DEVELOPMENT, VALIDATION AND APPLICATION OF ANALYTICAL METHODS TO MEASURE PROGNOSTIC BIOMARKERS IN PATIENTS RECEIVING ON-PUMP CORONARY ARTERY BYPASS GRAFTING SURGERY

by

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Abstract

Temporary interception and then re-infusion of oxygenated blood into heart during on-pump Coronary Artery Bypass Grafting (CABG) surgery causes ischemia-reperfusion injury (IRI). Propofol is an intravenous anesthetic agent that maybe potentially cardioprotective against IRI. This thesis presents the development, validation and application of analytical methods for monitoring propofol and a series of prognostic biomarkers in hope of identifying contributory factors to IRI and propofol cardioprotection.

I developed a Capillary Electrophoresis (CE) method to quantify propofol concentrations in blood. Propofol concentrations in the µg/mL range were measured from 400 µL samples. A dose-finding study using this method determined a practical infusion rate of 120 µg•kg⁻¹•min⁻¹ to achieve the target blood concentration of 5 µg•mL⁻¹.

To measure the oxidative stress biomarker, 15-F₂-isoprostane, the nitrosative stress biomarker, 3-nitrotyrosine, the myocardial protective factor, adenosine, and the cardiovascular risk factor, asymmetric dimethylarginine (ADMA), I developed simple, sensitive and robust Liquid Chromatography-Mass Spectrometry (LC-MS) or Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) methods. Briefly, a basic mobile phase and base-resistant column were used for the LC-MS analysis of 15-F₂-isoprostane. A one-step solid phase extraction and pentafluorophenyl (PFP) core-shell column were employed for the LC-MS/MS analysis of 3-nitrotyrosine. A Strong Cation Exchange (SCX) solid phase extraction and modifier-free mobile phase were implemented for the LC-MS/MS analysis of adenosine. A new derivatization method to enable baseline separation of ADMA and its regio-isomer symmetric dimethylarginine (SDMA) was developed for the ADMA LC-MS/MS quantitation. Performance parameters for these methods, including linearity, precision, accuracy, Limit of Detection (LOD), Limit of Quantitation (LOQ), Lower Limit of Quantitation (LLOQ) and stability were found satisfactory.

Concentrations of 15-F₂-isoprostane, 3-nitrotyrosine and adenosine were found to rise after on-pump CABG surgery. However, these changes were not able to explain the cardioprotective effect of propofol. Nonetheless, the correlations of 15-F₂-isoprostane with diabetes, glucose concentration and PTEN level were significant. Patients with low cardiac output syndrome experienced more 3-nitrotyrosine increase than patients without this syndrome. The basal adenosine level was found to increase more in patients with low left ventricular ejection fraction. These findings and the underlying methodologies are important for identifying new prognostic biomarkers.
Preface

I prepared this dissertation in accordance with the Structure of UBC Theses and Dissertations requirements of the Faculty of Graduate Studies, The University of British Columbia.

For each chapter of this dissertation, my roles were to: 1) design and implement method developments with a focus on instrumental analyses; 2) validate the analytical methods according to FDA and other regulatory guidelines; 3) perform quantitative analyses of patient plasma or whole blood samples and collect data for further analyses; 4) conduct data analyses and subanalyses to test statistical hypotheses for differences between treatment groups, patient groups and groups with different outcomes; 5) draft and edit manuscripts for method developments and biomarker quantitation; 6) outline, draft and edit this dissertation. Dr. David D. Y. Chen is my primary research supervisor and sponsor of this work. Dr. David M. Ansley, my research co-supervisor is the Principle Investigator of the Canadian Institute of Health Research Operating Grant #82757 that funded the majority of this work. Dr. Jennifer Love kindly shared her LC-MS/MS instrument for this research. Specifically, the authors and co-authors of published manuscripts underlying each chapter made the following contributions:

Chapter 2: I was mainly responsible for the method development, method validation and quantitation of patient samples. Dr. Koen Raedschelders did partial method validation and quantitative analysis of the patient samples and drafted the manuscript for submission. Dr. Hong Zhang carried out exploratory work on the method development. Dr. David M. Ansley and Dr. David D. Y. Chen contributed to revisions and provided guidelines.

Chapter 3: I was mainly responsible for the quantitation of patient samples. Dr. Koen Raedschelders performed data analysis and drafted the manuscript for submission. Dr. Hong Zhang carried out exploratory work on quantitative analysis of patient samples. Dr. David M. Ansley and Dr. David D. Y. Chen contributed to revisions and provided guidelines.

Chapter 4: I was mainly responsible for the method development, method validation and quantitation of 15-F2t-isoprostane in patient plasma samples. Dr. Baohua Wang et al performed western blotting and statistical analysis and drafted the manuscript for submission. Dr. David M. Ansley et al contributed to revisions and provided guidelines.

Chapter 5: I was mainly responsible for the method development, method validation and quantitation of free and protein bound 3-nitrotyrosine in patient plasma samples. I performed
statistical analysis and drafted the manuscript for submission. Michael Wong and Shuai Sherry Zhao assisted me develop the method. Dr. David M. Ansley and Dr. David D. Y. Chen contributed to revisions and provided guidelines.

Chapter 6: I was mainly responsible for the method development, method validation and quantitation of adenosine in patient plasma samples. I performed statistical analysis and drafted the manuscript. Shuai Sherry Zhao assisted me validate the method. Dr. David M. Ansley and Dr. David D. Y. Chen contributed to revisions and provided guidelines.

Chapter 7: I was mainly responsible for the method development, method validation and quantitation of asymmetric dimethylarginine in patient plasma samples. I performed statistical analysis and drafted the manuscript. Michael Wong carried out exploratory work on method development. Joo-oll Kim assisted the analyses of patient samples. Dr. David M. Ansley and Dr. David D. Y. Chen contributed to revisions and provided guidelines.

The PRO-TECH II clinical study was reviewed and approved by the UBC Clinical Research Ethics Board (Certificate number H04-70456) in compliance with the principles outlined in the Declaration of Helsinki.

This dissertation is based on the following manuscripts:


4. Yu Hui, Michael Wong, Shuai Sherry Zhao, Jennifer A. Love, David M. Ansley, and David D. Y. Chen. A Simple and Robust LC-MS/MS Method for Quantification of Free 3-Nitrotyrosine in


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List of Symbols and Abbreviations

•OH Hydroxyl radical

2-D 2-dimensional

2-ME 2-mercaptoethanol

AAPS American association of pharmaceutical sciences

ACN Acetonitrile

ADMA Asymmetric dimethylarginine

ADP Adenosine diphosphate

ALK Anaplastic lymphoma kinase

APCI Atmosphere pressure chemical ionization

APPI Atmospheric pressure photoionization

As Peak asymmetric factor

ATP Adenosine triphosphate

BSA Body surface area

CABG Coronary artery bypass grafting

CE Capillary electrophoresis

CE-MS Capillary electrophoresis mass spectrometry

cGMP-PKG Cyclic guanosine monophosphate-protein kinase G

CHD Coronary heart disease

CI Cardiac index

CID Collision induced dissociation
CK Creatine kinase
CK-MB Creatine kinase-MB (Muscle/brain subtype)
CO Cardiac output
CPB Cardiopulmonary bypass
cTnI Cardiac troponin I
DC Direct current
DDAH Dimethylarginine dimethylaminohydrolase
DNA Deoxyribonucleic acid
DPX Disposable pipette extraction
dSPE Dispersive SPE
EDTA Ethylenediaminetetraacetic acid
EDV End diastolic volume
ELISA Enzyme-linked immunosorbent analysis
EMA European Medicines Agency
eNOS Endothelial nitric oxide synthase
EPP Extraction and pre-purification
ESI Electrospray ionization
ESV End systolic volume
FAB Fast atom bombardment
FBG Fast blood glucose
FDA Food and Drug Administration
FIA Flow injection analysis

FID Flame ionization detector

FT-ICR Fourier transform-ion cyclotron resonance

GC Gas chromatography

GC-MS Gas chromatography

HILIC Hydrophilic interaction chromatography

HLB Hydrophilic lipophilic balance

HPLC High performance liquid chromatography

HR Heart rate

ICCF Intermittent cross-clamp fibrillation

ICU Intensive care unit

IHD Ischemia heart disease

iNOS Inducible nitric oxide synthase

iPF2α-III 8-epi prostaglandin F2α

IRI Ischemia Reperfusion Injury

IS Internal standard

iTRAQ Isobaric tags for relative and absolute quantitation

K-ATP Potassium-adenosine triphosphate

LC Liquid chromatography

LC-MS Liquid chromatography mass spectrometry

LC-MS/MS Liquid chromatography tandem mass spectrometry
LCOS Low cardiac output syndrome
LDI Laser desorption ionization
LLOQ Lower limit of quantitation
LOD Limit of detection
LOQ Limit of quantitation
LVEF Left ventricular ejection fraction
LVSWI Left ventricular stroke work index
MALDI Matrix-assisted laser desorption ionization
MAP Mean arterial pressure
ME Matrix effect
MEKC Micellar electrokinetic chromatography
MEPS Micro-extraction by packed sorbent
MI Myocardial infarction
MMA Monomethylarginine
MMP-9 Matrix metalloproteinase 9
mPTP Mitochondrial permeability pore
MRM Multiple reaction monitoring
mRNA messenger ribonucleic acid
MS/MS tandem mass spectrometry
MT Migration time
N Number of theoretical plates
NADPH Nicotinamide adenine dinucleotide phosphate
NCCLS National Committee for Clinical Lab Standards
NDA 2,3-Naphthalenedicarboxaldehyde
NF-H Hyperphosphorylated neurofilament
NMR Nuclear magnetic resonance
nNOS Neuronal nitric oxide synthase
NO• Nitric oxide
NPLA N-propyl-arginine
O• Superoxide
OLSPE on-line SPE
ONO• Nitrogen dioxide radical
ONOO¯ Peroxynitrite
ortho-phthalaldehyde OPA
PADP Pulmonary artery diastolic pressure
PAOP pulmonary artery occlusion pressure
PASP Pulmonary artery systolic pressure
PCR Polymerase chain reaction
PD Plasma desorption
PE Process efficiency
PFP Pentafluorophenyl
PGF₂α Prostaglandin F₂α
PI3K Phosphoinositide 3 kinase
PKC Protein kinase C
PMI Perioperative myocardial infarction
PRO-TECT II PROpofol cardioproTECTion for Type II Diabetes
PTCA Percutaneous transluminal coronary angioplasty
PTEN Tensin on chromosome 10
PVDF Polyvinylidene fluoride
QC Quality control
QIT Quadrupole ion trap
R Resolution
RAP Right atrial pressure
REs Relative errors
RF Radio frequency
RIA Radioimmunoanalysis
RISK Reperfusion injury salvation pathway
RNS Reactive nitrogen species
ROS Reactive oxygen species
RSD Relative standard deviation
RT Retention time
SBSE Stir bar sorptive extraction
SCX Strong cation exchange
SDMA Symmetric dimethylarginine

SILAC Stable isotope labeling with amino acids in cell culture

SPE Solid phase extraction

SPME Solid phase microextraction

SRM Selective monitoring mode

SV Stoke volume

SVI stroke volume index

SVR Systemic vascular resistance

SVRI Systemic vascular resistance index

TGF-β1 Transforming growth factor beta1

TID Thermionic detector

TMT Tandem mass tag

TOF Time of Flight

TSI Thermospray ionization

UV Ultraviolet
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Dedication

I dedicate this work to my wife and family with my heart-felt thankfulness.
1. Introduction: measurement of prognostic biomarkers to predict myocardial ischemia reperfusion injury (IRI) and pharmacological cardioprotection efficacy during on-pump cardiac artery bypass grafting (CABG)

Since this thesis is an interdisciplinary work between analytical chemistry and clinical science, background information from both sides will be imparted in this introduction to give a comprehensive context of this research. Section 1.1 and 1.2 outline the basic concepts of biomarker and biomarker research from a general clinical perspective, including biomarker definition and classification as well as preclinical and clinical biomarker research. Sections 1.3 through 1.5 give details of one important aspect in clinical biomarker research, the development and validation of analytical methods from a general analytical chemistry perspective. Sections 1.6 through 1.10 outline the biomarker research in our clinical study from a specific clinical perspective. Section 1.11 outlines my dissertation objectives.

As the readers of this thesis navigate to Chapter 2 through 7, development, validation and application of analytical methods in my biomarker research are presented in details only from a specific analytical perspective because clinical perspectives of this research are already described in this introduction.

1.1 Biomarker

1.1.1 Definition

A biological marker (biomarker), according to the definition made by the Biomarker Definition Working Group of the National Institute of Health, is “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention” [1]. By definition, a biomarker can be physical, chemical or biological in nature. For example, it may refer to a nucleic acid, peptide, protein, lipid, carbohydrate, amino acid, or metabolites. It may also refer to biophysical properties such as viscosity, pH, image, osmolarity, pressure, or size, etc.

1.1.2 Classification

Biomarkers may be classified as follows [1, 2]: 1) diagnostic biomarkers that are used to identify a disease or abnormal condition; 2) staging biomarkers to determine the stage or extent of
a disease; 3) prognostic biomarkers to predict the disease progression or remission; 4) predictive biomarkers for anticipating the clinical response to a therapeutic intervention.

The literature doesn’t clarify the key difference between these biomarkers. For example, diagnostic or staging biomarkers are sometimes called prognostic biomarkers. So as to eliminate confusion, I propose the following classification as shown in Figure 1. Diagnostic biomarkers are the downstream characteristics that result from a condition. Staging biomarkers are the special diagnostic biomarkers whose level is significantly correlated with the degree of a condition. They may be used to stratify the condition rankings. As opposed to the diagnostic biomarkers, prognostic biomarkers are the upstream characteristics that result in a condition. Their involvement in a condition is indicative (indicator), detrimental (a risk factor) or beneficial (a protective factor). If prognostic biomarkers are altered by a therapeutic intervention so that they have values in predicting a clinical response to such an intervention, they may be defined as the predictive biomarkers.

During a clinical trial, “a characteristic or variable that reflects how a patient feels, functions, or survives” is known as a clinical endpoint [1]. Clinical endpoints are the most reliable characteristics in evaluating the benefits and risks of a therapeutic intervention. Generally a clinical endpoint is not considered a biomarker since it is not always objectively measurable. Some of the clinical endpoints are very difficult or take enormous time to obtain, e.g. ultimate survival, kidney failure, heart attack, or stroke incidents [3]. Thus there is a good reason of using “surrogate endpoint” or “surrogate biomarker”, which are defined as “a biomarker that is intended to substitute for a clinical endpoint” and “expected to predict clinical benefit based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence” [1]. Either prognostic or diagnostic biomarkers may be chosen as surrogate endpoints if they quantitatively correlate with clinical endpoints.

There is substantial interest among many clinicians and drug developers to search for surrogate endpoints because of their substantial clinical significance. So far only a very small subset of biomarkers are validated surrogate endpoints, e.g. blood pressure, cholesterol concentration and intra-ocular pressure. Therefore the majority of biomarker research is the nonsurrogate biomarker research. Although nonsurrogate biomarkers are not as important as their surrogate counterparts, the use of nonsurrogate biomarkers in a clinical trial can provide: (1) a better understanding of a disease mechanism, therapeutic regimen and targets; (2) a better
methodology of alternative diagnoses; (3) a more rational inclusion or exclusion rule for future clinical trial conduct and design [4].

![Diagramatic Illustration of the Biomarker Classification System Proposed by the Author](image)

**Figure 1: Diagramatic illustration of the biomarker classification system proposed by the author**

**1.2 Biomarker research**

The last few decades have seen an explosive evolution of the discovery, validation and application of biomarkers for disease diagnosis, therapy and management. Biomarker research has helped translate laboratory findings to clinical applications in numerous therapeutic fields, such as cardiology [5], neurology [6] [7], nephrology [8], pulmonology [9], endocrinology [10] [11], gastroenterology [12], gynecology [13] and oncology [14] [15]. Both scientists and clinicians striving to provide optimal and innovative medicines for individual patients have broadly embraced this bench-to-bedside paradigm.
1.2.1 Biomarker discovery in preclinical biomarker research

Biomarker discovery is the first step of biomarker research. Most of the biomarker discovery uses animal or cell culture samples to identify candidate biomarkers.

Historically, biomarker discovery was focused on looking for one or two representative substances. In the postgenomic era, many emerging technologies have enabled biomarker discovery in a global manner. “Omics”, the high-throughput analyses of varieties of targets, including proteins (proteomics), gene expression (transcriptomics), genes (genomics), peptides (peptidomics) and metabolites (metabolomics), are widely employed technologies for biomarker discovery [16, 17].

Deoxyribonucleic acid (DNA) sequencing, real-time polymerase chain reaction (PCR) and DNA microarray are contemporary transcriptomics or genomics technologies used for high-throughput analyses of DNA or messenger ribonucleic acid (mRNA). In the postgenomic era, an emphasis is also placed on the use of computerized databases to search for clinical DNA or mRNA biomarkers [18].

Mass spectrometry is the primary proteomics technology to uncover novel protein or peptide biomarkers. Coupling liquid chromatography or gel electrophoresis with mass spectrometry further facilitates protein biomarker identification. As one example, “Shotgun proteomics” digests complex protein mixture to peptides and separates the peptide mixtures online using strong-cation exchange (SCX) followed by reverse phase chromatography prior to tandem mass spectrometry analysis (MS/MS) [19, 20]. Sequences of peptides can be deciphered by using MS/MS and genomic databases [21]. The utilities of these technologies may be extended to semi-quantitative analyses, where usually the isotope-coded tags are used to label the peptides. *In vivo* labeling (e.g. Stable Isotope Labeling with Amino Acids in Cell Culture or SILAC) [22] and *in vitro* labeling (e.g. Tandem Mass Tag or TMT [23], Isobaric Tags for Relative and Absolute Quantitation or iTRAQ [24], and Dimethyl Multiplexed Labeling [25]) are designed to identify the difference of protein expression between two or more samples in a single instrumental analysis.

Metabolomics is a global profiling study of metabolites. Again, mass spectrometry based technologies prevail in metabolomics because of their unparalleled sensitivity, selectivity and speed. Flow infusion analysis (FIA) delivers the sample directly to MS without a preceding separation. Despite being fast and convenient, FIA is not appropriate for the analysis of low
abundance metabolites, because it usually experiences substantial ion suppression. Hyphenated technologies such as LC-MS [26], CE-MS [27] and GC-MS [28] alleviate the concerns of matrix effect or ion suppression [29]. If background noise from chemical contaminations still confounds the identification of the biomarker of interest, tandem MS analysis can be used to increase the signal-to-noise ratio and percentage of positive identification.

Omics and biomarker discovery are the ongoing efforts attracting tremendous interests. New technologies have the far-reaching impact not only on preclinical biomarker research, but also on clinical biomarker research.

1.2.2 Analytical method development and validation in clinical biomarker research

Owing to the development of new technologies, each year hundreds of candidate biomarkers are found in potential correlation with a specific condition or treatment in preclinical biomarker research. Not all the candidate biomarkers can or will have clinical applications. Similar to drug development, multiple phases of clinical trials are conducted to validate biomarkers and apply them to real clinical scenarios. Regardless of the design and format, the clinical biomarker research put candidate biomarkers into vigorous test prior to turning them into diagnostic tools. Translation of a candidate biomarker from laboratory to clinics usually takes a long, uncertain and uneven path [30]. After the identification of a candidate biomarker in preclinical biomarker research, development and validation of analytical methods provide the feasibility and quality assurance of a sound clinical biomarker research. A poorly developed and validated analytical method could lead to false positive or false negative results, causing confusion and high costs for the healthcare system [31].

There are several challenges for developing and validating an analytical method: 1) the differences between biomarkers require the development of different analytical methods; 2) lack of reference substances makes definitive quantification difficult; 3) the heterogenous nature of biological matrixes presents a challenge to the accurate quantification; 4) diverse patient demographics may confound the changes of biomarkers in a patient population; 5) there is not any clear guideline how to validate analytical methods for biomarkers.

The confusion regarding analytical method validation for biomarkers is profound, underpinning the need for harmonization of validation approaches [32]. In 2001, Food and Drug Administration (FDA) issued analytical validation guidelines for small molecular drug analysis (www.fda.gov/downloads/Dru/.../Guidances/ucm070107.pdf). This document outlined the
validation steps for bioanalytical methods of exogenous analytes. However, when it comes to endogenous biomarkers, validation guidelines currently do not exist.

Following the American Association of Pharmaceutical Sciences (AAPS) 2003 Biomarker Workshop, analytical method validation in nonclinical and clinical samples was addressed [32, 33]. AAPS proposed a practical, iterative and “fit-for-purpose” approach with regard to biomarker analytical method development and validation. However, validation should be tailored to match the intended application while ensuring data quality. The experimental design should aim at using minimum but sufficient validation to address method reliability and data quality.

Many principles for developing and validating an analytical method originate from small molecule drug analysis. Definite quantitation for large molecule biomarkers has yet to be standardized and sound validation protocol remains elusive [34]. The following sections from 1.3 to 1.5 emphasize various aspects of developing and validating analytical methods for metabolite biomarkers, because most of my research projects are metabolite-related.

1.3 Pre-analytical aspects for analytical method development

Sample collection, quenching of reactive species, transportation and storage are likely to affect sample integrity; thus, they should be given high priorities in a method development [35, 36]. However, often times these pre-analytical steps are far from standardized. From a practical point of view, an effective collaboration between analysts and clinicians is rather critical because clinician take a high percentage of ownership of these pre-analytical steps from sample collection to storage to ensure appropriate collection and storage prior to analysis.

Biomarker-related chemical and biochemical reactions can remain active until they are inactivated with quenching. Timely sampling and quenching will characterize a target biological state and they should meet the following criteria [36]: 1) quenching is able to stop the biomarker changes instantaneously without incurring side reactions; 2) sampling and quenching should be mild enough to preserve biomarker’s chemical identity, physical property and concentration that are relevant to an analysis; 3) the collected and quenched sample should be compatible with the downstream sample preparation and analysis; 4) sampling should aim at the right compartment of the biological system so that the sample may accurately reflect the target physical or pathological state.
Lee et al have discussed a few aspects of sample collection that might be important to sample integrity and suitability [32]. First, the sample collection procedure should be consistent throughout a study so that the variations due to human factors are minimized. Second, the addition of anticoagulant or other substances into a sample could give rise to stability issues and might require extra investigation. Third, sample collection tubes, transfer pipettes, injection needles and storage containers may be sources of variability and bias because of biomarker-contact surface interaction. Fourth, serum is generally preferred over plasma due to serum’s simplicity. Fifth, ongoing reactions and heterogeneity of blood and tissue samples are of concern and may warrant a thorough investigation. Some of these issues are really difficult to address because of current methodology limitations but pre-knowledge about the interfering interactions or reactions, if any, could help prevent them from occurring.

Selection of which biological matrix is most suited for an analysis depends on the types of biomarkers under study. Sampling DNAs, mRNAs, proteins or peptides requires cell or tissue extraction. Metabolites are generally easier to sample because most of them are present in plasma, urine, serum or saliva. The uses of plasma, serum and urine as sample matrixes are preferred since they are easy to access in large quantity and can be quickly replenished by human body. Plasma or serum may reflect the “instantaneous” changes of a pathological or physical state while urine gives the “average” readout of polar biomarkers [35]. Other biological fluids such as saliva, cerebral spinal fluid, synovial fluid, stomach fluid, or exhaled breath can represent local conditions and thus provide valuable information for specific needs.

The most popular quenching measures following sample collection are pH and temperature modifications [36]. Additions of sodium hydroxide, potassium hydroxide, perchloric acid, hydrochloric acid, or trichloroacetic acid are plausible ways used to deactivate enzyme reactions in a pre-analytical sample. Cooling the sample to temperatures lower than -20 °C is a measure used to effectively corrupt enzyme activity and protect the sample from disintegrating. Quenching with organic solvents is another alternative. 60% cold-buffered methanol could reduce intracellular leakage of metabolites from yeast [37].

Keeping samples at low temperature is a widely accepted sample storage practice. General assessment of metabolite stability by Lauridsen et al showed that no changes in the H-1 NMR fingerprints occurred during the storage at or below -25 °C for 26 weeks [38]. On the other hand, the storage of samples at -20 or -80 °C could not stop endogenous B-type natriuretic peptide (BNP) degradation in one study [39].
1.4 Analytical method development

Analytical method development is concerned with three basic questions in order to achieve sufficient sensitivity and specificity: 1) what instrument is used for separation; 2) what detector is suitable; 3) what sample preparation is effective.

Around these questions, Table 1 summarized the tasks to be fulfilled, samples to be used and goals to be accomplished in analytical method development for biomarkers. Some of the performance parameters used in method validation should be preliminarily assessed when developing an analytical method prior to full validation. Most of these performance parameters will be described in detail in Section 1.5 Analytical method validation.
<table>
<thead>
<tr>
<th>Steps</th>
<th>Tasks</th>
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<tr>
<td>1. Selection of instrument for separation</td>
<td>Literature research; integration of resources; installing, qualifying and tuning instrument; preliminary evaluation of separation efficiency and system suitability</td>
<td>Internal standard and authentic standard in organic/aqueous solvent</td>
<td>Adequate separation and system suitability (e.g. resolution; number of theoretical plates; retention time or migration time %RSD; peak asymmetry factor; carryover effect)</td>
</tr>
<tr>
<td>2. Selection of Instrument Detector</td>
<td>Literature research; integration of resources; installing, qualifying and tuning detector; evaluation of sensitivity and selectivity</td>
<td>Internal standard and authentic standard in organic/aqueous solvent</td>
<td>Adequate sensitivity and selectivity and compatibility with separation technology (e.g. LOD and LOQ for standard solution; cross-talk between internal standard and authentic standard; selectivity over major known interfering substances if any)</td>
</tr>
<tr>
<td>3. Sample preparation</td>
<td><strong>Extraction</strong>: literature research; evaluation of different solvents, temperatures, extraction apparatus, protein precipitation methods, ultrafiltration methods;</td>
<td>Native biological sample spiked with internal standard; biological sample spiked with authentic standard and internal standard</td>
<td>Adequate crude separation of analyte from biological matrix (e.g. retention time shift from standard solution sample; %RSD of retention time and signal); Adequate sensitivity, selectivity and precision (e.g. estimated LOD and LOQ for real biological sample; estimated concentration of analyte in biological sample of interest; peak shape, intensity and discernibility; resolution factor; theoretical plates if possible; %RSD of authentic/internal standard ratio); Adequate recoveries (e.g. absolute recovery; SPE recovery);</td>
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1.4.1 Selection of instrument for separation

Biological samples contain numerous substances that may interfere with a downstream analysis. Without separation, the signal of low abundance analytes can be suppressed or enhanced by other high abundance components, causing inaccurate quantitation. Hence, pre-detection separation of analytes from interfering substances remains necessary in most analyses despite the development of more selective detectors [40, 41].

The quantitation of metabolite biomarkers poses great challenges for analysts. The number of metabolites in human body approximates 2500 to 3000 [42-44]. Further, the chemical and physical properties of metabolites are extremely divergent. They may be hydrophobic or hydrophilic, volatile or nonvolatile, water-soluble or not water-soluble, relatively small or large, ionic or nonionic. Therefore, method developments for metabolites have to be tailored case by case.

For volatile metabolites, most commonly used separation methods are based on gas chromatography (GC). In GC, volatile analytes are separated through their differential interactions with stationary phase. Mobile phase is a carrier gas, e.g. helium or hydrogen. Since the 1960s, GC-MS has been used to quantify a wide range of compounds of clinical interest. It was capable of quantifying analytes at picogram levels, requiring only 0.1-1.0 mL plasma or 1-5 mL urine samples [45-47]. GC is superior to LC in terms of resolution and retention time reproducibility. For example, GC-based methods offer superb sensitivity in the analyses of steroids [48], F2-isoprostane [49] and malondialdehyde [50]. However, the intrinsic drawback of a GC-based method is the need of converting nonvolatile to volatile analytes through derivatization. Further, GC-MS use is limited for thermal stable compounds [51, 52].

Liquid chromatography (LC) is the most suitable separation method for semi-polar metabolites [53]. In contrast to GC, LC is more versatile and can be applied to the separation and analysis of thermal labile and nonvolatile analytes. In LC, the differential partitioning of analytes between stationary phase and mobile phase results in the different retention times of the analytes and forms the basis of separation. While mobile phase plays a role in determining the partitioning coefficient of an analyte, the major determinant is the interaction of the analyte with stationary phase. Analytes may interact with stationary phase in adsorption, ion exchange, size exclusion, partition and affinity mode, by which LC is categorized.

Normal phase chromatography refers to a liquid chromatography system where the
mobile phase is less polar than the stationary phase. Normal phase chromatography is primarily used in separating organic compounds including chiral molecules on a preparative scale but its poor resolution power has limited its application in quantitative analysis [54]. In reverse phase chromatography, the mobile phase is more polar than the stationary phase. During a gradient elution program of the reverse chromatography, the organic content in the mobile phase, e.g., methanol or acetonitrile, increases gradually to disrupt the hydrophobic interaction between analyte and stationary phase and elute analytes out. Reverse phase chromatography is the most commonly used chromatography and account for the majority of LC analyses [55]. Although its resolution power is not as good as capillary electrophoresis or gas chromatography, reverse-phase chromatography offers great robustness, versatility, sample-loading capacity, separation capability, precision, speed and injection accuracy. It can also be easily interfaced with a variety of detectors. Reverse phase chromatography usually employs silica coated with C8 and C18 alkyl groups as stationary phase, and it is therefore able to separate the analytes according to their hydrophobicity. Despite its remarkable performance, reverse phase chromatography is not suited for the separation and analysis of ionic or highly polar analytes. Normal and reverse phase chromatography are mainly adsorption chromatography.

In 1990, hydrophilic interaction chromatography (HILIC), innovated by the Alpert group, revitalized the use of normal phase chromatography in quantitative analysis [56]. HILIC can be categorized as partition chromatography, where part of the mobile phase is believed to form a water-rich layer on the surface of a polar solid material and the rest of the mobile phase forms a water-deficient layer. Analytes with different partition coefficients between these two mobile phase layers can be separated from each other. Compared to reverse phase chromatography, HILIC still lacks stationary phase stability and robustness but recent development of new coating materials holds great promise to compensate for these deficiencies [53].

Ion exchange chromatography is another important type of liquid chromatography, which may separate a variety of biological molecules according to the differences of their charge states. The majority of biological molecules including drugs, metabolites, proteins, polypeptides, nucleic acids, polynucleotides, charged carbohydrates and polysaccharides are ions. Therefore, the utility of ion exchange chromatography is broad. During the sample-loading step of ion exchange chromatography, charged groups of an analyte interact with the stationary phase through ionic bonds. Then the molecules that don't pair with stationary phase may be removed during a washing step. Lastly, bound biological molecules are eluted out by a solution with either higher ionic strength or disruptive pH. Ion exchange chromatography provides high resolving power and
good recovery. However, ion exchange chromatography may not be compatible with the downstream online analysis (e.g. ESI mass spectrometry), because the elution solution may contain high concentration of salt. Ion exchange chromatography is widely applicable in analytical and preparative separations as long as a charge difference exists [55].

Since its inception in 1981 [57], capillary electrophoresis (CE) has become one of the most powerful separation techniques for ionic species, regardless of the size of a molecule. CE separates analytes on the basis of their charge-to-size ratios. The separation principle of CE is different from that of liquid chromatography or gas chromatography. So CE may provide complementary information. CE may also serve as an orthogonal method to verify quantitative information from LC analysis [58, 59]. CE offers several distinct advantages over LC or GC: 1) CE is highly amenable to analyzing large molecules such as proteins or DNA; 2) CE separation can be achieved in a fast and highly efficient manner (high theoretical plate counts); 3) the instrument and the separation capillary of CE are simple and inexpensive; 4) CE consumes very little solvent and most of the time organic solvent is unnecessary; 5) CE requires minimum sample pre-purification owing to its inherent compatibility with ionic analytes. Small loading volume tends to be a constraint on the use of CE in biomarker research. Fortunately, the recent development of a CE-MS interface by our lab and others [60] [61] largely offset this deficiency, thanks to the superior sensitivity and selectivity offered by the mass spectrometer.

As outlined in Table 1, the main goal in Step 1 of method development is to achieve adequate system suitability. By tuning instrument and analyzing standard samples prepared at nominal concentration in pure solvent, the analysts may assess the system suitability of a chosen instrument and method. The system suitability parameters that should be evaluated are defined as below [62]:

**Equation 1**

\[
Resolution (R) = \frac{(RT_{peak1} - RT_{peak2})}{1/2(Width_{peak1} + Width_{peak2})}
\]

**Equation 2**

\[
Number of Theoretical Plates (N) = 5.54 \times \left(\frac{RT}{Width_{half height}}\right)
\]

**Equation 3**
\[
\%\text{RSD of RT or MT} = \frac{\text{Standard Deviation of RT or MT}}{\text{Mean of RT or MT}} \times 100
\]

**Equation 4**

\[\text{Peak Asymmetry Factor (A}_2\text{)} = \frac{b}{a}\]

where RT is the Retention Time (often used in liquid chromatography); MT is the Migration Time (often used in CE); \%RSD is the percentage of relative standard deviation; b is the distance from the peak midpoint to the tailing edge measured at 10% of peak height; and a is the distance from the leading edge to the peak midpoint measured at 10% of peak height. A suitable separation system should have a Resolution equal or greater than 1, a Number of Theoretical Plates greater than 10,000 in liquid chromatography, \%RSD of RT less than 10%, and Peak Asymmetry Factor close to 1.

To qualify a separation system for quantitation, carryover effects should also be evaluated. Carryover is the contamination of a sample injection by the preceding injection and it has negative impact on the precision and accuracy of an analysis. The method to evaluate carryover effects is to run a high concentration sample prior to a blank sample and observe the presence of residual analyte peaks. Carryover effects may be attenuated by increasing the wash volume of separation column between the adjacent runs and flushing the autosampler needle [63].

**1.4.2 Selection of detector**

The selection of a detector depends on the chemical and physical properties of the target analyte(s), potential matrix interferences, availability of detectors, compatibility of a detector with the upstream separation techniques, predicted concentrations of analytes, required accuracy, precision, sensitivity, selectivity and the ultimate goal for the analysis.

Many reviews have been published on the working and selection principles of detectors [64-67]. A good detector should have the following characteristics: 1) high sensitivity and precision; 2) wide linear dynamic range (the range over which detector output changes linearly with the analyte quantity); 3) good response regardless of the nature and composition of mobile phase; 4) selective response to the analytes of interest; 5) no post-separation peak broadening; 6) robustness and easiness to use; 7) nondestructive to the analytes; 8) fast response and short duty cycle (the time interval used for acquiring, analyzing or transmitting data); 9) capability to generate qualitative and quantitative information of the analytes.
GC is usually coupled with a couple of unique detectors. A flame ionization detector (FID) converts carbon compounds to formylium ions \( \text{CHO}^+ \), which may react with water and break down to \( \text{H}_2\text{O}^+ \) and CO, and measures the resulted ion current [68]. The FID essentially counts the carbon numbers of a compound despite minor responses to other heteroatoms. It provides a large linear dynamic range \((10^6 \text{ to } 10^7)\) and good sensitivity (limit of detection \(~\text{pg}\)). Because it is universally applicable to nearly all organic compounds, it is the most widely used detector in GC analysis. An electron capture detector (ECD) contains a Ni-63 radioactive source of \( \beta \) particles (electrons), which may react with nitrogen to produce \( \text{N}_2^+ \) and one more additional electron. These electrons form a standing current between a pair of electrodes [69]. Organic compounds containing components with high electron affinity, e.g. halogen, phosphorus or nitro groups, may capture the electrons and reduce the standing current between electrodes to generate a signal. Hence, ECD is highly selective and sensitive towards trace amounts of organic compounds containing high electron affinity elements, with a limit of detection approaching low femto-grams [70].

Both LC and CE are usually coupled with a UV-Vis, fluorescence or electrochemical detector. Ultraviolet-visible (UV-Vis) is one of the most commonly used detectors in an analytical lab because it is cheap, robust, versatile, quantitative, qualitative, nondestructive and easy to use. However, a UV-Vis detector is not sensitive (limit of detection \(~\text{ng}\)) and selective enough for detecting low abundance biomarkers in a complex matrix. Many organic compounds respond to UV-Vis detection due to the electron transition from \( \pi \) to \( \pi^* \), \( n \) to \( \pi^* \), or \( n \) to \( \sigma^* \) molecular orbitals. For example, most aromatic compounds absorb UV at a wavelength <260 nm, compounds with double bonds at ~215 nm, and aliphatic compounds at ~205 nm. Since UV-Vis detection is universal for many compounds, mobile phase composition should be carefully selected not to interfere with the analyte detection. Three types of UV-Vis detectors, fixed wavelength detector, variable wavelength detector, and photodiode array detector (PDA), are commonly built into a LC or CE instrument for online detection. Fixed wavelength detector is cheap and simple but it is no longer routinely used in an analytical lab because the applicable scope of this detector is very limited. Variable wavelength detector places a grating plate and filtering slit before a flow cell and allows the adjustment of incident wavelength over time. PDA detector has a grating plate placed after the flow cell. In PDA, pass-through light composed of multiple wavelengths may be refracted to provide spectral information concurrently. PDA detector is not as sensitive as fixed and variable wavelength detectors but can be used for spectral fingerprinting [71, 72].
Fluorescence detector responds to the light emitted by analyte molecule upon the excitation by an incident light at a shorter wavelength. Only the analyte that is natively fluorescent or labeled with a fluorescent reagent may fluoresce at a specific wavelength. A fluorescence detector is usually arranged in a way such that emission light is perpendicular to excitation light. This configuration reduces the background noise effectively. The light source for excitation can be a broad-spectrum deuterium or xenon flash lamp or single wavelength laser. The broad-spectrum source doesn’t produce as sensitive detection as laser source. Fluorescence detector can be 100 times more sensitive than UV-Vis detector (limit of detection ~pg). Thus quantitation of biomarkers at low abundance is possible with a fluorescence detector. Further, fluorescence detector has a larger linear range than UV-Vis detector. Background fluorescence, a concern for quantitative analysis, might be reduced by degasing the mobile phase [71, 72].

Electrochemical detector can selectively measure the electrical current generated when an analyte is oxidized or reduced [73]. It is one of the most sensitive detectors for specific compounds, such as catecholamines or neurotransmitters. The mobile phase used in separation has to be conductive when an electrochemical detector is chosen. The working and reference electrodes configured in an electrochemical detector are usually applied with a fixed voltage. In addition to high sensitivity (limit of detection ~fg to pg) and selectivity, an electrochemical detector is easy to use and inexpensive, but may be susceptible to background noise and electrode fouling.

Corona charged aerosol detector, chemiluminescence, conductivity, light scattering and reflective index detectors are rarely used in the biomarker analyses because of either insufficient sensitivity (limit of detection at high ng or above) or special requirements of instrumentation [66].

A hyphenated system refers to a system made from two standalone systems. Mass spectrometer, a standalone detector, can be coupled with GC, LC or CE, or other separation or sample processing devices (e.g. MALDI), and is the cornerstone for the biomarker analysis.

Comprehensive reviews have been published for mass spectrometry instrumentation in many papers and book chapters [74, 75]. A mass spectrometer is composed of three major components: an ion source used to convert analyte molecules of various states to gaseous ions, a mass analyzer used to sort the ions according to their mass-to-charge ratio, and a transducer used to detect the presence of ions. Other components of mass spectrometers include vacuum systems, electric supply and control board, radiofrequency generator, ion lens for ion focusing, ion guide
for ion transportation, and computer hardware and software for acquiring and processing data.

Mass spectrometers are mainly categorized by the types of mass analyzers. Sector was the earliest mass analyzer [76], which filters ions and only passes the ions with the specific mass-to-charge ratios by varying the magnetic field of an electromagnetic sector and/or the acceleration potential for the gaseous ions. In the sector mass analyzer, the magnetic field is applied perpendicularly to the ion beam and makes the ions travel circularly. The principle for sector to separate ions may be summarized in:

**Equation 5**

\[
\frac{m}{q} = \frac{B^2 r^2}{2U_a}
\]

where \( \frac{m}{q} \) is the mass-to-charge ratio; \( B \) the magnetic field strength; \( r \) the radius of an ion circular trajectory and \( U_a \) the acceleration potential difference. Sector is the choice of high accuracy measurement, but because it is too bulky, sector has been largely replaced by TOF or FT-ICR.

TOF mass analyzer [77, 78] distinguishes the ions with different mass-to-charge ratios by measuring the times the gaseous ions travel from the ion source to the detector. The principle of how TOF mass analyzer separates the ions can be summarized in:

**Equation 6**

\[
t_{TOF} = \frac{L}{v} = L \sqrt{\frac{m}{2qU_a}}
\]

where \( t_{TOF} \) is the time a gaseous ion spend when it travels from the ion source to the detector; \( L \) is the length of the TOF mass analyzer tube; \( v \) is the velocity of a gaseous ion after acceleration; \( \frac{m}{q} \) is the mass to charge ratio of the ion; \( U_a \) is the potential difference of the acceleration region positioned before the mass analyzer tube. There is no need to have pre-knowledge regarding \( L \) and \( U_a \) since the unknown mass-to-charge ratio can be computed through the calibration with the ions of known mass-to-charge ratios. TOF offers low to high resolution (mass dependent, >35,000 for bovine insulin at 5734Da), high mass accuracy (<5ppm), very high m/z range (no theoretical limit), high sensitivity, medium dynamic range, medium to good quantification information, fast speed, pulsed or continuous ion-source, compact design and low to medium complexity for handling. TOF can be easily interfaced with GC, LC or CE or MALDI.
also coupled to TOF, ion traps, sector or quadrupole mass analyzers for tandem mass spectrometry. Quadrupole-TOF hybrid is one of most popular mass spectrometers for quantitative and qualitative analysis.

Quadrupole mass analyzer [79, 80] passes only the ions with specific mass-to-charge ratios through varying the direct current (DC) and radio frequency (RF) potentials applied on the four parallel medal rods, also known as electrodes, thereby stabilizing or destabilizing the ion trajectories according to the mass-to-charge ratios. In quadrupole mass analyzer, a voltage what equals to \(U+V\cos\omega t\) (\(U\) is the DC potential and \(V\) is the RF potential) is applied on one pair of electrodes opposite to each other, and a voltage that equals to \(-(U+V\cos\omega t)\) is applied on the other pair of electrodes. Only ions with the specific mass-to-charge ratios may pass through the quadrupole filter. Mathieu equation and stability diagram can be used to explain the principles underlying the quadrupole mass filtering [81, 82]. Quadrupole mass analyzer offers low to medium resolution (at best 25,000 at 1000Th), low mass accuracy (<5ppm might be possible), low m/z range (usually 25 to 2000Th, up to ~4000), high sensitivity, high dynamic range, good to very good quantification information, medium to fast speed (~6000Th/s), continuous ion-source, compact design, competitive low price and low complexity for handling. Quadrupole mass analyzer can be easily coupled with GC, LC or CE. Quadrupole mass analyzer is routinely used in quantitative analysis because of its good speed, sensitivity and precision for single ion monitoring. In fact, hybrid instrument such as triple quadrupole mass spectrometer is the gold standard for biomarker quantitation. With triple quadrupole mass spectrometer, tandem mass spectrometry [83] is commonly performed in a selective reaction monitoring mode (SRM, also called multiple reaction monitoring, MRM mode). Filled with inert gas, the second quadrupole functioning as a collision cell is used for the collision-induced dissociation (CID), which fragments the selected precursor ions to product ions. The third quadrupole selectively filters the product ions prior to electromultiplier detection.

Orbitrap and FT-ICR are designed for accurate mass determination and well suited for biomarker discovery. Orbitrap [84] offers very high resolution (~130,000 FWHM for m/z at 400Th), very high mass accuracy (~2ppm), low m/z range (50 to 4000Th), high sensitivity (sub femtomole), medium dynamic range (~5000), medium quantification information, slow to medium speed (~0.3s at 7500 FWHM or ~1.9s at 100,000FWHM), pulsed or continuous ion-source, and medium complexity for handling. Orbitrap can be easily coupled with LC, CE or MALDI. FT-ICR [85] offers highest resolution (~8,000,000 FWHM for bovine ubiquitin at 8569.6Da), very high mass accuracy (~0.1 to 1ppm), medium m/z range (~30 to 10,000), high
sensitivity (<100 zeptomole), medium dynamic range, medium quantification information, slow to medium speed, pulsed or continuous ion-source but demanding complexity for handling. FT-ICR can be coupled with LC, CE or MALDI. Orbitrap and FT-ICR are both expensive instruments and not readily accessible for routine analysis. The ion separation principle underlying FT-ICR mass analyzer can be summarized in:

**Equation 7**

\[ f_c = \frac{qB}{2\pi \cdot m} \]

where \( f_c \) is the cyclotron frequency of the ions circulating the trapping cell; \( B \) is the magnetic field strength; \( m/q \) is the mass-to-charge ratio.

Ion sources are also important to consider when analysts develop a mass spectrometry method for biomarker quantitation. The type of analyte determines the choice of ion source. If metal ions were to be quantified, an atomizing ion source (e.g. inductively coupled plasma [86]) would be a logic choice. If it were volatile molecules, an electron or photon induced ionization source (e.g. electron ionization, chemical ionization or photoionization) would be appropriate. The choices of ion sources for the analysis of small nonvolatile molecules are ESI [87], APCI [88], atmospheric pressure photoionization (APPI) [89], or thermospray ionization (TSI) [90] etc. In case of analyzing large fragile molecules such as proteins, nucleic acids, or noncovalently bound clusters, a soft ion source (e.g. MALDI [91-93], fast atom bombardment or FAB [94], plasma desorption or PD [95], or ESI [87]) would be reasonable choices.

ESI was invented in 1984 by Fenn et al [87]. In ESI, analytical samples are prepared in polar volatile solvents and infused to a spray emitter applied with a high positive or negative voltage. The high potential drop between the emitter and MS entrance aperture drives the charged species to enter the Taylor cone, a tapered protrusion of the polar solvent out of the emitter. Positive or negative analyte ions accumulate on the surface of Taylor cone under the electric field. Liquid droplets containing charged species are ejected out of the Taylor cone when the electrostatic field overcomes the surface tension of the solvent. The charged droplets will contract in size and/or split to small ones due to the loss of solvent through nitrogen-assisted evaporation. Finally the ions enter the gaseous phase due to the solvent vaporization or ion evaporation (a mechanism where the ions depart the surface of a small droplet prior to a complete solvent vaporization). The ionization of ESI may result in the gaseous ions of various charge states. ESI may be interfaced with different mass analyzers and offers high ionization efficiency. In case of
nano-LC-ESI for peptides analysis, the mass detection limit is in attomole to low femtomole range, implying a nearly 100% ionization. ESI is a soft ionization technique that induces very little in-source fragmentation.

APCI [88] is an ion source that sprays the analyte solution with a gas nebulizer and desolvates the solvent in a heated quartz tube. The nitrogen and oxygen molecules in air may interact with the corona discharge to create primary ions, which react with the vaporized solvent (e.g. water) to give secondary reactant gas ions (e.g. H$_2$O$^+$ and (H$_2$O)$_n$H$^+$). The resulted reactant gas ions then collide with neutral analyte molecules to produce analyte ions. Despite soft, APCI ionization may generate more in-source fragments than ESI. The sensitivity of APCI is usually not as good as that of ESI. APCI is less susceptible to the ion suppression than ESI and better suited for a high flow-rate LC. The performance of APCI is superior to ESI when analyzing the nonpolar molecules with low molecular masses.

Sensitivity and selectivity requirements determine the choice of detector for analytical method development. For the analyses of small ionizable biomarkers in low abundance, detectors with high sensitivity are recommended. However, sensitive detectors such as fluorescence or electrochemical detector offer limited selectivity. Mass spectrometer combining high sensitivity and selectivity turns out to be the most legitimate choice for ionizable biomarker quantitation. In fact, quadrupole or TOF mass spectrometer is routinely employed for the quantitative study of biomarkers, owing to their compact design, relatively low cost, high sensitivity, ease to be hyphenated with other separation instruments, medium to good linear dynamic range and ability to provide accurate quantitative information etc.

As outlined in Table 1, selected instrument detectors should provide adequate sensitivity and selectivity as well as the compatibility with separation instrument. Limit of Detection (LOD) and Limit of Quantitation (LOQ) should be evaluated in this method development step by running standard solution of internal standard and authentic standard [32]. LOD and LOQ are usually calculated by using the following equations:

**Equation 8**

\[
LOD = \frac{\text{Standard concentration}}{S/N} \times 3
\]
**Equation 9**

\[ \text{LOQ} = \frac{\text{Standard concentration}}{S/N} \times 10 \]

where S/N is the signal-to-noise ratio of the analyte peak relative to the background.

Cross-talk effect should be assessed when a mass spectrometer-based method and isotopic internal standards are used [96]. Both detection channels of an isotopic internal standard and an authentic standard should be monitored to ensure there is no cross-talk between these channels in two individual runs, one injection of the internal standard and one injection of the authentic standard.

In LC, GC or CE analyses, other interfering peaks can be assessed through a blank injection in comparison with a standard injection. Assurance of instrument selectivity over the interfering peaks is critical for the later method validation.

### 1.4.3 Sample preparation

Prior to analyte separation and detection, efficient release of the biomarkers from biological matrix and removal of interfering substances (e.g., salt or protein in small molecular biomarker analysis) are often necessary. These tasks are accomplished through various extraction procedures. An efficient extraction improves the sample’s compatibility with the downstream separation and detection, as well as pre-concentrates trace biomarkers for the enhancement of sensitivity. Bypassing sample preparation steps is always ideal for the high throughput analyses. But high salt, protein content, plastic tubes or anticoagulants may compromise the robustness, precision, specificity and sensitivity of the analyses because of the matrix effect and adduct formation. Thus, an appropriate extraction procedure is usually recommended for an analytical method development [36].

Selection of extraction procedures needs to take into account whether it is a liquid, tissue or cell culture sample. During extraction, a tissue sample is usually ground to homogeneous powder or emulsion. Mortar-pestle, ultrasound and Soxhlet extraction are reasonable choices for grinding. Sometimes liquid nitrogen is used to preserve the sample integrity. Cold or hot organic solvents (e.g., ethanol, methanol-water, methanol-water-chloroform, methanol etc.), acid or basic solutions (e.g., perchloric acid, acetic acid or KOH solutions) are commonly used to extract a cell culture sample.
Pre-purification procedures, including protein precipitation, liquid-liquid extraction, solid phase extraction and solid-phase microextraction, can remove the interfering substances and reduce the sample complexity for liquid samples [97]. Protein precipitation techniques, including lowering pH, use of organic solvents (e.g. acetone, methanol, acetonitrile, or ethanol), and addition of particular salts (e.g. zinc sulfate, or ammonium sulfate), can separate the protein or large peptides from other small molecule components. Liquid-liquid extraction (LLE) is one convenient and cost effective method for extracting metabolite biomarkers out of a biological matrix. LLE is well suited for the pre-purification of relatively nonpolar biomarkers in a large sample volume. LLE doesn’t produce solid waste and is fast and simple. However, compared to solid phase extraction, LLE has a few drawbacks: 1) phase separation is not complete; 2) recovery can be low; 3) solvent consumption is large; 4) automation is difficult. Solid-liquid extraction (or solid phase extraction, SPE) is a preferred method for pre-purifying and concentrating low abundance biomarkers. Drawbacks of SPE are: 1) it is more expensive; 2) it produces solid waste; 3) it requires sophisticated setup. Notwithstanding these drawbacks, SPE is one of the most efficient ways to pre-purify the target biomarkers with nearly quantitative recovery and complete phase separation. Furthermore, a large number of choices for SPE cartridge selection provide great flexibility [98, 99].

SPE can be categorized according to the types of packing materials. Reverse phase, normal phase, ion exchange and adsorption packing are the four most common ones. Reverse phase packing may extract organic analytes out of polar solvents (e.g. water). Hydrophobic interaction retains the organic analytes on the surface of reverse phase packing during sample loading. After interfering substances being washed away, analytes are eluted by a nonpolar solvent (e.g. acetonitrile). The major types of reverse phase SPEs include:

- C18 for nonpolar to moderately polar compounds;
- C8 for nonpolar to moderately polar compounds;
- C4 for extraction of peptides and proteins;
- Phenyl for reverse phase extraction of nonpolar to moderately polar compounds, especially aromatic compounds;
- HLB, which contains water-wettable polymers, for nonpolar to moderately polar compounds; theoretically HLB cartridges is easier to condition than C18 ones.
Normal phase packing retains polar analytes rather than nonpolar analytes. Hydrogen bonding, pi-pi interactions, dipole-dipole interactions and dipole-induced dipole interactions account for the retention of analytes by normal phase packing. Normal phase SPEs include:

- CN for moderately polar compounds, including weak cation exchange for carbohydrates and cationic compounds;
- Diol for polar compounds;
- NH$_2$ for polar compounds, including weak anion exchange for carbohydrates, weak anions and organic acids;

Ion exchange packing retains ionic analytes. The retention is based on the electrostatic interaction of the charges on the ionic analyte with the counterions on the ion exchange packing. A solution that neutralizes either the analyte or the ion exchange packing is used to elute the analyte. Alternatively, a solution with high ionic strength can be used in elution. Ion exchange SPEs include:

- SAX for anions, organic acids, nucleic acids, nucleotides and surfactants;
- SCX for strong cation exchange for cations, antibiotics, drugs, organic bases, amino acids, catecholamines, herbicides, nucleic acid bases, nucleosides and surfactants;
- WCX for weak cation exchange of cations, amines, antibiotics, drugs, amino acids, catecholamines, nucleic acid bases, nucleosides and surfactants;

Adsorption packing material retains nonpolar or polar analyte through adsorptive interaction (dispersion, pi-pi, dipole-dipole, induced dipole-dipole interactions etc.). Adsorption SPEs include:

- Silica and Florisil for nonpolar organic analyte to pass through while retaining polar compounds, known as sample cleanup;
- Alumina for polar compounds, e.g. vitamins, antibiotics, enzymes, glycosides and hormones;
- Carb for polar and nonpolar compounds, e.g. carbohydrates, polysaccharides, or polar aromatic compounds.
Different formats of SPE, including molecularly imprinted polymer SPE, monolith SPE device, dispersive SPE (dSPE), disposable pipette extraction (DPX), micro-extraction by packed sorbent (MEPS), solid phase microextraction (SPME), stir bar sorptive extraction (SBSE) and on-line SPE (OLSPE) are recent innovations that use one or multiple packing materials to lower elution volume, reduce sample preparation time or increase recovery [100]. They offer alternatives to conventional cartridges in many applications, e.g. extracting low abundance peptides or proteins from plasma or serum samples [101].

As outlined in Table 1, one goal in a sample preparation is to achieve crude separation of an analyte from a biological matrix. In order to achieve this goal, the retention time and signal intensity of an analyte are evaluated by running native or spiked biological samples on a chosen instrument and detector. The readout of retention time reproducibility and signal intensity may guide the optimization of the extractions and pre-purifications.

Adequate sensitivity for real biological sample reflects the overall performance of sample preparation, separation and detection. Analyst can estimate LOD and LOQ for real biological sample and the nominal concentration of an analyte in a biological matrix. Comparison of LOD/LOQ with the nominal concentration of the analyte may be used to guide sensitivity tuning. Observing a detectable endogenous signal is a milestone for all the sample preparation efforts. In case of no discernible analyte signal is detected, analyst can increase the pre-concentration factor or improve the SPE efficiency by altering the sample preparation procedure.

Further, analyst should reexamine selectivity to verify whether or not the previous separation and detection conditions for the standard solution remain suitable for the processed biological samples. The system suitability parameters for real biological samples, which speak of the cumulative selectivity, may be compared to those for the standard solutions. If the system suitability parameters deteriorate, adjustments of the separation, detection or sample preparation conditions are warranted. But the unknown nature of some interfering substances sometimes prevents a logic solution from being found. Therefore the identification of the interfering substances, e.g. through mass spectrometry characterization, is one way to troubleshoot a complex problem. Alternatively, adopting orthogonal purification and multi-dimensional separation programs may help resolve a selectivity issue.

Relative %RSD of the authentic standard to internal standard ratio from multiple injections of the same sample represents the precision of an instrumental analysis.
Relative %RSD during method development is indicative of the feasibility to perform a method validation.

Analysts should conduct preliminary recovery evaluations during method development. Absolute Recovery can be calculated by comparing the peak area of the authentic standard pre-spiked before Extraction and Pre-purification (EPP) with the peak area of the authentic standard in a standard solution [63]. Absolute Recovery is a cumulative performance parameter taking into account EPP Recovery and matrix effect. If the Absolute Recovery were not satisfactory, analyst could calculate the EPP Recovery by comparing the peak area of the authentic standard pre-spiked before EPP to the peak area of the authentic standard post-spiked after EPP. EPP Recovery precludes the matrix effect and solely represents the efficiency of extraction and pre-purification. Thus it tells analysts more definitive information about extraction and pre-purification process. How to evaluate these recoveries is further illustrated in Section 1.5 Analytical method validation.

1.5 Analytical method validation

1.5.1 Recoveries

Recoveries are the important performance parameters in analytical method validation, particularly for mass spectrometry-based analysis. The concepts and calculation of absolute, relative and EPP recoveries are more convoluting than they appear to be.

Two published papers written by Kollipara et al and Matuszewski et al have given different names to the same concepts [63, 96]. Absolute Recovery, Relative Recovery [63] and EPP Recovery used in this introduction are equivalent to Process Efficiency (PE), Matrix Effect (ME) and Recovery (RE) [96] respectively. If the peak area of an authentic standard in a pure solvent is denoted as Peak Area Standard Solution, the peak area of authentic standard post-spiked after EPP as Peak Area PostSpiked Extract, the peak area of authentic standard pre-spiked before EPP as Peak Area PreSpiked Extract, Absolute Recovery, Relative Recovery and EPP Recovery may be calculated as follows:

Equation 10

\[
\text{Absolute Recovery} \% = \frac{\text{Peak Area PreSpiked Extract}}{\text{Peak Area Standard Solution}} \times 100
\]
**Equation 11**

\[
\text{Relative Recovery} \% = \frac{\text{Peak Area PostSpiked Extract}}{\text{Peak Area Standard Solution}} \times 100
\]

**Equation 12**

\[
\text{EPP Recovery} \% = \frac{\text{Peak Area PreSpiked Extract}}{\text{Peak Area PostSpiked Extract}} \times 100
\]

Evaluating recoveries can be time consuming and laborious. 63 samples were used to get all the recovery information in a simple case where exogenous drugs were analyzed [96]. An alternative approach is suggested as follows to minimize these efforts but still maintain the legitimacy of method validation.

Full-scale evaluation of Absolute Recovery should be performed first during method validation. Absolute Recovery is an overall recovery taking into account the Relative Recovery as well as the EPP Recovery. If the Absolute Recovery is evaluated in different lots of matrix, the %RSD of the Absolute Recovery represents overall %RSD of the EPP recovery and Relative Recovery (matrix effect). Absolute Recovery doesn’t need to be 100%. As long as the Absolute Recovery remains consistent across different lots of biological matrix, a slight lower Absolute Recovery is considered acceptable because it won’t affect the final accuracy and precision of an analytical method.

Relative Recovery evaluation is necessary only if the Absolute Recovery is not satisfactory and matrix effect is suspected. Relative Matrix Effect is the %RSD of Relative Recovery evaluated using different lots of biological matrix. Absolute Matrix Effect is the absolute value of Relative Recovery evaluated in a given matrix. Compared to Absolute Matrix Effect, Relative Matrix Effect is far more important because when the former has the same degree of influence on the calibration sample and patient sample, the biases and errors are canceled out so that the accuracy and precision won’t be affected. If an isotopic internal standard is used, the evaluation of Relative Recovery is generally unnecessary, because the Relative Matrix Effect can be corrected due to the similar responses of the analyte and isotopic internal standard to matrix.

EPP Recovery evaluation is optional. If necessary, EPP Recovery ought to be evaluated during the method development rather than the method validation to save time and resources.

When a matrix containing endogenous substance is used for validation, the slopes of calibration curves constructed out of the post-spiked matrices or pre-spiked matrices may be
compared for evaluating the %RSD of Relative Recovery or Absolute Recovery, respectively. Alternatively, the following equations may be applied for Absolute Recovery calculation:

**Equation 13**

\[
\text{Absolute Recovery (\%)} = \frac{\text{Peak Area PreSpiked Extract} - \text{Peak Area Blank Extract}}{\text{Peak Area Standard Solution}}
\]

where Peak Area Blank Extract denotes the peak area of the endogenous analyte in an extract from the same matrix.

1.5.2 Calibration curve, linearity and linear Range

Calibration curve for biomarker analytical method validation is frequently constructed using samples in biological matrix pre-spiked with the authentic standard at a series of concentrations and internal standard at a constant concentration. The instrument response ratios of authentic standard to internal standard can be either plotted against various authentic standard concentration or the concentration ratios of authentic standard to internal standard. The analytical method validation guideline published by US FDA suggested the calibration standards include one blank sample (matrix without analyte or IS), one zero sample (matrix with only IS), and 6-8 non-zero standards covering the whole linear range. Usually analysis of low molecular weight, homogeneous biomarkers (i.e., <1,000 Da), produces consistent linearity data with a high degree of accuracy and precision, while linearity data of higher molecular weight, unknown or heterogeneous biomarkers is not as accurate and reproducible [32]. The estimated endogenous levels of analyte in biological samples during method development are benchmarks for choosing a proper range for the calibration. Alternatively, analysts may determine calibration range according to literature reports. In either case, the chosen range ought to bracket the high end and low end of the estimated concentrations. Linearity is the degree to which the calibration curve approximates a straight line [33]. The R square value obtained from linear regression is a widely accepted index for linearity.

Standard addition method uses authentic matrix that contains endogenous analyte to construct calibration curve. The advantages of using authentic matrix are: 1) it differs very little from the real samples; 2) it allows the estimation of nominal analyte range in an early method development; 3) no extra validation of the matrix is needed. The major disadvantage of using authentic matrix is the endogenous analyte concentration may vary from time to time due to instability. Stability of endogenous analyte can be usually improved by keeping the calibration...
matrix aliquots at -20°C.

Analyte-free matrix is an alternative to an authentic matrix [41]. Charcoal stripping, high-temperature incubation, acid or alkaline hydrolysis, or affinity chromatography may remove the endogenous analyte to afford an analyte-depleted matrix. The other alternative is an artificial surrogate buffer, which offers advantages over the analyte-depleted matrix including better stability, convenience and long-term consistency. Because none of these matrices is 100% equivalent to the authentic matrix, when they were used, parallelism study should be performed to prove their equivalency. In parallelism study, a real sample at a high concentration was diluted with an analyte-free matrix at various dilution factors, analyzed and back calculated against calibration curve constructed using the analyte-free matrix to result in observed concentrations. Multiplying these observed concentrations with dilution factors yield the Adjusted Observed Concentrations. If the %RSD of the Adjusted Observed Concentrations is within acceptable range (e.g. 15%), the analyte-free matrix has shown parallelism to the authentic matrix and the equivalency is justified [33, 102].

1.5.3 LOD, LOQ, LLOQ and ULOQ

LOD and LOQ are performance parameters defined in the 1.4.2 Selection of detector. National Committee for Clinical Lab Standards (NCCLS) recommends the use of LOC/LOQ in its Clinical Lab Improvement Amendments. Despite its value in quantitative analysis, LOQ is not as reliable as lower and upper limits of quantification (LLOQ to ULOQ), at which level analyte might be measured with a predetermined accuracy and precision [32, 33]. LLOQ is more important than ULOQ in a biomarker analysis because biomarker is generally present at an extremely low level. US FDA guidelines for analytical method validation, also known as Good Laboratory Practices, recommend that LLOQ be used instead of LOD/LOQ. According to FDA’s definition, LLOQ is the lowest concentration that can be measured with both Relative Error (%RE) and Relative Standard Deviation (%RSD) no greater than 20%. %RE is an accuracy parameter whereas %RSD is a precision parameter.

In pharmacokinetic (PK) analyses, scientists commonly ascribe a “zero” or “below quantifiable limits” value to results lower than the LLOQ. Because the quantitation of endogenous biomarker is more difficult, compromises may be made to maximize the data output for statistical analysis. In biomarker quantitation, when calculated concentrations are below LLOQ but above LOD, the results may be still reportable as quantitative estimates [32].
1.5.4 Validation samples, quality controls, precision and accuracy

Validation Samples (VS) and Quality Controls (QC) are the samples prepared through spiking authentic matrix or artificial matrix with various levels of authentic standards. Usually three authentic standard concentrations at a low, intermediate and high level are used to prepare the VS and QC samples. The VS samples are used for estimating the accuracy/precision in the pre-study validation, as opposed to the QC samples used for accepting or rejecting real sample data in the in-study validation. The VS and QC samples are the same except for the purpose of use.

Regression model for the calibration curve may factor into an analytical method validation. Ideally linear model is preferred. But because of the wide diversity of analytes and methods, logarithmic, polynomial, power and exponential models etc. are also plausible choices for a curve-fitting. Once a model is selected, the best-fitted equation of calibration curve will be taken to calculate performance parameters of a given analytical method, such as Relative Error (%RE).

Precision is the closeness of results from multiple individual measurements. Intra-day precision and inter-day precision are generally evaluated at three to four levels of VS with minimum three replicates. The repeats ought to be prepared independently from the beginning of sample preparation so that the final Relative Standard Deviation (%RSD) reflects the total error of sample preparation, separation and detection.

Accuracy is the closeness of the calculated mean value to the true value. Spiked concentration of authentic standard is perceived as the true value in an analytical method validation, whereas the instrument response is converted through the best-fitted equation to give the calculated value. %Relative Error is the relative difference between the calculated value and the true value and it reflects the accuracy of a method. The precision and accuracy calculations can be based on the data obtained by running the same VS samples. The calculations of %RSD and %RE are exemplified in Table 10 and Table 11.

1.5.5 Stability

US FDA suggests freeze-thaw and short-term room temperature stabilities be evaluated to determine the suitable storage and sample preparation conditions. Freeze-thaw stability may be evaluated by subjecting three sets of the low and high concentration QC samples in three freeze-
thaw cycles. The comparison of the analytical results after the third cycle with those of freshly prepared samples is recommended.

The short-term room temperature stability may be evaluated by subjecting three sets of the low and high concentration QC samples to room temperature for 4-24h. The samples are analyzed at the predetermined time points and the results are compared to those of freshly prepared samples. US FDA doesn’t specify the rejection or acceptance criteria for stability [63].

1.5.6 Pre-study vs in-study validation

Pre-study and in-study validation are performed before and within real sample analysis respectively. As summarized in Table 2, the pre-study validation determines performance parameters of a method while the in-study validation follows a Standard Operating Procedure (SOP) to ensure the analytical performance meets the predetermined acceptance criteria.

The in-study validation typically uses QC samples at three levels (low, intermediate and high concentration) with a minimum two replicates at each level. In-study validation scheme can be tailored to intended goals. In GLP, 4-6-X rule is widely adopted. 4-6-X rule requires at least 4 out of 6 QC samples meet acceptance criteria X, which usually defines the total error allowance of analysis (%RE + %RSD). It is worth noting that the acceptance criteria of most biomarker analytical methods depend on the physiological variability in the study population, sample availability, sample size, sample preparation, separation and detection method etc. To make the biomarker analysis fit-for-purpose, analysts should choose the acceptance criteria that are stringent enough to ensure data reliability but flexible enough to include sufficient data entries [32].
Table 2: Tasks, samples and goals for each step of analytical method validation

<table>
<thead>
<tr>
<th>Steps</th>
<th>Tasks</th>
<th>Sample(s)</th>
<th>Goals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-study validation</td>
<td>Determination of concentration range for method validation; Evaluation of the performance parameters set for the fit-for-purpose validation; adjustment of the separation, detection and sample preparation method for acceptable validation; assessment of stability</td>
<td>Native biological sample spiked with internal standard; biological sample spiked with authentic standard at series of concentrations and internal standard</td>
<td>Adequate recoveries (e.g. relative recovery or matrix effect); Adequate performance parameters (e.g. linearity; linear range; LOD, LOQ and LLOQ; intra- or inter-day accuracy in terms of %Relative Error or %RE and intra- or inter-day precision in terms of %RSD at low, middle and high concentrations); adequate stabilities (e.g. freeze and thaw; long term);</td>
</tr>
<tr>
<td>In-study validation</td>
<td>Insertion of Quality Control samples in the analysis sequence of real patient samples; setup of standard operation procedure (SOP) including rules for accepting or rejecting data entries</td>
<td>Patient samples spiked with internal standard; biological sample spiked with authentic standard at series of concentrations and internal standard</td>
<td>Adequate in-study validity of the developed method (4-6-X rule adopted from Good Laboratory Practice or GLP, which means 4 out of 6 QC samples should fall within the acceptance criteria X);</td>
</tr>
</tbody>
</table>

1.6 Cardioprotection against myocardial IRI during on-pump CABG

My PhD program is focused on developing and validating analytical methods for biomarkers and applying these methods to cardiovascular research. Section 1.6 through 1.10 will illustrate specific clinical perspectives of this program and how it is tied in with my clinical biomarker research. Coronary heart disease (CHD) is a leading cause of cardiovascular morbidity and mortality. While conservative methods are preferred to manage a mild or modest CHD, it is often necessary to perform a surgery in order to improve the life quality and long-term survival of a patient suffering from severe CHD [103].
1.6.1 Myocardial IRI

CABG is a surgical procedure that uses healthy vessels to bypass diseased coronary arteries. Because it is very risky for a surgeon to operate on a beating heart, the heart must be silenced momentarily during the surgery. In doing this, surgeons also cross clamp the aorta to isolate the silent heart from the systemic circulation, and a heart-lung machine is instituted to take over the heart and lung function and supply oxygenated blood to the vital organs. This is known as on-pump CABG or CABG with cardiopulmonary bypass (CPB). Patients at a moderate and high risk of CHD clearly benefit from the on-pump CABG, with improved long-term survivals and reduced ischemic syndromes.

Between cross-clamping and de-clamping, the arrested heart is rendered ischemic. Under this ischemia condition, the oxygen dependent ATP synthesis decreases as a direct result of anoxia. Many enzymes such as sodium/potassium ATPase rely on ATP to maintain their normal biochemical functions. Deprived of ATP, sodium ions accumulate in the cytosol and exchange with calcium ions through the sodium/calcium antiporters. Increased levels of cytosolic sodium and calcium ions may cause osmotic swelling and activate destructive proteases. Also in the ischemia phase, ongoing glycolysis and citric acid cycle generate excessive amounts of reductive species that are stored in the electron transport chain. At reperfusion of a bypassed heart, these electrons are transferred directly to the incoming oxygen to produce reactive oxygen/nitrogen species (ROS or RNS) and therefore oxidative and/or nitrosative stress. Furthermore, the protein, lipid and nucleic acid damages resulting from oxidative or nitrosative stress gives rise to inflammatory reactions, leading to the apoptosis and necrosis of the injured cardiac myocytes [104] [105, 106]. This is known as ischemia reperfusion injury (IRI). Myocardial IRI is associated with poor clinical outcomes including cardiac arrest, acute myocardial infarction and postoperative low cardiac output syndrome.

1.6.2 Ischemic preconditioning

In search of cardio-protective strategies to reduce myocardial IRI, “conditioning” the heart by applying either brief periods of ischemia or pharmacological agents, has demonstrated efficacy [107].

Ischemic preconditioning refers to the introduction of brief episodes of non-lethal myocardial ischemia and reperfusion prior to completely silencing the heart for CABG surgery to increase the heart’s tolerance to acute ischemia secondary to cessation of coronary blood flow.
during CPB [108]. In a systematic review, ischemia preconditioning reduced the incidence of ventricular arrhythmias, inotrope requirements and duration of intensive care unit stay [109].

The mechanisms underlying ischemic preconditioning are complex. It appears that ischemic preconditioning triggers the production of adenosine, bradykinin and opioids that bind to their respective G-protein coupled receptors on the cardiomyocyte surface and activates pro-survival signal transduction pathways such as Reperfusion Injury Salvation Pathway (RISK). Many protein kinase cascades including the phosphoinositide 3 kinase-Akt (PI3K-Akt), MEK1/2-Erk1/2 and cyclic guanosine monophosphate-protein kinase G (cGMP-PKG) are likely recruited in the RISK pathway [110, 111].

1.6.3 Volatile anesthetic preconditioning

Because ischemia preconditioning is an invasive procedure, pharmacological preconditioning was introduced to mitigate the substantial risks imposed by ischemic preconditioning. Following animal studies [112, 113], the preconditioning efficacy of volatile anesthetics, including sevoflurane, isoflurane and desflurane, was tested in multiple clinical trials. In one study, earlier assessment concluded that sevoflurane and desflurane reduced the postoperative rise of troponin but these benign effects were not translatable to favorable clinical outcomes [114]. In other studies, desflurane and sevoflurane reduced postoperative mortality, incidences of myocardial infarction, the need for inotropic support, time of mechanical ventilation, cardiac troponin release, time spent in the intensive care unit and overall hospital stay [115, 116].

The mechanisms accounting for volatile anesthetic preconditioning are similar to those ascribed to ischemic preconditioning. Generation of ROS [117], activation of PKC-epsilon [118], sensitization of K-ATP channels [119], translocation of PI3K [120], recruitment of Akt-Bcl2 pathway [120] and inhibition of mPTP [121] are associated with the volatile anesthetic preconditioning.

1.6.4 Antioxidative cardioprotection

The myocardial K-ATP channels are essential for cardio-protection conveyed by ischemic and volatile anesthetic preconditioning [111, 122]. In diabetes, sulphonylurea oral hypoglycemic agents can abolish ischemic preconditioning [110] and volatile anesthetic preconditioning [113] during coronary surgery by blocking K-ATP channel opening. Evidence that acute hyperglycemia and diabetes negated the preconditioning benefits of isoflurane [123]
Furthermore, volatile anesthetic preconditioning is only effective to protect the heart from contractile dysfunction and infarction for less than 25 to 40 min [125]. Such circumstances entail an alternative therapeutic approach to be considered.

Oxidative stress following reperfusion injury is greater in diabetes than in non-diabetes and causes the increase in vascular permeability and thus more complications [126]. ROS accounts for increased inflammatory responses to ischemia and reperfusion, causing exacerbated myocardial injury in diabetes [127]. Therefore, in diabetic patients who have defective oxidative defense, antioxidant therapy could be a viable remedy for IRI. Indeed, anti-oxidant agents have shown promising results. Chronic alpha-linolenic acid intake was cardioprotective in diabetic rats [128]. N-acetylcysteine and isosorbide 5-mononitrate showed short-term benefit in a cardiac ischemia-reperfusion model [129].

Our focus is using propofol (Figure 2), an intravenous anesthetic and antioxidant, to alleviate IRI. Anesthetic is readily accessible in CABG surgery. Our previous work has provided proof of concept that propofol conferred cardioprotection by neutralizing excessive oxidant in preclinical models [130-132]. Clinically, our preliminary data indicated that when a high dose propofol was administered during on-pump CABG, patients had shorter intensive care unit stay and improved cardiac index compared to other treatment groups including isoflurane treated patients [133]. Although conventional doses of propofol didn’t show the effectiveness in reducing IRI and improving clinical outcomes, our in vitro dose finding studies suggested that high dose range (≥10 to 25 µmol/L) was efficacious [132, 134, 135].

Figure 2: Chemical structure of propofol

1.7 Prognostic biomarkers for IRI in on-pump CABG

Investigation of biomarker changes during on-pump CABG will help illustrate the mechanism underlying IRI and/or the cardioprotection from IRI. Prognostic biomarkers are the focus of my PhD research, while the other biomarkers are routinely tested in clinical labs.
1.7.1 Oxidative stress indicator: $F_{2\alpha}$-isoprostanes

$F_{2\alpha}$-isoprostanes are a series of prostaglandin-like metabolites formed through \textit{in vivo} lipid peroxidation (Figure 3) [136]. There are four types of $F_{2\alpha}$-isoprostanes, 5, 12, 8, 15-$F_{2\alpha}$-isoprostanes, depending on the position of hydroxyl group. Among them, 15-$F_{2\alpha}$-isoprostane is a cis isomer of 15-$F_2$-prostaglandin. $F_{2\alpha}$-isoprostanes have been evaluated in varieties of disease states such as atherosclerosis [137], diabetes [138], obesity [139], and neurodegenerative disease [140]. They are regarded one of the “golden standards” in assessing oxidative stress [141]. In preclinical canine model of myocardial ischemia and reperfusion and patients with myocardial infarction, increases of isoprostane production were reported [142]. 15-$F_{2\alpha}$-isoprostane increases were also seen in coronary angioplasty and CABG procedures [143, 144].

15-$F_{2\alpha}$-isoprostane (or 8-epi-PGF$_{2\alpha}$) is one of the most abundant $F_{2\alpha}$-isoprostanes \textit{in vivo} that shows remarkable biological activities including induction of vasoconstriction, endothelin release and proliferation of smooth muscle cells [136]. As such, increased 15-$F_{2\alpha}$-isoprostane levels might aggravate the cardiac dysfunction after a myocardial ischemia and reperfusion [145-147]. Significant increase of 15-$F_{2\alpha}$-isoprostane production during coronary artery surgery accompanied by the myocardial IRI in an exploratory study indicated this injury was mediated by oxygen-derived free radicals [147].
Figure 3: Chemical structures of F_{2t}-isoprostanes, 15-F_{2t}-prostaglandin and their endogenous precursor arachidonic acid

1.7.2 Nitrosative stress indicator: 3-nitrotyrosine

Peroxynitrite (ONOO\(^-\)) is a product of superoxide (O_{2}\(^•-\)) reacted with nitric oxide (NO\(^•\)), and is a major source for nitrosative stress. Peroxynitrite might depress the mechanical functions of a rat heart [148], trigger cardiomyocyte apoptosis in rat and cell culture models [149], contribute to the development of post-bypass systemic inflammatory response in rats [150], and incur permanent and widespread damage to nucleic acids, lipids and proteins [151].

Several studies suggested that ischemia and reperfusion might give rise to increased O_{2}\(^•-\) and NO\(^•\) production, which would then react with each other to yield ONOO\(^-\) [152] [153] [154]. Under low pH condition as a result of intracellular acidosis, ONOO\(^-\) is immediately protonated to form its conjugate acid, ONOOH [155]. The free radicals hydroxyl (•OH) and nitrogen dioxide (ONO\(^•\)) as degradation products of ONOOH can react with many biomolecules [153, 156]. Monitoring peroxynitrite levels in clinical settings is not feasible because peroxynitrite is unstable and short-lived.

3-Nitrotyrosine is a stable end product of •OH and ONO• when they react with either tyrosine or tyrosine residues of proteins. As a result, free and protein-bound 3-nitrotyrosine are
both accepted as surrogate markers of peroxynitrite. It was shown that 3-nitrotyrosine levels were elevated upon myocardial ischemia and reperfusion after open-heart surgery [157], in the exhaled breath condensate of cystic fibrosis patients [158] and in allergic asthmatic children [159]. Therefore, measuring pre- and post-CPB 3-nitrotyrosine levels may help evaluate the extent of nitrosative stress and the potential prognostic value of 3-nitrotyrosine in myocardial ischemia and reperfusion.

1.7.3 Myocardial protectant: adenosine production and regulation

Adenosine, a hydrolysis product of intracellular adenosine monophosphate by a 5’-nucleotidase, is deemed cardioprotective during ischemia or hypoxia episodes [160]. Being a modulator for adenosine triphosphate (ATP) conservation and “retaliatory metabolite”, adenosine can dilate coronary arterioles, antagonize the inotropic effects and inhibit sympathetic output to correct energy imbalance [161]. Adenosine is produced and released in response to physiological or pathological signals primarily related to cellular energy state [162]. It was showed that adenosine concentration increased in an occlusion time-dependent manner in patients under percutaneous transluminal coronary angioplasty (PTCA) [163]. Patients who underwent aortic valve replacement for aortic stenosis had more adenosine in the coronary sinus than in the radial artery. The adenosine level was also higher at the end of cardiopulmonary bypass (CPB) than at the beginning of CPB [164].

Cardioprotection strategies have employed pharmacologic and physical means in an effort to maintain the cellular metabolic milieu, avoid depletion of cellular cardioprotective substrates or activate endogenous triggers of intracellular mediators of protection. Examination of adenosine and adenosine receptor pharmacology during myocardial ischemia-reperfusion *in vivo* and *in vitro* has attracted significant interests. Unfortunately, a major methodological challenge is the delineation of its role, given adenosine’s quick turnover in patient samples.

Sustained elevation of adenosine basal level might be an attractive alternative to overcome the practical challenges faced by clinicians. Although adenosine is rapidly produced and metabolized, it has been suggested that its regulatory effect is only activated when the buffering effect of red blood cells is overcome, and basal adenosine level is significantly increased [165]. Proof of concept studies in animals utilizing adenosine inhibitors suggests the utility of adenosine modulation to salvage myocardium and reduce myocardial stunning. The translation of such findings clinically, its roles as cardioprotectant and prognostic marker and the
analytical method of basal adenosine quantitation have yet to be established [163, 166].

1.7.4 Nitric oxide synthase inhibitor: asymmetric dimethylarginine

Arginine residues on constitutional proteins can be methylated by arginine methyltransferases [167]. Monomethylarginine (MMA), asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are released from these methylated arginine residues via normal proteolysis. ADMA is first metabolized to citrulline and dimethylamine via dimethylarginine dimethylaminohydrolase (DDAH) and then eliminated [168].

ADMA’s structural similarity with L-arginine makes it a natural inhibitor of nitric oxide synthase, a key enzyme converting L-arginine to nitric oxide and L-citrulline [169]. Besides its involvement in cardiovascular dysfunctions, increased ADMA concentration is also present in major depression [170], type 1 diabetes [171], type 2 diabetes mellitus [172], retinal venous occlusive disease [173], renal failure [174], and hyperlipidemia [174]. Evidences showed hypercholesterolemia and reduced DDAH activity might account for elevated ADMA [175].

ADMA has been evaluated as prognostic marker in various diseases. Due to the important role of nitric oxide in mediating vasodilation, an elevated concentration of ADMA is likely a risk factor for cardiovascular disease. Indeed, ADMA predicted the clinical outcome independent of other risk factors in a nested case-control cohort (N = 850) and might become a new prognostic marker of cardiovascular events [176]. For patients undergoing percutaneous coronary intervention, plasma ADMA levels independently predicted adverse cardiovascular events after this cardiac surgery and were utilized to guide risk stratification of patients prior to treatment [177]. During the followed-up of 1874 patients with coronary heart disease, median ADMA concentration of patients who died from cardiovascular causes or developed nonfatal myocardial infarction was significantly higher [178].

Various analytical methods such as ELISA [178], HPLC [179] or LC-MS/MS [180] were developed for ADMA’s quantitation. The major challenge of developing a quantitative method is the structural similarity between ADMA and SDMA. The development of an analytical method separating these regio-isomers is pivotal for evaluating the prognostic value of ADMA.

1.8 Diagnostic and staging biomarkers for IRI in on-pump CABG

Myocardial injury causes the release of myocardial structural proteins including cardiac troponin I and T (cTnI and cTnT) and creatine kinase (CK) into the plasma. Immunoanalysis of
cardiac troponins, revolutionized the clinical diagnosis of myocardial injury [181]. Immunoanalysis of creatine kinase MB (CK-MB) can yield complementary information for the diagnosis. Together they allow earlier diagnosis of myocardial infarction (MI), risk stratification of acute coronary syndrome and improvement of MI prevention and treatment [182].

Despite specific to myocardium, small percentage of CK-MB exists in skeletal muscle. So clear documentation for series of CK-MB measurements is important to guide diagnostic decision. Despite not as specific as troponin, the measurement for CK-MB remains important for detecting reinfarction after coronary interventions [183].

According to National Academy of Clinical Biochemistry Laboratory Medicine Practice Guidelines, biomarkers of myocardial injury should be measured in all patients who develop symptoms consistent with acute coronary syndrome (ACS). Cardiac troponins are the preferred markers for the diagnosis of myocardial infarction. The advantage of cardiac troponin over other biomarkers of myocardial injury was verified in many clinical studies [184].

Troponins are also used to stage perioperative myocardial infarction (PMI). In a prospective study, 94 out of the 3308 CABG patients undertook acute reangiography due to PMI. The elevation of troponin I after CABG was able to discriminate the graft-related PMI and non-graft-related PMI [185]. Furthermore, determination of troponin I 24h before CABG may be used to stratify the risk levels and identify the patients with increased risk of postoperative adverse outcome and in-hospital mortality [186].

1.9 Clinical endpoints and surrogate endpoints for IRI in on-pump CABG

Clinical endpoints are the characteristics or variables that reflect how a patient feels, functions or survives [1]. Composite of death, myocardial infarction and other ischemia heart diseases or syndrome (e.g. the presence and severity of angina) are deemed primary clinical endpoints because of their direct relationship with mortality or morbidity [103] [187-190]. Quality of life, exercise capacity [187], new revascularization, functional recovery (e.g. cardiac index and other hemodynamics) [188-193], use of health-care resources (e.g. Intensive Care Unit [ICU] length of stay, mechanical ventilation time, inotrope use, hospital length of stay) [190, 194] and biomarker level (e.g. troponin I and CK-MB level) [190, 195-198] are examples of secondary or surrogate endpoints.

Surrogate endpoints, the quantifiable measurements to predict the clinical endpoints, are
alternatives when primary clinical endpoints are not readily available. Troponins and CK-MB are established surrogate endpoints for CABG surgery [190, 195-198]; hemodynamics parameters, including cardiac output (CO), cardiac index (CI), systemic vascular resistance (SVR), stroke volume index (SVI), pulmonary artery occlusion pressure (PAOP), left ventricular stroke work index (LVSWI), are surrogate endpoints for the heart’s functional recovery after CABG [191-193]. These parameters are defined as below:

**Equation 14**

\[ CO = \frac{HR \times SV}{1000} \]

**Equation 15**

\[ CI = \frac{CO}{BSA} \]

**Equation 16**

\[ SVR = 80 \times \frac{MAP - RAP}{CO} \]

**Equation 17**

\[ SVI = \frac{CI}{HR} \times 1000 \]

**Equation 18**

\[ PAOP = PASP + \frac{2 \times PADP}{3} \]

**Equation 19**

\[ LVSWI = \frac{SVI}{MAP - PAOP} \times 0.0136 \]

where HR is the heart rate, SV the stroke volume, BSA the body surface area, MAP the mean arterial pressure, RAP the right atrial pressure, PASP the pulmonary artery systolic pressure, PADP the pulmonary artery diastolic pressure.

After CABG surgery, ischemia heart disease (IHD) contributed to more than 80% of the
deaths and the most frequent complications are hemorrhage and low cardiac output syndrome (LCOS) [199]. The prevalence of LCOS was 9.1% in one study of 4558 patients [200]. LCOS is defined as the need for postoperative intra-aortic balloon pump or inotropic support for longer than 30 minutes in the intensive care unit to maintain the systolic blood pressure greater than 90 mm Hg and the cardiac index greater than 2.2 L/min/m². A higher mortality rate was found in the patients with LCOS than in the patients without LCOS (16.9% vs 0.9%, p < 0.001) [200, 201].

1.10 Putative predictive biomarkers for propofol intervention in PRO-TECT II clinical trial

1.10.1 Mechanisms of propofol cardioprotection

Propofol (2,6-diisopropylphenol) has an intrinsic antioxidant capacity because of its disubstituted phenolic structure. Propofol can scavenge hydroxyl radical •OH [202]. Propofol exhibits antioxidant potency greater than butylated hydroxyanisole, butylated hydroxytoluene and alpha-tocopherol. Propofol was able to inhibit 100% of lipid peroxidation at 75µg/mL, a level that exceeds maximum clinical relevant concentration [203]. The antioxidant properties of propofol can be also attributed to its scavenging ability of peroxynitrite, which mediates DNA damage and apoptosis [204, 205]. In cardiac surgery, propofol showed cardioprotective effects by attenuating the free radical mediated lipid peroxidation and systemic inflammation in patients with intact or impaired myocardial function [133, 206-208].

The PI3K/Akt prosurvival pathway is vital to the physical and pharmacologic preconditioning. Once activated, Akt induces the cytoprotection by activating eNOS and anti-apoptotic Bcl-2 and antagonizing the mitochondrion-directed cell death [209, 210]. However, the diabetic myocardium is resistant to Akt-dependent physical preconditioning [211, 212]. The negative regulators of the PI3K/Akt pathway are phosphatase and tensin on chromosome 10 (PTEN) [213]. Hyperglycemia-induced reactive oxygen or nitrogen species production could potentiate the PTEN antagonism of Akt [214, 215].

In a cell culture model, our research group demonstrated that propofol protects cardiac H9C2 cells from hydrogen peroxide-induced injury by activating Akt, upregulating Bcl-2 and blocking apoptosis. We further showed the protection depended on the activation of PI3K-Akt-Bcl-2 signal transduction pathway because a PI3K inhibitor obliterated the propofol cardioprotection [216]. In our earlier reports, the high dose propofol at 50 µM might also increase eNOS expression via the Akt-independent pathway and suppress caspase-3 activity [134].
1.10.2 PROpofol Cardioprotection for Type II Diabetics (PRO-TECT II) study

So which cardioprotective approach, propofol or volatile anesthetic, offers greater cardioprotection for CABG patients? Several studies indicated that volatile anesthetic preconditioning was associated with shorter ICU or hospital stays, improved myocardial functions and less troponin I release compared to propofol [217-221]. In contrast, other studies failed to demonstrate the superiority of volatile anesthetic preconditioning over propofol cardioprotection [222]. Yet another study concluded that volatile anesthetic preconditioning was superior to propofol in the patients with minor ischemic heart disease, whereas propofol was better in the patients with severe ischemia disease or cardiovascular instability [223]. A number of factors might have attributed to these discrepancies: 1) the absence of standard volatile anesthetic preconditioning maneuvers; 2) the lack of measurements of achieved propofol concentrations in whole blood and the uses of different propofol regimens; 3) the selections of different clinical/surrogate endpoints; 4) different exclusion and inclusion criteria for study subjects.

As mentioned in section 1.6.4 Antioxidative cardioprotection, diabetic patients are the high-risk population that requires alternative therapeutic approaches other than volatile anesthetic or ischemic preconditioning. Nearly 30% of patients receiving on-pump CABG surgery are diabetic [224]. These patients have higher rate of mortality, renal failure, wound infection, angina, mediastinitis, stroke, longer length of stay, prolonged ventilation and worse 10-year survival than the nondiabetic population after cardiac surgery [225-228]. In one study, the adjusted risk of morbidity, infections and the composite outcomes in diabetes is 35% higher than that in non-diabetes, particularly among insulin-treated diabetes [229]. In particular, diabetic patients are at an elevated risk for low cardiac output syndrome [200, 230], where the prolonged use of high doses of inotropes, vaspressors, and/or intra-aortic balloon counterpulsation are required.

Owing to the propofol’s unique mechanisms of action, we postulate that high-dose propofol attenuate IRI in on-pump CABG by counteracting the oxidative or nitrosative stress. The PRO-TECT II study was carried out by our group in this context [231]. It is a Phase II randomized, controlled trial comparing high-dose propofol cardioprotection with isoflurane preconditioning in diabetic and nondiabetic patients with respect to a series of clinical endpoints, surrogate endpoints, prognostic biomarkers, diagnostic biomarkers and putative predictive biomarkers. The results from PRO-TECT II study will be used to determine the feasibility and sample size of a Phase III randomized, controlled trial.
1.10.3 Putative predictive biomarkers for propofol intervention

Predictive biomarkers for propofol intervention are selected in regard to the propofol antioxidation effects. 15-F₂t-isoprostane and 3-nitrotyrosine are chosen to predict the propofol’s antioxidive and antinitrosative capability respectively.

1.11 Dissertation objectives and outline

In the context given in sections 1.1 through 1.10, the objectives of this dissertation are: 1) to develop and validate a simple and reliable analytical method for propofol quantitation; 2) to compare propofol infusion rate with achieved propofol concentration for the purpose of finding a translatable dosing regimen resembling the in vitro propofol cardioprotective concentration; 3) to develop and validate a reliable and robust analytical method for 15-F₂t-isoprostane quantitation; 4) to develop and validate a reliable and robust analytical method for 3-nitrotyrosine quantitation; 5) to develop and validate a reliable and robust analytical method for basal level adenosine quantitation; 6) to develop and validate a reliable and robust analytical method for separating ADMA and SDMA chromatographically and quantifying them individually.

The organization chart is outlined in Figure 4 for this PhD dissertation. Chapters 2 and 3 establish the pharmacological intervention protocol for propofol cardioprotection trial, which strives to offer patients benefits in on-pump CABG, an IRI trigger. Patient profiles are pre-determinants of IRI. Chapters 4, 5 and 6 describe the analytical methods for measuring prognostic biomarkers including 15-F₂t-isoprostane, 3-nitrotyrosine and adenosine for IRI. PTEN-Akt and nitric oxide are the prognostic markers measured via analytical methods not described in this dissertation. Chapter 7 describes the analytical method for measuring ADMA, an on-pump CABG-independent prognostic marker for IRI. CK-MB and troponin are monitored during our clinical trial, and the analytical methods for measuring them are not in the scope of this thesis. Clinical endpoints such as LCOS and myocardial infarction are reported in relation to these prognostic markers as considered appropriate.
Figure 4: Organization chart for this PhD dissertation
2. Quantitative analysis of propofol in whole blood using capillary electrophoresis coupled with ultraviolet detection

2.1 Introduction

Propofol (2,6-diisopropyl phenol) is a frequently used intravenous drug for the introduction and maintenance of anesthesia. Our research group is interested in the potential cardioprotective effects of propofol during cardiopulmonary bypass surgeries. Target controlled infusion devices, which predict whole blood propofol concentrations based on mathematical algorithms that link patient characteristics and pharmacokinetics with infusion rates, are currently in use. Unfortunately, there are often discrepancies between predicted and achieved concentrations, and target controlled infusion devices are not universally approved for clinical use. In order to overcome these limitations, techniques and devices capable of determining actual drug concentrations in whole blood are required. In order to maximize their clinical usefulness, analytical devices should be simple, highly automated, fast, accurate, and precise.

Several methods for the quantitative determination of propofol in biological samples have been reported, examples include high performance liquid chromatography–UV spectrophotometry (HPLC–UV) [232-235], gas chromatography with flame ionization detection (GC-FID) [235] [236], and chromatography techniques coupled with mass spectrometry detectors [234],[235-238]. Capillary electrophoresis (CE) is one of the most powerful tools for chemical separation. The advantages of high resolution, short analysis time, low cost, and small buffer and solvent volume requirements make CE an attractive technique to analyze complex matrices [239, 240]. The similarities shared by CE and microfluidic devices could foster the development of target achieved drug delivery strategies, in which drug dosing is dictated by actual concentrations achieved in whole blood, that are better suited to routine use in clinical settings.

The aim of the present study was to develop and validate a CE-based method capable of quantitative propofol analysis in whole blood. Propofol has limited water solubility, strongly binds to plasma proteins and cellular blood constituents, and has a mean free fraction in plasma of 1–3% [241]. Capillary electrophoresis with micellar additives, also called micellar electrokinetic chromatography (MEKC), first introduced by Terabe, et al. [242], has been widely used for the analysis of biological samples [243], particularly where neutral and hydrophobic compounds are concerned. We have developed and validated a fast and highly selective MEKC based method for the quantitative analysis of propofol in whole blood using commercially available CE system.
This method was used to determine the concentration of propofol in whole blood samples obtained from patients undergoing coronary artery bypass grafting (CABG) with cardiopulmonary bypass (CPB).

2.2 Materials and methods

2.2.1 Apparatus

All experiments were carried out on a Beckman Coulter P/ACE MDQ System (Beckman Coulter Inc., Fullerton, CA, USA) with a UV absorption detector. The detection wavelength was 200 nm. An uncoated fused-silica capillary (50 cm total length, 40 cm length to detector, 50 µm inner diameter, 360 µm outer diameter) (Polymicro Technologies, Phoenix, AZ, USA) was used throughout. A separation temperature of 25°C was maintained for all CE experiments.

2.2.2 Chemicals and reagents

Borax and sodium dodecyl sulfate were purchased from Sigma-Aldrich (Oakville, ON, Canada). Separation buffer consisted of an aqueous solution containing 50 mM sodium dodecyl sulfate and 15 mM borax. This separation buffer was sonicated and filtered through a 0.45 µm membrane filter. Tetramethylammonium hydroxide (25% in methanol) was purchased from Alfa Aesar (Ward Hill, MA, USA) and diluted before use with HPLC grade 2-propanol (Fisher Scientific, Ottawa, ON, Canada) (3:37 v/v). Cyclohexane, acetonitrile, and methanol were purchased from Fisher Chemicals (Fisher Scientific, Ottawa, ON, Canada). Deionized water was obtained using a NANOpure Infinity Reagent Grade Water System (Apple Scientific Inc., Chesterland, OH, USA). All solutions were filtered through 0.45 µm membrane filters (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) prior to use. Propofol (2,6-diisopropylphenol), 97% purity, was purchased from Sigma-Aldrich. Thymol, 99% purity, was purchased from Acros Organics (Morris Plains, NJ, USA) and used as internal standard. Durapore centrifugal filters with 0.1 µm pores for the liquid-liquid extracted solutions, were purchased from Millipore, Inc. (Bedford, MA, USA).

2.2.3 Preparation of standard solutions

Initial stock solutions of propofol and thymol were prepared in 50% acetonitrile at 800 µg•mL\(^{-1}\) propofol and 100 µg•mL\(^{-1}\) of thymol, respectively. Propofol was subsequently serially diluted with 6% acetonitrile to desired concentrations: 16, 8, 4, 2, 1, 0.5 µg•mL\(^{-1}\). Two additional
concentrations (0.3, 0.1 µg•mL⁻¹) were prepared from 1 µg•mL⁻¹. These solutions were spiked with thymol stock solution for a final internal standard (IS) concentration of 7 µg•mL⁻¹.

2.2.4 Patients and Sampling

This investigation conforms to the principles outlined in the Declaration of Helsinki. Following institutional approval and written informed patient consent, 30 patients scheduled for primary CABG surgery were enrolled in an ongoing parallel study investigating the short-term application of propofol during CPB. All patients received a 1.0 mg•kg⁻¹ bolus dose of propofol at heparinization (approximately 10 minutes prior to aortic crossclamp placement) followed by an infusion of 120 µg•kg⁻¹•min⁻¹ for the duration of CPB. A blood sample of 5 mL was withdrawn from the central venous line 15 minutes after aortic declamping, and stored in 1.25 mL aliquots at -80°C for subsequent CE analysis. Patient and operative characteristics are listed in Table 3. Perioperative patient care was administered according to the routine clinical practice at Vancouver General Hospital.

Table 3: General patient and operative characteristics

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2.2.5 Sample extraction

A liquid-liquid extraction procedure described by Plummer [244] is frequently used for the analysis of propofol in blood samples. We have modified this procedure to suit the CE analysis. In brief, 30 µL of thymol internal standard stock solution (100 µg•mL⁻¹), 200 µL of deionized water, and 25 µL of 1 M NaH₂PO₄ (pH 4.2) were added to a 400 µL aliquot of whole blood. The sample mixture was then vortexed for 1 min, after which 650 µL of cyclohexane was added, followed by at least 2 minutes of inverting and vortexing. Organic and aqueous layers were separated by centrifugation (1200 g for 1 minute at 4°C). A 500 µL aliquot of the cyclohexane layer was transferred through a 0.1 µm centrifugal filter into a clean tube containing
10 µL of diluted Tetramethylammonium hydroxide solution (2% v/v in propanol). The solvent was evaporated to dryness under speedvac (down to 0.6 torr) and the residue was re-dissolved in 80 µL of 6% acetonitrile in water.

2.2.6 Capillary electrophoresis

Prior to daily use, the capillary was conditioned with 0.1 M NaOH, methanol, purified water, and separation buffer in successive rinses of 20 minutes each. Individual CE runs were preceded by four successive 3-minute rinses using 0.1 M NaOH, methanol, purified water, and separation buffer. A pressure difference of 20 psi was used in all rinse procedures.

Upon filling the capillary with separation buffer, conventional hydrodynamic sample injection was performed at 0.5 psi (3447 Pa) for 5 seconds. A separation voltage of 25 kV under normal polarity was applied continuously for 12 minutes. Propofol and thymol were detected with UV absorbance at λ = 200 nm.

2.3 Results and discussion

Propofol is a highly lipophillic compound [245] with a pKa of 11. We developed a MEKC-based separation to overcome the low aqueous solubility of the drug in its neutral form. Specific aspects of the method development process for the quantitative analysis of propofol in whole blood are described below.

2.3.1 Specificity

In order to identify the thymol and propofol peaks, and to match them with specific migration times and apparent electrophoretic mobilities, we spiked the blank matrix and blank whole blood matrix with either propofol or thymol reference materials. The peak identities were further confirmed during the construction of the calibration curve, in which a series of standard solutions were prepared with increasing propofol concentrations but one consistent thymol concentration. Accordingly, the propofol peak could be distinguished from the thymol peak because the former had an incrementally increasing peak area.

In CE analysis, apparent electrophoretic mobility (μep) is more representative of a given analyte than migration time because it normalizes the rate of migration with respect to both the medium and the electric field strength, and is independent of electroosmotic flow. Accordingly, our group always converts migration times to electrophoretic mobility when conducting CE. The
apparent electrophoretic mobilities of thymol and propofol always have coefficients of variation below 0.7 (Table 4, Table 5, Table 7). Based on specific apparent electrophoretic mobility, we can assign thymol and propofol peaks in different samples without ambiguity. In a similar vein, there appears to be a difference between the migration times of propofol and thymol from Figure 5. These electropherograms were produced with two separately prepared capillaries. This difference may manifest itself in the electroosmotic flow, and therefore alter the migration times. Calculating apparent electrophoretic mobilities reveals the magnitude of the actual difference (Figure 5A: $\mu_{ep}^T = -22.08 \text{ cm}^2\text{kV}^{-1}\text{min}^{-1}$, $\mu_{ep}^P = -23.08 \text{ cm}^2\text{kV}^{-1}\text{min}^{-1}$; Figure 5B: $\mu_{ep}^T = -22.19 \text{ cm}^2\text{kV}^{-1}\text{min}^{-1}$, $\mu_{ep}^P = -23.22 \text{ cm}^2\text{kV}^{-1}\text{min}^{-1}$).

The specificity of the method was also assessed by its ability to separate propofol and thymol from other nonspecific blood components, and to resolve the peaks from one another. In brief, the optimized method produced sharp, gaussian peaks for both propofol and thymol with baseline resolution ($R_s \geq 2.6$) at concentrations of 2, 4, and 8 µg•mL$^{-1}$ in both standard and sample extracted from whole blood matrices. Additionally, blank blood samples did not produce any detectable signals that interfered with the propofol and thymol peaks. Figure 5 shows the representative electropherograms for propofol and thymol from a) 6% acetonitrile and b) whole blood matrices.
Figure 5: Representative electrophoregrams of standard solution of propofol and thymol (Panel A) and whole blood containing propofol and thymol (Panel B)

Run conditions: Sample injection: 0.5 psi for 5 s. Run buffer: 50mM SDS, 15mM borax separation: 25 kV normal polarity over 12 min across an uncoated capillary (ID = 50 mm; LT = 50 cm; LD = 40 cm). Detection: UV absorbance at λ = 200 nm.

2.3.2 Selection of buffer type and separation voltage

The solubility of propofol is proportional to the concentrations of sodium dodecyl sulfate in the buffer, particularly because propofol is exists almost entirely in its protonated and neutral in this buffer (pH=8.5). This limitation could be overcome by the addition of organic solvent in the running buffer, but this significantly compromised the consistency of run-times. The varying migration times reflect the difficulty of accurately controlling and reproducing the organic
content in the buffer. The increased ionic strength of higher borax concentrations in the separation buffer translated into longer migration times. Alternatively, a lower ionic strength separation buffer resulted in broader peaks, lower resolution, and an inferior limit of detection. The composition of the separation buffer represents a compromise between the solubility and stacking efficiency of the analyte, and the running time. A 15 mM borax buffer (60 mM borate) with 50 mM sodium dodecyl sulfate was chosen as optimum condition based on the solubility of propofol, as well as the precision and length of the runs.

The separation voltage of 25 kV across a 50 cm capillary of 50 µm inner diameter was chosen based on the highest potential difference within the linear region in the Ohm’s law plot. The optimized separation conditions produce propofol and thymol peaks in less than 8 min (thymol $t_{mig}$: 6.72±0.02 min; $\mu_{ep}^T$: $-22.05\pm0.06 \text{ cm}^2\text{kV}^{-1}\text{min}^{-1}$; propofol $t_{mig}$: 7.34±0.02 min; $\mu_{ep}^P$: $-22.05\pm0.06 \text{ cm}^2\text{kV}^{-1}\text{min}^{-1}$). Values are in mean±SD).

2.3.3 Linearity

The clinically relevant concentration range of propofol was estimated between 1.5 and 10 $\mu$g•mL$^{-1}$ (whole blood) [130, 246]. The linearity of the current method was assessed using the corrected area of standard propofol solutions between 0.1 and 16 $\mu$g•mL$^{-1}$ relative to that of a fixed concentration of 7 $\mu$g•mL$^{-1}$ thymol. This ratio was plotted against the propofol concentration. This curve has a goodness of fit of $r^2 = 0.9995$ throughout the tested range, with the equation: $y = 0.0740x + 0.0019$ (slope: 0.00064, 95% CI slope: 0.0725 to 0.0760; SD y-intercept: 0.0039, 95% CI: -0.0075 to 0.0112, n = 15 concentrations).

2.3.4 Precision

Precision was assessed by injecting 3 clinically relevant concentrations of propofol (2, 4, and 8 $\mu$g•mL$^{-1}$) dissolved in 6% acetonitrile, and injected 3 times on the same day and on 3 successive days. The electrophoretic mobilities of propofol and thymol were calculated to assess the precision and specificity of the separation, while the corrected area ratio of the propofol and thymol peaks was used to measure the quantitative precision.

Table 4 and Table 5 summarize the results of the precision analyses for propofol at 2, 4 and 8 $\mu$g•mL$^{-1}$ on the same day and on 3 successive days, respectively. The apparent electrophoretic mobilities of thymol and propofol had acceptable consistency within a given day and throughout a three-day interval for each of the tested concentrations ($\%\text{RSD} \leq 0.7$). The
consistency of the corrected area ratio was also acceptable on the same day and over the three days across the three tested concentrations (% RSD ≤ 4.3).

**Table 4: Precision of three clinically relevant standard propofol concentrations in the same day**

<table>
<thead>
<tr>
<th>Concentration (µg/mL)</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>Mean</th>
<th>SD</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 µg/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area&lt;sub&gt;corr&lt;/sub&gt;</td>
<td>P/T</td>
<td>0.149</td>
<td>0.138</td>
<td>0.148</td>
<td>0.145</td>
<td>0.006</td>
</tr>
<tr>
<td>µ&lt;sub&gt;ep&lt;/sub&gt;&lt;sup&gt;A&lt;/sup&gt;</td>
<td>T</td>
<td>-21.99</td>
<td>-22.07</td>
<td>-22.10</td>
<td>-22.05</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>-23.01</td>
<td>-23.08</td>
<td>-23.11</td>
<td>-23.07</td>
<td>0.06</td>
</tr>
<tr>
<td>t&lt;sub&gt;mig&lt;/sub&gt;</td>
<td>T</td>
<td>6.70</td>
<td>6.71</td>
<td>6.73</td>
<td>6.72</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>7.33</td>
<td>7.34</td>
<td>7.36</td>
<td>7.34</td>
<td>0.02</td>
</tr>
<tr>
<td>4 µg/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area&lt;sub&gt;corr&lt;/sub&gt;</td>
<td>P/T</td>
<td>0.301</td>
<td>0.316</td>
<td>0.298</td>
<td>0.305</td>
<td>0.010</td>
</tr>
<tr>
<td>µ&lt;sub&gt;ep&lt;/sub&gt;&lt;sup&gt;A&lt;/sup&gt;</td>
<td>T</td>
<td>-21.86</td>
<td>-21.89</td>
<td>-21.95</td>
<td>-21.90</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>-22.87</td>
<td>-22.88</td>
<td>-22.94</td>
<td>-22.90</td>
<td>0.04</td>
</tr>
<tr>
<td>t&lt;sub&gt;mig&lt;/sub&gt;</td>
<td>T</td>
<td>6.77</td>
<td>6.89</td>
<td>6.93</td>
<td>6.86</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>7.40</td>
<td>7.53</td>
<td>7.58</td>
<td>7.50</td>
<td>0.09</td>
</tr>
<tr>
<td>8 µg/mL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area&lt;sub&gt;corr&lt;/sub&gt;</td>
<td>P/T</td>
<td>0.603</td>
<td>0.621</td>
<td>0.587</td>
<td>0.604</td>
<td>0.017</td>
</tr>
<tr>
<td>µ&lt;sub&gt;ep&lt;/sub&gt;&lt;sup&gt;A&lt;/sup&gt;</td>
<td>T</td>
<td>-21.89</td>
<td>-21.93</td>
<td>-22.01</td>
<td>-21.95</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>-22.91</td>
<td>-22.94</td>
<td>-23.03</td>
<td>-22.96</td>
<td>0.27</td>
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<tr>
<td>t&lt;sub&gt;mig&lt;/sub&gt;</td>
<td>T</td>
<td>6.93</td>
<td>6.78</td>
<td>7.00</td>
<td>6.90</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>7.59</td>
<td>7.40</td>
<td>7.68</td>
<td>7.56</td>
<td>0.14</td>
</tr>
</tbody>
</table>

P = Propofol; T = Thymol; Area<sub>corr</sub> = corrected area; µ<sub>ep</sub><sup>A</sup> = apparent electrophoretic mobility (cm<sup>2</sup>•kV<sup>-1</sup>•min<sup>-1</sup>); t<sub>mig</sub> = migration time (min).
Table 5: Precision of three clinically relevant standard propofol concentrations across three days

<table>
<thead>
<tr>
<th></th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Mean</th>
<th>SD</th>
<th>%RSD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2 µg/mL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area corr</td>
<td>P/T</td>
<td>0.145</td>
<td>0.145</td>
<td>0.145</td>
<td>0.145</td>
<td>0.004</td>
</tr>
<tr>
<td>µ\textsubscript{ep}^A</td>
<td>T</td>
<td>-22.05</td>
<td>-22.11</td>
<td>-21.82</td>
<td>-22.01</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>-23.07</td>
<td>-23.11</td>
<td>-22.84</td>
<td>-23.02</td>
<td>0.16</td>
</tr>
<tr>
<td>τ\textsubscript{mig}</td>
<td>T</td>
<td>6.72</td>
<td>6.70</td>
<td>6.61</td>
<td>6.68</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>7.34</td>
<td>7.32</td>
<td>7.22</td>
<td>7.29</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>4 µg/mL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area corr</td>
<td>P/T</td>
<td>0.305</td>
<td>0.287</td>
<td>0.301</td>
<td>0.300</td>
<td>0.011</td>
</tr>
<tr>
<td>µ\textsubscript{ep}^A</td>
<td>T</td>
<td>-21.90</td>
<td>-22.03</td>
<td>-21.89</td>
<td>-21.96</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>-22.90</td>
<td>-23.03</td>
<td>-22.93</td>
<td>-22.97</td>
<td>0.06</td>
</tr>
<tr>
<td>τ\textsubscript{mig}</td>
<td>T</td>
<td>6.86</td>
<td>6.76</td>
<td>6.58</td>
<td>6.73</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>7.50</td>
<td>7.38</td>
<td>7.20</td>
<td>7.36</td>
<td>0.15</td>
</tr>
<tr>
<td><strong>8 µg/mL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area corr</td>
<td>P/T</td>
<td>0.604</td>
<td>0.605</td>
<td>0.626</td>
<td>0.609</td>
<td>0.015</td>
</tr>
<tr>
<td>µ\textsubscript{ep}^A</td>
<td>T</td>
<td>-21.95</td>
<td>-22.11</td>
<td>-21.98</td>
<td>-21.99</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>-22.96</td>
<td>-23.09</td>
<td>-23.01</td>
<td>-22.99</td>
<td>0.07</td>
</tr>
<tr>
<td>τ\textsubscript{mig}</td>
<td>T</td>
<td>6.90</td>
<td>6.77</td>
<td>6.60</td>
<td>6.76</td>
<td>0.15</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>7.56</td>
<td>7.38</td>
<td>7.21</td>
<td>7.38</td>
<td>0.17</td>
</tr>
</tbody>
</table>

\( P = \text{Propofol; } T = \text{Thymol; } Area_{corr} = \text{corrected area; } \mu_{ep}^A = \text{apparent electrophoretic mobility (cm}^2\text{kV}^{-1}\text{min}^{-1}); \tau_{mig} = \text{migration time (min).} \) *Day 1 data represents an average of 3 consecutive runs.

2.3.5 Optimization of the liquid-liquid sample extraction

The procedure used to extract propofol from whole blood was adapted from the liquid-liquid extraction first described by Plummer [244]. We have modified this method to suit the requirement for CE analysis. The first modification was to run the liquid-liquid extracted organic layer through a syringe filter of 0.1 µm pore diameter in order to prevent the capillary from becoming plugged and to reduce the variation in the migration time. Additionally, rather than using a stream of N\textsubscript{2(g)} to dry the extracted organic layer, we were able to achieve better precision using a vacuum centrifuge at 0.6 torr. We found that the dry pellet could be stably stored under N\textsubscript{2(g)} at -80 °C until subsequent resuspension and analysis with minimal degradation (data not shown).
In order to control the volatility and reduce the loss of propofol during the drying process, tetramethylammonium hydroxide needs to be added to the organic layer prior to drying and resuspension. In order to optimize the tetramethylammonium hydroxide content for subsequent CE analysis, we added 5, 15, 25, 35, and 45 µL of 1% tetramethylammonium hydroxide (in methanol) to the organic layer prior to drying and resuspension. The best electropherograms, in terms of resolution, peak height, and baseline stability, were obtained with 15 or 25 µL of 1% tetramethylammonium hydroxide. We subsequently made all of our tetramethylammonium hydroxide dilutions at 2% v/v in propanol because of its superior solubility with cyclohexane compared to methanol. Indeed, we found this change to yield better run-to-run precision. The pH of the resulting resuspended sample solutions was consistently between 11 and 12.

We chose to use 400 µL blood and 650 µL cyclohexane to constitute the extraction system which satisfied the need for both accuracy and operability. 500 µL of organic phase out of 650 µL was then aspirated to preclude aqueous contamination. The smaller total volume of this extraction system (<1.5 mL) than that of the original design is not only more convenient from a clinical perspective, but also in the laboratory because common and disposable lab supplies and equipment such as eppendorf tubes, spin filters and microcentrifuges can be used. The precision and accuracy of the separation and recovery requires that errors in all upstream steps be minimized. Pipetting errors, weighing errors, and inconsistencies in the extraction steps tend to propagate throughout subsequent steps. This is especially true when smaller starting volumes of blood are used, as with our method, because the relative error is larger. Weighing small quantities of solid should be avoided because they tend to yield relatively large sampling errors.

2.3.6 Optimization of the sample resuspension solution

We investigated the effects of the organic content and the ionic strength in the resuspension solution. Pellets were resuspended in aqueous solutions containing 6%, 30%, or 60% acetonitrile. 6% acetonitrile was a practical organic content from an analytical perspective because it was capable of keeping the analyte homogeneously dissolved without negatively affecting the peak shape. Higher organic contents tended to produce poor peak shapes. In order to optimize the ionic strength of the resuspension solution, we resuspended pellets in three buffers of increasing ionic strength. Solutions containing 6% acetonitrile with 0%, 1%, or 10% running buffer were studied. We found that the lowest ionic strength resuspension solution (6% acetonitrile with 0% running buffer) yielded the best resolution and peak shapes. We found that the main determinant for the pH of the extracted and prepared sample ready to be injected into the
capillary was the amount of tetramethylammonium hydroxide that was added to the organic phase prior to drying under vacuum centrifuge, as opposed to the amount of borax buffer contained within the resuspension solution. These modifications enabled us to quantitatively analyze propofol from a 400 µL starting volume of whole blood.

2.3.7 On-line pH-difference induced focusing

The resuspended solutions consistently had a pH of between 11 and 12, which is similar to the pKa of both propofol (pKa = 11) and thymol (pKa = 10.5). Accordingly, propofol and thymol are likely fractionally ionized in the injected sample, establishing equilibria between their respective anionic and neutral species. The analytes in the sample plug could experience an effect similar to pH junction velocity-difference induced focusing [247]. In order to verify this effect we separated propofol and thymol in two distinct sample solutions: the first sample consisted of propofol and thymol resuspended in an aqueous solution containing 6% acetonitrile adjusted to a final pH of 12 with tetramethylammonium hydroxide. The second sample consisted of an equal concentration of propofol and thymol as condition 1, resuspended in 6% acetonitrile aqueous solution, with a final pH of 6.

Table 6 describes the peak width, migration time, velocity, and end length of the propofol and thymol peaks derived from these samples, as well as the resolution between them. These results, which show improved peak width, length, and resolution, suggest that our method induces an online focusing effect. We were able to achieve sufficient sensitivity for routine clinically relevant propofol concentrations, but this focusing effect could be further investigated to increase the sensitivity if needed.

Table 6: Contribution of sample solution pH to the resolution and focusing effect of the separation

<table>
<thead>
<tr>
<th></th>
<th>Sample Solution pH 12</th>
<th>Sample solution pH 6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Thymol</td>
<td>Propofol</td>
</tr>
<tr>
<td>Peak Width (min)</td>
<td>0.13</td>
<td>0.12</td>
</tr>
<tr>
<td>Migration time (min)</td>
<td>6.70</td>
<td>7.30</td>
</tr>
<tr>
<td>Analyte Velocity (mm/min)</td>
<td>59.70</td>
<td>54.79</td>
</tr>
<tr>
<td>Peak length (mm)</td>
<td>7.76</td>
<td>6.58</td>
</tr>
<tr>
<td>Resolution</td>
<td>4.80</td>
<td></td>
</tr>
</tbody>
</table>
2.3.8 Indices of precision and recovery of whole blood analysis

In order to assess the precision of the propofol analysis from whole blood, we spiked propofol into blank whole blood to obtain one of three clinically relevant final concentrations (2, 4, and 8 µg·mL⁻¹). Thymol was subsequently added to a final concentration of 7 µg·mL⁻¹. We performed the extraction, and analyzed the sample using our optimized CE method. Each concentration was independently prepared and analyzed in triplicate. The percent-recovery was calculated using the standard curve to convert the propofol/thymol (P/T) corrected area ratio of each sample to its corresponding propofol concentration, and expressing this value as a percentage of the actual spiked propofol concentration (Equation 20).

\[ R = \frac{(P/T - b_{\text{std.curve}}) m_{\text{std.curve}}}{P_{\text{spike}}} \]

\( P/T \) denotes the corrected area ratio of propofol to thymol; \( b_{\text{std.curve}} \) and \( m_{\text{std.curve}} \) denote the y-intercept and slope of the standard curve; and \( [P_{\text{spike}}] \) denotes the concentration of propofol supplemented into the blank blood sample.

Table 7 shows indices of precision for propofol at 2, 4 or 8 µg·mL⁻¹. The electrophoretic mobilities of propofol and thymol were consistent for each of the tested propofol concentrations (% RSD ≤ 0.2). The migration times of extracted samples were similar to those achieved using standard prepared solutions containing propofol and thymol, suggesting that the extraction and residual whole blood components did not significantly alter the separation process. Migration times of propofol and thymol peaks derived from extracted samples all have % RSD ≤ 0.1, indicating that the sample preparation step consistently produces samples of similar composition that do not negatively influence the regeneration of the capillary after each separation process.
Table 7: Indexes of precision of three independently prepared clinically relevant propofol concentrations spiked into and extracted from whole blood

<table>
<thead>
<tr>
<th></th>
<th>2 µg/mL</th>
<th>4 µg/mL</th>
<th>8 µg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>% RSD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Area&lt;sub&gt;corr&lt;/sub&gt;</td>
<td>P/T</td>
<td>0.182</td>
<td>0.181</td>
</tr>
<tr>
<td>% R</td>
<td>P/T</td>
<td>124.0</td>
<td>123.8</td>
</tr>
<tr>
<td>µ&lt;sub&gt;ep&lt;/sub&gt;</td>
<td>T</td>
<td>-22.02</td>
<td>-21.97</td>
</tr>
<tr>
<td>P</td>
<td>-23.01</td>
<td>-22.95</td>
<td>-22.98</td>
</tr>
<tr>
<td>t&lt;sub&gt;mig&lt;/sub&gt;</td>
<td>T</td>
<td>6.72</td>
<td>6.72</td>
</tr>
<tr>
<td>P</td>
<td>7.33</td>
<td>7.33</td>
<td>7.31</td>
</tr>
<tr>
<td>Area&lt;sub&gt;corr&lt;/sub&gt;</td>
<td>P/T</td>
<td>0.359</td>
<td>0.356</td>
</tr>
<tr>
<td>% R</td>
<td>P/T</td>
<td>121.6</td>
<td>120.4</td>
</tr>
<tr>
<td>µ&lt;sub&gt;ep&lt;/sub&gt;</td>
<td>T</td>
<td>-22.07</td>
<td>-22.04</td>
</tr>
<tr>
<td>P</td>
<td>-23.06</td>
<td>-23.02</td>
<td>-22.97</td>
</tr>
<tr>
<td>t&lt;sub&gt;mig&lt;/sub&gt;</td>
<td>T</td>
<td>6.72</td>
<td>6.73</td>
</tr>
<tr>
<td>P</td>
<td>7.32</td>
<td>7.34</td>
<td>7.34</td>
</tr>
<tr>
<td>Area&lt;sub&gt;corr&lt;/sub&gt;</td>
<td>P/T</td>
<td>0.717</td>
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</tr>
<tr>
<td>% R</td>
<td>P/T</td>
<td>121.0</td>
<td>124.3</td>
</tr>
<tr>
<td>µ&lt;sub&gt;ep&lt;/sub&gt;</td>
<td>T</td>
<td>-22.04</td>
<td>-22.03</td>
</tr>
<tr>
<td>P</td>
<td>-23.03</td>
<td>-23.01</td>
<td>-23.05</td>
</tr>
<tr>
<td>t&lt;sub&gt;mig&lt;/sub&gt;</td>
<td>T</td>
<td>6.70</td>
<td>6.69</td>
</tr>
<tr>
<td>P</td>
<td>7.30</td>
<td>7.29</td>
<td>7.31</td>
</tr>
</tbody>
</table>

P = Propofol; T = Thymol; Area<sub>corr</sub> = corrected area; %R = % recovery; µ<sub>ep</sub> = apparent electrophoretic mobility (cm<sup>2</sup>•kV<sup>-1</sup>•min<sup>-1</sup>); t<sub>mig</sub> = migration time (min).

The consistency of the P/T corrected area ratio and the percent recovery reflect the cumulative error of the operator, the extraction, and the instrument. The coefficients of variation associated with these values are therefore expected to be elevated. Accordingly, the P/T corrected area ratio derived from propofol in whole blood has % RSD values at or below 5.2%, while the percentage recovery has % RSD values at or below 5.1%.

We report percent recovery values that consistently exceed 100% over the concentration range (2, 4, or 8 µg•mL<sup>-1</sup>) we examined (120.3%, 123.8% or 120.8%). The distribution and partition coefficients (log D at pH of 3-7, and log P, at 25°C) were calculated using Advanced Chemistry Development software (V8.14 for solaris ACD/Labs). Thymol has log D and log P...
values of 3.28, while those of propofol are 4.16. Accordingly, the efficiency with which propofol is extracted into cyclohexane is greater than that of thymol, and the P/T signal ratio subsequent to the extraction is therefore elevated. This elevated P/T signal ratio is used to derive the numerator in Equation 20, the percent recovery therefore exceeds 100%.

2.3.9 Limit of detection and quantitation

The limit of detection of a standard solution of propofol using our method was determined using the signal-to-noise ratio of 3 relative to the signal of the standardized thymol concentration (7 µg•mL\(^{-1}\)). The slope from the standard curve was used to translate this P/T ratio to the corresponding propofol concentration. The limit of quantitation was determined similarly, using a signal-to-noise ratio of 10. The current method has a limit of detection of 0.07 µg•mL\(^{-1}\) and a limit of quantitation of 0.24 µg•mL\(^{-1}\). The above values are determined using thymol prepared in a neat solution, and therefore describe the limits of detection and quantitation for the instrument.

A second series of calculations were done using electropherograms derived from whole blood samples. The signal-to-noise ratio from these electropherograms includes the contribution of the extraction and the original whole blood matrix, and therefore more closely reflects the conditions for which the method was intended. The limit of detection and limit of quantitation values were determined as described above, but included a correction for the recovery. The resulting limit of detection and limit of quantitation values from electropherograms of whole blood samples were 0.07 µg•mL\(^{-1}\) and 0.23 µg•mL\(^{-1}\), respectively. The similarity between the noise from neat and extracted injections testifies to the effectiveness of the modified sample extraction procedure. Although these values are higher than what has been achieved with some other methods, we found that the sensitivity is sufficient for clinically relevant propofol concentrations with acceptable % RSD.

2.3.10 Patient samples

We used our method to analyze the concentration of propofol in the blood of 30 patients receiving propofol as the primary anesthetic during CPB. Whole blood samples were drawn 15 minutes after aortic declamping. Figure 6 shows the concentration distribution of these 30 patients. The average concentration of propofol was 5.36 µg•mL\(^{-1}\) (95% CI 4.48 to 6.24 µg/mL). This concentration of propofol reflects the infusion rate of 120 µg•kg\(^{-1}\)•min\(^{-1}\). The distribution in Figure 6 shows that propofol infusions normalized to patient weight results in a considerable...
amount of variation in the concentration achieved in whole blood. The magnitude of this distribution is in agreement with previous reports [238, 246, 248] and likely reflects both population variance and altered pharmacokinetics during CPB. Whatever the reason, the data show the need for a reliable method to monitor the actual whole blood concentrations achieved in patients during surgery. Such techniques will improve patient safety by reducing the likelihood of complications related to elevated concentrations, including the rare but fatal “propofol infusion syndrome” [249].

Figure 6: Concentration distribution of propofol in whole blood of 30 patients undergoing aortocoronary bypass surgery

Samples were obtained 15 min after aortic declamping under a propofol infusion rate of 120 µg·kg⁻¹·min⁻¹. Run conditions: sample injection: 0.5 psi for 5 s. Run buffer: 50 mM SDS, 15 mM borax separation: 25 kV normal polarity over 12 min across an uncoated capillary (ID = 50 µm; LT = 50 cm; LD = 40 cm). Detection: UV absorbance at λ = 200 nm.
2.4 Conclusion

We describe a MEKC-based method capable of quantitative propofol analysis in whole blood over a clinically relevant concentration range with acceptable precision. This method is capable of producing sharp, baseline-resolved analyte peaks in less than 8 minutes from 400 µL of whole blood. More importantly, the method is robust and accurate enough to provide reliable propofol concentrations from patient samples collected in clinic settings. The procedures described in this work that were aimed at improving the precision and robustness should be applicable to method development for other pharmaceutical analysis. Target achieved type techniques will require analytical methods with a high degree of automation, fast run times, and small and simple instrumentation. We recognize that the current method does not meet all of these criteria, especially insofar as the sample preparation step is concerned. However, this accurate and reliable method will provide a foundation for the development of other types or devices that more closely meets this clinical demand.
3. Target achieved propofol concentration during on-pump CABG surgery: a pilot dose finding study

3.1 Introduction

Ischemia-reperfusion injury during CABG is a source of intraoperative cardiac injury [250]. Therapeutic pharmacologic options during surgery to preserve the viability of ischemic myocardium include volatile anesthetic pre- and post-conditioning and antioxidant therapies [114, 190, 218, 251]. Unfortunately, the clinical precision and effectiveness of volatile anesthetic preconditioning has recently come into question [252, 253]. Anesthetic preconditioning has not translated easily to the clinical scenario and is not universally effective. Patient factors including diabetic status [124, 254] and aortic cross-clamp intervals exceeding 30 to 40 minutes [125] could mitigate the effects of the preconditioning stimulus. Research into alternative approaches of cardioprotection is required.

Conditions at reperfusion significantly contribute to tissue injury and repair [255]. The antioxidant [256] and cell signaling properties [134, 257] of propofol, as well as its ability to inhibit mitochondrial permeability transition [258, 259], are well suited to reduce reperfusion injury. Unfortunately, clinical anesthetic conditioning studies have demonstrated that target controlled infusion devices, which predict whole blood propofol concentrations based on mathematical algorithms that link patient characteristics and pharmacokinetics with infusion rates, set to a target propofol concentration from 1 to 4 µg•mL⁻¹ failed to protect against myocardial injury [218, 260]. Based on work from our lab and others using both simulated models of ischemia-reperfusion injury and studies in patients [131, 261-266], we postulate that propofol confers cardioprotection when a target range of 4.5-8.9 µg•mL⁻¹ (25 to 50 µM) is achieved.

There have been limited in vivo studies evaluating the effect of increased propofol dosing to achieve the therapeutic concentration range defined in vitro. Recently, a clinically relevant swine model of normothermic blood cardioplegic arrest with CPB demonstrated that a 1 mg•kg⁻¹ bolus followed by a 100 µg•kg⁻¹•min⁻¹ continuous propofol infusion was cardioprotective without negative hemodynamic consequences [264]. The authors estimated whole blood propofol concentration of 3.7 µg•mL⁻¹ based on other clinical studies with similar operative procedures [248, 267, 268]. We previously found that a 2 to 2.5 mg•kg⁻¹ bolus of propofol followed by an infusion of 200 µg•kg⁻¹•min⁻¹ produces drug concentrations associated with increased antioxidant capacity (8.2+/− 2.1 µg•mL⁻¹), but showed signs of intraoperative cardiac depression compared to
conventional propofol or isoflurane anesthesia maintenance [130]. More recently, increasing propofol anesthetic maintenance from 60 to 120 µg•kg⁻¹•min⁻¹ intraoperatively was associated with a reduction in biomarkers of cardiac injury and oxidative stress, although the range of values was consistent with those expected during cardiac surgery [133]. Clinically relevant differences in hemodynamics and left ventricular function were not detected in this study and drug concentrations were not measured. Further systematic investigations are needed to evaluate the role of propofol in cardiac surgery.

It is unknown if experimental cardioprotective propofol concentrations can routinely be achieved at reperfusion during CABG with CPB using short-term continuous infusion, or if such concentrations are associated with an increased risk of cardiac instability upon emergence from CPB. To address this question, we conducted a pilot dose finding study and developed a predictive mathematical modeling for optimal dosing in patients.

In this pilot study, we hypothesize that a whole blood propofol concentration of 5 µg•mL⁻¹ can be achieved clinically with continuous drug delivery during CABG with CPB. We focused our treatment interval to the CPB interval of CABG, and measured the resulting propofol concentrations in whole blood 15 minutes after reperfusion. We also sought to identify any evidence of clinically significant cardiac depression upon separation from bypass, and measured intraoperative hemodynamic performance using cardiac index (CI), systemic vascular resistance index (SVRI), and left ventricular stroke work index (LVSWI).

3.2 Materials and methods

3.2.1 Study design

We report on two successive studies, with the aim of establishing a clinical anesthetic maneuver that reliably yields a target whole blood propofol concentration of 5 µg•mL⁻¹.

The first study (Study 1) was an open label pilot dose finding study in 24 patients who received one of three propofol doses by continuous infusion during CPB. Propofol concentrations were mathematically described as a function of the infusion rate with an empirical line of best fit, constructed using nonlinear regression followed by Akaike’s Information Criteria comparison. The pharmacokinetics of propofol are most accurately described by a three compartment model [246]. True pharmacokinetic steady state for propofol under a 3-compartment model would require in excess of 3 days for non-obese patients, and beyond 10 days for obese patients [269].
Given these timescales, it is unreasonable to anticipate steady state conditions for propofol during cardiac surgery –let alone during the ischemic or reperfusion phase. For these reasons, the mathematical function describing the relationship between the infusion rate and the propofol concentration achieved at reperfusion was not modeled on pharmacokinetic or physiological principles. We used this mathematical function solely to determine the infusion rate predicted to yield our target propofol concentration.

The infusion rate derived from Study 1 was employed in a subsequent and ongoing randomized controlled trial, entitled PRO-TECT II (www.clinicaltrials.gov NCT00734383) [231]. We planned an analysis of propofol concentrations at the midpoint of the PRO-TECT II trial (n=72) in those patients randomized to the propofol treatment arm (n=30). The purpose of this interim analysis (Study 2) was to assess the reliability with which our clinical maneuver achieves our target propofol concentration at reperfusion.

Both studies focus on propofol concentrations in whole blood sampled 15 minutes after reperfusion in vacutainer tubes containing EDTA as the anticoagulant (Becton Dickinson, NJ). This operative timeframe precludes pharmacokinetic analysis. Hemodynamic measures of CI, SVRI, and LVSWI were also recorded prior to CPB, upon separation from CPB, and prior to patient transfer to the intensive care unit in both studies.

3.2.2 Study population

This investigation conforms to the principles outlined in the Declaration of Helsinki. Following institutional approval and informed patient consent, we enrolled hemodynamically stable patients scheduled for revascularization of 3 or more coronary vessels where a minimum continuous aortic cross-clamp time of 60 minutes was anticipated. We excluded patients who were less than 18 or greater than 80 years of age, those who refused consent, and those who had co-existing valvular heart disease, an acute or evolving myocardial infarction, or a history of hypersensitivity to propofol or any formulation component.

3.2.3 Perioperative procedures

Perioperative monitoring (arterial, central, and pulmonary catheterization), surgical, and cardioplegia techniques (warm, intermittent, antegrade delivery of blood:crystalloid (8:1 ratio)) were standardized. The antifibrinolytic therapy of choice was tranexamic acid 0.05 mg•kg⁻¹ then 0.10 µg•kg⁻¹•min⁻¹. CPB was conducted at 34-37 °C. Intraoperative hematocrit was maintained at
0.25 to 0.27 during CPB and facilitated by retrograde autologous prime procedure [270].

3.2.4 Anesthesia protocol

Anesthesia was standardized to induction with fentanyl 10-15 µg•kg, midazolam 2-4 mg and sodium thiopental as required for loss of consciousness. Muscle relaxation and tracheal intubation were achieved with rocuronium 0.1 mg•kg⁻¹. Anesthesia was maintained with isoflurane (0.5-2%, end tidal), except during CPB when propofol was administered. Post-CPB anesthesia was as per clinical practice of the attending anesthesiologist.

3.2.5 Experimental maneuver: application of propofol during CPB

Delivery of isoflurane was discontinued approximately ten minutes prior to aortic cross-clamp. Propofol was then applied as a 1.0 mg•kg⁻¹ bolus followed by a continuous infusion of 50 (n=8), 100 (n=9) or 150 µg•kg⁻¹•min⁻¹ (n=7) in Part One, or 120 µg•kg⁻¹•min⁻¹ (n=30) in Part Two, until 15 minutes after release of the aortic cross-clamp (reperfusion).

3.2.6 Measurement of propofol concentration

Four milliliters of whole blood was sampled from the central venous line 15 minutes after reperfusion to accommodate the surgeon after cross-clamp removal. Whole blood was sampled using vacutainer tubes containing EDTA as the anticoagulant (Becton Dickinson, NJ), then stored as ~1.25 mL aliquots and stored at -80 °C for subsequent quantitative propofol analysis by capillary electrophoresis [271] (see Chapter 2).

3.2.7 Hemodynamic data collection

Intraoperative central venous pressure and mean pulmonary catheter wedge pressure were maintained to within ± 20% of baseline values by volume transfusion from the CPB reservoir. Transesophageal echocardiography was employed during the perioperative period to facilitate volume loading, and to rule out cardiac tamponade, pneumo- or hemothorax as possible causes of cardiac depression. Intraoperative cardiac function (CI, SVRI, and LVSWI) was measured and derived at three timepoints: pre-CPB, post-CPB emergence, and just prior to admission to the intensive care unit (pre-ICU).

3.2.8 Inotropic and vasoactive drug protocol

Intraoperative hemodynamic management included use of phenylephrine (1-2 µg•kg⁻¹
for blood pressure below 85 systolic or mean arterial pressures below 50 mmHg. Systolic blood pressure greater than 140 mmHg or mean arterial pressures above 80 mmHg was treated by deepening anesthesia by using fentanyl (1-2 µg/kg) followed by the vasodilator of choice prn at the discretion of the attending anesthesiologist. If pre-CPB heart rate was above 85 beats per minute, and if the attending anesthesiologist thought at his/her discretion that adequate anesthesia and analgesia had been achieved, patients were treated with metoprolol intravenous prn.

Systolic blood pressure below 90 mmHg and/or a CI below 2.2 L/min/m², despite a PCWP range of 12-15 mmHg at the time of separation from CPB, was treated with dopamine or dobutamine (> 4 µg•kg⁻¹•min⁻¹), epinephrine or norepinephrine (> 0.04 µg•kg⁻¹•min⁻¹), alone or in combination with milrinone (0.25 to 0.75 µg•kg⁻¹•min⁻¹) at the discretion of the attending anesthesiologist. Inotropic support, as described in Table 8, exceeding 30 minutes in duration was considered clinically significant.

Table 8: Suggested dose regimen for attending anesthesiologist

<table>
<thead>
<tr>
<th>Inotrope</th>
<th>Starting dose</th>
<th>Dose range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine</td>
<td>0.5-2 mg•min⁻¹</td>
<td>0-8 mg•min⁻¹</td>
</tr>
<tr>
<td>Milrinone</td>
<td>0.125-0.25 mg•kg⁻¹•min⁻¹</td>
<td>0-0.75 mg•kg⁻¹•min⁻¹</td>
</tr>
<tr>
<td>Dobutamine</td>
<td>3.5-7.5 mg•kg⁻¹•min⁻¹</td>
<td>0-10 mg•kg⁻¹•min⁻¹</td>
</tr>
<tr>
<td></td>
<td>1.5-3.5 mg•kg⁻¹•min⁻¹</td>
<td>0-10 mg•kg⁻¹•min⁻¹</td>
</tr>
<tr>
<td>SVR &lt; 600</td>
<td>2-4 mg•min⁻¹</td>
<td>0-8 mg•min⁻¹</td>
</tr>
<tr>
<td>SVR &gt; 1200</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Milrinone</td>
<td>0.125-0.75 mg•kg⁻¹•min⁻¹</td>
<td></td>
</tr>
<tr>
<td>mPAP &gt; 25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NTG and/or milrinone</td>
<td>0.125-0.75 mg•kg⁻¹•min⁻¹</td>
<td></td>
</tr>
</tbody>
</table>

SVR = systemic vascular resistance; mPAP = mean pulmonary arterial pressure; NTG = nitroglycerine.

3.2.9 Sample size and statistical analysis.

Based on two of our previous studies [130, 256], we anticipated a one-tailed difference in whole blood propofol concentrations of 2.2 µg•mL⁻¹ between doses in study 1, with a standard deviation of 1.4 µg•mL⁻¹. The type 1 error rate was set at α = 0.05 and the power at 0.9.
Accordingly, we determined a minimum sample size of seven patients per group. At the midpoint of PRO-TECT II, 72 patients had been randomized, 30 to the propofol treatment arm. This comprises the sample available for analysis for Study 2; no formal sample size calculation was performed.

All data are reported and presented as the mean and standard deviation except for predicted values and constant of proportionality, which are described using 95% confidence intervals. Hemodynamic parameters from Part 1 were analyzed using a two-way repeated-measures analysis of variance. Bonferroni/Dunn post-tests for pair-wise comparisons of averages for doses across time were performed when the variance of the dose-time interaction reached a significance level of p<0.05. Hemodynamic parameters from Part 2 are presented descriptively. All analyses were performed using GraphPad Prism 4.0c software (San Diego, CA, USA).

3.3 Results and discussion

3.3.1 Patient and operative characteristics

Patient and operative characteristics according to experimental group are described in Table 9. Insufficient anesthesia, as evidenced clinically by elevated mean arterial pressure (exceeding 80 mmHg) and low mixed venous oxygenation (less than 65%) on CPB, was suspected by the attending anesthesiologist in three patients who had received a propofol infusion of 50 µg•kg⁻¹•min⁻¹ during CPB. These patients received supplemental isoflurane. Two patients receiving a propofol infusion of 150 µg•kg⁻¹•min⁻¹, and one receiving a propofol infusion of 100 µg•kg⁻¹•min⁻¹, were described as clinically unstable at separation from CPB. They required two or more inotropes, alone or in combination with norepinephrine, for hemodynamic stabilization prior to intensive care unit transfer.
# Table 9: Patient demographic and perioperative characteristics

<table>
<thead>
<tr>
<th></th>
<th>Experimental group</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 µg•kg⁻¹•min⁻¹</td>
</tr>
<tr>
<td>N</td>
<td>8</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>59±7</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71.0±17.0</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>162.5±8.5</td>
</tr>
<tr>
<td>BSA (m²)</td>
<td>1.78±0.23</td>
</tr>
<tr>
<td>Gender (m/f)</td>
<td>4:4</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>50±17</td>
</tr>
<tr>
<td>ACC (min)</td>
<td>65±29</td>
</tr>
<tr>
<td>CPB (min)</td>
<td>89±35</td>
</tr>
</tbody>
</table>

Data are expressed as mean± standard deviation or patient numbers. BSA = body surface area; LVEF = left ventricular ejection fraction; ACC = aortic cross-clamp interval; CPB = cardiopulmonary bypass interval.

## 3.3.2 Propofol concentrations in whole blood

**Part One**

The whole blood concentrations of propofol in patients treated with infusion rates of 50, 100, and 150 µg•kg⁻¹•min⁻¹ were 2.10 (1.20), 2.96 (1.87) and 14.28 (4.79) µg•mL⁻¹, respectively (Figure 7). The empirical line of best fit for the relationship between propofol concentrations and infusion rates was determined using non-linear curve fitting. According to an Akaike’s Information Criteria comparison, an exponential growth nonlinear model was preferred over the alternative power series model. The equation is described mathematically by

**Equation 21**

\[ y = a * e^{Kx} \]

where \( y \) represents the achieved whole blood concentration, the \( x \) represents the infusion rate, and \( a \) (0.215; 95%CI = -0.088 to 0.519) and \( K \) (0.0279; 95%CI = 0.0181 to 0.0376) are constants of
proportionality. The line had a coefficient of determination of \( r^2 = 0.781 \), and predicted that 113 \( \mu g \cdot kg^{-1} \cdot min^{-1} \) was required to achieve a mean concentration of 5 \( \mu g \cdot mL^{-1} \).

**Figure 7**: Propofol concentrations in whole blood at reperfusion during CABG-CPB in 24 patients receiving one of three infusion rates: 50, 100 or 150 \( \mu g \cdot kg^{-1} \cdot min^{-1} \).

The solid line represents the empirical line of best fit \( (r^2 = 0.781) \); the dotted line represents its 95% confidence interval. All concentrations were determined using capillary electrophoresis from 400 \( \mu L \) of whole blood sampled 15 min post-reperfusion during CABG.

**Part Two**

There were 4 study protocol violations; two patients received Propofol 2.0 \( mg \cdot kg^{-1} \) bolus
(propofol concentrations: 10.6, 11.0 µg•mL⁻¹); two additional patients received no loading dose (propofol concentrations: 2.6, 2.6 µg•ml⁻¹). Three cases had operative aortic crossclamp intervals below 60 minutes (propofol concentrations: 2.4, 4.0, 6.5 µg•mL⁻¹), thus intraoperatively violating study inclusion criteria. These seven patients were excluded from subsequent analysis.

The whole blood propofol concentration from the remaining 23 patients was 5.39 ± 1.45 µg•mL⁻¹, with a range of 2.60 to 7.54 µg•mL⁻¹. The 25%, 50%, and 75% quartiles were 4.36 µg•mL⁻¹, 5.63 µg•mL⁻¹, and 6.34 µg•mL⁻¹, respectively.

Propofol levels were 4.45 µg•mL⁻¹ (25 µM) or higher in 18/23 patients (78%), and above 5 µg•mL⁻¹ 15/23 patients (65%). Propofol concentrations showed no apparent correlation with patient age, weight, body surface area, or aortic cross-clamp duration in this series.

3.3.3 Intraoperative hemodynamic function

Intraoperative profiles such as CI, SVRI and LVSWI were compared in patients receiving propofol infusions at 50, 100, 150 µg•kg⁻¹•min⁻¹ along side those from patients receiving infusion at 120 µg•kg⁻¹•min⁻¹. We did not detect significant differences in the dose-time interaction for any of these hemodynamic parameters. The hemodynamic profiles were similar for patients in Study 1 and Study 2.

3.3.4 Principle findings and their implications

The current study describes conditions under which laboratory-based propofol mediated cardioprotection was translated to an experimental clinical maneuver. In order to minimize alterations to the operative procedure, and to facilitate clinical investigation, the method employed a loading bolus followed by constant infusion focused to the CPB interval. The primary research question relates to whether cardioprotective concentrations could be reliably achieved in vivo without undue risk of cardiac depression. The principle findings of this study are: (1) the constant infusion rate predicted to achieve a mean propofol concentration of 5 µg•mL⁻¹ in whole blood was 113 µg•kg⁻¹•min⁻¹; (2) the propofol concentration achieved with the nearest practical rate of 120 µg•kg⁻¹•min⁻¹, was 5.39 (1.45 µg•mL⁻¹), with quartiles of 25% = 4.36 µg•mL⁻¹; 50% = 5.63 µg•mL⁻¹; and 75% = 6.34 µg•mL⁻¹. Our model predicted a concentration of 6.10 (± 1.76) µg/ml at this infusion rate; (3) patient age, weight, body surface area, or aortic cross-clamp duration were not found to influence propofol concentration at reperfusion; (4) There was no evidence of depressed left ventricular function at emergence from CPB in patients receiving 120
µg•kg⁻¹•min⁻¹ propofol infusions during CPB.

The dosing groups in Part One of our study were partly modeled after a pharmacokinetic study, conducted in a nonsurgical setting, by Gepts et al [272]. The mathematical model we used represents an empirical means to fit our data in order to predict the infusion rate most likely to produce a given propofol concentration under similar operative and anesthetic conditions. As a result, a 1 mg•kg⁻¹ propofol bolus followed by a 120 µg•kg⁻¹•min⁻¹ continuous infusion was chosen for our PRO-TECT II protocol. Given its primary importance to tissue injury and repair, our sampling coincides with the early stage of reperfusion.

The method of drug application in our study produced a wide range of blood concentrations for a given infusion rate. This variability appears to be in line with that of several other studies where propofol concentrations were measured under similar operative conditions [248, 273-276]. It is clear that steady state conditions were not achieved, but it is also clear that steady state conditions for propofol cannot reasonably be anticipated within the context of cardiac surgery. We suggest that a significant reduction in the variance of propofol concentrations in the absence of steady state conditions will require monitoring of the concentration achieved during the course of surgery. By extension, drug level monitoring may be required to appropriately evaluate the role of propofol in cardioprotection, and its absence in experimental clinical studies makes interpretation of findings difficult.

We are satisfied that the experimental clinical maneuver derived in this study is capable of producing a propofol concentration associated with laboratory based cardioprotection. Indeed, the effect of increased propofol dosing to achieve the therapeutic concentration range associated with laboratory based propofol mediated cardioprotection (25-50 µM) was achieved clinically in 78% of patients in our study. The highest level we measured was approximately 7.5 µg•mL⁻¹ (45 µM) as seen in 17% of cases. This concentration is clinically and experimentally relevant, given these levels have been previously associated with the range expected to inhibit both lipid peroxidation [277] and mitochondrial permeability transition [259]. The absence of high drug levels among patients in study 2 suggests that a large dose of propofol, applied during CPB, has no detrimental effect on early post-bypass functional recovery relative to lower infusion rates. This contrasts with reports where total intravenous anesthesia with propofol and remifentanil was used for cardiac surgery [260].

We did not observe a decrease in cardiac index, consistent with our definition of cardiac
depression, upon emergence from CPB across dosing groups. By extension, elevated doses of propofol during cardioplegic arrest do not appear to increase the risk of cardiac instability on emergence from CPB. The benefit of this method with respect to clinical outcomes and cardioprotection cannot be extrapolated in the current study, and remains to be determined.

Patient characteristics of age, disease state, and weight have been identified as significant covariates that influence propofol pharmacokinetics. Their effect on data spread effects is likely to be amplified in non-steady state conditions. We did not find any systematic influence of these parameters on propofol concentrations at reperfusion, suggesting that non-steady state conditions and variability in total infused drug volume have a larger influence.

We used capillary electrophoresis to quantitatively analyze propofol in whole blood [271] (see Chapter 2). The separation is completed in less than 8 minutes, but the length of the preparative step still precludes its use for point of care target-achieved type dosing. Quantitative analysis that provides target-achieved drug infusion would likely facilitate perioperative care of high-risk patients. Technologies that enable target achieved dosing could then be adopted for routine use in studies designed to determine clinical outcomes.

There are limitations to the present study. Propofol concentrations were only measured in central venous blood collected at one time point, which limits any pharmacokinetic interpretations of the data. Central venous sampling was used for quantitative propofol analysis. Site-effect studies have confirmed that venous sampling is equally representative of arterial drug concentrations provided the infusion interval prior to sampling is longer than 20 minutes [246, 278-282]. Second, the mathematical model described in this study is inherently susceptible to changes in the anesthetic maneuver, and is incapable of predicting propofol concentrations in routine clinical practice, or beyond the infusion rates used in our study. In the absence of controls that omit propofol anesthesia, we are unable to attribute either the magnitude or the pattern of hemodynamic changes to the administration of propofol. The volume of propofol delivered in our study prior to sampling is entirely dependent on patient weight and crossclamp interval. Crossclamp intervals are neither consistent between surgical cases nor sufficient to establish near-steady state pharmacokinetic conditions [269, 272]. For these reasons, our line of best fit has no pharmacokinetic basis, its constants are not known to represent any physiological parameters, and there is no known basis for the apparent log-linear relationship between the infusion rate and the concentration that Equation 21 suggests. The current study focused on the intraoperative interval. Any patterns of hemodynamic performance are not known to extend to the postoperative period.
Finally, our hemodynamic findings are not known to apply to patients with severe ventricular dysfunction and profoundly low cardiac output, or to patients treated with drugs used to treat low cardiac output, such as milrinone.

3.4 Conclusion

The current study introduces an experimental clinical maneuver focused to the CPB interval, capable of yielding an elevated propofol concentration at reperfusion. In summary, the administration of a 1 mg·kg⁻¹ bolus dose of propofol followed by a continuous infusion of 120 µg·kg⁻¹·min⁻¹ during CPB produced relevant cardioprotective drug concentrations in whole blood at reperfusion. These concentrations were associated with an increase in cardiac index at emergence from CPB, in the absence of additional inotropic support. The achieved drug concentrations have previously been associated with enhanced red cell and tissue antioxidant capacity in vitro and in vivo [202, 256, 283], reduced dysfunction subsequent to experimental ischemia-reperfusion injury [264], and reduced endothelial and cardiomyoblast apoptosis [134, 216]. Failure to prevent cardiac injury with conventional propofol doses could be explained by inadequate concentrations and timing of administration. It remains to be determined if achieving a target concentration of 5 µg·mL⁻¹ will improve clinical outcomes (morbidity and mortality) in high-risk patient populations undergoing cardiac surgery.
4. Development and application of a LC-MS method with one-step pre-purification for quantitation of 15-F$_{2\alpha}$-isoprostane in human plasma

4.1 Introduction

As described in Chapter 1, 15-F$_{2\alpha}$-isoprostane, also know as 8-isoprostane, or 8-isoprostaglandin F$_{2\alpha}$, or 8-epi prostaglandin F$_{2\alpha}$, or iP$_{2\alpha}$-III, is a validated biomarker and gold standard for assessing oxidative stress.

A few analytical methods were developed to quantify the 15-F$_{2\alpha}$-isoprostane level in biological fluids. The analysis of 15-F$_{2\alpha}$-isoprostane was considered unreliable through the ELISA, which may overestimate of 15-F$_{2\alpha}$-isoprostane concentrations. Furthermore, ELISA results are not consistent with each other or with LC-MS/MS data. The use of mass spectrometry-based bioanalysis was advised [284]. MS-based methods GC-MS [49] [285] and LC-MS [286] [287] [288] [289] [290] have shown good specificity and sensitivity and are suitable for the analysis of 15-F$_{2\alpha}$-isoprostanes, which is at pg/mL level in the human plasma and can be complicated by the existence of many regioisomers, stereoisomers and prostaglandin-like structures.

Although GC-MS and LC-MS are generally more reliable to use, most of them require multiple steps for sample preparation. Recently, Sircar etc. reported a method combining affinity chromatography of 15-F$_{2\alpha}$-isoprostane and LC-MS [286]. This procedure involved only one step sample preparation and reduced the artifacts and interferences of other prostaglandin-like compounds.

They used acetic acid as modifier in the mobile phase, and each run took more than 45 min. To detect 15-F$_{2\alpha}$-isoprostane, the electron spray ionization was applied in negative mode. However, in my exploratory work, due to the lower negative ionization efficiency under acidic condition, acetic acid compromised the sensitivity of the analysis. Because I need to process a large number of patient samples, 45 min run doesn’t meet throughput need either.

Given the importance of 15-F$_{2\alpha}$-isoprostane as a prognostic biomarker in assessing oxidative stress, which attributes to the IRI, and the limitations imposed by available literature methods, the goals of this method development work are to 1) reproduce and improve reported LC-MS method for quantitation of 15-F$_{2\alpha}$-isoprostane; 2) shorten the analysis time and adapt his
developed method for moderate-throughput quantitation; 3) achieve sufficient sensitivity to determine 15-F_{2\alpha}-isoprostane concentration in patient plasma; 4) compare 15-F_{2\alpha}-isoprostane levels in different patient groups, treatment groups, and outcome groups; and 5) contrast 15-F_{2\alpha}-isoprostane concentration variation with other biological or clinical findings to cast light on future directions for cardioprotection, especially for high risk patient groups (e.g. diabetic patients).

4.2 Materials and methods

4.2.1 Instrument systems

The method development experiments and patient data acquisition were performed on a Hewlett Packard Series 1100 liquid chromatography system coupled with an Esquire-LC ion trap mass spectrometer (Bruker Daltonics, Leipzig, Germany). Data processing software DataAnalysis and QuantAnalysis are used for quantitative analysis. Prism and Excel were employed for statistical analysis.

4.2.2 Chemicals, reagents and consumables

15-F_{2\alpha}-isoprostane reference substance, internal standard 15-F_{2\alpha}-isoprostane-D₄ reference substance, 4 mL 8-isoprostane affinity column, 5x eicosanoid affinity column buffer (0.5 M phosphate solution, pH 7.4, containing 2.5 M sodium chloride and 0.25% sodium azide) and eicosanoid affinity column elution buffer (95% ethanol) were purchased from Cayman Chemical (Ann Arbor, MI, USA). HPLC grade acetonitrile and ACS grade concentrated ammonium hydroxide were purchased from Fisher Scientific (Nepean, ON, Canada). Ultrapure water was purchased from Cayman Chemical (Ann Arbor, MI, USA). Potassium hydroxide and potassium dihydrogen phosphate were purchased from Sigma Aldrich (Oakville, ON, Canada). Commercial pooled human plasma was purchased from Innovative Research (Novi, Michigan, USA).

4.2.3 Preparation of calibration standards

Initial stock solutions of 15-F_{2\alpha}-isoprostane and the internal standard (IS) 15-F_{2\alpha}-isoprostane-D₄ were both prepared in 15% acetonitrile in ultrapure water at a concentration of 100 ng•mL⁻¹ and stored in a freezer at -20 °C. The 100 ng•mL⁻¹ 15-F_{2\alpha}-isoprostane stock solution was then diluted with 2% acetonitrile in ultrapure water supplemented with 0.01% ammonium hydroxide to a series of desired concentrations: 100, 50, 20, 5, 2.5 and 1.25 ng•mL⁻¹ as calibration working solutions. To construct a calibration curve, 10µL of the calibration working standards and 4µL of the internal standard stock solution were spiked into 500µL of commercial pooled
human plasma before sample extraction and LC-MS analysis. Zero samples were prepared and included in the calibration curve by substituting only calibration working solution with ultrapure water. Blank samples were prepared and included in the calibration curve by substituting both calibration and internal standard working solutions with ultrapure water.

4.2.4 Patients and sampling

This investigation conformed to the principles outlined in the Declaration of Helsinki. Upon institutional approval and written informed consent, 92 patients enrolled for primary on-pump CABG surgery at Vancouver General Hospital were studied. Standardized anesthetic care and monitoring techniques were utilized (intra-arterial blood pressure monitoring, central venous and pulmonary artery catheterization, and transesophageal echocardiography). Subjects underwent intravenous anesthetic induction with fentanyl 10-15 $\mu$g•kg$^{-1}$, midazolam 0.15-0.25 mg•kg$^{-1}$, and sodium thiopental 1-2 mg•kg$^{-1}$ followed by muscle relaxation using rocuronium 1-1.5 mg•kg$^{-1}$ to enable tracheal intubation. Prior to CPB, anesthesia was maintained with 0.5 to 1.5 isoflurane for all patients. Subjects received phenylephrine (1-2 $\mu$g•kg$^{-1}$), fentanyl (1 to 2 $\mu$g•kg$^{-1}$), or vasodilator therapy (e.g., nitroglycerin 0.125 to 0.25 $\mu$g•kg•min$^{-1}$) to maintain their systolic and mean arterial blood pressures between 85 to 140 mmHg and 50 to 80 mmHg respectively. Subjects were given metoprolol if their pre-CPB heart rates exceeded 85 bpm. Tranexamic acid (0.05 mg•kg$^{-1}$ then 0.01 mg•kg$^{-1}$•h$^{-1}$) was used as an antifibrinolytic agent to reduce the risk of bleeding. Following a median sternotomy, the left and right internal mammary and radial arteries were dissected for grafts depending on the sites of coronary artery lesions. Subjects were given intermittent, antegrade blood cardioplegia during continuous aortic cross clamping. The temperature of the cardioplegia was left to surgical preference. Cardiopulmonary bypass was conducted at temperatures between 34 to 37 °C. Intraoperative hematocrit fraction was maintained between 0.25 and 0.27 during CPB. From ten minutes before CPB until fifteen minutes after CPB, subjects received either a 1 mg•kg$^{-1}$ propofol IV bolus followed by a propofol IV infusion at 120 $\mu$g•kg$^{-1}$•min$^{-1}$, or a 2.5% isoflurane bolus followed by 0.5–1.0% isoflurane. Two blood samples of 5 mL each were withdrawn from the coronary sinus 10 minutes before aorta clamping (pre-CPB sample) and 15 minutes after declamping (post-CPB sample). The blood samples were treated with potassium EDTA and centrifuged at 4000 rpm for 15min. Plasma was then separated from blood cells and stored in 1.25 mL aliquots at -80°C for subsequent analysis.
4.2.5 Sample preparation

To 0.5 mL freshly thawed plasma was spiked 400 pg (4 µL of 100 ng•mL⁻¹) internal standard. This spiked plasma sample was treated with 0.3 mL 15% potassium hydroxide followed by incubation at 40 °C for 1 h. The alkali solution was neutralized with 1 mL 1 M KH₂PO₄ to give a solution at pH 7.2 to 7.4.

The affinity column was conditioned with 2.5 mL 1x column buffer for two times. Upon the buffer passed through the affinity column by gravity, the neutralized sample was loaded onto the column, which was then washed with 5 mL 1x column buffer and 5 mL ultrapure water. The isoprostane analyte and internal standard were eluted with 2 mL 95% ethanol. The collected elute was reduced to dryness by SpeedVac. The residue was suspended in 30 µL 2% acetonitrile in water supplemented with 0.01% ammonium hydroxide.

4.2.6 Liquid chromatography

Mobile phase A was 0.01% ammonium hydroxide in ultrapure water while mobile phase B was 0.01% ammonium hydroxide in acetonitrile. The total run time, including column equilibration time was 25 minutes and comprised of 3 linear gradient components: from 0 to 12 min, 2 to 20%B; from 12 to 12.5 min, 20 to 2%B; from 12.5 to 25min, 2%B. A Gemini-NX 100x2.0 mm 3.0 µm C18 column from Phenomenex (Torrance, CA, USA) was used throughout the chromatographic separation. During the chromatographic separation, the C18 column was heated and the column temperature was kept at 40 °C. A C18 guard column from Phenomenex was installed to protect the analytical column all the time. For each run 20 µL of pre-purified sample was injected. Mobile phase flow rate was set at 0.2 mL•min⁻¹.

4.2.7 Mass spectrometry

Sensitivity was tuned in accordance with manufacturer’s recommendation by using direct infusion and auto-tune function of the software. The chosen conditions are as follows: capillary - 3900 V; end plate offset -500 V; nebulizer gas 20 psi; dry gas 10.00 L/min; dry temperature 360 °C; skim 1 25.0 V; skim 2 -5.0 V; cap exit offset -75.0 V; octopole -1.90 V; octopole A -1.90 V; oct RF 120.00; lens 1 5.0 V; lens 2 45.0 V; trap drive 35.5; detector +1700 V; dynode +7.0 V; skimmer 1 -100.0 V; slimmer 2 -300.0 V; max accumulation time 20 ms. Precursor ion scan mode was used to monitor m/z 350.0 to 360.0. Extracted ion chromatograms for m/z 353.1 (and m/z 357.1 were used for post-run data processing. Channel m/z 353.1 corresponds to [M-1] of
15-F_{2\tau}-isoprostane and channel m/z 357.1 corresponds to [M-1] of 15-F_{2\tau}-isoprostane-D$_4$.

4.3 Results and discussion

4.3.1 Mass spectrometer parameters

The compound-dependent MS parameters (e.g. skimmer voltage, cap exit offset, octopole voltage and lens voltage) were optimized for maximum sensitivity by infusing a 100 ng•mL$^{-1}$ 15-F$_{2\tau}$-isoprostane standard solution at constant volumetric flow rate via a syringe pump. The source-dependent MS parameters (e.g. sprayer capillary position and voltage, nebulizer pressure, and dry gas flow rate and temperature) were optimized for maximum sensitivity by flow injecting 20 µL of a 100 ng•mL$^{-1}$ 15-F$_{2\tau}$-isoprostane standard solution via a post-column Rheodyne valve. All the MS parameters were altered systematically to achieve best detection sensitivity.

4.3.2 Mobile phase and liquid chromatography

Mobile phase containing 0.01% ammonium hydroxide was compared with mobile phase containing acetic acid and modifier-free mobile phase with regard to throughput and detection sensitivity. With the same gradient program, mobile phase containing acetic acid and modifier-free mobile phase resulted in analyte peaks appearing at a retention time of 34.0 min and 23.3 min respectively. Mobile phase containing 0.01% ammonium hydroxide gave the shortest retention time (14.5 min) and better sensitivity. To separate the analyte from other interfering substances, I started with a mobile phase containing low percentage of organic solvent (2% ACN). 15-F$_{2\tau}$-isoprostane is hydrophobic compound bearing acidic functionality. Under basic condition, this compound is mostly ionized in liquid phase prior to entering gaseous phase, giving rise to less retention time and better sensitivity.

A big concern, though, is that basic condition is in fact prone to deteriorate the C18 packing material of the chromatographic column. One way to avoid this damage is to keep pH lower than 10. Another way to avoid this damage is to choose appropriate column that is resistant to pH-dependent column deterioration. 0.01% ammonium hydroxide gives a pH at 9.60, within the stability range (pH 1 to 12) of the selected Gemini-NX C18 column.

Using the conditions depicted in Section 4.2.5 Sample preparation, 4.2.6 Liquid chromatography and 4.2.7 Mass spectrometry, we obtained the representative chromatograms of plasma samples with or without spiked 15-F$_{2\tau}$-isoprostane (Figure 8).
Figure 8: Representative chromatograms of plasma samples without (Panel A) or with (Panel B) 100 pg•mL⁻¹ over-spiked 15-F₂t-isoprostane

Methods and conditions applied for the analyses are described in 4.2.5 Sample preparation, 4.2.6 Liquid chromatography and 4.2.7 Mass spectrometry.

4.3.3 Affinity column purification

I have adopted the method first reported by Sircar et al to purify 15-F₂t-isoprostane prior to LC-MS analysis [286]. After each purification, the affinity column was rinsed with 2.5 mL 95% ethanol, 5 mL ultrapure water and 5 mL 1x column buffer successively. This washing sequence may be used to regenerate the affinity column and allow three purifications in total. The affinity column should be stored at 4 °C with 2 mL of column buffer remaining and never let dry.

4.3.4 Linearity

The estimated total 15-F₂t-isoprostane (a sum of free and esterified 15-F₂t-isoprostane) concentration was in the range of 30-400 pg•mL⁻¹ in human plasma according to literature reports [49, 285, 286, 291-293]. Therefore, the linearity of this method was assessed by over-spiking 15-
F₂₉-isoprostane at concentrations between 0 and 1000 pg•mL⁻¹ (seven point validation). The peak area ratios of 15-F₂₉-isoprostane over internal standard were plotted against over-spiked 15-F₂₉-isoprostane concentrations. This calibration curve has a goodness of fit of $r^2 = 0.9991$ across the tested range, with the equation: $y = 1.4418x + 0.9972$. I found that the commercial plasma level is between 200 pg•mL⁻¹ to 300 pg•mL⁻¹, consistent with what is reported in the literature.

4.3.5 Precision

Precision was assessed by over-spiking four clinically relevant concentrations of 15-F₂₉-isoprostane (1000, 100, 50 and 25 pg•mL⁻¹) into the commercial human plasma, followed by affinity column extraction and LC-MS analysis in negative ESI mode. Intraday precision was determined by using five individually prepared plasma samples over-spiked with known amount of 15-F₂₉-isoprostane standard and internal standard 15-F₂₉-isoprostane-D₄ during the same day. Interday precision was determined by using six individually prepared over-spiked plasma samples during three consecutive days in duplicates. Intraday precision and interday precision are intended to meet the FDA Guidance for Industry Analytical Method Validation. The precision determined at each concentration level should not exceed 15% of relative standard deviation (RSD) or coefficient of variation (CV). Our determined values conform to the FDA requirements for analytical method validation, with percentage of RSD below 8.0% in all cases (Table 10).

Table 10: Intraday and interday precision parameters of the LC-MS method for 15-F₂₉-isoprostane quantitation

<table>
<thead>
<tr>
<th>Conc (pg•mL⁻¹)</th>
<th>Average Ratio</th>
<th>SD</th>
<th>%RSD</th>
<th>No. of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraday</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>2.793</td>
<td>0.03448</td>
<td>1.23</td>
<td>5</td>
</tr>
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<td>5</td>
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<td>1.099</td>
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<td>2.82</td>
<td>5</td>
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<td>1.050</td>
<td>0.03353</td>
<td>3.19</td>
<td>5</td>
</tr>
<tr>
<td>Interday</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>2.836</td>
<td>0.21784</td>
<td>7.68</td>
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</tr>
<tr>
<td>100</td>
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<td>0.04080</td>
<td>3.44</td>
<td>6</td>
</tr>
<tr>
<td>50</td>
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<td>0.02359</td>
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<td>25</td>
<td>1.053</td>
<td>0.02615</td>
<td>2.48</td>
<td>6</td>
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</tbody>
</table>

All samples were prepared by individually over-spiking 15-F₂₉-isoprostane standard solution and internal standard 15-F₂₉-isoprostane-D₄ into commercial human plasma followed by one-step
affinity column purification and LC-MS analysis. Five samples at each concentration prepared in the same day were used to assess the intraday precision. Two samples at each concentration prepared for three consecutive days were used to assess the interday precision.

4.3.6 Accuracy

The accuracy of an analytical method is defined to be the closeness of mean concentration determined by the analytical method to the true concentration of the analyte. Accuracy of this method was assessed by testing four concentrations in the range of expected 15-F_2t-isoprostane basal concentration and using five to six samples per concentration. According to the FDA Guidance for Industry Analytical Method Validation, the mean value should be within 15% of the true value at tested concentrations. Our calculated concentrations are reasonably close to the known concentrations, with relative error or inaccuracy no greater than 13% when the spiked concentration is above 50 pg·mL\(^{-1}\) and no greater than 25% when the spiked concentration is at 25 pg·mL\(^{-1}\) (Table 11).

Table 11: Intraday and interday accuracy parameters of the LC-MS method for 15-F_2t-isoprostane quantitation

<table>
<thead>
<tr>
<th></th>
<th>Conc (pg·mL(^{-1}))</th>
<th>Conc Cal Aver (pg·mL(^{-1}))</th>
<th>%RE</th>
<th>No. of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intraday</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>1000</td>
<td>996.49</td>
<td>-0.35</td>
<td>5</td>
</tr>
<tr>
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<td>100</td>
<td>102.89</td>
<td>2.89</td>
<td>5</td>
</tr>
<tr>
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<td>50</td>
<td>56.31</td>
<td>12.62</td>
<td>5</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>29.41</td>
<td>17.64</td>
<td>5</td>
</tr>
<tr>
<td><strong>Interday</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>1000</td>
<td>1020.06</td>
<td>2.01</td>
<td>6</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>105.38</td>
<td>5.38</td>
<td>6</td>
</tr>
<tr>
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<td>50</td>
<td>56.44</td>
<td>12.88</td>
<td>6</td>
</tr>
<tr>
<td>25</td>
<td>25</td>
<td>30.95</td>
<td>23.79</td>
<td>6</td>
</tr>
</tbody>
</table>

Methods of sample preparation and analysis as in Table 10. Calculated concentration is derived by using: [Mean Cal Conc] (pg·mL\(^{-1}\)) = (Mean Peak Area Ratio – a)/b, where a is the y-intercept and b is the slope of the calibration curve. Relative error is derived by using equation: Relative Error % = ([Mean Cal Conc] – [True Conc])/[True Conc] * 100%.
4.3.7 LOD, LOQ and LLOQ

To obtain LOD and LOQ applicable to human plasma matrix and take into account every step in sample pre-treatment and analysis. We first measured the peak areas of 15-F_{2t}-isoprostane and its internal standard 15-F_{2t}-isoprostane-D_4 in the zero sample. We then used the slope from the calibration curve to translate the peak area ratio of 15-F_{2t}-isoprostane over internal standard to endogenous 15-F_{2t}-isoprostane concentration. From the signal to noise ratio at this endogenous concentration we determined the LOD and LOQ of this method were 3.06 pg•mL\(^{-1}\) and 10.21 pg•mL\(^{-1}\) respectively.

LLOQ is defined to be a concentration at which the analyte peak is identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120%. Experimentally, we proved the LLOQ was between 25 and 50 pg•mL\(^{-1}\) since at 50 pg•mL\(^{-1}\) level precision and accuracy were tested to be acceptable (<15%) but at 25 pg•mL\(^{-1}\) level accuracy was out of 15% range.

4.3.8 Elevation of 15-F_{2t}-isoprostane level upon ischemia reperfusion

15-F_{2t}-isoprostane levels before and after cardiopulmonary bypass were compared (Figure 9). The significant elevation of 15-F_{2t}-isoprostane level in coronary sinus plasma supports that ischemia reperfusion procedure during cardiopulmonary bypass is accompanied with a substantial degree of oxidative stress. This is consistent with other literature reports [143-145]. However, no statistical difference of 15-F_{2t}-isoprostane levels was found between treatment groups (propofol versus isoflurane), outcome groups (low cardiac output syndrome versus non-low cardiac output syndrome) and patient groups (diabetics versus non-diabetics).
Figure 9: 15-F2\textsubscript{t}-isoprostane changes over the course of ischemia reperfusion during cardiopulmonary bypass surgery

Patient plasma LC-MS analysis revealed that 15-F2\textsubscript{t}-isoprostane concentration increased significantly after CABG surgery. No treatment effect (propofol versus isoflurane) on 15-F2\textsubscript{t}-isoprostane elevation has been noticed. Nor is there any correlation of outcome or diabetic condition with degree of increment of 15-F2\textsubscript{t}-isoprostane level.

4.3.9 15-F2\textsubscript{t}-isoprostane baseline level in relation to patient diabetic conditions and apoptosis pathway

Hyperglycaemia-induced reactive oxygen or nitrogen species generation could upregulate PTEN antagonism of Akt [214, 215]. To investigate whether diabetic patients presenting for CABG surgery had increased PTEN level, and to find evidence of its relationship with plasma 15-F2\textsubscript{t}-isoprostane levels, we evaluated PTEN expression by Western blot and performed a correlation analysis between PTEN and the 15-F2\textsubscript{t}-isoprostane levels using linear regression.
The baseline concentration of 15-F$_{2\alpha}$-isoprostane was significantly greater in coronary sinus plasma from diabetic patients compared with nondiabetic patients (Figure 10 Panel A). Correlation analysis between 15-F$_{2\alpha}$-isoprostane and Fasting Blood Glucose levels was performed using linear regression. This analysis revealed a significant positive correlation ($p = 0.02$, $r = 0.3869$) (Figure 10 Panel B). As shown in Figure 10 Panel C, biopsies from diabetic patients had significantly higher PTEN protein expression than those from nondiabetic patients (nondiabetic: 0.50 ± 0.50, diabetic: 1.01 ± 0.64, $p = 0.005$, Mann-Whitney test). In addition, the linear regression analysis revealed a significant positive correlation between PTEN/β-actin and 15-F$_{2\alpha}$-isoprostane levels ($p = 0.008$, $r = 0.4445$) with a slope of 0.007 (95% CI: 0.002-0.001) (Figure 10 Panel D).
Figure 10: Correlation of 15-F_2t-isoprostane level with patient baseline diabetic profile

15-F_2t-isoprostane level is higher in diabetic patients than non-diabetic patients (Panel A). The correlation between 15-F_2t-isoprostane level and fasting blood glucose concentration in clinical subjects is statistically significant (p = 0.02) (Panel B). PTEN/β-actin ratio is higher in diabetic patients than non-diabetic patients (Panel C). The correlation between PTEN/β-actin ratio and 15-F_2t-isoprostane level is statistically significant (p = 0.008) (Panel D).

Oxidative stress is a hallmark of type 2 diabetes. 15-F_2t-isoprostane is surrogate biomarker of oxidative stress in type 2 diabetes [294]. The level of 15-F_2t-isoprostane in coronary sinus blood reflects the amount of reactive oxygen species in coronary circulation. In this study,
the baseline level of plasma 15-F_{2\alpha}-isoprostone in coronary sinus blood was significantly higher in patients presenting type 2 diabetes (Figure 10 Panel A). Acute hyperglycaemia stimulates isoprostone generation and decreases plasma antioxidant defenses [293, 295]. The level of isoprostone is reported to vary with the degree of glucose fluctuation, even in the absence of an increase in HbA1c in healthy patients or those with type 2 diabetes [296]. This is consistent with our present findings, which demonstrated a significant correlation between fast blood glucose (FBG) and coronary 15-F_{2\alpha}-isoprostone level (Figure 10 Panel B).

PTEN is a dual protein lipid phosphatase that dephosphorylates the secondary messenger produced by PI3K and interrupts the downstream activation of Akt. It plays a significant role in regulating the balance between survival and death in many cell types, including cardiomyocytes. Indeed, PTEN is associated with a reduction in preconditioning efficacy [297], and its inactivation may increase myocardial survival following an ischaemic episode and ischaemia-reperfusion injury [298]. Mocanu et al. reported an increased PTEN level in the Goto-Kakizaki rat heart model and suggested diabetes may be associated with increased PTEN level, at least in the myocardium [297]. We detected PTEN expression was significantly higher in atrial tissue from patients with type 2 diabetes (Figure 10 Panel C), supporting their finding. Chronic pathological reactive oxygen species production induces PTEN expression [213, 214]. In our patient series, increased FBG levels correlated with increased levels of 15-F_{2\alpha}-isoprostone in coronary sinus blood (Figure 10 Panel B). Increased cardiac 15-F_{2\alpha}-isoprostone generation was highly correlated with PTEN in human diabetic myocardium (Figure 10 Panel D), indicative of isoprostone’s potential role in its generation.

PTEN negatively regulates insulin signaling via action on Akt. Suppression of PTEN has been shown to restore glycaemic control [299]. PTEN is modulated during myocardial ischaemia and reperfusion, and its loss is involved in the induction of the ischaemic preconditioning stimulus, via the transient upregulation of Akt at reperfusion [300]. Chronic Akt activation may be maladaptive and associated with cardiac dysfunction and failure, but its acute enhancement is associated with cyto- and cardioprotection and improved contractility [301]. The correlation of this PTEN-Akt signaling transduction pathway with 15-F_{2\alpha}-isoprostone provides additional evidence that oxidative stress may antagonize the salvation of myocytes and suggests the prognostic value of 15-F_{2\alpha}-isoprostone in predicting IRI and patient clinical outcome.
4.4 Conclusion

In conclusion, we have developed a simple, sensitive, selective, and fast LC-MS method for 15-F₂tı-isoprostane determinations in plasma samples of patients receiving on-pump CABG surgery. From this study, it appears that oxidative stress might play a role in triggering apoptosis through PTEN-Akt pathway. Antioxidative therapy lowering the generation of 15-F₂tı-isoprostance may improve the survival of myocytes via the suppression of PTEN and activation of Akt-mediated salvaging mechanism.
5. Development and application of a simple and robust LC-MS/MS method for quantitation of free 3-nitrotyrosine in human plasma

5.1 Introduction

Despite the biological importance of 3-nitrotyrosine, a simple, robust, reliable, sensitive and selective method has yet to be developed for large-scale evaluation of clinical samples. Several analytical methods have been developed for free 3-nitrotyrosine determination, including liquid chromatography using ultraviolet, fluorescence and electrochemical detectors, GC-MS/MS, and LC-MS/MS methods [302]. Among these methods, liquid chromatography with ultraviolet [157], fluorescence [303], and electrochemical detection [304, 305] do not typically provide sufficient selectivity. GC-MS/MS methods [306, 307] often require sample derivatization and may be error-prone [308]. The available LC-MS/MS methods either could not identify a detectable plasma 3-nitrotyrosine signal [308, 309] and is limited to exhaled breath condensate samples [159, 310, 311], or required a significant amount of time for each individual run [312, 313]. There are two additional challenges in 3-nitrotyrosine analysis: 1) the physiological concentration of 3-nitrotyrosine is found at the nanomolar range [302]; 2) strong acid treatment of plasma samples should be avoided because 3-nitrotyrosine artifacts might be generated by reacting nitrite or nitrate with tyrosine under acidic conditions [308, 309].

Here we have developed a simple, robust and reliable LC-MS/MS method to evaluate 3-nitrotyrosine levels in patients receiving on-pump CABG surgery in order to further test the utility of 3-nitrotyrosine in assessing nitrosative stress. This method has been systematically developed to maximize recovery and sensitivity.

5.2 Materials and methods

5.2.1 Instrument and software

All experiments were performed on a Varian 1200L LC-MS/MS system (Agilent Technologies, Palo Alto, CA, USA) with a ProStar 210 Dynamax solvent delivery system and a triple quadrupole mass analyzer. Varian Mass Spectrometry Workstation Version 6 was employed to control the LC-MS/MS instrument, as well as acquire the spectral and chromatographic information. Excel and Prism were used for statistical analysis.
5.2.2 Chemicals, reagents and consumables

HPLC grade acetonitrile, HPLC grade methanol, ACS grade acetic acid and 88% formic acid were purchased from Fisher Scientific (Nepean, ON, Canada). Ultrapure water was purchased from Cayman Chemical (Ann Arbor, MI, USA). 3-Nitro-L-tyrosine standard (>98.0%) was purchased from Acros (Morris Plains, NJ, USA). O-methyl-L-tyrosine standard (>98.0%), and sodium nitrate (>99.0%) from Sigma Aldrich (Oakville, ON, Canada); Sep-Pak Vac C18 Cartridges (3 mL, 500 mg, Part No. WAT020805), Oasis HLB Cartridge 6 mL (500 mg) 60 µm (Part No. 186000115) and Oasis HLB cartridge 6 mL (150 mg) 30 µm (Part No. 186003365) were purchased from Waters Inc. (Milford, MA, USA). SPEC SCX column, 3 ml, with monolithic solid phase extraction disk (Cat. No. A5310420) was purchased from Varian Inc. (Palo Alto, CA, USA). Ultrafree Durapore PVDF 0.1 µm centrifugal filters (Cat. No. UFC30VV00) were purchased from Millipore (Billerica, MA, USA). Sodium nitrite (>99.0%) was from VWR (Edmonton, AB, Canada). Commercial pooled human plasma was from Innovative Research (Novi, Michigan, USA). The SAX (strong anion exchange) cartridge (Cat. No. SA-020-3) was a gift from PromoChrom Technologies (Richmond, BC, Canada).

5.2.3 Preparation of calibration standards

Initial stock solutions of 3-nitro-L-tyrosine and the internal standard (IS) O-methyl-L-tyrosine were both prepared in 1:1 ultrapure water/acetonitrile at a concentration of 1 mM and stored in a refrigerator. The 1 mM 3-nitrotyrosine stock solution was then diluted with ultrapure water to a series of desired concentrations: 400, 200, 100, 50, 25, and 12.5 nM as calibration working solutions. Internal standard stock solution was diluted with ultrapure water to give a 200 nM working solution. The internal standard working solution was dispensed in 1 mL aliquots to Eppendorf tubes and stored under -20 °C. To construct a calibration curve, 50 µL of the calibration working standards and 50 µL of the internal standard stock solution were spiked into 900 µL of commercial pooled human plasma before sample exaction and LC-MS/MS analysis. Zero samples were prepared and included in the calibration curve by substituting only calibration working solution with ultrapure water. Blank samples were prepared and included in the calibration curve by substituting both calibration and internal standard working solutions with ultrapure water.
This investigation conformed to the principles outlined in the Declaration of Helsinki [314]. Upon institutional approval and written informed consent, 45 patients enrolled for primary on-pump CABG surgery at Vancouver General Hospital were studied [231]. Standardized anesthetic care and monitoring techniques were utilized (intra-arterial blood pressure monitoring, central venous and pulmonary artery catheterization, and transesophageal echocardiography). Subjects underwent intravenous anesthetic induction with fentanyl 10-15 µg•kg⁻¹, midazolam 0.15-0.25 mg•kg⁻¹, and sodium thiopental 1-2 mg•kg⁻¹ followed by muscle relaxation using rocuronium 1-1.5 mg•kg⁻¹ to enable tracheal intubation. Prior to CPB, anesthesia was maintained with 0.5 to 1.5 isoflurane for all patients. Subjects received phenylephrine (1 to 2 µg•kg⁻¹), fentanyl (1 to 2 µg•kg⁻¹), or vasodilator therapy (e.g., nitroglycerin 0.125 to 0.25 µg•kg⁻¹•min⁻¹) to maintain their systolic and mean arterial blood pressures between 85 to 140 mmHg and 50 to 80 mmHg respectively. Subjects were given metoprolol if their pre-CPB heart rates exceeded 85 bpm. Tranexamic acid (0.05 mg•kg⁻¹ then 0.01 mg•kg⁻¹•h⁻¹) was used as an antifibrinolytic agent to reduce the risk of bleeding. Following a median sternotomy, the left and right internal mammary and radial arteries were dissected for grafts depending on the sites of coronary artery lesions. Subjects were given intermittent, antegrade blood cardioplegia during continuous aortic cross clamping. The temperature of the cardioplegia was left to surgical preference. Cardiopulmonary bypass was conducted at temperatures between 34 to 37 °C. Intraoperative hematocrit fraction was maintained between 0.25 and 0.27 during CPB. From ten minutes before CPB until fifteen minutes after CPB, subjects received either a 1 mg•kg⁻¹ propofol IV bolus followed by a propofol IV infusion at 120 µg•kg•min⁻¹, or a 2.5% isoflurane bolus followed by 0.5–1.0% isoflurane. Two blood samples of 5 mL each were withdrawn from the coronary sinus 10 minutes before aorta clamping (pre-CPB sample) and 15 minutes after declamping (post-CPB sample). The blood samples were treated with potassium EDTA and centrifuged at 4000 rpm for 15 min. Plasma was then separated from blood cells and stored in 1.25 mL aliquots at -80 °C for subsequent analysis.

5.2.5 Sample Preparation

Each of the 900 µL patient plasma samples was spiked with 50 µL of 200 nM IS working solution and 50 µL of water. A solid phase extraction procedure was used to process the human plasma samples including calibration samples and patient samples, which had been all spiked...
with IS. No acid or heat treatments were performed to avoid the generation of 3-nitrotyrosine artifacts.

The SPE procedure using C18 cartridge is as follows. The cartridge was 1) rinsed with 5 mL of 1% formic acid in methanol; 2) rinsed with 5 mL of 1% formic acid in ultrapure water; 3) loaded with 1 mL IS spiked plasma samples; 4) washed with 1 mL 1% formic acid in water then 1 mL water; and 5) eluted with 1 mL 50% ACN in water. Positive air pressure was used to enable faster cartridge wash or elution in each step.

The eluted solution was concentrated by SpeedVac and the residue was re-suspended in 60 µL of 2% acetonitrile aqueous solution. The sample was filtered through Millipore Durapore PVDF 0.1 µm centrifugal filters and the filtrate was submitted to LC-MS/MS analysis.

5.2.6 Liquid chromatography

During method development, the following columns were screened: Altima HP 100x2.1 mm, 3 µm C18 column from Grace (Deerfield, IL, USA) and Kinetex 100x2.1 mm, 2.6 µm XB-C18 column from Phenomenex (Torrance, CA, USA).

During method validation, the following conditions were consistently used. Mobile phase A was 0.01% acetic acid in ultrapure water while mobile phase B was 0.01% acetic acid in acetonitrile. The total run time, including column equilibration time was 20 minutes and comprised of 5 linear gradient components: from 0 to 9.5 min, 2 to 20%B; from 9.5 to 12 min, 20 to 90%B; from 12 to 15 min, 90%B; from 15 to 15.5 min, 90 to 2%B; from 15.5 to 20 min, 2%B. A Kinetex 100x2.1 mm 2.6 µm PFP column from Phenomenex (Torrance, CA, USA) was used throughout the chromatographic separation. A Krudkatcher inline filter was installed to protect the analytical column all the time. For each run 10 µL of pre-purified sample was injected. Mobile phase flow rate was at 0.2 mL·min⁻¹.

5.2.7 Tandem mass spectrometry

Sensitivity was tuned in accordance with manufacturer’s recommendation. The chosen conditions are as follows: needle voltage was at 4500 V; shield at 400 V; capillary at 40 V; nitrogen as drying gas at 300 °C and 21 psi; nitrogen as nebulizer at 50 psi; electromultiplier at 1700 V; collision energy at -12.0 V for 3-nitrotyrosine and -7.0 V for internal standard; ultrapure
argon as collision gas at 2.00 mTorr. Multiple reaction monitoring (MRM) was used to monitor 3-nitrotyrosine (m/z 227.1->181.1) and O-methyl-tyrosine (m/z 196.0->179.0).

5.3 Results and discussion

5.3.1 Mass spectrometer parameters

All MS parameters were adjusted to achieve optimal sensitivity according to manufacturer’s recommendation. The optimal conditions are listed in Section 5.2.7 Tandem mass spectrometry. The product ion spectra of 3-nitrotyrosine and O-methyltyrosine were acquired through selecting only specific precursor ions (m/z at 227.1 and 196.0) by filtering them in the first quadrupole, fragmenting them through collisional induced dissociation in the second quadrupole, and scanning the product ions (m/z from 50 to 250) in the third quadrupole. Base peaks after gas collision by ultrapure argon at 2.00 mTorr for 3-nitrotyrosine and O-methyltyrosine were m/z 181.1 and 179.0, respectively, corresponding to neutral loss of HCOOH or NH₃ during fragmentation (Figure 11).

Figure 11: Product ion spectra of 3-nitrotyrosine (Panel A) and O-methyl-tyrosine (Panel B)

*Base peaks were identified to be m/z 181.1 and 179.0 for 3-nitrotyrosine and O-methyl-tyrosine, respectively.*
5.3.2 Mobile phase

Mobile phases containing different concentrations of acetic acid were compared. As shown in Figure 12, a 0.01% concentration of acetic acid was superior to 0.1% and to mobile phase containing no acetic acid in terms of signal intensity and signal to noise ratio. In order to avoid ion suppression and matrix effect, we chose to start the gradient with very low percentage of organic solvent (2% ACN). Relative long elution time (~6.7 min) allows the analyte to reach the ESI interface much later than solvent front carrying matrix ions (~1 min). To prevent contamination of mass spectrometer, the first two minutes of elution was diverted to waste.

![Figure 12: Comparison of mobile phases containing acetic acid at different concentrations](image)

Plasma samples over-spiked with 10 nM 3-nitrotyrosine and prepared following Section 5.3.3 Solid phase extraction (SPE)

5.3.3 Solid phase e xtraction (SPE) used. Mobile phase pairs used for chromatogram A, B, C were pure water and acetonitrile (Panel A); 0.1% acetic acid in water and 0.1% acetic acid in acetonitrile (Panel B); 0.01% acetic acid in water and 0.01% acetic acid in acetonitrile (Panel C) respectively. Altima HP 100x2.1 mm 3 µm C18 column was used for mobile phase optimization.

5.3.3 Solid phase e xtraction (SPE)

In the beginning of method development, we examined several types of SPE cartridges. Anion exchange cartridges and cation exchange cartridges did not provide consistent and acceptable recoveries for plasma samples. In contrast, both large size HLB and C18 cartridges
were capable of trapping 3-nitrotyrosine and facilitating consistent recoveries. However, further comparison suggested that under the same condition, HLB cartridges gave only 32.4% and 89.7% of recoveries as C18 cartridges did for 3-nitrotyrosine and IS, respectively.

Comparing the untreated neutral and formic acid treated C18 cartridges indicated that acidification of the cartridge was necessary for retaining 3-nitrotyrosine during sample loading and pre-wash, because only 9.4% of 3-nitrotyrosine can be recovered using neutral cartridge compared to that using acidified cartridge. Further, a first wash with 10% acetonitrile carries away 3-nitrotyrosine pre-maturely and results in more than 15% loss of recovery. In order to minimize the loss of analyte, water in place of 10% acetonitrile was used in the pre-wash step. The overall absolute recovery of 3-nitrotyrosine at 1.25 nM level by using method described in Section 5.3.3 Solid phase extraction (SPE) was 96.3 ± 4.0% (n = 6). This excellent recovery is also a suggestion that ion suppression or matrix effect is negligible.

There were no heat or strong acid involved in any of the sample preparation steps to prevent the formation of 3-nitrotyrosine artifacts. Indeed, we did not observe any notable change of 3-nitrotyrosine signal intensity after over-spiking 10 µM of nitrate and nitrite solution into the plasma samples prior to sample preparation.

### 5.3.4 Analytical column selection

Several columns have been examined for performance in terms of sensitivity and selectivity. The column performances of Kinetex columns (C18 and PFP columns) were much better than that of Altima HP column for this application, with almost three times increase in peak height and three to five times increase in signal to noise ratio (Figure 13). Kinetex columns are packed with core-shell particles that lead to smaller band broadening due to uniform particle size and shape as well as increased column efficiency enabled by faster mass transfer.
Figure 13: Performance comparison of different columns

Chromatographic conditions: same as in Section 5.2.6 Liquid Chromatography. One 1 μM 3-nitrotyrosine and 1 μM internal standard in water were used as testing samples. Column for Panel A: Kinetex 100x2.1 mm 2.6 μm XB-C18 column from Phenomenex; column for Panel B: Kinetex 100x2.1 mm 2.6 μm PFP column from Phenomenex; column for Panel C: Altima HP 100x2.1 mm 3 μm C18 column.

The Kinetex C18 column was superior to PFP column with respect to sensitivity enhancement and was initially chosen to analyze patient samples. However, over continued use the backpressure increased and retention times shifted significantly after 10 to 20 consecutive runs. We suspect that it is due to the incompatibility of our sample preparation method and this type of column.

Switching to Kinetex PFP column resolved the high backpressure and inconsistent retention time problems completely. We were able to run over 80 real patient and QC samples on the PFP column without any increase of backpressure, quantitation parameters or significant shift of retention times. The separation by PFP column is based on a combination of hydrophobicity, hydrophilicity, and aromaticity of an analyte, and the separation mechanism is orthogonal to the sample preparation based on C18 cartridge and may improve selectivity in theory. In fact, we noticed less interference of 3-nitrotyrosine peaks during real sample analyses (e.g. comparing Figure 12 and Figure 13). Kinetex PFP column was eventually selected for method validation.

5.3.5 Linearity

The estimated basal 3-nitrotyrosine concentration was in the range of 1-10 nM in human plasma according to literature reports [302, 312, 313]. Therefore, the linearity of this method was
assessed by over-spiking 3-nitrotyrosine at concentrations between 0 and 20 nM (seven point validation). The peak area ratios of 3-nitrotyrosine over internal standard were plotted against over-spiked 3-nitrotyrosine concentrations. This calibration curve has a goodness of fit of $r^2 = 0.9972$ across the tested range, with the equation: $y = 0.0104x + 0.0101$.

5.3.6 Precision

Precision was assessed by over-spiking four clinically relevant concentrations of 3-nitrotyrosine (10, 2.5, 1.25 and 0.625 nM) into the commercial human plasma, followed by C18 solid phase extraction and LC-MS/MS analysis. Intraday precision was determined by using five individually prepared plasma samples over-spiked with known amount of 3-nitrotyrosine and internal standard during the same day. Interday precision was determined by using six individually prepared over-spiked plasma samples during three consecutive days in duplicates. Intraday precision and interday precision are intended to meet the FDA Guidance for Industry Analytical Method Validation. The precision determined at each concentration level should not exceed 15% of relative standard deviation (RSD) or coefficient of variation (CV) (Table 12). Our determined values conform to the FDA requirements for analytical method validation, with percentage of RSD below 6.30% in all cases.

Table 12: Intraday and interday precision parameters of the LC-MS/MS method for 3-nitrotyrosine quantitation

<table>
<thead>
<tr>
<th>Conc (nM)</th>
<th>Mean Peak Area Ratio</th>
<th>SD</th>
<th>%RSD</th>
<th>No. of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraday</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>0.112</td>
<td>0.00427</td>
<td>3.8</td>
<td>5</td>
</tr>
<tr>
<td>2.5</td>
<td>0.036</td>
<td>0.00225</td>
<td>6.3</td>
<td>5</td>
</tr>
<tr>
<td>1.25</td>
<td>0.024</td>
<td>0.00039</td>
<td>1.6</td>
<td>5</td>
</tr>
<tr>
<td>0.63</td>
<td>0.013</td>
<td>0.00052</td>
<td>4.2</td>
<td>5</td>
</tr>
<tr>
<td>Interday</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10.0</td>
<td>0.132</td>
<td>0.00507</td>
<td>3.8</td>
<td>6</td>
</tr>
<tr>
<td>2.5</td>
<td>0.036</td>
<td>0.00187</td>
<td>5.2</td>
<td>6</td>
</tr>
<tr>
<td>1.25</td>
<td>0.024</td>
<td>0.00098</td>
<td>4.1</td>
<td>6</td>
</tr>
<tr>
<td>0.63</td>
<td>0.016</td>
<td>0.00080</td>
<td>5.0</td>
<td>6</td>
</tr>
</tbody>
</table>

All samples were prepared by individually over-spiking 3-nitrotyrosine standard solution and O-methyl-tyrosine internal standard into commercial human plasma followed by solid phase

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extraction and LC-MS/MS analysis. Five samples at each concentration prepared in the same day were used to assess the intraday precision. Two samples at each concentration prepared for three consecutive days were used to assess the interday precision.

5.3.7 Accuracy

The accuracy of an analytical method is defined to be the closeness of mean concentration determined by the analytical method to the true concentration of the analyte. Accuracy of this method was assessed by testing four concentrations in the range of expected 3-nitrotyrosine basal concentration and using five to six samples per concentration. According to the FDA Guidance for Industry Analytical Method Validation, the mean value should be within 15% of the true value at tested concentrations (Table 13). Our calculated concentrations are reasonably close to the known concentrations, with relative error or inaccuracy no greater than 7.36% in all cases.

Table 13: Intraday and interday accuracy parameters of the LC-MS/MS method for 3-nitrotyrosine quantitation

<table>
<thead>
<tr>
<th>Conc (nM)</th>
<th>Mean Cal Conc (nM)</th>
<th>%RE</th>
<th>No. of Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraday</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>9.79</td>
<td>-2.08</td>
<td>5</td>
</tr>
<tr>
<td>2.5</td>
<td>2.47</td>
<td>-1.31</td>
<td>5</td>
</tr>
<tr>
<td>1.25</td>
<td>1.32</td>
<td>5.40</td>
<td>5</td>
</tr>
<tr>
<td>0.63</td>
<td>0.60</td>
<td>-4.76</td>
<td>5</td>
</tr>
<tr>
<td>Interday</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>9.94</td>
<td>-0.55</td>
<td>6</td>
</tr>
<tr>
<td>2.5</td>
<td>2.48</td>
<td>-0.69</td>
<td>6</td>
</tr>
<tr>
<td>1.25</td>
<td>1.34</td>
<td>7.36</td>
<td>6</td>
</tr>
<tr>
<td>0.63</td>
<td>0.58</td>
<td>-7.35</td>
<td>6</td>
</tr>
</tbody>
</table>

Methods of sample preparation and analysis as in Table 12. Calculated concentration is derived by using: \([\text{Mean Cal Conc (nM)}] = (\text{Mean Peak Area Ratio} - a)/b\), where \(a\) is the y-intercept and \(b\) is the slope of the calibration curve. Relative error is derived by using equation: Relative Error \(\% = \left(\frac{[\text{Mean Cal Conc}] - [\text{True Conc}]}{[\text{True Conc}]}\right) * 100\%.\)
5.3.8 Stability

The stabilities of standard and processed samples have been assessed. Storage of standard samples at 4 °C and processed samples at -20 °C for one week didn’t cause significant changes in signal intensity of overspiked 3-nitrotyrosine.

5.3.9 LOD, LOQ and LLOQ

LOD and LOQ are defined to be the concentrations where signal to noise ratios are 3 and 10, respectively. To obtain LOD and LOQ applicable to human plasma matrix and take into account every step in sample pre-treatment and analysis. We first measured the peak areas of 3-nitrotyrosine and its internal standard in the zero sample. We then used the slope from the calibration curve to translate the peak area ratio of 3-nitrotyrosine over internal standard to endogenous 3-nitrotyrosine concentration. From the signal to noise ratio at this endogenous concentration we determined the LOD and LOQ of this method were 0.034nM and 0.112nM respectively.

LLOQ is defined to be a concentration at which the analyte peak is identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120%. Experimentally, we proved the LLOQ was below 0.625 nM since 0.625 nM was tested to be acceptable regarding precision and accuracy (<15%).

5.3.10 Robustness

The robustness of this method has been tested with over 80 consecutive real sample analyses. Retention times were consistent across all the analyses (6.732 ± 0.043 min for analyte; 6.947 ± 0.045 min for internal standard).

5.3.11 Elevation of 3-nitrotyrosine level upon ischemia reperfusion

Analysis of protein bound 3-nitrotyrosine has been difficult with complicated protein digestion and sample preparation [315-318]. Further, most of the reports could not achieve clean separation of 3-nitrotyrosine from co-eluting substances. Affinity columns may help ameliorate the interference from sample digestion, but the preparation procedure of affinity column is not trivial [318]. Analysis of 3-nitrotyrosine in plasma is most preferred because plasma is best suited for a wide range of clinical investigations. Ultrafiltration combined with hypercarb chromatography has been used for plasma sample preparation and LC-MS/MS quantitation [312,
and it appears that the usage of hypercarb column has improved selectivity and sensitivity of 3-nitrotyrosine analysis. But the tendency of hypercarb to strongly retain polar compound has prolonged the analysis time significantly and for some reason, the three reports using similar methods gave quite diverse results (6.5, 9.4, and <0.4 nM).

Analysis of 3-nitrotyrosine at nanomolar concentration requires not only removal of salts and proteins, but also pre-concentration of sample from a larger volume. Thus, we have used 1mL of plasma to ensure sensitivity and taken every necessary measure to maximize recovery in this study. Orthogonal PFP column provides additional selectivity to baseline separate 3-nitrotyrosine from other sources of background noise in a short run time (<10 min), enabling accurate quantification. Figure 14 shows the representative chromatograms of patient plasma samples with or without spiked 3-nitrotyrosine by using methods described in Section 5.2.5 Sample Preparation, 5.2.6 Liquid chromatography and 5.2.7 Tandem mass spectrometry.

![Figure 14: Representative chromatograms of patient plasma samples without (Panel A) or with 10 nM over-spiked 3-nitrotyrosine (Panel B)](image)

*Kinetex 100x2.1 mm 2.6 µm PFP column from Phenomenex was used as separation column.*

There is a lack of agreement about the normal average concentration of free 3-nitrotyrosine in human plasma. Four independent studies employing two different GC-MS/MS strategies have reported a ranges from 0.64 to 2.8 nM [302], while LC-MS/MS in three
independent studies have given a number between 0.4 and 9.4 nM [313, 319]. Our results (1.494 ± 0.1065 nM and 2.167 ± 0.1770 nM for pre-CPB and post-CPB coronary sinus plasma) generally agree with those previously reported values. Although significant, approximately 45% increase of 3-nitrotyrosine level is at most modest as compared to what have been seen in Hayashi’s report 8. By using HPLC-UV methods, Hayashi et al. have noticed dramatic elevation of 3-nitrotyrosine to tyrosine ratio (1.72% to 3.16% in coronary sinus effluent) after CPB procedure. The lack of selectivity and sensitivity made HPLC-UV method less favorable and often inadequate to analyze clinical samples. LC-MS/MS and GC-MS/MS remain the methods of choice for nitrosative stress biomarker validation and application since they have combined selectivity and sensitivity and would likely give more consistent results.

The validated method was used to analyze samples from 45 patients. Free 3-nitrotyrosine concentrations in coronary sinus plasma from pre-CPB and post-CPB were evaluated. The mean concentration of pre-CPB samples was significantly lower than that of post-CPB samples. (95% CI -1.084 to -0.2063; p = 0.0016; two-tailed t-test). The pre-CPB and post-CPB concentrations of 3-nitrotyrosine in patient plasmas were 1.494 ± 0.1065 nM and 2.167 ± 0.1770 nM (Mean ± SEM) respectively. This result confirmed the hypothesis that nitrosative stress level experienced by patients would increase when subjected to CPB-related ischemia and reperfusion.

5.3.12 3-Nitrotyrosine level in relation to treatment groups, patient diabetic conditions and patient outcome

Analysis of patient plasma 3-nitrotyrosine unveiled some interesting trends. 3-nitrotyrosine level is significantly increased during CABG surgery (Figure 15). Propofol and isoflurane treatments, however, don’t seem to affect 3-nitrotyrosine production. Patients with low cardiac output syndrome experienced more 3-nitrotyrosine increase than patients without this syndrome. Diabetic patients are more susceptible to 3-nitrotyrosine elevation than non-diabetic patients. From this study, it appears that nitrosative stress might contribute to low cardiac output syndrome and put diabetic patients at risk. Prevention or mitigation of nitrosative stress is a promising approach to improve clinical outcome for patients undertaking CABG surgery.
Figure 15: 3-nitrotyrosine level in relation to treatment groups, patient profile and clinical outcomes

3-Nitrotyrosine level is significantly higher in post-CPB samples than pre-CPB samples (Panel A); there is no significant difference between the changes of 3-nitrotyrosine level in propofol-treated patients and those in isoflurane-treated patients (Panel B); the changes of 3-Nitrotyrosine level are significantly greater in patients with low cardiac output syndrome than patients without low cardiac output syndrome (Panel C); the changes of 3-nitrotyrosine level are greater in diabetic patients than non-diabetic patients but the difference is not statistically significant (Panel D);

5.4 Conclusion

In conclusion, we have developed a simple, sensitive, selective, and robust LC-MS/MS method for free 3-nitrotyrosine determinations in plasma samples of patients receiving on-pump CABG surgery. The results suggested that 3-nitrotyrosine levels increased during cardiopulmonary bypass procedure. Furthermore, analysis of free 3-nitrotyrosine in a larger group
of patients using this method revealed the changes of 3-Nitrotyrosine level are significantly greater in patients with low cardiac output syndrome than in patients without low cardiac output syndrome.
6. Development and application of a LC-MS/MS method for quantitation of basal adenosine concentration in human plasma

6.1 Introduction

As explained in Chapter 1, quantitative method to determine basal levels of adenosine has yet to be established and its role in cardioprotection remains to be identified [163, 166, 320].

High performance liquid chromatography (HPLC) and radioimmunoanalysis techniques to quantify low basal adenosine levels (80-100 nM) have proven to be insufficient and associated with limitations to use. Analytical methods based on separation are highly preferable and several separation methods have been developed for adenosine quantitation. They include capillary electrophoresis [321, 322], high performance liquid chromatography with UV absorbance detection (HPLC-UV) [165, 323-326], HPLC-fluorescence detection, liquid chromatography-mass spectrometry (LC-MS) [322, 327, 328], and liquid chromatography-tandem mass spectrometry (LC-MS/MS) [329-334].

LC-MS/MS is generally considered to be the method of choice in analytical field, offering superior selectivity and sensitivity to detect low concentration analytes in complex matrices, such as blood, plasma and tissue extracts. LC-MS/MS methods have been published for measuring adenosine in microdialysate [329], mouse kidney perfusate [334], urine [332], and cell culture medium [333]. An exploratory method for the analysis of adenosine in plasma has been described [331]. A fully validated method for detecting basal adenosine level in human plasma with adequate sensitivity and robustness is still needed.

The goals of this study were: 1) to develop a more sensitive, selective, and robust LC-MS/MS method for adenosine determination in CABG patient plasma; 2) use the fully validated method to monitor the adenosine changes throughout the on-pump CABG surgery; and 3) describe potential clinical relevance of basal adenosine levels in cardioprotection.

6.2 Materials and methods

6.2.1 Instrument and data processing software

All experiments were conducted on a Varian 1200L LC-MS/MS system (Varian Inc. now a part of Agilent Technologies, Palo Alto, CA, USA) with a triple quadrupole detector. The solvent delivery system consists of two single-piston rapid-refill pumps (ProStar 210 Dynamax...
System). Varian Mass Spectrometry Workstation Version 6 was employed to control and monitor the LC-MS/MS instrument, as well as acquire and process the spectra and chromatography information. Excel and Prism were used for statistical analysis.

6.2.2 Chemicals, reagents and consumables

HPLC grade acetonitrile and methanol were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure water was purchased from Cayman Chemical (Ann Arbor, MI, USA). ACS grade acetic acid was purchased from Fisher Scientific (Nepean, ON, Canada). Adenosine standard was purchased from Sigma-Aldrich (St Louis, MO, USA). $^{13}$C$_5$-Adenosine standard (>98%) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA). Redistilled trichloroacetic acid was purchased from Sigma-Aldrich (St Louis, MO, USA). 3mL SPEC SCX column with monolithic solid phase extraction disk (Cat. No. A5310420) were purchased from Varian Inc. (Palo Alto, CA, USA). Pooled human plasma was purchased commercially from Innovative Research (Novi, Michigan, USA).

6.2.3 Preparation of calibration standards

Concentrations of calibration working solutions were 5000, 1000, 500, 100, 50, and 10 nM. Concentration of internal standard working solution was 2000 nM. To construct a calibration curve, 50 µL of the calibration working standards and 50 µL of the internal standard stock solution were spiked into 400 µL of commercial pooled human plasma before sample extraction and LC-MS/MS analysis. Zero concentration samples and blank samples were also prepared for the calibration.

6.2.4 Patients and sampling

This investigation conforms to the principles outlined in the Declaration of Helsinki [314]. The surgery was performed according to previously described procedure [231]. Two blood samples of 5 mL each were withdrawn from the coronary sinus 10 minutes before aorta clamping (denoted as C2 blood sample) and 5 minutes after declamping (denoted as C3 blood sample). Radial artery blood samples were obtained at the same time points (denoted as A2 and A3 blood samples respectively). The blood samples were treated with potassium EDTA and centrifuged at 4000 rpm for 15 min at 4 °C. Plasma was then quickly separated from blood cells and stored in 1.25 mL aliquots at -80 °C for subsequent adenosine analysis.
6.2.5 Sample pre-purification

Each of the 400 µL patient plasma samples was spiked with 50 µL of 2000 nM internal standard (IS) working solution. A solid phase extraction procedure was used to process the human plasma samples including calibration samples and patient samples spiked with IS before they were submitted to LC-MS/MS analysis. To avoid enzymatic degradation of adenosine, 20% trichloroacetic acid was added to the plasma samples at 2:10 (v/v). The mixtures were vortexed and then centrifuged at 13000 rpm for 15 min. Supernatants were aspirated with a pipette and loaded to pretreated SCX cartridge.

The SPE procedure using SCX cartridge is as follows. The cartridge was 1) rinsed with 250 µL of methanol under gravity for 1 min; 2) rinsed with 250 µL of 0.1 M acetic acid under gravity for 1 min; 3) loaded supernatant under positive air pressure; 4) washed with 0.1 M acetic acid/methanol (50:50) under positive air pressure for 1 min; and 5) eluted with 250 µL of methanol/concentrated ammonium hydroxide (98:2) under positive air pressure.

Eluted methanol solution was concentrated by SpeedVac and the residue was resuspended in 70 µL 2% acetonitrile aqueous solution and submitted to LC-MS/MS analysis.

6.2.6 Liquid chromatography

A modifier-free mobile phase strategy was used in liquid chromatography. Mobile phase A contained 100% ultrapure water while mobile phase B contained 100% acetonitrile. The total run time was 12.5 min including column equilibration time with 3 linear gradient components: from 0 to 9.5 min, 2% to 15% B; from 9.5 to 10 min, 15% to 2% B; from 10 to 12.5 min, 2% B, where %B is a volume percentage of total mobile phase. A Gemini-NX 3 mm C18 100x2.0 mm column from Phenomenex (Torrance, CA, USA) was applied in the chromatographic separation. A guard cartridge system from Phenomenex, SecurityGuard™, was installed to protect the analytical column at all times. For each run a 25 µL of processed sample was injected. The flow rate was set to 0.2 mL/min.

6.2.7 Tandem mass spectrometry

The sensitivity was tuned according to manufacturer’s recommendations. We have used positive electrospray ionization (ESI) as LC-MS interface. The specific conditions after tuning were as follows: needle voltage 3750 V; shield 375 V; capillary 30 V; drying gas, nitrogen at 300 °C and 21 psi; nebulizer, nitrogen at 50 psi; electromultiplier 1700 V; collision energy -12.0 V;
collision gas, ultrapure argon at 2.00 mTorr. Multiple reaction monitoring (MRM) was used to monitor the adenosine (m/z 268.0->136.1) and $^{13}$C$_5$-adenosine (m/z 273.0->136.1).

6.3 Results and discussion

A reliable and robust analytical method requires full validation that addresses selectivity, linearity, precision, accuracy, limit of detection (LOD), limit of quantitation (LOQ), lower limit of quantitation (LLOQ), and stability. The method development aspects for the quantitative analysis of adenosine in human plasma followed by its application to adenosine level determination for patient plasma are described below. Statistical analysis revealed significant changes of adenosine level during CABG surgery.

6.3.1 Optimization of mass spectrometer parameters

All MS parameters were adjusted to achieve optimal sensitivity according to manufacturer’s recommendation. The optimal conditions are listed in Section 7.2.7 Tandem mass spectrometry. The product ion spectra of adenosine and $^{13}$C$_5$-adenosine were acquired through a product ion scan mode, where specific precursor ion (m/z at 268.0 or 273.0) was transmitted through the first quadrupole and a mass range was swept (m/z from 50.0 to 380.0). Major product ion peaks after gas collision by ultrapure argon at 2.00 mTorr for adenosine and internal standard were both at m/z 136.1, corresponding to a purine moiety of adenosine (Figure 16).
Figure 16: Product ion spectra of $^{13}\text{C}_5$-adenosine and adenosine

Base peaks were identified to be m/z 136.1 for both $^{13}\text{C}_5$-adenosine (Panel A) and adenosine (Panel B). Asterisk * denotes the position of $^{13}\text{C}$.

6.3.2 Modifier free mobile phase

Since the amino group on the purine ring of adenosine is easily protonated under acidic conditions, which is beneficial for electrospray ionization and subsequent MS/MS analysis, an acidic modifier was added to the mobile phase in the hope of achieving higher signal intensity and hence sensitivity. However, as shown in Figure 17, the addition of 0.1% acid modifier (e.g. formic acid) did not provide a significant increase in sensitivity but led to shorter retention time of the analyte and higher background level compared to the modifier free mobile phase.

Adenosine is a relatively polar molecule, given that it bears three hydroxyl groups and one aromatic amine. Under acidic conditions, more amine groups of adenosine are protonated, making the analyte and nonpolar reverse stationary phase interact to a lesser degree. Although we have adapted a very low elution gradient, adenosine still comes out the column rather quickly (retention time around 3 min). At this retention time, three factors may come into play to cause reduced signal-to-noise ratio: ionization suppression, low organic content, and high noise level from other polar compounds. Plasma samples contain numerous polar organic metabolites and
inorganic salts. During the first few minutes of reverse phase elution, these polar contaminants could foul the mass spectrometer’s heated capillary, and cause severe ionization suppression.

Matrix effects incurred by these endogenous substances are also a concern for the accuracy and precision for LC-MS/MS methods, particularly rapid high-throughput analysis. Organic content is known to significantly influence the ionization and higher organic content is usually preferred [335]. In this scenario, at an already low gradient, eluent with a lower retention time is accompanied with a low organic content at the point of analyte ionization, resulting in a low sensitivity. Lastly, the presence of an acid modifier increases the ionization efficiency of impurities, causing a higher background signal, as seen in Figure 17 Panel A.

Figure 17: Mobile phase with 0.1% formic acid versus modifier-free chromatograms of adenosine SPE extract

Chromatography condition: from 0 to 9.5 min, 2% to 15% B; from 9.5 to 10 min, 15% to 2% B; from 10 to 12.5 min, 2% B. A Gemini-NX 3 mm C18 100x2.0 mm column from Phenomenex (Torrance, CA, USA) was applied in the chromatographic separation. For Panel A, mobile phase A contains 0.1% formic acid in water; mobile phase B contains 0.1% formic acid in acetonitrile. For Panel B, mobile phase A contains ultra pure water; mobile phase B contains HPLC grade acetonitrile.

The modifier-free mobile phase provided superior performance compared to with modifiers, including acetic acid, ammonium acetate, and ammonium formate. On average, a 5 to
10 fold improvement in S/N ratio was observed for the modifier-free mobile phase system compared to those modified with an acid or acidic salt when analyzing SPE extracts spiked with equal amounts of adenosine. Despite lacking buffer capacity, modifier free mobile phase does not cause peak deterioration in essentially all the performed analyses. Retention times were consistent across the tested samples from various sources (\(\%\text{RSD} < 1\%, n = 15\)). An additional benefit for modifier-free mobile phase system is that it does not require any work associated with mobile phase preparation. Representative chromatograms of adenosine and \(^{13}\text{C}_5\)-adenosine are shown in Figure 18.
Figure 18: A representative chromatogram of a patient sample spiked with 200nM internal standard

Panel A chromatogram, the SRM m/z 268.0->136.1, represents endogenous adenosine from patient plasma sample; Panel B chromatogram, the SRM m/z 273.0->136.1, represents the spiked $^{13}$C$_5$-adenosine. The basal adenosine level in this patient is calculated to be 44.8 nM.
6.3.3 Solid phase exaction

Although solid phase exaction is not always required prior to quantitative analysis of adenosine by LC-MS/MS [333], bypassing this step in our experience caused a significant increase in column pressure, leading to deviations of retention times. This could be a consequence of particle accumulation on the column head if no preventive extraction steps were used. In order to prevent irreversible damage to the expensive analytical column, and to keep retention times constant, we incorporated solid phase extraction into the protocol. Furthermore, unprocessed sample gave lower signal intensity than processed sample (Figure 19). The difference in retention times between Panel A and B in Figure 19 is possibly caused by different acid content in the suspension buffer. The sample without SPE contains high percentage of trichloroacetic acid while the sample with SPE contains no acid. Trichloroacetic acid drops the pH considerably so that the retention of adenosine in the beginning of chromatography by C18 column is predicted to be poor. Thus we concluded that it is important to carry out sample pre-treatment before the analysis for complicated matrices such as plasma.
Figure 19: The comparison of chromatograms of plasma samples spiked with same amount of IS before and after SPE

Chromatography condition: from 0 to 9.5 min, 2% to 15% B; from 9.5 to 10 min, 15% to 2% B; from 10 to 12.5 min, 2% B. Mobile phase A contains ultra pure water; mobile phase B contains HPLC grade acetonitrile. Panel A shows chromatogram before SPE and Panel B shows chromatogram after SPE.
Varian SPEC SCX monolithic disk cartridges were chosen for adenosine analysis because they have uniform flow properties, low bed mass, high mass transfer efficiency, and small processing volume. Their uniform flow properties allow viscous, particle-laden samples such as plasma to be processed quickly without clogging the disk. Low bed mass resulted in less retention of interfering particles or contaminants, hence a cleaner sample. High mass transfer efficiency allows faster processing time with this type of SPE. Finally, only 250 µL of solvent in each individual step is needed to run the SPE due to its small processing volume, saving time, solvent and labor for sample pre-purification. After monolithic disk purification and further centrifuge sedimentation as described in the method Section 6.2.5 Sample pre-purification, the samples submitted to LC-MS/MS did not cause any build-up of backpressure or shift of retention time after repeated continuous runs (n > 80). The absolute recovery of adenosine off the SCX disc is 84.5 ± 1.56% (mean ± SD; n = 12).

6.3.4 Selectivity

We assigned the adenosine and $^{13}$C$_5$-adenosine peaks and matched them with their specific retention times by spiking either adenosine or $^{13}$C$_5$-adenosine reference substances into the human plasma matrix. Isotopic internal standard is always the analytical choice in terms of its ability to improve repeatability and accuracy, because the peak area ratio of adenosine and $^{13}$C$_5$-adenosine is independent upon differential ion suppression and variation of sample extraction. Furthermore, co-elution of adenosine along with its isotopic internal standard facilitates unambiguous peak identification and integration. Cross-talk issue was examined to avoid selectivity problem [96]. For standard aqueous samples, no appreciable peak in ion channel m/z 273.0->136.1 (for $^{13}$C$_5$-adenosine) was observed at the retention time of $^{13}$C$_5$-adenosine (~7.0 min) if only adenosine is spiked and vice versa.

6.3.5 Linearity

The basal adenosine concentration range was estimated to be between 20 nM and 200 nM in human plasma according to literature reports [165, 331, 336]. The linearity of the current method was assessed using the peak area of standard adenosine solutions between 1 nM and 500 nM relative to that of 200 nM internal standard. This ratio of peak areas of adenosine over that of its internal standard was plotted against the ratio of spiked adenosine concentrations over spiked internal standard concentration. This calibration curve has a goodness of fit of $r^2 = 0.996$ across the tested range, with the equation: $y = 1.1513x + 0.1399$. 
6.3.6 Precision

Precision was assessed by spiking three clinically relevant concentrations of adenosine (10, 50, and 500 nM) into the commercial human plasma, followed by trichloroacetic acid treatment, SCX cartridge extraction, and LC-MS/MS analysis. Intraday precision was determined by using six individually prepared plasma samples spiked with known amounts of adenosine and internal standard during the same day. Interday precision was determined by using eight individually prepared spiked plasma samples during four days in duplicates. Our determined values conform to the FDA requirements for analytical method validation, with percentage of RSD below 6.33% in all cases (Table 14).

**Table 14**: Intraday and interday precision parameters of the LC-MS/MS method for adenosine quantitation

<table>
<thead>
<tr>
<th>Conc. (nM)</th>
<th>Area Ratio</th>
<th>SD</th>
<th>%RSD</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intraday</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.76</td>
<td>0.17</td>
<td>6.33</td>
<td>6</td>
</tr>
<tr>
<td>50</td>
<td>0.44</td>
<td>0.02</td>
<td>3.80</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>0.20</td>
<td>0.01</td>
<td>3.33</td>
<td>6</td>
</tr>
<tr>
<td><strong>Interday</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>500</td>
<td>2.76</td>
<td>0.14</td>
<td>4.92</td>
<td>8</td>
</tr>
<tr>
<td>50</td>
<td>0.45</td>
<td>0.03</td>
<td>6.00</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>0.20</td>
<td>0.01</td>
<td>3.78</td>
<td>8</td>
</tr>
</tbody>
</table>

All the samples were prepared by individually spiking standard solution and internal standard into commercial human plasma followed by acidic protein precipitation, solid phase extraction and LC-MS/MS analysis. Basal adenosine concentration in the commercial plasma is calculated to be 30.39±2.72nM.

6.3.7 Accuracy

The accuracy of an analytical method is defined to be the closeness of mean test results determined by the analytical method to the true concentration of the analyte. The accuracy of this method was assessed by testing three concentrations in the range of expected adenosine basal concentration and using six to eight samples per concentration. According to the FDA Guidance for Industry Analytical Method Validation, the mean value should be within 15% of the true value at tested concentrations. Our calculated concentrations are reasonably close to the known concentrations, with relative error or inaccuracy no greater than 10% in all cases (Table 15). It is
seemingly unexpected that at 500 nM level the calculated value deviates from the true value more than those at 50 nM or 10 nM level. However, this may be explained by the fact that the mass spectrometry response is non-linear at high concentrations. This is also known as high concentration related analyte “saturation” phenomena [337, 338].

Table 15: Intraday and interday accuracy parameters of the LC-MS/MS method for adenosine quantitation

<table>
<thead>
<tr>
<th></th>
<th>True Conc (nM)</th>
<th>Calculated Conc (nM)</th>
<th>%RE</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intraday</td>
<td>500</td>
<td>454.70</td>
<td>-9.06</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>53.05</td>
<td>6.10</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.34</td>
<td>3.43</td>
<td>6</td>
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<tr>
<td>Interday</td>
<td>500</td>
<td>455.06</td>
<td>-8.99</td>
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<tr>
<td></td>
<td>50</td>
<td>51.57</td>
<td>3.15</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.93</td>
<td>9.29</td>
<td>8</td>
</tr>
</tbody>
</table>

Calculated concentration is derived by using: \(\text{[Mean Cal Conc] (nM)} = (\text{Mean Peak Area Ratio} – a)/b\), where \(a\) is the \(y\)-intercept and \(b\) is the slope of the calibration curve. Relative error is derived by using equation: \(\text{Relative Error \%} = (\text{[Mean Cal Conc]} – [\text{True Conc}])/[\text{True Conc}] * 100\%\). Basal adenosine concentration in the commercial plasma is calculated to be 30.39±2.72nM.

6.3.8 LOD, LOQ and LLOQ

The LOD and LOQ are defined as the concentration at which the signal is 3 times and 10 times the standard deviation of the background, respectively. We determined the LOD and LOQ were 0.257 nM and 0.857 nM, respectively, for this method. Our method is approximately 100-400 fold more sensitive than LC-MS methods previously developed in human synovial fluid [328] or in the venoms from viperinae snakes [322], and 6 fold more sensitive than LC-MS/MS method [339] developed in Cordyceps sinensis, based on this estimate of the LOD.

LLOQ is defined to be a concentration at which the analyte peak is identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120%. Experimentally, we determined that the LLOQ was below 10 nM since 10 nM was tested to be acceptable with precision and accuracy (<15%). This is at least 6 times lower than what is a recently reported LC-MS/MS method [333] developed in serum-free cell culture medium.
6.3.9 Stability

The stability of adenosine under conventional storage conditions was assessed by subjecting the spiked samples to three freeze-thaw cycles as well as 24h room temperature storage. Six high concentration samples and six low concentration samples were prepared in parallel. To check the freeze-thaw stability, one of the three samples of each group was analyzed immediately, freeze-thawed once, or freeze-thawed three times. To check the room temperature storage stability, one of the remaining three samples of each group was analyzed immediately, 8 hours later, or 24 hours later. The RSD percentages of the freeze-thaw series were 4.94 and 9.31 for high and low concentrations respectively, while the RSD percentages of the prolonged room temperature storage series were 2.55 and 6.57 for high and low concentrations, respectively. Overall, no obvious changes were observed for area ratio of adenosine/IS under freeze-thaw test and prolonged time test.

6.3.10 Adenosine basal level in A2, C2, C3 and A3 samples

We used this LC-MS/MS method to analyze the concentration of adenosine in the plasma of 84 patients receiving either propofol or isoflurane during cardiopulmonary bypass. The mean plasma concentration of adenosine in coronary sinus blood prior to and following CPB was 36.02 ± 2.02 nM vs 45.92 ± 2.49 nM (mean value ± SEM) respectively (two-tailed Student’s t-test; p = 0.0024), as shown in Figure 20.
Figure 20: The comparison of adenosine levels in A2, C2, C3 and A3 plasma samples

C2 and C3 plasma samples were obtained from the coronary sinus 10 minutes before aorta clamping and 5 minutes after aorta declamping respectively. A2 and A3 samples drawn from radial artery were obtained at the same time points. Asterisk ** denotes statistical significance between C2 and C3 (p = 0.0024; two-tailed t-test). Ampersand sign & denotes statistical significance between C3 and A3 (p = 0.0409; two-tailed t-test).

Due to adenosine’s ultrafast turnover in human blood, stopping solution was often immediately added into blood sample at collection [163, 340-342]. However, even with stopping solution to trap adenosine instantly, Becker et al still couldn’t detect any change of adenosine in coronary sinus or arterial blood (n = 11) after CABG surgery [342]. In contrast, Kerbaul et al noticed a 50% increase in the concentration of adenosine following CABG-CPB compared to at the beginning of this surgery (n = 30) [164]. This disagreement reflected considerable variations in patient profiles, analytical methods, sample number and sampling techniques. Our results from testing relatively large number of samples are in agreement with Kerbaul’s findings.

At the point of aorta declamping, the average coronary adenosine level is also significantly higher than the average radial artery adenosine level (two-tailed Student’s t-test; p =
suggested adenosine changes are cardiogenic. Our results are consistent with Kerbaul’s findings [164] that upon CABG-CPB, plasma adenosine level is higher in coronary sinus than in radial artery.

Adenosine production and release have been associated with ischemia following coronary occlusion or stenosis [163, 343]. Increased plasma adenosine levels in mixed venous blood have also been observed during CABG-CPB surgery [340]. The inverse proportional relationship between oxygen supply and adenosine production [163] might be one of the reasons accounting for the increased coronary sinus adenosine level. Other factors such as elimination rate can also contribute to an altered adenosine balance equation. Although large amounts of adenosine output may not be detected using our method because of adenosine’s ultrafast turnover, this sensitive LC-MS/MS method has allowed us to see small but significant changes of adenosine basal level.

6.3.11 Sensitive measurement of adenosine basal level without stopping solution

The detection limit of most HPLC methods is at best approximately 20 nM if using 2 mL of plasma sample. These methods also require affinity gel pre-purification involving tedious washing steps, identification of the adenosine peak by adding deaminase, and sometimes two columns in tandem for cleaner separation [325, 341, 344]. Adenosine basal levels ranging from 130 to 360 nM in human plasma have been reported in literature [345]. Here we observed a lower basal adenosine level than what has been seen with HPLC-UV methods. However, more sensitive methods such as LC-MS/MS [331] or radioimmunoanalysis [336] give similar ranges to this method. The discrepancy can be explained by the fact that HPLC-UV methods are usually not very selective and co-eluting interfering substance might have contributed to a higher baseline [341].

It has been shown that the change of basal adenosine level at steady state was minimal even when incubating plasma sample at 37 °C for 1 hour and plasma adenosine in freshly drawn human blood (~80-100 nM) was at a similar level as adenosine sample collected in dipyridamole stopping solution (72 nM). The in vitro and in vivo measurements agreed with each other because the formation of adenosine is mostly from extracellular AMP degradation and adenosine elimination is mostly through red blood cell uptake, which might reach the equilibrium extremely fast 2. In the same paper, the half-life of radiolabeled adenosine was about 1.5 s and this fast kinetics has made stopping solution a popular choice when adenosine release needs to be instantly captured to prevent elimination. However, there are three limitations to studying kinetic changes of adenosine: 1) researchers have used variety of cocktails [163, 340-342] to inhibit either red
blood cell uptake or deaminase degradation of adenosine so that the results are difficult to compare; 2) the ultrafast kinetic change of adenosine makes sampling and timing extremely challenging [340-342]; 3) the presence of a short adenosine spike after ischemia condition might not be pharmacologically meaningful. In contrast, determination of the sustained elevation or depression of adenosine basal level by sensitive LC-MS/MS methods may provide consistent results and meaningful insights regarding adenosine steady state, regulation and sustained action on heart.

Furthermore, the application of LC-MS/MS to monitor adenosine basal level may find wide spread applications in relating patient characteristics or clinical outcome with basal adenosine concentration, optimizing the CABG-CPB maneuver by regulating adenosine level via pharmacological intervention, and differentiating adenosine’s contribution to cardioprotection from those of other modulatory factors. For example, quantitation of basal adenosine level can give accurate feedback to surgeons in cardioprotective dosing finding studies of either adenosine [345-351] or adenosine modulators, [166, 320, 330, 352, 353] as well as help explain clinical outcome with other cardioprotective measures.

6.3.12 Adenosine level in relation to treatment groups, patient diabetic conditions and patient outcome

Subanalysis found treatment didn’t have impact on adenosine regulation. Nor did outcome (Figure 21). But a statistical analysis suggested the physiological adenosine-dependent vasodilation was activated to compensate left ventricular dysfunction because the patients with such dysfunction tended to have higher adenosine basal level after on-pump CABG surgery. On the other hand, diabetic patients seemed to have lower adenosine basal level than diabetic patients, but the difference was not significant.
Figure 21: Adenosine level in relation to treatment groups, patient profile and clinical outcomes

There is no significant difference between the changes of adenosine level in propofol-treated patients and those in isoflurane-treated patients (Panel A); the changes of adenosine level are greater in non-diabetic patients than diabetic patients but the difference is statistically insignificant (Panel B); the changes of adenosine level are significantly greater in patients with low left ventricular ejection fraction than in patients with normal left ventricular ejection fraction (Panel C); There is no significant difference between the changes of adenosine level in patients with low cardiac output syndrome and patients without low cardiac output syndrome (Panel D).

6.4 Conclusion

In conclusion, we have developed a simple, sensitive, selective, and robust LC-MS/MS method for the determination of basal adenosine level in patients undergoing on-pump CABG surgery. Due to its high sensitivity, the method may be also applied to detect trace adenosine in other biological fluids.
Without using stopping solution, this method might be useful to resolve the conflicting findings around adenosine changes during on-pump CABG surgery. The increase of basal adenosine level in coronary sinus and different basal adenosine levels detected between the coronary sinus and radial artery plasma at reperfusion imply that the equilibrium of adenosine production and elimination has been altered in the heart upon CPB. These global changes can further help to understand the sustained action of adenosine on the heart and delineate cardioprotective strategies via adenosine modulation against IRI.
7. Development and application of a new derivatization method coupled with LC-MS/MS to enable baseline separation and quantitation of dimethylarginines in human plasma

7.1 Introduction

There are several analytical methods available for determining ADMA and SDMA concentrations in human plasma, including HPLC-UV [354], HPLC-fluorescence [173, 355-359], CE-fluorescence [360], CE-MS/MS [361], and LC-MS/MS [180, 362-370]. Tandem mass spectrometry-based methods are generally regarded as the methods of choice because of their superior selectivity and sensitivity. Most analyses of biological samples require mass spectrometry coupled with liquid chromatography to alleviate the matrix effect [40] and isobaric (same mass) interferences. However, separation of ADMA and its region-isomer, SDMA, by chromatography is quite challenging because of their striking structural similarity. So far, a popular strategy to separately quantify these two isomers is to identify their specific mass product ions, m/z 203->46 for ADMA and 203->172 for SDMA, after collisional gas fragmentation [362-364, 367-369]. Since ADMA and SDMA are very polar molecules, commonly used reverse phase columns are not able to retain them such that normal phase chromatography is often applied. While the advantages of using differential spectral analysis are obvious, there are three inherent deficiencies associated with this type of analysis: 1) the distinct product ions for ADMA and SDMA are in small abundance compared to major product ions; 2) m/z 46 is a secondary product ion and its abundance during fragmentation may not be consistent; and 3) normal phase chromatography, e.g. HILIC, usually requires lengthy method development while offering poorer reproducibility than reverse phase chromatography.

Analysts have developed several derivatization methods for ADMA and SDMA analysis, especially with fluorescence detection [173, 355-359]. After derivatization, ADMA and SDMA can be chromatographically separated from each other before they reach the fluorescence detector. In order to achieve baseline separation, a prolonged run time (up to 70 min) is sometimes needed. Fluorescence detection is also fraught with interferences inherent in many biological samples, necessitating a careful selection of solvent system and solid phase extraction protocol. To our knowledge, there is only one report of derivatization coupled with LC-MS/MS analysis, which used ortho-phthalaldehyde (OPA)-2-mercaptoethanol (2-ME) to label ADMA and SMDA for LC-MS/MS analysis [180].
Here we report a highly sensitive and selective LC-MS/MS method for ADMA and SDMA quantitation. Following a new derivatization reaction, we can achieve baseline separation of ADMA and SDMA on a reverse phase column within a reasonable time frame. Furthermore, this method requires only 50 µL of patient plasma. The results may help shed light on clinical outcomes associated with CABG surgery and guide clinicians to tailor cardioprotective measures before and after the operation.

7.2 Materials and methods

7.2.1 Instrument

All experiments were performed on a Varian 1200L LC-MS/MS system (Agilent Technologies, Palo Alto, CA, USA) with a ProStar 210 Dynamax solvent delivery system and a triple quadrupole mass analyzer. Varian Mass Spectrometry Workstation Version 6 was employed to control the LC-MS/MS instrument, as well as acquire the spectral and chromatographic information.

7.2.2 Chemicals, reagents and consumables

HPLC grade methanol, HPLC grade acetonitrile (ACN), ACS grade concentrated ammonium hydroxide and ACS grade acetic acid were purchased from Fisher Scientific (Nepean, ON, Canada). Ultrapure water and N-propyl-arginine (NPLA, internal standard or IS, >98.0%) were purchased from Cayman Chemical (Ann Arbor, MI, USA). N\textsuperscript{G}, N\textsuperscript{G'}-L-dimethylarginine (symmetric dimethylarginine or SDMA, >99.0%), N\textsuperscript{G}, N\textsuperscript{G}-dimethyl-L-arginine (asymmetric dimethylarginine or ADMA, >99.0%), potassium cyanide (KCN, >99.0%), 2-mercaptoethanol (2-ME, >99.0%), 3-mercaptopropionic acid (3-MPA, >99.0%), 2,3-naphthalenedicarboxaldehyde (NDA, >98.0%), ortho-phthaldialdehyde (OPA, >98.0%), potassium cyanide (>99.0%), L-alanine (>98.0%), L-aspartic acid (>98.0%), L-asparagine (>98.0%), L-glutamic acid (>99.0%), glycine (>99.0%), proline (>98.0%), serine (>98.0%), taurine (>99.0%) and trichloroacetic acid were purchased from Sigma Aldrich (Saint Louis, MO, USA). 3 mL SPEC SCX columns with monolithic solid phase extraction disk (Cat. No. A5310420) were purchased from Varian Inc. (Palo Alto, CA, USA). Commercial pooled human plasma was from Innovative Research (Novi, Michigan, USA).
7.2.3 Preparation of calibration standards

Calibration working solutions of ADMA and SDMA at 2.500, 1.000, 0.500, 0.250, 0.100, and 0.050 µM were prepared in ultrapure water. Internal standard working solution at 10 µM was also prepared in ultrapure water. The internal standard working solution was dispensed in 1mL aliquots to Eppendorf tubes and stored at -20 °C. To construct a calibration curve, 25 µL of the calibration working standards and 25 µL of the internal standard stock solution were spiked into 50 µL of commercial pooled human plasma followed by sample extraction and LC-MS/MS analysis. Zero samples were prepared and included in the calibration curve by substituting only calibration working solution with ultrapure water. Blank samples were prepared and included in the calibration curve by substituting both calibration and internal standard working solutions with ultrapure water. The sample solutions were then extracted as described in Section 7.2.5 Sample preparation and analyzed by LC-MS/MS to construct a calibration curve.

7.2.4 Patients and sampling

This investigation conformed to the principles outlined in the Declaration of Helsinki. Upon institutional approval and written informed consent, patients enrolled for primary on-pump CABG surgery at Vancouver General Hospital were studied. Arterial blood samples were collected before the surgery for the evaluation of ADMA and SDMA concentrations. The blood samples were treated with potassium EDTA and centrifuged at 4000 rpm for 15 min. Plasma was then separated from blood cells and stored in 1.25 mL aliquots at -80°C for subsequent analysis.

7.2.5 Sample preparation

25 µL of 10 µM IS working solution and 25 µL of water were spiked into each of the 50 µL patient plasma samples. This mixture was treated with trichloroacetic acid followed by centrifugation at 13,000 rpm for 15 min to precipitate plasma proteins. A solid phase extraction procedure was used to further process the supernatant of both calibration and patient samples.

The SPE procedure using SCX cartridge is as follows. The cartridge was 1) rinsed with 250 µL of methanol; 2) rinsed with 250 µL of 0.1 M acetic acid in water; 3) loaded with plasma supernatant; 4) washed with 250 µL of 0.1 M acetic acid in 1:1 methanol/water; and 5) eluted with 1.5 mL 10% NH₄OH in 1:1 methanol/water. Positive air pressure was used to enable faster cartridge wash or elution in each step.
The eluted solution was concentrated using SpeedVac and the residue was re-suspended in 30 µL water. The solution was then treated with 5 µL of 15 mM 2-mercaptoethanol in water and 5 µL of 15 mM 2,3-naphthalenedicarboxaldehyde in ACN. The mixture was incubated for 10 min at room temperature. The sample was centrifuged at 13,000 rpm for 15 min and the supernatant was submitted to LC-MS/MS analysis.

7.2.6 Liquid chromatography

Mobile phase A was ultrapure water while mobile phase B was HPLC grade methanol. The total run, including column equilibration time, was 23 min, comprising of 8 linear gradient components: from 0 to 2 min, 10 to 30% B; from 2 to 12 min, 30% B; from 12 to 14 min, 30 to 50% B; from 14 to 14.5 min, 50% B; from 14.5 to 15 min, 50 to 90% B; from 15 to 16 min, 90% B; from 16 to 16.05 min, 90 to 10% B; from 16.05 to 23 min, 10% B. A Kinetex 75 x 3.0 mm, 2.6 µm C18 column from Phenomenex (Torrance, CA, USA) was used throughout the chromatographic separation. A Krudkatcher inline filter was installed to protect the analytical column at all times. For each run, 2.0 µL of pre-purified sample was injected. Mobile phase flow rate was at 0.3 mL/min.

7.2.7 Tandem mass spectrometry

Sensitivity was tuned in accordance with manufacturer’s recommendations. The chosen conditions are as follows: needle voltage, 6000 V; shield, 450 V; capillary, 40 V; drying gas, nitrogen at 300 °C and 21 psi; nebulizer gas, nitrogen at 50 psi; electromultiplier, 1700 V; collision energy, -20.0 V; collision gas, ultrapure argon at 2.00 mTorr. Multiple reaction monitoring (MRM) was used to monitor ADMA and SDMA (m/z 369.3->282.0) and NPLA (m/z 383.3->282.0).

7.3 Results and discussion

7.3.1 Chromatographic separation of derivatives from three labeling reactions

A mixture of ADMA and SDMA was derivatized with three sets of labeling reagents. The first set of reagents contained OPA and 2-ME. Through chromatography, OPA-2-ME derivatives of ADMA and SDMA could be partially separated (Figure 22 Panel A). The second set of reagents contained NDA and KCN. Through chromatography, ADMA and SDMA NDA-KCN derivatives could nearly be baseline separated (Figure 22 Panel B). The third set of reagents containing NDA and 2-ME gave the best results among the three sets of labeling reagents with
regard to chromatographic separation (Figure 22 Panel C). With the NDA and 2-ME derivatization, full baseline separation was readily achieved within 15min.

![LC-MS chromatograms](image)

**Figure 22: LC-MS chromatograms of ADMA and SDMA derivatives from three types of amino group labeling reactions**

*Panel A shows the separation of ADMA and SDMA OPA-2-ME derivatives; Panel B shows the separation of ADMA and SDMA NDA-KCN derivatives; Panel C shows the separation of ADMA and SDMA NDA derivatives with 2-ME as catalyst. A Kinetex 75x3.0mm, 2.6μm C18 column*
from Phenomenex (Torrance, CA, USA) was used for all chromatographic separations. Chromatographic conditions have been optimized for each separation within a 20 min run.

7.3.2 Derivatized products from labeling reactions

The proposed structures of derivatized products between three sets of labeling reagents and ADMA are illustrated in Figure 23. Upon completion of the reaction between ADMA, OPA and 2-ME, 2-ME was incorporated into the final product to form 1-mercapto-2H-isoinindole (Figure 23 Panel A). The reaction between ADMA, NDA and KCN gave 1-cyano-2H-benzo[f]isoinindole as major product (Figure 23 Panel B). In contrast to these two reactions, in the reaction of ADMA with NDA and 2-ME, 2-ME moiety does not appear in the final product (Figure 23 Panel C). Instead, 2,3-dihydro-benzo[f]isoinol-1-one was produced, as evidenced by the peak at m/z at 369.2, rather than 429.2. Further investigation revealed that incubating NDA directly with ADMA in the absence of 2-ME generated the same product as in presence of 2-ME. The retention time and m/z ratio of a reaction product without addition of 2-ME are both identical to those with addition of 2-ME. However, based on a side-by-side comparison, the reaction rate was much slower if no 2-ME was added to this labeling reaction. Furthermore, after 20 min of reaction, the mixture without 2-ME produced only 10% at 60°C, or 1%, at room temperature compared to the mixture with 2-ME incubated at 20 °C. Therefore, we can conclude that 2-ME acts as a catalyst for NDA derivatization of ADMA. Although the product of NDA-derivatization with ADMA or other amino acids may not be suitable for fluorescence detection, it is a good candidate for LC-MS analysis. The signal intensity of the NDA derivative of ADMA was actually much stronger than its OPA-2-ME or NDA-KCN derivatized counterparts when the same conditions were applied.
Figure 23: Structure of protonated ADMA derivatives from three different types of reactions

Products are shown when reacting ADMA with OPA and 2-ME (Panel A); NDA and KCN (Panel B); NDA catalyzed with 2-ME (Panel C). Panel D shows the protonated product of reacting NPLA with NDA catalyzed with 2-ME.

7.3.3 Application scope of the new derivatization reaction

We have tested reactions of other selected amino acids with NDA and 2-ME. Most reactions proceeded in the same fashion as with AMDA. As shown in Figure 24, reacting NDA with alanine (Panel A), asparate (Panel B), asparagine (Panel C), glutamate (Panel D), glycine (Panel E), serine (Panel G), and taurine (Panel H) in the presence of 2-ME gave major products.
with m/z ratios at 254.1, 298.1, 315.1, 312.1, 240.1, 270.1 and 290.1, respectively, corresponding to 2,3-dihydro-benzo[f]isoindol-1-ones in the negative ionization mode. No reaction occurred between proline and NDA, according to the LC-MS analysis, since NDA is predicted to react with primary amino acids only. LC-MS analysis has not found any m/z ratios corresponding to NDA 2-ME adducts, further proving that 2-ME is a catalyst as opposed to a reactant in NDA labeling reactions with amino acids. Here we have demonstrated that this type of labeling reaction might be used for general amino acid qualification and quantitation under negative MS mode. Coupled with negative MS detection, it has two significant merits: 1) it allows concurrent analysis of amino acids with negatively charged species such as lipids or fatty acids; 2) it will increase the hydrophobicity of amino acids to facilitate the extraction and chromatography of these highly polar molecules. However, we were not able to detect 2,3-dihydro-benzo[f]isoindol-1-ones using positive MS mode, due to the lack of functionalities for protonation. This might be a limitation for using this labeling reaction. To expand the scope of application, we propose to design and synthesize a dialdehyde reagent bearing a basic functionality so that the resulting derivatives may be used for both positive and negative ion detection.
Figure 24: LC-MS chromatograms of 2,3-dihydro-benzo[f]isoindol-1-ones obtained by reacting NDA with amino acids other than dimethylarginine

Product peaks for alanine (Panel A), asparate (Panel B), asparagine (Panel C), glutamate (Panel D), glycine (Panel E), proline (Panel F), serine (Panel G), and taurine (Panel H) with 2-ME as catalyst are shown.
This derivatization reaction in conjunction with LC-MS/MS analysis may help open a venue to explore alternative chemical labeling modes for LC-MS/MS applications. The potential of combining chemical labeling with LC-MS/MS is tremendous, evidenced by many recent techniques made available in quantitative proteomics, such as tandem mass tagging (TMT), isotopic dimethylation and isobaric tag for relative and absolute quantitation (iTRAQ) [371]. Our novel derivatization reaction may have an impact on quantitative proteomics on peptides and proteins, given the structure similarity between amino acids, peptides and proteins. Additionally, the easy, fast, and quantitative nature of this reaction is a tremendous advantage. We plan to make a series of analogs of NDA, as well as applying this reaction to peptides and proteins. Quantitation of ADMA and SDMA is the beginning of this effort and provides initial proof of concept of this framework. Further, this reaction can also give rise to a facile analysis of many polar metabolites. Analysis of polar metabolites will continue to present a challenge to bioanalysts, because many metabolites tend to co-elute in a short chromatographic separation and suppress each other during ESI. This challenge can be overcome through a chemical derivatization, which may produce remarkable results [372-374]. On this note, many metabolites bearing primary amines can be easily converted into nonpolar derivatives through the reaction presented in this ADMA manuscript. Metabolites that have been modified to their nonpolar counterparts are separable by reverse chromatography, without fear of degradation of labeling reagents and products. This work aims to inspire readers to give more thoughts into the utility of chemical labeling reactions and consider beyond simply fast analyses.

7.3.4 Fragmentation of precursor ion

The product ion spectra of the NDA-derivatives of ADMA, SDMA and NPLA were acquired through a product ion scan mode, where specific precursor ions (m/z at 369.2, 369.2 or 383.0) were transmitted through the first quadrupole and a mass range was swept (m/z from 50.0 to 400.0). Base product ion peaks of the derivatives were all at m/z 282 (Figure 25 Panel A and B) after collision with ultrapure argon at 2.00 mTorr. This result shows that NDA derivative of NPLA takes the same fragmentation pathway as that of ADMA. We propose that 2,3-dihydrobenzo[f]isoindol-1-ones undergo loss of an alkylated guanidine moiety and formation of six-member ring lactone to give product ion at m/z 282 (Figure 26).
Figure 25: Representative LC-MS/MS product ion spectra of NPLA (Panel A) and ADMA (Panel B) NDA derivatives catalyzed by 2-ME
Figure 26: Proposed major fragmentation pathway of ADMA NDA derivatives with gas collision

SDMA and NPLA NDA derivatives follow a similar fragmentation pathway with gas collision.

7.3.5 Solid phase extraction (SPE)

We were able to derivatize the endogenous ADMA and SDMA after protein precipitation without solid phase extraction, and run LC-MS/MS analysis on the labeled products. However, our previous experiences have taught us that simple protein precipitation could hardly remove all the contaminants for an analytical column. The remaining trace amounts of proteins or phospholipids may clog or damage the analytical column over time. Therefore, we opted to use a simple solid phase extraction in order to maintain the robustness of this method and to avoid unnecessary and expensive damage to the analytical column, as bypassing this step may cause irreversible elevation of column pressure and deviation of retention time. Varian SPEC SCX monolithic disc cartridges were selected for ADMA and SDMA pre-purification because this type of SPE cartridge has uniform flow properties, low bed mass, high mass transfer efficiency, and small processing volume. During the elution step, 10% NH$_4$OH had to be prepared freshly to recover ADMA, SDMA and NPLA from the SCX cartridge.

7.3.6 Chromatography

Kinetex C18 and PFP columns were tested for effective separation of the NDA derivatives of ADMA and SDMA. The separation performance of Kinetex C18 column was
superior, with resolution factor $R_s = 1.76$ between ADMA and SDMA, theoretical plate number $N = 222,432$ and tailing factor $T = 1.01$ (USP method) for ADMA. Kinetex columns are packed with core-shell particles. This new technology allows decreased band broadening with uniform particle size and shape as well as increased column efficiency enabled by faster mass transfer. Only pure water and MeOH need to be used for this separation, saving time and effort compared to the preparation of mobile phases containing buffer systems. Obviating the need for a buffer has an additional advantage of improved consistency of chromatography. The chromatography conditions described in section 7.2.6 Liquid chromatography gave baseline separation of these two analytes in both standard samples and extracted samples from patient plasma (Figure 27).

Figure 27: Representative LC-MS/MS chromatograms of ADMA and SDMA NDA derivatives catalyzed by 2-ME

Panel A: standard ADMA and SDMA mixture spiked with NPLA as internal standard; Panel B: endogenous ADMA and SDMA in a typical patient sample spiked with NPLA as internal standard.

7.3.7 Linearity

Typical ADMA and SMDA concentrations in human plasma found in the literature were between 0.10 µM and 1.00 µM. Thus, the linearity of this method was validated through overspiking ADMA and SDMA at six levels (equivalent concentrations at 0.025, 0.050, 0.125, 0.250, 0.500 and 1.250 µM). The method of standard addition was performed with authentic standard, accurately known concentration of authentic analytes, and authentic matrix, pooled human
plasma from healthy individuals. Addition of increased concentration of authentic standards to equal aliquot of authentic matrix allows us to construct calibration curve. Endogenous concentrations of ADMA and SDMA were determined from the intercept of the calibration curve. The calibration curves were constructed by plotting the peak area ratios (ADMA or SDMA over the internal standard) against the corresponding ADMA or SDMA over-spiked equivalent concentrations. The linear best-fit equations for ADMA and SDMA were \( y = 0.1607x + 0.038 (r^2 = 0.9965) \) and \( y = 0.1899x + 0.0514 (r^2 = 0.9932) \). The calculated endogenous ADMA and SDMA concentrations were 236.44 ± 5.61 nM and 270.87 ± 7.79 nM respectively. Four standard curves were constructed and average of these four sets were reported. For ADMA, the Slope ± SD was 0.1607 ± 0.0023 and for SDMA, the Slope ± SD was 0.1899 ± 0.0038.

7.3.8 Precision

Precision was assessed by over-spiking four clinically relevant concentrations of ADMA and SDMA (0.500, 0.250, 0.125 and 0.025 µM) into commercial human plasma, followed by C18 solid phase extraction and LC-MS/MS analysis. Intraday precision was determined by using four individually prepared plasma samples over-spiked with known amounts of ADMA, SDMA and internal standard during the same day. Interday precision was determined by using six individually prepared plasma samples during three consecutive days in duplicates. Intraday precision and interday precision are intended to meet the FDA Guidance for Industry Analytical Method Validation. The precision determined at each concentration level should not exceed 15% of relative standard deviation (RSD) or coefficient of variation (CV) (Table 16). Our determined values conform to the FDA requirements for analytical method validation, with percentage of RSD below 6.80% in all cases.
Table 16: Intraday and interday precision parameters of the LC-MS/MS method for asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) quantitation

<table>
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<tr>
<th></th>
<th>Conc (nM)</th>
<th>Mean Peak Area Ratio</th>
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<th>%RSD</th>
<th>No. of Samples</th>
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All the samples were prepared by individually over-spiking ADMA and SDMA standard solutions and internal standard N-propyl-arginine (NPLA) into commercial human plasma followed by strong cation exchange (SCX) purification and LC-MS/MS analysis. Four samples at each concentration prepared in the same day were used to assess the intraday precision. Two samples at each concentration prepared for three consecutive days were used to assess the interday precision.

7.3.9 Accuracy

The accuracy of an analytical method is defined to be the closeness of the mean concentration determined by the analytical method to the true concentration of the analyte. The
accuracy of this method was assessed by testing four concentrations in the range of expected 3-nitrotyrosine basal concentration and using four to six samples per concentration. According to the FDA Guidance for Industry Analytical Method Validation, the mean value should be within 15% of the true value at tested concentrations (Table 17). Our calculated concentrations are reasonably close to the known concentrations, with relative error or inaccuracy no greater than 15% in all cases.

Table 17: Intraday and interday accuracy parameters of the LC-MS/MS method for asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) quantitation

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<tr>
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<th>Mean Cal Conc (nM)</th>
<th>%RE</th>
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Methods of sample preparation and analysis have been already described in Table 16 legend. Calculated concentration is derived by using: \([\text{Mean Cal Conc} (\text{nM}) = (\text{Mean Peak Area Ratio} - a)/b]\) where \(a\) is the \(y\)-intercept and \(b\) is the slope of the calibration curve. Relative error is derived by using equation: \(\text{Relative Error \%} = ((\text{Mean Cal Conc}) - \text{[True Conc]})/\text{[True Conc]} \times 100\%\).
7.3.10 LOD, LOQ and LLOQ

The LOD and LOQ are defined to be the concentrations at which ratios of the analyte signal to the background noise are 3 and 10, respectively. To obtain values applicable to human plasma and take every step into consideration, including sample pre-treatment, we first measured the peak areas of ADMA, SDMA and their internal standard in the zero sample. We then used the slope from the calibration curve to translate the peak area ratio of ADMA or SDMA over internal standard to their endogenous concentrations. From the signal to noise ratio at this endogenous concentration we determined that the LOD and LOQ of this method were 2.6 nM and 8.7 nM for ADMA, and 2.5 nM and 8.3 nM for SDMA, respectively.

LLOQ is defined to be a concentration at which the analyte peak is identifiable, discrete, and reproducible with a precision of 20% and accuracy of 80-120%. Experimentally, we proved the LLOQ was below 25 nM since 25 nM was tested to be acceptable regarding precision and accuracy (<15%).

7.3.11 Absolute recoveries and matrix effects

Evaluation of matrix effects is important because matrix composition differences existing among biofluidic samples may cause pronounced variations in ionization and thereby in the LC-MS/MS response. Although experimental design for assessing matrix effects for exogenous analyte has been published [96], there lacks a guideline to evaluate matrix effect (or relative recovery) [63] for endogenous analyte. Evaluation of matrix effect for endogenous analyte is difficult since the matrix itself contains unknown amount of endogenous analyte. Preparing analyte-free authentic matrix or using surrogate matrix [41] is possible, but neither of them represents the real matrix and the procedure to prepare them is error prone.

Absolute recovery [63] or process efficiency (PEs) [96] is the product of SPE recovery and matrix effect. It is the overall performance index reflecting the efficiency of SPE and the influence of matrix effect. Coefficient of variation or relative standard deviation of absolute recovery is therefore usually larger than that of matrix effect [96] or SPE recovery individually. On the other hand, absolute recovery acts as a more comprehensive and accurate validation index of a analytical method than SPE recovery or matrix effect alone. From a practical point of view, evaluating absolute recovery, SPE recovery and matrix effect all together is very tedious and probably unnecessary. For instance, 105 samples had been used to evaluate all these parameters in a study [96] for a single exogenous analyte. In our opinion, evaluation of absolute recovery
alone reduces a lot of unnecessary validation work but remains sufficient to validate a analytical method.

Here we modified the literature procedure [96] and adapted it to evaluate the absolute recovery of endogenous analyte. First, we obtained the peak area of an analyte in a native matrix after SPE, derivatization and LC-MS/MS analysis. Then the peak area of an analyte in a matrix spiked with authentic standard before SPE was determined. We subtracted the peak area of the analyte in native matrix from the total peak area of the analyte in matrix pre-spiked with authentic standard. This difference was divided by the peak area of authentic standard suspended in water to yield absolute recovery. Five lots of plasma samples were used to assess absolute recoveries. The absolute recoveries for ADMA were 93.97 ± 7.05% at 0.500 µM and 93.79 ± 6.41% at 0.125 µM, while the absolute recoveries for SDMA were 97.66 ± 9.56% at 0.500 µM and 105.07 ± 10.30% at 0.125 µM. Although we employed non-isotopic internal standard, the matrix effect can be contained pretty well owning to careful chromatographic elution and selective derivatization reaction. Theoretically, this derivatization reaction may suppress the primary amine signals under positive ionization mode and eliminate most ionization competition from matrix components. Changing NPLA to isotopic internal standard is predicted to give better results, but the high cost of preparing isotopic internal standard, concerns about isotopic internal standard purity and likely “cross-talk” between isotopic internal standard and analyte are intrinsic drawbacks of using an isotopic internal standard.

7.3.12 Stability

Although the method using OPA-2-ME as a derivatizing reagent, reported by Martens-Lobenhoffer et al. [180], gave good precision and accuracy when applied to ADMA and SDMA analysis, the stabilities of these derivatives have not been evaluated. Here, we assessed the stabilities of NDA products of ADMA and SDMA in comparison with the stabilities of their OPA-2-ME products. An exponential model was used to determine the best fit, giving an acceptable $R^2$ value for all regressions (Figure 28). The degradations of those products conform to first order kinetics, in which case, the half lives of these products can be derived from $t_{1/2} = \frac{\ln(2)}{k}$. From the best-fit exponential equations, the elimination constant ($k$) values of ADMA-NDA, SDMA-NDA, ADMA-OPA-2-ME, and SDMA-OPA-2-ME derivatives are 0.008, 0.008, 0.325 and 0.316 respectively. Accordingly, the half-lives of the NDA products were both 86.6 hours while the half lives of the OPA-2-ME products of ADMA and SDMA were 2.2 hours and 2.1 hours, respectively. In conclusion, the NDA products have much better stabilities than their
OPA-2-ME counterparts. We can anticipate that the analysis of these NDA products would offer improved accuracy and precision over that of OPA-2-ME products, especially when running multiple samples.

Figure 28: Stability comparison between ADMA OPA-2-ME derivative and AMDA NDA derivative

*Panel A*: the stability chart of ADMA and SDMA OPA-2-ME derivatives; *Panel B*: the stability chart of ADMA and SDMA NDA derivatives.
7.3.13 Patient samples

The ADMA and SDMA concentrations in patient plasma were quantified in 123 patients. The calculated concentrations were largely consistent with the low-end values reported in literature [173, 180, 355-359, 362-366, 375], with ADMA concentration averaging at 298.1 ± 11.2 nM (Mean±SEM) and SDMA concentration averaging at 457.7 ± 19.8 nM (Mean±SEM), respectively. Retrospectively, the average human plasma concentrations quantitated by LC-MS/MS are lower than those quantitated by other methods. This is probably due to the superior selectivity provided by LC-MS/MS compared to other alternative methods.

ADMA’s inhibitory effect of nitric oxide production might be a significant contributing factor in increased morbidity and mortality after on-pump CABG surgery. ADMA concentrations in patient plasma quantified by a robust, simple and accurate method prior to on-pump CABG surgery may be used for guiding clinician’s post-surgical care decisions and predicting patients’ clinical outcome. While it is important to relate patients’ clinical outcomes with ADMA predetermined concentrations, at the present time we are not able to unblind this clinical trial. Further statistical correlation of patients’ characteristics and clinical outcomes with ADMA concentration is warranted for a better understanding of ADMA’s clinical significance as a risk factor for on-pump CABG surgery.

7.4 Conclusion

In conclusion, we have developed a new derivatization method to label ADMA and SDMA. The derivatized products can be baseline separated within 15 min on a C18 reverse phase column. Labeling ADMA and SDMA with NDA in the presence of 2-ME as a catalyst gives corresponding 2,3-dihydro-benzo[f]isoindol-1-ones, which exhibit superior stabilities compared to OPA/2-ME derivatives. LC-MS/MS analysis of these 2,3-dihydro-benzo[f]isoindol-1-ones was used to determine ADMA and SDMA plasma concentrations in 123 patients prior to undergoing on-pump CABG surgery. Overall, this method is simple, sensitive, selective, reproducible, and accurate under LC-MS/MS mode. ADMA’s significance and implication in relation to clinical outcomes following on-pump CABG surgery will be the subject of future investigations.

There are certain limitations of this method. First, if more expensive isotopic internal standards had been employed, authors should have been able to shorten the time of analysis. Second, if a higher HPLC flow rate had been used (e.g 0.8 to 1.0 mL/min), we would have significantly shortened the time of analysis. However, as we were limited by the flow rate of
Varian 1200 L that has a maximum of 0.4 mL/min, other instruments such as Sciex API 4000 are recommended. Third, we may explore the upper limit of quantitation (ULOQ) in the future if such concentrations are relevant in other biological fluids. Comparison of this very method with other published works is also important to undertake because cross-lab variations can undermine the quality of patient data if care is not taken.
8. Closing remarks and future perspectives

In the last decade, biomarker research has been a focus of biomedical research to meet urgent clinical diagnostic and prognostic needs for thousands of patients. “Omics”, including genomics, proteomics and metabolomics, provide technologies through which hundreds of biomarkers are discovered. Despite the success of omics to identify the biomarker leads in preclinical stage, testing the biomarkers in real patients clinically is largely stymied by the lack of standardization of analytical method validation protocols. The development, validation and application of suitable analytical methods for biomarkers are essential elements to assure the quality of clinical programs, whether they are the clinical studies for drug therapy, surgery intervention or mechanism investigation.

Coronary artery bypass grafting (CABG) surgery proves to be the most effective way to reduce the mortality and morbidity of the patients with severe coronary heart diseases (CHD). Cardiopulmonary bypass (CPB) in CABG, also known as on-pump CABG, creates a motionless field for surgeons to operate an electrolyte-silenced heart. The interception then re-establishment of oxygenated blood into heart trigger a cascade of biochemical and biophysical events that cause ischemia reperfusion injury (IRI). The identification and quantitation of biomarkers that respond to on-pump CABG, prevent or aggravate IRI are instrumental to the prognosis of this injury and patient clinical outcome.

Ischemia preconditioning, volatile anesthetic preconditioning and anti-oxidant cardioprotection are therapies that protect the heart under on-pump CABG from IRI. Propofol is an anesthetic and antioxidant that exhibits cardioprotective properties in on-pump CABG surgery when given at a high dose. Investigation of prognostic biomarker changes may elucidate the mechanisms underlying the propofol cardioprotective efficacy.

Propofol concentrations that produce routine anesthetic effect during on-pump CABG surgery are not sufficient to provide cardioprotective benefits. Our previous laboratory research showed that only a propofol concentration at 5 μg•mL\(^{-1}\) or above might effectively protect myocardial cells from oxidative damages. Thus achieving 5 μg•mL\(^{-1}\) of propofol concentration in patients through continuous infusion is a pre-requrement to assess propofol cardioprotective effect against IRI during on-pump CABG. Finding a practical infusion rate that produces a cardioprotective propofol concentration without inducing hemodynamic instability relies on the accurate measurements of propofol concentrations in patient blood.
I developed a simple CE-UV method to measure clinically relevant propofol concentrations in 400 µL of whole blood from on-pump CABG patients. The method was able to detect propofol at a low µg/mL level with acceptable precision and accuracy. The development and validation work of this method was presented and discussed. All the performance parameters during validation are found satisfactory. I further applied the method to quantify propofol concentrations in blood samples from 30 patients. The method showed good robustness in the preliminary clinical application [271].

Following the development and validation of CE-UV method for propofol quantitation, twenty-four patients were randomized to receive one of the three propofol infusion rates; 50, 100, or 150 µg•kg⁻¹•min⁻¹ prior to the on-pump CABG in an open-label pilot study. Cardiac index (CI), systemic vascular resistance index (SVRI), and left ventricular stroke work index (LVSWI) were monitored to ensure hemodynamic stability. The propofol concentration (y) was plotted as a function of infusion rate (x) to give the best-fitted equation of $y = 0.215e^{0.0279x}$ ($r^2 = 0.781$). From this equation, a concentration of 5 µg•mL⁻¹ entails an infusion rate at 113 µg•kg⁻¹•min⁻¹. Based on this prediction, a practical infusion rate of 120 µg•kg⁻¹•min⁻¹ was chosen in a confirmative study. This rate produced a concentration of 5.39 ± 1.45 µg•mL⁻¹ and stable hemodynamics, meeting the initial goals of this study [376].

Several prognostic biomarkers were selected to investigate the mechanism underlying the cardioprotective effect of propofol against IRI. Independent development and validation of the analytical methods used to evaluate these prognostic biomarkers provide feasibility and quality assurance of the mechanism investigation. For a better understanding of IRI, prognostic marker levels were also assessed against patient profiles and clinical outcomes.

To evaluate oxidative stress biomarker, 15-F₂-isoprostane, I developed a LC-MS method modified from a literature procedure. 15-F₂-isoprostane in human plasma is selectively trapped by an immuno-affinity cartridge and then eluted with 95% ethanol prior to SpeedVac concentration. The length of each run is considerably shortened due to the use of basified mobile phase and base-resistant Gemini-NX C18 column. Isotopic internal standard 15-F₂-isoprostane-D₄ and authentic standard 15-F₂-isoprostane are monitored in MS channels with mass to charge ratios (m/z) at 353.1 and 357.1 respectively. The analytical performance of this method is generally satisfactory. The calibration curve has a coefficient of determination: $r^2 = 0.9991$ across the tested range, with the equation: $y = 1.4418x + 0.9972$. Precision and accuracy are evaluated on the over-spiked of authentic 15-F₂-isoprostane at clinical relevant concentrations (1000, 100, 50 and 25
pg•mL\(^{-1}\)) into commercial human plasma. Limit of detection (LOD), limit of quantitation (LOQ) and lower limit of quantitation (LLOQ) are determined to be 3.06, 10.21 and 50.0 pg•mL\(^{-1}\). The baseline concentration of 15-F\(_2\)-isoprostane was significantly greater in coronary sinus plasma from the diabetic patients compared with the nondiabetic patients. The linear regression analysis between the 15-F\(_2\)-isoprostane and Fasting Blood Glucose levels revealed a significant positive correlation \((p = 0.02, r = 0.3869)\). Also found is a significant positive correlation between the PTEN/β-actin and 15-F\(_2\)-isoprostane levels \((p = 0.008, r = 0.4445)\). However, no statistical difference in 15-F\(_2\)-isoprostane levels was seen between the different treatment groups (propofol versus isoflurane), clinical outcomes (low cardiac output syndrome versus non-low cardiac output syndrome) and patient profiles (diabetics versus non-diabetics) [377].

To evaluate nitrosative stress biomarker, 3-nitrotyrosine, I developed a sensitive and selective LC-MS/MS method to measure the free 3-nitrotyrosine concentrations in the human plasma of patients receiving on-pump CABG surgery. The analyte at low nM concentrations was pre-purified and enriched by a one-step SPE procedure. The pre-purified analytes were separated from other interfering substances on a 2.1x100 mm Kinetex PFP column and then detected by a triple quadrupole mass spectrometer. I validated the method at 3-nitrotyrosine concentrations near its nominal plasma concentrations. The RSDs or REs of the intraday and interday determinations were no more than 10%. LOD, LOQ and LLOQ were 0.034 nM, 0.112 nM and less than 0.625 nM, respectively. Neither the column performance nor precision was compromised after running 80 patient samples. The elevation of 3-nitrotyrosine concentrations in coronary sinus plasma samples was noticed after the on-pump CABG procedure. The concentrations of 3-nitrotyrosine prior to and post to CABG procedure were 1.494 ± 0.1065 nM and 2.167 ± 0.1770 nM (Mean ± SEM), respectively. Propofol and isoflurane treatments didn’t seem to affect 3-nitrotyrosine concentrations. Patients with low cardiac output syndrome experienced more 3-nitrotyrosine increase than patients without this syndrome. Diabetic patients were more susceptible to 3-nitrotyrosine elevation than non-diabetic patients. From this study, it appeared that nitrosative stress might be contributing to low cardiac output syndrome (LOCS) and put diabetic patients at risk. Prevention or mitigation of nitrosative stress is a promising strategy to improve clinical outcome for the patients undertaking on-pump CABG surgery [378].

To evaluate the change of myocardial protective factor, adenosine during CABG-CPB, I developed a sensitive and selective LC-MS/MS method to measure the basal adenosine concentrations in the human plasma of patients receiving on-pump CABG surgery. The analyte was pre-purified and enriched by a SCX monolithic cartridge to increase robustness and reduce

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analytical complexity. The mobile phase employed was free of any modifier to improve sensitivity and precision. The method demonstrated acceptable precision and accuracy with %RSDs or %REs of intraday and interday determinations all within 10%. The linearity ranging from 1 nM to 500 nM was satisfactory \( (r^2 = 0.996) \). LOD, LOQ and LLOQ were 0.257 nM, 0.857 nM and < 10 nM, respectively. Changes of adenosine basal concentrations in the plasmas of the patients receiving on-pump CABG surgery were monitored by this method. It was found that the mean plasma concentration of adenosine was significantly more elevated in coronary sinus after surgery as compared to in coronary sinus before the surgery \( (p = 0.0024; \) two-tailed \( t \)-test) and in radial artery after the surgery \( (p = 0.0409; \) two-tailed \( t \)-test). These findings implied that the equilibrium between the adenosine production and elimination might shift towards the adenosine production during on-pump CABG surgery and the adenosine equilibrium shift is heart specific. Subanalysis found treatment doesn’t have impact on adenosine regulation. Nor does outcome. But patients with abnormal left ventricular function tend to have higher adenosine output after the surgery, suggesting an adenosine-dependent compensatory vasodilation may exist for patients with left ventricular dysfunction. On the other hand, diabetic patients seem to have lower adenosine output than diabetic patients, but the difference was not significant [379].

To evaluate ADMA, a risk factor of cardiovascular events, I developed a new method to separate ADMA and its regio-isomer SDMA within 15 min on a C18 reverse phase column after derivatizing them with naphthalene-2,3-dicarboxylaldehyde (NDA). Catalyzed by 2-mercaptoethanol (2-ME), the reaction of ADMA and SDMA with NDA produces corresponding 2,3-dihydro-benzo[f]isoindol-1-ones that showed greater stability than previously reported ortho-phthaldialdehyde (OPA) and 2-ME derivatives. The separation and detection of these derivatives by LC-MS/MS can be used to measure ADMA and SDMA concentrations in only 50 µL of plasmas of patients receiving on-pump CABG surgery. The LOD, LOQ and LLOQ of this method were 2.6 nM, 8.7 nM and 25 nM for ADMA, and 2.5 nM, 8.3 nM and 25 nM for SDMA, respectively. The %RSDs and %REs of the intraday and interday determinations are no more than 15%. The ADMA and SDMA concentrations in patient plasma were determined to be 298.1 ± 11.2 nM (Mean ± SEM, \( n = 123 \)) and 457.7 ± 19.8 nM (Mean ± SEM, \( n = 123 \)) respectively. The determined values of ADMA will be assessed against the patient clinical outcome and their responses to pharmacological interventions upon the unblinding of our clinical trial [380].

To this end, all the oxidative stress, nitrosative stress, and adenosine regulation failed to explain the cardioprotective effect of propofol. But during this study, the elevations of oxidative
stress and nitrosative stress biomarkers, along with the upregulation of adenosine are found during on-pump CABG surgery, confirming that the occurrence of oxidative stress, nitrosative stress and retaliatory cardioprotection in response to the surgery.

I realize that there are certain limitations associated with this research. First, the size of the study subjects is only approximately 100 for most statistical analyses. Therefore small differences might not be detected during this study. Second, the criteria I used to identify the clinical outcome groups might not be reflective of true clinical endpoints. There are other criteria I can use to categorize the clinical outcome groups. For example, I divided the patients into LCOS or Non-LCOS groups in this study. But other serious adverse events secondary to the surgery or drug therapy should be also taken into account. Third, I used average values to compare the biomarker levels in patients. However, these values sometimes could not represent the biological significance. In some situations, a threshold value above a certain physiological range is clinically meaningful. For example, 15-F₂-isoprostane might not indicate oxidative stress unless it is greater than a threshold value. These limitations warrant more comprehensive statistical analyses within a clinical context. In addition, I was not shown all of the clinical results while I was doing the analyses, therefore, it is anticipated that more useful discoveries might be forthcoming once all of the analytical and clinical information is combined.

Ideally, the proteins, peptides and small molecule metabolites ought to be comprehensively cataloged with untarget proteomics, peptidomics and metabolomics prior to targeted quantifications. High-throughput omics may generate an initial list of targets in a timely and comprehensive fashion and allow researchers to get to the bottom of a research question. We chose a targeted approach owing to the limited access of these technology platforms for the time being as well as a limited sample repository in clinical studies. To overcome the first limitation, we can extend the collaboration with other research labs or take steps to build up our own capability. To address the later limitation, we can use cell or animal models for preliminary testing and strategically preserve precious clinical samples for turn-key experiments.

LC-MS and CE-MS are universal platforms for preclinical biomarker research as well as clinical biomarker research. The experience gained in one stage will benefit the other. For example, when hyphenated with mass spectrometer, nano-LC offers better sensitivity because of its enhanced ESI efficiency. Nano- or micro-bore columns that were previously used for biomarker discovery in preclinical biomarker research were recently applied for the absolute quantitation of a number of proteins clinically [381]. In the future, we will strive to innovate LC-
MS and CE-MS instrumentation that is well suited for speed, sensitivity and selectivity requirements of both preclinical and clinical biomarker research. Streamlining biomarker discovery in preclinical biomarker research and the development, validation and application of analytical method in clinical biomarker research on the same technology platform will help reduce cost, improve efficiency and generate unbiased biomedical knowledge. Fundamental breakthrough in instrumentation will ultimately have a far-reaching impact on biomarker research that revolutionizes disease diagnosis, prognosis, prevention and treatment.
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