ions at the surface of the droplets to be ejected into the gaseous phase. Desolvated ions are thus formed after numerous repeat of this process^{133, 123}. The ionization of molecules directly from solution makes it compatible with liquid separation methods such as reverse phase chromatography. Electrospray process can be performed at different flow rates depending on the geometry of the ion source. Moreover, ESI uniquely enables the formation of multiply charged ions species which is beneficial for peptide analysis.

1.7.1.1.2 Matrix-assisted laser desorption ionization

In matrix-assisted laser desorption ionization technique, samples are co-crystallized with an organic matrix on a metal target. Excitation of the matrix is executed by a pulsed laser which causes thermal heating of the molecules and desorption of ions into the gas phase afterwards. The application of pulsed laser in MALDI results in the production of packets of ions rather than a continuous beam, hence, usually coupled with mass analyzers that measure a complete mass spectrum without scanning the mass range or trapping all ions for subsequent mass analysis. Advantages of this technique include the formation of singly charged molecular weight (MW) ion species, tolerate reasonable amount of impurities to be analyzed, rapid process of different samples in an automatic manner, easy re-analysis of samples, simple sample preparation, minimal sample volume, etc.

1.7.2 Separation of ions (mass analyzer) and detection

After the ionization of the sample at the gas phase, the ions are guided into the mass analyzer where they are separated based on their mass to charge ratio prior to detection. There are different kinds of mass analyzers currently available including sector instruments (magnetic and electric), time of flight (TOF), ion traps (3-D quadrupole, linear quadrupole, and Orbitrap ion traps), and Fourier transform ion cyclotron resonance and hybrids. The main characteristics for measuring the performance of mass analyzers are mass limit, transmission, the mass accuracy, the analysis speed, dynamic range, resolution and resolving power, and sensitivity. Orbitraps and TOF analyzers have a high accuracy, high sensitivity and a good dynamic range whilst LTQ have high speed and sensitivity. Hybrid analyzers combine the performance characteristics of the individual analyzers to meet specific purpose. One of these is LTQ-Orbitrap mass analyzers which combine the sensitivity and speed of the LTQ with the high mass accuracy and high resolution of Orbitrap mass analyzers^{135, 136}. As shown in Figure 1.7, the linear trap quadrupole (LTQ, known as linear ion trap) is used as the front end for ion trapping, ion selection, fragmentation reactions and low resolution ion detection, whereas the Orbitrap is used for high resolution ion selection, high mass accuracy ion detection^{137, 138, 139}. It provides a powerful platform for proteomic applications¹³⁶. LTQ-Orbitrap mass analyzer was used to perform most of the experiments in this thesis.

A linear ion trap uses a set of quadrupole rods to confine ions radially and static electric potential on end electrodes to confine the ions axially^{140, 125}. Once the ions are trapped, the amplitude of the RF (radio frequency) voltage is applied that resonates with the frequency of the trapped ions. After this occurs, the ions are ejected in an order based on their m/z value^{141, 125}. The Orbitrap mass analyzer uses electrostatic fields to trap and analyze ions which comprise one central spindle and one outer barrel-like electrode¹⁴². Ions injected into the Orbitrap are electrostatically trapped while rotating and oscillating along the central electrode. Oscillating ions induce an image current signal on the outer electrodes. Image current signals are converted into frequencies by Fourier transformation. The frequencies, which are characteristic of each ion m/z value, are finally converted into a mass spectrum^{138, 143}. They can be operated in two different

modes. In first mode, the Orbitrap mass analyzer acquires the full MS spectra and the LTQ the MS/MS fragment ion, also called FT-IT mode. In the alternative mode, both the MS and MS/MS scans are analyzed in the Orbitrap detector. This mode is referred to FT-FT. In short-gun proteomics experiments, the FT-IT mode is widely employed ¹³⁷.

Once ions are separated by mass analyzer, ions are detected and transformed into usable signal by a detector. Detectors are able to generate a current signal from the incident ions that is proportional to their abundance. The detectors used for MS include electron multipliers, Faraday cups, ion-to-photon detectors, or microchannel plate detectors.

Figure 1.7: Thermofischer LTQ-Orbitrap mass spectrometer ¹³⁷. (Adapted and modified from reference)

1.8 Paper Spray Mass Spectrometry (PS-MS)

Paper Spray (PS) ionization technique is a simple mass spectrometry based method, introduced by Cooks and co-workers in 2010 that combines the characteristic features of both ESI and ambient ionization methods ^{109, 106}. Paper spray has been demonstrated to be useful for fast, qualitative and quantitative analysis of various compounds in complex mixtures ^{109, 110}. For instance, using both PS–MS and PS–MS/MS analysis, a wide range of compounds, from small molecules to large molecules in DBS and dried spots have been analyzed for applications in therapeutic drug and substance abuse monitoring, food safety, neonatal screening and tissue analysis ^{7, 8, 9, 10}.

In PS, dried spots are prepared by spotting few microliters of the sample (whole blood, plasma, urine and tissue homogenates) onto a paper cut into an isosceles/equilateral triangle with sharp tip and allowed to dry for about 2 h. Alternatively, the sample can be loaded onto the paper triangle by incorporating the sample into the spray solvent, transfer from solid surfaces using the paper as a wipe or the sample is blotted from another surface ¹⁰⁹. The paper triangle is positioned in front of the MS with sequential application of solvent and voltage to initiate the ionization of the sample.

The paper spray process is considered as a simplified model comprising three step processes which include extraction, transportation-separation and ionization of the analytes in the raw sample as shown in Figure 1.8. At the extraction step, target analyte(s) together with chemical compounds in the sample matrix are extracted by the spray solvent from the sample (dried blood spot ^{7, 144} or tissue sample ¹⁰) on the paper substrate ¹⁴⁵. Extracted chemical compounds and the analyte(s) are then transported toward the spray tip, driven by solvent migration along the on-paper electric field. Due to the differences in the chemical-substrate interactions, chromatographic separation can occur during transport ¹⁴⁵. Ionization of the analytes in the gas phase occurs through electrospray process after the solvent reaches the tip of the paper substrate. The ions produced are then transferred into the analyzer for MS analysis ¹⁴⁵.

The ionization process of paper spray is highly dependent on the experimental conditions; hence, initial optimization studies for these parameters are essential in paper spray mass spectrometry and are discussed below.

Figure 1.8: Schematic of the paper spray ionization process. Three proposed steps which include extraction, transportation-separation and ionization for the process ¹⁴⁵. (Adapted and modified from the reference)

1.8.1 Experimental conditions that influence paper spray ionization mass spectrometry

1.8.1.1 Paper type and geometry

As in chromatography where the choice of column is critical for the analysis, so is the choice of paper type for paper spray experiments. Extraction and separation of molecules occur in the paper matrix as the solvent moves through. In one study, paper types of different pore sizes were examined. They include grade 1 (11 μm), grade 2 (8 μm), grade 595 (4-7 μm), grade 6 (3 μm), glass microfiber filter paper and chromatography ¹⁰⁹. It was found that, the paper types had different levels of background peaks due to the composition of the paper type. Also, chromatography paper with a thickness of 0.18 mm obtained the best quality spectrum with highest S/N, which was selected for further experiments. This paper type has been extensively utilized for paper

spray analysis. In other experiments, slightly thicker paper types such as ET31 paper (0.5 mm) and grade 4 (0.21 mm) were used. These thick paper types enable larger dynamic range and lower detection limits. Moreover, the affinity of the analyte to a paper substrate also affects the extraction efficiency and causes loss of analyte during transport to the paper tip and inefficiency in ionization. In some of the experiments performed in the literature, modified paper types are used for specific purposes. For instance, silica-coated chromatography paper of 0.27 mm thickness was found to be effective for analysis of small molecules from DBS. This paper type is coated with silica, hydrophilic, which reduces the binding of non-polar small molecules to the paper matrix thereby maximizing their elution and response ¹⁴⁶. In certain applications where the purpose was to detect polar analyte(s) like peptides or proteins, the surface hydrophobicity of the paper type was increased by modifying the hydroxyl groups on the cellulose by perfluoroalkylation to produce better signals for protein hemoglobin ¹⁴⁵.

Furthermore, the geometry of the paper triangle used is another factor that impacts the ionization efficiency of paper spray. The paper tip angle plays a role in spray generation. This was investigated by designing the front end of the paper substrates with different tip angles of 30°, 60°, 90°, 120° and 150°. It was found that the diameter of the spray plume increased as the paper tip angle increased and no spray was observed for the highest tip angle of 150°. Moreover, the spray voltage also increased as the tip angle increased ¹⁴⁷. There is also a relationship between the sample load, the size of the paper substrate and the solvent applied. This was investigated in a study where different blood amounts 0.5, 1.25 and 2.5 µL were spotted on three homologues paper substrates with dimensions of T1 (7.5 mm × 8 mm), T2 (11.9 mm × 12.7 mm) and T3 (16.8 mm × 17.9 mm) bearing a ratio of 1:2.5:5. After drying, spray solvents of 10, 25 and 50 µL in proportion to their area was applied to the paper substrates ¹⁴⁷. It was found that the peak signal intensity increased proportionally with the increase in volume of sample load and paper substrates size. Moreover, with the 1.25 µL sample load, the signal intensity dropped as the size of paper substrate increased due to the larger volume of spray solvent which diluted the blood sample and lowered the signal intensity. An opposite trend was observed for 2.5 µL sample load in which the highest signal intensity was observed for

T3 paper substrate. In addition, when the same sample-to-solvent ratio of 0.5 was used, the larger paper tips yielded much improved signal intensities.

1.8.1.2 Spray solvent, volume and mode of application

The type of solvent applied for the paper spray plays an important role in the analyte elution, transportation and spray ionization ¹⁴⁵. It also depends on chemical nature of the analyte ¹⁴⁶. Therefore, the selected spray solvent should enable elution of the analyte from the paper and enhance the ionization of the analyte. In literature, spray solvent of low surface tension (e.g. pure methanol) is ideal for negative mode experiments whilst methanol/water mixture of high surface tension is normally used for positive mode experiments ^{123, 144}. The addition of water to polar organic solvents like methanol tends to increase its surface tension. The onset spray voltage is proportional to the surface tension of the spray solvent. Also, in other experiments performed in literature, methanol/water/formic acid mixture was used for serum samples whilst acetonitrile/water mixture was utilized for blood samples ¹⁴⁸. The selection of the paper substrate and the solvent system also influence the performance of PS process. In one study, the combination of silica-coated paper and dichloromethane/isopropanol was identified as optimal for the analysis of various small molecule drug types as compared to that of chromatography paper and methanol/water combination ¹⁴⁶. Also, the use of non-polar solvents such as hexane, toluene, dioxane, pentane, and heptane for PS analysis of polar analytes has been reported ¹⁴⁹. The non-polar solvents tend to have small surface tensions than most polar solvents; therefore, experiments were performed with low onset spray voltage to analyze peptides, phospholipids, nucleotides and pharmaceuticals.

Moreover, the volume of the solvent used for the PS affects the size of the sprayed droplets ¹⁵⁰ and therefore, the optimum volume to be applied is crucial. In addition, this parameter is directly related to the size of the paper substrate ¹⁰⁹ as already mentioned. Ample solvent volume is applied to enable stable spray for the required period of time. However, too much spray solvent applied to the paper substrate may affect the signal intensity of the analyte due to poor elution of the analyte ¹⁴⁴. In this situation the solvent

tends to travel over the spotted sample instead of travelling within it to enable analyte elution. Inadequate application of spray solvent will not also be able to initiate the spray. Furthermore, the spray solvent volume is directly related to the spray duration. For instance, application of spray solvent volumes of 5, 10 and 25 μL produced spray signal duration of 30, 60 and 90 s respectively ^{109, 7}.

The mode of solvent application also affects the chemical elution pattern as well as the ion signal intensity ¹⁴⁵. In literature, two modes of solvent application have been described. In one mode, the solvent is deposited at once on the paper substrate whilst the solvent can also be added continuously or in slow wicking mode. In some of the experiments, a well-defined eluted peak was observed for the one time solvent application as compared to the wicking mode ¹⁴⁴. However, in another study, a reverse trend was observed where continuous supply at a controlled speed improved the stability of the ion signal in paper spray ^{149, 151}.

1.8.1.3 MS instrument settings

Similar to ESI-MS experiments where the MS settings are essential for obtaining good detection and sensitivity, so is PS process. MS in-source parameters such as capillary voltage, capillary temperature and tube lens settings are optimized for particular analysis.

1.8.1.3.1 Spray voltage

The typical onset spray voltage for PS is between 2 kV and 3 kV, and relates directly to the surface tension of the solvent, a similar concept well known in ESI ¹⁴⁴. In most experiments described in literature, onset spray voltage of (-3 kV to -3.5 kV) and (3.5 kV to 4.5 kV) was usually used for negative and positive modes experiments respectively ^{152, 10, 8}. Corona discharge could occur at both positive and negative modes where there is an increase in spray current and a reduction or elimination of analyte signal ¹⁴⁴. This occurs

in positive mode if the solvent is not replenished or at high spray voltages whilst in negative mode, it occurs at low spray voltages ¹⁴⁴. In most of the initial paper spray experiments described in literature, the spray voltage was applied before the solvent application ^{109, 110, 10, 144, 152}. However, recent PS quantitation studies have indicated that, reproducible results could be obtained when the spray voltage is applied after the solvent application ^{7, 9}. In a recent reported study, PS process was achieved with zero volt, however, the signal intensity obtained was lower than voltage PS ¹⁵³.

1.8.1.3.2 Position of paper tip to MS inlet

The distance of the paper tip to the MS inlet is important factor which affects the signal intensity and reproducibility. In literature, the paper tip positions to the MS inlet used are 4mm, 5 mm and 1 cm ^{109, 9, 110}. During the PS set-up, the paper substrates are manually positioned to the MS inlet which is likely to result into random and systematic errors.

1.8.1.4 Sample preparation

1.8.1.4.1 Concentration

The paper spray ionization process is concentration dependent with low recovery of the analyte(s) from the paper substrate ¹⁵⁰.

1.8.1.4.2 Matrix effects and ionization suppression

The elimination of sample preparation and chromatographic separation in the PS process exposes it to matrix effects as commonly observed in ESI-MS experiments. In PS study, an analyte was spiked into both blood and clean matrix ¹⁴⁴. The authors observed that, ionization suppression occurred as indicated by a lower analyte signal in blood

relative to the clean matrix. In another study, relative matrix effect was accessed by constructing standard lines in different lots of biofluid ⁷.

1.8.1.4.3 Internal standard addition

In quantitative PS experiments, internal standards, ideally isotopically labeled are added to offset the matrix effect on the analyte (s). One of the analytical challenges presented by the use of DBS rather than liquid plasma or blood is the addition of the internal standard (IS). For PS, the following methods for adding IS to DBS were investigated by Manicke *et al.*; pre-treating the paper with IS prior to blood spotting, spiking the IS into the elution solvent, adding an IS solution to a dried spot ¹⁴⁴. In this experiment, acceptable precision was obtained for all except when the IS was spiked into the elution solvent as compared to the pre-mixed which was used as the control.

Furthermore, there is lack of methods to introduce internal standards to microvolume samples (< 10 µL) e.g. plasma or blood prior to transferring it onto substrates. The introduction of internal standard is particularly important in quantitative bioanalysis. A method using glass capillary coated with internal standard for microsampling purpose has been reported. It is used to collect a small volume (1 µL) of sample with automatic mixing occurring simultaneously within the glass capillary before the sample is transferred onto a substrate to produce dried spot prior to MS analysis. The sampling method was tested successfully with paper spray for quantitation of a pharmaceutical in DBS sample ¹⁵⁴.

1.8.1.5 Data acquisition and analysis

For quantitative PS analysis, selected reaction monitoring mode (SRM) using collision induced dissociation (CID) ^{9, 7, 155, 150, 146, 156} or in only CID ^{147, 109} was performed. In this method of analysis, the signal intensities from the product ions were used in quantitation. Inherently, the signal stability of PS-MS is less than conventional

ESI-MS method ¹²⁵. Therefore, the portion of the ion chromatogram to select for averaging is very essential. The area under the curve (AUC) method is used when a well-defined peak shape is produced consistently ^{7, 150}. This is estimated for each ion of interest by generation of extracted chronograms (EIC). The other method is to use the average intensity of each ion for a particular window when the peak shapes produced are inconsistent ⁷.

1.9 Research objectives

The main goal of this research project was to evaluate paper spray method for direct analysis of small molecule drugs and peptide markers from dried matrix spots (DMS).

The first aim is to evaluate paper spray mass spectrometry (PS-MS) method for *in vivo* bioanalysis of small molecule drugs for preclinical studies. Specific goals include:

- Develop and validate a paper spray method.
- Develop a method for internal standard addition to microvolume matrix and demonstrate its application for direct microvolume sampling.
- Compare PS-MS and LC-ESI-MS/MS method performance for the analysis of small drug molecules and evaluate the potential application of PS-MS assay for preclinical pharmacokinetics study.

Secondly, evaluate the PS-MS method for biomarker analysis using Hb CS as the disease model. Specific goals include:

- Develop DBS sampling method for the analysis of endogenous Hb CS-specific peptide markers.
- Investigate the possibility of applying paper spray technology for direct MS analysis of the peptide markers from DBS samples.

Chapter 2

2.0 Development and validation of Paper Spray Mass Spectrometry (PS-MS) method

2.1 Chapter overview

Paper spray (PS) ionization technique was introduced by Cooks and coworkers which combines the characteristic of both ESI and ambient ionization methods¹⁰⁹. Also, as a substrate spray-based method, ionization and transportation of the analyte(s) in the DBS samples are carried out by applying a voltage to the wet filter paper substrate, positioned in front of a mass spectrometer. Paper spray has been used as ambient ionization method for small molecule analysis in a range of applications including therapeutic drug monitoring, food safety, neonatal screening and tissue analysis^{7, 8, 9, 10}. However, the potential application of PS in preclinical PK studies has so far not been harnessed. PS method has the ability to enable serial blood sampling during PK studies coupled with fast and inexpensive analysis. The conventional analytical method applied in preclinical pharmacokinetic studies is LC-MS/MS interfaced with either ESI or APCI. The stability or efficacy of novel small molecule drugs is evaluated using animal models, where rodents (mice/rats) are mostly utilized at the early stage preclinical studies. But the plasma or serum sample volume required by the analytical methods for drug quantitation is relatively high (> 100 uL) in order to reach the desired sensitivity (for instance 1 ng/mL). Upon availability of high sensitive mass spectrometer detectors and application of microsampling, DBS sampling technique was introduced to curtail this problem. However, it required extensive sample pre-treatment prior to separation and MS analysis.

Current bioanalytical methods applied in preclinical pharmacokinetic studies demand significant amount of time and resources, hence, the need to develop alternative methods. Paper spray mass spectrometry is simply, the direct analysis of dried spot samples without any extensive sample pre-treatment or chromatographic separation, usually

requiring minimal sample preparation only and small sample volume. Therefore, the current study aims to extend PS-MS application in preclinical studies.

The complexity of biological samples such as urine, plasma or whole blood poses significant challenge due to the presence of large amount of interfering components including phospholipids, salt, and proteins. Without sample preparation and chromatography prior to analysis, may cause ion suppression due to the competition for ionization by the analyte of interest and the components in the whole blood resulting in matrix effects in mass spectrometry^{157, 158, 159}. Another potential issue for PS-MS is the carry-over due to the reusable PS metal clip. The paper substrates are placed in-between a metal clip to which a high voltage is applied to enable the movement of the analyte to the MS after prior application of spray solvent which may cause cross-contamination.

Microsampling has recently emerged as successful way to reduce and refine the use of small animals in preclinical PK studies. It has been demonstrated to have the capacity to remove or reduce the need for satellite PK groups and also refine the sampling procedure. Apart from the ethical advantage it serves, it also provides the possibility of obtaining full PK data from a single small animal instead of composite sampling thereby increasing the scientific value^{160, 161}. The microsampling method is compatible with dried blood spot (DBS) sample technique. Even though direct analysis has been made possible by ambient ionization methods such as desorption electrospray ionization (DESI), paper spray ionization, etc. however, internal standard introduction to microvolume samples is one of the key challenges currently facing ambient ionization techniques including paper spray¹¹⁶. Some procedures to introduce stable isotope labeled internal standard (SIL-IS) to the matrix required for quantitative analysis of small molecule drugs have been reported. One way is to introduce the SIL-IS to the matrix prior to spotting. The use of glass capillary tubes for collecting microvolume of blood has been described in literature¹⁶². In a study by Peng *et al.*, a sample volume of 10 μ L plasma for HPLC-MS/MS assay was obtained from a microvolume mouse blood of 30 μ L. They demonstrated the feasibility of serial bleeding in mice and the obtained results were in agreement with the conventional methods³⁶. A recent study demonstrated the use of internal standard coated capillary tubes for sampling micro volume blood and the application of this method was

shown with paper spray analysis ¹⁵⁴. Though these aforementioned methods provide reproducible results; however, they come with their associated disadvantages. They include education and practical training of personnel to use capillary tubes for sampling, required washout and dilution steps, provision of limited information on the assay performance due to limited incorporation of internal standard (IS) with the matrix components and sample paper, and cost. Therefore, alternative method that applies IS to matrix prior to spotting and reduce/eliminate these aforementioned disadvantages will be of great benefit. Therefore, based on the work of Liu *et al.* ¹⁵⁴ and the principle of sample evaporation and concentration, an alternative method was developed. Solvent removal from sample is an important process in conventional bioanalytical methods. The traditional sample pre-concentration procedures involve sample evaporation and concentration step which enables evaporation of the solvent (methanol, acetonitrile, etc) and drying of the sample into a solid form. Centrifugal vacuum concentrators are mostly utilized for this purpose in conventional laboratories. In principle, they induce solvent boiling under vacuum and ensure that the solvent boils from the sample surface downwards to minimize boiling over, solvent bumping, sample loss and cross-contamination ¹⁶³. Removal of solvents from variety of formats such as tubes, microplates and vials can be achieved.

Therefore, this part of the thesis comprises series of experiments conducted to develop and fully validate the PS-MS assay according to U.S. Food and Drug Administration's (FDA) Guidance for industry on Bioanalytical Method validation ¹⁶⁴ and a Conference Report from the workshop held on the same subject ¹⁶⁵. Important validation parameters such as matrix effect, selectivity, accuracy, precision, linearity, sensitivity and stability were established using dried matrix spot (DMS) samples. DMS refers to dried spot samples prepared from plasma, serum or blood. Furthermore, alternative method to add internal standard to microvolume samples was developed and demonstrated for direct microvolume sampling.

The findings of this work have been published. The chemical structures of the model compounds are shown in Appendix 2.

2.2 Experimental section

2.2.1 Materials and equipment

Sunitinib and Benzethonium chloride were purchased from Abblis Scientific Research Chemicals Inc. (Houston, TX, USA). The internal standards, sunitinib-d10 (lot number 593, 96% pure by HPLC) and benzethonium-d7 chloride (lot number 128, 98.6% pure by HPLC) were obtained from Medical isotopes (Pelham, NH, USA). Pipette tips (10 µL) and 0.2 mL PCR tubes were purchased from Axygen Scientific In. (CA, USA). Centrifugal vacuum concentrator 5301 was purchased from B. Braun Singapore Pte Ltd (Singapore). Acetic acid, acetonitrile and methanol (98.9%) were purchased from Merck KGaA, (Germany) and HPLC grade water was purchased from Merck KGaA, (Germany). Dipotassium ethylene diamine tetra acetic acid (K₂EDTA), blank human plasma and rat plasma and serum were obtained from Sigma-Aldrich (Singapore). Deionized water from Sartorius deionization system (Sartorius, Singapore) was used for all aqueous solutions. Whatman Grade SG81 ion exchange (product code: 3668-915), Grade ET31 (product code: 3031-681), Grade 1 (product code: 3001-861) chromatography papers and Grade 1 qualitative filter paper (product code: 1001-125) were all purchased from GE healthcare UK Limited (Buckinghamshire, England). Blood was collected from healthy rats and mice purchased from Invivos (Singapore). Blood collection and experiment were conducted in accordance with the Nanyang Technological University Institutional Animal Care and Use Committee (IACUC).

Table 2.1: General information on the paper types tested

Paper type	Thickness (mm)	Flow rate (mm/30min)	Properties	Principal application
SG 81 ion exchange paper	0.27	110	Surface of cellulose coated with silica	Partition and adsorption separations
Grade ET31Chr	0.5	225	Thick chromatography paper with the highest flow rate	Electrophoresis of large molecules
Grade 1 Chr	0.18	130	Smooth surface chromatography paper	General analytical separations
Grade 1 qualitative filter paper	0.18	-	Standard grade filter paper, 125 mm, circles	Routine qualitative analytical applications

2.2.2 Fabrication of paper spray prototype

The device was made by using a flexible wire connected to metal clip and power source where high voltage was tapped from the MS instrument to the paper substrate. Before the final setup, various designs were made and tested. Improvement was made by including a 3D movement of the platform in x, y, z directions making it able to obtain a good position of the paper substrate on the platform (Figure 2.1A and B). Moreover, the metal clip attached to the flexible wire required manual positioning to the direction of the MS inlet to prevent it from moving away during set up. The platform was further modified by attaching the metal clip directly to the platform and connected to power source within a square box below it (Figure 2.1C). Another problem solved with this new modification was the issue of laboratory air which caused quick evaporation of the spray solvent. Since a lower detection limit was required, an attempt was made to solve it. Two plastic wedges were placed at both side of the platform upon which a tissue paper was placed to cover the upper part of the device. The tissue paper was finally replaced with plastic. Shown in Figure 2.1D and 2.2 is the final modified paper spray prototype platform used for the experiments in this thesis.

Figure 2.1: Paper spray platform modification at different stages. The stage 4 platform was chosen for further works.

Figure 2.2: The paper spray prototype. (A) Closed with plastic case to prevent excessive drying of samples due to the air (B) opened to display the triangular paper fixed to the clipper and directed towards the MS inlet at an angle of 45 °.

Upon obtaining the right design of the paper spray prototype, further studies involving the optimization of paper spray analysis conditions was done.

2.2.3 Preparation of samples

Stock solutions of model compounds and their internal standards (IS) were prepared at concentration of 1 mg/mL in methanol or DMSO.

Dilutions from the individual stocks were made in methanol: water (1:1, v/v) to yield a working stock solution of 50 µg/mL. The working stock solutions of the compounds were then used to prepare calibration standards and quality control (QC) samples in both human and mouse plasma. The calibration standards for sunitinib and benzethonium chloride were prepared by spiking the working stock solutions of the drugs and IS into blank drug free plasma. The concentration of calibrators was 1, 5, 10, 50, 100, 150, 300 and 500 ng/mL. The quality control samples were independently prepared in blank plasma by diluting another working solution in order to obtain 1, 5, 15, 250 and 400 ng/mL. The concentration of the internal standards was 100 ng/mL.

Due to the sensitivity of sunitinib to light, all preparations were made under light protected conditions. All prepared solutions were kept in the dark at -20 °C.

Each dried plasma spot was prepared by spotting a fixed volume of 2 µL spiked plasma onto the tip of a silica-coated paper substrate, excised into an isosceles triangular shape, and allowed to dry at room temperature for 2 h. They were stored at room temperature in a sealed plastic bag containing desiccant or analyzed immediately by PS-MS.

2.2.4 Paper spray ionization and mass spectrometry

An LTQ linear ion trap mass spectrometer (Thermo Scientific, San Jose) was used to perform all experiments using the selected reaction monitoring (SRM) mode to obtain

mass spectra at a spray voltage of 4 kV ^{7, 150, 166}. Capillary temperature of 275 °C was maintained throughout the runs. Data analysis and acquisition were performed using Xcalibur software, version 2.0.7. The precursor/product ion pairs for quantitation of the compounds and their internal standards are listed in Table 2.2.

Paper spray ionization was carried out by using a previously described method with some modifications ^{107, 144, 146}. Silica-coated paper was laser-cut into an isosceles triangle with 9 mm base width × 17.1 mm height, with a tip angle of 30°. About 2 µL of the sample was spotted to the paper tip and dried. Spray solvent composition of methanol: acetic acid (90:0.2) and acetonitrile: acetic acid (80:0.2) was used for DPS and DBS respectively. The solvent was applied continuously through a silica capillary (100 µm ID) on the dried spot with initial flow rate of 200 µL/min for 6 s and then reduced to 15 µL/min to keep the paper moist for the remaining period of analysis before halting the flow. In all experiments, the spray voltage was set at 4 kV. The overall analysis time for each dried spot sample was 1 min.

Table 2.2: Tandem mass spectrometry setting for selected reaction monitoring.

Name of compound	Parent ion (<i>m/z</i>)	Fragment ion (<i>m/z</i>)
Sunitinib	399	326
Sunitinib-d10	409	326
Benzethonium	412	320
Benzethonium-d7	419	320
Imatinib	494	394
Imatinib-d8	502	394
Dasatinib	488	401
Dasatinib-d8	496	406
Nilotinib	530	289
Nilotinib-d3	533	289
Mass-to-charge ratio: <i>m/z</i>		

2.2.4.1 Data acquisition, processing and analysis

Data acquisition was performed using Xcalibur software, version 2.0.7. The area under the curve (AUC) method of quantitation was calculated for each ion, by generation of extracted ion chromatograms of the analyte and the SIL internal standard ⁷.

Paper spray analysis conditions were evaluated based on signal stability, signal intensity and reproducibility of mass spectrum. The signal stability is a measure of ion intensity over a period of time and was assessed using a percent coefficient of variation (% CV). A lower % CV indicates higher stability since least variation is desirable. As shown in Figure 2.3, the average intensity of a selected ion across the entire chromatogram (AUC) is recorded and used for the % CV estimation.

Figure 2.3: Total ion chromatogram. The entire window under the area (shaded portion) selected for spectrum averaging to determine signal stability expressed as % CV.

Calculations were performed with Microsoft Excel 2007 from Microsoft Co. and SPSS statistical packages, version 16.0 for statistical analysis. The graphs were generated with GraphPad Prism, version 5.01.

2.3 Validation studies

The PS-MS assay was validated for bioanalytical application by assessing important performance parameters such as matrix effect, selectivity, linearity, sensitivity, precision, accuracy and stability.

2.3.1 Matrix effect

Evaluation of matrix effect (paper and blood matrices) on the PS-MS assay was studied by analyzing dried spots from pure, whole blood and plasma samples. For dried plasma spot (DPS), test compounds at concentration of 1 $\mu\text{g/mL}$ and their SIL internal standards (0.1 $\mu\text{g/mL}$) were spiked into drug free blank plasma and pure solvent (50 % methanol) respectively. DBS and dried pure spot samples were also prepared at the concentration of 1 $\mu\text{g/mL}$ for sunitinib only using four types of paper types including silica coated paper, Grade ET31 and Grade 1 Chromatography (Chr) papers, and Grade 1 qualitative filter paper. Pooled rat whole blood or plasma was utilized for this experiment.

The effect of the matrix in different lots of blood (DBS) was also evaluated by using different lots of rat whole blood. Seven point calibration curves were developed by spiking calibration standards (1, 5, 10, 50, 100, 250 and 500 ng/mL) into different lots of blood free from drug. The matrix effect was determined by the percent coefficient of variation (% CV) within the slopes of the calibration curves. Samples prepared in the first lot of blood were analyzed first followed by samples from consecutive lots. Slopes of

calibration curves were determined from the non-weighted linear regression analysis after plotting peak area ratios of drug/IS versus analyte concentrations.

2.3.2 Linearity

Experiments to construct linear calibration curves of the compounds were carried out with calibration standards described in Section 2.2.3. Calibration curves were plotted using the ratio of the peak area of the analyte to that of the internal standard for each compound in Microsoft Excel and the data were subject to $1/x^2$ weighted linear least squares using SPSS statistical packages, version 16.0. The linear range was evaluated by determination of the correlation coefficient (R^2) and the acceptance criteria for each back calculated standard was set within ± 15 % deviation from the nominal value (± 20 % deviation from the nominal value for the low limit of quantitation (LLOQ)). Each calibration curve parameters were used to determine the concentration of the unknown samples by back-calculation.

2.3.3 Precision and accuracy

Method qualification for precision and accuracy of drug concentration measurement in pure human and mouse plasma was assessed with the analysis of quality control samples including LLOQ (1 ng/mL). For the intraday accuracy and precision, six replicates of each level of the QCs were analyzed with calibration curve to assess intra-assay precision and accuracy. Inter-day accuracy was evaluated over two analysis days; with two batches on the same day and another batch on a separate day. Each batch contained calibration standards and six replicate of each QC sample. The accepted criteria for each back-calculated quality control samples was set at ± 15 % deviation from the nominal value at each concentration ($20 \pm$ % deviation from the nominal value for LLOQ). The percent coefficient of variation (% CV) for each QC concentration must be ≤ 15 % (≤ 20 % for LLOQ) to be accepted. At least 50 % of the quality control samples

at each concentration must pass this criterion and overall at least 2/3 of the QC samples must pass. Accuracy and precision were calculated using the following equations: $[\text{mean measured concentration} - \text{nominal concentration}] / \text{nominal concentration} \times 100$ and $[\text{standard deviation} / \text{mean measured concentration}] \times 100$ respectively.

2.3.4 Low limit of quantification

The accuracy and precision of the LLOQ was assessed through the analysis of replicate QCs at the LLOQ concentration during the inter-assay precision and accuracy study.

2.3.5 Carryover

The test compounds (sunitinib, benzethonium and imatinib) were spiked into blank rat plasma individually at concentrations of 10 and 500 ng/mL. No internal standards were included in this experiment. Dried plasma spots (DPS) at different concentrations of the test compounds and blanks were prepared on Grade 1 Chromatography paper. The prepared DPS samples were sequentially analyzed by PS-MS/MS as follows: 10 ng/mL, 500 ng/mL and blank.

2.3.6 Preparation of calibration and validation of dried blood spots

The expected hematocrit level in Sprague Dawley rats is within the range of 40 to 50 %¹⁶⁷. A hematocrit level of 45 % was selected for the preparation of control DBS samples to represent the average value expected in the Sprague Dawley rats used in this study¹⁶⁸. Blank rat whole blood was centrifuged at $7000 \times g$ for 4 min and the plasma generated transferred to a clean eppendorf. The red blood cell (RBC) suspension and plasma was mixed in proportions (45:55, v/v) to give whole blood with an adjusted

hematocrit of 45 %. Calibration and validation DBS samples were prepared fresh by diluting 4 μ L of each sunitinib spiking solution with 200 μ L of control rat whole blood adjusted to a hematocrit of 45 %. The final concentration of calibration standards were 1, 5, 10, 50, 100, 200, 500 and 1000 ng/mL. About 5 μ L aliquots of spiked whole blood were spotted onto Grade ET31 chromatography paper and allowed to dry for an hour at room temperature prior to PS-MS/MS analysis.

2.3.7 Evaluation of storage effect on analyte stability and elution from dried matrix spot

The stability of sunitinib on dried plasma spots (DPS) on silica coated paper was assessed by storing the dried plasma spots at concentration of 5, 250 and 400 ng/mL for 24 h and 6 days at room temperature (22-26 °C) and 37 °C. Furthermore, the stability of sunitinib on DBS was also assessed by preparing (5 and 100 ng/mL) DBS samples on Grade ET31 chromatography paper and storing for 9 days and 1 month at room temperature (22-26 °C) and 37 °C. In both experiments, the samples were kept in a sealed plastic bag containing desiccant.

For each QC sample concentration used, six replicate was made and analyzed by paper spray mass spectrometry. The results were compared to those of freshly prepared DBS samples. Stability of analyte was established when the precision of QC concentrations were within ≤ 15 % whilst the accuracy were within ± 15 % of the nominal value after the storage periods. The accuracy and precision were assessed by calculating the percent deviation from the nominal concentration and reported as % bias whilst precision was assessed by calculating the percent coefficient of variation (% CV) for each set of replicates.

2.4 Results and discussion

2.4.1 Optimization of paper spray mass spectrometry conditions

Experimental conditions which influence quantitative paper spray ionization were optimized to achieve a sensitivity of 1 ng/mL in plasma. Model compounds were selected based on their relatively low LOD in dried blood spots reported ⁷. Parameters or experimental conditions reported to have significant impact on PS-MS analysis in terms of its signal duration, signal intensity and sensitivity include the type of paper and its geometry, mode of solvent application, spray voltage, sample amount and type of solvent and composition, etc.^{107, 145}. During the optimization process, optimal parameters were selected based on signal stability expressed as percent relative standard deviation (% RSD) and ion intensity/signal response.

2.4.1.1 Paper substrate, size and dimension

Grade SG81 (Silica coated) paper was selected based on information from literature where a lower background and good sensitivity was reported for small molecule drugs in biological sample ¹⁴⁶. The paper substrate serves as a ‘column’ of separation in paper spray ionization, therefore selection of paper type for a specific purpose is paramount. The selected paper type is coated with silica, hydrophilic, which reduces the binding of non-polar small molecules to the paper matrix thereby maximizing their elution and response ¹⁴⁶. For instance, paper spray analysis was performed for glucosamine, an amino sugar, using silica coated paper as substrate. As shown in Figure 2.4, the signal response of glucosamine in this paper type was the lowest as compared to hydrophobic papers due to its polar nature.

The size and the shape of the paper type have been reported to have significant effect on the spectra and the signal intensity ¹⁴⁷. Based on this information, the paper was cut into an isosceles triangle with 9 mm base width × 17.1 mm height, with a tip angle of 30°.

Figure 2.4: Glucosamine signal response in different paper types. The signal response was lowest in silica coated paper and best in other non-coated paper types. Highly polar glucosamine interacts more strongly to the silica coating on the surface.

2.4.1.2 Spray solvent type and composition

The spray solvent depends on the chemical nature of the analyte of interest and it's responsible for the electrospray at the tip of the paper substrate ¹⁴⁵.

In this experiment, four pure organic solvents including ethanol, acetonitrile, methanol and isopropanol were tested. As shown in Figure 2.5A&B, the signal response produced from methanol was relatively higher with corresponding acceptable variation in A/IS ratios.

The amount of water to be added to methanol was also tested. As shown in Figure 2.5C&D, the highest signal intensity for the test compound was obtained from a spray solvent composed of 90 % methanol and was further selected as the optimal spray solvent.

Figure 2.5: Optimization of experimental conditions for quantitative paper spray mass spectrometry. Effect of spray solvent on (A) signal intensity and (B) the ratio of analyte to internal standard (A/IS) of sunitinib. Effect of methanol percentage in water on (C) the signal intensity and (D) the ratio of the analyte to internal standard (A/IS) of sunitinib. Effect of sample load on (E) the signal intensity and (F) the ratio of the analyte to internal standard (A/IS) of sunitinib.

2.4.1.3 Sample load

Effect of sample load on sunitinib signal intensity and reproducibility was also optimized. Dried plasma spots of volumes 1, 1.5, 2 and 2.5 μL were prepared and analyzed for sunitinib (Figure 2.5 E&F). High signal response with good reproducibility was obtained for the 2 μL sample load and was therefore chosen as the optimal sample load for further studies.

2.4.1.4 Spray solvent volume and mode of application

In this study, spray solvent volume was optimized by applying at once different volumes (5, 10, 20 and 30 μL) of the spray solvent to a 2 μL blood spot on a isosceles paper triangle with dimension 9 mm base width $\times$ 17.1 mm height. The spray solvent volume directly relates to the size of the paper triangle and spray duration¹⁰⁹, hence, 30 μL spray volume produced longer spray duration of about 40 s. The mode of spray solvent application influences the elution pattern of the analytes and also the signal intensity¹⁴⁵. The spray solvent was applied to the paper substrate at once to obtain the optimum spray volume. Continuous supply of the optimum solvent volume at a controlled speed improved the stability and intensity of the ion signal. Therefore, the spray solvent was delivered onto the dried spot on the paper substrate following a sequential solvent application program which initiates with a flow rate of 200 $\mu\text{L}/\text{min}$ from 0.002 to 0.095 min, with estimated spray solvent volume of about 18.6 μL delivered to the paper substrate, referred as A on Figure 2.6. The flow rate was then reduced to 15 $\mu\text{L}/\text{min}$ from 0.096 to 1 min, with estimated spray solvent of 13.6 μL delivered, referred as B on Figure A total spray volume of 32 μL was delivered to the paper substrate within 1 min. This sequential solvent application program was designed to produce gradual solvent introduction to maximize analyte extraction.

Moreover, this mode of solvent introduction enabled similar stable spray duration and spectra to be obtained for analytes and samples; hence, the entire AUC was selected

for averaging. The difference in maximums and minimums of the spectra did not affect the averaging.

Figure 2.6: Sequential solvent application program. The delivery of the spray solvent initiates with a flow rate of 200 $\mu\text{L}/\text{min}$ from 0.002 min to 0.095 min (A) and reduced to 15 $\mu\text{L}/\text{min}$ from 0.096 to 1 min (B). A total spray volume of about 32 μL was delivered onto the paper substrate.

Shown in Figure 2.7 are five total ion chromatograms (TICs) generated by five separate analyses using 32 μL spray solvent sequentially applied to 2 μL dried plasma spot on a paper substrate with dimension 9 mm base width $\times$ 17.1 mm height. The signal was stable throughout the 1 min analysis time and extracted ion chromatograms generated were similar in shape. Therefore, entire area under the curve (AUC) was selected and used for the calculations for the ion of interest.

Figure 2.7: Total ion chromatograms generated from five separate analyses. About 32 μL of spray solvent (90% MeOH, 0.4% acetic acid) was applied continuously onto 2 μL dried plasma spot on a paper substrate with dimension 9 mm base width $\times$ 17.1 mm height. The signal was stable throughout the 1 min analysis time and extracted ion chromatograms generated were similar in shape. Therefore, entire AUC was selected and used for the calculations for the ion of interest.

2.4.1.5 Amount of acid to be added to spray solvent

Additives such as acid or base added to mobile phases are known to improve ionization process and depend on the nature of compounds under study as already established in LC-MS analytical platforms. Formic and acetic acids are commonly used additives for basic compounds whilst ammonium formate is employed for acidic compounds. Since basic compounds are proton acceptors, the presence of acid in the solvent system might enable the protonation of these basic compounds, hence, improve their ionization and sensitivity.

Figure 2.8: Amount of acid added to solvent system. (A) Signal response of sunitinib in 80 % acetonitrile (B) signal response of sunitinib in 90 % methanol (C) signal response of benzethonium in isopropanol: dichloromethane (DCM) (95:5, v/v). An amount of 0.2 % acetic acid was found to be optimal to enhance sensitivity of analytes tested in different solvent systems.

However, the addition of more of these additives could also be detrimental to the ionization of the basic compounds and reduce their ion intensities ¹⁶⁹. Therefore, optimal amount of the additive added to the solvent system is essential. This phenomenon applies to the spray solvent system used for paper spray analysis.

Therefore, the amount of formic or acetic acid added was investigated. The solvent systems selected for this experiment include 80 % acetonitrile (v/v) and 90 % methanol (v/v) for sunitinib, and isopropanol: dichloromethane (DCM) (95:5, v/v) for benzethonium. To these solvent systems were added different amounts of acetic acid ranging from 0.1 to 1 %. As shown in Figure 2.8A&B, 0.2 % acetic acid added to 80 % acetonitrile and 90 % methanol increased the sunitinib ion intensity. Moreover, 0.2 % acetic acid in isopropanol: DCM (95:5, v/v) solvent showed similar increase in ion intensity for benzethonium (Figure 2.8C). The same optimal amount was observed when formic acid was used instead. As seen literature, this current study also shows that addition of additives to the spray solvent systems could affect the sensitivity of some analytes in paper spray process, similar to conventional ESI systems.

2.4.1.6 Spray voltage

This parameter relates to the surface tension of the spray solvent composition, therefore was also optimized. The paper substrate was placed 5 mm from the MS inlet and the spray solvent applied whilst the spray voltage was varied between 3 to 5 kV at 0.5 increments. An optimal spray voltage of 4 kV gave the best reproducibility in both sunitinib and its internal standard (sunitinib-d10) signal intensity as shown in Figure 2.9A&B. The presence of internal standard was able to compensate for the variability during ionization, thereby obtaining acceptable precision within 2.0 to 4.5 % for all spray voltages applied (Figure 2.9C). Moreover, the analyte to internal standard average ratios were 0.48, 0.53 and 0.51 for spray voltages of 3.5, 4 and 4.5 kV respectively, as shown in Figure 2.9C. Applied voltage of 4 kV was selected since the variability in the analyte signal response was comparatively lower.

Figure 2.9: Effect of spray voltage on (A) sunitinib) signal intensity (B) internal standard (sunitinib-d10) signal intensity (C) ratio of sunitinib to sunitinib-d10 signal intensity.

2.4.1.7 Position and angle of inclination of the paper tip to MS inlet

The paper triangle tip was placed at a distance of 5 mm from the MS inlet in all the experiments ¹⁰⁹. Successful positioning of the paper substrate to the MS inlet at this distance is essential due to the potential effect on the assay precision and signal intensity. Hence, by using the MS inlet as the reference, a distance marker (5 mm length mark on piece of straight and hard paper board) was then used to gauge the spray tip position to the MS inlet.

In addition, for better sensitivity, the silica coated paper tip was initially oriented in an inclined position (around 45°) towards the MS inlet (Figure 2.2B). This enabled gravity-driven solvent movement towards the spray tip which could compensate the loss of solvent due to evaporation, thereby enhancing stable sunitinib detection and

sensitivity. This observation was thoroughly investigated with the addition of other paper types such as ET 31 and Grade 1 Chr.

Figure 2.10: Angle of inclination of paper tip to MS inlet. (A) ET31 Chr paper (B) Chr paper (C) silica coated paper. The increase in sunitinib signal response depends on the solvent speed of the paper type when the spray tip is inclined to MS inlet. An optimum angle of 0, 10 and 20° was obtained for ET 31, Grade 1 and silica coated paper respectively.

All the paper types were cut into isosceles triangle of dimension 9 mm base width × 17.1 mm height. Each paper type was positioned at different angles (0, 10, 20, 30 and 40 °) to the MS inlet and the same solvent delivered to the plasma spot on the paper in sequential mode with a constant flow rate, as described in Section 2.4.1.4. These paper types have been used in paper spray experiments reported in literature and therefore selected for this study. Figure 2.10 shows the signal response of sunitinib in dried plasma spots on paper substrates of variable thickness and flow rate. As shown in Table 2.1, Grade ET31 paper has a thickness of 0.5 mm with a flow rate of 225 mm/30 min whilst silica and Grade 1 Chr papers have 0.27 and 0.18 mm thickness with flow rates of 110 and 130 mm/30 min respectively. The flow rate or solvent speed is defined as the time

required at 26 °C for water: phenol solvent to ascend to a specific distance within the paper type^{170, 170a}. The solvent speed is shown in increasing order for the paper types: ET 31 > Grade 1 > silica. Hence, there seems to be a relationship between the inclined angle and the solvent speed of the paper type. As the solvent speed increases, the optimum angle to the MS inlet decreases. This means the signal response for sunitinib tend to decrease when the spray tip of ET 31 paper with the highest flow rate/solvent speed is inclined to the MS inlet. Grade 1 paper with second highest solvent speed enabled improvement in sunitinib signal when inclined to 10 ° whilst silica coated paper with the smallest solvent speed produced greater response in sunitinib when inclined to 20 °. This could probably be due to the high solvent flow rate at the spray tip which causes the ejection of large droplets from the tip without proper evaporation. Therefore, the increase in sunitinib signal response could depend on the solvent speed of the paper type when the spray tip is inclined to MS inlet. The optimum angle for the paper substrates was 0, 10 and 20° for ET 31, Grade 1 and silica coated paper respectively.

2.4.1.8 Position to spot plasma sample on paper substrate

The position to spot the plasma samples on the paper triangle substrate was also investigated. The samples were spotted at the center and tip of the paper substrates in two different experiments. As shown in Figure 2.11A, the spotting of the plasma samples at the tip of the paper triangle obtained better signal stability for the test compound. In most of the reported studies, samples were spotted at the center of the paper substrate^{147, 144, 7}. This is based on the principle for better dissolution and extraction of the analyte from the paper substrate and then move towards the paper tip for electrospray. Hence, better signal stability and high intensities obtained for sunitinib and its internal standard show that dissolution and extraction of analyte(s) at the spray tip was sufficient to enable efficient ionization of the sample.

Moreover, as shown in Figure 2.11B, the signal intensity of sunitinib was higher when the sample was spotted on the surface of paper substrate which confirmed well with that in literature¹⁴⁶. The surface of the paper is coated with silica to decrease the analyte

to paper matrix binding of non-polar compounds such as sunitinib and provide a smooth surface for the analyte to be eluted efficiently. The back of the paper is non-coated.

Figure 2.11: Optimization of sample spot position on the paper triangle. (A) Middle vs. tip (B) surface versus back. Higher signal intensity and stability was achieved when the sample was spotted at the surface and tip of the paper triangle.

2.4.1.9 Quantitation

2.4.1.9.1 Internal standard addition optimization

The effect of internal standard (sunitinib-d10) on sunitinib signal response was investigated by spiking blank rat plasma at two different concentration levels. Figure 2.12A shows the result of sunitinib at 1000 ng/mL in the presence of internal standard (IS) concentrations of 100 ng/mL and 500 ng/mL. The signal response for sunitinib was higher at the presence of 100 ng/mL internal standard (IS) concentration with lower variability of 10.6 %.

The mode of internal standard (IS) introduction into the plasma sample prior to spotting was also optimized. In this experiment, the IS was either pre-mixed with the plasma samples prior to spotting or pre-spotted onto the paper substrate. As shown in Figure 2.12B, the plasma sample spiked with 250 ng/mL sunitinib and pre-mixed with 100

ng/mL of sunitinib-d10 obtained the best reproducibility as compared with the pre-spotted method.

Figure 2.12: Internal standard optimization. (A) Concentration to be added (B) mode of internal standard introduction. Signal intensity of sunitinib (1000 ng/mL) in the presence of 100 ng/mL IS concentration was higher whilst the mode of IS introduction with acceptable accuracy and reproducibility was pre-mix.

This could be due to the poor dissolution and extraction of the IS from the paper. In addition, standard curve plot using the pre-mix method showed good linearity with acceptable precision and accuracy for the calibration standards as compared with that of pre-spot method. Therefore, based on this information, method performance will be assessed using an internal standard concentration of 100 ng/mL with pre-mix method of introduction. Alternative procedure to introduce internal standard to blood samples that could enable direct microvolume sampling from study animals will be explored.

2.4.2 Summary of experimental conditions optimization studies for quantitative paper spray mass spectrometry

After the fabrication of the paper spray (PS) prototype device, the experimental conditions which influence PS ionization were investigated. The optimal parameters obtained are summarized in Table 2.3., and were used for dried plasma or blood spots experiments. DPS samples were used for the optimization process; hence, any change made during DBS experiments was noted during the discussion.

Table 2.3: Summary of the optimal conditions for paper spray mass spectrometry.

Parameter	Optimal
<i>Paper</i>	
Type	Grade SG81 paper (Silica coated)
Dimension	Isosceles triangle, 9 mm base $\times$ 17.1mm height
Angle of spray tip	30 °
Distance from MS inlet	5 mm
<i>Spray solvent</i>	
Composition	90 % Methanol: 0.2 % acetic acid
Volume	32 μ L
Mode of application	Continuous through silica capillary
<i>Sample load</i>	
Volume	2 μ L
Position spotted	Surface and tip
<i>Applied spray voltage</i>	4 kV
<i>Internal standard</i>	
Concentration	100 ng/mL
Mode of introduction	Pre-mix

2.4.3 Method performance

Following the development of PS-MS method, the assay was validated using U.S. Food and Drug Administration's (FDA) Guidance for industry on Bioanalytical Method validation ¹⁶⁴ and a conference Report from the workshop held on the same subject ¹⁶⁵. Other tests specific to DBS analysis such as the influence of hematocrit on analyte recovery were performed to provide an assessment of the developed method for sunitinib quantification and its suitability for application to preclinical PK studies.

2.4.3.1 Matrix effects and ion suppression

Matrix effect impacts important validation parameters such as precision, selectivity and sensitivity of the analytical method ^{159, 158}. The need to assess it during method development and validation has been included in both U.S. Food and Drug Administration's (FDA) Guidance for industry on Bioanalytical Method validation ¹⁶⁴ and a conference Report from the workshop held on the same subject ¹⁶⁵. In most cases, signal suppression is often observed in MS based techniques which can have profound effect on the detection sensitivity ¹⁷¹.

To assess suppression or enhancement of the paper substrate and plasma components on the detection of the test drug compounds, the AUCs of each compound in the plasma spots were compared to those at the same concentration (1 µg/mL) in pure samples prepared in methanol: water, (50:50, v/v) when spotted on silica coated paper. Test drug compounds such as nilotinib, dasatinib, benzethonium, imatinib and sunitinib were examined in dried plasma (DPS) samples prepared from silica-coated paper. Figure 2.13 demonstrates the matrix effect on peak area responses of each compound. The variability of suppression among the compounds in both pure and plasma samples were the same ($\leq 15\%$). Moreover, the magnitude of suppression when comparing the peak area responses for each of the compounds in DPS and pure samples varied among the compounds. For instance, the signal suppression due to the plasma matrix for dasatinib was as large as 10 fold whilst that of imatinib was lower.

Figure 2.13: Matrix effect evaluation in dried plasma spot. Log signal responses (AUCs) for the test compounds on in dried plasma spot (DPS) (brown) and methanol/water (50:50, v/v) (cream). The concentration of each analyte is 1 μ g/mL.

2.4.3.2 Linearity and LLOQ

All procedures used for qualification requires the study of the linearity range, therefore calibration curves were prepared. The standards were analyzed by PS-MS in SRM mode using the conditions stated earlier. The calibration curves were linear in both human and mouse plasma over a concentration range of 1 – 500 ng/mL for sunitinib and 5 – 500 ng/mL for benzethonium in human plasma. The correlation coefficients (R^2) were constantly > 0.99 with reproducible calibration curves between days for the small molecule drugs. Moreover, the back-calculated concentrations of the calibration standards had less than $\pm 15\%$ deviation from the nominal values ($\pm 20\%$ for LLOQ)

therefore, met the requirement. The linear range of the method for sunitinib was considered acceptable for pharmacokinetic studies.

The LLOQ of the method was established by spiking blank plasma with sunitinib and benzethonium at the concentration of 1 ng/mL and analyzed with the QC samples during the inter-assay precision and accuracy study. The precision of the back-calculated concentration of the mean value was less than 20 % and the mean percent deviation was also within ± 20 % of the nominal value. Furthermore, in each run, at least 50 % of the back-calculated concentrations of the LLOQ met this requirement. For benzethonium, the back-calculated concentration of the LLOQ in human plasma did not meet the criteria, obtaining accuracy between -36.7 to 46.5 % and precision between 2.2 to 42.6 % as shown in Table 2.5. Therefore, LLOQ for benzethonium in human plasma was established at 5 ng/mL. A similar procedure was used to establish the LLOQ in both human and mouse plasma using sunitinib. The LLOQ of sunitinib was established at 1 ng/mL in both plasma samples as indicated in Table 2.4.

2.4.3.3 Precision and accuracy

The precision and accuracy studies were carried out by spiking both sunitinib and benzethonium in human plasma and only sunitinib in mice plasma at four levels of concentrations (number of replicates (n) = 6) and analyzed at three separate analytical batches. For sunitinib, the intra- and inter-day precision of the method in both matrices was between 11.7 to 1.1 % and the accuracy for the concentration levels for sunitinib was between -11.4 to 19.0 % of the nominal value (Table 2.4). For benzethonium, the precision of the method in the standard human plasma was between 14.2 and 2.2 % and the accuracy was between -14.2 to 13.0 % (Table 2.5). The precision and accuracy of the method met the acceptance criteria and were therefore in agreement with FDA guidelines. This indicates the suitability of the assay in terms of precision and accuracy for bio-analytical purposes.

Table 2.4: Intraday and Interday accuracy and precision data for sunitinib in human and mouse plasma (n=6).

Nominal concentration (ng/mL)		Human plasma				Mouse plasma			
		1	5	250	400	1	5	250	400
Run 1	Mean conc. (ng/mL)	1.2	4.9	234.0	408.5	1.1	5.0	260.4	416.0
	Accuracy (%)	19.0	-1.4	-6.4	2.1	8.3	1.0	4.1	4.0
	Precision (CV %)	11.7	3.0	1.5	3.6	6.1	3.5	2.6	2.6
Run 2	Mean conc. (ng/mL)	1.1	5.6	247.3	444.6	1.1	5.3	265.8	409.2
	Accuracy (%)	10.9	11.9	-1.1	11.2	13.2	6.0	6.3	2.3
	Precision (CV %)	4.9	5.0	3.3	2.3	7.9	3.3	1.7	1.9
Run 3	Mean conc. (ng/mL)	1.0	5.2	221.5	395.3	1.1	5.3	258.2	422.8
	Accuracy (%)	-0.1	3.8	-11.4	-1.2	5.5	5.7	3.3	5.7
	Precision (CV %)	5.8	3.1	1.2	1.8	5.4	3.8	1.6	1.5
Interday									
	Mean conc. (ng/mL)	1.1	5.2	234.3	416.1	1.1	5.2	261.5	416.1
	Mean accuracy (%)	9.9	4.7	-6.3	4.0	9.0	4.2	4.6	4.0
	Overall precision (CV %)	7.5	3.7	2.0	2.6	6.5	3.5	2.0	2.0

Table 2.5: Intraday and interday accuracy and precision data for benzethonium in human plasma (n=6).

Intraday		human plasma				
Nominal conc. (ng/mL)		1	5	15	250	400
Run 1	Mean conc. (ng/mL)	0.6	4.5	15.5	235.1	379.0
	Accuracy (%)	-36.7	-10.0	3.4	-6.0	-5.3
	Precision (CV %)	20.7	8.3	3.0	14.2	4.0
Run 2	Mean conc. (ng/mL)	1.5	5.5	17.0	214.5	388.1
	Accuracy (%)	46.5	9.0	13.0	-14.2	3.1
	Precision (CV %)	42.6	4.0	4.0	3.1	2.2
Run 3	Mean conc. (ng/mL)	1.5	5.3	16.9	231.0	387.6
	Accuracy (%)	45.3	6.9	12.6	-7.6	-3.1
	Precision (CV %)	17.6	2.7	5.7	6.8	2.6
Interday						
Overall Mean conc. (ng/mL)		1.2	5.1	16.5	226.9	384.9
Mean accuracy (%)		18.4	2.0	9.7	-9.3	-3.8
Overall precision (CV %)		26.9	5.0	4.2	8.0	2.9

2.4.3.4 Carryover

Carryover is assumed to be significant in paper spray ionization process due to the reuse of the same metal clip to hold each paper substrate and the subsequent wetting with the spray solvent. In the previous study, the possibility of cross-contamination was averted through the use of blanks in-between samples. Therefore, to evaluate carryover on PS-MS system, plasma samples of the test compounds at relatively high concentration (500 ng/mL) were analyzed after low concentration plasma samples (10 ng/mL) followed by blank plasma samples using the same metal clip. In all the analysis, 8 replicates were made for each. The AUC observed in each of the blank samples were compared with the mean AUC of the low concentration (10 ng/mL, n=8) plasma samples that were analyzed before the high concentration samples. Any value higher than 20 % of the lowest concentration would be considered significant carry over ¹⁷².

Of the three compounds tested, benzethonium and sunitinib showed no significant carry over whilst imatinib demonstrated some level of carryover as listed in Table 2.6. Sunitinib showed a minor AUC (4.6 %) in the first blank, and the AUC soon decreased in the subsequent analysis up to the eighth blank with the lowest AUC area of 0.7%. For benzethonium, a rather different trend was observed. It started with a minor AUC of 8.1 % in the first blank which then changed to an undulating fashion in blank samples. However, at the eighth blank, all the AUCs were lower than 20 % of the low concentration samples. Imatinib showed significant carryover in all the blank samples with the exemption of the seventh blank which showed an AUC of 14 %. Even at the eighth blank, it still showed AUC higher than 20 % of the low concentration samples. This demonstrated that carryover on PS-MS could be minimal for some compounds even when the same metal clip is utilized.

By using the same metal clip without cleaning or running blanks, we present here the worst case scenario where carryover was manageable for the test compounds, even without changing the metal clip or using disposable cartridges which may add to the total cost of analysis ¹⁴⁷. Due to the fast analysis time, extra blanks can be inserted after high concentration samples to manage the carryover without adding much extra analysis time.

In the method validation, blanks were included in sunitinib and benzethonium samples to reduce any possible cross contamination. However, it has been shown in this experiment that even without the insertion of blanks in-between samples, both compounds did not show significant carryover. Moreover, for certain compounds that tend to show high or persistent carryover, cleaning of the metal clip before each analysis plus blanks or disposable cartridges could be used to potentially reduce the cross contamination.

Table 2.6: Carryover of test compounds in blanks following analysis of high concentration samples (% of the mean AUC of 8 analyses of 10 ng/mL samples).

Blank no.	Benzethonium	Imatinib	Sunitinib
1	8.1	34.8	4.6
2	1.4	28.2	2.7
3	8.0	26.1	0.9
4	5.4	28.5	1.5
5	9.6	27.0	1.3
6	17.2	24.7	1.2
7	2.0	14.1	1.4
8	3.4	30.3	0.7

2.4.3.5 Validation of dried blood spot

The PS-MS assay, previously validated according to FDA guidelines for DPS, was also validated for dried blood spots (DBS). The adoption of DBS sampling instead of DPS during preclinical studies could eliminate the need for sample processing at the site of blood collection and enable direct spotting to the paper. Therefore, additional DBS validation steps such as effect of hematocrit, blood-to-plasma partition, matrix effect and stability will be evaluated to ascertain if this sampling method can be a suitable matrix for the measurement of sunitinib during preclinical PK studies.

2.4.3.6 Hematocrit

Hematocrit (Hct) which is a measure of the proportions of blood that constitutes RBC has the potential to affect drug concentration when DBS sampling is adopted¹⁷³. Its effect directly relates to the viscosity and the resulting spreadability of blood on cellulose based paper. For methods which require a disc to be taken from within a DBS sample, the uneven spread of blood due to Hct effect can impact the accuracy of the concentration measurement⁸⁸. Hct range varies according to age, gender and species¹⁷⁴. For healthy male and female rats, it is 34 to 57 %, depending on age. As observed in many other species, females have lower Hct than do males and this feature is generally true for various breeds and all ages¹⁶⁷.

Since adult rat was used for this study, Hct levels of around 40 to 50 % are expected^{167, 168}. The evaluation of Hct on the assay performance was performed at 50, 200 and 1000 ng/mL in replicates (n = 4) using 5 μ L blood spots with an adjusted Hct of 40, 45 and 50 %. The whole blood was separated into RBC and plasma components as previously described in Section 2.3.6 and recombined in correct proportions to give bloods of differing Hct percentage. The prepared DBS samples were directly analyzed with PS-MS/MS assay. Calibration standards were prepared with 45 % Hct blood and linear regression equation used to determine the concentrations. Results were considered acceptable within the Hct range tested if the mean concentration of the spiked samples had a percent RSD of ≤ 15 % and mean accuracy (RE %) within the range of ± 15 % of the nominal value.

As shown in Table 2.7, the results from the Hct effect evaluation gave accuracy and precision values within the pre-defined limit of ± 15 % and ≤ 15 % at all Hct levels for each tested sunitinib concentration. Unlike in other methods where a disc is removed from within a DBS sample, the entire DBS spot on the Grade ET 31 Chr paper was utilized during paper spray process which might be the reason for this positive correlation. Winogradova *et al.* studied the effect of Hct on sunitinib measurements over a hematocrit range of 20 to 60 % with normalization at 40 %. The study showed no Hct influence on sunitinib accuracy at all levels¹⁷⁵.

Table 2.7: Hematocrit effect on the accuracy (RE %) of sunitinib measurements presented as the difference from the analyte/IS peak area ratio at the 45 % hematocrit level. Precision (CV %) are indicated, n = 4.

Nominal conc. (ng/mL)	Hematocrit					
	40 %		45 %		50 %	
	RE %	CV %	RE %	CV %	RE %	CV %
50	-0.2	4.7	Normalized	5.7	3.7	4.9
200	-1.1	1.4	Normalized	2.2	2.1	3.2
1000	6.4	1.6	Normalized	1.4	2.5	1.1

2.4.3.7 Red blood cell association

The measurements of drug concentrations in both plasma and DBS is the sum of bound and unbound fractions, however, it is the unbound drug or free fraction in plasma that exerts a pharmacological effect ⁴⁰. The relationship between bound and unbound drug in plasma and RBCs is shown in Figure 2.14.

Figure 2.14: An illustration of drug analyte distribution in whole blood.

Due to the difficulty in measuring unbound drug, majority of drug assays either from plasma or whole blood matrices measure the total concentration (unbound plus bound drug). Hence, in most cases, the total drug concentration is measured and considered a surrogate for the unbound drug. At equilibrium, the total plasma drug concentration (C_p), total blood drug concentration (C_b) and unbound drug concentration (C_u) are related by the equations below ⁴⁰.

$$C_p = \frac{C_u}{f_u} \quad \text{Equation 1}$$

$$C_b = \left[1 - \frac{H}{f_u} + H \times \rho \right] C_u \quad \text{Equation 2}$$

Where, H = hematocrit

f_u = fraction unbound in plasma; ρ = red blood cell to plasma ratio

In plasma, the total drug concentration (C_p) is proportional to the unbound drug concentration (C_u) if the fraction unbound in plasma (f_u) remains constant. Hence, factors which may alter the extent of plasma protein binding would need to be considered when using plasma. Likewise, in blood, total blood drug concentration (C_b) directly relates to the unbound drug concentration (C_u) provided the fraction unbound in plasma (f_u) does not change and remains constant. Under these conditions, either blood or plasma could be used to measure drug concentrations. However, measuring total blood drug concentration (C_b) to represent the unbound drug concentration when there is variability in these factors has the potential to affect PK and PD data interpretation. Hence, to adopt DBS, it is necessary to understand the distribution kinetics of a drug in whole blood ⁴⁰. The idea of blood-to-plasma ratio is vital for understanding the distribution of a given drug between RBCs and plasma. It can also be used to predict the variable (H , f_u , ρ) likely to govern the ratio of total drug concentration to unbound drug concentration in plasma or whole blood and the suitability of each respective matrix.

Furthermore, this value is essential in situations where comparison or conversion between plasma and whole blood drug concentration data is required ⁸⁸.

To evaluate the partitioning of sunitinib into RBCs, a conventional *in vitro* method was performed using spiked rat whole blood ^{176, 177, 88}.

The rate of partitioning was initially evaluated to determine equilibrium time between RBCs and plasma under controlled conditions. The determined time period was then used to evaluate the extent of partitioning (ρ) with changes in sunitinib concentration.

2.4.3.8 Time to reach equilibrium

Whole unadjusted blood of hematocrit 45 % was spiked with sunitinib to produce a single concentration of 250 ng/mL and incubated at 37 °C with gentle agitation for 60 min. At 0, 1, 15, 30 and 60 min, a 50 μ L aliquot was removed to determine the blood-to-plasma ratio. Whole blood was spotted onto ET 31 Chr paper in replicate to produce 5 μ L DBS samples and the remaining whole blood was centrifuged at 7, 000 $\times$ g for 2 min. The plasma generated was applied onto ET 31 Chr paper in replicates to produce 5 μ L dried blood spot (DBS) samples. Once dried, the DBS samples were directly analyzed by PS-MS assay. The equilibration was selected when no further increases in blood-to-plasma ratio were observed ⁸⁸. The blood-to-plasma ratio was calculated by the equation:

$$\text{Blood-to-plasma ratio} = \frac{PAb}{PAp} \quad \text{Equation 3}$$

Where PAb = Peak area ratio of the analyte to the *IS* in blood, PAp = Peak area ratio of the analyte to the *IS* in plasma. *IS* = internal standard

Table 2.8: Effect of incubation time on blood-to-plasma ratio of sunitinib

	Time (min)				
	0	1	15	30	60
Blood-to-plasma ratio	0.87	0.91	0.90	0.89	0.89
SD	0.09	0.05	0.07	0.02	0.04
Precision (CV %)	10.09	5.64	7.62	2.08	4.21

As observed from Table 2.8, there was increase in blood-to-plasma ratio between 0 and 1 min after which no further increases were observed. Sunitinib seems to rapidly reach equilibrium between plasma and blood cells within 1 min.

2.4.3.9 Extent of red blood cell partitioning

The time to reach equilibrium of drugs between plasma and blood cells and the distribution of drugs to blood cells are important in PK studies ¹⁷⁸. The extent of RBC partitioning was evaluated using blood spiked with sunitinib working solution to produce concentrations of 50, 200 and 1000 ng/mL. The liquid blood samples were incubated at 37 °C for 30 min (pre-determined time) with mild gently agitation. After the incubation period of 1 min, 5 µL DBS and DPS samples of replicate (n = 3) were prepared for each concentration and allowed to dry at room temperature prior to PS-MS/MS analysis. The whole blood-to-plasma ratio was estimated as described in Section 2.4.3.6 and the equation below was used for the RBC association calculation ¹⁷⁹.

$$\text{Fraction of drug in red blood cells} = \left[1 - \left(cp \frac{(1-H)}{cb} \right) \right] \quad \text{Equation 4}$$

Where C_b = blood drug concentration, C_p = plasma drug concentration, H = hematocrit

By inserting into the equation hematocrit value of the control blood (packed cell volume, 0.45) and calculated concentration, and multiplying the fraction with 100 to obtain the percentage association value, the equation now becomes:

$$\text{Fraction of drug in red blood cells} = \left[1 - \left(cp \frac{(1-0.45)}{cb} \right) \right] \times 100 \quad \text{Equation 5}$$

The extent of RBC partitioning could be influenced by chemical nature of the compound, pH, temperature, concentration and drug lipohilicity¹⁷⁶. Sunitinib is a lipophilic compound ($\log P$, 5.2) and therefore would be expected to permeate RBCs¹⁸⁰. From Table 2.9, the blood-to-plasma ratio determined for sunitinib was close to one indicating a degree of association with RBCs. *In vitro* studies in mice showed sunitinib preferentially partitioned into RBCs but no solid conclusion was made for that of rats due to discrepancy in the *in vitro* and *ex vivo* data. However, based on their available data the authors speculated that sunitinib might distribute equally between plasma and RBCs or preferentially uptake into RBCs¹⁸¹. The observation from this current study shows that there is an equal distribution of sunitinib between plasma and RBC components of whole blood.

All tyrosine kinase inhibitors are known to exhibit similar pharmacokinetic characteristics and are highly bound to proteins¹⁸². In certain situation, a decrease in albumin concentration resulted in decrease in protein binding and therefore an increase in an unbound fraction of the drug. In an *in vitro* protein binding study by Toyoma *et al.*, the investigators observed that 65 % of sunitinib bound to proteins, mainly albumin in the plasma component of whole blood and that the protein binding kinetics for sunitinib was linear in plasma. Moreover, the unbound fraction in analbuminemic rats was significantly larger than that of SD rats without significant differences in the unbound plasma concentration between SD and analbuminemic rats¹⁸³.

Table 2.9: Concentration effect on the extent of sunitinib partitioning

	Nominal conc. (ng/mL)		
	50	200	1000
Blood-to-plasma ratio	0.93	0.99	0.95
SD	0.03	0.06	0.04
Precision (CV %)	3.7	6.3	3.8
RBC association (%)	41.0	44.4	41.8

In vitro protein binding study for another tyrosine kinase inhibitor imatinib and its active metabolite CGP74588 produced contrary results to sunitinib in terms of the unbound fraction. It was found that 65 % of imatinib bound to plasma proteins, whilst the unbound fraction of imatinib and its active metabolite were found to be lower in plasma ¹⁸⁴. Therefore, plasma protein binding may be associated with a decreased partitioning into blood cells. For imatinib, plasma protein binding appears to be a driving force for its blood distribution and also the most important factor in interpreting PK data when using either plasma or whole blood measurements. In the current study, the percentage of sunitinib associated with RBCs did not change significantly with increasing concentration indicating linear distribution kinetics over the calibration range of the assay (Table 2.9). Therefore, either blood or plasma could potentially be used to measure sunitinib drug concentrations provided there are no significant changes in plasma protein binding. However, it has been shown by Manicke *et al.* that the degree of protein binding in the blood does not affect the detection of the drug in paper spray process. This is because the drying process and analyte extraction with polar organic solvent probably denature the plasma proteins and disrupt the noncovalent interactions respectively, enabling paper spray to measure the total drug concentration ¹⁴⁴. DBS PK estimates can be compared with those in plasma without prior need for conversion of concentration measurements.

2.4.3.10 Matrix effects and ion suppression

To assess matrix effects of the detector response due to constituents within DBS matrix, sunitinib spiked pooled rat whole blood at a concentration of 2 $\mu\text{g/mL}$ was used to produce replicate DBS samples. These samples were compared to standard of the same concentration spiked into pure methanol: water (50:50, v/v). Dried spot samples for whole blood and pure solvents were prepared on different paper substrates such as silica-coated paper, Grade ET31 and Grade 1 Chr and qualitative filter papers (Table 2.1). The paper types were selected due to their difference in thickness, physical properties and information from literature. Silica-coated paper with a 0.27 mm thickness has been demonstrated in a previous study to improve sensitivity and recovery of analytes such as sunitinib in DBS ¹⁴⁶. The inner surface of the paper is filled with silica to improve elution and recovery by providing a smooth surface for analytes to move whilst reducing the analyte to paper interaction for hydrophobic compounds. It has been reported that both the spray solvent and paper substrate had a significant influence on the elution and recovery of analytes from complex sample matrices (blood) and ion formation for MS ¹⁴⁶. Both Grade ET31 and Grade 1 Chr papers are non-coated with 0.5 and 0.18 mm thickness respectively. Both have been demonstrated for use in DBS therapeutic drug monitoring and forensic applications, achieving good sensitivities for the test compounds ^{110, 185}. Standard qualitative filter paper with 0.18 mm thickness has not been applied so far in paper spray but was included to add to the variability in physical properties.

As shown in Figure 2.15, the peak ion intensities of sunitinib in DBS versus the pure sample spots varied slightly among the paper type. Comparatively, sunitinib response was not significantly different across the paper types and signal suppression due to the blood matrix within the paper types was much larger. It was observed in this current study that although the magnitude of suppression was not significantly different among the paper types, the variability of suppression in Grade ET31 Chr paper was the least (10.9 %). Therefore, Grade ET31 Chr paper was selected for DBS sample preparation for the model compound.

Figure 2.15: Matrix effect evaluation in DBS. Log signal responses (AUCs) for sunitinib on qualitative filter, Grade 1 Chr, silica coated and ET31 Chr papers in DBS (brown) and methanol/water (50:50, v/v) (cream).

2.4.3.11 Matrix effect in different lots

The evaluation of matrix effects by the determination of standard line slopes in different sources (lots) of a biofluid serve as a good quantitative indicator of the presence or absence of a relative matrix effect in bioanalytical methods¹⁸⁶. In order for the method to be considered practically free from any matrix effect liability, the precision (CV %) value of standard line slopes constructed in five different lots of a biofluid should not exceed 3-4 %¹⁸⁷. In addition, the use of stable isotope-labeled (SIL) analogues as internal standards is recommended when available to effectively eliminate relative matrix effect liability¹¹⁶. In quantitative bioanalysis, concentration determination of analytes in biofluid is usually obtained from standard curves prepared in a single lot of a biofluid

which is also used for analyte concentration determination in biofluid samples originating from different rodent lots, at various time points after dosing and from different population pools. The quest to study, identify and eliminate relative matrix effect in the bioanalytical assay procedures is of critical importance since its presence on the relative MS/MS response will invalidate the final PK data obtained ¹⁸⁷.

Therefore, a single analyte (sunitinib) was selected to evaluate the extent of matrix effect in different lots of rat blood on the PS method. Moreover, its SIL (sunitinib-d10) was used as the internal standard throughout the experiment. As demonstrated in Table 2.10 and in Figure 2.16, the precision value of standard line slopes constructed in six different lots of rat blood was 2.2 %, less than the cut-off value of < 3-4 %. This high precision value (small slope precision value) indicates the reliability of the assay since matrix effect on the MS/MS response of the analyte and the SIL-IS is insignificant. Moreover, the slope difference which represents the difference between the highest and the lowest slope values divided by the lowest value and expressed in percentage was 6.3 %. It is practically impossible for the slopes of standard lines constructed in different blood lots to be the same and there is direct proportional relationship between the slope precision value and that of the slope difference value ¹⁸⁷. Therefore, a smaller slope precision value of 2.2 % produced a smaller slope difference of 6.3 %, cementing the reliability of the method and free from matrix effect liability. In addition, the information obtained from the difference in slope value is important since it indicates the extent to which relative matrix effect accounts to the PK data alone without the effect from drug interaction. For instance, due to the relative matrix effect, the calculated concentration of sunitinib in for example Lot 1 was 6.3 % higher/lower than when standard line was constructed in different lot (Lot 2). However, this difference in slope value obtained in this study is acceptable and the assay considered reliable to be used for PK studies.

The use of SIL-IS (sunitinib-d10) managed the variability in different matrix lots by efficiently tracking the analyte (sunitinib) response in complex biological matrix. However, in situation where these SIL-ISs are not commercially available, the necessity to synthesize them is inevitable but is costly and time consuming. The alternative economical option is the use of structural analogues of the analytes but unable to

compensate for ion suppression in crude biological matrix like that exhibited through the use of SIL-ISs. Paper spray ionization mass spectrometry like any other ambient ionization MS assay aims to enable high throughput analysis by eliminating sample pre-treatment or chromatographic separation thereby making it more prone to matrix effects compared to conventional LC-MS/MS methods. Therefore, the utilization of SIL-ISs by direct analysis assays for quantitative bioanalysis to overcome matrix effects in complex matrices seems indispensable for now ¹¹⁶. Also, incorporation of sample pre-treatment into the ionization step to further enable reduction in matrix effect and improve sensitivity has been reported for some ambient ionization methods including paper spray ionization method ^{188, 189}.

Table 2.10: Matrix effect evaluation in different lots of blood.

Lot number	Slope	Slope CV (%) ^a	Slope difference (%) ^b
Lot 1	0.013802	2.2	6.3
Lot 2	0.013034		
Lot 3	0.013528		
Lot 4	0.013540		
Lot 5	0.013388		
Lot 6	0.013851		

^a Precision value (percent coefficient of variation, % CV) of slopes of standard lines constructed in six different lots of blood.

^b Maximum difference between the highest and the lowest slope values divided by the lowest slope value and multiplied by 100.

Figure 2.16: Matrix effect evaluation in different lots of blood. Standard line curves obtained from the plot of peak area ratios of sunitinib/sunitinib-d10 in six different rat blood lots versus sunitinib concentrations.

2.4.3.12 Evaluation of storage effect on analyte stability and elution from dried matrix spot

This study was carried out to evaluate storage effect on analyte stability and elution during paper spray analysis. Hence, both sunitinib dried plasma and whole blood spots were stored under different conditions. Sunitinib in dried human and mouse plasma spots on the silica coated paper were kept at room temperature (22-26 °C) and 37 °C for a period of 24 h and 6 days. Stability of the analyte was established when the precision of QC concentrations was within $\leq 15\%$ whilst the accuracy was within $\pm 15\%$ of the nominal value after the storage periods. Sunitinib was found to be stable in dried human and mouse plasma spots on silica coated paper at these conditions for 24 h and 6 days when compared with fresh samples (Table 2.11).

The ability of the paper substrate to be utilized as a storage device for an extended period of time was also evaluated using DBS samples prepared from pooled rat whole blood. One of the merits of DBS sampling procedure is its ability to stabilize the analyte(s) within the dried blood matrix due to the inactiveness of endogenous enzymes mostly responsible for the degradation and also enable the release of the analyte(s) from the paper during processing. Therefore, the selected paper type for the paper spray ionization should be able to possess these vital characteristics. As already mentioned, silica coated paper type enabled the stability and elution of sunitinib after 6 days storage period. In this experiment, the effect of longer storage periods (9 days and 1 month) on sunitinib stability and elution were assessed under the same storage conditions in dried blood spots prepared on ET31 Chr paper. As shown in Table 2.12, the variations were contained within $\pm 15\%$ of the nominal concentration, indicating the stability of sunitinib in DBS samples at both storage conditions. Hence, the ET31 Chr paper type possessed the analyte stability feature. However, the stability of analyte on DBS is analyte dependent; therefore, stability of each analyte in DBS samples should be investigated when intended to be kept for longer periods prior to analysis or future reanalysis.

Table 2.11: Storage effect on sunitinib precision and accuracy in dried mouse and human plasma spots on silica coated paper (n=6) at room temperature (RT) and 37 °C within 24 h and up to 6 days.

		Accuracy (%)					Precision (%)				
		24 h			6days		24 h			6 days	
Nominal Concentration (ng/mL)		fresh	RT	37 °C	RT	37 °C	fresh	RT	37 °C	RT	37 °C
Mouse plasma	5	5.7	-5.1	-3.5	-6.8	-5.9	3.8	2.3	3.2	2.8	2.2
	250	3.3	-1.1	-0.5	-3.3	-4.0	1.6	1.6	2.1	1.0	2.9
	400	5.7	-1.1	-1.1	-4.8	-3.7	1.5	1.5	1.8	1.8	2.6
Human plasma	5	1.8	-10.7	-6.4	-3.9	-10.1	2.4	5.7	4.9	5.0	1.7
	250	-3.3	2.7	-5.3	-2.4	-3.1	4.2	1.8	5.8	2.9	2.5
	400	2.4	3.6	4.4	4.1	7.1	2.0	3.6	2.3	3.4	4.3

Table 2.12: Storage effect on sunitinib precision and accuracy on ET31 Chr dried blood spots (n=6) at room temperature (RT) and 37 °C within 9 days and 1 month respectively.

		Imprecision (% CV)				Accuracy (% bias)				
		9days		1month		9days			1month	
nominal conc. (ng/mL)	Fresh	RT	37 °C	RT	37 °C	Fresh	RT	37 °C	RT	37 °C
5	1.6	2.6	8.2	3.7	6.2	4.9	5.3	3.8	2.1	-3.8
100	3.0	5.3	2.7	2.2	3.6	-4.3	-6.2	-6.4	2.6	-2.3

Moreover, conventional sample pre-treatment procedure for DPS or DBS samples requires punching and extraction of analyte from small discs of dried spot from the paper. The extraction in solvent medium of the multiple punched discs enables the moistening of the paper substrate and subsequent release of the analyte from the paper matrix after a period of rigorous agitation. Paper spray ionization envisages on the elimination of these extensive sample preparation steps and embarks on direct analysis of the DBS samples. However, the analyte recovery of paper spray is not high ¹⁵⁰ and any condition which affects the recovery will eventually reduce the sensitivity of the method. After a longer storage period of DBS samples for 1 month and PS-MS analysis, a dramatic loss of sensitivity was observed when immediately analyzed without proper moistening, probably due to stronger binding of analyte to the paper matrix.

Figure 2.17: Schematic representation of pre-moistening process before paper spray. (A) An Off-line and (B) on-line procedure. Spray solvent is applied directly onto the dried spot in two stepwise processes with 10 s delay before paper spray.

Figure 2.18: Storage effect on analyte elution from DBS. (A) Analyte signal response without pre-moistening step (B) analyte signal response with either off-line or on-line pre-moistening step. The signal response of the test compound and its internal standard was improved after the dried spot was moistened sufficiently to reduce the analyte interaction with the paper substrate after storage.

Therefore, proper wetting of the dried blood spots on paper substrate was required to improve the elution of the analyte for ionization and MS analysis. This was achieved by a two stepwise spray solvent application (32 μL), on top of the blood spot, with a ten second delay between injections either off-line or on-line (Figure 2.17). For off-line procedure, spray solvent was manually applied to the dried spot whilst sequential solvent-injection program was designed for on-line procedure. The additional moistening of the blood spot prior to paper spray was able to improve analyte extraction and signal response (Figure 2.18). So far, there is no report in literature regarding storage effect on analyte stability and elution on paper spray. Since most of the studies gears towards immediate analysis, this effect has been overlooked thus far. This experiment is particularly important in the case of reanalysis, sudden breakdown of instrument or collection of samples from distance place to analytical lab, etc.

2.4.4 Method development for addition of internal standards

In all the experiments discussed so far in this thesis, the pre-mix method of internal standard introduction to the sample was employed where the internal standard was pre-mixed with the matrix containing the analyte prior to spotting. Therefore, a method of adding internal standard to the matrix using SIL-IS coated PCR sampler was investigated. Sample evaporation and concentration technique was used to coat SIL-IS inside the PCR tube.

The method of IS addition was tested with the already determined optimum internal standard concentration (100 ng/mL). The required internal standard concentration (C_{IS}) solution was prepared in 100 % methanol. A volume (V_C) of this solution was pipetted into a 0.2 mL PCR tube and evaporated to dryness to form a solid coating at the inside bottom of the tube using centrifugal concentrator. A volume of sample equivalent to V_C is taken for the sample containing the analyte at a concentration of (C_A) and transferred into the IS-coated tube. Quick mixing is done and then transferred to the paper substrate to

form dried spots. Similar to the principle in the reported work by Liu *et al.*, “as shown with the **Equation 6**, the ratio of I_A/I_{IS} is proportional to the ratio of the total amounts of the analyte and the internal standard dissolved from the coating, which is equal to the ratio of the original concentrations of the analyte in the sample and that of the internal standard in the coating preparation solution”¹⁵⁴.

$$\frac{I_A}{I_{IS}} \propto \frac{A_A}{A_{IS}} = \frac{C_A V_C}{C_{IS} V_C} = \frac{C_A}{C_{IS}} \quad \text{Equation 6}$$

The ability to use tube sampler for the quantitation of sunitinib in blood samples was evaluated. The time of evaporation under different temperature conditions of the centrifugal vacuum concentrator was first examined. Pure methanol of 5 μ L was added to 0.2 mL PCR tubes and placed into the concentrator. Complete drying of the methanol under evaporation condition (ambient, 45 and 60 °C) was examined. As shown in the Table 2.13, it took about 120 s for the methanol in the PCR tubes to be completely dry under both evaporation temperatures of 45 and 60 °C. An additional 60 s was required for the pure methanol in the PCR tube under ambient temperature to dry up. Moreover, drying times for different volumes of pure methanol were also accessed at 60 °C evaporation temperature and shown in Table 2.13.

The next phase of this experiment involved the coating of the inner bottom surface of the tube with the internal standard (sunitinib-d10). To each tube was pipetted into 5 μ L of pure methanol solution containing sunitinib-d10 (100 ng/mL). The tubes were then transferred to the concentrator for evaporation of the methanol at temperature of 60 °C, resulting in a solid coating of 0.5 ng (Figure 2.17A).

Figure 2.19: The tube sampler procedure for adding internal standard to matrix. (A) A tube sampler prepared by transferring specific volume of the internal standard solution to the tube and drying through evaporation and concentration process to form a solid IS coating at the bottom of the tube. (B) Blood sample is pipetted into the tube sampler, mixed quickly and deposited onto the paper triangles for air drying and subsequent analysis by paper spray ionization with mass spectrometry as detector.

Table 2.13: Drying time for pure methanol

Temperature °C	Evaporation time (s)
Ambient	180
45	120
60	120
Volume of methanol (µL)	
0.5	30
1	40
3	90
4	90

The 60 °C evaporation temperature was selected for further studies due to the fast solvent removal. Secondly, boiling of methanol under vacuum provides additional protection for IS from any degradation or sublimation. After preparation of the tube samplers, a group of dried blood spots were prepared and analyzed by paper spray in SRM mode to check the variation within the ratios of the signals recorded for the analyte and its internal standard transitions. Into each tube was transferred 5 µL of blood spiked with 10, 100 and 1000 ng/mL of sunitinib and mixed briefly (Figure 2.17B). As shown in Figure 2.18A, the variations of the measured ratios for the analyte and its internal standard at different concentrations were < 5 % (RSD, n = 5). Therefore, the tube sampler was precised at different concentrations tested.

Figure 2.20: Peak ratio accuracy for quantitation. (A) Quantitation of sunitinib in blood at 10, 100 and 1000 ng/mL using tube samplers. 0.2 mL PCR tubes were coated with sunitinib-d10 (from 100 ng/mL methanol solution) as internal standard. (B) Intensity ratios of sunitinib and sunitinib-d10 (A/IS), paper spray of DBS (100 ng/mL sunitinib for whole blood, 500 ng/mL sunitinib for plasma & serum) prepared using IS coated tube sampler (bottom) and premixing the IS using pipetting (top).

Peak ratio accuracy for quantitation was also investigated by comparing the results obtained from tube sampling method to that of conventional pre-mix method (control).

A group of dried blood spots were prepared by transferring 5 μ L of sunitinib spiked whole blood (100 ng/mL), or plasma/serum (500 ng/mL) into each tube, mixed briefly and immediately spotted onto the chromatography paper. Another group of dried blood spots on chromatography paper were prepared using conventional laboratory procedure (pre-mix). From a bulk whole blood, plasma/serum sample (0.5 mL) was pre-mixed with sunitinib concentration at either 100 ng/mL or 500 ng/mL and sunitinib-d10 at 100 ng/mL. Paper spray mass spectrometry was used to analyze both sets of samples. Shown in Figure 2.18B is the ratio of m/z 326 intensities for sunitinib and sunitinib-d10 obtained from using the tube sampler and the conventional lab procedures. As shown in Table 2.14, the ratio values and variations (RSD of about ≤ 8.6 % vs. ≤ 7.7 , $n \geq 5$ for pre-mixed and tube sampler respectively) observed for the tube sampler were identical to those from conventional premixed procedure (control), hence, good mixing of the internal standard into the blood sample was achieved with the tube sampler procedure.

Table 2.14: Peak ratio accuracy for tube internal standard addition with pre-mix as control

Method of IS addition	Mean sun/sun-d10 (AUC, $N=6$ for blood, $N=5$ for plasma & serum)	Std	CV%
Blood			
Pre-mix	0.49	0.04	7.5
Tube sampler	0.49	0.02	5.0
Plasma			
Pre-mix	2.28	0.09	3.9
Tube sampler	2.30	0.18	7.7
Serum			
Pre-mix	2.22	0.19	8.6
Tube sampler	2.46	0.18	7.4

IS: Internal standard, sun/sun-d10: sunitinib/sunitinib-d10, Std: standard deviation,

CV %: percent coefficient of variation, N : number of replicate

The PCR tube sampler has been demonstrated for sampling and IS mixing for microvolume (5 μ L) of serum, plasma and blood. The results show that this method can enhance homogenous mixture of the IS with the matrix components and the sample paper due to the brief mixing after matrix addition prior to spotting. The preparation of the tube samplers required the use of conventional centrifugal concentrator mostly available in bioanalytical labs where many tubes can be prepared within a period of 120 s. Moreover, the sampling process simply required the use of laboratory pipette tips and pipettes without any special education or training requirement. Physical properties of the liquid samples did not affect their sampling process due to the utilization of pipettes for sampling and mixing.

It is relevant to note that similar performance was also observed when HPLC vials were coated with IS instead of PCR tube. In view of this, other format inserts for centrifugal concentrators, especially microplates can also be optimized for internal standard coating since they are more compatible with most of the recent automated instruments.

Furthermore, at the blood collection site, the pipette tips can be rinsed with EDTA solution prior to blood sampling from the animal and transferring into the PCR tube sampler, thereby introducing anticoagulant to the blood sample so that it will be similar to anticoagulant containing matrix used to prepare standards and quality control samples. In addition, stabilizers can also be introduced in similar fashion to prevent degradation of some prodrugs during the drying process of dried blood spot preparation. This method can be potentially used for direct microvolume blood collection from mice/rat or for microsample analysis in the conventional laboratories.

2.5 Summary and conclusion

PS-MS which enables direct MS analysis of dried matrix spot was developed and validated. Initially, experimental conditions which influence the ionization parameters were optimized using small molecule drugs in dried plasma spots (DPS). It was also found that increase in analyte signal response could be increased when the spray tip inclined to the MS inlet but generally depends on the solvent speed of the paper type. Hence, there was inverse relationship between the solvent speed of paper type and the spray tip angle to the MS inclined. Grade ET31 with the highest flow rate produced a decreased signal response when the spray tip was inclined whilst there was improvement in analyte signal response when the spray tips of Grade 1 Chr and silica coated paper types were inclined to the MS inlet due to their relatively lower solvent speeds.

Important validation parameters which characterize the performance of analytical method for bioanalysis were investigated. The results from this study indicate that matrix effect was prevalent for the test compounds, but it could be effectively managed by using a stable isotope labeled internal standard (SIL-IS). Moreover, PS-MS assay was found to be linear (1 – 500 ng/mL), precise and accurate obtaining a LLOQ of 1 and 5 ng/mL for sunitinib and benzethonium respectively in 2 μ L dried plasma spots (DPS), similar to those already reported. Carryover was found to be minimal for some compounds and variable for others and could generally be overcome by simple clean-up and inserting matrix blanks without compromising with the analysis time. The PS-MS assay was also validated for dried blood spots (DBS), since the adoption of DBS sampling instead of DPS during preclinical studies could eliminate the need for sample processing at the site of blood collection and enable direct spotting to the paper substrate. The results from the hematocrit effect and blood-to-plasma partition experiments indicated that, DBS can be a suitable matrix for the measurement of sunitinib during preclinical PK studies. The evaluation of storage effect on analyte stability and elution also showed that, the test compound was stable in both DPS and DBS samples under the various conditions stored. However, it was found that longer storage caused dramatic loss in sensitivity compared to fresh samples when analyzed immediately. Hence, either off-line or online wetting prior to direct analysis could be adopted to improve sensitivity. Online wetting of the dried

spot on the paper substrate was achieved through the use of a two stepwise spray solvent application (32 μL), on top of the blood spot, with a ten second delay between injections which improved overall analyte extraction and sensitivity.

Moreover, in order to enable direct introduction of SIL-ISs to microvolume samples during quantitative bioanalysis, alternative method to introduce SIL-IS to sample matrix was investigated by coating the bottom of a PCR tube with SIL-IS using sample evaporation and concentration technique commonly used in the sample pre-treatment procedure. The tube sampler results were found to correlate well with those from a conventional laboratory pre-mixed procedure and comparable accuracy and precision were achieved with a 5 μL plasma, serum and whole blood spots. Hence, < 10 μL blood could be sampled directly from small mammals (e.g. mice, rats) during preclinical studies.

Chapter 3

3.0 Comparison of Paper Spray Mass Spectrometry (PS-MS) and LC-ESI-MS/MS

3.1 Chapter overview

This chapter of the thesis is the extension of the previous work and the experiments discussed here were performed to ascertain that PS-MS assays will provide comparable data to LC-MS/MS assay when used for *in vivo* bioanalytical analysis. In the previous work, the experimental parameters which influence the ionization process of PS were thoroughly optimized. Furthermore, important validation parameters were established using dried plasma spot samples. Adoption of DBS samples could eliminate the need for centrifugation; hence, DBS was validated for measurement of the model compound, sunitinib. The PS-MS method performance showed a LLOQ of 1 ng/mL of sunitinib in dried plasma spot (DPS). In order to establish the performance of PS-MS assay also for DBS samples analysis, important validation parameters such as linearity, precision, accuracy, lower limit of quantification (LLOQ) were investigated in this part of the thesis and the results were compared to those obtained by conventional LC-MS method.

Furthermore, the elimination of chromatographic separation may also increase the potential effect from interfering components present in *in vivo* samples such as metabolites that may compete with the parent compound for ionization through coelution and cause ion suppression or may also increase the response of the parent compound through in-source conversion of the metabolites particularly from in-source breakdown of liable metabolites such as acyl glucuronides, sulfates and N-oxides to yield the protonated molecule of the parent drug thereby giving rise to artificially high plasma concentrations^{157, 190}. Hence, potential interference from metabolites was also investigated. Finally, the potential application of PS-MS assay for *in vivo* high throughput bioanalysis in preclinical PK studies was evaluated. The chemical structures of the model compounds are shown in Appendix 2.

3.2 Experimental section

3.2.1 Chemicals and materials

Sunitinib was purchased from Abblis Scientific Research Chemicals Inc. (Houston, TX, USA). The internal standard, sunitinib-d10 (lot number 593, 96% pure) was obtained from Medical isotopes (Pelham, NH, USA) whilst N-desethyl sunitinib (lot number 1-WHH-100-3, 96% pure) was obtained from Toronto Research Chemicals (Toronto, ON, Canada). Acetonitrile, methanol, formic acid (98.9% pure) and HPLC grade water were purchased from Merck KGaA, (Germany). Dipotassium ethylene diamine tetra acetic acid (K₂EDTA), ammonium formate and blank rat plasma were purchased from Sigma-Aldrich (Singapore). Deionized water from Sartorius deionization system (Sartorius, Singapore) was used for all aqueous solutions. Whatman Grade SG81 ion exchange (product code: 3668-915) and Grade ET31 (product code: 3031-681) chromatography papers were all purchased from GE healthcare UK Limited (Buckinghamshire, England).

3.2.2 Preparation of stock, working and spiking solutions

Stock solution of test compounds and isotope labeled analogue internal standard were prepared at concentration of 1mg/mL in methanol. Dilutions from the individual stocks were made in methanol: water (1:1, v/v) to yield a working stock solution of 50 µg/mL for each. These working solutions were further diluted with blank drug free rat plasma or blood to make the analytical samples.

Solution preparation for light the test compounds were made under light protected conditions and all prepared solutions were kept at -20 °C.

3.2.3 Sample preparation

3.2.3.1 Sample preparation for PS-MS/MS and LC-MS/MS comparison studies

The stock working solutions of sunitinib was used to prepare calibration standards and quality control (QC) samples in blank rat plasma or whole blood. The calibration standards for sunitinib was prepared by spiking the working stock solutions of the drug and IS into blank drug free rat plasma or blood. The concentration of calibrators was 1, 5, 10, 50, 100, 250, 500 and 1000 ng/mL. The quality control samples were independently prepared in blank plasma or blood by diluting another working solution in order to obtain 1, 5, 100 and 1000 ng/mL. The concentration of the internal standard was 100 ng/mL. DBS samples of 5 μ L were prepared after air drying for two hours at room temperature. Plasma samples were prepared using protein precipitation procedure as described in Section 3.2.4.2.

Experiment to construct linear calibration curves of sunitinib was carried out with calibration standards. Calibration curves were constructed by plotting analyte/internal standard peak area ratios versus the nominal concentrations of sunitinib in Microsoft Excel and the data were subject to $1/x^2$ weighted linear least squares using SPSS statistical packages, version 16.0. The linear range was evaluated by determination of the correlation coefficient (R^2) and the acceptance criteria for each back calculated standard was set within ± 15 % deviation from the nominal value (± 20 % deviation from the nominal value for the low limit of quantitation (LLOQ)). Each calibration curve parameters were used to determine the concentration of the unknown samples by back-calculation.

Method validation for precision and accuracy of sunitinib concentration measurement in DBS and rat plasma was assessed with the analysis of quality control samples including LLOQ (1 ng/mL). For the intraday accuracy and precision, six replicates of each level of the QCs were analyzed with calibration curve to assess intra-assay precision and accuracy. Inter-day accuracy was evaluated over two analysis days; with two batches on the same day and another batch on a separate day. Each batch contained calibration standards and six replicates of each QC concentration level. The

accepted criteria for each back-calculated quality control samples was set at $\pm 15\%$ deviation from the nominal value at each concentration ($20\% \pm$ deviation from the nominal value for LLOQ). The percent coefficient of variation (%CV) for each QC concentration must be $\leq 15\%$ ($\leq 20\%$ for LLOQ) to be accepted. At least 50 % of the quality control samples at each concentration level must pass this criterion and overall at least 2/3 of the QC samples must pass. Accuracy and precision were calculated using the following equations: $[\text{mean measured concentration} - \text{nominal concentration}] / \text{nominal concentration} \times 100$ and $[\text{standard deviation} / \text{mean measured concentration}] \times 100$ respectively.

3.2.3.2 Sample preparation for *N*-desethyl sunitinib assay samples

The calibration standards (1-1000 ng/mL) and QC samples (5, 100 and 1000 ng/mL) were prepared by serially diluting the sunitinib stock solution with rat plasma and whole blood. The metabolite, *N*-desethyl sunitinib, at 1000 ng/mL was spiked into each QC samples. The plasma samples were prepared and subjected to LC-MS/MS analysis. DBS samples were prepared and directly analyzed by PS-MS/MS.

3.2.3.3 Paper spray ionization and mass spectrometry

An LTQ linear ion trap mass spectrometer (Thermo Scientific, San Jose) interfaced with paper spray ionization prototype was used to examine dried blood samples in the selected reaction monitoring (SRM) mode to obtain mass spectra. Data analysis and acquisition were performed using Xcalibur software, version 2.0.7. Calculations were performed with Microsoft Excel 2007 from Microsoft Co. The precursor/product ion pairs for quantitation of the compounds and their internal standards are listed in Table 2.2 and 3.1. For paper spray ionization, the paper types were cut into an isosceles triangle with 10 mm base width $\times$ 19 mm height, with a tip angle of 30° . The paper triangle was fixed to the metal clip and positioned at 5 mm away from the MS inlet. Spray voltage of

4 kV was applied to the metal clip after optimum spray solvent (64 μ L of 80 % acetonitrile with 0.2 % acetic acid) was applied to dried blood spots to enable dissolution and extraction of the analytes for MS detection.

3.2.4 Conventional liquid chromatography electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) analysis

The analytical technique for the quantification of sunitinib and its metabolite was selected on the basis of the relatively good LLOQ (1ng/mL) obtained in plasma ¹⁹¹.

The analysis was performed on Thermo scientific Accela[®] 1250 pump equipped with Thermo scientific Accela[®] autosampler coupled to ion trap mass spectrometer interfaced with electrospray ionization (ESI) source. The development of a working LC-MS method was undertaken using standard solutions of sunitinib in methanol: water (50:50, v/v). Effects of chromatographic parameters such as column temperature, injection volume, mobile phase composition and flow rate were investigated on the resulting chromatographic signal. The mass spectrometer conditions were first optimized using flow injection analysis on the standards, without the column.

3.2.4.1 Preparation of rat plasma samples

Whole blood was collected from rat in EDTA containing eppendorf tubes. The tubes were centrifuged immediately at $2,100 \times g$ for 10 min at 4 °C to separate the plasma. The plasma was transferred to fresh eppendorf tubes and stored at -80 °C or prepared immediately.

3.2.4.2 Sample preparation and analysis by LC-ESI-MS/MS

Plasma samples were thawed to room temperature. Aliquots of plasma (100 μ L) were placed in 1.5 mL microcentrifuge tubes, spiked with internal standard and vortex mixed. Extraction was carried out by the addition of 200 μ L of acetonitrile to each tube and vortex mixed for 10 min. After automatic vortexing, each sample was centrifuged at $6000 \times g$ for 15 min at 4 $^{\circ}$ C. A volume of 100 μ L of the supernatant was diluted 2-fold using the mobile phases A and B in a 50/50 (v/v) ratio. The samples were centrifuged at $1,5871 \times g$ for 10 min at 4 $^{\circ}$ C and the supernatant was transferred into HPLC vials. These vials were placed into the LCMS autosampler and 5 μ L injected for LC-ESI-MS/MS analysis.

The chromatographic system consisted of Thermo scientific Accela[®] 1250 pump equipped with Thermo scientific Accela[®] autosampler. Sunitinib, sunitinib-d10 and N-desethyl sunitinib were analyzed on a Luna C18 column (Phenomenex Luna 5 u C18 100 Å pore size, 50×2.00 mm) protected by C18 guard cartridge (Phenomenex). The column temperature was maintained at 40 $^{\circ}$ C. The mobile phase consisted of 10 mM ammonium formate buffer containing 0.1 % (v/v) formic acid (mobile phase A), and acetonitrile with 0.1 % (v/v) formic acid (mobile phase B). Gradient elution program was performed as follows: 0 - 0.5 min 5 %B, 2.0 – 4.0 min 90 %B, 4.01 – 7 min 5%B. The flow rate was 0.2 mL/min. The eluates from the analytical column was directed to waste for the first 2 min via a divert valve and then introduced directly into the ESI source which was operated in positive ionization mode. High purity nitrogen gas (SOXAL, Singapore) was used for nebulization and desolvation.

ESI-MS experiments were performed on a LTQ XL ion trap (Thermo Scientific, San Jose) equipped with heated electrospray source. The general MS settings and analyte specific parameters for the assay are summarized in Table 3.1. The compound-dependent parameters including collision energy, capillary voltage and tube lens were individually optimized for all compounds by flow injection analysis. For these experiments, an HPLC pump was used at a flow rate of 0.2 mL/min with an eluent of acetonitrile-water (80:20,

v/v), and 5 μ L of standards at 1 μ g/mL were injected directly into the MS using a syringe pump without a column.

Quantification was performed using selected reaction monitoring (SRM) of the transitions of the compounds (Table 2.2 & 3.1).

3.2.4.3 Data acquisition and analysis

Data acquisition was performed using Xcalibur software, version 2.0.7. Calculations were performed with Microsoft Excel 2007 from Microsoft Co. and SPSS statistical packages, version 16.0 for statistical analysis. The graphs were generated with GraphPad Prism software, version 5.01.

Table 3.1: General settings and analyte specific parameters for LC-MS

General setting	
Spray voltage (V)	5
Capillary temperature ($^{\circ}$ C)	275
Sheath gas pressure	30
Auxiliary gas pressure	10
Vaporizer temperature ($^{\circ}$ C)	300
Tube lens (V)	75
Capillary voltage (V)	19
Analyte specific parameters	
<i>N</i> -desethyl sunitinib	
Parent mass (m/z)	371.1
Product mass(m/z)	283.1
Collision energy	49
Mass-to-charge ratio (m/z)	

3.2.5 Sunitinib administration in mice, sample collection and analysis

The PS-MS assay was used to demonstrate its feasibility for the pharmacokinetic study of sunitinib in male ICR mice (5-6 weeks old with an average weight of 30 g) purchased from Invivos (Singapore). The experiment was conducted in accordance with the Nanyang Technological University Institutional Animal Care and Use Committee (IACUC). The mice were allowed to acclimatize to their surroundings prior to the start of experiments. They were kept in a room at constant temperature and humidity and were fed with standard pellet and water *ad libitum*. The experimental mice were assorted into Groups A and B, each consisting of 6 and 4 mice respectively.

Plasma profile was determined following an oral or intravenous dose administered as 0.1 M citrate buffer (pH 5) suspension^{192, 193}. An oral dose level of 15 mg/kg sunitinib was given to the mice in Group A by gastric gavage. Serial blood samples were taken from the tail vein of the mice before dose and 0.5, 1, 3, 6, 8, 12, 24 h after oral administration. For Group B, the mice were administered with a single bolus of sunitinib at the nominal dose level of 5 mg/kg via tail vein injection. Blood samples were also taken before dose and 0.08, 0.25, 1, 3, 6, 12, 24 h after intravenous dosing. Serial blood samples were collected using 20 uL capillary tubes and 0.2 mL eppendorf tubes with EDTA (0.5M). Mouse blood samples were immediately separated by centrifugation and the plasma stored at -80 °C until paper spray mass spectrometry analysis.

On the day of analysis, the plasma samples were thawed on ice and 4 uL aliquot was taken. To each aliquot, 1 uL of the internal standard solution (500 ng/mL of sunitinib-d10 in methanol: water, 1:1) was added followed by vortex-mixing. Dried plasma spots were prepared, as described above, followed by quantification of sunitinib using paper spray mass spectrometry.

Pharmacokinetic data analysis

Pharmacokinetic analysis of sunitinib concentration vs. time data and determination of pharmacokinetic parameters were based on the non-compartmental approach using PK solutions 2.0 (Summit Research Services, CO, USA ¹⁹⁴) that yielded the following parameters: the terminal elimination half-life ($t_{1/2}$), the area under the sunitinib plasma concentration-time curve (AUC_{0-24h}), the time (t_{max}) to reach peak plasma concentration (C_{max}), total clearance (CL) and the apparent volume of distribution (Vd).

3.3 Result and discussion

3.3.1 Bioanalytical assay performance

The feasibility of utilizing PS-MS assay for *in vivo* bioanalysis in drug discovery and development was evaluated by using spiked samples and study samples. The results were compared to those obtained by conventional LC-MS method. Important validation parameters which characterize the performance of bioanalytical assay such as linearity, precision, accuracy, lower limit of quantification (LLOQ), sensitivity and potential interference from metabolites were evaluated using DBS samples.

As stated under the experimental section, calibration standards and quality control samples were prepared by spiking sunitinib into rat plasma and blood for LC-MS and PS-MS respectively. DBS samples of 5 μ L were prepared and directly analyzed by PS-MS whilst the plasma samples were extracted using protein precipitation procedure followed by LC-MS analysis. Also, in both situations the same pooled blank rat whole blood was used as the matrix whilst SIL analogue, sunitinib-d10, was used as the internal standard.

3.3.1.1 Linearity

The linearity of the method was evaluated by analyzing calibration standards (1 – 1000 ng/mL) for quantitation of sunitinib in DBS using PS-MS/MS. The assay was found to be linear in DBS over the concentration range studied and the correlation coefficients (R^2) were constantly > 0.99 with reproducible calibration curves between days for sunitinib, comparable to that from LC-MS analysis. As shown in Table 3.2, 1 ng/mL lower limit of quantification (LLOQ) was obtained for sunitinib in DBS and extracted plasma samples for PS-MS/MS and LC-MS/MS respectively. As already demonstrated in the previous study, a LLOQ of 1 ng/mL was obtained for sunitinib in dried plasma spot (DPS) by PS-MS/MS assay. Also, it is essential to note that the dynamic range of the method was extended to 1000 ng/mL, twice higher than that established in DPS in the

previous study. PK studies in drug development relies on the dynamic range of the concentrations, hence, the wider the range, the better.

3.3.1.2 Accuracy and precision

The precision and accuracy of the method were evaluated by analyzing replicates QC samples (1, 5, 100 and 1000 ng/mL) for sunitinib quantitation with PS-MS/MS analyses at three separate analytical batches. The accuracy and precision were assessed by calculating the percent deviation from the nominal concentration and reported as % bias whilst precision was assessed by calculating the percent coefficient of variation (% CV) for each set of replicates. DBS and extracted plasma samples were analyzed in duplicates by PS-MS/MS and LC-MS/MS respectively and the results are summarized in Table 3.2. The mean % CV obtained from the PS-MS/MS and LC-MS/MS methods for the four levels of QC were within 3.87 to 9.83 % and 3.44 to 7.65 % respectively. The % bias obtained was within -3.2 to 4.8% and -9.1 to 1.9 % of the nominal value for PS-MS/MS and LC-MS/MS methods respectively. These results showed that, precision and accuracy of PS-MS/MS assay was largely comparable to that obtained using LC-MS/MS. Moreover, low QC demonstrated similar variability on PS-MS/MS to that of the conventional LC-MS/MS, indicating that the coeluting matrix did not have impact on the assay at the lower concentration level. The results demonstrated that precision and accuracy of the assay met the acceptance criteria under the FDA guidelines¹⁶⁴ and suggested the suitability of the PS-MS/MS assay for bioanalytical purposes when SIL analogue is employed as internal standard.

The conventional LC-MS/MS required sample pre-treatment of the plasma samples prior to chromatographic separation. Hence, determination of optimized sample preparation and chromatographic steps was necessary. Sample preparation per sample by protein precipitation was about 40 min followed by LC separation which took about 8 min (7 min analysis time, plus 1 min injection time). Comparably, the data acquired by PS-MS/MS required no sample pre-treatment or chromatographic separation. Sample analysis was achieved by simple direct analysis of DBS samples after optimization of MS

instrument conditions and experimental conditions of PS ionization source. MS spectrum for each sample was acquired within a total analysis time of 1 min which was about 48 times faster on PS-MS/MS. In addition, 5 μ L rat whole blood was required for DBS preparation as compared to about 300 μ L of blood required by LC-MS/MS method in order to obtain the required sample volume of 100 μ L plasma per sample.

Table 3.2: PS-MS/MS and LC-MS/MS method performance data for sunitinib in rat plasma and DBS samples (n = 6)

	Nominal conc. (ng/mL)	Method	mean accuracy	%CV	%bias
LLOQ	1	LC-MS	90.9	5.60	-9.1
		PS-MS	100.0	9.83	0.0
Low QC	5	LC-MS	94.7	6.88	-5.3
		PS-MS	104.8	6.90	4.8
Mid QC	100	LC-MS	92.5	7.65	-7.5
		PS-MS	96.8	3.87	-3.2
High QC	1000	LC-MS	101.9	3.44	1.9
		PS-MS	103.8	4.73	3.8

3.3.1.3 Metabolite effect

The elimination of chromatographic separation in the analytical procedure increase the potential effect from interfering components present in *in vivo* samples that may compete with the parent compound for ionization through coelution and cause ion suppression.

To evaluate the metabolite effect on parent molecule quantitation on the PS-MS/MS assay, *N*-desethyl sunitinib, a major pharmacologically active metabolite of sunitinib was selected for this experiment. Both calibration standards and QC samples were prepared by spiking sunitinib into rat plasma and blood. The metabolite, *N*-desethyl sunitinib, was spiked into three levels (low, mid and high) QC samples at a relatively high concentration (1000 ng/mL), more than the expected normal physiological levels to evaluate the effect on parent molecule quantitation using the assay.

Chromatographic separation of sunitinib and *N*-desethyl sunitinib was achieved on the conventional LC-MS with acceptable bioanalytical assay performance for quantitation of sunitinib. Without chromatographic separation on the PS-MS/MS, coelution of the metabolite with the parent compound had minimal impact on the assay and similar performance was achieved (Table 3.3 and Figure 3.1). Furthermore, the variability at all concentration levels was comparatively lower. The absence of the metabolite interference could be attributed to the use of SIL as the internal standard which could have compensated for ion suppression caused by the coeluting metabolites if present¹⁹⁵. Equivalent sensitivity and linearity were achieved on both methods with fast analysis time by PS-MS/MS assay.

Table 3.3: Metabolite effect evaluation on the PS-MS/MS assay (n = 6)

		PS-MS		LC-MS	
	nominal conc. (ng/mL)	% Bias	% CV	% Bias	% CV
low QC	5	-7.4	2.86	-9.5	5.53
mid QC	100	-2.1	1.96	-9.8	4.82
High QC	1000	-9.2	2.14	-3.0	8.70

Figure 3.1: Metabolite effect evaluation on the PS-MS/MS assay. Representative SRM chromatograms for sunitinib (at 100 ng/mL) and its active metabolite, *N*-desethyl sunitinib (at 1000 ng/mL) in (A) DBS analyzed by PS-MS and (B) plasma analyzed by LC-MS. Separation of sunitinib and its metabolite was achieved on LC-MS (retention time difference of 0.09 min) whilst in PSMS, complete coelution was observed. The two coeluted peaks observed in both SRM channels of LC-MS chromatograms represent the light induced *trans* and *cis*-isomers of both sunitinib and *N*-desethyl sunitinib. The sum areas of both isomers of sunitinib and *N*-desethyl sunitinib were used for quantification.

3.3.1.4 Application of PS-MS assay for preclinical pharmacokinetics study

The validated PS-MS assay was successfully used to determine pharmacokinetic (PK) parameters in plasma samples from male ICR mice which had already received respective amount of 15 mg/kg orally and 5 mg/kg sunitinib intravenously. Serial blood samples (20 μ L) were collected at predetermined time points and analyzed using this assay. Plasma samples obtained from intravenous administration were diluted 10 times with mouse plasma prior to analysis, since some of them exceeded the calibration range. Figure 3.3 shows typical MRM chronogram for a plasma sample from male mouse obtained at 3 h after 5 mg/kg intravenous administered sunitinib, resulting in a sunitinib concentration of 103 ng/mL.

The results yielded from non-compartmental approach are summarized in Table 3.4. Plasma concentration versus time curves for sunitinib obtained after serial bleeding from tail vein are presented in Figure 3.2. After i.v. administration, biphasic elimination was observed with an elimination half-life of 2 ± 0.5 h. The tissue distribution of sunitinib was extensive with volume of distribution of 15.6 ± 9.0 L/Kg. The total clearance value was 0.15 ± 0.05 L/h/Kg and the AUC_{0-24h} was 1.03 ± 0.29 μ g/mL.h. After oral administration, plasma levels reached maximal concentrations of 187.5 ± 65.5 ng/mL at 2.3 ± 1.0 h. The terminal elimination half-life was 2.7 ± 0.4 h, which is similar to those reported in literature^{196, 181} and the AUC_{0-24h} was 1.04 ± 0.34 μ g/mL.h.

Investigation of pharmacokinetic parameters requires blood sampling of several time points over an appropriate time period³⁶. Mice has been one of the species of choice in PK evaluations due to many advantages it offers over others but the volume of blood that can be collected per time point is restricted due its body size. In most conventional LC-MS assays, relatively large blood volume (> 100 μ L) of whole blood is required in order to produce the required sample volume for analysis. However, obtaining this large volume from smaller mammals such as mice is difficult and therefore composite sampling is mostly employed in such cases. The use of this sampling approach reduces the quality of data and simultaneously increases associated costs and resources involved in the preclinical PK study. Also, large number of animals has to be euthanized in such

experiment which is usually not encouraged by the ethical authorities for animal welfare. Over the years, new analytical methods have been introduced within the bioanalytical phase of PK study, but in situation where the use of animals is reduced due to the adoption of DBS sampling, other issues, e.g. sample pre-treatment and chromatographic separation tend to increase analysis time and reduce throughput. Developing high throughput analytical method is of high importance in PK study where time and resources is of great interest.

Figure 3.2: Concentration versus time curve. (A) Obtained after oral administration of sunitinib (15mg/kg) (B) obtained after intravenous administration of sunitinib (5mg/kg) after serial bleeding from tail vein in 6 and 4 mice respectively. Values from both represent the mean of 6 and 4 mice respectively $\pm$ SD.

Figure 3.3: Ion chromatogram for the samples. (A) blank mouse plasma (B) blank mouse plasma spiked with 1 ng/mL (LLOQ) of Sunitinib (C) and plasma sample from a male mouse obtained at 3 h after 5 mg/kg intravenous administered sunitinib, resulting in a concentration of 103 ng/mL sunitinib.

Table 3.4: Pharmacokinetic parameters obtained in male ICR mice after single administration of 5 mg/kg and 15 mg/kg sunitinib intravenously and orally estimated using non-compartmental model (6 and 4 mice for p.o and i.v respectively).

PK parameter	male (i.v)	male (p.o)
$C_{\max}$ (ng/mL)	NA	187.5 ± 65.5
$t_{\max}$ (h)	NA	2.3 ± 1.0
$AUC_{0-\infty}$ (μ g/mL.h)	1.03 ± 0.29	1.04 ± 0.34
$t_{1/2}$ (h)	2.0 ± 0.5	2.7 ± 0.4
CL/kg (L/h/kg)	5.1 ± 1.5	N/A
Vd/kg (L/kg))	15.6 ± 9.0	N/A

NA, not applicable, CL, clearance; Vd, apparent volume of distribution; $t_{1/2}$, terminal elimination half-life;

$AUC_{0-\infty}$, the area under the sunitinib plasma concentration-time curve.

The present study provides a potential alternative to conventional bioanalytical methods used in preclinical PK studies. Each analysis requires a sample volume of 4 μL for quantitation of small molecule drugs within an overall analysis time of 1 min, with minimal sample preparation and no sample pre-treatment or chromatography. Moreover, full pharmacokinetic profile of the model compound, sunitinib, can be obtained from a single mouse. Tail-bleeding method was employed for blood collection because it is less expensive requiring only a mouse restrainer and also enables the possibility of obtaining parameters with the same conscious mice without the use of anesthetics during sampling and further enhances reuse of animals without euthanasia. It must be noted that, upon collection of 20 μL whole blood, microfluidic device could then be further used for fast blood separation and sample pre-treatment. This procedure adopts the multi-step sample pre-treatment process and reduces sample handling^{197, 198}. We attempted to develop such device to be coupled to PS-MS assay. The microfluidic card comprised two components, namely; the filtration and the solid phase extraction (SPE) component. The blood sample is diluted and passed through the filtration membrane to initially remove the red blood cells. The plasma is then passed through the SPE component for sample clean-up and concentration of the analyte of interest with consecutive mixing of internal standard prior to spotting on paper substrate. Upon various testing and investigation of the experimental conditions, poor analyte recovery was observed and the device could not be used.

3.3.1.5 Current limitations and potential applications

In view of the limitations of PS-MS assay for direct *in vivo* bioanalysis, it is important to recognize the potential opportunities it could offer in this field. The elimination of sample pre-treatment and chromatographic separation in this assay increases the potential effect from interfering components in the samples, particularly from in-source breakdown of liable metabolites such as acyl glucuronides, sulfates and N-oxides to yield the protonated molecule of the parent drug thereby giving rise to artificially high plasma concentrations. The use of stable isotopes as internal standards can be adopted to compensate for the ion suppression effect. However, it cannot resolve

in-source conversion issue. Another potential issue is the problem with possible cross-contamination due to the use of the same fixed metal clip. During paper spray analysis, the paper substrates are fixed to a metal clipper connected to the MS high voltage source where a spray is initiated after addition of spray solvent. In this study, this problem was minimized by running blank samples in-between samples. The extent of cross-contamination must be assessed to determine the level of carry-over and decide whether to clean the metal clipper and run blanks in-between samples or resort to the development of disposable cartridges. Finally, the conversion of manual operation of this paper spray prototype to automated control could reduce the workload with minimal sample handling and also improve the required high throughput. Currently, this is a major limitation to this in-house prototype.

Given the unique advantages and some limitations, we think the PS-MS assay could potentially be a useful tool for high throughput MS quantitation of *in vivo* samples where reduce and reuse of animals is required and very small sample volume is available. In this study, a full PK profile was obtained from a single mouse unlike the conventional methods which mostly requires more than one mouse per time point. Also, the sample volume used for the analysis was just 4 μ L. The PS-MS assay enabled the direct analysis of dried plasma spots by combining sampling and ionization process into a single step with minimal sample preparation, thereby reducing the time and cost of analysis. In this regard, it is obvious that the exemption of HPLC equipment which comes with high mobile phase consumption and LC waste stream generation could significantly reduce the overall cost in preclinical studies within the drug discovery and development process.

3.4 Summary and conclusion

The PS-MS assay was evaluated in this study to ascertain that it will provide comparable data to LC-MS/MS assay when used for *in vivo* bioanalytical analysis. Validation parameters and potential interference from metabolite were investigated and compared to results from a conventional LC-MS system. The assay was found to be linear in dried blood spot (DBS) over the concentration range of 1 – 1000 ng/mL and the correlation coefficients (R^2) were constantly > 0.99 with reproducible calibration curves between days for sunitinib, comparable to that from LC-MS analysis. The dynamic range of the PS-MS method was extended to 1000 ng/mL, twice higher than that established in DPS in the previous study. PK studies in drug discovery relies on the dynamic range of the concentrations, hence, the wider the range, the better. A lower limit of quantification (LLOQ) of 1 ng/mL was obtained for sunitinib in DBS and extracted plasma samples for PS-MS/MS and LC-MS/MS respectively. Based on this, quantitative bioanalysis of sunitinib can be carried out using either dried plasma or whole blood spots. The sensitivity of PS-MS assay for sunitinib in DBS was similar to those reported. Furthermore, the PS-MS assay results were found to correlate well with those from conventional LC-MS system, and comparable accuracy, precision and sensitivity were achieved with analysis speed of about 48-fold higher. The presence of stable metabolite in the samples showed no impact on the parent quantitation for a test compound in PS-MS assay. The PS-MS assay was further applied to preclinical PK studies and it was found that, the assay was simple and rapid with overall analysis time of 1 min and a full PK pharmacokinetic profile of sunitinib was obtained from a single mouse. Furthermore, this assay requires small sample volume for analysis reducing the overall cost and the use of animals in the preclinical study. This current work has for the first time showed that, PS-MS could be a potential tool for high throughput *in vivo* bioanalysis in preclinical studies with minimal sample preparation and no chromatographic separation, as most of the reported work in literature so far mainly focus on *in vitro* bioanalysis of small molecule drugs. However, full automation of the PS-MS system in the future will greatly improve the throughput of the assay and is currently a major limitation. Given the unique

advantages and some limitations, the PS-MS system could potentially be a useful tool for high throughput MS quantitation of small molecule drugs in *in vivo* samples.

Chapter 4

4.0 DBS method development for the analysis of Hb CS peptide markers

4.1 Chapter overview

This chapter of the thesis focuses on the method development for the analysis of endogenous peptide markers for hemoglobin Constant Spring (Hb CS) in dried packed red cell spot samples.

In a previous study in our lab, peptide markers for inherited blood diseases were identified using peptidomic approach⁵⁵. Briefly, endogenous peptides that originated from hemoglobin degradation due to protease activity or instability of hemoglobin were identified as potential markers. During the validation studies, peptides with specific mutation sites were considered as markers with ultimate specificity. Hemoglobin constant spring (Hb CS) was among these inherited blood diseases that were studied and the targeted peptides as markers are shown in Figure 4.1.

1	MVLSPADKTNVKA	AWGKVG	AHAGEYGA	EALERMFLS	FPTTKTYF	PHFDLSHG	SAQVKGHG	60
61	KKVADALTNA	VAHVDDMP	NALSALSD	LHAHKLRV	DPVNFKLL	SHCLLVTL	AAHLPAEFTP	120
121	AVHASLDKFL	ASVSTVL	TSKYR	QAGASVAV	PPARWAS	QRALLPSL	HRPFLVFE	173
							[M+H]	
							RPFLVFE -	907
							HRPFLVFE -	1044
							SLHRPFLVFE -	1244
							LPSLHRPFLVFE -	1454
							LLPSLHRPFLVFE -	1567
							ALLPSLHRPFLVFE -	1638
							SQRALLPSLHRPFLVFE -	2010

Figure 4.1: The alpha globin chain of Hb CS with a mutated stop codon at position 142. The peptides used as biomarkers with their molecular masses are listed, all originating from the tail portion of the alpha globin chain.

In the discovery and validation studies, a packed red cell volume of 100 μL was utilized, which might require about 300 μL of capillary venipuncture blood collection. Adopting DBS as alternative blood sampling method to reduce the required sample volume will be of great benefit and can extend this novel Hb CS peptide markers for diagnostic purpose in population screening, especially new born gene screening or seriously sick anemic patients. It will also reduce the pressure on volunteers who donate the blood samples for the research study and further reduce the total cost of shipment as well. Although, DBS sampling method could offer many advantages such as sample volume reduction, ease of transportation and cost, its requirement for extensive sample pre-treatment might not provide the required high throughput analysis demanded in clinical settings. Therefore, direct analysis of the DBS samples will be pursued.

Therefore, using Hb CS as the disease model, this part of the thesis aim to develop a DBS sampling method to detect endogenous Hb CS peptide markers and to further investigate the possibility of direct DBS analysis to detect these biomarkers using paper spray technology to enable high throughput analysis.

4.2 Experimental section

4.2.1 Chemicals, materials and equipment

Harris Micro-punch and cutting mat were purchased from United Scientific Equip. (Singapore). C18 (1-10 μ L) Nutip was obtained from Glygan Corp. (MD, USA). Whatman S&S 903 and Grade 1 (product code: 3001-861) chromatography paper were purchased from GE healthcare UK Limited (Buckinghamshire, England). Centrifugal vacuum concentrator 5301 was purchased from B. Braun Singapore Pte Ltd (Singapore). Centrifuge 5415R was obtained from Eppendorf (Hamburg, Germany). Ethanol (99.9%), acetonitrile (99.9%), formic acid (98.9%), formic acid (98.9%) and HPLC grade water were all purchased from Merck KGaA; and α -cyano-4-hydroxycinnamic acid from Sigma Aldrich (Singapore). Distilled water was obtained from Sartorius purification system (Sartorius, Singapore).

4.2.2 Sample collection and preparation

Normal and Hb CS samples were collected from patients in Thalassemia Clinic, Nakhon Pathom hospital, Thailand. The approval for this study protocol was obtained from the Ethical committee in Mahidol University Institutional Review Board, Thailand (MU-IRB 2012/195.3011). The Ethylene diamine tetra acetic acid (EDTA) blood samples were diagnosed for hemoglobinopathy and thalassemia.

Whole blood (1 mL) was collected in EDTA tube, and then centrifuged at $18407 \times g$ at 4 °C. Plasma and buffy coat were removed whilst the red blood cell was washed thrice with PBS buffer. The washed packed red cell was processed immediately by the addition of cocktail of protease inhibitors. All blood samples were kept at -80 °C for further experiment.

4.2.3 Preparation of dried blood spot samples

About 10 μL volume of packed red cells was spotted on Schleicher & Schuell (S&S) 903 paper and air-dried for 4 h. After drying, eight spot punches were removed from each DBS by using 1.2 mm Harris-Micro punch into a 0.2 mL tube and 100 μL of 50 % ACN: 0.1 % formic acid added. It was vortex mixed and sonicated for 30 min, centrifuged for 30 min at 4 °C, ($15871 \times g$) and the supernatant transferred into fresh 0.2 mL tubes. The collected supernatant was dried by vacuum centrifugation. The samples were reconstituted with 10 μL of 0.1 % formic acid in HPLC grade water. Then 90 μL of 0.1 % formic acid in acetonitrile added and incubated for 1 h on ice. The content was transferred into a new 0.2 mL tube and centrifuged at 4°C, ($15871 \times g$) for 15 min. The supernatant was transferred into a new 0.2 mL tube, dried by vacuum centrifuge and then re-suspended in 20 μL of 0.1% FA HPLC water. After the reconstitution, the sample was further desalted and concentrated by using Millipore C18 Ziptip. The ziptip was first rinsed with 100 % acetonitrile 10 times followed by washing using 100 % HPLC water by pipetting 10 times. Samples were loaded into the ziptip by pipetting 20 times followed by desalting using 100 % water by pipetting 10 times. Then the samples were eluted from ziptip using 50 % acetonitrile first and 100 % acetonitrile one more time. Final extracts were dried through speedvac under room temperature and reconstitute with HPLC water containing 0.1 % formic acid. A 5.5 μL aliquot of the sample was used for LC-MS/MS analysis and 0.5 μL was spotted onto a MALDI target for MALDI-TOF MS analysis.

4.2.4 Matrix assisted laser desorption ionization (MALDI) MS analysis

Alpha-cyano-4-hydroxycinnamic acid (CHCA) was prepared through dissolving 4 mg of CHCA powder into a solution mixture of acetonitrile: HPLC water: formic acid (50:50:0.1, v/v) followed by vortex and brief centrifugation. The supernatant was taken and used.

Equal volume (0.5 μL) of the purified sample and matrix (α -cyano-4-hydroxycinnamic acid) were deposited on Applied Biosystem Opti-TOF 384 Well Insert

plate. The spots were mixed immediately by pipetting up and down and allowed to air dry. After the spots were dried, the plate was loaded into AB 4800 MALDI TOF/TOF for analysis. Acquisition was made using positive reflectron mode with laser intensity set between 2500 and 3500. The detection range was set between 800 m/z and 3000 m/z . Manual checking of the spectra was made to identify typical markers.

4.2.5 LC-MS/MS analysis

Vials containing 5.5 μ L purified samples were loaded into Dionex Ultimate 3000 UHPLC auto-sampler system. The UHPLC system was coupled with Thermo Finnegan LTQ-Orbitrap mass spectrometer through nano-sprays system which enables high detection sensitivity. About 5 μ L of the sample were loaded into Dionex pepmap C18 peptide traps for brief online purification, and then the samples were eluted through gradient of 3.2 % acetonitrile to 40 % acetonitrile in 30 min as illustrated in Figure 5.2, using 15 cm length Dionex pepmap peptide column.

The MS detection method include one MS1 scan under high resolution FT mode for precursor scanning, followed by 5 MS2 scans in ion trap mode on the most intensive 5 peaks detected in MS1 scan. Dynamic exclusion which prevent major peaks from monopolizing the scans and mask off the weaker signals was set to 7.5 s due to typical peak width of the UPLC-MS system determined before. The acquired .Raw files were later submitted to database search due to high complexity.

Figure 4.2: LC gradient for the nano flow LC-MS/MS analysis. Buffer A is 100 % water with 0.1 % formic acid and buffer B is 100 % acetonitrile with 0.1 % formic acid. A Flow rate of 0.3 $\mu\text{L}/\text{min}$ was used throughout the gradient.

4.2.5.1 Database search

Due to the complexity of the LC-MS result, database searching is preferred to manual checking. The .Raw files were first converted into mass generic files using Rawdistiller before submitted to database search engines. The Global Proteome Machine, GPM, a free database search engine utilizing Tandem algorithm were used in this research due to its fast searching speed. The databases used in this research were human protein database and customized databases called HbVar website. Predefined method for Orbitrap was used for other parameters. Because samples were peptides resulted from unspecific proteolytic activity; semi-cleavage and unanticipated cleavage were enabled during the search.

4.2.6 Paper spray mass spectrometry

All experiments were performed using Thermo Finnegan LTQ Orbitrap mass spectrometer (Thermo scientific, San Jose, CA) in full scan mode. Capillary temperature of 275 °C was maintained throughout the experiments. Paper spray ionization was carried out by using Grade 1 chromatography paper cut into an isosceles triangle with 9 mm base width $\times$ 17.1 mm height. A metal clipper was used to hold the paper substrate during the paper spray with a dc voltage of 3.5 kV applied to the metal clipper and 32 μ L of spray solvent (90 % methanol) was used.

4.3 Results and discussion

Briefly, the biomarker candidates for Hb CS were initially identified by analyzing liquid packed red cell Hb CS samples of different genotypes by both MALDI-TOF MS and LC-MS/MS utilizing sample volume of 100 μ L.

The purified extracted sample was subjected to MALDI-MS analysis where unique peaks were observed in the Hb CS samples spectra but not the normal blood spectrum as shown in Figure 4.3. In Figure 4.3B, the singly protonated peptides $[M+H]^+$ present in the blood was matched to the elongated region of Hb CS due to the likely degradation of the tail region according to their masses and therefore had the potential to be reliable markers.

Although the sequences matching on MS1 level looks promising, information from MS/MS spectrum was still required as it provides information on amino acid sequences. To address this, Hb CS and normal samples were subjected to LC-MS/MS analysis and the detected peptides with +2 charge states are shown in Appendix 3. Peptide marker candidates that were identified from both MALDI and LC-MS/MS analyses are summarized in Table 4.1.

Table 4.1: The Hb CS peptide markers with their sequences

$[m/z]$	Peptide sequence
907	¹⁶⁷ RPFLVFE ¹⁷³
1044	¹⁶⁶ HRPFLVFE ¹⁷³
1244	¹⁶⁴ SL HRPFLVFE ¹⁷³
1454	¹⁶² LPSL HRPFLVFE ¹⁷³
1567	¹⁶¹ LLPSL HRPFLVFE ¹⁷³
1638	¹⁶⁰ ALLPSL HRPFLVFE ¹⁷³
2010	¹⁵⁷ SQRALLPSL HRPFLVFE ¹⁷³

Figure 4.3: MALDI-MS spectra of liquid packed red cell samples. (A) Normal packed red cell sample with the peptides candidates absent. (B) Hb CS packed red cell sample with the peptide candidates indicated with rectangular bars.

4.3.1 Dried blood spot method development for Hb CS peptide analysis

With the fore-knowledge of these peptide markers, their detection in dried blood spots was pursued using smaller sample volume. Therefore, initial part of the work involved the optimization of various parameters that will enable maximum recovery of these peptide markers for detection whilst utilizing minimum sample volume. Qualitative evaluation based on replicate analysis was used for the selection of the optimum condition for each parameter.

A minimum blood volume spot of 10 μ L was enough to detect these peptide markers in dried blood using the optimal extraction parameters obtained. Shown in Figure 4.4 are different blood volumes spotted on S&S 903 filter paper. After spotting, it was allowed to dry at ambient temperature for a period of 4 h. In addition, as shown in Figure 4.5, different spot punches were made including the optimum spot punch of eight from the dried spot which represented a less volumetric measurement of the 10 μ L spotted.

Figure 4.4: Optimization of sample volume. Dried blood spots prepared with volumes of 10 and 30 μ L having diameters of 0.7 and 1.1 cm respectively. The packed red cell of different volumes were spotted on S & S 903 filter paper and allowed to dry at ambient temperature for 4 h period.

Figure 4.5: Optimization of spot punches number to be taken. Dried packed red cell spot of 10 μ L with different spot punches using 1.2 mm Harris micro-punch after drying. Spot punches of 4, 6 and 8 from the filter paper represent a volumetric measurement that is similar to the application of a pipette for liquid measurements.

After the extraction of the DBS with optimal solvent (0.1 % formic acid in 50 % acetonitrile), hemoglobin and impurities must be precipitated with organic solvent with simultaneous extraction of peptides from the packed red cell. Hence, the effect of different organic solvents including 100 % cold ethanol and 0.1 % formic acid in acetonitrile was tested. Both organic solvents are miscible with water in all ratios and low molecular weight proteins, and especially peptides, are soluble in both acetonitrile¹⁹⁹ and ethanol buffers. Therefore, these organic solvents can manifest some degree of selectivity in precipitating hemoglobin and other impurities whilst extracting more peptides. The use of both solvents for precipitation resulted in recovery of peptide markers. However, 0.1 % formic acid acetonitrile was selected for precipitation for a number of reasons. It did not require the use of lab-grade freezers (-80 °C) unlike in ethanol precipitation but only ice. In addition, no occasional blockage of the LC trap was observed. An hour precipitation period could be exempted to reduce the analysis time by employing molecular weight cutoff kits instead, however, based on previous observation; they tend to retain some amount of sample which may lead to the loss of some particular peptide markers. The use of plastic materials were practically reduced during the sample pre-treatment stage, hence, glass vials were mostly utilized.

The optimal conditions obtained for the investigated parameters were used to prepare the dried packed red cell spots samples. Figure 4.6 represents a MALDI spectrum obtained from a dried 10 μ L packed red cell spot and extracted with the optimum conditions. All potential markers were confidently detected from the extracted Hb CS

DBS samples as shown in Figure 4.6B; different from the dried spot from the normal sample Figure 4.6A.

After the successful detection of the peptide markers from Hb CS DBS samples, stability of these markers in DBS was also evaluated. DBS sampling method is famously known for stabilizing analytes within the paper matrix for an extended period of time. Therefore, stability of these peptide markers in DBS under different storage conditions was investigated. Group of DBS samples were prepared and stored at 4 °C and room temperature for a period up to 12 months. In addition, another group of DBS samples were also prepared and stored under moderate temperatures of 37 °C and 40 °C for 1 month period. These temperatures were investigated to mimic the transportation conditions in normal courier services or shipment. At the end of the storage period, the DBS samples were analyzed and the results showed that peptide markers were stable under all conditions within the period validated.

Finally, the optimum conditions of DBS preparation and extraction was used to analyze peptide markers in all Hb CS samples of different genotypes using MALDI-MS and LC-MS/MS analyses. The result (Table 4.2) showed detection of peptides markers in all the Hb CS genotypes by both analytical methods used. However, it is important to note that the information obtained from these analyses was not enough to differentiate the different genotypes of Hb CS but can be used for the diagnosis of Hb CS in population screening, especially new born gene screening.

Figure 4.6: MALDI-MS spectra of DBS samples. (A) Normal DBS extracted sample with peptides candidates absent. (B) Hb CS DBS extracted sample with the peptide candidates indicated with rectangular bars.

Table 4.2: Summary of peptide markers in different heterozygote CS DBS samples obtained from both LC-MS/MS and MALDI- MS analysis. Bottom row represents normal DBS sample as negative control from which no peptide markers were identified.

Sample		Peptides						
Code	Genotype	907	1044	1244	1454	1567	1638	2010
1	HCS	✓	✓	✓	✓	✓	✓	✓
2	HCS	✓	✓	✓	✓	✓	✓	✓
3	HCS	✓	✓	✓	✓	✓	✓	✓
4	HCS	✓	✓	✓	✓	✓	✓	✓
5	HCS	n.d	✓	✓	✓	✓	n.d	✓
6	HCS	✓	✓	✓	✓	✓	✓	✓
7	HCS	n.d	✓	✓	n.d	✓	n.d	n.d
8	HCS	✓	✓	✓	✓	✓	✓	✓
9	HCS	✓	✓	✓	✓	✓	✓	n.d
10	HCS	✓	✓	✓	✓	✓	✓	✓
11	HCS	✓	✓	✓	✓	✓	✓	✓
12	CSCS	✓	✓	✓	✓	✓	✓	✓
13	CSCS	✓	✓	✓	✓	✓	✓	✓
14	EFCS	✓	✓	✓	✓	✓	✓	n.d
15	Hb H-CS	n.d	✓	✓	n.d	n.d	✓	n.d
	Normal	n.d	n.d	n.d	n.d	n.d	n.d	n.d

HCS- Hb CS trait, CSCS – homozygous of Hb CS, Hb H-CS – Hb H-CS disease, EFCS - EFBart's disease, n.d – not detected, ✓ - detected.

Similar result to the conventional venipuncture sampling adopted in the discovery and validation study was obtained for the DBS sampling method and indicates the possibility of using this sampling method in diagnostic applications. Packed red cell occupies about 50 % of normal blood whilst in the disease Hb CS patients; it is reduced to 30 %. Hence, more whole blood is to be sampled in case conventional venipuncture sampling method is to be adopted in clinical/research settings to obtain the required sample volume for analysis.

In addition, apart from the smaller blood volume collection demonstrated by DBS method, the use of peptide markers for Hb CS diagnostics provides several advantages to the traditional methods currently utilized. For instance, blood smear method is error prone whilst ELISA requires the use of antibodies which is usually costly. Also, longer analysis time and use of carcinogenic reagents for HPLC-UV and PCR-Gel respectively is a concern. Unfortunately, these two methods are the most common diagnostic tools widely utilized in Thailand, where thalassemia is prevalent. Contrary to these aforementioned disadvantages of the conventional methods, this new method can be easily adopted for diagnostics purposes due to its requirement for less labor and expertise. Moreover, during the discovery studies, MRM method was demonstrated for three selected peptide markers using high flow analysis (Figure 4.7). The total analysis time for these selected peptide markers was 35 min. Hence, the availability of triple quadrupole instruments with MRM capability in clinical settings in the future will enable Hb CS population screening. Also, unlike most peptide-based approach for hemoglobinopathy screening where peptide markers for relevant hemoglobin variants are generated through trypsin digestion of DBS samples, the current method avoids digestion step.

However, one significant drawback of sampling by means of DBS in this study is the processing step required prior to chromatographic separation. Additional sample preparation steps also common to liquid sampling such as protein precipitation and sample desalting and concentration, altogether limit the required high throughput analysis needed in research or clinical settings.

In view of these drawbacks, direct analysis of the peptide markers in DBS samples was pursued through the application of paper spray ionization technology. The ability to

detect the peptide markers directly from the DBS samples without any traditional sample preparation and chromatographic separation will provide the required high throughput analysis platform. Therefore, 10 μ L dried packed red cell spot samples were prepared using grade 1 chromatography paper and subjected to PS analysis after optimization.

Figure 4.7: Elution times of the three peptide markers in LC-MS. Total ion chromatography spectrum of sample with code name MS01. 1637.93Da, 1566.90Da and 1453.81Da correspond to peptide sequence **ALLPSLHRPFLVFE**, **LLPSLHRPFLVFE** and **LPSLHRPFLVFE**, respectively. Difference of one amino acid between these samples renders 1 min difference in retention time ⁵⁵.

4.3.2 Direct analysis of Hb CS DBS samples by paper spray mass spectrometry

The choice of the paper substrate for blood collection in this study depends on the hydrophobicity of the paper substrate. This is to enable a less binding interaction between the proteins/peptides and the celluloses in the paper matrix. Both Grade 1 and Silica coated Chr papers are less hydrophobic but Grade 1 Chr paper is comparatively more hydrophobic; hence, was selected for this experiment.

Dried blood spots for the HB CS samples were made by spotting 10 μ L of the packed red cell onto the paper and allowed to dry at room temperature. The DBS samples were analyzed directly by adding the spray solution at once to the dried spot. One representative PS-MS spectrum obtained is shown in Figure 4.8. The protein hemoglobin and some lipids could be observed without the Hb CS peptide markers. The peak at m/z 616 was due to the heme group which indicates that there was unfolding of hemoglobin during the paper spray process. In addition, the less hydrophobicity of the paper substrate enabled the extraction of lipids from the paper substrate as observed by the presence of peaks in the m/z 700 – 900 range. This observation suggests that, there was extraction of lipids and proteins from the DBS with the spray solvent system. However, the absence of the peptide markers could be due to the low percentage of Hb CS peptide markers in the peripheral blood makes which could make it difficult to be detected without sample preparation and concentration. Also, the presence of high molecular weight hemoglobin in the MS spectrum could mean it was extracted from the DBS during paper spray process which could suppress the ionization of the peptides through charge competition in the ionization phase.

Another likely reason could be the use of chromatography paper as substrate which could not enable the elution of the peptide markers and subsequent ionization and detection. A report published after the completion of this project confirmed this hypothesis where a specialized paper, silanized, was used to produce better signals for protein hemoglobin ¹⁴⁵. The surface hydrophobicity of the paper type was increased by modifying the hydroxyl groups on the cellulose by perfluoroalkylation ²⁰⁰. This

silanization process reduced the binding of hydrophilic hemoglobin proteins to cellulose¹⁴⁵. In the same study, Grade 1 Chr paper, less hydrophobic, was used and resulted in detection of the heme groups and lipids without hemoglobin. This finding further confirmed the reasons for not detecting any peptide markers in this work when this paper type was utilized.

Figure 4.8: PS-MS mass spectrum of Hb CS DBS sample using Grade 1 Chr paper. Hb CS peptide markers were not observed.

So far, analyses of proteins using ambient ionization methods have been demonstrated in various studies^{201, 202, 109, 115}. However, applications of direct analysis methods in proteomics or peptidomics approaches have not been documented. This is because; most of these markers exist in small concentration in the blood, so sample pre-treatment and pre-concentration seems to be inevitable. However, the rise of ambient ionization methods with sample pre-treatment steps incorporated into the ionization process may further improve the detection of these low level protein or peptide markers in the future.

4.4 Summary and conclusion

Dried blood spot (DBS) sampling technique was developed as an alternative sampling method for the analysis of biomarkers in DBS samples and further investigated the direct analysis of these biomarkers in DBS using paper spray mass spectrometry method. The disease model used for this study was hemoglobin Constant Spring (Hb CS) which was among a group of inherited blood diseases that were previously studied in our lab and the potential peptide markers identified using peptidomic approach. Therefore, optimum parameters needed for DBS preparation and extraction of endogenous peptide markers prior to chromatographic separation and mass analysis were investigated. A DBS prepared by spotting 10 μ L packed red cell on S & S 903 filter paper, dried for 4 h and punched-out spots of eight taken with subsequent solvent extraction and precipitation with 0.1 % formic acid in acetonitrile was sufficient to detect all Hb CS specific peptide markers. The eight punched-out spots represented a less volumetric measurement of the 10 μ L spotted. The stability studies showed that peptide markers were stable in the DBS at 4 °C and room temperature for a period up to 12 months. In addition, the peptide markers were found to be stable at 37 °C and 40 °C for 1 month period. Analysis of Hb CS DBS samples of different genotypes by MALDI and LC-MS methods showed detection of peptide markers in all the Hb CS genotypes comparable to that obtained when liquid packed red cell (100 μ L) was used. Practical advantages of DBS such as requirement of microvolume sample, analyte stability during storage and ease of sample

handling relative to the traditional liquid biological sample matrices ¹⁰⁶ have been demonstrated in this study. Hence, this DBS sampling method can be potentially utilized in the Hb CS diagnosis in population screening especially newborn gene screening or enable the collection of blood samples from remote regions for clinical Hb CS diagnostics. However, in most clinical laboratories where population screening are carried out, high throughput analytical methods are preferable; hence, the elimination of the DBS sample preparation prior to chromatographic separation and MS analysis will be of great interest.

Therefore, direct analysis of the DBS samples by paper spray mass spectrometry method was investigated. Unfortunately, this direct analysis approach was unsuccessful. Paper spray method is concentration dependent and the relatively low percentage Hb CS peptide markers present in the peripheral blood might make it difficult to be detected. Moreover, the presence of high molecular weight hemoglobin could suppress the ionization of the peptides during elution from the paper matrix. Another likely reason could be the use of chromatography paper as substrate which could not enable the elution of the peptide markers and subsequent ionization and detection. The chromatographic paper has low surface hydrophobicity which might bind the hydrophilic peptide markers to the cellulose matrix. Therefore, the existence of peptide markers at extremely low concentration in the blood makes sample cleanup prior to chromatographic separation and mass analysis inevitable.

Final Conclusion & Future recommendation

Part I

- *In vitro* investigations have shown developed PS-MS assay to be linear, precise, accurate and sensitive, in accordance with regulatory acceptance criteria on the validation of bioanalytical. Longer storage effect on the analyte stability and elution in dried plasma and whole blood spots showed that the test compound, sunitinib, was stable in all conditions stored. It was also observed that storage could impact the analyte extraction and detection sensitivity; hence, either off-line or online wetting of DBS prior to MS analysis could minimize the effect on analyte signal.
- Investigation into the procedure of adding SIL-IS directly into microvolume sample during quantitative *in vivo* bioanalysis showed that the results from SIL-IS coated PCR tube sampler correlate well with those from a conventional laboratory pre-mixed procedure and comparable accuracy and precision were achieved with a 5 μ L plasma, serum and whole blood spots.
- Evaluation of the PS-MS assay to ascertain that it will provide comparable data to LC-MS/MS assay when used for *in vivo* bioanalytical analysis have shown that PS-MS assay results correlate well with those from conventional LC-MS system, and comparable accuracy, precision and sensitivity were achieved with analysis speed of about 48-fold higher. Also, the presence of stable metabolite in the samples showed no impact on the parent quantitation for a test compound in PS-MS assay. Sensitivity of 1 ng/mL was obtained in both dried plasma and blood spots, similar to that obtained from conventional LC-MS assay, with minimal sample preparation and no chromatographic separation.

- The application of PS-MS assay to preclinical PK study found that, the assay was simple and rapid with overall analysis time of 1 min and a full PK pharmacokinetic profile of sunitinib could be obtained from a single mouse. The assay required small sample volume, thereby reducing the overall cost and the use of animals in the preclinical study.
- Conversion of this in-house prototype from manual operation to automatic control in the future could reduce contamination, sample handling and improve the throughput of the assay, as this is currently identified as a major limitation in this study.
- Future studies will look into the translation of this study to real case drug discovery studies in our lab where we aim to develop peptide drug analogues with better stability than cetorelix (commercialized peptide drug for prostate cancer treatment). Serum testosterone levels are measured to determine our peptide drug analogues' stability using conventional liquid chromatography electrospray tandem mass spectrometry (LC-MS/MS) method. The alternative application of paper spray ionization for the analysis of testosterone can provide enormous advantages in terms of its simplicity and high throughput analysis required for the quick selection of potential peptide drug candidates. Microvolume serial blood sampling could be achieved using mice instead of rats to reduce the amount of peptide drug analogues dosed and the overall cost of the study. Due to the poor ionization of testosterone and crude sample matrix, reactive paper spray can be used. This is a sample preparation step that is incorporated into the ionization process without compromising with the analysis time. Therefore, an online derivatization procedure can be used for the paper spray ionization of testosterone.

Part II

- A 10 μ L packed red cell on S & S 903 filter paper was sufficient to detect all Hb CS specific peptide markers.
- The eight punched-out spots represented a less volumetric measurement of the 10 μ L spotted.
- The stability studies showed that Hb CS peptide markers were stable in the DBS at 4 °C and room temperature for a period up to 12 months and at 37 °C and 40 °C for 1 month period.
- DBS coupled with MALD-MS and LC-MS/MS analysis enabled the detection of Hb CS peptide markers in different genotypes with results obtained comparable to that of packed red cell liquid (100 μ L).
- Direct analysis of Hb CS DBS samples by paper spray mass spectrometry method was unsuccessful probably due to low percentage Hb CS peptide markers present in the peripheral blood, presence of high molecular weight hemoglobin that could suppress the ionization of the peptide markers or poor elution from the paper matrix. Further investigation will be necessary to comprehend the impact of these factors on the peptide markers detection.
- The results from this study indicate that the use of DBS samples could enable microvolume sample use, Hb CS peptide markers stability and ease of sample handling compared to the liquid packed red cell. Hence, this DBS assay can be potentially utilized in the Hb CS diagnosis in population screening especially newborn gene screening or enable the collection of blood samples from remote regions to the site of clinics for Hb CS diagnostics.
- Future studies may look into the use of different paper types with high surface hydrophobicity. Moreover, detection of peptide markers may be improved by

performing blood component separation, sample pre-treatment and concentration *in situ* in a microfluidic device prior to spotting on the paper substrate. Also, detection sensitivity of the markers can also be improved online by incorporating these sample preparation procedures into the paper spray ionization process without compromising with the analysis time. Finally, the use of pipette-tip as ambient ionization method has been reported ²⁰³. Hence, specialized pipette tip with C18 adsorbent able to reduce the sample complexity and direct peptide markers analysis can be investigated.

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Appendices

Appendix 1

Standard deviation

The standard deviation of the method is calculated from:

$$S_{xo} = \frac{S_y}{b}$$

Coefficient of variance

The coefficient of variance of the method expressed as a percentage is given by the equation;

$$V_{xo} = \frac{S_{xo}}{\bar{x}} \times 100\%$$

Where;

$\bar{x}$ = mean

S_{xo} = standard deviation

Appendix 2

Nilotinib

Dasatinib

Sunitinib

Imatinib

Figure. 1. Chemical structures of test compounds.

Appendix 3

MS29-10ph #3139 RT: 35.65 AV: 1 NL: 4.06E6
F: FTMS + p NSI Full ms [300.00-2000.00]

MS29-10ph #2926 RT: 33.97 AV: 1 NL: 9.29E6
F: FTMS + p NSI Full ms [300.00-2000.00]

MS29-10ph #2453 RT: 30.42 AV: 1 NL: 2.10E6
F: FTMS + p NSI Full ms [300.00-2000.00]

MS29-10ph #2228 RT: 28.78 AV: 1 NL: 7.95E6
F: FTMS + p NSI Full ms [300.00-2000.00]

MS29-10ph #2801 RT: 33.05 AV: 1 NL: 2.84E5
F: FTMS + p NSI Full ms [300.00-2000.00]

MS29-10ph #3274 RT: 36.98 AV: 1 NL: 6.06E6
F: FTMS + p NSI Full ms [300.00-2000.00]

MS29-10ph #2855 RT: 33.45 AV: 1 NL: 1.97E5
F: FTMS + p NSI Full ms [300.00-2000.00]

Figure 2: Zoom in LC-MS/MS spectra of the Hb CS peptide markers with +2 charge states indicated with the arrows.

List of publications and presentations during thesis

Publications

1. Takyi-Williams, J.; Dong, X.; Gong, H.; Wang, Y.; Jian, W.; Liu, C.-F.; Tang, K., Application of paper spray–MS in PK studies using sunitinib and benzethonium as model compounds. *Bioanalysis* **2015**,7 (4), 413-423.
2. Takyi-Williams, J.; Liu, C.-F.; Tang, K., Ambient ionization MS for bioanalysis: recent developments and challenges. *Bioanalysis* **2015**,7 (15), 1901-1923.

Poster presentations

1. Takyi-Williams, J., Tang, K., Liu, C.-F. Evaluation of a Paper Spray Mass Spectrometry for direct analysis of Dried Plasma Spots for In Vivo Bioanalytical Studies. BioPharma Asia Convention, Singapore, March **2015**.
2. Takyi-Williams, J. Dong, X., Gong, H Tang, K., Liu, C.-F. Paper spray mass spectrometry for the quantification of small molecule drugs in plasma. 62nd ASMS Conference, Baltimore, MD, USA, June **2014**.
3. Takyi-Williams, J., Dong, X.; Gong, H.,Tang, K. Optimization of Paper Spray Mass Spectrometry for Quantification of Small Molecules in Plasma. 4thAOMSC & 10thTSMS Annual Conference, Taipei, Taiwan, July **2013**.