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INVESTIGATION OF INOSINE AND HYPOXANTHINE AS BIOMARKERS OF CARDIAC ISCHEMIA IN PLASMA OF NON-TRAUMATIC CHEST PAIN PATIENTS AND A RAPID ANALYTICAL SYSTEM FOR ASSESSMENT

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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Inspiration and Dreams

"The best and most beautiful things in the world cannot be seen, nor touched…but are felt in the heart." Helen Keller (1880-1968)

"The future belongs to those who believe in the beauty of their dreams." Eleanor Roosevelt (1884-1962)

It has always been a dream of mine to make a memorable contribution to this life's journey. The notable and excellent quotes above best describe what this particular research has meant to me. I have always wanted to use what knowledge and experiences I have attained in school and life, to contribute to the well being of others in need, hence this research helped fulfill a sense of purpose and contribution as part of my life's journey. So to be able to follow one of my dreams and finish this most intriguing research, which may someday benefit society, has been a great honor and experience for me. Several individuals close to me are primarily responsible for inspiring me to finish this graduate school and I would like to recognize them. The person most responsible for me finishing this graduate work is my mother, Setsuko aka "Momason", whom I owe sincere thanks for her constant words of motivation and encouragement.

Another individual who also inspired me is our daughter, Christine, who through her years of a serious medical ailment has shown me what real strength, determination, and the passion to succeed in life are all about. She will graduate from college Virginia

Commonwealth University (VCU) in the coming year and is already looking at professional or graduate school; which is a remarkable feat given what she had gone through for many years. And many accolades to her brother Bryan, who has shown me what sibling love and support are all about, as he stood strongly by his sister and family. We are very blessed to have two wonderful children to watch grow up, have their own dreams and to make contributions to their journey in life. I end with the encouragement to others, to follow your dreams and use your instincts and heart to help you make good choices in life. If you have a passion for something, pursue it vigorously, and give it your best efforts. Isn't that what life and this part of our journey are truly all about?

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I would also like to acknowledge my parents and family members. I probably would never have decided to pursue a graduate degree without the strong push from my parents. Our parents instilled to all their children to pursue continuous self improvement throughout life, which is why I'm here at the tender age of 49. My mother (Setsuko) and father (Harold) deserve all the credit, and they were always there to support their kid's academic efforts; and for this I can not thank them enough. Other immediate family members (Carol, Christine, Bryan, Daniel, Donna and Debby) are each thanked for their continued support of me finishing school, no matter how old I am. Of course, there has to

be some family competition in this matter, as our daughter (Christine, senior at VCU) and son (Bryan, sophomore at VCU) may graduate before me. So there is added pressure for me to finish graduate school before they do; this I believe is called family pride, but it really has helped me to remain focused on the goal of finishing up now.

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Table of Contents

Page

List of Tables

Page

List of Figures

Page

List of Abbreviations

ACB	albumin cobalt binding
ACS	American Chemical Society
ADA	adenosine deaminase
ANOVA	analysis of variance
ANP	atrial natriuretic peptide
ASA	acetyl salicylic acid
ATP	adenosine triphosphate
AUC	area under the curve
BD	Becton Dickinson
Blk	blank
BNP	brain natriuretic peptide
°C	Celsius (degree)
CID	collision induced dissociation
CK-MB	creatine kinase - muscle brain
COX	cyclo-oxygenase
CRP	C-reactive protein
cTnI	cardiac troponin I
cTnT	cardiac troponin T
Ctrl	control

DAD	diode array detector
Dalton	Da
DF	developed force (cardiac)
DHBA	dihydroxybenzoic acid
DI	deionized water
ECG	electrocardiogram
ED	emergency department (hospital)
EDTA	ethylenediaminetetraacetic acid
EMS	Emergency Medical Services
ESI	electrospray ionization
FDA	Food and Drug Administration
FFA	free fatty acid
GNC	General Nutrition Centers, Inc.
HA	heart attack
hCG	human chorionic gonadotropin
HIPAA	Health Insurance and Portability Accountability Act (1996)
HPLC	high pressure liquid chromatography
HR	heart rate
HRP	horseradish peroxidase
Hypo	hypoxanthine
ICR	Institute of Cancer Research
IMA	ischemia modified albumin

Ino	inosine
IRB	Institutional Review Board
kDa	kilo dalton
LC-MS	liquid chromatography mass spectrometer
LOD	limit of detection
MI	myocardial infarction
MW	molecular weight
MWCO	molecular weight cutoff (filter)
ND	none detected
·OH	hydroxyl free radical
Myo	myoglobin
PMT	photomultiplier tube
PNP	purine nucleoside phosphorylase
POC	point-of-care
RBC	red blood cell
RFP	rate-force product (cardiac)
RLU	relative luminescence unit
ROS	reactive oxygen species
RSD	relative standard deviation
RT	retention time
Rxn	reaction

SA	salicylic acid
SAR	super oxide anion radial
SAX	strong anion exchange
SEM	standard error of the mean
SST	serum separator tube
TCA	trichloroacetic acid
TFA	trifluoroacetic acid
U/ml	Units per ml (enzyme)
US	United States
UV	ultraviolet
VCU	Virginia Commonwealth University
WHO	World Health Organization
Xan	xanthine
XO	xanthine oxidase

Abstract

INVESTIGATION OF INOSINE AND HYPOXANTHINE AS BIOMARKERS OF CARDIAC ISCHEMIA IN PLASMA OF NON-TRAUMATIC CHEST PAIN PATIENTS AND A RAPID ANALYTICAL SYSTEM FOR ASSESSMENT

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Virginia Commonwealth University, 2008

Major Director: H. THOMAS KARNES, Ph.D.
PROFESSOR, DEPARTMENT OF PHARMACEUTICS

Each year in the U.S., approximately 7-8 million patients with non-traumatic chest pain visit hospital emergency departments (ED) for medical evaluation. It is estimated that approximately 2-5% of these patients are experiencing acute cardiac ischemia, but due to the shortcomings of current test methods, they are incorrectly diagnosed and discharged without appropriate treatment provided, thus leading to poor patient outcome and potential medical malpractice litigation. The goals of this research were to evaluate plasma samples

for potential biomarker(s) of acute cardiac ischemia prior to heart tissue necrosis, and to ultimately develop a rapid method for detection of the potential biomarker(s) in human plasma. Initial experiments were performed using the mouse model, with subsequent evaluations on human plasma samples using high performance liquid chromatographic ultraviolet detection (HPLC-UV). The final phase of this research involved the development of a rapid luminometer test method (<10 min analysis time objective) to be potentially used in the clinical laboratory environment.

An HPLC-UV detection method was developed and utilized for inosine, hypoxanthine and other adenosine triphosphate (ATP) catabolic by-products in Krebs-Henseleit (Krebs) buffer solution, with analysis on perfusate samples from isolated mouse hearts undergoing 20 min acute global ischemia. The HPLC-UV method was modified for subsequent use on human plasma samples, obtained from hospital emergency department (ED) patients presenting with non-traumatic chest pain (potential acute cardiac ischemia) and from healthy normal individuals. The HPLC-UV (component quantification) and HPLC-MS (component identification) test methods utilized C_{18} column technology, mobile phases consisting of aqueous trifluoroacetic acid (0.05% TFA in deionized water pH 2.2, v/v) and methanol gradient to achieve component separation, with both utilizing simple sample preparations (e.g. direct injection of Krebs perfusate samples and centrifugal membrane filtration on plasma samples).

Results of the animal experiments using isolated mouse hearts undergoing 20 min acute global ischemia demonstrated significant levels of endogenous inosine effluxed from the heart tissue, indicating its use as a potential candidate biomarker of acute cardiac

ischemia. The HPLC results from human plasma representing ED non-traumatic chest pain patients demonstrated elevated levels of inosine (hypoxanthine precursor) and significant levels of hypoxanthine, which provided additional support for the use of these candidate biomarker(s) as a potential diagnostic tool for the initial acute cardiac ischemic event, prior to heart tissue necrosis.

The final phase of this research focused on the development of a rapid, simple and sensitive chemiluminescence test method. Using a microplate luminometer with direct injectors and continuous mixing, the measurement of inosine and hypoxanthine in human plasma was achieved for healthy normal individuals and on patients with confirmed acute MI, with an analysis time of less than 5 minutes. The utility of this rapid luminescence technique would be the potential use at point-of-care (POC) services (e.g. hospital clinical laboratory or emergency medical services) as part of the initial ED treatment protocol on patients presenting with non-traumatic chest pain and signs/symptoms of acute myocardial ischemia or acute MI.

CHAPTER 1. Background and Significance

1.1 Introduction and Request for Additional Biomarkers

Cardiovascular diseases (e.g. acute myocardial infarction (MI)) are the leading cause of mortality in the world [Naudziunas et al., 2005; Okrainec et al., 2004; Dorner et al., 2004, AHRQ, 2000]. Each year in the US, approximately 7-8 million patients present with non-traumatic chest pain and seek emergency medical treatment [Morrow et al., 2007]. Current emergency medical evaluation on these patients suspected of having acute MI includes obtaining patient history, signs and symptoms, vitals, electrocardiogram (ECG) and blood evaluation for specific cardiac biomarkers [Beyerle, 2002; A.D.A.M. Inc., 2005; Lees, 2000]. However, the percent diagnostic accuracy of acute MI when using patient signs and symptoms, ECG and c-troponin is only approximately 50%. With the addition of the recently FDA cleared albumin cobalt binding assay, the diagnostic accuracy improves to approximately 70%; hence the need for additional research for biomarkers of acute cardiac ischemia to further improve patient diagnostic accuracy is important.

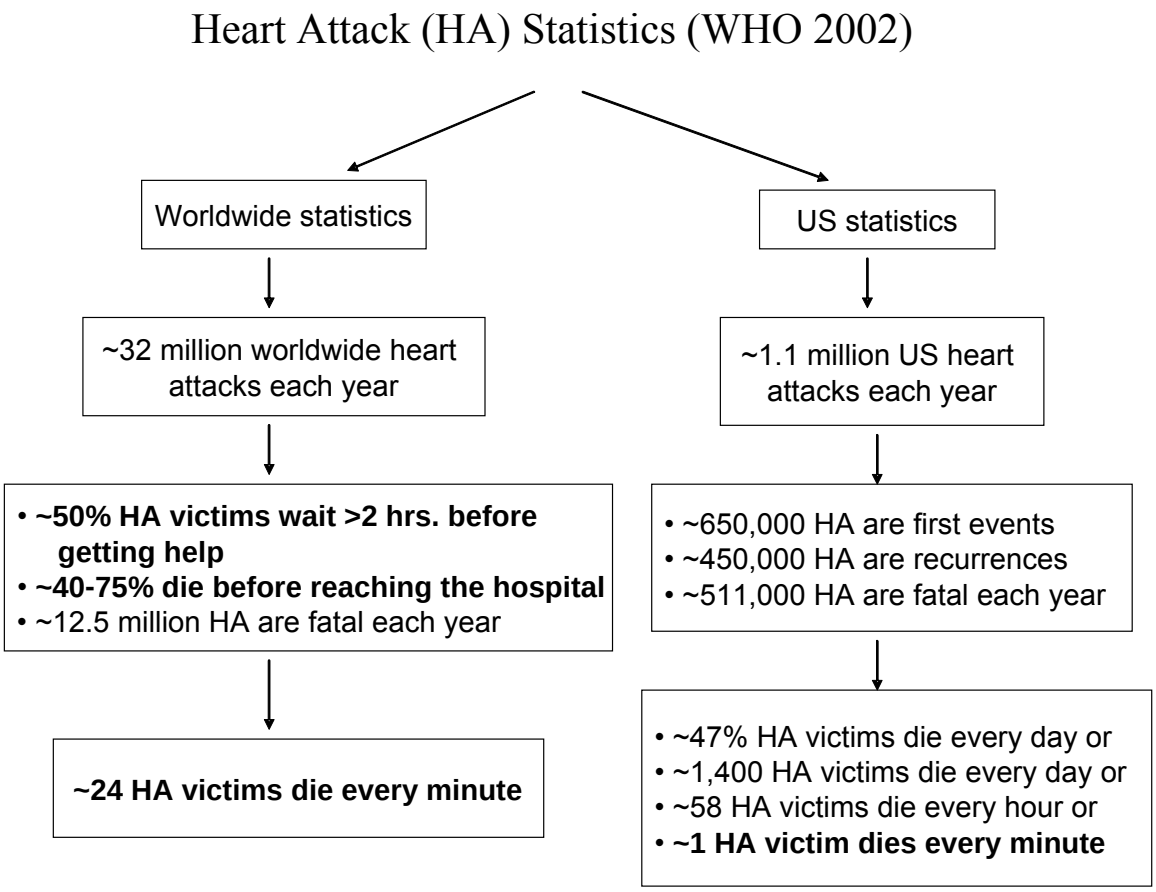
The hospital emergency department blood evaluation determines levels of several specific endogenous cardiac protein biomarkers (e.g. cardiac troponin I and T (cTnI, cTnT), creatine kinase-MB (CK-MB) isoform, and myoglobin). However, these protein biomarkers are indicative of cardiac tissue necrosis, and are typically detected hours after

the acute cardiac event (infarct), and not at the time of acute cardiac ischemia, which may include angina (stable or unstable, but non-necrotic).

One recent published scientific editorial requested the need for early onset biomarkers of acute cardiac ischemia prior to cardiac tissue necrosis [Morrow et al., 2003]. Ideally, these early onset biomarkers would aid emergency medical services (EMS) personnel in the rapid diagnosis and treatment of initial acute cardiac ischemia (potentially acute MI), thus increasing the survival rate of acute MI victims every year. One research group [Bhagavan et al., 2003] addressing the scientific editorial request, describes a blood measurement for ischemia modified albumin (IMA), which appears at an elevated level in the bloodstream from patients undergoing an ischemic cardiac event; however the author's state that the colorimetric test would not discriminate between cardiac ischemic patients with and without acute MI (e.g. angina), and recent clinical evaluations of the test assay have reported significant false positive results.

Table 1 depicts the US and world wide heart attack (HA) statistics compiled in 2002 and clearly demonstrates the critical need for additional endogenous biomarkers of early onset cardiac ischemia [World Health Organization (WHO), 2002]. As can be seen from the WHO heart attack statistics table, approximately 24 individuals die every minute in the world from heart attacks, with many individuals waiting more than two hours before seeking medical attention. The American Heart Association cites that approximately 75-80% of the heart attacks occur at home, with up to 95% dying before ever reaching a hospital [American Heart Association Statistics, 2007]. Thus, the need for continued

Table 1. World Health Organization (WHO) 2002 Heart Attack Statistics.



additional research for additional biomarkers of acute cardiac ischemia is critical in addressing the health care community requests [Maisel et al., 2005, Morrow et al., 2007, Apple et al., 2005].

1.2 Biomarkers, Use and Requirements

Biomarker is a term defined by the U.S. Food and Drug Administration (FDA) as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic or pathogenic processes or pharmacological responses to a therapeutic intervention." [FDA, Rockville, USA]. Examples of current biomarkers in human blood that are commonly used to aid in diagnosis and treatment of medical conditions are hemoglobin-A1c and glucose levels for diabetes, cardiac protein troponin I (cTnI) and creatine-kinase MB (CK-MB) isoenzyme (both being very specific to cardiac tissue) for acute MI, and hormone human chorionic gonadotropin (hCG) for pregnancy testing [Bloom et al., 2003].

A biomarker is classified as a surrogate endpoint, which is an objective characteristic, intended to substitute for a clinical (and sometimes subjective) endpoint [Biomarker World Congress 2007, USA]. For example, one clinical endpoint for acute MI can be how the person feels (e.g. chest pain, nausea, fatigue), which can be subjective and caused by other various medical conditions (Table 2). However, using current biomarker surrogate endpoints for acute MI (elevated plasma concentrations of cardiac proteins myoglobin, CK-MB and cTnI or cTnT) significantly improves patient diagnostic accuracy, and may led to prompter emergency medical intervention (treatments), and improved

Table 2. Listing of various medical conditions causing non-traumatic chest pain other than acute cardiac ischemia and acute MI. Information tabulated from Prehospital Emergency Care, 7th Edition, Mistovich et al., 2004.

Condition	**Symptom profile**
Gastroesophageal reflux	Dull to sharp chest pain, chest pressure, nausea and vomiting
Acid Reflux	Dull chest pain, chest pressure, heartburn
Angina	Breathlessness or choking feeling, heavy weight or tightening across the upper chest
Musculoskeletal	Sharp pains confined to a specific area of the chest
Pneumonia	Sharp pain on side of the chest, anxiety, rapid breathing
Aortic Dissection	Ripping or tearing pain, shortness of breath, abdominal pain, fainting
Nerve Impingement	Shooting, burning pain, numbness
Pulmonary Embolus	Shortness of breath, rapid breathing and sharp pain in the mid chest
Spontaneous Pneumothorax	Shortness of breath, sharp chest pain, rapid heart rate, dizziness
Acute Pericarditis	Sharp or stabbing pain in the mid chest
Heartburn	Burning pain in chest, nausea, vomiting
Asthma	Shortness of breath, wheezing or cough
Anxiety	Palpitations, sweating, muscle tension, fatigue
Peptic Ulcer	Abdominal pain below the sternum, nausea, vomiting

patient outcomes. A "routine biomarker" is currently defined as the use of FDA approved commercial kits and assays for testing in diagnostic labs (e.g. cTnI and CK-MB are routine biomarkers used as indicators of acute MI). A "novel biomarker" is currently defined as a non-routine kit or assay "for research only," and typically utilized in biotechnology (discovery) or academic environments [Biomarker World Congress 2007, USA].

An example of how one recent novel biomarker progressed into a routine biomarker is the current use of ischemia modified albumin as an indicator for acute cardiac ischemia. A detailed description of this test will be later described in Section 1.4. Many years of research (>10 years) went into the development of using ischemia modified albumin as a potential biomarker of acute cardiac ischemia, with the first US patent being filed in 1991 [Bar-Or et al, 1991], and subsequent FDA clearance for use 12 years later in 2003 (assay was initially called albumin cobalt binding (ACB) and is currently called ischemia modified albumin (IMA)).

Some considerations are important in the discovery and development of a potential endogenous plasma biomarker. If the biomarker is endogenous to plasma, it may exhibit circadian fluctuations in basal concentrations levels. For example, C-reactive protein (CRP), which is currently recognized as an endogenous plasma biomarker of inflammation, has both seasonal and diurnal variation in a patient's basal plasma concentration. Thusly, if measuring CRP, it would have been important to document the date and time of the blood sample draw, as it may have affected the diagnostic interpretation of the laboratory sample results.

Several other important biomarker considerations are determining (or sourcing from reported literature) the half-life and whether co-existing medical conditions (e.g. kidney disease) affects the plasma biomarker concentrations. Knowing the component half-life is important as some plasma biomarkers have very short half-lives (e.g. inosine is less than 5 minutes), while others may have longer half-lives (e.g. cTnI can be detected for several days). It is well documented that patients diagnosed with chronic renal failure typically have high plasma levels of many constituents due to their kidney clearance problems. Thus the need for their frequent hemodialysis treatments, and potential errors in interpreting their plasma test results. Typically, plasma biomarker discoveries involve the use of techniques such as LC-MS, with subsequent clinical use of the FDA cleared biomarker typically employing automated clinical platforms utilizing immunoassay or enzymatic techniques [Biomarker World Congress, 2007].

Use of endogenous plasma biomarkers as medical diagnostic tools is a relatively new field in industry, as compared to discovery and development of drugs (pharmaceutical industry); however both industries are regulated by the US FDA. One important difference between these two regulated industries is that when using endogenous plasma biomarkers as a diagnostic tool, it is important to demonstrate the proof-of-concept (proof-of-biology) on how the level of the biomarker relates to the associated disease condition. Approval of pharmaceutical drugs typically requires clinical studies demonstrating patient safety and efficacy evaluations; however the exact biological mechanism-of-action is not always known (e.g. some cancer drugs are known to be efficacious, without fully understanding the exact mechanism of action).

For medical diagnostic biomarkers, it is important to link the biomarker with the disease condition and patient clinical outcome. This requires either published scientific knowledge or demonstration of the mechanism-of-action on how the endogenous plasma biomarker change is associated with the disease condition. For example, the human plasma level of cTnI is normally found at <0.05 μg/L in healthy normal individuals, but under conditions of acute cardiac MI, the levels of cTnI are significantly elevated (≥0.05 μg/L) due to the affected heart tissue (necrotic) being diffused into the bloodstream, which causes the increase in the concentration of cardiac troponins (e.g. cTnI, cTnT). Chapter 2 of this research utilizes the mouse model to demonstrate the proof-of-concept (proof-of-biology) that one ATP catabolic by-product, inosine, may be a potential candidate biomarker of acute global cardiac ischemia.

Current requirements for proposed biomarker test methods to address are component specificity and sensitivity. Specificity is a term defined as the uniqueness of the biomarker for the disease or condition for which it will be used to aid in patient diagnosis. Specificity is the test procedure's ability to measure a negative response (minimize false positives). Conversely, sensitivity is a term defined as the ability of a test procedure to measure a positive response. For many analytical measurements, sensitivity is directly related to the method detection limit and slope of the detector response relative to the concentration level of the analyte (e.g. low method detection limit and large positive slope typically indicates a sensitive test method). The test method detection limit is an important parameter to determine and is defined as the ratio of analyte signal to matrix and instrument electronic noise. The goal of a good biomarker test method is to possess both a

high specificity (reduce the number of false positive results) and high sensitivity (reduce the number of false negative results). Insufficient evaluation of these criteria may lead to poor patient outcomes (e.g. false positives may lead to erroneous medical treatment(s) and psychological stress and false negatives may lead to poor patient prognosis, especially for cancer patients).

As the field of medical diagnostics and use of biomarkers continues to grow, it is important to standardize the method development approach and validation requirements of biomarker assays. In recent years, representatives from pharmaceutical, biotechnology, and medical diagnostic companies have jointly collaborated, and drafted initial guidelines to be used for biomarker method development and validation, covering both qualitative and quantitative assays [Lee et al., 2006]. Conferences such as the Biomarker World Congress have several meetings annually with attending representatives (domestic and international) from industry, biotechnology, academia, and government (e.g. FDA).

Biomarker discovery and development success rate to the market place (i.e. clinical laboratory or point-of-care) is very similar to the pharmaceutical industry, with just a fraction of the discovered biomarkers being successfully utilized in the clinical laboratory. Although there has been approximately 4,000 biomarkers reported in the literature, less than 100 have been cleared for use by the FDA, and less than 10 are routinely used in the clinics [Biomarker World Congress, 2007]. Of the endogenous biomarkers that are currently being used, concentration level changes in plasma are all less than 10-fold increases, with most biomarkers having less than a 5-fold increase in concentration level [Biomarker World Congress, 2007]. As will be reported in Chapter 4 of this dissertation,

our proposed candidate biomarker(s) inosine and hypoxanthine from hospital room emergency room patients exhibiting non-traumatic chest pain; had plasma concentrations (called total hypoxanthine as inosine is converted to hypoxanthine using purine nucleoside phosphorylase enzyme) above the 5-fold increase typically found in the currently used FDA cleared biomarkers.

1.3 Biomarkers of Acute Myocardial Infarction

The biomarkers that are currently used for detection of acute MI are myoglobin, CK-MB and either cTnI or cTnT. These are biomarkers of cardiac tissue necrosis and can be used individually, but more emphasis has been placed on using panels of these cardiac biomarkers together (e.g. simultaneous evaluation of myoglobin, CK-MB and cTnI). The rationale for using a cardiac panel of biomarkers is that it improves the diagnostic accuracy, as ultimately more information is gathered to use for patient diagnosis. There has been other cardiac biomarkers of acute MI over the years, many being replaced as new discoveries find better biomarkers demonstrating higher specificity and sensitivity (e.g. lactate dehydrogenase replaced by current acute MI biomarkers).

As can be seen in Table 3 (Cortez Diagnostics, CA, USA) and Figure 1, the cardiac biomarkers have completely different onset and duration profiles (leading to different half-lives). Myoglobin (MW ~17 kDa) is found in muscle tissue and transports cellular oxygen consumed by muscle mitochondria. Although it is a very sensitive biomarker of muscle tissue necrosis, it is not totally specific to heart tissue and can also be found in other muscle tissue (e.g. skeletal). In a medical situation consisting of an ischemic event involving both cardiac and skeletal muscle, elevated levels of myoglobin may be present

Table 3. Cardiac tissue necrosis biomarker detection time after AMI onset, normal individual plasma range, and the biomarker duration time (Information from Cardiac Panel Test Strip Information Sheet, Cortez Diagnostics, CA, USA).

Marker	Detection time after onset of AMI	Normal range	Remain elevated	Test Strip cut-off level
Myo	1-4 hrs	30 - 90 ng/ml	< 12 hrs	70 ng/ml
CK-MB	4-6 hrs	< 5 ng/ml	~24 hrs	5 ng/ml
cTnI	10-12 hrs	ND	60-80 hrs	1 ng/ml

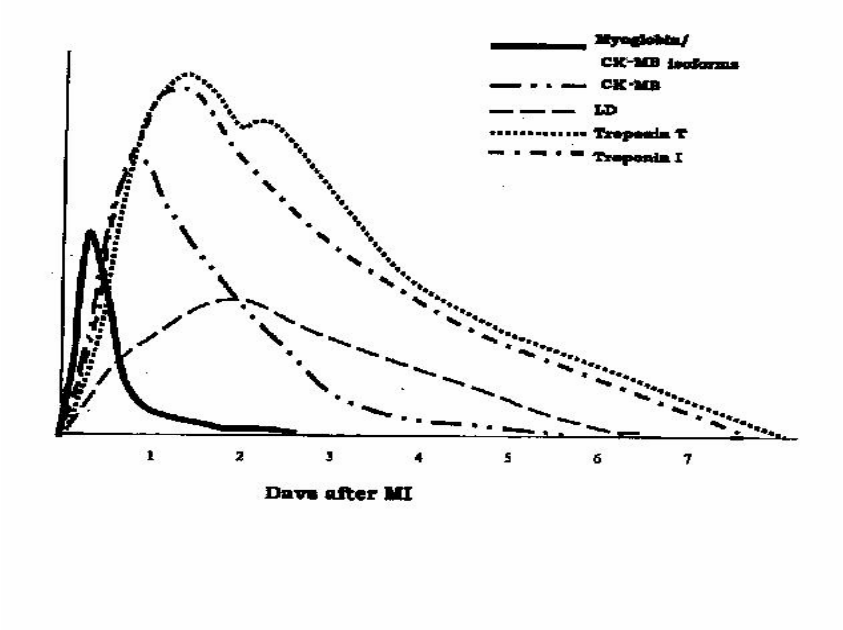


Figure 1. Serum profile of plasma biomarkers (myoglobin, CK-MB, troponins, and lactate dehydrogenase (LD)) representing human cardiac tissue necrosis. Profile depicts differing onset and duration of cardiac biomarkers after an acute MI event. Picture with permission from Futura Publishing Company, Inc., NY, USA.

from either form of muscle, thus leading to potential confounding results if only using myoglobin as the cardiac biomarker.

Instead, many clinical labs utilize multiple cardiac biomarkers (currently called cardiac panels) to improve patient diagnosis (increases predictive value). CK-MB (MW ~ 41 kDa) is an isoenzyme of the creatine kinase (CK) family of enzymes and is primarily found in heart muscle tissue. Although it is more specific to heart muscle tissue than myoglobin, it can not be detected until approximately 4-6 hours after the acute MI event. The latest cardiac biomarkers to be utilized are the cardiac troponins (cTnI, MW 24 kDa and cTnT, MW 34 kDa). These biomarkers are highly specific to cardiac muscle tissue, but like CK-MB, are not readily detected until 4-8 hours after the acute MI event. The high specificity of the troponin proteins can be explained as both are structural components of the thin filament of cardiac muscle tissue; hence necrosis of the affected cardiac tissue followed by the body's elimination of the necrotic tissue into the bloodstream, would cause a significant elevation of these biomarkers (which neither is normally found at detectable levels in the bloodstream with the currently used immunoassay techniques).

1.4 Biomarkers of Acute Myocardial Ischemia

There are several plasma biomarkers currently under evaluation for acute myocardial ischemia; however only one to date has been cleared for use by the FDA. The current biomarker assays are ischemia modified albumin (IMA), unbound free fatty acids (FFA_u) and choline [Apple et al., 2005]. Of the three, only IMA has received FDA clearance (2003) for marketing as a diagnostic cardiac biomarker. It is worthwhile to briefly discuss each of these candidate biomarkers as to their proof of biology (if known)

and why they appear elevated in the bloodstream under conditions of acute cardiac ischemia.

Choline ($HOCH_2CH_2N^+(CH_3)_3$) is one major product formed from the phosphodiesteric cleavage of membrane phospholipid phosphatidylcholine. During an ischemic event, phospholipase D (PLD) is activated and cleaves membrane phosphatidylcholine into phosphatidic acid and choline, which is subsequently released into the blood stream and can be detected. Evaluation of choline was made using LC-MS technology. At the time of the review article describing its potential use as a biomarker, it was recommended that the development of rapid point-of-care and central laboratory assay were still needed to further evaluate this biomarker's usefulness [Apple et al, 2005]. This recommendation is not surprising as most clinical laboratories utilize commercially bought kit assays, which are typically immunological techniques (e.g. enzyme linked immunosorbent assay (ELISA)).

Ischemia modified albumin (IMA) is the term given to serum albumin that has been modified by the reaction with free radicals generated by heart tissue undergoing acute cardiac ischemic conditions. The premise of this biomarker candidate is that free radials (e.g. hydroxyl) generated by ischemic heart tissue can react with albumin in the bloodstream forming a "modified albumin", which can be subsequently detected using a colorimetric technique. The exact mechanism is still unclear, but it is hypothesized that free radicals attach irreversibly to the amino end terminus of the albumin protein, creating a modified albumin moiety. Briefly, the assay utilizes an aqueous cobalt chloride solution, with cobalt typically able to bind to the amino terminus of serum albumin (which normally

binds and transports transition metals like cobalt). However, under conditions of acute cardiac ischemia and the formation of the modified albumin moiety, the cobalt can not bind to the n-terminus of albumin, and the resulting solution will have an intense blue color (as cobalt will instead bind with the test reagent dithiothreitol); which is measurable using a spectrophotometer. A spectrometer can be found used in a routine chemistry analyzer typically found in clinical laboratories.

Several IMA method conditions are important to list: the avoidance of sample collection using chelators (e.g. EDTA) as it would interfere with the analysis by binding to divalent cations (e.g. calcium, cobalt, magnesium), and to analyze the sample within 2.5 hrs of the blood draw (unless stored frozen at -20°C). Currently, there are clinical chemistry platforms for this analysis with on-going investigations on the potential use of immunoassay techniques. Although this biomarker has been cleared by the FDA for use, several clinical studies have reported the test to have significant false positive results (e.g. increased IMA values may be found in patients with cancer, liver disease, brain ischemia, end-stage renal disease, and infections [Apple et al., 2005; Asian D. et al., 2005; Wu, 2003]) and the analysis cost is fairly high (2006 US Medicaid reimbursement cost was ~$47 per test). As the diagnostic predictive accuracy of using IMA results alone is <50% [Sinha et al, 2003, Bagavan et al., 2003], it is important to mention that the FDA clearance of the IMA test was based on its use in conjunction with two other acute MI diagnostic tests (e.g. electrocardiogram (ECG) and cardiac troponin (e.g. cTnI)), which significantly increased the overall patient diagnostic accuracy.

Free fatty acids (FFA) are typically found in serum bound to albumin with a small amount typically found in the unbound form (FFA_u, free). However, under conditions of cardiac ischemia, the levels of FFA_u are found elevated, with the exact understanding of why (mechanism-of-action) the FFA_u is found elevated remaining unclear. The FFA_u analysis consists of the binding the unbound FFA to a protein labeled with a fluorescent tag, and subsequent measurement using a fluorometer. It was recommended that additional clinical evaluations were necessary to further evaluate this candidate biomarker [Apple et al., 2005].

1.5 Past Research on Inosine and Hypoxanthine

Inosine and hypoxanthine are found in human plasma at low basal concentrations (inosine 0.75-1.49 μM, hypoxanthine 1.47-2.94 μM) normally resulting from purine metabolism [Feng et al., 2000]. Past research has been performed evaluating inosine and hypoxanthine in humans to explore their possible roles in response to ischemia and to also determine what role inosine may have as an immunosuppressant. Several research groups have published articles evaluating nucleotide breakdown products in blood during ischemic cardiac events (e.g. pacing induced angina, acute MI) and utilized HPLC test methods for analysis.

For example, one group studied the role of xanthine oxidase in purine metabolism in ischemic humans [Kock et al., 2003]. They evaluated components such as hypoxanthine, xanthine and uric acid, but these authors did not measure inosine and reported insignificant differences in hypoxanthine concentrations (mean [μM], SD) between normal male individuals (11.9 μM, 4.1), and patients with MI (16.2 μM, 7.1) and

other ischemic diseases (e.g. angina pectoris, 11.9 μM, 5.0). Our research in Chapter 4 (Section 4.4) focused on addressing the differences in hypoxanthine results from our experiments using plasma (lithium heparin) and Kock's group; which used serum with gel (SST) for their experiments.

Another group studied normal volunteers and patients with documented ischemic heart disease; utilized an atrial pacing stress test and reported elevated levels of hypoxanthine using HPLC, but insignificant levels of adenosine, inosine and xanthine [Harmsen et al., 1981]. While these authors evaluated cardiac ischemic patients and found hypoxanthine concentrations to be elevated, they also reported insignificant levels of inosine (hypoxanthine precursor); which our Chapter 4 research reports significant concentrations of both hypoxanthine and inosine from our study patients. The differences in inosine results may be explained by differences in the cardiac patient types. Harmsen's group used ischemic heart disease patients and performed the atrial pacing stress test, which may end after a brief period of time (e.g. 20-30 min). This is somewhat different than what our cardiac patient samples represent; which were all from a local hospital emergency department's group of non-traumatic chest pain patients. These patients, which may have potential acute cardiac ischemia, had reported non-traumatic chest pain that may have been occurring for many hours (e.g. 2 to >10 hrs.), thus allowing concentrations of the ATP catabolic by-products (e.g. inosine, hypoxanthine) to elevate in the blood stream.

Toguzov's group reported a rise in purine metabolic end products in patients with angina and MI [Toguzov et al., 1989]. Their overall conclusion was that plasma concentrations of xanthine and uric acid were better indicators of the severity of cardiac

ischemia than were inosine and hypoxanthine. Since xanthine and uric acid can both be elevated by other patient medical conditions (e.g. elevated xanthine with xanthine oxidase deficiency and elevated levels of uric acid with gout); these two substances may have false positives associated with their use as potential cardiac biomarkers.

Although other research groups have worked with patients with cardiac disease (e.g. ischemia, acute MI, angina), there have been no reported studies on nucleotide catabolic by-products in plasma from patients (hospital emergency department) presenting with non-traumatic chest pain and potential acute cardiac ischemia, nor have any groups published a simple, rapid and sensitive test method (goal of <10 min and for clinical laboratory) for nucleotide catabolic components inosine and hypoxanthine in plasma.

Animal research in the area of cardiac ischemia has also been performed with publications demonstrating inosine and hypoxanthine cardiac efflux utilizing experiments with animal models such as the dog and pig [Jennings et al., 1981; Backstrom et al., 2003], with one group using the mouse model and adenosine metabolism inhibitors to study the mechanism underlying inosine cardio protection [Peart et al., 2001]. Jennings et al. used the dog (healthy mongrel) model for their experiments. In brief, these investigators performed in-vitro (excised heart) and in-vivo experiments, inducing ischemia and then evaluating the eluted nucleotides and their by-products (e.g. inosine, hypoxanthine). They concluded that when inducing cardiac ischemia on in-vitro (total ischemia) and in-vivo (severe ischemia) heart tissue, that elevated levels of inosine and hypoxanthine were detected (after 15 min for in-vivo, after 60 min for in-vitro). Peart et al. used isolated mouse hearts to evaluate adenosine metabolism inhibitors during periods of cardiac

ischemia. Their conclusion was that adenosine deaminase and kinase inhibition (via enzyme inhibitors) provided cardioprotection (from reducing the xanthine/xanthine oxidase reaction and the generation of damaging free radicals). Although their research goal (enzyme inhibitor evaluations using wild type C57/BL6 and transgenic mice) was somewhat different than our animal model research goal (cardiac ischemia marker proof of concept using ICR heterogeneous mice), both groups used isolated mouse hearts for the experiments. To meet the goal of cardiac ischemia marker proof of concept, the isolated mouse heart tissue represented less potential confounding results (e.g. clean matrix from Krebs perfusate and removal of body compensations to ischemia) plus the mouse is easy to use and relatively inexpensive to purchase/maintain.

Backstrom et al. used the pig model for their experiments, with microdialysis catheterization at several different blood vessel sites (e.g. great cardiac vein, pulmonary artery), and then used arterial occlusion to induce cardiac ischemia for various times (0, 10, 15 and 60 min). They reported significant dialysate concentrations of inosine and hypoxanthine at ischemic time points of 15 and 60 min relative to baseline concentrations (ischemic time point 0 min), and concluded that there was a graded outflow of amino acids and purines in response to ischemic conditions.

1.6 Research Hypothesis and Significance

The research hypothesis is that during periods of acute cardiac ischemia in patients presenting with non-traumatic chest pain (hospital emergency department), plasma concentrations of inosine (hypoxanthine precursor) and hypoxanthine would be significantly elevated above their normally low endogenous plasma concentrations, and

potentially becoming candidate biomarkers indicative of the pre-necrotic acute cardiac ischemic event. As acute cardiac ischemia is an event prior to potential acute myocardial infarction, the affected heart tissue in the ischemic area is still functional and not yet necrotic, as are tissue from an acute myocardial infarction (AMI).

The current biomarkers indicative of AMI are relatively large protein components (e.g. myoglobin, CK-MB, and troponins with each larger than 10,000 Da) which are released from necrotic heart tissue. However, inosine and hypoxanthine; which are ATP catabolic by-products and small polar substances (MW inosine 268 Da, MW hypoxanthine 136 Da), are transported by passive diffusion from affected heart tissue into the bloodstream. Since an acute cardiac ischemic event occurs prior to potential AMI, it is hypothesized that the concentrations of inosine and hypoxanthine should appear elevated in the bloodstream prior to elevated concentrations of protein biomarkers indicative of AMI (which are detected several hours after the AMI event).

The significance of this research would be the identification of potential candidate biomarker(s) of acute cardiac ischemia and the development of a rapid analysis (<10 min) to use for the biomarker measurement. Ideally, EMS personnel would benefit from this biomarker and rapid test method to guide in their diagnostic and treatment steps for non-traumatic chest pain patients, as there are more than ten medical conditions other than acute MI that may cause non-traumatic chest pain (e.g. anxiety attack, acid reflux, angina). However, one limitation of using elevated concentrations of inosine and hypoxanthine as a biomarker of acute cardiac ischemia is these biomarkers may not differentiate a patient

experiencing acute cardiac ischemia prior to AMI, or experiencing cardiac angina without AMI.

1.7 Overall Research Experimental Design

The research is divided into three study phases, with each study phase being completed prior to beginning the next (i.e. each subsequent study phase builds from the prior study phase). The study phases are briefly described below with a general outline of all three phases listed afterwards.

Phase I (Chapter 2) study has the overall goal of comparing control and ischemic isolated mouse hearts to identify potential biomarkers of acute cardiac ischemia. HPLC methodologies (DAD and MS detection) will be developed and used for all sample analysis from Phase I experiments. As aspirin (ASA) is currently used for human patients suspected of undergoing acute myocardial infarction (a condition that may occur after an acute cardiac ischemic event), it will be investigated (Chapter 3). Since aspirin is rapidly metabolized to salicylic acid (SA) in the body; SA will be investigated and used for the additional isolated mouse heart experiments undergoing ischemic conditions. The goal is to determine the effect of SA on the biomarkers from Chapter 2 experiments.

Phase II (Chapter 4) study has the goal of evaluating blood samples to determine if the biomarker identified in Chapter 2 is present in human plasma and then measure the concentrations. HPLC methodology (UV detection) will be used for all sample analysis in Phase II studies. IRB approval will be obtained for the use of blood samples represented healthy normal individuals and non-traumatic chest pain patients (from a local hospital emergency department). Additional experiments will be performed using different types of

sample collection additives (e.g. serum separator tube (SST), matched sets of heparin plasma and SST). The goal is to determine if the SST additive (e.g. gel) causes potential artifacts in biomarker concentration and state the preferred blood sample collection additive for any future work on the potential biomarkers.

Phase III (Chapter 5) study has the overall goal of developing a rapid and simple screening assay (i.e. potential diagnostic tool) to use in a clinical environment. A microplate luminometer will be purchased, setup and utilized for plasma (lithium heparin) analysis. Evaluations of enzyme (e.g. xanthine oxidase, purine nucleoside phosphorylase) concentration and incubation times will be performed to optimize assay conditions and achieve a rapid analysis time. The screening assay will be tested on plasma from healthy normal individuals and cardiac patients with acute myocardial infarction (as defined by elevated levels of cardiac troponin I). The goal is to determine if potential biomarker concentrations are significantly different between these two groups (normal subjects versus acute MI patients).

Each of the three phases of studies can also be listed as follows:

Phase I - Animal studies to identify potential biomarkers of acute cardiac ischemia and demonstrate biomarker proof-of-concept

- Evaluate potential biomarker(s) from animal model (isolated mouse hearts) undergoing 20 min acute global cardiac ischemic conditions. Evaluate test (ischemic) and control animal perfusate samples (n=6 each group) using HPLC methodology.
- Evaluate effects of salicylic acid (aspirin metabolite) in Krebs buffer solution on the potential biomarker(s) levels, as aspirin (platelet inhibitor) is currently used for initial

treatment on patients presenting with non-traumatic chest pain and potential acute myocardial infarction.

- Develop quantitative HPLC-DAD (diode array detection) test method for evaluation of Krebs perfusate samples from the animal experiments. Utilize LC-MS method for biomarker component identification.
- Calculate total biomarker efflux (area under the curve, AUC) and utilize statistics (e.g. t-test, ANOVA) for data analysis on control and test group results.

Phase II - Human plasma evaluation for identified biomarker(s) from Phase I studies

- Obtain human plasma samples (n=20 each group) from emergency department (ED) non-traumatic chest pain patients and healthy individuals (controls, non-symptomatic for acute cardiac ischemia) following institutional review board (IRB) and hospital departmental approvals.
- Develop and validate HPLC-UV method for quantitative evaluation of inosine and hypoxanthine in human plasma.
- Statistically evaluate HPLC plasma results using parametric techniques (e.g. t-test) using $\alpha=0.05$ with $p<0.05$ demonstrating statistical significance between test and control group plasma samples.
- Evaluate effects of sample collection matrix (e.g. lithium heparin plasma, serum separator tube) on levels of inosine and hypoxanthine from human samples.

Phase III – Develop rapid test method for biomarker(s) from Phase II studies

- Develop a rapid, simple, and sensitive plasma luminescence test method (<10 min analysis time) for potential use by emergency medical services (EMS) or clinical lab environment.
- Evaluate plasma samples (n=6 each) from healthy individuals and hospital acute MI patients (hospital documented elevated cTnT levels). Compare results statistically using t-test and using a calculated biomarker cut-off limit (e.g. 99th percentile).

CHAPTER 2. Animal Studies to Demonstrate Proof-of-Concept, "HPLC Determination of Inosine, a Potential Biomarker for Initial Cardiac Ischemia using Isolated Mouse Hearts"

2.1 Introduction

In this chapter of research, animal studies were performed to identify potential biomarker(s) of acute cardiac ischemia and to demonstrate the potential biomarker(s) proof-of-concept (via mechanism of action). The rationale of using isolated mouse hearts for these experiments was to eliminate potential confounding factors (e.g. body metabolism, body compensatory mechanisms) that may occur with in-vivo type experiments. Ischemia is defined as a reduced blood flow and results in reduced oxygen supply to the affected site (tissue). As a consequence to the lack of oxygen in heart muscle tissue, adenosine triphosphate (ATP) production in the cardiac cell's mitochondria would be limited to anaerobic production.

Thus, it is important to understand how the heart utilizes ATP, a high energy phosphate molecule, to perform its circulatory function. The heart is highly energy-dependent on ATP, which is made in cardiac cellular mitochondria by either aerobic (oxidative phosphorylation via electron transport chain) or anaerobic (glycolysis) processes. The aerobic process is heavily oxygen dependent and generates approximately 80% of cardiac cellular ATP. The anaerobic process is independent of oxygen and produces approximately 20% of the cardiac cellular ATP; with lactic acid as a by-product.

For production of large quantities of ATP, human cardiac cells have an abundance of mitochondria which comprise approximately 40-50% of the cardiac cellular mass. When cardiac tissue is subjected to periods of constant oxidative stress (e.g. cardiac ischemia), insufficient oxygen is available for cardiac mitochondria to aerobically synthesize the ATP required for normal cardiac function. This causes a cellular accumulation of ATP metabolic by-products (e.g. adenosine diphosphate (ADP), adenosine monophosphate (AMP)) and activates normally dormant enzymes (e.g. 5′-nucleotidase, adenosine deaminase, purine nucleoside phosphorylase and xanthine oxidase) to catabolize the ATP by-products to substances such as adenosine, inosine, hypoxanthine, xanthine and uric acid for cardiac cellular elimination [Abd-Elfattah et al., 2001]. In human cardiac tissue, another source of ATP metabolic by-products is through metabolism of diadenosine polyphosphates, which are released from cardiac-specific secretory granules during periods of cardiac metabolic or ischemic stress to provide cellular protective functions [Luo et al., 2004].

Inosine (9-β-D-ribofuranosylhypoxanthine) is an endogenous purine nucleoside normally found in the human body as a degradation component of purine metabolism. In human plasma, inosine is metabolized in red blood cells with a reported half-life of <5 min with endogenous plasma levels found in trace amounts (e.g. low ng/mL) [Viegas et.al., 2000]. In humans, nature has provided a cellular biochemical mechanism to help conserve energy in producing the required large quantities of ATP for cardiac cellular use (called salvage pathway), which can convert cellular inosine back to ATP via several enzymatic steps; thus recycling cellular inosine [Nelson et al., 2000]. However, in periods of constant

cardiac oxidative stress (e.g. 20 min), cardiac cells buildup significant amounts of ATP metabolic by-products, which activate normally dormant enzymes to catabolize ATP by-products, which then become systemically available prior to their elimination.

The research hypothesis is that prior to extra-cellular biomarkers (e.g. free radical modified serum albumin) appearing in the bloodstream from cardiac ischemic events, plasma inosine levels should be elevated above normally low endogenous levels, thus becoming a useful biomarker of pre-necrotic acute cardiac ischemia. Adenosine, another nucleoside metabolic by-product of ATP catabolism is metabolized by red blood cells and has a very short plasma half-life (e.g. ~15 sec); thus making it more difficult to quantitatively measure in plasma [Mei et.al., 1996]. The three other metabolic by-products (xanthine, hypoxanthine and uric acid) are normally found at higher levels in the plasma, but would lack the necessary specificity due to potential contributions from other human disease state conditions (e.g. plasma uric acid levels elevated in gout; plasma xanthine levels elevated in xanthine oxidase deficient individuals).

The ICR (Institute of Cancer Research) outbred mouse [Dohm, 2004] was used as the animal model for all isolated heart experiments and utilized a Langendorff apparatus [Xi et al., 1998]. For sample analysis, a developed analytical method utilized a high performance liquid chromatographic diode array detection (HPLC-DAD) for the detection and quantification of inosine in Krebs-Henseleit (Krebs) buffer solution. A Synergy C_{18} column (hydrophobic/hydrophilic reversed phased retention) at a flow rate of 0.6 ml/min with an aqueous mobile phase of trifluoroacetic acid (0.05% TFA in deionized water pH 2.2, v/v) and methanol gradient was used for component separation. The assay detection

limit for inosine in Krebs buffer solution was 500 ng/mL using a 100 µL neat injection. The HPLC results were used to determine total cardiac effluxed inosine (AUC) into the Krebs effluent for each mouse during acute global cardiac ischemia (oxidative stress) and compared to percent cardiac ventricular functional recovery to determine if a relationship exists amongst this cardiovascular parameter during periods of acute global cardiac ischemia.

2.2 Experimental

2.2.1 Chemicals, Mobile Phase and Krebs Buffer Solution

Hypoxanthine and xanthine were purchased from Acros Organics (Fair Lawn, NJ, USA). 2,3-dihydroxybenzoic acid (DHBA), 2,5-dihydroxybenzoic acid, salicylic acid (SA), adensosine, inosine and uric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sodium chloride, sodium bicarbonate, potassium chloride, magnesium sulfate, monobasic potassium dihydrogen phosphate, dextrose and calcium chloride were used to prepare the Krebs buffer solution and all were purchased from Sigma-Aldrich. All purchased chemicals were ACS reagent grade or better. The Krebs buffer solution [118.5 mM NaCl, 25.0 mM $NaHCO_3$, 11.1 mM $C_6H_6O_6$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$ and 2.5 mM $CaCl_2$] was prepared in ultrapure deionized water with pH 7.4 and 95%O_2:5%CO_2. For mobile phase preparation, trifluoroacetic acid (TFA) was reagent grade and methanol was Optima HPLC grade and both were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure distilled and deionized water (18 MΩ-cm) used for HPLC work was prepared in-house using Purelab Ultra deionized water system (US Filter, Lowell, MA, USA) and filtered prior to use.

2.2.2 Preparation of Standard Solutions

Stock standards of 93.6 µM adenosine, 93.2 µM inosine, 183.7 µM hypoxanthine, 164.4 µM xanthine and 148.7 µM uric acid were prepared in deionized water and stored at 4°C. Working standards (1:10 dilution of stock standard) of each component were prepared in Krebs buffer solution and maintained at -20°C along with the mouse Krebs buffer eluant samples. The working standards stored at -20°C were stable for at least 6 months.

2.2.3 HPLC-UV and HPLC-MS Equipment Set Points

For inosine quantification and diode array spectral purity, the HPLC equipment consisted of Agilent Model 1100 Quaternary HPLC-DAD and Chemstation software (Palo Alto, CA, USA). The DAD was set to acquire complete UV spectrum for component specificity with 240 nm used for quantification of inosine and the other ATP metabolic by-products. For inosine confirmation, LC/MS was used and the equipment consisted of a Shimadzu LCMS-2010A HPLC coupled to a single quadrapole mass spectrometer using LCMS Solutions software (Columbia, MD, USA). The HPLC-MS conditions consisted of using ESI with the following instrument set points (heating block at 300°C, nebulizer at 4 L/min nitrogen, interface voltage at 2 kV) and full scan acquisition using positive ion mode.

The analytical column for both HPLC-DAD and HPLC-MS analysis was a Synergi™ Hydro-RP C_{18}, 150 mm x 3 mm I.D., 4 µm packing, 80 Å (Phenomenex®, Torrance, CA, USA). The C_{18} guard column was a 30 mm x 4.6 mm I.D., 40-50 µm pellicular packing (Alltech, Deerfield, IL, USA). The mobile phase consisted of aqueous

trifluoroacetic acid (0.05% TFA in deionized water, v/v, and pH 2.2) and methanol gradient. The mobile phase gradient was linear with time course as follows (95:5 0.05% TFA in deionized water: methanol, v/v at 0 min; 70:30 0.05% TFA in deionized water: methanol, v/v at 12 min; 10:90 0.05% TFA in deionized water: methanol, v/v at 13 min and held 3 min, and 95:5 0.05% TFA in deionized water: methanol, v/v at 17 min).

The mobile phase was degassed automatically using an Agilent 1100 membrane degasser with a flow-rate of 0.6 ml/min. An injection volume of 100 µl of the Krebs buffer eluant was made using an autosampler. Typical HPLC operating pressure was approximately 150 bar with ambient column oven temperature and 345 kPa back-pressure regulator (SSI, State College, PA, USA) to prevent mobile phase outgassing in the detector.

2.2.4 ICR Mouse Experiment Conditions

ICR mice were used for all isolated mouse heart experiments with morphometric characteristics and baseline cardiac function of the adult mice (ICR strain) provided in Table 4. The mice were anaesthetized; hearts surgically removed and isolated using the Langendorff apparatus (Figure 2). Global cardiac oxidative stress was accomplished by adjusting the Krebs buffered solution to zero flow through the heart for 20 min. Upon heart reperfusion, approximately 1.5 mL samples of Krebs buffered eluant from the isolated mouse hearts were collected at predetermined time-points (0, 1, 3, 5, 10 and 20 min) in plastic bullet centrifuge tubes and frozen at -20°C until HPLC-DAD analysis.

To evaluate the effects of oxidative stress on the mouse heart, established cardiovascular measurements (e.g. ventricular functional recovery) were performed on

Table 4. Morphometric characteristics and baseline cardiac function of the adult mice (ICR strain).

	Control (n = 6)	Ischemia-Reperfusion Test (n = 6)
Body Weight (g)	42.2 ± 1.3	38.7 ± 2.1
Heart Wet Weight (mg)	258 ± 6	242 ± 14
Heart Rate (bpm)	368 ± 23	345 ± 23
Developed Force (g)	0.81 ± 0.19	1.12 ± 0.12
Rate-Force Product (g x bpm)	308 ± 80	372 ± 49
Coronary Flow (ml/min)	2.3 ± 0.2	1.7 ± 0.1

Values are mean ± SEM. No significant difference ($P>0.05$) between the groups was found for the listed parameters, except coronary flow.

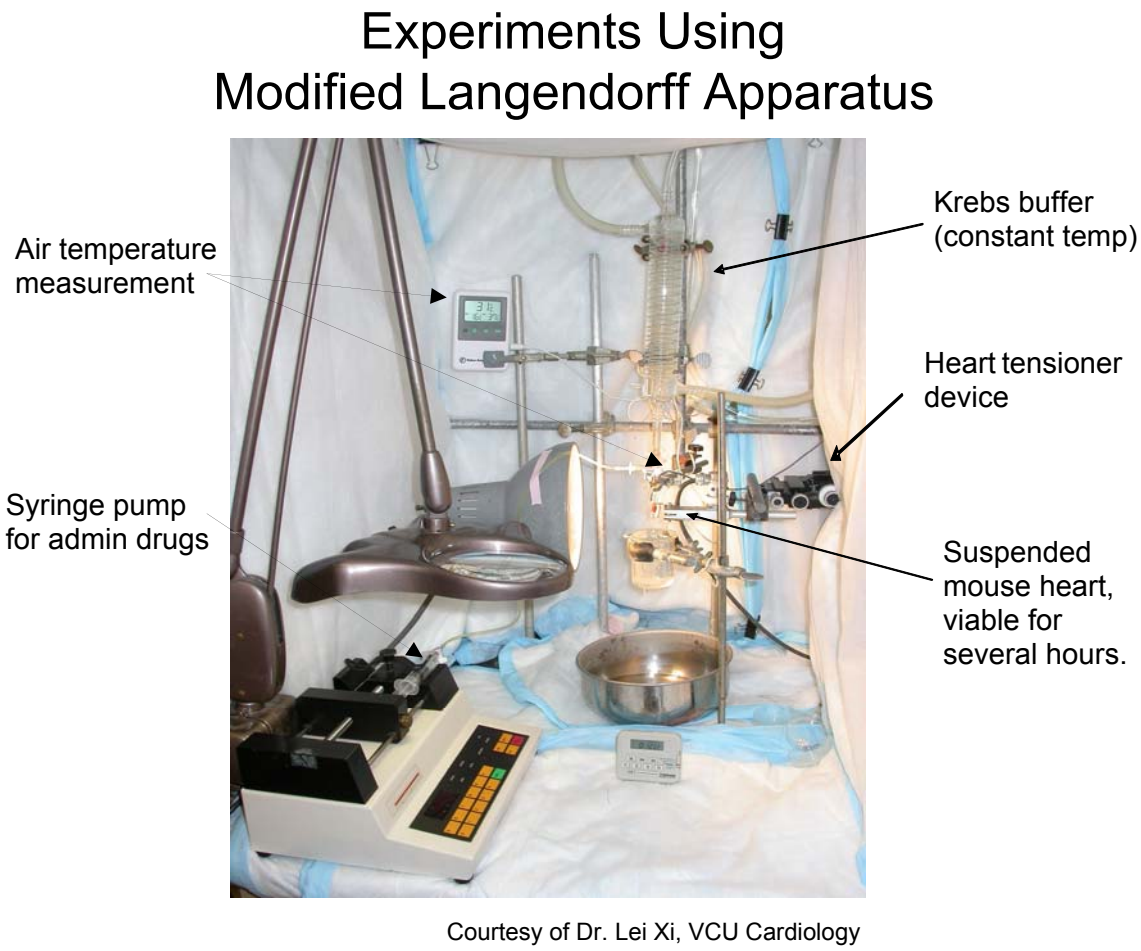


Figure 2. Modified Landendorff apparatus used for all animal (mouse) experiments. The suspended isolated heart has Krebs buffer solution perfusing through it to provide sufficient nutrients (e.g. buffer salts, glucose, oxygen etc.) for experiments.

both control (non-ischemic conditions) and test (ischemic conditions) animals (n=6 for each group). Our methodology for evaluating the isolated perfused mouse heart has been previously described [Xi et al., 1998]. In brief, animals are anesthetized with an intraperitoneal injection of pentobarbital sodium ([100 mg/kg] with 33 IU heparin added). The heart was removed and immediately placed in ice-cold Krebs buffer. The aorta was cannulated within 3 min onto the Langendorff perfusion system and the heart was perfused in a retrograde fashion at a constant pressure of 55 mmHg with Krebs buffer gassed with 95%O_2 and 5%CO_2. The pH of the buffer and the heart temperature were maintained at 7.35-7.50 and 37±0.5ºC, respectively. A force-displacement transducer (Grass, FT03) was attached to the apex via a metal hook/surgical thread/pulley system to continuously record and measure ventricular contractile force and heart rate. For each heart the resting tension was set at ~0.3 g in the beginning of the experiment.

The protocol for the test group consisted of 30 min of stabilization, 20 min of zero-flow global ischemia, and 30 min of reperfusion [Xi et al., 1998]. Time-matched normoxic perfusion was carried out for the control group. At the end of each experiment, the heart was removed from the Langendorff system, quickly weighed and stored at -20°C.

2.2.5 Sample Preparation, Stability, and Instrument Precision Evaluation

Prior to HPLC analysis, perfusate samples frozen at -20°C were thawed to ambient temperature, mixed thoroughly by inversion and transferred to plastic autosampler vials for subsequent direct injection into the HPLC-DAD system. To evaluate sample stability in the perfusate solution and instrument precision; prepared samples in autosampler vials

were stored at ambient lab temperature overnight and re-injected (n=3 times) into the HPLC for analysis.

2.2.6 Component Retention Times, Inosine Calibration and AUC Calculations

During HPLC method development and validation, standards of adenosine (3.7 to 93.6 μM), inosine (3.7 to 93.2 μM), hypoxanthine (7.3 to 183.7 μM), xanthine (6.6 to 164.4 μM) and uric acid (5.9 to 148.7 μM) were prepared in Krebs buffer solution. Standard curve linearity (non-weighted) of all components was acceptable with all correlation coefficients >0.995. During subsequent analytical runs, a single point calibration standard mixture (9.3 μM inosine, 18.4 μM hypoxanthine, 9.4 μM adenosine, 16.4 μM xanthine and 14.9 μM uric acid) was prepared in Krebs buffer solution and was used for identifying component retention times and quantification of inosine found in test samples. Using UV detection at 240 nm, component peak area and external standardization were used for inosine computations. For determining inosine AUC on test samples, the trapezoidal rule computation using Excel software was performed on inosine sample values from 0 to 20 min.

2.3 Results and Discussion

2.3.1 Additional Evaluation for Hydroxyl (·OH) Free Radicals

During periods of cardiac oxidative stress (e.g. acute myocardial infarction), the heart is deprived of the oxygen needed for ATP synthesis. In the absence of oxygen dormant enzymes activate whereby ATP is sequentially converted to ADP, AMP, adenosine, inosine and hypoxanthine. Upon reperfusion of the heart with oxygenated blood or oxygenated Krebs solution, additional cellular enzymatic conversions transpire

with xanthine oxidase converting hypoxanthine to xanthine and uric acid. A metabolic by-product of xanthine oxidase is the formation of hydrogen peroxide (H_2O_2) which is normally converted by glutathione peroxidase to H_2O. However, in the presence of Fe^{2+}, H_2O_2 may be converted to a hydroxyl free radical (·OH) via the Fenton and Haber-Weiss reactions (Figure 3) [IUPAC, 1997].

The (·OH) is a known potent reactive oxygen species (ROS) and can cause damage to cellular components (e.g. lipids, proteins, nucleic acids) [Tardif and Bourassa 2000]. To investigate the formation of ROS, one research objective was to evaluate and estimate the amount of (·OH) generated from 20 min of global cardiac ischemia using isolated mouse hearts. In several of the initial experiments, SA [1 mM] was fortified in the Krebs buffer solution (pH adjusted 7.4) to react with (·OH) and form the reaction products of 2,3 and 2,5-DHBA isomers [Onodera et al., 1991; Coudray et al., 2000].

The HPLC-DAD conditions that were used for inosine determination resolved prepared standards [13 ng/mL or 86 nM] of the 2,3 and 2,5-DHBA isomers from other Krebs eluant sample components (e.g. SA, adenosine, inosine, hypoxanthine etc.). However, in our experiments performed using SA, we did not observe either the 2,3 or 2,5-DHBA isomers in the sample chromatograms from mouse hearts that were subjected to global cardiac ischemia. It is possible that the initial level of SA [1 mM] added to the Krebs buffer solution increased the total solute concentration to a level, which reduced the solubility of 2,3 and 2,5-DHBA isomers and therefore made each analytically undetectable. Lower concentrations of SA [e.g. ≤ 1 μM] may in theory resolve this aspect of ROS generation from mouse acute global cardiac ischemia.

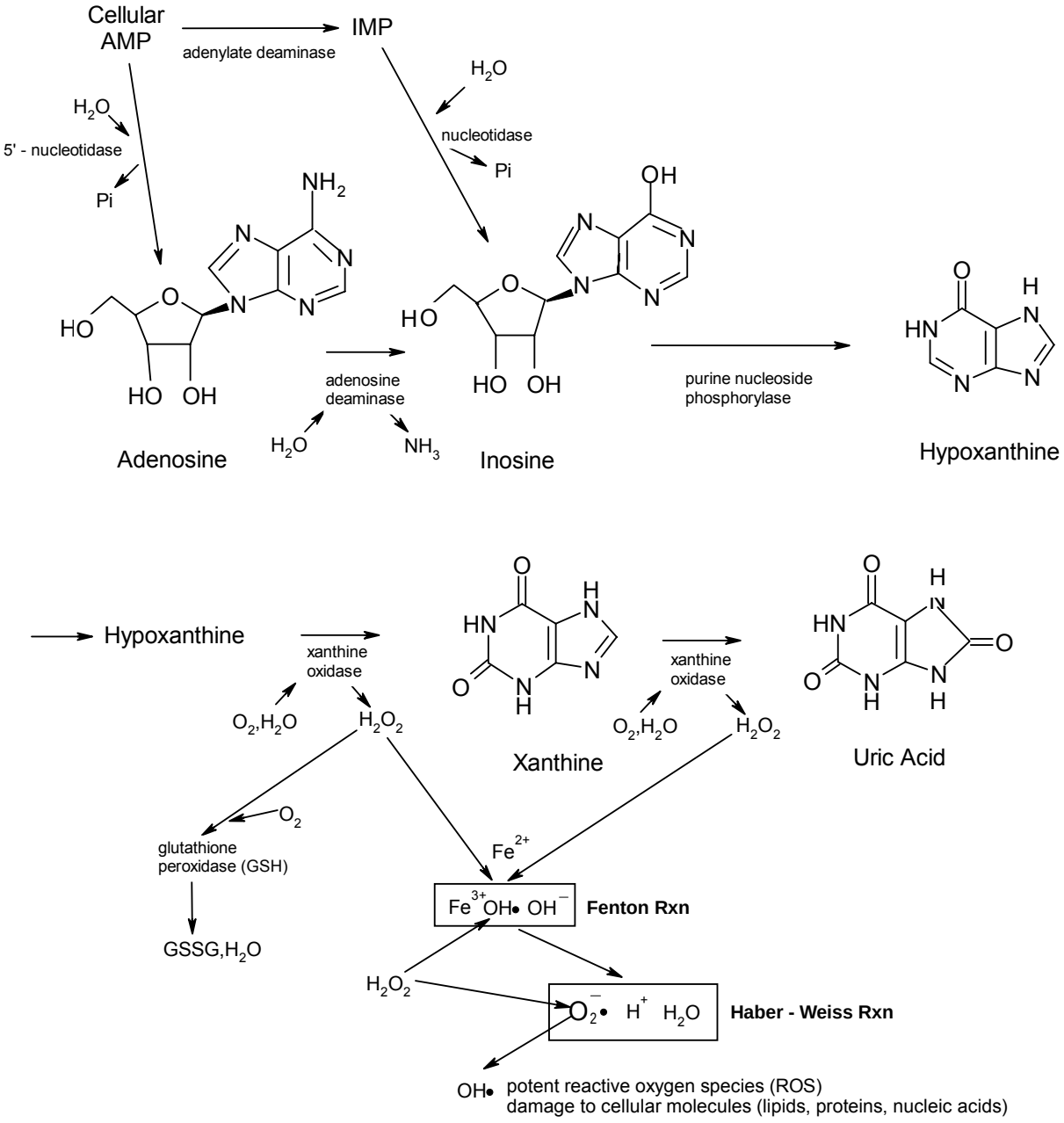


Figure 3. Schematic drawing of cardiac cellular ATP catabolism due to oxidative stress and potential oxygen reperfusion injury due to (·OH) free radical generation.

2.3.2 HPLC-DAD and HPLC-MS Identification

The HPLC-DAD method was used for determining all of the following components (adenosine, inosine, hypoxanthine, xanthine, uric acid, 2,3-DHBA and 2,5-DHBA isomers). The mobile phase aqueous component 0.05% TFA in deionized water was chosen as pH ~2.3 provided good peak shapes on all components and a low pH was necessary to reduce peak tailing on the acidic components (e.g. 2,5-DHBA has pKa ≈ 2.9). The Synergi™ Hydro-RP C_{18} (polar endcapped) and Synergi™ Polar-RP C_{18} (ether-linked phenyl) columns of identical dimensions were evaluated for use. While both columns worked well for inosine and polar components (e.g. adenosine), the Synergi™ Hydro-RP C_{18} was selected for overall analysis as it provided good component peak shape and sufficient resolution of all components.

Other components evaluated using this method have HPLC retention times as follows (uric acid 2.8 min, hypoxanthine 3.9 min, xanthine 4.2 min, adenosine 5.7 min, CK-MB 8.2 min, 2,3-DHBA 8.4 min, 2,5-DHBA 10.2 min, myoglobin 14.1 min, atrial natriuretic peptide 14.5 min, brain natriuretic peptide 15.0 min and salicylic acid 15.4 min). Both cardiac troponin I and troponin T were not detected using this HPLC method. An HPLC-DAD chromatograms overlay from a mouse subjected to 20 min acute global cardiac ischemia and a control mouse (non-ischemia) are presented in Figure 4 with inosine elution at 5.9 min.

To evaluate perfusate sample stability, the prepared samples were initially injected and analyzed by HPLC-DAD. The samples were subsequently stored overnight on the autosampler at ambient lab temperature and re-injected (n=3 times) to evaluate both for

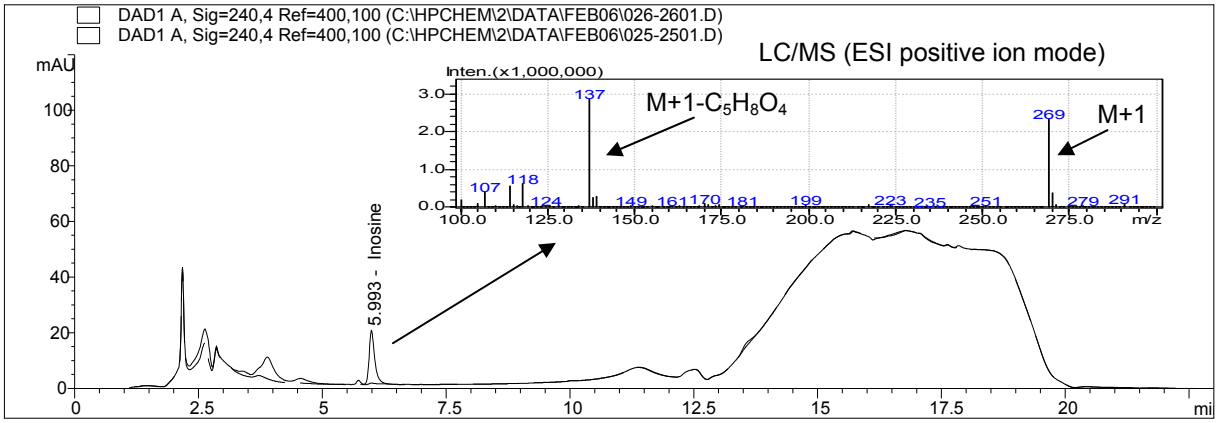


Figure 4. HPLC-DAD chromatograms overlay of control (025-2501.D) and 20 min global cardiac ischemia (026-2601.D) mouse perfusate samples. Inosine (RT 5.9 min) and HPLC-ESI/MS mass spectrum identifying inosine (MW 268 Da) as a potential early biomarker of global cardiac ischemia is demonstrated in the ischemic mouse heart perfusate. The chromatogram overlay between control and 20 min global cardiac ischemia also demonstrates consistent gradient repeatability.

changes in component levels due to possible synthesis or degradation reactions from potential enzymes eluted in the perfusate and to evaluate instrument precision. In all re-injected perfusate samples, component levels remained constant (≤ 4% RSD) indicating stability overnight at ambient temperature and the absence of appreciable levels of nucleoside and purine converting enzymes in the perfusate.

2.3.3 HPLC-MS Identification of Inosine as Potential Initial Ischemia Biomarker

An HPLC-MS was used to identify inosine at retention time 5.9 min in samples from test mice subjected to oxidative stress. The HPLC analytical column, mobile phase gradient and flow rate were identical to that used in the HPLC-DAD method. The mass spectrum for inosine (MW 268 Da) is presented in Figure 4. It was acquired using the MS positive ion mode, which provided a good mass spectral quality match against a prepared standard of inosine in Krebs buffer solution. The full scan spectrum was achieved using up-front collision induced dissociation (CID) and nitrogen as the collision gas. The mass spectrum base peak (137 Da) represents the cleavage of the ribose entity from inosine leaving a protonated hypoxanthine (MW 136 Da) (Figure 5).

2.3.4 Evaluation of Inosine AUC and Other Cardiovascular Parameters

Initially, the focus was on identifying cardiac protein or peptide biomarkers (e.g. ANP, BNP) that may be released from ischemic myocardium; however in comparison with non-ischemic mouse hearts only inosine (22 to 69 fold) and xanthine-like products (e.g. hypoxanthine (>7 fold), xanthine (~3 fold), uric acid (~3 fold)) were found at higher levels in globally ischemic mouse hearts. Figure 6 is a profile of mouse hearts subjected to

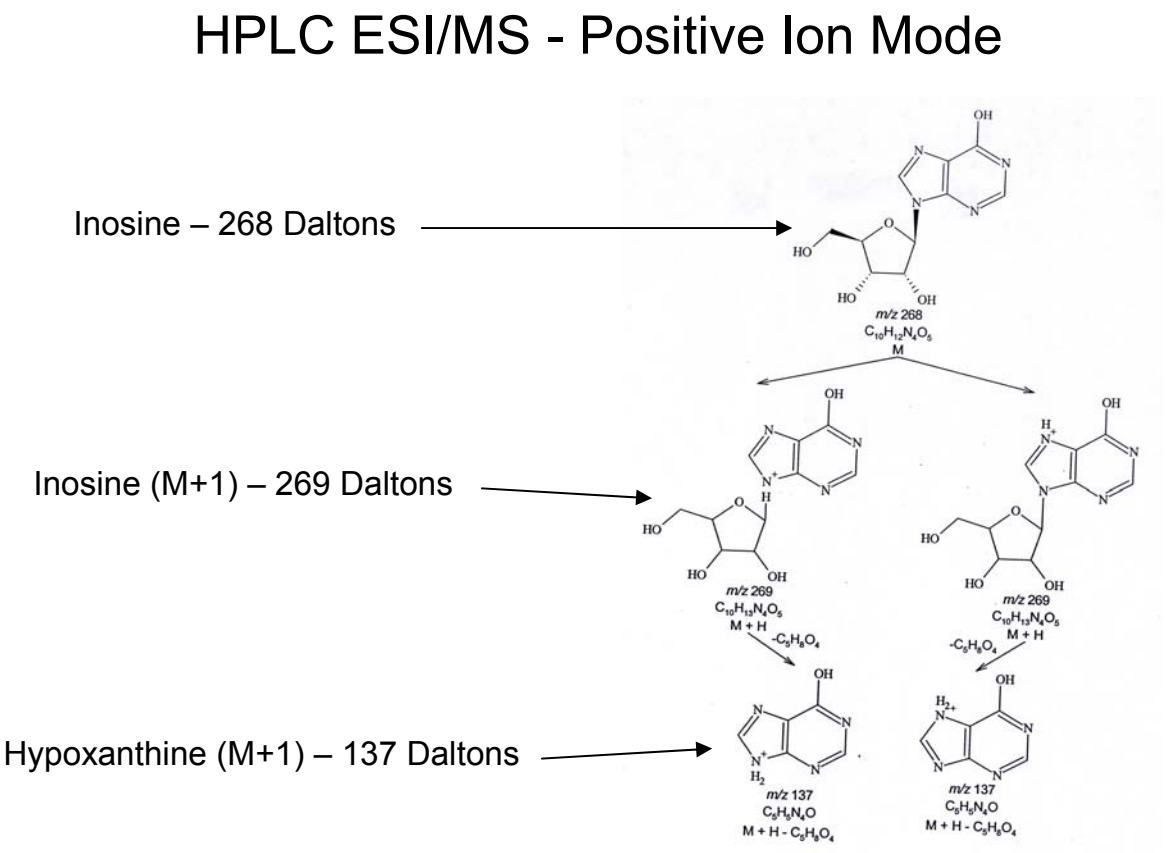


Figure 5. HPLC-ESI/MS positive ionization mode of inosine (MW 268 Da) with fragmentation to hypoxanthine (MW 136 Da).

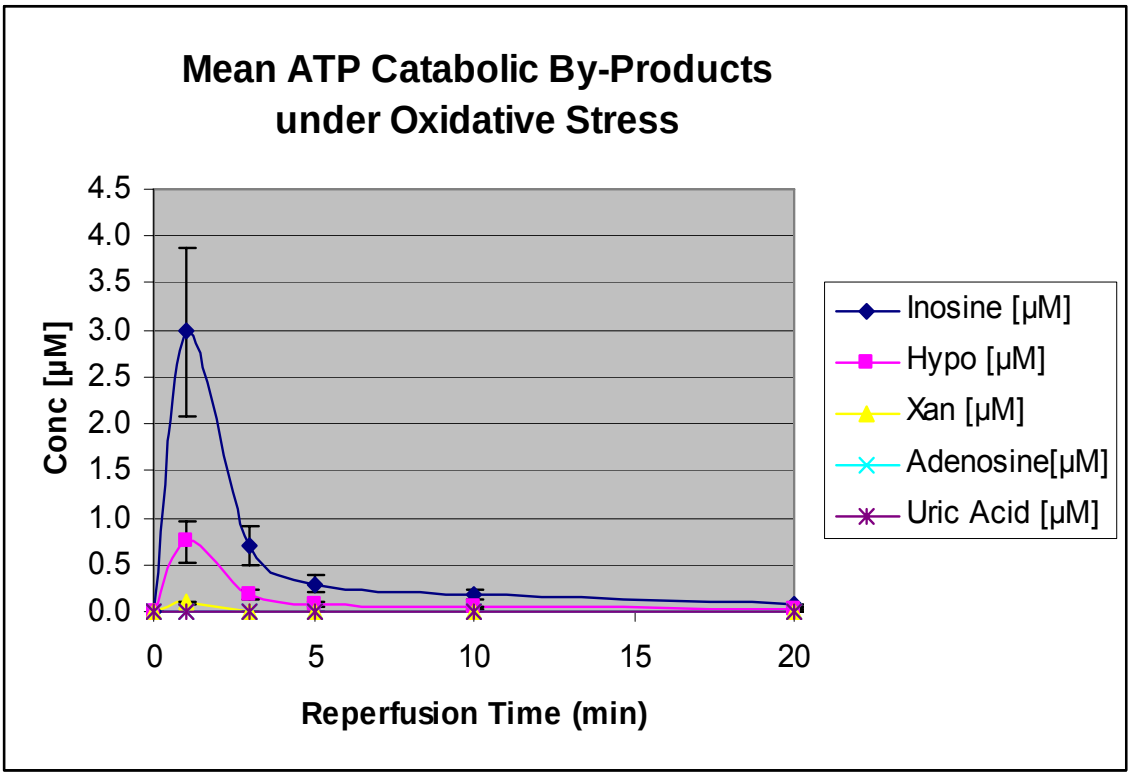


Figure 6. Profile of the mean (±SD) ATP catabolic by-products detected in Krebs solution versus reperfusion time after 20 min mouse global cardiac ischemia (n=6 mice). Control mice (n=6, non-ischemia) are not plotted with undetectable levels of ATP catabolic by-products in all sample time points (0, 1, 3, 5, 10 and 20 min).

oxidative stress with individual ATP degradation by-product components (mean ± SD) plotted against Krebs buffer reperfusion time. As can be seen in Figure 6, inosine was the component which had the highest response with detectable component amounts found at low μM levels, after 20 min global cardiac ischemia (n=6 mice). The control mice (n=6, non-ischemia) are not shown, as there were undetectable levels of all ATP catabolic by-products in each sample time point (0, 1, 3, 5, 10 and 20 min).

Cardiovascular parameter (e.g. percent cardiac ventricular functional recovery) was measured and reported with the calculated inosine AUC results (Table 5). As can be seen from Table 5, inosine efflux was present in test mouse heart perfusate samples which were subjected to oxidative stress and was not detected in control mouse heart perfusate samples. However, for both controls and test mice, the percent cardiac functional recovery ranged from 39 to 92% with the lowest measured cardiac functional recovery being in test mouse hearts which had the largest amount of inosine present in the Krebs buffer solution (e.g. test mouse with 2, 469 ng min ml^{-1} AUC inosine effluxed with 39% cardiac functional recovery). This may indicate that mouse hearts which are injured to a greater degree from the effects of oxidative stress efflux more inosine from ATP by- product degradation. Further studies with larger test and control mouse sample size (e.g. n=10) would be necessary to statistically interpret this observation.

2.4 Conclusion

The preliminary results suggest that the concentration of inosine found in test animals subjected to cardiac oxidative stress may serve as a potential biomarker indicative

Table 5. Inosine washout and cardiac ventricular functional recovery in Langendorff mouse hearts following aerobic perfusion and 20 min global ischemia.

Sample Type	Inosine AUC 0-20 min [ng min / mL]	% Cardiac Functional Recovery
Control	N.D.	70
Control	N.D.	72
Control	N.D.	74
Control	N.D.	82
Control	N.D.	81
Control	N.D.	64
Test	653	92
Test	962	84
Test	954	77
Test	1,003	53
Test	2,469	39
Test	2,583	52

of early cardiac ischemia. This can be explained by ischemic myocytes undergoing nucleotide purine catabolism in the absence of oxygen with subsequent activation of dormant cellular enzymes and generation of degradative breakdown products of ATP. Preliminary human studies will need to be undertaken to determine the validity of this hypothesis.

Additional research from our group will be performed on plasma samples obtained from hospital admitted patients suspected of undergoing acute myocardial infarction to determine if plasma levels of endogenous inosine are significantly elevated during periods of cardiac oxidative stress. If inosine plasma levels are found to be significantly elevated in samples obtained from patients undergoing acute myocardial infarction, inosine should be recommended as a potential biomarker for the initial cardiac ischemic event, and may be useful in indicating the need for immediate medical treatment, potentially improving patient outcome.

2.5 Acknowledgements

For this chapter of research, I would like to acknowledge the co-authors of our manuscript "High-performance liquid chromatography (HPLC) determination of inosine, a potential biomarker for initial cardiac ischemia using isolated mouse hearts," which was published in Biomarkers (2006, 11(5), 449-459). The co-authors were Dr. Lei Xi[3], Dr. Lynne Gehr[4], Dr. Domenic Sica[2], Terri Larus[2] and Dr. H. Thomas Karnes[1] ([1]Departments of Pharmaceutics, [2]Clinical Pharmacology and Hypertension, [3]Cardiology, and [4]Pediatrics, VCU Medical Center, Virginia Commonwealth University, Richmond, VA 23298, USA). A technical poster on this research was presented at the 29th International Symposium on

High Pressure Liquid Phase Separations and Related Techniques (Stockholm, Sweden, June 2005) (Appendix A). I would also like to thank Dr. Bill Gardner and Dr. Dongliang Zhan from Philip Morris Research and Development, Richmond, VA, USA for their technical expertise using HPLC-MS and Philip Morris, Richmond, VA, USA for the use of HPLC-DAD equipment for the analysis of Krebs perfusate samples. We thank Mr. Brian Berger (VCU Medical Center, Richmond, VA USA) for his technical assistance on initial animal experiments using isolated rat hearts undergoing acute global ischemic conditions. Dr. Lei Xi was supported by a grant from the American Heart Association, National Center (0530157N).

CHAPTER 3. Animal Studies to Evaluate Salicylic Acid, "Effects of Salicylic Acid on Post-Ischemic Ventricular Function and Purine Efflux in Isolated Mouse Hearts"

3.1 Introduction

Aspirin (acetyl salicylic acid, ASA) is one of the most widely used drugs in the world and has been used for many years for its analgesic, anti-pyretic, anti-inflammatory and anti-platelet (blood thinning) properties (Vane et al., 2003; Schror, 1997; Evans et al., 1968). ASA (160 or 325 mg dose) is routinely administered with other cardiac medications as part of initial emergency treatments to patients presenting with chest pain and potential acute myocardial infarction (MI) to inhibit platelet aggregation at the site of cardiac thrombosis (Feldman et al., 1999; Antman et al., 2004; Abarbanell et al., 2001; Kosowsky, 2006). A 325 mg dose of aspirin can have plasma concentration maximums (Cmax) of 67 μM acetyl salicylic acid and 188 μM salicylic acid (Merck 11th Edition, 1989). Aspirin is also used chronically by many individuals for other medical conditions, with plasma salicylic acid concentrations ranging from 145 to 725 μM (analgesia) and 1,086 to 2,172 μM (anti-inflammatory) (Moffat et al. 1986).

Aspirin's mechanism of action is well documented with interactions causing irreversible inhibition of both cyclooxygenase isoenzymes (COX-1, COX-2) (Smith et al., 1971; Schror, 1997). The mechanism for ASA inhibiting platelet aggregation is through irreversible acetylation of the COX-1 enzyme which blocks synthesis of thromboxane A_2,

a platelet aggregator and vasoconstrictor (Vane et al., 2003). Other reported medical uses of ASA require higher blood concentrations (e.g. a rheumatoid arthritis patient may require 1.5 to 2.5 mM ASA and an anti-inflammatory dose may require sodium salicylate levels of 0.5 to 5 mM) (Nulton-Persson et al., 2004; Smith et al., 1971). In humans, ASA is rapidly metabolized (half-life ~3-4 hrs) and excreted via phase I metabolism (60% via deacetylation to salicylic acid (SA)) and phase II metabolism (~ 40% via conjugated products) (Rowland et al., 1968).

In addition a recent publication reported inhibitory effects of both ASA and SA on rat cardiac mitochondrial respiration (Nulton-Persson et al., 2004). These authors found that ASA and SA both could reduce NADH supply to the electron transport chain in isolated rat cardiac mitochondria, thus reducing ATP synthesis (via inhibition of oxidative phosphorylation). They also demonstrated a negative dose-response effect from both ASA and SA (0-10 mM concentration range) on cardiac mitochondrial respiration under non-ischemic conditions. Other research was performed demonstrating the inhibitory effects of ASA and SA on xanthine oxidase, which would inhibit enzymatic conversion of hypoxanthine to xanthine and uric acid (Carlin et al., 1985; Masuoka and Kubo, 2003).

To this context, the present study was designed to further examine the effects of SA (0, 0.1 and 1.0 mM) on ATP catabolic by-products (e.g. inosine and hypoxanthine) along with an indirect evaluation of purine nucleoside phosphorylase (PNP) enzyme activity (indicated by inosine/hypoxanthine conversion ratio). We hypothesized that higher concentrations of SA (e.g. 1.0 mM) coupled with periods of acute cardiac ischemia may potentiate the ischemic adverse effects on heart tissue via increased uncoupling of

oxidative phosphorylation and subsequently enhanced efflux of ATP catabolic by-products such as inosine, which has been proposed in our recent publication (Farthing et al., 2006) as a potential biomarker of acute cardiac ischemia. The morphometric characteristics and baseline cardiac function data from controls (non-ischemic and without SA) and test (ischemic and without SA) animals from Chapter 2 research were used for statistical comparisons to the experimental groups exposed to SA in this chapter of research.

3.2 Experimental

3.2.1 Chemicals, Standards and Krebs Buffer Solution

All experimental chemicals were purchased and solutions prepared as per our recent published work (Farthing et al., 2006). Briefly, ACS grade or better purity hypoxanthine, xanthine, trifluoroacetic acid (TFA) and methanol (Optima) were purchased from Acros Organics (Fair Lawn, NJ, USA). Salicylic acid, adenosine, inosine, uric acid, sodium chloride, sodium bicarbonate, potassium chloride, magnesium sulfate, monobasic potassium dihydrogen phosphate, dextrose, ethylenediaminetetraacidic acid (EDTA) and calcium chloride were all purchased from Sigma-Aldrich (St. Louis, MO, USA).

Stock and working standards of adenosine (374.2 µM), inosine (372.8 µM), hypoxanthine (734.7 µM), xanthine (657.4 µM) and uric acid (594.8 µM) were prepared in deionized water and stored at 4°C as per work (Farthing et al., 2007). The working standards were maintained at -20°C along with the mouse Krebs buffer perfusate samples and demonstrated stability for at least 6 months. The Krebs buffer solution consisted of either 0, 0.1 or 1.0 mM SA and 118.5 mM NaCl, 25.0 mM $NaHCO_3$, 11.1 mM $C_6H_6O_6$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.5 mM EDTA and 2.5 mM $CaCl_2$ using in-

house prepared deionized water (final pH 7.4 and continuously gassed with 95%O_2:5%CO_2 during the isolated heart experiments). For HPLC analysis, deionized water (18 MΩ-cm) used was produced and filtered using US Filter Purelab Ultra deionized water system (Lowell, MA, USA).

3.2.2 Langendorff Isolated Mouse Heart Preparation and Experimental Protocols

All animal experimental conditions were similar to our published work (Farthing et al., 2006; Xi et al., 1998). Adult male mice (ICR strain) were used for all cardiac ischemia experiments with morphometric characteristics, baseline cardiac function, and ANOVA results presented in Table 6. The protocol for Groups II, III, and IV consisted of 30 min of stabilization, 20 min of zero-flow global ischemia and 30 min of reperfusion (Figure 7). Time-matched normoxic perfusion (for 80 min) was carried out for the Control group and Group I. Briefly, the mice were anaesthetized; hearts quickly isolated and cannulated onto a Langendorff apparatus within 3 min. Following the 30 min of stabilization period, global ischemia was accomplished by stopping heart perfusion inflow for 20 min. Upon heart reperfusion, approximately 1.5 mL samples of Krebs buffered perfusate from the isolated mouse hearts were collected at predetermined time-points (0, 1, 3, 5, 10 and 20 min) into plastic bullet centrifuge tubes and immediately frozen at -20°C. At the end of each experiment, the heart was removed from the Langendorff system and quickly weighed.

The present study measured simultaneously both cardiovascular parameters (e.g. coronary flow rate, heart rate and cardiac developed force) and efflux of ATP catabolic by-products (e.g. inosine and hypoxanthine evaluated in Krebs perfusate samples) for each of the experimental groups. The control group consisted of six aerobically perfused hearts

Table 6. Morphometric characteristics and baseline cardiac function of the adult mice (ICR strain).

	Control (n = 6)	Group I (n = 6)	Group II (n = 6)	Group III (n = 6)	Group IV (n = 6)
Body Weight (g)	42.2 ± 1.3	37.0 ± 0.9	38.7 ± 2.1	41.5 ± 2.7	36.1 ± 1.3
Heart Wet Weight (mg)	258 ± 6	243 ± 16	242 ± 14	252 ± 18	258 ± 20
Heart Rate (bpm)	368 ± 23	340± 26	345 ± 23	368 ± 37	373± 17
Developed Force (g)	0.81 ± 0.19	1.06 ± 0.27	1.12 ± 0.12	1.09 ± 0.23	0.77 ± 0.16
Rate-Force Product (g x bpm)	308 ± 80	361 ± 109	372 ± 49	411 ± 107	287 ± 65
Coronary Flow (ml/min)	2.3 ± 0.2	2.3 ± 0.3	1.7 ± 0.1	2.1 ± 0.2	2.7 ± 0.3

Values are mean ± SEM. No significant difference ($P<0.05$, one-way ANOVA) between the groups were found for the listed parameters.

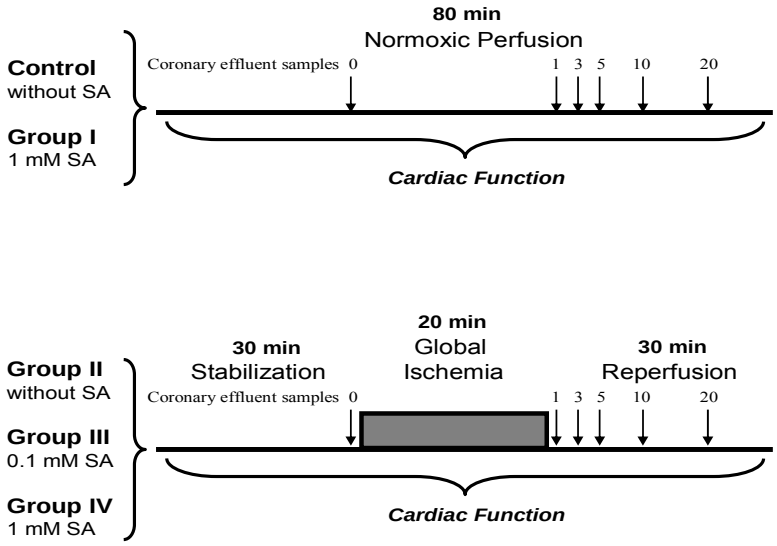


Figure 7. Protocol for animal experiments depicting experimental groups, salicylic acid (SA) level, ischemic condition and coronary effluent sample time points.

which were not subjected to ischemia and used non-SA Krebs buffer (Farthing et al., 2006). Group I consisted of six hearts which were not subjected to global cardiac ischemia; however the Krebs buffer solution contained 1.0 mM SA to evaluate the effects of the highest tested concentration of SA on ATP catabolism and PNP activity. Group II consisted of six hearts which were subjected to global cardiac ischemia with the Krebs buffer solution not containing SA to evaluate the effects of ischemic conditions on ATP catabolic by-products. To determine if the effects of SA are dose-dependent in the hearts undergoing global cardiac ischemia, Group III consisted of six hearts which were subjected to Krebs buffer solution containing 0.1 mM SA and Group IV consisted of six hearts subjected to 1.0 mM SA.

3.2.3 HPLC-UV Conditions and Mobile Phase

All instrument conditions were performed as previously reported (Farthing et al., 2007). Briefly, the HPLC-UV method used a Phenomenex® Onyx™ monolithic C_{18} analytical column (20 cm x 4.6 mm I.D., 130 Å) and Onyx™ C_{18} guard column (5 cm x 4.6 mm I.D.) (Torrance CA, USA). The mobile phase gradient consisted of aqueous trifluoroacetic acid (0.1% TFA in deionized water, pH 2.2, v/v) and methanol with time course (1 to 20% methanol linear gradient over 10 min). The mobile phase flow-rate was 1.0 ml/min with operating pressure of ~84 bar at ambient column temperature. A 15 µl direct injection of the Krebs buffer perfusate sample was made with optimal UV wavelength absorption of 250 nm used for inosine and hypoxanthine detection.

3.2.4 Data Computation and Statistics

The HPLC data acquisition and component computations were performed using TotalChrom™ Workstation software (Perkin Elmer™, Norwalk, CT, USA). Statistics utilizing ANOVA to compare the multi-group experimental results was performed using MS Excel (Microsoft®, Seattle, WA, USA) and post hoc analysis (Dunnett, Tukey) using JMP 6.0 (SAS Institute Inc., Cary, NC, USA) with α=0.05 and $P<0.05$ demonstrating significance. The Dunnett post hoc analysis was performed to compare each experimental test group's mean result to a control group's mean result. In addition, the Tukey post hoc analysis was also performed as it compares each group's mean result to each of the other group's mean result. However, it should be emphasized that for these non-parametric analysis, the small sample size (n=6 each group) for these animal experiments may be a limitation when interpreting the results. Correlation evaluation was performed using GraphPad Prism 4 (San Diego, CA, USA). For determining total effluxed inosine, the area under the curve (AUC) was calculated utilizing trapezoidal rule computations and MS Excel on HPLC results (0 to 20 min time point perfusate samples).

3.3 Results and Discussion

3.3.1 Chromatography and Method Validation

Figure 8 shows representative chromatograms for: (A) low standard of hypoxanthine (1.8 µM) and inosine (0.9 µM) in deionized water; (B) standard of hypoxanthine (14.7 µM, RT ~5.2 min), uric acid (11.9 µM, RT ~5.6 in), xanthine (13.1 µM, RT ~6.7 min), adenosine (7.5 µM, RT ~10.3 min) and inosine (7.5 µM, RT ~10.5 min) in deionized water; (C) Group I (no ischemia + 1.0 mM SA) perfusate sample

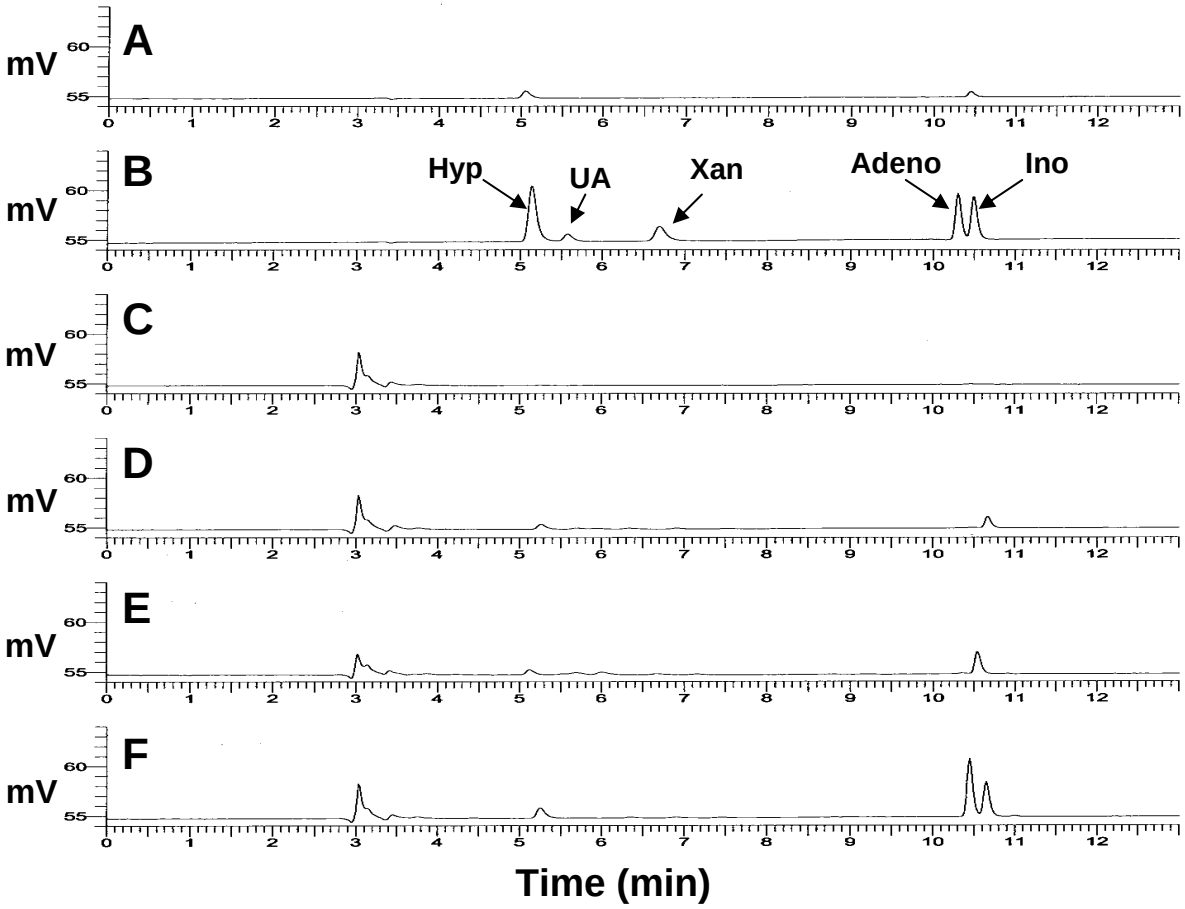


Figure 8. Chromatograms representing (A) low standard of 1.8 µM hypoxanthine (Hypo, RT 5.2 min) and 0.9 µM inosine (Ino, RT 10.6 min) in deionized water, (B) standard of 14.7 µM hypoxanthine, 11.9 µM uric acid (UA, RT 5.6 min), 13.1 µM xanthine (Xan, RT 6.7 min), 7.5 µM adenosine (Adeno, RT 10.3 min) and 7.5 µM inosine in deionized water, (C) Group I mouse perfusate sample (no ischemia and 1.0 mM SA), (D) Group II perfusate sample (20 min global ischemia and no SA), (E) Group III mouse perfusate sample (20 min global ischemia and 0.1 mM SA) and (F) Group IV mouse perfusate sample (20 min global ischemia and 1.0 mM SA).

collected at 1 min of aerobic perfusion, (D) Group II (ischemia + 0 mM SA) perfusate sample collected at 1 min of reperfusion; (E) Group III (ischemia + 0.1 mM SA) perfusate sample collected at 1 min of reperfusion; and (F) Group IV (ischemia + 1.0 mM SA) perfusate sample collected at 1 min of reperfusion.

As demonstrated by the chromatograms, the HPLC method provided sufficient sensitivity (Figure 8A) and selectivity (Figure 8B) for each of the ATP catabolic by-products. Figures 8C, 8D, 8E and 8F demonstrate how changes in experimental conditions (ischemia and SA concentrations) resulted in increased concentrations of inosine and hypoxanthine effluxed from the heart. Particularly Figures 8E and 8F demonstrate higher levels of ATP catabolic by-products suggesting the presence of SA in the Krebs buffer exacerbates ATP uncoupling only in the ischemic mouse hearts. It is also noteworthy that the heart subjected to ischemia under 1.0 mM SA (Group IV, Figure 8F) had elevated levels of ATP catabolic by-product adenosine, which is the nucleoside precursor to inosine and indicative of the largest total amount of ATP catabolic by-products effluxed due to the effect of 1.0 mM SA on the ischemic heart. All chromatograms obtained from animal reperfusates using SA in the Krebs buffer solution lacked detectable levels of xanthine and uric acid components, thus supporting published research citing ASA and SA inhibitory effects on the XO enzyme (Carlin et al., 1985; Masuoka et al., 2003).

Method validation was performed as described in our previous publication (Farthing et al., 2007) and in summary, the method demonstrated sufficient linearity of the calibration standards (inosine curve 0.9-18.6 μM, hypoxanthine curve 1.8-36.7 μM, with

R>0.9990 for each component) and method detection limits of 0.4 µM (inosine) and 0.7 µM (hypoxanthine). Method accuracy and precision for inosine and hypoxanthine was determined using quality control samples (n=15) with acceptable combined intra-day and inter-day component accuracy (±6 % error) and precision (±8.1 % CV). To demonstrate component stability, re-injections of the animal perfusate samples were made after sitting on the HPLC autosampler overnight at lab ambient temperature and again after long term storage. Both inosine and hypoxanthine in animal perfusate demonstrated excellent stability overnight on the HPLC autosampler and for more than 6 months when stored at -20°C.

3.3.2 Effect of Salicylic Acid on Purine Efflux

Table 7 lists mean (SEM) inosine and hypoxanthine concentrations for the mouse hearts representing each of the 5 experimental groups. As shown in Figure 9, Control group without both cardiac ischemia and SA (Farthing et al., 2006) and Group I (non-ischemic hearts but using 1.0 mM SA, the highest tested SA concentration) did not have detectable amounts of ATP catabolic by-products. However, inosine levels for Groups II, III and IV (all underwent global ischemia) had significantly elevated total inosine efflux of 1,437±348, 3,872±900, and 12,575±3319 ng/mL/min respectively (mean ± SEM, Figure 9), i.e. Groups II ($P<0.05$), III ($P<0.05$), and IV ($P<0.01$) as compared with the Control group (Dunnett test). These inosine results demonstrate that SA concentrations increased ATP catabolism under our conditions of acute global cardiac ischemia with groups III and IV mean total effluxed inosine levels potentiated above group II by approximately 2.7-fold and 8.8-fold, respectively.

Table 7. Table with mean (SEM) inosine and hypoxanthine concentrations and inosine/hypoxanthine ratios under various experimental conditions (control and global cardiac ischemia) and concentrations of salicylic acid in Krebs buffer (0, 0.1 and 1.0 mM).

Sample	Ischemia	Reperfusion Time (min)	Mean Hypoxanthine [μM]	SEM Hypoxanthine	Mean Inosine [μM]	SEM Inosine	Inosine/Hypoxanthine ratio	Krebs Salicylic Acid Level
Control	No	0	0	0	0	0	N/A	0 mM SA
(n=6)		1	0	0	0	0	N/A	
		3	0	0	0	0	N/A	
		5	0	0	0	0	N/A	
		10	0	0	0	0	N/A	
		20	0	0	0	0	N/A	
Group I	No	0	0	0	0	0	N/A	1.0 mM SA
(n=6)		1	0	0	0	0	N/A	
		3	0	0	0	0	N/A	
		5	0	0	0	0	N/A	
		10	0	0	0	0	N/A	
		20	0	0	0	0	N/A	
Group II	Yes	0	2.8	2.0	1.2	2.0	0.4	0 mM SA
(n=6)		1	111.5	0.6	200.9	0.6	1.8	
		3	29.4	0.6	41.7	0.6	1.4	
		5	9.7	2.0	16.9	1.0	1.7	
		10	9.7	2.0	15.5	1.2	1.6	
		20	2.8	2.0	2.6	1.2	0.9	
Group III	Yes	0	0	0	0	0	N/A	0.1 mM SA
(n=6)		1	230.7	0.7	391.5	0.5	1.7	
		3	83.0	0.4	117.8	0.4	1.4	
		5	38.9	1.4	53.7	0.8	0	
		10	38.9	1.4	49.6	1.0	0	
		20	11.0	1.4	4.5	1.4	0	
Group IV	Yes	0	15.4	1.5	12.9	1.5	0.8	1.0 mM SA
(n=6)		1	183.2	1.1	761.3	1.1	4.2	
		3	89.2	1.2	240.0	1.5	2.7	
		5	46.3	1.4	85.4	1.6	1.8	
		10	20.9	1.4	19.1	1.3	0.9	
		20	14.2	1.5	10.7	1.5	0.8	

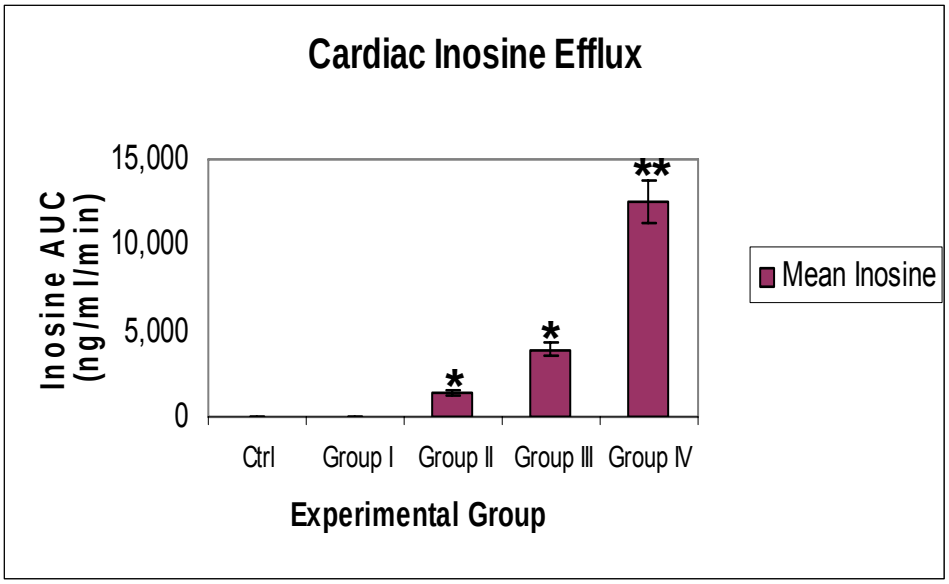


Figure 9. Bar chart representing total effluxed inosine (mean + SEM) for control (Ctrl), Group I, Group II, Group III and Group IV experimental conditions. Groups II, III and IV demonstrated statistical significance (* is $p<0.05$, ** is $p<0.01$) from Ctrl (Dunnett test) and Group I (Tukey test). Ctrl (no ischemia and no SA), Group I (no ischemia and 1.0 mM SA), Group II (20 min global ischemia and no SA), Group III (20 min global ischemia and 0.1 mM SA) and Group IV (20 min global ischemia and 1.0 mM SA).

It should be noted that we did not observe any ATP catabolic by-products from group I animals (1.0 mM SA and non-ischemic). Based on published work (Nulton-Persson et al., 2004; Cronstein et al., 1994), we suspected that in the presence of 1.0 mM SA we might observe low concentrations of ATP catabolic by-products even under non-ischemic conditions. However, we did not observe either inosine or hypoxanthine in detectable levels from any perfusate samples from this experiment group. One possible explanation is that these authors used cardiac mitochondrial preparations for their studies and not the entire heart tissue as did our study. They reported 1.0 mM SA uncoupling oxidative phosphorylation by approximately 20% (using oxygen electrode and α-ketoglutarate dehydrogenase enzyme analysis) which may not produce high enough concentrations of ATP metabolites to activate the normally dormant adenosine deaminase and PNP enzymes, thus inosine and hypoxanthine would not be produced and detected. Using SA and ischemic conditions together would combine the effects of SA (ATP decoupling) and ischemia (blocks ATP synthesis) and lead to significant reduction in cellular ATP concentration and the potentiated effluxed levels of inosine observed in our present study.

3.3.3 Effect of Salicylic Acid on Cardiac Function

Figure 10 bar charts (mean ± SEM) demonstrate the effects of SA on cardiac functional parameters (DF, HR, RFP). No significant differences were found in HR for all experimental conditions. For DF and RFP, significance ($P<0.05$, Tukey test) was demonstrated between Groups III and IV relative to each other but neither group was statistically different than control (Dunnett test). Cardiac DF and RFP were slightly

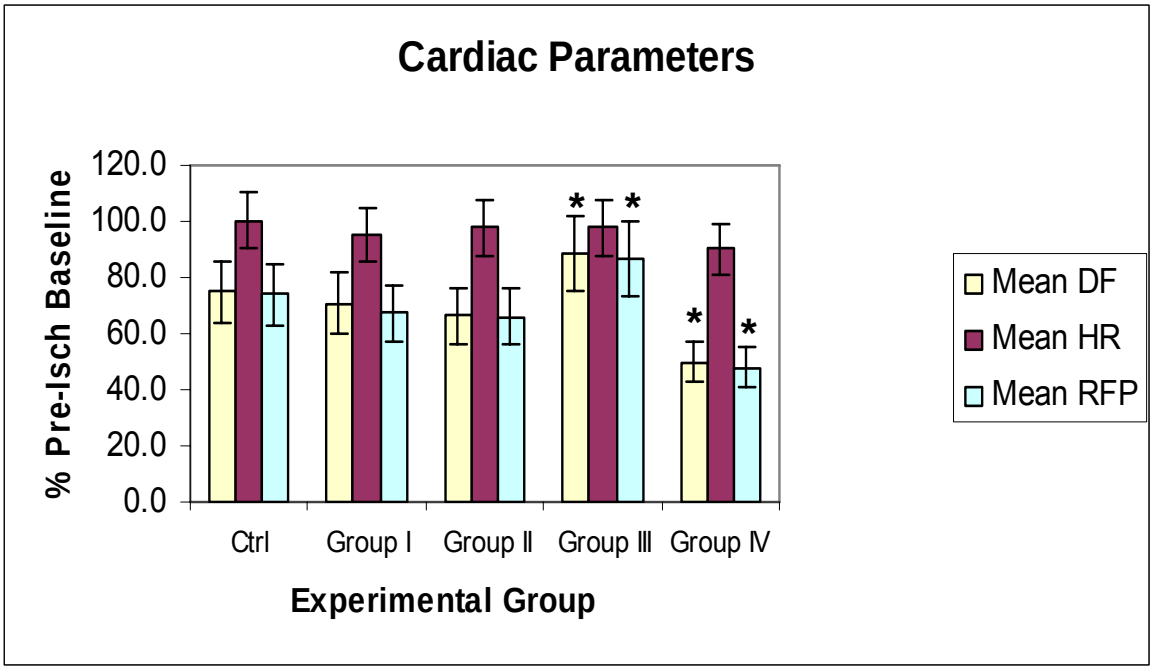


Figure 10. Bar chart representing mean ± SEM for cardiac developed force (DF), heart rate (HR) and rate-force product (RFP) at the end of 30 min reperfusion for each of the experimental groups. Developed force and RFP on Groups III and IV demonstrated statistical significance (* is $p<0.05$, Tukey test) between each other, however neither demonstrated significance from control (Ctrl) (Dunnett test) nor Group I (Tukey test). Control (no ischemia and no SA), Group I (no ischemia and 1.0 mM SA), Group II (20 min global ischemia and no SA), Group III (20 min global ischemia and 0.1 mM SA) and Group IV (20 min global ischemia and 1.0 mM SA).

increased (beneficial) at the 0.1 mM SA concentration, but adversely affected at 1.0 mM SA. Theses results demonstrate that under conditions of acute global cardiac ischemia, higher levels of SA in the Krebs buffer exhibited an increasing relationship on effluxed inosine; however a beneficial then adverse effect on DF and RFP was observed. These results have confirmed the previously reported positive inotropic effects of ASA on cardiac contractility through inhibiting COX enzymes and in turn prostaglandin synthesis (Karmazyn, 1986) and through its modulating effects on cellular calcium levels (Molderings et al., 1987).

3.3.4 Non-Linear Relationship of Inosine Efflux and Cardiac Contractile Function Following Ischemia

Figure 11 is a correlation plot which demonstrates the lack of correlation between total effluxed inosine (in Groups II, III and IV) and DF or FRP, with r^2 (coefficient of determination) values of 0.52 and 0.59, respectively. This non-linear relationship can be explained by an apparent beneficial effect of 0.1 mM SA on heart contractility, yet an adverse effect at 1.0 mM SA, while total effluxed inosine increased with increasing SA concentrations. Therefore, whereas the inosine efflux concentration could serve as a sensitive biomarker for acute cardiac ischemia, it appears to be a poor predictor of the individual post-ischemic cardiac functional recovery, at least in this ex vivo model.

3.3.5 Effect of Salicylic Acid on Purine Nucleoside Phosphorylase Activity

Figure 12 illustrates the ATP catabolic by-products resulting from acute cardiac ischemic conditions. Normally dormant enzymes in heart tissue (e.g. adenosine

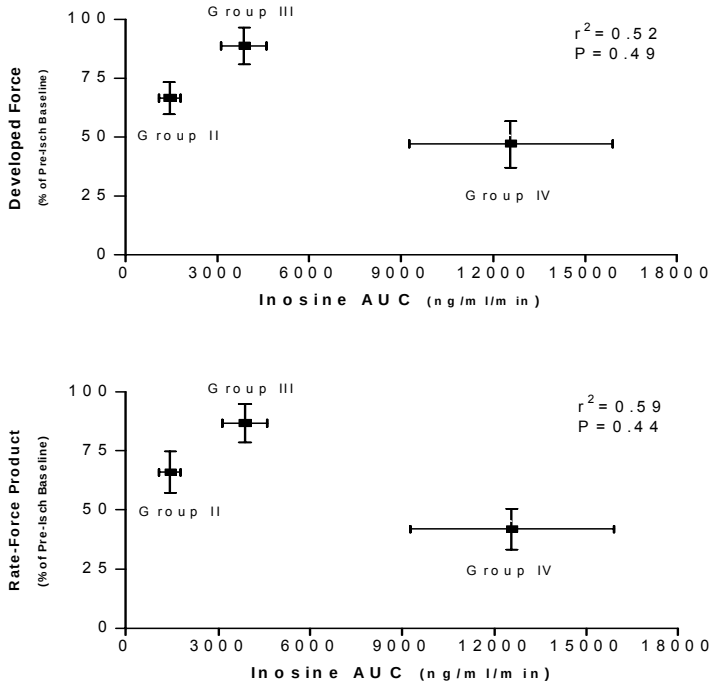


Figure 11. Correlation plot demonstrating lack of significant linear correlation between total effluxed inosine (AUC) and DF or RFP in the ischemic mouse hearts under various SA concentrations (0, 0.1 and 1.0 mM). Group II (20 min global ischemia and no SA), Group III (20 min global ischemia and 0.1 mM SA) and Group IV (20 min global ischemia and 1.0 mM SA).

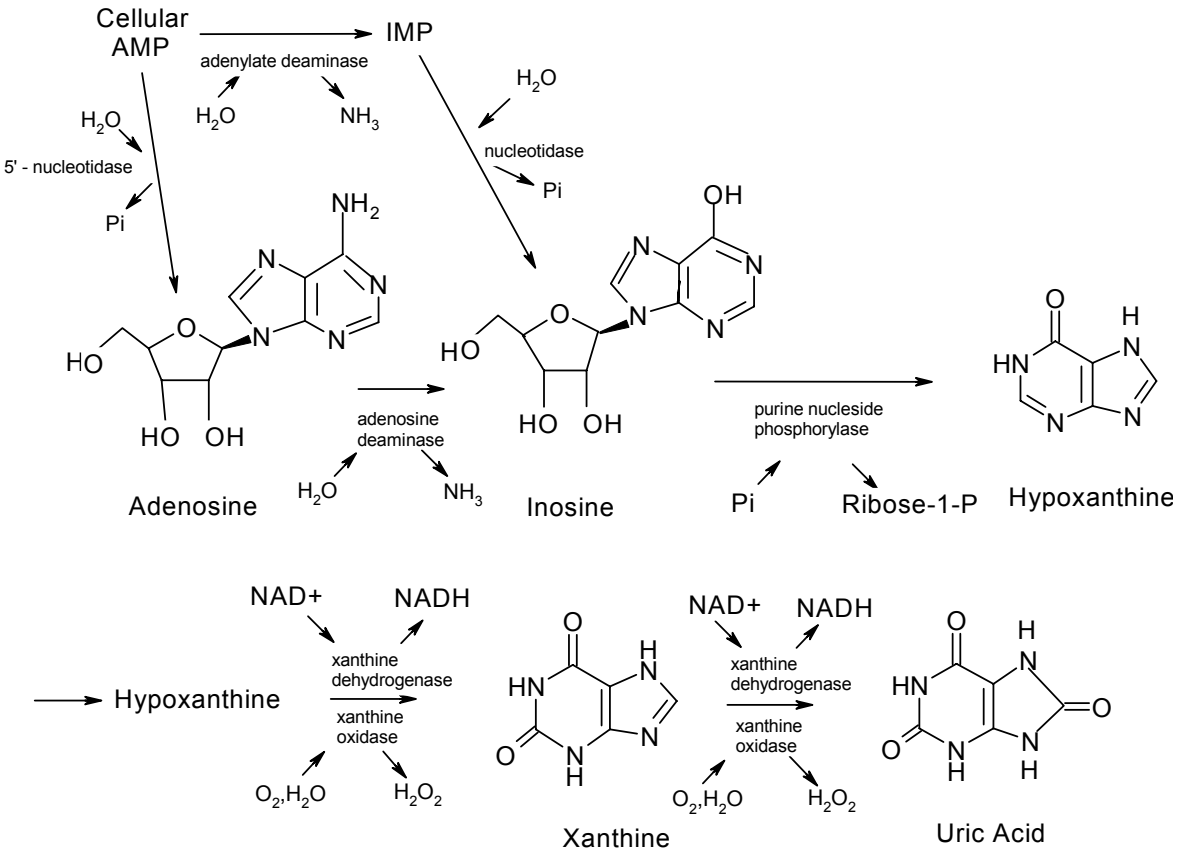


Figure 12. Biochemical pathway of ATP catabolic by-products resulting from global acute cardiac ischemia.

deaminase, purine nucleoside phosphorylase) are activated due to cellular buildup of ATP catabolic by-products (e.g. ADP, AMP). Under our experimental conditions, inosine and hypoxanthine were the primary effluxed by-products of ATP catabolism. In observation of the larger amounts of total inosine effluxed in our 1.0 mM SA perfusate samples, we wanted to investigate if SA had a negative effect (inhibitory) on PNP enzyme activity which might account for the potentiated effluxed inosine levels.

The PNP enzyme activity under ischemic conditions can be indirectly determined by calculating the conversion of inosine to hypoxanthine ratio on each perfusate sample. Table 7 lists the inosine [μM]/hypoxanthine [μM] (ino/hypo) conversion ratio for the experimental groups. In Group II (ischemia, 0 mM SA), the ino/hypo mean conversion ratio was approximately ~1.3. Under Group IV experimental conditions (ischemia, 1.0 mM SA), the ino/hypo conversion ratios were not constant with the perfusate sample collected at 1 min of reperfusion having the largest ino/hypo ratio, and at the subsequent time points returning to a more constant ino/hypo conversion ratio (~0.9) (Figure 13).

These results can be explained by two effects occurring simultaneously when using both SA and ischemia conditions. Cardiac ischemic (anaerobic) conditions can cause ATP catabolism as demonstrated in our previous work (Farthing et al., 2006), while SA has been reported to cause decoupling of cardiac mitochondrial respiration (Nulton-Persson et al., 2004). Both situations may contribute to the increase in ATP catabolic by-products as heart muscle contractions require significant amounts of ATP as its primary energy source. This leads to cellular buildups of ADP and AMP metabolic by-products as ATP is not able to be regenerated (via salvage pathway) or synthesized, thus ATP catabolism occurs to

eliminate the cellular buildup of by-products. As aerobic conditions to the heart tissue was reestablished via reperfusion, the ino/hypo conversion ratio returns to a more constant conversion ratio (e.g. ~0.9). The conversion ratio drops to zero as aerobic conditions should deactivate ADA and PNP enzymes in the cardiac myocytes, thus inhibiting ATP catabolism (Figure 13).

3.3.6 Potential Clinical Relevance

Since both ASA and SA have been reported to uncouple mitochondrial respiration under aerobic conditions and in turn to inhibit ATP synthesis (Nulton-Persson et al., 2004) and our current study demonstrates a potentiated ATP catabolism by SA under cardiac ischemia, it is possible that current use of ASA for medical emergency treatment in acute cardiac ischemic situations (e.g. acute MI) may potentially increase the ischemia-caused ATP catabolism and inosine efflux.

While standardized doses of ASA (160 to 325 mg) are used to inhibit platelet aggregation at the site of thrombus as part of the treatment for acute MI, the higher 325 mg dose equates to a potential Cmax of 67 μM acetyl salicylic acid and 188 μM salicylic acid blood concentrations. Even with its significant protein binding (e.g. albumin), the augmented free drug levels of ASA and its metabolite SA may have an inhibitory effect on cardiac ATP production during the periods of acute cardiac ischemia. The SA-induced enhancement of inosine efflux should also be taken into consideration when we analyze and interpret the patient's plasma inosine level as a potential biomarker for acute cardiac ischemia (Farthing et al. 2006, 2007).

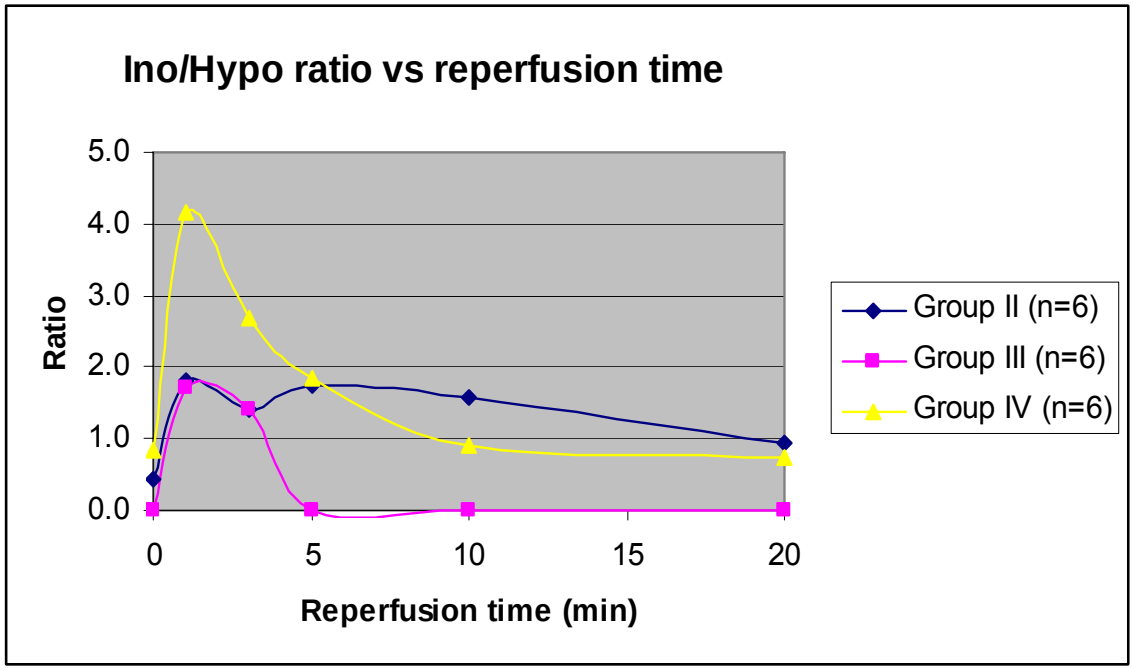


Figure 13. Plot of inosine [μM] to hypoxanthine[μM] (ino/hypo) conversion ratio versus reperfusion time (min). The plot represents mean data from experimental conditions. Ino/hypo conversion ratio is highest in the 1 min reperfusion sample and returns to a constant ratio before dropping as aerobic conditions presumably deactivate ADA and PNP enzymes in the cardiac myocytes.

Whereas there are certainly positive benefits associated with using ASA as part of the treatment for acute MI patients to inhibit platelet aggregation at the site of thrombus, higher doses of ASA used for other medical conditions including analgesia (0.5 mM plasma salicylate) and rheumatoid arthritis (1.5 to 2.5 mM plasma salicylate) may actually aggravate the ischemic effects on heart tissue metabolism and ventricular contractile function, if the patients who have been using higher dose of ASA suddenly encounter acute cardiac ischemic events. This animal research on isolated mouse hearts utilized SA levels of 0.1 and 1.0 mM, which were slightly lower but similar to expected blood concentrations in human patients utilizing aspirin as treatment for the above described medical conditions (e.g. acute MI, rheumatoid arthritis). We suggest that further laboratory and clinical studies are warranted on the apparent adverse effects of higher concentrations of ASA and SA on ATP catabolism under acute cardiac ischemia, given the knowledge of how widespread ASA is used for its other medical benefits (e.g. analgesia, rheumatoid arthritis) and the possibility of those patients one day experiencing an acute cardiac ischemic event.

3.4 Acknowledgements

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ischemia. This cost of the animal experiments was partially supported by a grant from American Heart Association, National Center (0530157N to Dr. Lei Xi).

As this chapter of research was published in Biomarkers (2007, 12(6), 623-634), I would like to sincerely thank and acknowledge the manuscript co-authors for their technical expertise and valuable scientific discussions (Dr. Lynne Gehr[2], Dr. H. Thomas Karnes[1], Dr. Domenic Sica[3], Dr. Todd WB Gehr[3], Terri Larus[3], Christine Farthing[3], and Dr. Lei Xi[4]) at [1]Departments of Pharmaceutics and [2]Pediatrics, Department of Internal Medicine, Divisions of [3]Nephrology and [4]Cardiology, VCU Medical Center, Virginia Commonwealth University, Richmond, VA 23298, USA. A technical poster presentation of this work entitled "Effect of salicylic acid on ischemia-reperfusion injury in isolated mouse hearts" was presented at Pittcon 2006 Analytical Exposition (Orlando, Florida, March 2006) (Appendix B).

CHAPTER 4. Human Plasma Sample Evaluation, "An HPLC method for determination of inosine and hypoxanthine in human plasma from healthy volunteers and patients presenting with potential acute cardiac ischemia"

4.1 Introduction

The mouse model results from Chapter 2 research demonstrated significant levels of inosine and elevated levels of hypoxanthine effluxed from cardiac tissue subjected to constant conditions of oxidative stress (e.g. acute cardiac ischemia or myocardial infarction) (Farthing et al., 2006). For this chapter of research, the HPLC method from Chapter 2 was modified and validated for the evaluation of human plasma samples, which are significantly more complex than Krebs perfusate samples from Chapter 2. The purpose for the development of a plasma test method was to facilitate evaluation of the research hypothesis that non-traumatic chest pain patients potentially undergoing acute cardiac ischemia; should have elevated blood levels of ATP catabolic products (e.g. inosine and hypoxanthine) in their bloodstream, until medical treatment can succeed in restoring adequate blood flow to the oxygen deprived myocardium.

Samples representing healthy individuals, hospital non-traumatic chest pain patients as well as other common vascular disease (e.g. congestive heart failure (CHF), hypertension (HT)) conditions were evaluated for inosine and hypoxanthine concentration

levels. In addition, subject matched sample sets comprised of consecutive blood draws for plasma (heparin) and serum separator tube (SST), from healthy normal individuals were evaluated to determine if the blood draw matrix (e.g. anticoagulants, SST gel) affected inosine and hypoxanthine concentrations.

Current test methods for endogenous cardiac biomarkers (e.g. cardiac troponin I, creatine kinase-MB and myoglobin) include LC-MS analysis (Bunk et al., 2000; Mayr et al. 2006) and fluorescence immunoassay (Apple et al., 1999; Heeschen et al., 1999; Apple et al., 2000; McCord et al., 2001); however elevation of these protein biomarkers reflect some level of myocardial necrosis, and are typically elevated in a diagnostic range several hours after acute myocardial infarction. Current methods for plasma level measurement of selected ATP catabolic by-products such as inosine, hypoxanthine, xanthine and uric acid, in plasma utilize HPLC-UV with sample preparation steps including solid phase extraction (Feng et al., 2000), protein precipitations (e.g. ethanol or TCA) as well as some methods requiring use of an internal standard (Boulieu et al., 1983; Boulieu et al., 1984). HPLC with ion pairing reagents (Scott et al., 1992; Furst et al., 1992; Tavazzi et al., 2005) or protein precipitation and enzyme catalyzed luminescence detection (Jabs et al., 1990) have also been used. One HPLC method utilized centrifugal filtration for sample preparation; however their method did not completely resolve hypoxanthine and xanthine components at concentrations five times lower than our patient hypoxanthine concentrations, and with reported column degradation after three months of use (Severini et al., 1987).

None of these techniques, however, offers as simple a determination for inosine and hypoxanthine (can also evaluate uric acid, adenosine, and xanthine) in human plasma

as the method detailed in this chapter of research. The method utilizes centrifugal membrane filter technology and does not require the use of an internal standard. In addition, this method employs a recently introduced HPLC column technology (Onyx™ monolithic column, Phenomenex® Inc. 2005 market introduction) (Phenomenex Inc.), which provided sufficient component resolution and sensitivity for measurement of inosine and hypoxanthine in human plasma samples, from healthy volunteers and emergency department patients presenting with chest pain with and without acute cardiac ischemia.

4.2 Human Plasma and Serum Sample Procurement

To obtain human plasma samples used for analytical evaluations (HPLC-UV and Luminometer), approvals from Virginia Commonwealth University (VCU) Institutional Review Board (IRB) (Appendix C) and Chippenham Hospital (Richmond, VA) Clinical Chemistry Department were obtained. Plasma samples (lithium heparin) from normal healthy individuals (non-symptomatic for cardiac disease) were acquired from VCU Medical Center and plasma samples (lithium heparin) from non-traumatic chest pain patients were acquired from Chippenham Hospital Clinical Chemistry Department. All obtained samples were frozen (-20°C or below) after draw and prior to analysis.

In addition, purchased human plasma (EDTA and lithium heparin anticoagulant) from cardiac diseased patients (e.g. hypertension, acute myocardial infarction), human blank plasma (lithium heparin anticoagulant) and serum samples (SST and non-SST) were commercially obtained from a FDA certified biorepository, ProMedDx (Norton, MA, USA) (Appendix D), which utilized an IRB protocol for their sample procurements. The

ProMedDx samples were stored frozen (-20°C or below) after draw and during storage prior to analytical evaluations.

4.3 HPLC-UV Assay Development

4.3.1 Experimental

4.3.1.1 Chemicals and Blank Plasma

Hypoxanthine and xanthine were purchased from Acros Organics (Fair Lawn, NJ, USA) and adenosine, inosine and uric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA) with all chemicals being ACS reagent grade or higher purity. For mobile phase preparation, trifluoroacetic acid (TFA) was reagent grade, methanol was Optima HPLC grade and both were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Ultrapure distilled and deionized water (18 MΩ-cm) used for all HPLC work was prepared in-house using PureLab® Ultra water purification system (US Filter, Lowell, MA, USA) and 0.2 µm filtered prior to use. Blood bank human blank plasma (acid citrate) used for preparation of controls was provided by VCU Medical Center, Richmond, VA USA.

4.3.1.2 HPLC Equipment and Mobile Phase

The HPLC-DAD (diode array detector) equipment consisted of a Hewlett Packard (HP) Model 1090 HPLC system (Agilent Technologies, Palo Alto, CA, USA). The analytical column used was a Phenomenex® Onyx™ monolithic C_{18}, 20 cm x 4.6 mm I.D., 130 Å column coupled to an Onyx™ C_{18} guard column, 5 cm x 4.6 mm I.D. (Torrance CA, USA). The guard column was replaced after each analytical run of approximately 50 samples. The mobile phase consisted of aqueous trifluoroacetic acid (0.1% TFA in

deionized water, pH 2.2, v/v) and methanol gradient. The mobile phase gradient was programmed with time course as follows (99:1 0.1% TFA in deionized water:methanol, v/v at 0 min and held for 3 min; 70:20 0.1% TFA in deionized water:methanol, v/v at 10 min; 5:90 0.1% TFA in deionized water:methanol, v/v at 11 min and held 2 min, and 99:1 0.1% TFA in deionized water:methanol, v/v at 14 min).

The mobile phase was continuously degassed using helium sparging and used at a flow-rate of 1.0 ml/min. Typical HPLC operating pressure at gradient time 0 min conditions was approximately 84 bar with ambient column temperature. An injection volume of 15 µl of the prepared plasma sample was accomplished using the HP Model 1090 autosampler. Component detection was achieved using the HP Model 1090 DAD detector with data collection at the optimal UV wavelength absorption of 250 nm for both inosine and hypoxanthine. The detector was operated at high sensitivity set point with a 1 s response time. A 345 kPa back-pressure regulator (SSI, State College, PA, USA) was coupled to the detector outlet to prevent mobile phase out-gassing. Data acquisition and component computations were performed using TotalChrom™ Workstation software (Perkin Elmer™, Norwalk, CT, USA).

4.3.1.3 Standard and Control Preparation, Freeze-Thaw Study

Stock standards of adenosine (374.2 µM), inosine (372.8 µM), hypoxanthine (734.7 µM), xanthine (657.4 µM) and uric acid (594.8 µM) were prepared in deionized water and stored at 4°C. Working standards to establish HPLC retention times of adenosine (9.4 µM), xanthine (16.4 µM) and uric acid (14.9 µM) components were prepared in deionized water. Working standards of inosine (0.9, 1.9, 3.7, 11.2 and 18.6 µM) and hypoxanthine

(1.8, 3.7, 7.3, 22.0 and 36.7 µM) were prepared in deionized water. All working standards were stored at -70°C and stable for at least 6 months. Working controls of inosine (0.9, 7.5 and 14.9 µM) and hypoxanthine (1.8, 14.7 and 29.4 µM) were prepared using pooled hospital blood bank blank plasma (n=3 donated lots) which were evaluated individually and confirmed to lack detectable levels of inosine and hypoxanthine components.

It is possible the levels of inosine and hypoxanthine in blood bank plasma were not detectable due to the time (>10 days) the plasma was stored refrigerated (4°C) prior to expiration and availability for laboratory experimental use. Without freezing the plasma or utilizing plasma enzyme inhibitors, xanthine oxidase and purine nucleoside phosphorylase found in plasma may metabolize the normally low levels of inosine and hypoxanthine to their end product uric acid. Following preparation of control samples, they were immediately frozen at -70°C, to prevent endogenous plasma purine nucleoside phosphoryase from converting inosine to hypoxanthine prior to formal sample analysis.

To demonstrate inosine and hypoxanthine freeze-thaw stability, control samples at each concentration were freeze-thawed (n=2 times) and evaluated by HPLC for degradation. The lack of inosine and hypoxanthine degradation from freeze-thaw from -70˚C was demonstrated by inosine and hypoxanthine concentrations being consistent (<6.0% error) with the results from the accuracy and precision evaluation (Table 8).

4.3.1.4 Sample Conditions

Following hospital approval, blood was obtained from hospital emergency room patients (n=20), in vacutainer™ tubes containing plasma (lithium heparin) as per hospital emergency room protocols for patients presenting with non-traumatic chest pain and

potential MI or acute myocardial ischemia. Sample tubes were centrifuged at ~1000 g for 10 min with plasma drawn off and split into tubes for hospital clinical testing and one tube immediately frozen at -20°C (transferred to -70°C for storage) for inosine and hypoxanthine analysis. Plasma (lithium heparin) samples from healthy blood donors (male and female, both genders >18 years of age) were purchased from ProMedDx (Norton, MA, USA) which used an IRB approved specimen collection protocol and stored frozen at -70°C. Prior to HPLC analysis, plasma samples were thawed to ambient temperature, mixed thoroughly by inversion and centrifuged at 1000 g for 10 min to eliminate fibrous material.

4.3.1.5 Sample Preparation

Samples were prepared for HPLC analysis by pipetting 250 µl of plasma into a polypropylene Microcon® YM-10 (10,000 molecular weight cutoff, MWCO) centrifugal filter tube (Millipore, Bedford MA, USA). The sample tubes were capped and centrifuged at 14,000 g for 15 min at ambient lab temperature. The clear filtrates were transferred to deactivated glass HPLC autosampler vials (Waters®, Milford MA, USA) with 15 µl injected into the HPLC system for analysis.

4.3.2 Results and Discussion

4.3.2.1 HPLC Conditions Optimizations

Several types of C_{18} columns were evaluated for resolving adenosine, inosine, hypoxanthine, xanthine and uric acid from other plasma components. Due to minimal sample preparation using the centrifugal membrane filter, the ideal HPLC column should have high efficiency for resolving inosine and hypoxanthine components from components

in the plasma matrix. Conventional HPLC columns such as Synergi Polar-RP C_{18} (15 cm x 3.0 mm ID x 4 µm packing) and Hypersil ODS C_{18} (15 cm x 3.2 mm ID x 3 µm packing) were evaluated versus the recently marketed HPLC column technology, the Onyx monolithic C_{18} column (10 cm x 4.6 mm ID).

The monolithic column provided superior chromatographic resolution of components as described later in section 4.3.2.4 with a low system backpressure of approximately 84 bar (gradient time zero conditions and flow rate of 1 ml/min). It should be emphasized that both conventional HPLC columns were evaluated at operating flow rates of ~0.6 ml/min and with system pressures that were approximately twice as high as when using the monolithic column. The supplier of the monolithic column cited advantages of high component efficiencies (resolution) and low system backpressure with use of the new monolithic column technology. We observed that both of these stated advantages over the two conventional mid-bore diameter HPLC columns evaluated were clearly demonstrated. The mobile phase aqueous component, 0.1% TFA in deionized water, provided a pH of 2.2 which also provided good peak shape (e.g. uric acid component, pKa ~5.8) from components of interest from the endogenous plasma components (MW <10,000 Da) obtained from the YM-10 sample preparation.

Optimization and adjustment of the acid strength improved the separation between hypoxanthine (RT 5.2 min) and uric acid (RT 5.7 min). Initial use of aqueous 0.05% TFA did not provide component baseline resolution while aqueous 0.1% TFA offered complete component baseline resolution at the expense of increased column retention times. The mobile phase organic modifiers (e.g. acetonitrile versus methanol) were evaluated to

determine which organic solvent would provide the best chromatographic separation from endogenous plasma components and at the same time being most cost effective. Methanol was chosen as the organic modifier as it provided symmetrical component peak shapes and good selectivity from other endogenous plasma components; however the HPLC system backpressure was somewhat higher when using methanol with the methanol gradient increasing from 1 to 90%. Methanol is also more cost effective for routine HPLC analysis because of its lower procurement cost.

A mobile phase gradient was used for reproducible separations of the structurally similar purines (hypoxanthine, uric acid) and nucleosides (inosine, adenosine). Since the mobile phase organic constituent is critical to controlling component elution times (initial 1% methanol composition at gradient time zero), the use of protein precipitation technique using solvents such as acetonitrile or methanol (typically 1:1 or 2:1, organic:plasma ratio) was eliminated from consideration.. The structurally similar components injected using organic solvent precipitation were not chromatographically resolved due to band broadening effects from the added organic modifier. Different column oven temperatures (e.g. ambient lab of 20°C, 30°C and 40°C) were evaluated without significant chromatographic improvement (component resolution, peak shape), thus ambient temperature was utilized for the analysis. At higher column temperatures (e.g. 40°C), component co-elution for both early (hypoxanthine, uric acid) and late components (inosine, adenosine) was observed.

4.3.2.2 Linearity, Limits of Quantitation and Detection, Computations

The plasma method was linear throughout the concentration range of 0.9 to 18.6 µM for inosine (mean correlation coefficient of 0.9991, n=10) and 1.8 to 36.7 µM hypoxanthine (mean correlation coefficient of 0.9998, n=10) with all standard back-calculated values within 5% of their nominal amount. The limit of detection (LOD) for inosine (0.4 µM) and hypoxanthine (0.7 µM) was determined using a fortified amount of each component in pooled blood blank plasma (n=3 each) and calculation from each component's standard curve (component peak heights had greater than 3 times s/n than blank plasma background). For plasma component calculations and reporting results, normal linear regression utilizing external standardization and peak height was used with the lowest calibration standard as the limit of quantification (defined as combined accuracy and precision within 20% of the nominal amount).

4.3.2.3 Accuracy, Precision and Recovery

The accuracy and precision for the method was determined by evaluation of replicate prepared plasma control samples (inosine at 0.9, 7.5, 14.9 µM and hypoxanthine at 1.8, 14.7, and 29.4 µM) (Table 8). The combined intra-day (within day) and inter-day (between day) accuracy of the method was reported as the percent error of nominal fortified amounts versus measured component concentrations. The combined intra-day and inter-day precision of the method was reported as percent relative standard deviation (% RSD). The method demonstrated sufficient accuracy (±6%) and precision (±8.1) for both components in plasma (n=15 at each component concentration level). Absolute recovery for the plasma method was evaluated by comparing the extracted fortified control

Table 8

Combined intra and inter-day accuracy and precision for inosine and hypoxanthine in plasma controls. Controls demonstrated excellent accuracy ±6% and precision ±8.1% throughout the plasma concentration range.

Component	Fortified Concentration (µM) n=15	Calculated Mean Concentration (µM) n=15	% Error	% RSD
Inosine	0.93	0.91	-2.8	8.1
Inosine	7.5	7.3	-1.7	4.9
Inosine	14.9	14.6	-2.2	3.6
Hypoxanthine	1.84	1.95	6.0	7.5
Hypoxanthine	14.7	15.0	2.2	5.5
Hypoxanthine	29.4	28.9	-1.7	2.2

samples prepared in pooled blood blank plasma versus unextracted standards prepared in deionized water (n=3). The absolute recovery for the plasma method was determined to be >98% for both inosine and hypoxanthine. In addition, the standards and controls used for all HPLC analysis were prepared and handled identical to patient and volunteer subject samples, thus controlling for potential errors in sample handling, micropipetting and YM-10 component extraction recovery.

4.3.2.4 Chromatography

Figure 14 illustrates chromatograms of 14.7 µM hypoxanthine (RT ~5.3 min), 11.9 µM uric acid (RT ~5.8 min), 13.1 µM xanthine (RT ~7.2 min), 7.5 µM adenosine (RT ~10.7 min) and 7.5 µM inosine. patient. (RT ~10.9 min) in deionized water for marking component retention times; limit of quantitation and lowest plasma standard of 1.84 µM hypoxanthine and 0.93 µM inosine; pooled blank plasma (acid citrate) from the VCU Health Systems Hospital blood bank; prepared plasma (lithium heparin) from a healthy female subject; and prepared plasma (lithium) from a hospital emergency room female patient exhibiting symptoms of chest pain and acute myocardial ischemia (Figures A, B, C, D and E, respectively).

The method demonstrated good component chromatographic selectivity with no endogenous plasma interferences at the retention times of hypoxanthine and inosine. This method also provided sufficient sensitivity for both components of interest using conventional UV detection with an analytical run time of ~21 min (allows mobile phase gradient equilibration). To extend column lifetime, the analytical column was flushed after

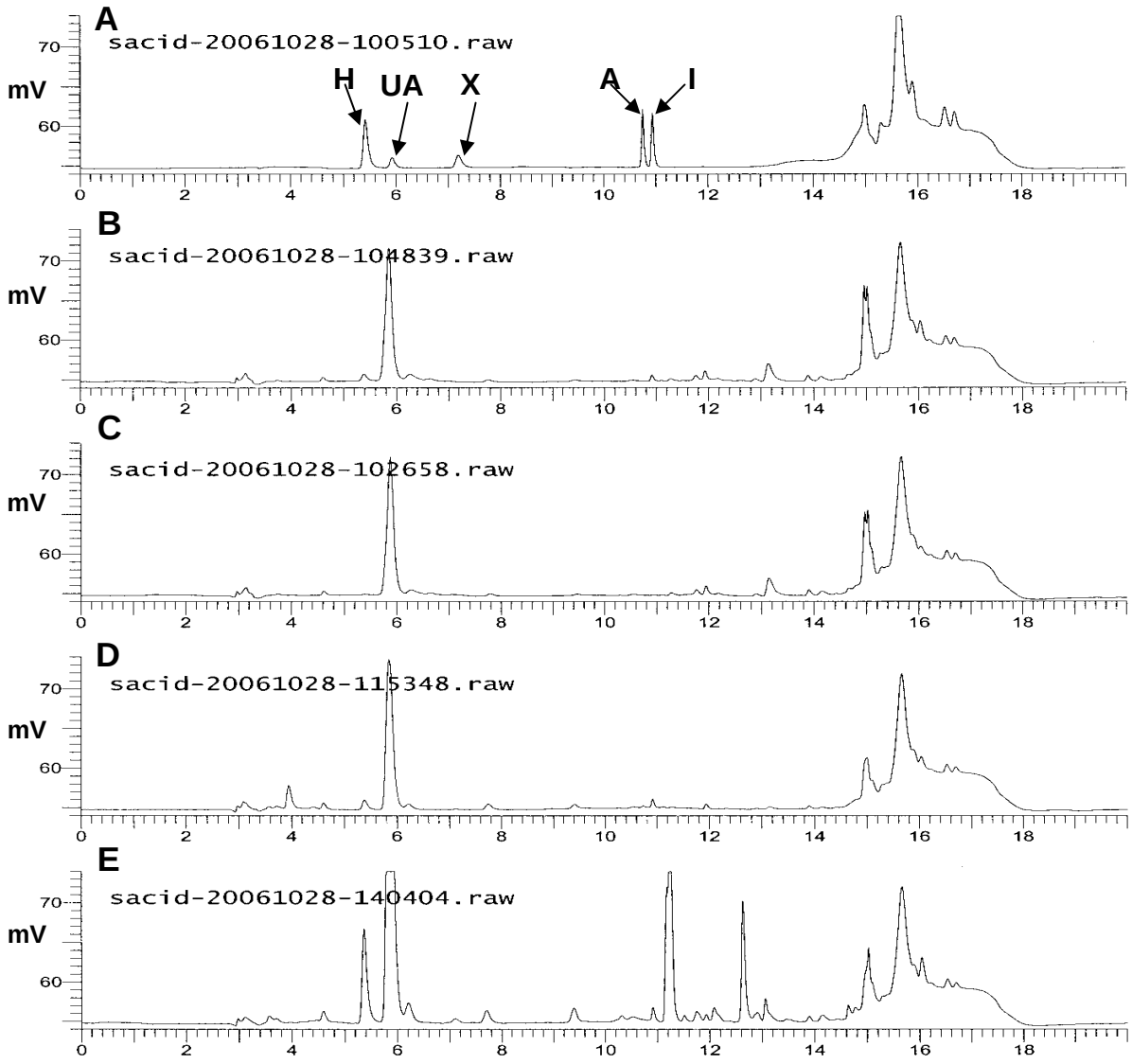

Figure 14. Chromatograms illustrating (a) 14.7 µM hypoxanthine (RT ~5.3 min), 11.9 µM uric acid (RT ~5.8 min), 13.1 µM xanthine (RT ~7.2 min), 7.5 µM adenosine (RT ~10.7 min) and 7.5 µM inosine (RT ~10.9 min) in deionized water, (b) low standard of 1.84 µM hypoxanthine and 0.93 µM inosine in blank plasma, (c) blank plasma, (d) plasma sample from healthy female subject and (e) plasma sample from hospital emergency room female

each analytical run (~50 injections) for 1 h at 1.0 ml/min with acetonitrile: deionized water (90:10, v/v) to eliminate potential retained non-polar substances from the column.

4.3.2.5 Sample Preparation Optimization and Filtrate Stability

Sample preparation evaluations using protein precipitation and centrifugal membrane filters were conducted. As previously described in Section 4.3.2.1, organic solvent precipitation was not useful due to resulting poor chromatographic resolution of structurally similar components. TCA (trichloroacetic acid) was not evaluated due to the hazards of using the strong acid and the resulting sample dilution effect potentially affecting overall method sensitivity. The centrifugal membrane filter is commonly used to concentrate peptides, proteins and nucleic acids for proteonomic and genomic determinations [Microcon Centrifugal Filter Devices].

Since the molecular weights of our components are all less than 300 Da, our approach to using this technique was to inject the filtrate which would contain the low molecular weight components that transfers across theYM-3 or YM-10 cellulose membrane cutoff filters. This essentially removes most peptides and all proteins from the sample to be injected as they are retained by the cellulose membrane cutoff filter, thus improving method selectivity. Method sensitivity is also improved because there is no sample dilution effect since there is no solvent added.

Evaluations to optimize sample preparation conditions using the YM-10 (10,000 Da MWCO) and YM-3 (3,000 Da MWCO) centrifugal filter were conducted. With the centrifugal force set at 14,000 g (recommended by YM-10 supplier) and using 250 uL of prepared plasma control samples, the centrifuge spin time was varied from 5, 15, 30 and 60

min. The five min spin time did not provide enough time to adequately separate plasma proteins from the aqueous matrix (salts, small peptides and substances less than 10,000 Da) with an insufficient amount of sample filtrate recovered. The 15, 30 and 60 min centrifugal spin times resulted in maximum recovery of sample filtrate. However the 60 min spin filtrate samples were significantly warmer than ambient lab temperature most likely due to warming effects of the sample tubes friction with air from the centrifugal spin. Thus to eliminate potential component degradation due to heat from spinning 60 min and to shorten sample preparation time, a spin time of 15 min was used for all analyses as described in section 3.3.

Results for the YM-3 filter evaluation demonstrated longer spin times were required (~45 to 60 min) at 14,000 g versus the 15 min spin using the YM-10 filter. The YM-3 filtrate did not offer better filtration of smaller plasma peptides (<10K Da), as observed on chromatograms, than was already achieved using the YM-10 filter. However, using either YM-3 or YM-10 filter effectively removed the purine nucleoside phosphorylase enzyme (nominal weight ~90-94 KDa protein, [Cook et al., 1981; Osborne, 1980]) thus eliminating the potential for inosine to hypoxanthine metabolism in the sample filtrate. The filtrates were stored frozen (-70˚C) after HPLC analysis with both inosine and hypoxanthine components demonstrating stability for greater than 3 months.

4.3.2.6 Plasma Purine Nucleoside Phosphorylase Activity

Purine nucleoside phosphorylase (PNP, EC 2.4.2.1) is an enzyme that rapidly metabolizes inosine to hypoxanthine in blood ($t_{½}$ <5 min due to red blood cells). This enzyme has low activity in plasma and is normally found in human cardiac muscle, GI

tract, spleen, brain and red blood cells [Viegas et al., 2000; Yamamoto et al., 1995]. Therefore, to better estimate an ischemic heart's effluxed inosine during periods of acute cardiac oxidative stress, venous blood samples should be kept cold (ice) and prepared immediately. Either the blood sample should be immediately inhibited (e.g. peldesine, competitive inhibitor (Viegas et al., 2000)) or the metabolite hypoxanthine should be simultaneously determined with inosine to better estimate the level of acute cardiac ischemia.

In whole blood or plasma samples, hypoxanthine will not be further metabolized to xanthine as the human enzyme xanthine oxidase (XO), which is require for hypoxanthine to xanthine conversion, has low activity in plasma (Yamamoto et al., 1996) and is typically found in human tissue (liver, small intestine) and other bodily fluids (milk and colostrum). A plasma (heparinized) sample is recommended for inosine and hypoxanthine determination in that the approximate 30 min clot time required for a serum sample would allow significant conversion of inosine to hypoxanthine in the collection tube, which would contain PNP from the red blood cell and plasma matrix.

Several evaluations (n=3 samples at each condition) were performed to evaluate inosine metabolism by PNP activity in plasma stored at 4˚C (refrigerator), -20˚C and -70˚C. Results of the evaluation can be seen in Figure 15; plasma fortified with inosine only at 7.46 µM and without PNP enzyme inhibitor is metabolized rapidly to hypoxanthine (~70% in 24 hrs); plasma fortified with 0.93 µM of inosine and 1.84 µM hypoxanthine and without PNP enzyme inhibitor is also metabolized rapidly to hypoxanthine (~70% in 24 hrs); however the plasma fortified with 7.46 µM of inosine and 14.69 µM hypoxanthine

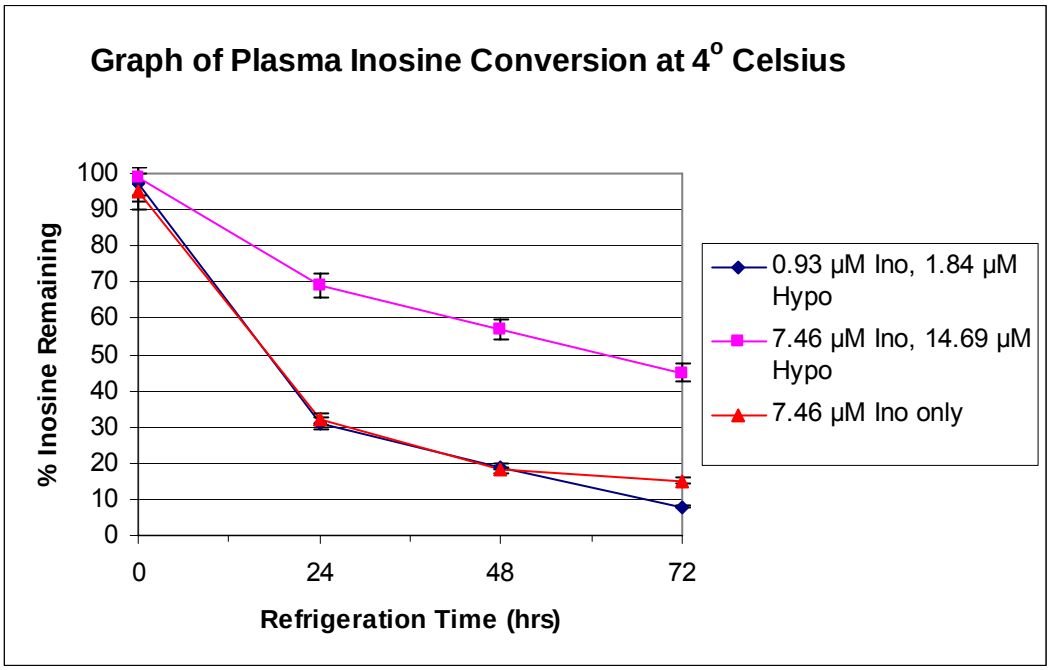

Figure 15. Graph of mean percent inosine remaining after plasma PNP metabolism when stored at 4˚C. Square symbols represent fortified amounts of 7.46 µM inosine and 14.69 µM hypoxanthine in blank plasma (n=3), diamond symbols represent fortified amounts of 0.93 µM inosine and 1.84 µM hypoxanthine in blank plasma (n=3), and triangle symbols represent fortified amount of 7.46 µM inosine only in blank plasma (n=3).

and without a PNP enzyme inhibitor, is metabolized less rapidly to hypoxanthine (~30% in 24 hrs) and slightly less than 50% after 72 hrs. Results of storing fortified plasma samples at -20˚C immediately after preparation indicated a reduced rate of inosine to hypoxanthine conversion (~30% after 8 months) with storage at -70˚C almost completely deactivating the PNP enzyme (<5% inosine conversion after 3 months). A possible explanation for the plasma hypoxanthine-concentration dependence for the conversion rate of inosine to hypoxanthine, would be product inhibition (PNP K_{eq}≈0.04 mM) (Brenda Enzyme Database). This low K_{eq} indicates that thermodynamically, inosine synthesis is favored over product conversion to hypoxanthine.

When the venous sample plasma concentration of hypoxanthine is present at higher levels (e.g. 14.69 µM), the conversion of inosine to hypoxanthine by plasma PNP decreases in the absence of significant XO enzyme activity, which converts hypoxanthine to xanthine and uric acid for biological elimination (therefore XO activity ultimately increases PNP activity as it reduces hypoxanthine product inhibition of PNP). It was also determined that the total amount of inosine and hypoxanthine fortified into the pooled plasma was recovered, thus verifying the lack of significant XO activity in human plasma and supports our recommendation of simultaneous determination of both inosine and hypoxanthine components.

A preliminary investigation to show the utility of the method is shown in Figures 14D (healthy control with 1.3 µM inosine and 2.74 µM hypoxanthine) and 14E (acute cardiac ischemia patient with 2.39 µM inosine and 29.3 µM hypoxanthine). These figures demonstrate an increase in both inosine and hypoxanthine concentrations (as well as final

ATP catabolic by-product uric acid) in one patient having presented with non-traumatic chest pain and undergoing evaluation for acute cardiac ischemia. Figure 16 demonstrates significant levels ($P<0.05$) of inosine, hypoxanthine and a combined total inosine plus hypoxanthine concentration level in plasma from ED non-traumatic chest pain patients and healthy normal controls.

It is important to discuss the time course of the ED patient's blood sample draw. These patients arrive at the hospital emergency department with the chief complaint of non-traumatic chest pain. Each patient's time of the non-traumatic chest pain may vary (minutes to hours) depending on the severity of their chest pain. Although the plasma samples from the ED that were evaluated for this research represent each patient's first blood sample drawn for standard hospital clinical chemistry analysis (e.g. troponin I, CK-MB), the samples themselves represent a range of time (minutes to hours) of potential patient acute cardiac ischemia. Therefore, the interpretation of these ED results may have this as a potential confounding factor or limitation.

4.4 Human Plasma (Heparin) versus Serum (SST) Sample Evaluation

Using the validated HPLC-UV method as described in Section 4.3, an evaluation was performed on commercially purchased human plasma (heparin) and serum (SST) samples. There are many techniques used for the specimen collection of biological fluids (e.g. blood component). Examples are collection of plasma, serum or serum using an inert gel barrier (SST) etc. As reproducibility of test results is a critical method validation parameter, it becomes important to determine the proper specimen matrix for analysis and

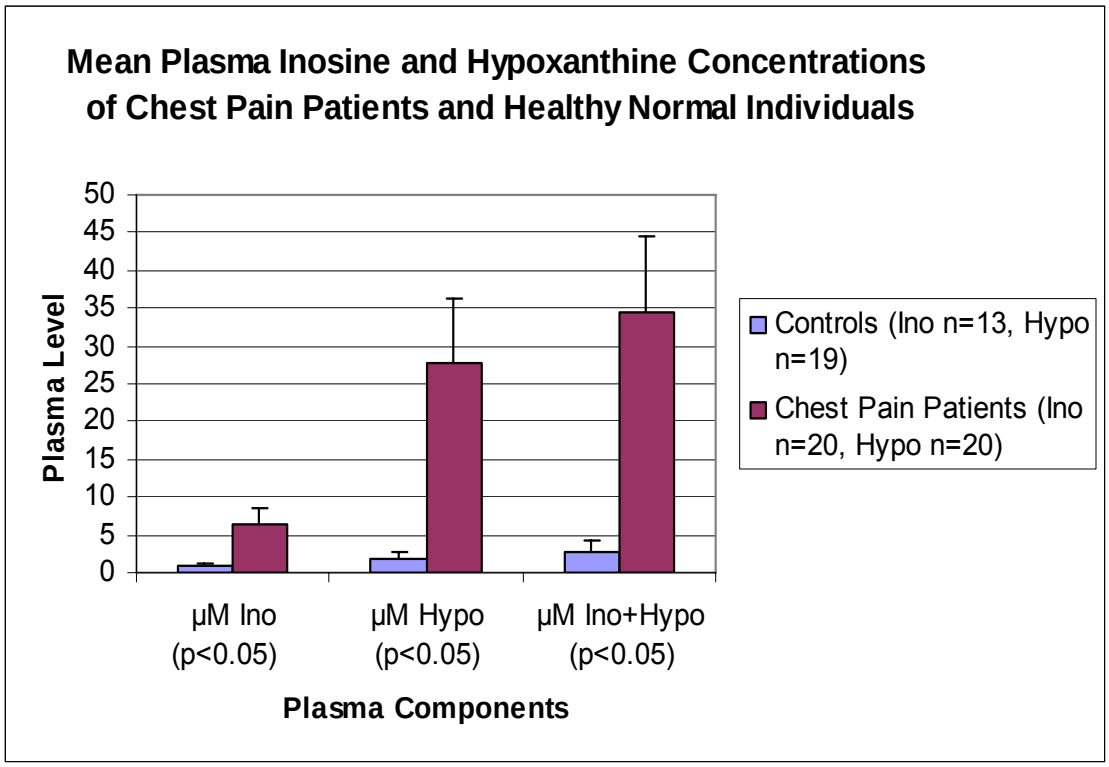

Figure 16. Chart depicting inosine, hypoxanthine, and total inosine plus hypoxanthine concentrations (mean + SEM) in plasma from ED non-traumatic chest pain patients and healthy normal individuals.

to determine if the sample collection matrix affects the component(s) of interest (e.g. resulting in artifacts in component concentrations) (Magee, 2005).

For this research, plasma (lithium) was initially selected for evaluation as many US hospitals typically use plasma for measurement of cTnI and CK-MB analysis (e.g. Chippenham Hospital). Serum (SST) is also being evaluated to investigate why one past research group (Kock et al., 1994) reported hypoxanthine levels which were not statistically significant in their patients that were undergoing acute myocardial infarction and other ischemic diseases. As this research reports elevated levels of inosine and significant levels of hypoxanthine in plasma from non-traumatic chest pain patients from a local hospital emergency department, it is important to determine and explain how the differences in reported hypoxanthine concentrations exist (if possible) between Kock et al. and this research since we evaluated similar type of patients (e.g. acute cardiac ischemia).

Studies on sample collection matrices effects have been performed by other investigators with reported differences in analyte levels based on the sample matrix (Doumas, 1989), thereby necessitating this matrix effect study. Samples were acquired from normal healthy volunteers (n=6) from a local blood bank (contracted with ProMedDx for this sample requirement), with each subject's blood acquisition being obtained sequentially (first serum SST vacutainer sample draw followed by heparin vacutainer sample draw), to minimize potential inosine and hypoxanthine concentration level changes with time and phlebotomy techniques. When drawing multiple sample types from the same subject, sample order of draw is documented as red top (serum) prior to green top (heparin) to prevent potential anti-coagulant (heparin) contamination in the serum sample

(ABP, Inc. 2004). Per ProMedDx, all collected samples were processed and handled according to BD Vacutainer® inserts (e.g. centrifugation at 1200 g for 15 min) (BD Vacutainer®, 2007), with the serum SST sample taken first followed by the heparin sample (prevents potential anticoagulant carryover effect).

As some biomarkers are diurnal and time sensitive (e.g. some endogenous hormones and proteins (e.g. CRP)), it supports the importance of requiring sequential samples for this small study to eliminate potential errors, as inosine and hypoxanthine are also endogenous to plasma and may possibly fluctuate throughout the day. After thawing samples to ambient temperature, each was prepared using the 30K centrifugal membrane filter and micro-centrifuged at 14,000 x g for 5 min, with all preparations analyzed by HPLC (single injection of each sample) on the same analytical run date.

As demonstrated in Figure 17, there were differences observed in the hypoxanthine concentrations from the same subject, with the serum (SST) samples generally higher in hypoxanthine concentrations. A possible reason for observing the higher hypoxanthine levels when using serum (SST) can be caused by red blood cell hemolysis during preparation. It is also known that hemolysis is a common occurrence when using serum samples (Arzoumanian, 2003) with another published article reporting significant differences between 10 of 17 plasma and serum measured analytes (Hrubec et al., 2002).

When using the SST tube for collection, the BD package insert directions state to gently invert the tube 5 times and let the tube sit 30 min prior to centrifugation. During this time period, the blood clots (as no anticoagulant is used) with a formed thrombus. With centrifugation, the formed thrombus remains near the bottom of the tube with the

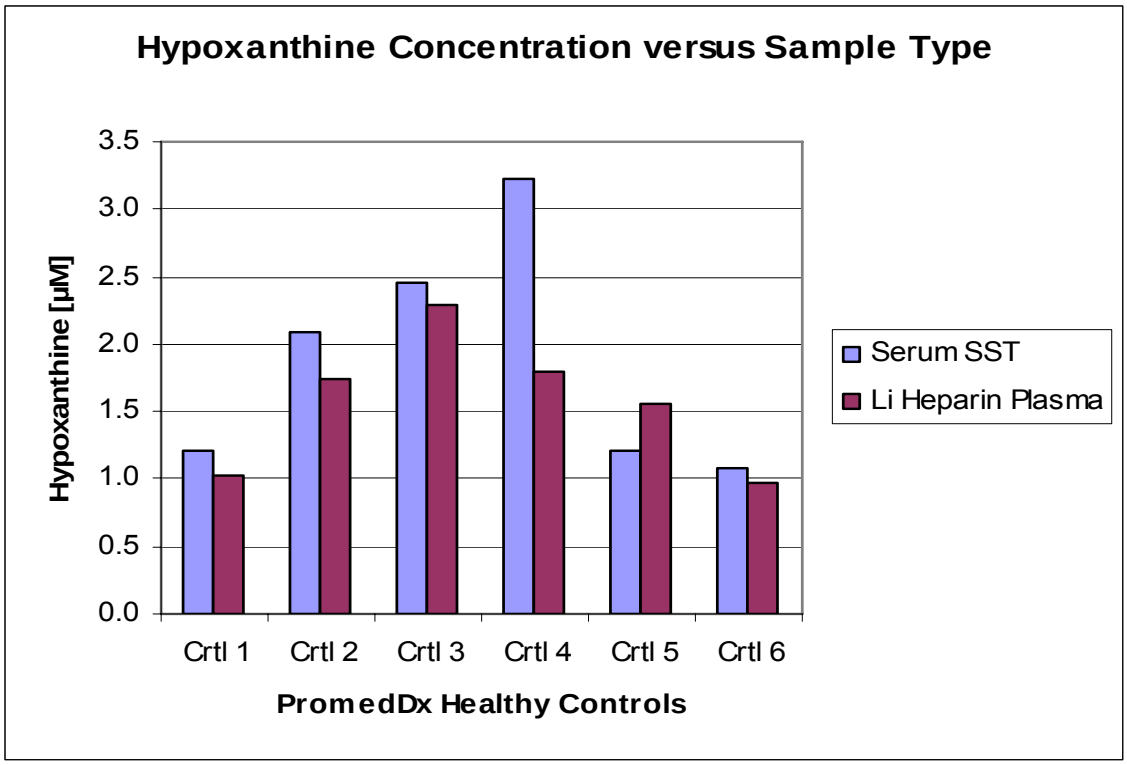

Figure 17. Bar chart depicting differences in hypoxanthine concentrations between plasma (lithium heparin) and serum (SST) from normal healthy individuals. Overall, SST samples demonstrated ~19% positive bias relative to the plasma (lithium heparin) samples.

other matrix components migrating upwards and around the inert gel (found initially at the bottom of the tube but migrates upwards based on material density).

It is feasible that during this clotting time and subsequent centrifugation step, that some red blood cells may leak some of its contents or hemolyze (rupture) due to mechanical deformation, which has been recently reported with RBC (Sprague et al., 2001, Sprague et al., 1996). It has also been reported that inside red blood cells, ATP is typically found in significant amounts (mM levels) as the RBC also functions as an ATP storage vesicle (Dietrich et al., 2000). If ATP is released from the RBC into the blood sample matrix, it is enzymatically converted several times to form hypoxanthine (Heptinstall et al., 2005, Coade et al., 1989), which may cause falsely elevated hypoxanthine results (artifact).

4.5 Other Cardiovascular Disease Matched Sample Set Evaluations

Further investigations into the effect of sample matrix were performed using additional commercially purchased matched sample sets (contract with ProMedDx for n=10 matched sets for each requested sample group). For this study, additional normal healthy (non-symptomatic for ischemic cardiac disease) individual samples of plasma (heparin) and serum (no gel) were collected and frozen at -20°C. The results of these additional normal individuals can be used for comparison to the results obtained in Section 4.4 (i.e. healthy normals serum with gel (SST) versus healthy normals serum without gel).

In addition, matched sample sets representing two cardiovascular disease states (e.g. hypertension, acute myocardial infarction) were also purchased from ProMedDx for evaluation to determine patient basal concentrations of inosine and hypoxanthine in these two cardiovascular disease states; however these matched sample sets were only available

in plasma (heparin) and serum (SST). The plasma (lithium) and serum (SST) samples from hypertension and acute myocardial infarction patients were collected at a hospital in contractual agreement with ProMedDx with serum (SST) being the hospital normal sample protocol for collecting serum samples and the collected samples frozen at -20°C prior to HPLC-UV analysis. Clinical diagnosis and demographic information (e.g. age, gender, medications) on patients with hypertension and acute myocardial infarction were provided by the hospital where the matched sample sets were obtained.

Appendix D list the sample sets and HPLC-UV results for this section. Several observations can be seen from the results. The matched sets for normal healthy individuals had similar plasma and serum (without the inert gel, not SST) concentrations for inosine and hypoxanthine. These results suggest that the initial results from using serum (SST) from normal healthy individuals may have gel contributed artifacts affecting the component concentrations (more notably hypoxanthine). In addition, the matched set samples from hypertension and acute myocardial infarction had notable differences in plasma and serum (SST) concentrations, somewhat for inosine but mainly for hypoxanthine (mostly higher hypoxanthine results, but with some variable results in the acute myocardial infarction samples). However, it is important to note that the hospital patient samples may also have medications which contribute to the observed phenomena.

Based on these studies, it should be recommended that plasma (lithium heparin) be used as the sample collection matrix for the analysis of human hypoxanthine and inosine. While serum (without gel) appears to be an acceptable matrix, it does require waiting for 30 min for the blood to clot prior to centrifugation, thereby adding to the total time from

patient sampling to analytical results to be returned from the clinical laboratory to the emergency room physician.

The HPLC results from blood samples collected using different vacuutainer additives (e.g. lithium heparin, SST) may help to explain differences in hypoxanthine concentrations from the Kock et al. study (1994 article) and this research work. Kock et al. reported no significant differences in hypoxanthine concentrations from their study patients (e.g. acute MI patients versus healthy controls), whereas this research reports significant differences from ED non-traumatic chest pain patients versus healthy normal individuals. Two possible reasons may account for the reported differences in hypoxanthine concentrations.

Firstly, the Koch et al. group used SST collection tubes and this study used lithium heparin for collecting plasma; thus the blood collection additives were different. Secondly, Koch et al. did not use ED non-traumatic chest pain patients (non acute MI when the blood sample was drawn) for their study as were used in this research work. Since there are no published kinetic studies of hypoxanthine in blood from non-traumatic chest pain or acute myocardial infarction patients, it is possible that the concentrations of hypoxanthine in the blood may be very different in these two groups of cardiac patients (e.g. patient chest pain is a typical symptomatic event preceding an acute myocardial infarction).

When collecting blood from an individual, the choice of blood collection additives can be just as important as the analytical test method. For this research, the differences between collecting plasma (lithium heparin) and SST from whole blood demonstrated variable hypoxanthine concentrations (artifacts). For example, in Figure 17, a consistent

positive bias in hypoxanthine concentrations from SST samples relative to lithium heparin plasma samples from normal healthy individuals (called matched sets) was demonstrated. However variable hypoxanthine concentrations were observed in patients with heart disease conditions (e.g. PromedDx lithium heparin plasma and SST matched set samples representing hypertension (HT) and acute myocardial infarction (cTnI) patients are listed in Appendix D), which indicates that hypoxanthine concentration artifacts may occur if using SST collection tubes for hypoxanthine analysis.

Thus, in order to obtain reproducible inter-laboratory hypoxanthine concentration results, lithium heparin should be used as the additive and not serum (SST), as the SST gel appears to cause spurious artifacts. Artifacts using SST gels are not unique, as several other investigators have reported similar findings when using serum (SST) for their studies (e.g. progesterone, (Ferry et al., 1999); free triiodothyronine (FT3), Kilinc et al., 2002).

4.6 Conclusion

A sensitive and selective method has been developed for evaluation of inosine and hypoxanthine in human plasma. The method employed a one step sample preparation for plasma (no organic solvents or solid phase extraction cartridges required) with high analyte recoveries, which eliminated the need for an internal standard. In addition, this method utilized recently introduced HPLC monolithic column technology, which provided sufficient selectivity and sensitivity for measurement of these components. Subject matched set sample evaluation indicated a consistent positive bias in hypoxanthine levels from use of SST collection tubes relative to plasma (heparin), thus the recommendation of using plasma (heparin) collection to eliminate any potential artifacts and reduce total

analysis time (serum and SST samples require ~30 min clotting time prior to centrifugation). The method was employed without significant methodological problems in the evaluation of plasma samples obtained from healthy individuals and hospital emergency department patients presenting with non-traumatic chest pain with significant levels of both inosine and hypoxanthine effluxed from the patient samples. These results, albeit from a small sample size (n=20 each group), further support the research hypothesis that these components may qualify as potential candidate biomarkers of acute cardiac ischemia.

4.7 Acknowledgements

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CHAPTER 5. Rapid Chemiluminescence Detection of Inosine and Hypoxanthine using Microplate Luminometer

5.1 Introduction

The goal of this chapter of research is the development of a rapid chemi-luminescence test method for determination of inosine and hypoxanthine in human plasma. The purpose is to allow for rapid patient screening capability (diagnostic tool for acute cardiac ischemia) for potential use in hospital emergency department environments. The luminescence method will be utilized on samples from healthy individuals and hospital patients with confirmed acute MI (hospital documented elevated levels of cTnT). To be effective, the method will need to be rapid (defined as less than 10 min analysis time), sensitive and specific for inosine and hypoxanthine to reduce the potential errors in interpreting sample test results (e.g. goal is to minimize false positive and false negative results).

Currently, there are no published articles or U.S. patents for a rapid test method to determine inosine and hypoxanthine in plasma, which can meet the stringent sample turnaround time requirements of an emergency medical services (EMS) environment. The rationale for using chemiluminescence technology over commonly used LC and immunoassay technologies are as follows: LC and immunoassay methods are both very sensitive and specific techniques (e.g. monoclonal antibodies for immunoassay and mass spectrometer detection for LC); however, an LC-MS system is expensive to purchase and

operate, both techniques require technical expertise to perform, and both lack a rapid enough turnaround time needed by an EMS facility analyzing a priority "stat" type sample. However, a luminometer can measure chemiluminescent light, is relatively inexpensive to purchase, currently used in clinical labs (microplate capability), and can provide high component sensitivity.

Luminescence technology is well established with many instrument vendors (e.g. BMG LabTech Inc. Lumistar Optima (Durham, NC, USA), BioTek Synergy HT (Winooski, Vermont, USA), Thermo Fisher Scientific Luminoskan (Waltham, MA, USA)) and suppliers (e.g. Corning Life Sciences, Lowell, MA, USA) of luminescence supplies and reagents available worldwide. It is known to be one of the most sensitive techniques, with one recent publication on its application for low ng/ml concentrations of ATP in human plasma [Gorman et al., 2007]. The high sensitivity of luminescence is primarily due to its high analyte signal to noise (s/n) ratio, with reported detection levels at low picogram and femtogram levels.

To address biomarker specificity requirement, the developed luminescence test method will utilize biological enzymes purine nucleoside phosphorylase (PNP) and xanthine oxidase (XO), which are specific for enzymatic conversions of inosine and hypoxanthine, respectively. The PNP enzyme converts inosine to hypoxanthine and XO converts hypoxanthine to xanthine, followed by XO conversion of xanthine to final product uric acid (in human species). Each time XO reacts with one mole of hypoxanthine, and subsequently with one mole of xanthine, the metabolic by-products of each XO enzymatic turnover is the production of one mole of hydrogen peroxide and two

moles of superoxide anion radical ($O_2^{-}\cdot$), which both of these by-products can become substrates for luminescence type reactions. Several commonly used luminescent materials (e.g. luminol (oxidation), lucigenin (reduction), and pholasin (oxidation)) were considered for this research. If using luminol or lucigenin as the luminescent material, the hydrogen peroxide (which has both oxidizing and reducing capabilities) can react with the horseradish peroxidase (HRP) enzyme, luminol, and signal enhancers to generate measurable blue light ~ 450 nm, thus an amplification of signal effect (one mole of hypoxanthine and xanthine can generate two moles of hydrogen peroxide) (Figure 18).

However, to achieve even greater sensitivity as low concentration (ng/ml or μM) levels of inosine and hypoxanthine are typically found in human plasma, another luminescence approach was investigated, which utilizes a highly sensitive photoprotein (pholasin®). Since one mole of hypoxanthine will generate 4 moles of superoxide anion radicals (SAR) as a by-product of XO activity, using a chemiluminescent material that reacts with SAR should theoretically provide even more luminescence signal, thus potentially increasing the sensitivity twice fold over using the hydrogen peroxide/horseradish peroxidase/luminol approach. One article cited pholasin having more than 100 fold sensitivity than lucigenin (Knight, 1997).

Pholasin®, a photoprotein isolated from the bi-valve mollusk, has been reported to be a very sensitive chemiluminescent material (called lucidalin®) for SAR and other reactive oxygen species (ROS) such as the hydroxyl free radical (Knight, 1988). Pholasin® has been extensively studied and patented by Knight Scientific, Plymouth, UK. It is an approximately 34-36 kDa glycoprotein, which can be made excitable by several ROS,

Luminol and Lucigenin Chemiluminescence Measurement Approach

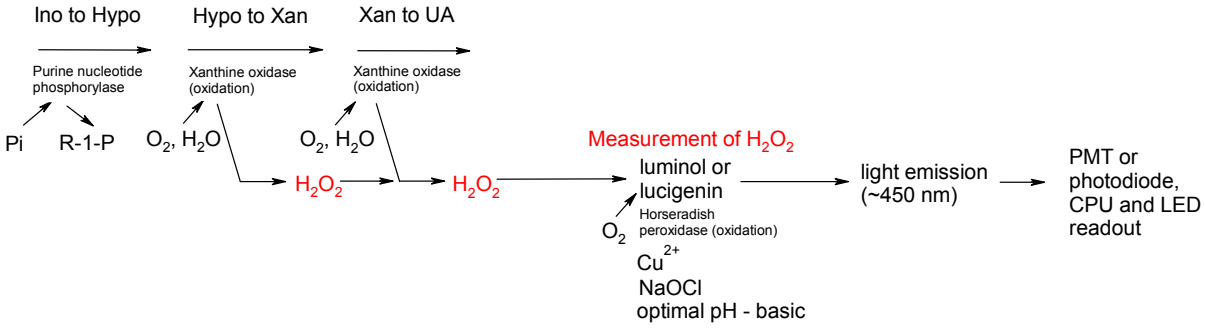


Figure 18. Diagram of enzymatic conversions of inosine and hypoxanthine components with generation of hydrogen peroxide as a by-product, which can react with luminol or lucigenin and HRP to generate visible blue light (chemiluminescence).

emitting blue-green light, and it has been reported to not have fluorescent properties. The presence of SAR can react with the pholasin photoprotein to generate measurable light (~490 nm) (Figure 20), thus an amplification of signal effect (one mole of hypoxanthine can generate four moles of SAR), which should increase sensitivity and provide lower component detection limits. The reaction of pholasin with SAR can be very quick (flash type technique, typically seconds) and may be made even more sensitive with use of signal enhancers (e.g. Adjuvant-k (proprietary) from Knight Scientific).

The Lumistar Optima Microplate Reader (BMG LabTech, Durham, NC, USA) was used for all luminescence evaluations. The instrument has temperature control, supports the use of 96 well plates (opaque white) which were purchased from Corning Life Sciences (Lowell, MA, USA), and is capable of variable microplate mixing speeds with flash and glow luminescence capabilities. The instrument is fitted with two direct injectors capable of rapid injections (e.g. 310 ul/sec), thus micropipetting assay reagents into the sample wells was automatically performed, which may help to reduce potential errors from manual pipetting.

5.2 Experimental

5.2.1 Chemicals, Reagents and Materials

Hypoxanthine, xanthine and ethyl alcohol (HPLC grade, denatured) were purchased from Acros Organics (Fair Lawn, NJ, USA). Inosine, dibasic sodium hydrogen phosphate, and uric acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzymes xanthine oxidase (isolated from bovine milk, Grade III, ammonium sulfate suspension, enzymatic activity ~1.3 units/mg protein, storage temp 2-8°C),

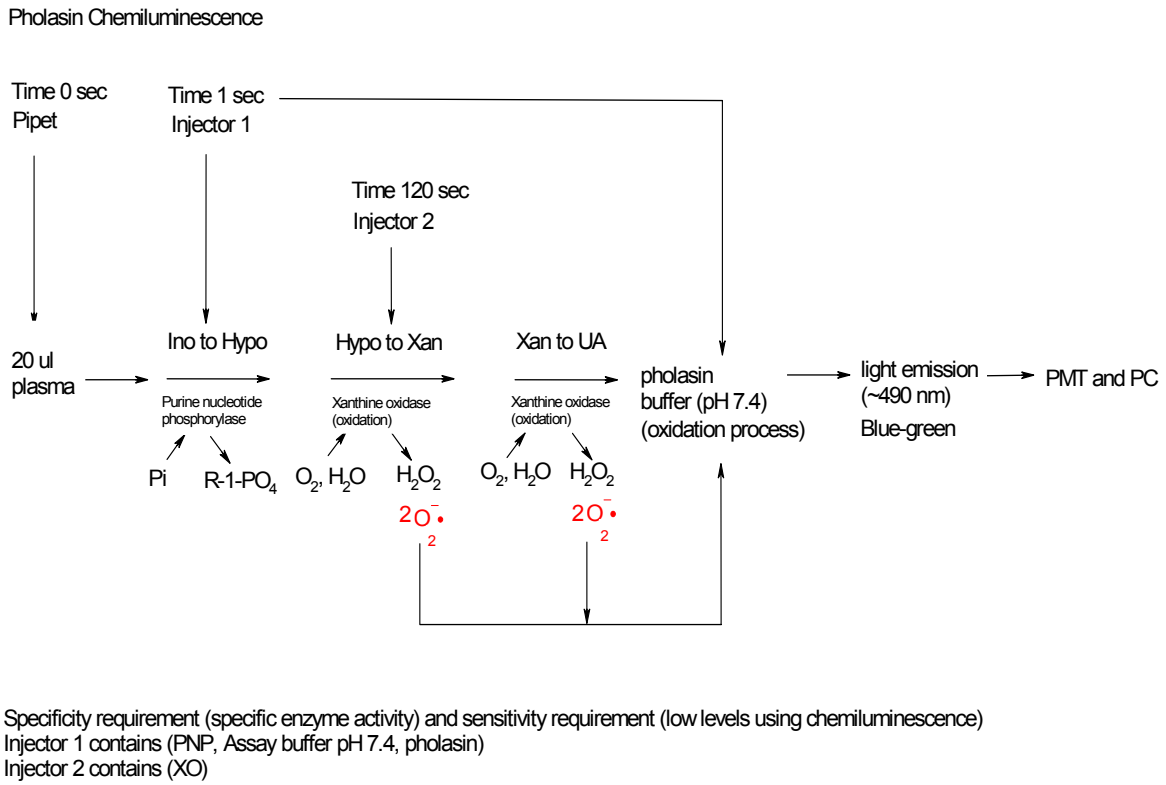


Figure 20. Diagram of typical reagent addition, injector time points and resulting pholasin emission (chemiluminescence).

purine nucleoside phosphorylase (isolated from human blood, lyophilized powder, enzymatic activity ~19 units/mg protein., storage -20°C) and uricase (isolated from Arthrobacter globiformis, lyophilized powder, ~19.7 units/mg protein, storage -20°C) were all purchased from Sigma-Aldrich.

A commercial test kit used for antioxidant evaluations was purchased for initial setup of the luminometer and included an assay utilizing xanthine/xanthine oxidase plate mode kinetics (glow technique). The kit included pholasin (50 µg), xanthine, xanthine oxidase [~10.25 mU/ml] and buffer (proprietary) for plate mode kinetics and was purchased from Knight Scientific (Plymouth, UK). The luminometer instrument was qualified using the commercial antioxidant test kit and by successful replication of the xanthine/xanthine oxidase plate mode kinetics profile from Knight Scientific. For all experiments following instrument qualification, the reagents and enzyme solutions were prepared accordingly. Dibasic sodium hydrogen phosphate was used to prepare the 20 mM assay buffer solution with ultrapure deionized water as the diluent (final pH 7.4 using concentrated phosphoric acid). Ultrapure deionized water (~18 MΩ-cm) used for all reagent solutions was filtered (0.2 µm) prior to use.

The luminometer rinse solution for the direct injector syringes was prepared using ethyl alcohol:deionized water mixture (75:25%, v/v). Weekly rinses were performed to reduce potential material (e.g. protein and enzyme residue) buildup in the syringes, reagent tubing and injector needles. Opaque 96 well microplates were purchased from Corning Life Sciences (Lowell, MA, USA) and stored in the dark at ambient temperature. Blank human plasma (lithium anticoagulant) from one healthy volunteer (250 ml), an additional

six healthy volunteers plasma (lithium heparin) samples (1 ml each), and six patient's plasma (lithium heparin) samples with confirmed acute MI (hospital reported elevated cTnT, 1 ml each) were purchased from ProMedDx (Norton, MA, USA) and stored at -20°C prior to use.

5.2.2 Preparation of Standards, Enzymes and Pholasin Solutions

Stock standards of inosine (25 µg/ml, 93.2 µM), hypoxanthine (25 µg/ml, 183.7 µM), xanthine (25 µg/ml, 164.4 µM) and uric acid (25 µg/ml, 148.7 µM) were prepared in deionized water, stored at 4°C with stability greater than 3 months. Working calibration standards for each component were prepared in deionized water immediately prior to use.

For experiments, the working xanthine oxidase solution was prepared by pipetting 40 µL of the aqueous stock XO (from bovine milk) suspension into 2.0 ml of assay buffer (pH 7.4) resulting in ~676 mU XO/ml. The working XO solution was stable at ambient laboratory temperature (22°C) and could be stored at 4°C overnight with minimal loss in enzyme activity; however the working XO solution should not be stored frozen (e.g. -20°C), as a complete loss of enzyme activity was observed upon freeze-thaw and subsequent use.

To prepare PNP and uricase solutions from solid and lyophilized purine nucleoside phosphorylase and uricase, 1.0 ml of assay buffer (pH 7.4) was pipetted directly into the vendor container bottle with gentle vortexing into solution. After reconstitution using 1 ml of assay buffer (pH 7.4), the PNP stock concentration was ~18.7 Units PNP/ml and uricase stock concentration was ~110 Units uricase/ml. A working solution of PNP [~701 mU PNP/ml] was prepared by pipetting 75 µL of the aqueous stock material into 2.0 ml of

assay buffer (pH 7.4). A working solution of uricase [~1.1 U uricase/ml] was prepared by pipetting 20 µL of the aqueous stock material into 2.0 ml of assay buffer (pH 7.4). Both working solutions of PNP and uricase were stable at ambient laboratory temperature and could be stored at 4°C overnight with minimal loss in enzyme activity.

For preparation of the pholasin luminescent material, 5.0 ml of assay buffer (pH 7.4) was pipetted directly into the vendor container bottle containing 50 µg pholasin with gentle vortexing, resulting in a ~10 µg/ml solution. The prepared pholasin reagent was stable at ambient laboratory temperature and 4°C, and was stored protected from light to eliminate potential basal luminescence as it is an excitable photoprotein. The reconstituted pholasin solution was transferred and stored in plastic screw top tubes (~1 ml aliquots stored at -20°C).

5.2.3 Luminometer Equipment and Set Points

The luminometer equipment consisted of a BMG LabTech Inc. Lumistar Optima and Optima software (version 2.1) (Durham, NC, USA) and Dell Optiplex 745 PC (Dell, TX, USA). The luminometer was equipped with temperature control (8°C to 45°C), two direct injectors (minimum injection volume of 3 µl) with variable injection speeds (100 µ/s to 420 µ/s), and microplate shaking (orbital, linear, figure eight) capability. The luminometer listed specifications for the limit of detection (<50 amol / well ATP), spectral range (240 – 740 nm) and dynamic range (9 decades). All luminescence assays utilized opaque 96 well plates, an incubation temperature of 25°C, lens mode (no emission filter) and a photo-multiplier (PMT) gain setting of 3900 volts. Equipment set points for all experiments in the flash mode are listed in Figure 21.

OPTIMA
BMG LABTECH

Testname: XO (WELL MODE) 2007/11/17 13:16:17
ID 1,2,3: XO (WELL MODE) Luminescence 11/17/2007,1:16:13 PM 304.dbf

Luminescence, well mode
Plate type: COSTAR 96

Kinetic window	1	2	3	4
No. of intervals	112	-	-	-
Kin. interval time [s]	2.00	-	-	-
Meas. start time [s]	0.0	-	-	-
Meas. interval time [s]	1.00	-	-	-

To allow comparison of different kinetic windows all measurement values are normalized to 1 sec.

Gain: 3900
Emission filter: lens

Required value [%]: 0

Volume group	1	2	3	4
Volume [µl]	20	-	-	-
Used pump	1	-	-	-
Pump speed [µl/s]	310	-	-	-
Smart dispensing used	X	-	-	-
Pump syringe vol. [ml]	0.5	-	-	-
Injection start time [s]	120.0	-	-	-
Shaking after inject. [s]	92	-	-	-

Positioning delay [s]: 0.2
Shaking width [mm]: 1
Shaking mode: orbital
Additional shaking: 2s before plate reading

Target temperature [°C]: 25

Reading direction: 3
None

Calculation Start1: 55 Stop1: 59 Start2: 60 Stop2: 76

Comment:

Software version control: 2.10 R2
Software version evaluation: 2.10 R2 B:0004
Serial number: 413-3190
User: USER

Audit trail

Saturday, November 17, 2007 - 2:03:28 PM, User 'USER': Data record created by performing test protocol 'XO (WELL MODE)' (started: Saturday, November 17, 2007 - 1:16:17 PM) using reader 413-3190.
Saturday, November 17, 2007 - 2:03:40 PM, User 'USER': Automatic initialization of evaluation settings done during first opening of test run.
Saturday, November 17, 2007 - 2:37:39 PM, User 'USER': Settings have been changed.
Saturday, November 17, 2007 - 2:52:19 PM, User 'USER': Settings have been changed.
Monday, December 03, 2007 - 7:57:52 AM, User 'USER': Settings have been changed.

Signatures

Figure 21. BMG luminometer set points used for flash mode experiments.

5.2.4 Method Development and Optimization

Development and optimization of the luminescence test method included evaluation of parameters such as determining hypoxanthine concentration level range, adjustment of XO enzyme concentration level to reduce analysis time, and enzyme incubation time (e.g. PNP) to maximize sensitivity and repeatability and to minimize turnaround time (<10 min analysis). All plasma analysis utilized 20 µl of sample in a final microplate well volume of 200 µl (effectively making a 1:10 dilution of the plasma sample). Potential endogenous interference (e.g. uric acid) was evaluated to determine quenching effects as this substance has antioxidant capacity and is typically found in plasma at high concentrations (e.g. 350-450 µM), especially in gout patients.

The HPLC results from normal volunteers (ProMedDx plasma) and non-traumatic chest pain patients (Chippenham Hospital ED plasma) from Chapter 4 were used to estimate expected plasma concentrations of inosine and hypoxanthine for this chapter of research (Table 9). Since the luminometer is a detection device and will not separate a mixture of components (as does HPLC), it was necessary to utilize the PNP enzyme and convert component inosine to hypoxanthine, and then measure the resulting total plasma hypoxanthine (inosine plus hypoxanthine) concentration. Using the XO enzyme, hypoxanthine converts to xanthine, and xanthine to uric acid. The luminometer measures the light signal generated from the XO reaction with hypoxanthine and xanthine (XO generates superoxide anion radicals which react with the luminescent material pholasin). Using a µg/ml to µM (micro molar) conversion table (Excel formula computations, Table 10), a standard curve of hypoxanthine was prepared at concentration range of

Table 9. Estimated inosine, hypoxanthine, xanthine and uric acid concentrations in healthy normal individuals and non-traumatic chest pain patients. Values for normal individuals are based on published literature and the HPLC results using PromedDx healthy normal individuals. Values for potential acute cardiac ischemia are from the HPLC results using Chippenham Emergency Department plasma (heparin) samples.

	Plasma [ug/mL]	Plasma [uM]	Comments
Estimated lowest inosine level =	0.10	0.4	
Estimated lowest hypoxanthine level =	0.10	0.7	
Assume 100 % ino to hypo conv	hypo =	1.1	Estimated levels (normals), n=20 from PromedDx
Estimated (normals) inosine level =	0.30	1.1	
Estimated (normals) hypoxanthine level =	0.30	2.2	
Assume 100 % ino to hypo conv	hypo =	3.3	Estimated levels (normals) from Feng et al, Ther Drug Mon (2000) 22:177-183.
Estimated (ischemic) inosine level =	0.3	1.1	Lowest chest pain patient value
Estimated (ischemic) hypoxanthine level =	2.0	14.7	
Assume 100 % ino to hypo conv	hypo =	15.8	Ischemic (based on Chippenham ED data).
Estimated (ischemic) inosine level =	7.8	29.1	Highest chest pain patient value
Estimated (ischemic) hypoxanthine level =	9.7	71.3	
Assume 100 % ino to hypo conv	hypo =	100.3	Ischemic (based on Chippenham ED data).
Estimated (normals) xanthine level =	0.9	5.9	Estimated xanthine levels (normals) from Feng et al, Ther Drug Mon (2000) 22:177-183.
Estimated (normals) uric acid level =	60.0	356.9	Potential XO inhibitor and luminescence quenching (anti-oxidant).
Estimated (normals, high) uric acid level =	80.0	475.9	Potential XO inhibitor and luminescence quenching (anti-oxidant).
Estimated uric acid highest level (gout) =	100.0	594.8	Potential XO inhibitor and luminescence quenching (anti-oxidant).

Table 10. Component µg/ml to µM conversion table.

For Experiments Compound	Weight mg	Volume Ml	Conc µg/ml	Conc µM
Adenosine	25.0	1000.0	25.0	93.6
Inosine	25.0	1000.0	25.0	93.2
Hypoxanthine	25.0	1000.0	25.0	183.7
Xanthine	25.0	1000.0	25.0	164.4
Uric Acid	25.0	1000.0	25.0	148.7

2.3 to 30.3 µM. The initial hypoxanthine concentration range was set to focus on hypoxanthine concentrations to maximize the luminescence method sensitivity and detect concentration differences between healthy normal individuals and non-traumatic chest pain patients (e.g. ~3 µM for normal individual and ~15 µM for lowest observed chest pain patient). Plasma samples above the highest standard can be diluted with deionized water. The initial range incorporated total inosine and hypoxanthine concentrations from both healthy normal individuals and non-traumatic chest pain patients (based on n=20 for each group from Chapter 4 research). However it is important to note that this small set of plasma samples may not cover the entire range of hypoxanthine concentrations from a larger patient population, and further research work is necessary to optimize the hypoxanthine standard curve, as the goal of this chapter of research is the development of a rapid and simple method.

Xanthine was found to be at a constant concentration (~6 µM) in both normal individuals and non-traumatic chest pain patient samples. It is important to discuss why a standard curve of xanthine would not be used for this assay. To prepare a standard curve of xanthine for computation of inosine and hypoxanthine concentrations would report erroneously low results, as xanthine only activates the XO enzyme once (xanthine to uric acid), whereas hypoxanthine activates the XO enzyme twice (hypoxanthine to xanthine to uric acid). Since we are only interested in inosine and hypoxanthine concentrations for this research, and with xanthine levels constant, it was appropriate to prepare hypoxanthine standards (which incorporated total inosine to hypoxanthine conversion) for this research project.

Typical spreadsheets used for luminescence experiments on inosine, xanthine, and hypoxanthine evaluations include each reagent preparation, volume pipetted into the well, and target concentrations and are listed in Tables 11, 12, and 13, respectively. Using the experimental spreadsheet for each component standard concentration range, plasma (20 µl) was pipetted into the microplate well with reagents (e.g. assay buffer, phosalin, PNP, uricase) either manually pipetted or injected using one direct injector; with the other direct injector used to inject the XO solution to start the reaction with pholasin and subsequent luminescence emission.

5.2.5 Luminescence Computations

All computations were performed using BMG Excel software (with built in macros) and data processing set points as defined by the method. Figure 22 and 23 represents a scan (plasma with 10 µM hypoxanthine) and the RLU tabulated results (e.g. BMG Excel Table 1, 2, and 3 in Figure 23) from raw data acquired over the analytical run and with data acquisition set at one data point per second. The background (baseline) luminescence signal (labeled as Range 1 and presented in BMG Excel Table 1) can be caused by reagents (e.g. buffer, pholasin, PNP, plasma) and electronic noise and was calculated as the maximum RLU signal between scan times 100-118 seconds. It would have been more appropriate to average the background RLU signal; however the BMG Excel software was written to have the same computation applied to both table ranges and does not currently allow the flexibility of independent computations on each individual table.

Table 11. Typical spreadsheet used for inosine luminescence experiments.

Final Inosine Conc. (200 ul well volume) [uM]	Inosine (ul)	Standard Inosine WS [uM]	Pholasin (ul)	Assay buffer (ul)	PNP (ul)	XO (ul)	Total well volume (ul)
0.0	0	0	50	70.0	40	40	200
1.0	21.5	9.32	50	48.5	40	40	200
2.5	53.6	9.32	50	16.4	40	40	200
5.0	10.7	93.2	50	59.3	40	40	200
10.0	21.5	93.2	50	48.5	40	40	200
20.0	42.9	93.2	50	27.1	40	40	200
30.0	64.4	93.2	50	5.6	40	40	200
Example	**Plasma (ul)**						
If plasma sample 1:10 dilution	20	0	50	50.0	40	40	200

Notes:

1. Stock inosine [93.2 uM or 25 ug/ml] in DI. Prepared by adding 25 mg in 1000 mL DI (or assay buffer).

2. Working stock (WS) **WS-1 (9.32 uM)** 100 ul stock inosine
   (1:10 stock) 900 ul assay buffer

3. Final total inosine conc based on 200 ul well volume.

4. Pholasin conc [10 ug/ml]. Add 5 ml assay buffer to vial (50 ug pholasin from mollusca, Knight Scientific). Store frozen.

5. XO conc [~676 mU XO / ml]. Pipet 40 ul stock (XO from bovine milk, Sigma) to 2 ml assay buffer. Store refrigerated.

6. PNP conc [~701 mU PNP / ml]. Pipet 75 ul stock (PNP from human RBC, Sigma) to 2 ml assay buffer. Store refrigerated.

Table 12. Typical spreadsheet used for xanthine luminescence experiments.

Final Xanthine Conc. (200 ul well volume) [uM]	Xanthine (ul)	Standard Xanthine WS [uM]	Pholasin (ul)	Assay buffer (ul)	XO (ul)	Total well volume (ul)
0.0	0	0	50	110.0	40	200
1.0	12.2	16.4	50	97.8	40	200
2.5	30.5	16.4	50	79.5	40	200
5.0	61.0	16.4	50	49.0	40	200
10.0	12.2	164.4	50	97.8	40	200
20.0	24.3	164.4	50	85.7	40	200
30.0	36.5	164.4	50	73.5	40	200
Example	**Plasma (ul)**					
If plasma sample 1:10 dilution	20	0	50	90	40	200

Notes:

1. Stock xanthine [164.4 uM or 25 ug/ml] in DI. Prepared by adding 25 mg in 1000 mL DI (or assay buffer).

2. Working stock (WS) — **WS-1 (16.4 uM)** (1:10 stock): 100 ul stock xanthine + 900 ul assay buffer

3. Final xanthine conc based on 200 ul total well volume.

4. Pholasin conc [10 ug/ml]. Add 5 ml assay buffer to vial (50 ug pholasin from mollusca, Knight Scientific). Store frozen.

5. XO conc [~676 mU XO / ml]. Pipet 40 ul stock (XO from bovine milk, Sigma) to 2 ml assay buffer. Store refrigerated.

Table 13. Typical spreadsheet used for hypoxanthine luminescence experiments.

Final Hypoxanthine Conc. (200 ul well volume) [uM]	Hypoxanthine (ul)	Standard Hypoxanthine WS [uM]	Pholasin (ul)	Assay buffer (ul)	XO (ul)	Total well volume (ul)
0.0	0	0	50	110.0	40	200
0.1	10.9	1.84	50	99.1	40	200
0.2	21.8	1.84	50	88.2	40	200
0.5	54.4	1.84	50	55.6	40	200
1.0	10.9	18.37	50	99.1	40	200
2.0	21.8	18.37	50	88.2	40	200
5.0	5.4	183.7	50	104.6	40	200
10.0	10.9	183.7	50	99.1	40	200
Example	**Plasma (ul)**					
If plasma sample 1:10 dilution	20	0	50	90	40	200

Notes:

1. Stock hypoxanthine [183.7 uM or 25 ug/ml] in DI. Prepared by adding 25 mg in 1000 mL DI (or assay buffer).

2. Working stock (WS)

WS-1 (18.37 uM)	100 ul stock hypoxanthine
(1:10 stock)	900 ul assay buffer
WS-2 (1.84 uM)	100 ul WS-1 hypoxanthine
(1:10 WS-1)	900 ul assay buffer

3. Final total hypoxanthine conc based on 200 ul well volume.

4. Pholasin conc [10 ug/ml]. Add 5 ml assay buffer to vial (50 ug pholasin from mollusca, Knight Scientific). Store frozen.

5. XO conc [~676 mU XO / ml]. Pipet 40 ul stock (XO from bovine milk, Sigma) to 2 ml assay buffer. Store refrigerated.

6. Target range of nucleoside/purine assay (includes xanthine plus ino and hypo conversion to xanthine) is ~2 uM (normals) up to ~100 uM (ischemic).

7. Sensitivity and linearity of the nucleoside/purine assay (if 1:10 dilution of plasma) needs to be ~0.1 up to ~10 uM.

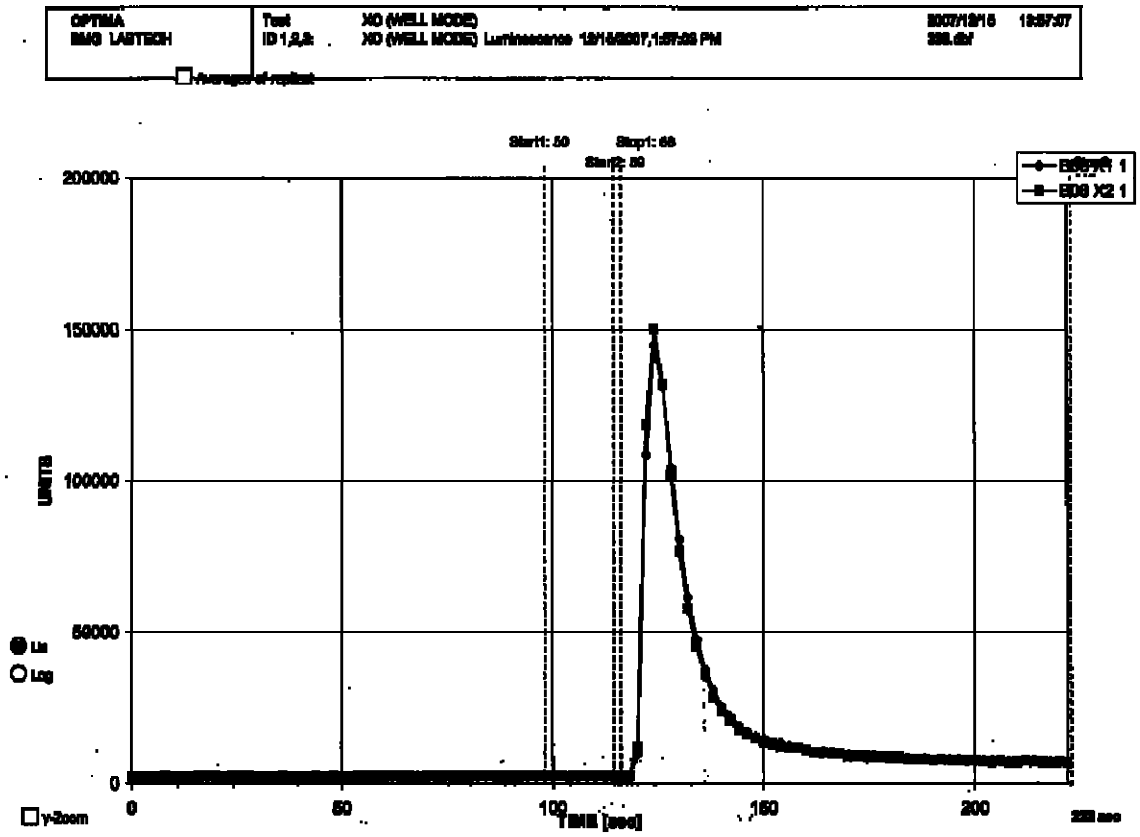


Figure 22. Typical BMG output luminescence scan for sample analysis of 10 µM hypoxanthine in plasma. Range one (background RLU measurement between 100 and 120 sec) and range two (peak height RLU measurement between 120 sec and 222 sec).

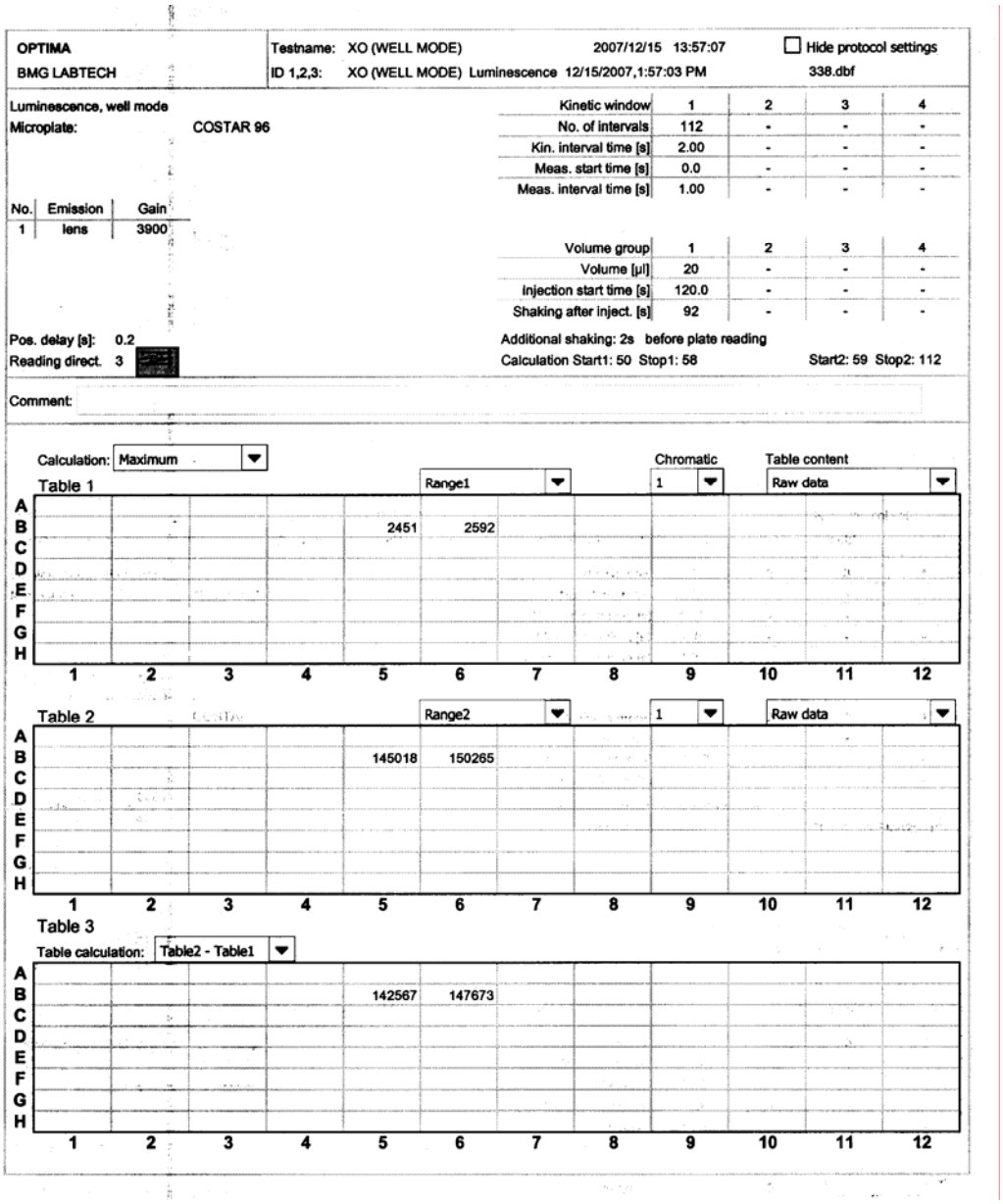


Figure 23. BMG Excel computations, method and data processing set points, and file name are documented for GLP compliance. Results are reported in excel cells based on microplate sample well location (96 well plates).

The peak luminescence signal from the generation of light from pholasin (labeled as Range 2 and presented in BMG Excel Table 2) and superoxide anion radicals was calculated as the maximum RLU peak height signal between scan times 119-222 seconds. BMG Excel Table 3 represents the net RLU and is calculated by subtracting the background signal (BMG Excel Table 1) from the peak luminescent signal (BMG Excel Table 2). The use of the peak height response of the RLU was used for the computations on these plasma samples, as some patient plasma samples RLU responses were very slow to return to background (baseline) RLU levels. The cause of the slow RLU signal return to baseline is unknown, but may be due to patient medications (e.g. vasodilators, salicylic acid) used for treatment of acute MI patients.

As this luminescence method was set up for rapid screening purposes on potential non-traumatic chest pain patient samples from the ED, it was not developed to be a quantitative assay as was the validated HPLC-UV method (Chapter 4). However, the luminescence method needed to be rapid (<10 min analysis), sensitive (use 20 ul plasma), specific (use of enzymes) and have high precision (repeatability) to be useful in the ED environment. This luminescence method was developed to compare the RLU differences between healthy normal individual plasma samples (negative control) and samples from ED non-traumatic chest pain patients that may be experiencing acute cardiac ischemia.

A comparison was made of the net RLU value between the non-traumatic chest pain patient and negative control sample, using a calculated 99% RLU reference cut-off value generated from healthy normal individuals, as the decision making RLU cut-off level. Determining the 99% RLU cut-off value (which is beyond the scope of this

research) would best be determined using a large number of healthy normal individuals (e.g. >>100)) and calculated using the RLU mean value plus the 2.326 standard deviations (α=0.01, one tail, 99% confidence interval), and would be used to determine whether the patient has acute cardiac ischemia causing the reported chest pain.

For example, if a non-traumatic chest pain patient net RLU was similar to a negative control sample net RLU, then the patient was most likely not having an acute cardiac ischemic event, but had some other type of medical condition (e.g. anxiety, heartburn) causing the reported chest pain. However, if a patient's net RLU was above the 99% RLU reference cut-off value for healthy normal individuals, then the patient was probably experiencing an acute cardiac ischemic event, and would require immediate medical attention, as it may lead to acute MI and potential adverse outcome.

5.3 Results and Discussion

To setup the new luminometer equipment, a standardized plate mode luminescence test kit was bought (ABEL 61M Antioxidant Test Kit, Knight Scientific, Ltd) which evaluates antioxidant capability using xanthine/xanthine oxidase and pholasin. This test kit was used to qualify the new luminometer equipment using a standardized plate mode (glow technique). However, method modifications were necessary as the plate mode analysis run time was approximately 30 min and had low sensitivity (Figure 24) as it is developed primarily for antioxidant and glow kinetic type studies, which would be insufficient for our research objective of a rapid and sensitive assay.

Adjustments were made to the level of XO used for analysis to increase the reaction rate (flash mode) and the incubation time of PNP enzyme for plasma inosine

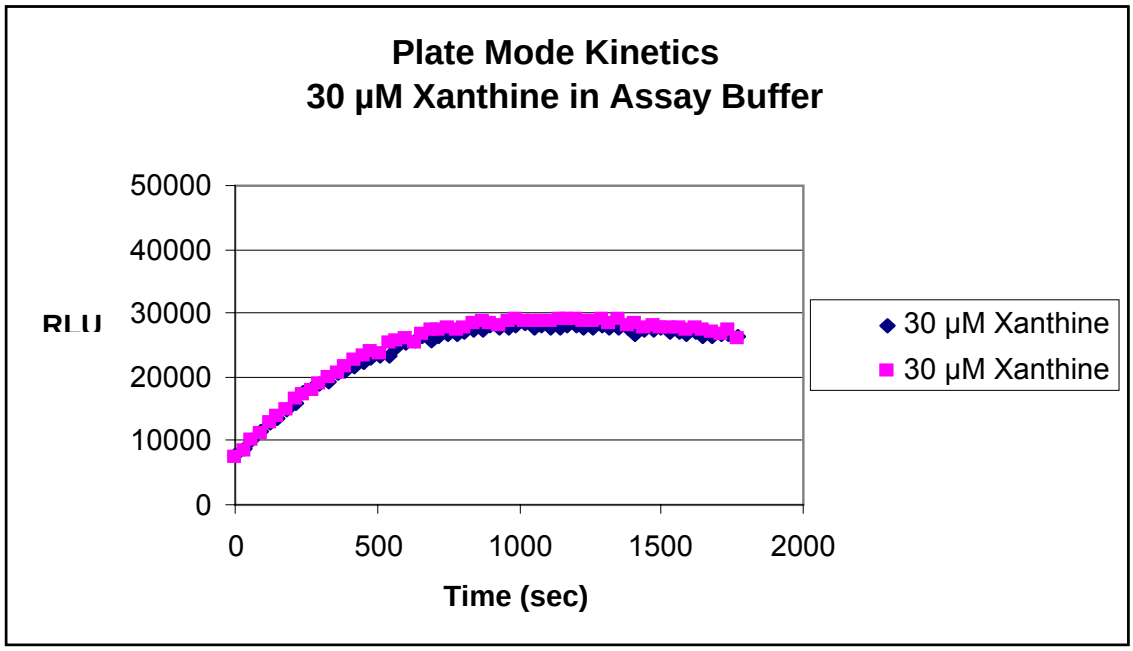


Figure 24. Chart of relative light units (RLU) versus time (sec) for 30 µM xanthine/XO plate mode kinetics. The profile demonstrates successful new equipment setup and operation using a commercial test kit for antioxidant evaluation (ABEL 61-M, Knight Scientific). Two individual samples overlay with analysis time ~30 min.

conversion to hypoxanthine. The starting level of XO enzyme level for the plate mode was approximately 10.25 mU/ml after reconstitution with assay buffer. With adjustment of XO to increase the concentration, the final working concentration was approximately 676 mU/ml. This resulted in an analysis time reduction from approximately 30 min to 5 min (Figure 25). Since the commercial kit from Knight Scientific (plate mode) was set up for xanthine/xanthine oxidase analysis and studies on material antioxidant capabilities, it was necessary to increase the XO level to additionally incorporate plasma hypoxanthine levels, but more importantly to reduce the time of analysis to under 10 min (i.e. switch from glow mode to flash mode kinetics).

A standard curve of hypoxanthine was evaluated at concentrations from 2.3 to 30.3 μM and demonstrated sufficient linearity (normal linear regression) with correlation coefficient >0.9990 (n=2). The incubation time of purine nucleoside phosphorylase was evaluated at 60 and 120 second equilibration times using 10 μM inosine as the substrate with the monitoring of hypoxanthine level (Figure 26). Therefore, the PNP incubation time should remain set at 120 sec to allow for complete inosine to hypoxanthine conversion, with subsequent XO injection to start the luminescence reaction. For future work, one possible way to reduce the overall analysis time would be to add the PNP enzyme to the sample collection tube (e.g. BD vacuutainer), with inosine conversion then occurring during the whole blood to plasma centrifugation step. If this centrifugation technique utilizing PNP in the vacuutainer results in complete inosine to hypoxanthine conversion, it would eliminate the need for the 120 sec PNP incubation time; and reduce

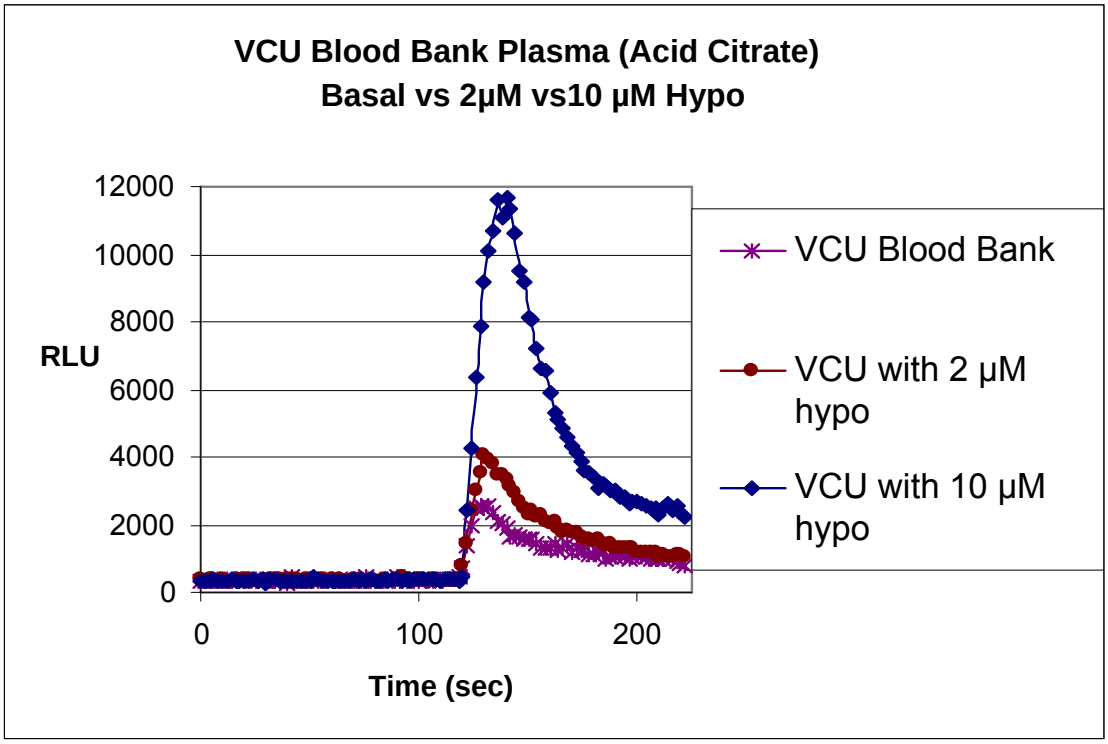
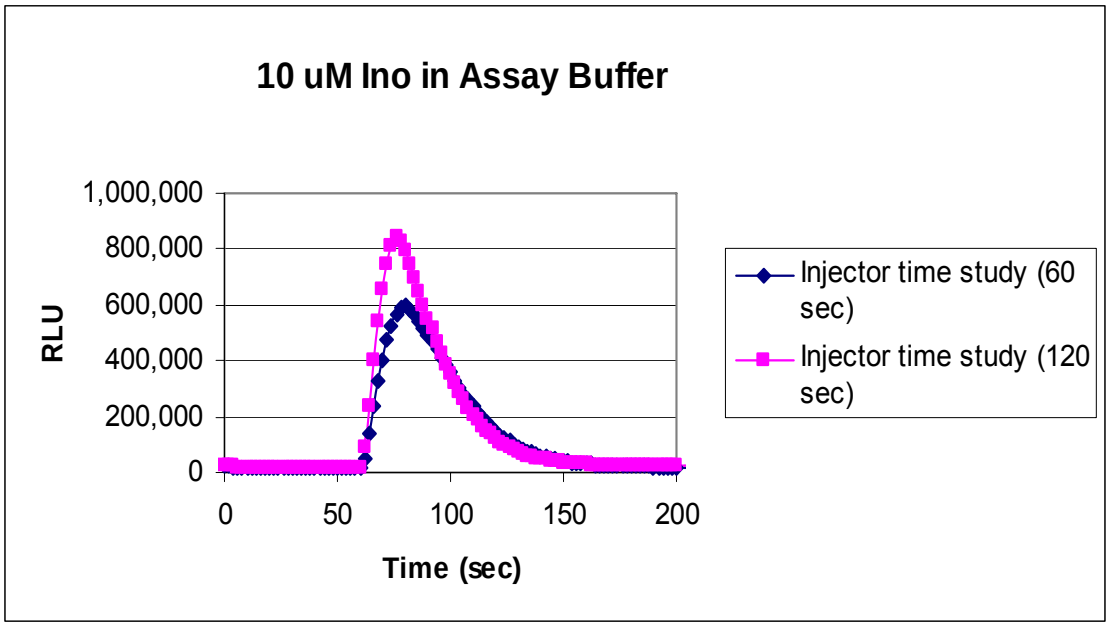


Figure 25. Chart demonstrating a significantly reduced analysis time by utilizing increased amounts of XO (from ~10.3 mU/ml to ~676 mU/ml) and continuous microplate mixing. Analysis time ~3.7 min.



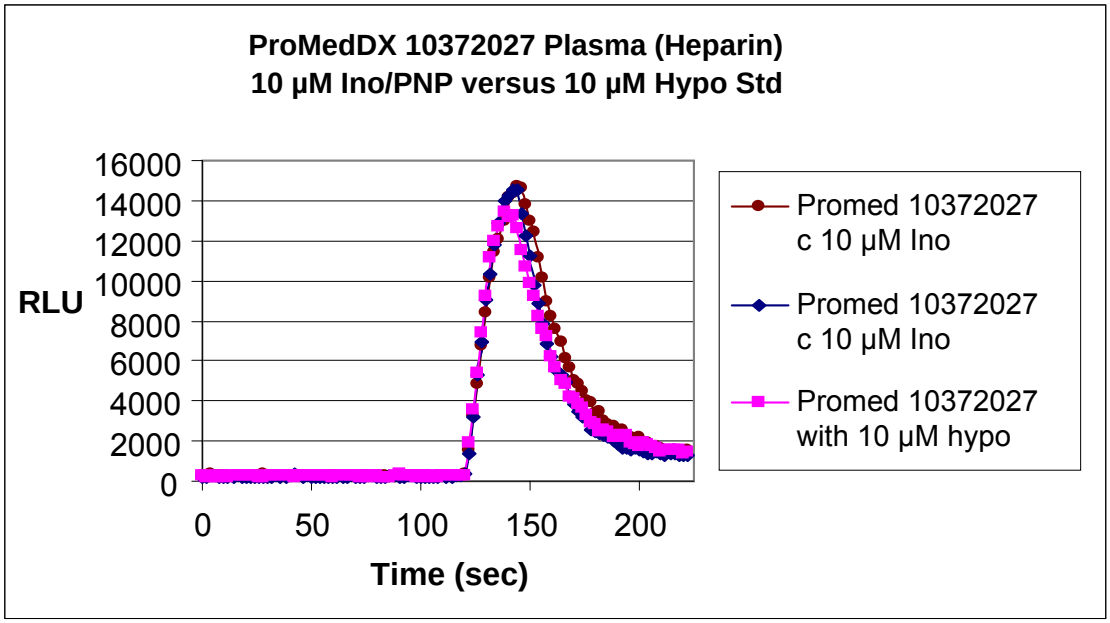
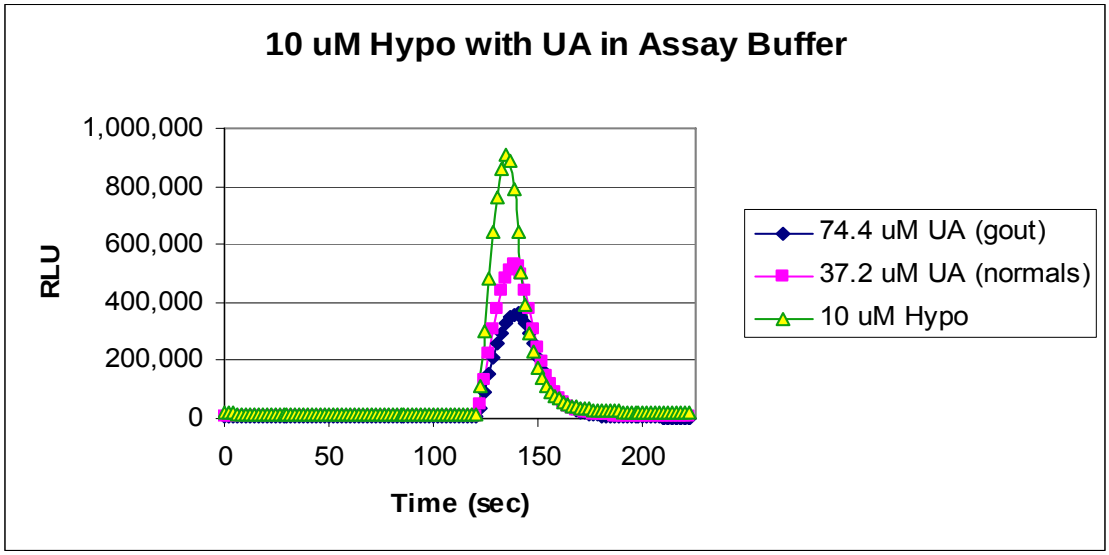


Figure 26. Charts depicting inosine and PNP incubation time and conversion study. Evaluation of 60 and 120 sec PNP incubation times, with 120 sec demonstrating the complete conversion of inosine to hypoxanthine. The 10 µM inosine with PNP conversion RLU responses (n=2) overlays completely against the 10 µM hypoxanthine standard.

the analysis time to only 30 seconds (assumes injection of XO at 0.1 sec and measurement of peak height RLU response).

A study of the effect of plasma uric acid on luminescence response was performed. Since uric acid is found in plasma at relatively higher concentrations (normal range ~350-475 μM) and is a known antioxidant, it was important to evaluate its potential effect on the luminescence signal. As seen in Figure 27, the uric acid's antioxidant affects decreases the luminescence signal (~50% quenching). To address the uric acid, an experiment was performed using strong anion exchange (SAX) resin to remove organic anions from the plasma matrix. Also seen in Figure 27 is a 1:100 dilution of plasma and subsequent use of the SAX pipet tip (Varian, Inc, CA, USA); both demonstrated that removal of potential interfering organic acids (e.g. urate at pH 7.4) resulted with an increase in luminescence response and sensitivity. Since the blank plasma used had approximately 500 nM hypoxanthine, the 1:100 dilution using deionized water and subsequent use of SAX sorbent makes detection levels of hypoxanthine at the pM levels attainable.

A second approach to eliminate the uric acid was to utilize uricase (~1.1 U/ml from Arthrobacter globiformis bacteria, Sigma, USA) during the PNP incubation time in an attempt to eliminate the endogenous uric acid. As seen in Figure 28, it appears that the XO enzyme is deactivated (product inhibition) by the presence of large amounts of by-product hydrogen peroxide that is generated by uricase activity. As one by-product of XO activity is the production of hydrogen peroxide, this finding was not completely surprising due to the effects of product inhibition on XO enzyme turnover. One possible solution to eliminate the generated hydrogen peroxide is to use horseradish peroxidase, which



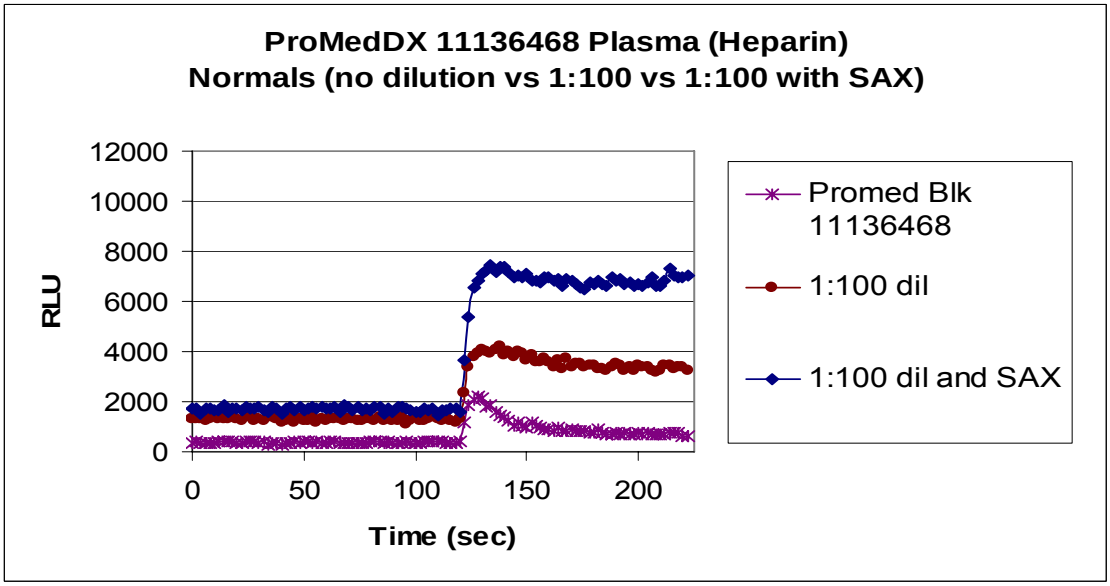


Figure 27. Charts demonstrating effects of uric acid (human physiological levels) on pholasin luminescence signal. High levels of uric acid (in buffer) can quench the luminescence by more than 50%. Treatment of plasma (1:100 dilution) and use of strong anion exchange (SAX) can reduce antioxidant effect on the luminescence signal and increase method sensitivity.

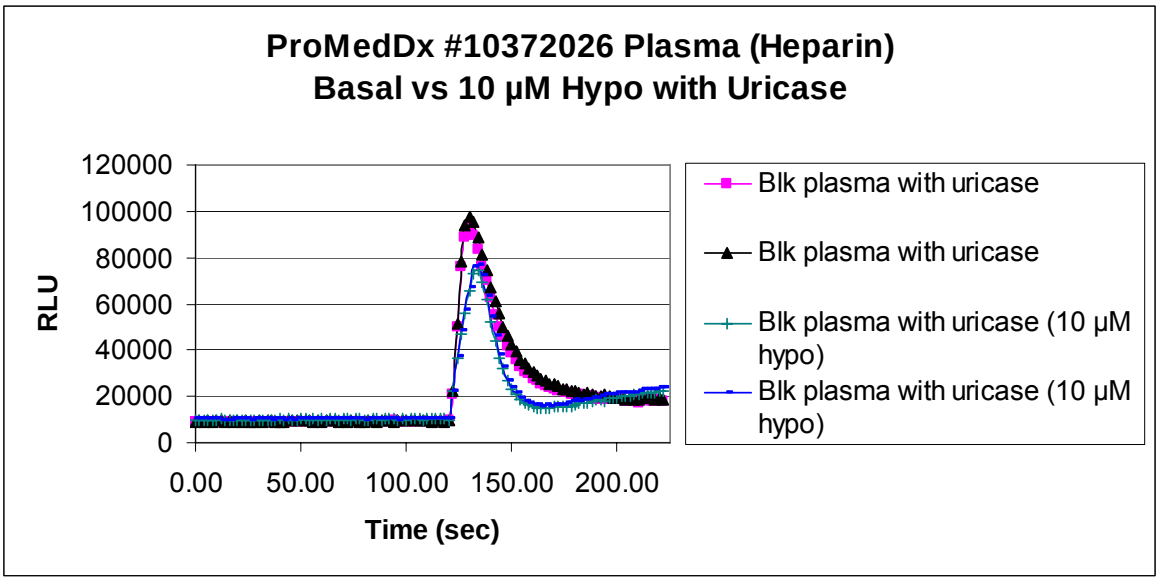


Figure 28. Chart demonstrating the effect of uricase on basal uric acid levels (normal healthy individual) and with fortification of 10 µM hypoxanthine. The generation of hydrogen peroxide (by-product) from uricase enzymatic conversion of uric acid to allantoin caused XO inactivity (potentially from hydrogen peroxide product inhibition on XO effect).

catalyzes hydrogen peroxide to products water and oxygen, but this additional enzyme would only add to the complexity and cost of the analysis and therefore not evaluated.

However, the use of the both uricase and SAX pipet tip technology to eliminate organic acids (e.g. uric acid) was probably not necessary to use, as differences in luminescence response between the healthy normal individuals and confirmed acute MI patients (elevated cTnT levels) was significant (t-test, $\alpha=0.05$, $p<0.01$), when using the 99% percentile ($\alpha=0.01$, one tail, 2.326 standard deviations for n=6) as the calculated biomarker cut-off reference value for acute cardiac ischemia (Figure 29). Even though this research utilized a small sample set for evaluation (n=6 for each group), the 99% cut-off for healthy normal individuals was 5,946 RLU, with all six cTnT patients clearly above this calculated decision point cut-off RLU level. The luminescence method was optimized for rapid evaluation of hypoxanthine in plasma to potentially be used in an ED clinical type environment. For this research study, method parameters such as calibration, repeatability and limit of detection were evaluated using hypoxanthine standards.

The method demonstrated linearity from 2.3-30.3 μM hypoxanthine in assay buffer ($R=0.9990$, n=2) (Figure 30). This range covered the low and midpoint total hypoxanthine concentrations of samples from HPLC analysis of healthy individuals and non-traumatic chest pain patients (Figure 16), and focused on the potential biomarker cut-off concentration for this small group of samples (n=20). Repeatability (n=3) was evaluated by fortification of plasma at basal (~0.5 μM) and 1.5 μM hypoxanthine concentrations (final well levels) and demonstrated by consistent RLU overlays (Figure 31).

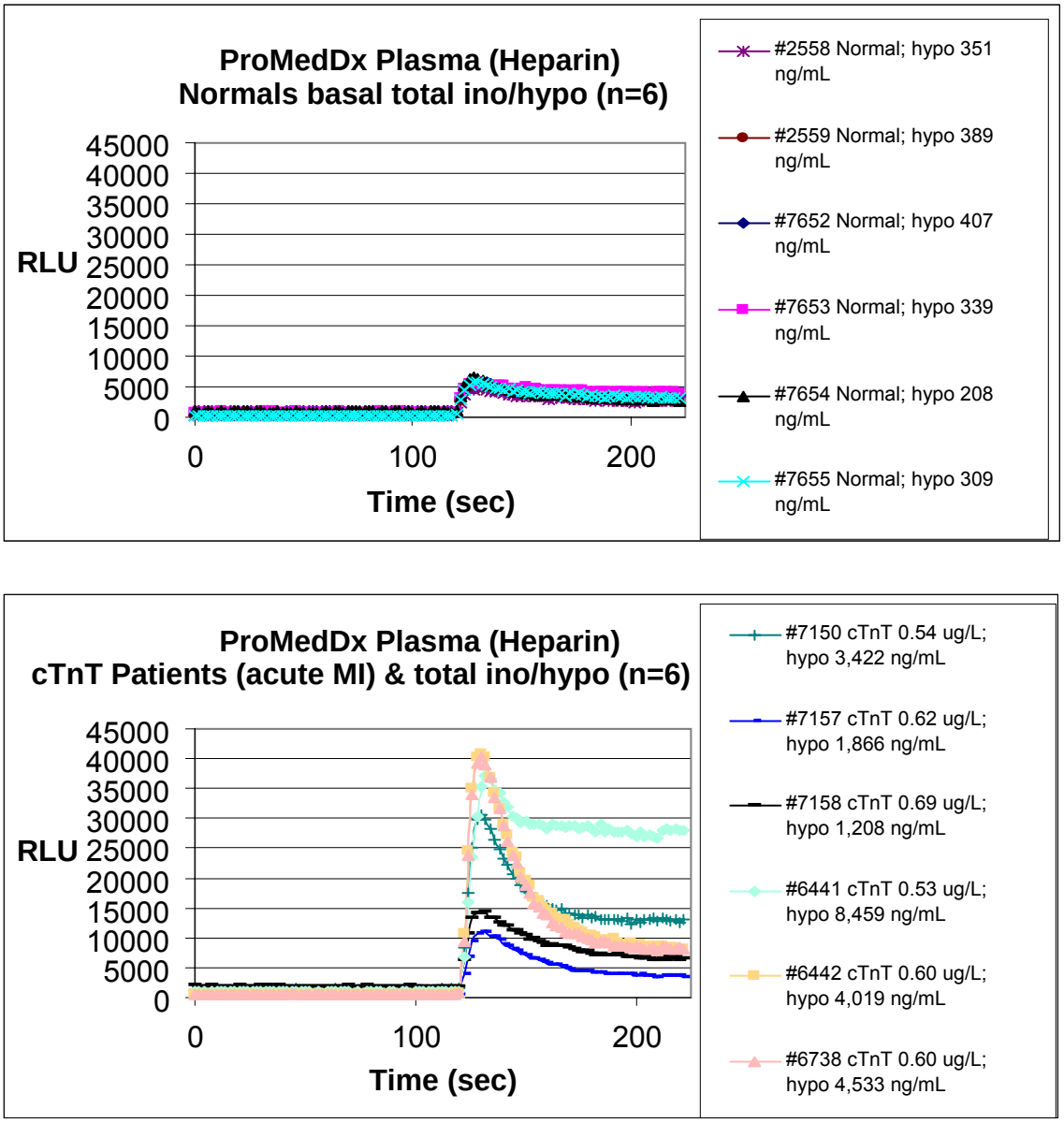
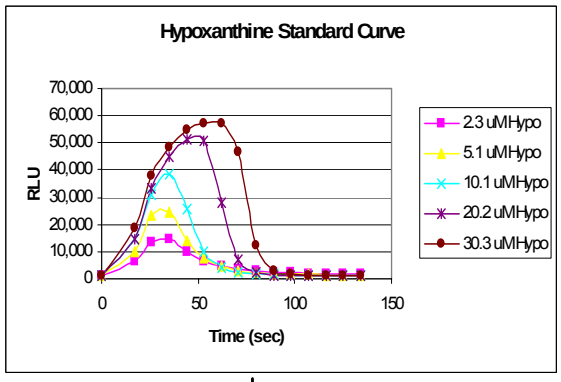
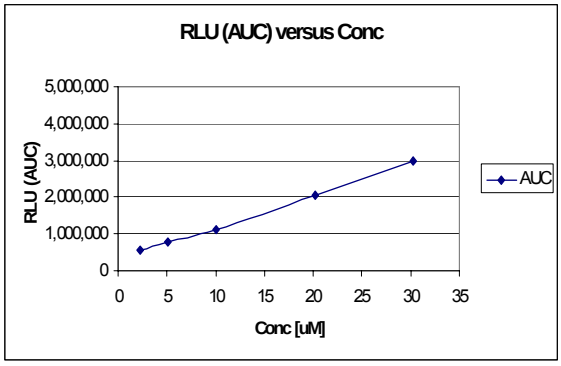


Figure 29. Charts demonstrating healthy normal individuals and patients with confirmed acute MI (hospital documented elevated levels of cTnT). All cTnT patient samples RLU response were clearly above the calculated 99% cut-off reference value (5,944 RLU) for healthy normal individuals (n=6 for each group). HPLC values for total hypoxanthine (from Chapter 4) and cTnT values (from ProMedDx) are listed in the legend.



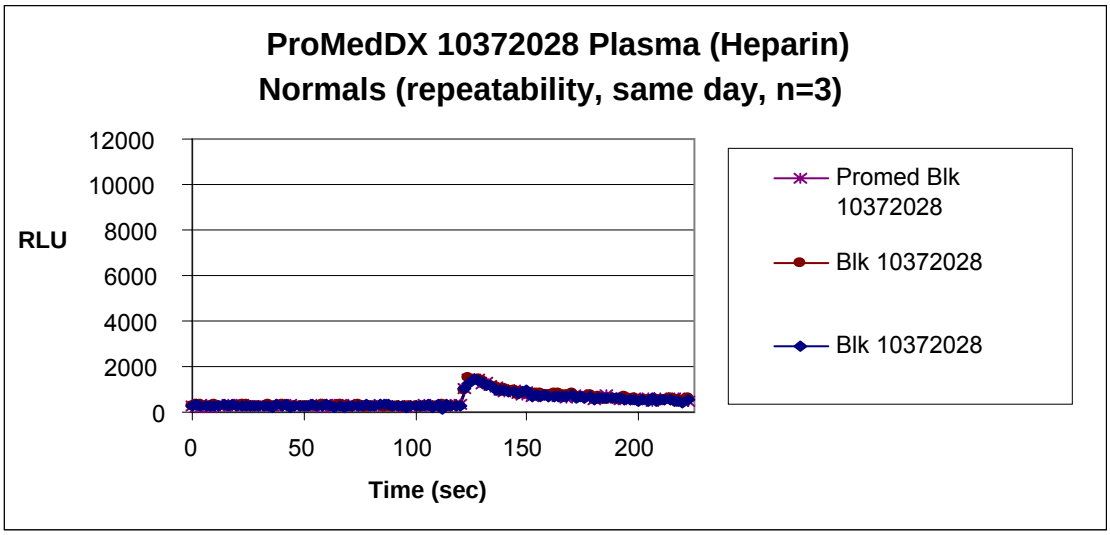


Hypo Conc [uM]	AUC	Hypo Back Calc [uM]
2.3	541,233	2.7
5.1	770,674	5.3
10.1	1,109,493	9.2
20.2	2,070,677	20.1
30.3	2,984,817	30.6

SUMMARY OUTPUT

Regression Statistics	
Multiple R	0.9990
R Square	0.9979
Adjusted R Square	0.997
Standard Error	53664.02
Observations	5

Figure 30. Hypoxanthine standard curve in assay buffer ranging from 2.3 to 30.3 µM demonstrating sufficient linearity and back-calculated hypoxanthine concentrations.



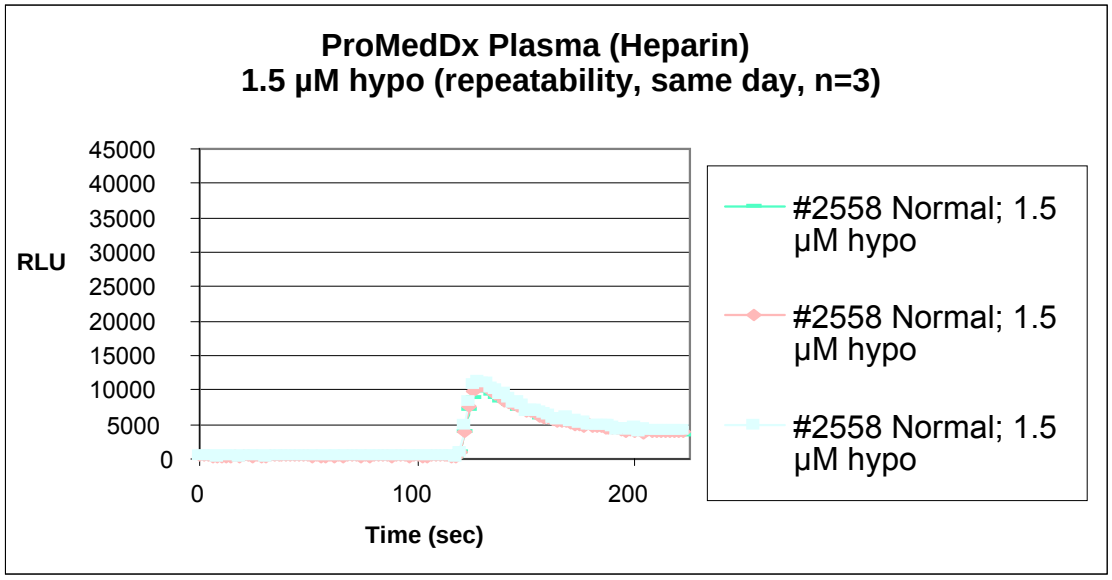


Figure 31. Charts demonstrating repeatability of the luminescence assay. Healthy normal individual (basal level, ~0.5 µM hypoxanthine) and fortified sample (1.5 µM hypoxanthine) assayed three consecutive times. Overlay of profiles demonstrate plasma sample repeatability.

5.4 Conclusion

A rapid luminescence method was developed for the detection of inosine and hypoxanthine in human plasma. Using only 20 ul of plasma (heparin) and instrument direct injectors, the method allowed for the rapid (<5 min) detection of total hypoxanthine (as inosine is converted to hypoxanthine using enzyme PNP) concentrations, which may potentially be used as a biomarker of acute cardiac ischemia. The use of a hypothetical cut-off level (e.g. 99% confidence) relative luminescence unit (RLU) for decision making (i.e. positive level, negative level) may be the most effective use of this rapid screening assay. The method was utilized for evaluation of plasma samples from healthy individuals and cardiac patients with confirmed acute myocardial infarction (hospital documented elevated plasma cTnT levels), and demonstrated the potential of this rapid assay to be used as a diagnostic tool, for use by emergency department services personnel on non-traumatic chest pain patients suspected of undergoing acute cardiac ischemia.

5.5 Acknowledgements

I would like to acknowledge several individuals for their support of the research in this chapter. Dr. Jan Knight from Knight Scientific, Ltd is thanked for her group's technical advice and support on the use of pholasin for luminescence experiments. Dr Knight is world renown for her expertise with pholasin and her company has knowledgeable and kind employees. I would also thank Valerie Mulligan, Dr. Mohammad Hajaligol, Dr. George Patskan and PM USA for the purchase of the luminometer equipment, and all reagent and supplies used for these luminescence analyses. BMG technical support is thanked for their kind assistance in setup and operation of the

luminometer equipment and software. Christine Farthing and Terri Larus are thanked for their technical assistance and demonstrated skills in the operation of the microplate luminometer.

A technical poster presentation of this work entitled "Rapid Luminescence Method for Determination of Inosine and Hypoxanthine in Human Plasma" will be presented at Pittcon 2008 Analytical Exposition (New Orleans, LA, March 2008). I thank the co-authors for their technical contributions (Dr. H. Thomas Karnes[1], Dr. Domenic Sica[2], Dr. Todd Gehr[2], Terri Larus[2], and Christine Farthing[2]) at [1]Departments of Pharmaceutics and [2]Department of Internal Medicine, Division of Nephrology, VCU Medical Center, Virginia Commonwealth University, Richmond, VA 23298, USA.

CHAPTER 6. Summary Research Conclusions and Potential Limitations

With cardiovascular disease (e.g. acute myocardial infarction (MI)) being one of the leading causes of mortality in the world, a rapid patient assessment, diagnosis and treatment is important to improving patient outcomes. This research investigation focused on the use of endogenous plasma levels of inosine (hypoxanthine precursor) and hypoxanthine, as a potential diagnostic tool for use in the evaluation of emergency department non-traumatic chest pain patients. The research was divided into three phases with highlighted research goals as follows: Phase I. Animal (mouse) experiments to identify potential biomarker(s) and demonstrate biomarker proof-of-concept and disease condition (acute cardiac ischemia); Phase II. Evaluation of healthy normal individuals and ED non-traumatic chest pain patients plasma samples for the identified biomarker(s) from Phase I studies; Phase III. Development of a sensitive and rapid clinical assay (<10 min analysis goal) for detection of inosine and hypoxanthine in human plasma.

Phase I research utilized the ICR mouse model for all experiments. Briefly, the mice were anaesthetized; hearts removed and isolated onto a Langendorff apparatus, with Krebs buffer solution providing physiological nutrients. For ischemic conditions, the isolated heart experiments consisted of 30 min stabilization, 20 min zero-flow global ischemia, followed by 30 min of Krebs buffer reperfusion. Time-matched studies were carried out for control (non-ischemic) and test (ischemic) experimental groups. Upon heart

reperfusion, approximately 1.5 ml samples of the Krebs buffered perfusate from isolated mouse hearts were collected at time-points (0, 1, 3, 5, 10 and 20 min) and stored frozen (-20°C) until analysis. An HPLC-UV method was developed and validated for sample analysis with LC-MS used for biomarker identification. Results from this phase of research demonstrated the significant increase in levels of inosine (t-test, $p<0.05$) and elevated levels of hypoxanthine from isolated mouse hearts undergoing 20 min acute global ischemia relative to the non-ischemia control group.

An evaluation of the effects of salicylic acid (SA) in Krebs buffer solution was made, as aspirin (ASA, salicylic acid precursor) is used as part of the initial treatment for patients suspected of undergoing acute MI, to reduce platelet formation at the site of the thrombus (clot). Results of mouse hearts undergoing 20 min acute global ischemia and exposure to 0.1 and 1.0 mM SA concentrations both demonstrated a potentiation of effluxed inosine from the affected heart tissue. As any further work using SA was beyond the scope of this research project, however it is strongly recommended that additional research using animal models are performed on the apparent adverse effects of ASA and SA on ATP catabolism under conditions of acute cardiac ischemia. Knowing how widespread ASA is used for other medical treatments (e.g. analgesia, rheumatoid arthritis) and the possibility of those patients one day experiencing an acute cardiac ischemic event, supports the recommendation of further research on aspirin and its metabolite, salicylic acid on ATP catabolism.

Phase II research evaluated plasma samples representing healthy normal individuals and local hospital emergency department non-traumatic chest pain patients.

Samples were evaluated using a modified and validated HPLC-UV method from Phase I studies. An evaluation of sample collection (matched subject sets of plasma (heparin) and serum separator tube (SST) samples) was performed to determine if inosine and hypoxanthine component differences exist between these two frequently used blood collection techniques. Results of this phase of research demonstrated elevated amounts of hypoxanthine concentrations (~19% positive bias) from blood samples collected using SST tubes, relative to the collection of plasma (heparin). The use of the anticoagulant heparin for obtaining blood samples for these test procedures is recommended; as it eliminates the potential positive bias in hypoxanthine concentrations, and it ultimately saves time (SST requires a recommended clotting time of 30 min prior to the centrifugation step). Significant differences (t-test, $p<0.05$) were found for plasma concentrations of inosine and hypoxanthine detected from emergency department patients presenting with non-traumatic chest pain relative to healthy normal individuals.

Phase III research focused on the initial development of a rapid (<10 min analysis goal) luminescence assay for plasma inosine and hypoxanthine, to potentially be used in hospital clinical laboratory or point-of-care environments. Briefly, the assay utilized 20 ul plasma with enzymes purine nucleoside phosphorylase (PNP) used for conversion of plasma inosine to hypoxanthine, and xanthine oxidase (XO) used for conversion of hypoxanthine to xanthine, and xanthine to uric acid. One metabolic by-product of the XO enzymatic conversion is the generation of highly reactive superoxide anion radicals, which can react with pholasin® (a photoprotein) to generate blue-green light measurable using a luminometer.

The luminescence assay was utilized for rapid detection of plasma concentrations of inosine and hypoxanthine obtained from healthy normal individuals and hospital acute MI patients (hospital documented elevated cTnT plasma levels). Using a calculated 99th RLU (relative luminescence unit) percentile reference cut-off level from the healthy normal individuals (n=6), all six acute MI patients with elevated levels of cTnT had hypoxanthine levels above the 99th RLU percentile cut-off level. These results, although having limited data (n=6 for each group), demonstrate the potential utility of using total hypoxanthine (inosine plus hypoxanthine levels) as a biomarker of cardiac ischemic conditions and the luminescence method as a rapid, simple and sensitive measurement technique.

For evaluation of human plasma samples, the use of the HPLC-UV or microplate luminometer both demonstrated the necessary sensitivity and specificity for determination of inosine and hypoxanthine components in human plasma. However, each technique offered certain specific advantages on its particular use. The HPLC-UV technique offers complete separation and quantification of each component of interest without interferences from endogenous uric acid, but the analytical run time of approximately 20 min may be too long, if used in a hospital emergency department environment. The luminometer technique offered a rapid analysis (<5 min), which is ideal for the emergency department environment, but it does not individually measure inosine and hypoxanthine components, and may have potential interferences (e.g. high plasma uric acids in gout patients or individuals which may take antioxidant type GNC supplements such as ascorbic acid or

polyphenols) that quench the luminescence emission from pholasin (i.e. signal suppression with potential false negative results).

Since biomarker sensitivity and specificity are important parameters to evaluate, it is important to briefly discuss them relative to this research project's results. Total hypoxanthine (inosine and hypoxanthine combined) is found endogenously in the body from normal purine metabolism, thus making the specificity of using total hypoxanthine as a biomarker of acute cardiac ischemia low, as it is not totally specific to heart tissue (as are the cardiac troponins). However, under patient conditions of acute cardiac ischemia, the increase in ATP catabolic by-products found in the bloodstream can certainly be attributed to the heart cell mitochondria's inability to produce sufficient amounts of ATP; thus ultimately resulting in the efflux of ATP catabolic by-products inosine and hypoxanthine out of the heart tissue and into the bloodstream. This higher concentration level of total hypoxanthine (inosine and hypoxanthine) and the ED patient's chief complaint of non-traumatic chest pain; potentially indicates an acute cardiac ischemic condition and warrants immediate medical treatment. It should be mentioned that total hypoxanthine plasma levels may also be significantly elevated from other potential ischemic conditions (e.g. ischemic stroke, angina).

For biomarker sensitivity, luminescence technology is one of the most sensitive analytical techniques currently available, and the results from Phase III demonstrated sufficient sensitivity for total hypoxanthine in normal healthy individuals and cTnT patients, even without plasma sample treatment (no extractions or preparation steps were necessary). The recently FDA cleared IMA test for cardiac ischemia has the same

biomarker properties (low specificity, high sensitivity), and was approved to be used in conjunction with other test (ECG and cardiac troponin) to improve patient diagnostic accuracy. Our proposed candidate biomarker may follow the same path as the FDA cleared IMA biomarker; however it should be emphasized that one major difference between these two biomarkers is the albumin that is modified from the ischemic heart tissue is found in the bloodstream, and not effluxed from the cardiac tissue itself (thus the low specificity). From the findings of this research, the total hypoxanthine levels that are found elevated from acute cardiac ischemic conditions would be effluxed from the affected heart issue into the bloodstream, thus potentially making hypoxanthine a more specific biomarker than IMA for acute cardiac ischemia.

Some other potential sources of error in test results are shown in Table 14. Individuals born with enzyme deficiencies (e.g. adenosine deaminase (ADA), purine nucleoside phosphorylase) may cause erroneous results (e.g. false negative and false positive results), however these individuals should also have immunological problems which are associated with these enzyme deficiencies, and therefore communicated to emergency department personnel as part of obtaining the patient medical history.

Kidney disease and kidney failure are two medical conditions which may lead to potential errors in inosine and hypoxanthine test results. As elimination of most substances from the blood stream is severely compromised in these patient populations, even small polar components such as inosine and hypoxanthine may be retained in the blood stream, leading to elevated blood concentrations and potential false positive results. Kidney disease typically requires chronic treatments (e.g. medication) and individuals

Table 14. Listing of enzyme deficiencies, food sources and medical conditions which many contribute to errors in interpreting test results.

Potential Errors in Results

- ADA Deficiency - ↑ Adeno, ↓ Ino and ↓ Hypo (causes immune problems) – potential false negative
- PNP Deficiency - ↑ Ino, ↓ Hypo (causes immune problems) – potential false positive
- XO Deficiency - ↑ Xan, slight ↑ Hypo (may cause kidney problems)
- Kidney failure or disease
- Food sources containing purines – organ meats, mushrooms, spinach, yeast, peas, beer
- Inosine – GNC (enhance athletic performance)

having this disease are typically aware of their medical condition. However, kidney failure may occur acutely from a traumatic event (e.g. acute kidney failure resulting from a ladder fall), with the individual unaware of an acute kidney failure condition that can rapidly lead to a significant buildup of waste products in their blood stream (thus leading to potential erroneous test results). Individuals with XO deficiency or taking allopurinol (medication commonly used for gout treatment), should have elevated levels of xanthine in the blood potentially causing false positive results. Other sources of exogenous purines that may cause potential errors in test results; would be from a high consumption of purine containing food sources (e.g. organ meats, spinach, and beer) and the use of GNC supplements (e.g. inosine advertised to enhance athletic performance).

In conclusion, it has been demonstrated using our animal model that inosine, a potential biomarker of acute cardiac ischemia, was significantly effluxed from isolated mouse hearts undergoing 20 min acute global ischemia (Phase I results). The significant levels of effluxed inosine may best be explained by the acute cardiac ischemic event, which causes ATP by-product catabolism and the formation and efflux of inosine by the affected cardiac tissue (proof-of-biology). Subsequent work (Phase II), using human plasma samples obtained from hospital emergency department patients with non-traumatic chest pain, indicated elevated levels of inosine and significant levels of hypoxanthine (inosine metabolism occurs immediately in red blood cells), thus supporting the results from Phase I studies.

In Phase III and finishing the research project objectives, the development of a potential clinical assay was achieved to rapidly measure inosine and hypoxanthine levels

using only 20 µl of human plasma, in less than 5 minutes. As there are more than 10 medical conditions that may cause non-traumatic chest pain (e.g. angina, anxiety, heartburn, acid reflux, etc.), it would benefit emergency medical service providers to have additional biomarker(s) of acute cardiac ischemia and a rapid clinical diagnostic assay to assist in patient diagnosis to either rule-in (RIMI) or rule-out (ROMI) acute MI. The results of this work may one day help to answer the medical communities request for additional biomarker(s) of acute cardiac ischemia, and ultimately lead to prompter treatment for the millions of non-traumatic chest pain patients that visit hospital emergency departments every year.

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Young G.P., Green T.R., 1993. The role of single ECG, creatine kinase, and CK-MB in diagnosing patients with acute chest pain. American Journal of Emergency Medicine, 11(5), 444-449.

APPENDIX A

Poster presented at the 29th International Symposium on High Pressure Liquid Phase Separations and Related Techniques (Stockholm, Sweden, June 2005).

HPLC identification of a potential biomarker for initial cardiac ischemia using isolated mouse hearts

Farthing D.[1,2], Xi L.[3], Gehr L.[4], Sica D.[2], Larus T.[2], Kames H.T.[1]

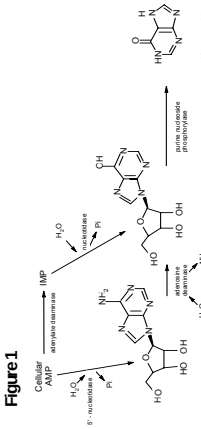
Departments of [1]Pharmaceutics, [2]Clinical Pharmacology and Hypertension, [3]Cardiology, [4]Pediatrics, VCU Medical Center, Virginia Commonwealth University, Richmond, VA 23298-0533, USA

INTRODUCTION

Each year in the US, 7-8 million patients are evaluated with non-traumatic chest pain. It is estimated that approximately 2-5% of these patients are experiencing myocardial infarction but are misdiagnosed and discharged. Emergency medical services would benefit by having an early biomarker to differentiate initial cardiac ischemia from other non-cardiac illnesses.

When cardiac tissue is subjected to oxidative stress (e.g. cardiac ischemia), less oxygen is available for cardiac mitochondria cells to synthesize the required ATP for normal cardiac tissue function. This results in cardiac cellular buildup of ATP metabolic by-products (e.g. ADP, AMP) and activates normally dormant enzymes (5'-nucleotidase, adenosine deaminase, purine nucleoside phosphorylase) to catabolize ATP by-products to substances such as adenosine, inosine, hypoxanthine, xanthine and uric acid for biological elimination (Fig 1). Our research, using the ICR outbred mouse model, indicates inosine may be a potential endogenous biomarker of initial cardiac ischemia.

Figure 1



METHODS

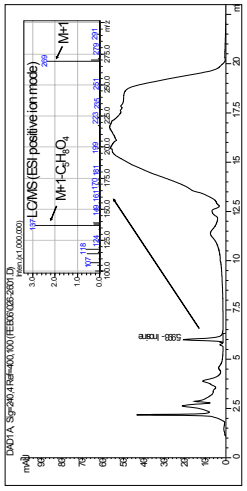
Experiments were conducted to identify potential biomarkers from perfusate of ICR outbred mouse hearts to which global ischemia had been induced. The mice were anaesthetised, hearts removed, and isolated for experiments using a Langendorff system. The hearts were perfused using Krebs buffered solution (pH 7.4) which contained 95% O_2:5% CO_2. Global cardiac ischemia was initiated by adjusting the Krebs buffered solution to a zero flow rate for 20 min. Upon heart reperfusion, samples of the perfusate were collected at 1, 3, 5, 10 and 20 min and frozen prior to HPLC analysis.

For HPLC analysis, 100 µL of the perfusate samples were injected neat onto a Synergi™ Hydro-RP C_{18} column. A gradient consisting of 0.05% TFA in deionized water:methanol (5% to 90%) was used for component separation with diode array detection (DAD) used for inosine quantification and LC/MS for inosine identity confirmation. Area under the curve (AUC) computations were performed using the trapezoidal rule.

Table 1

Sample Type	Inosine AUC 0-20 min [ng min / mL]	% Infarct Size	% Cardiac Functional Recovery
Control	0	9	70
Control	0	21	72
Control	0	31	74
Control	0	25	82
Test	653	24	92
Test	962	26	84
Test	954	12	77
Test	1,003	5	53
Test	2,469	28	39
Test	2,583	19	52

Figure 2



RESULTS

- Inosine and xanthine-like products (e.g. hypoxanthine, xanthine, uric acid) were identified by retention time and DAD spectrum match and were found to be increased 22 to 69, >7, ~3 and ~3 fold, respectively.
- Inosine levels were detected in all test (ischemic) samples and not in the control (non-ischemic) samples (Table 1).
- Inosine levels were quantified using absorbance at 240 nm (Fig 2, RT 5.9 min) and LC/MS was used to confirm inosine (MW 268 Daltons) identity in the mouse perfusate test (ischemic) samples.
- Heart % infarct size and % cardiac functional recovery was determined for test and control mouse hearts (Table 1).
- A higher AUC for inosine was observed in test (ischemic) animals with a lower % cardiac functional recovery than control animals, suggesting a possible relationship between the two variables (Table 1).

CONCLUSION

These results, using the ICR mouse model, suggest that levels of endogenous inosine found in human plasma during periods of cardiac oxidative stress can serve as a potential biomarker for the onset of initial cardiac ischemia.

Further research from our group will be performed on plasma samples obtained from hospital admitted patients undergoing myocardial infarction to determine if plasma levels of endogenous inosine are significantly elevated during initial periods of cardiac oxidative stress. If inosine plasma levels are found to be significantly elevated, this biomarker may be useful to indicate the need for immediate medical treatment, potentially improving patient outcomes.

The authors would like to acknowledge Dr. Bill Gardner (PM, USA) for his LC/MS technical support.

APPENDIX B

Poster presented at Pittcon 2006 Analytical Exposition (Orlando, Florida, March 2006).

Effect of salicylic acid on ischemia-reperfusion injury in isolated mouse hearts

Farthing D.[1,2], Sica D.[2], Gehr L.[4], Larus T.[2], Karnes H.T.[1], Xi L.[3]

Departments of [1]Pharmaceutics, [2]Clinical Pharmacology and Hypertension, [3]Cardiology, [4]Pediatrics, VCU Medical Center, Virginia Commonwealth University, Richmond, VA 23298-0533, USA

INTRODUCTION

Each year worldwide, approximately 32 million individuals experience myocardial infarction. Emergency medical services to patients experiencing myocardial infarction typically include treatment using aspirin® (acetyl salicylic acid, ASA) to reduce platelet formation at the site of cardiac thrombosis. Salicylic acid (SA) is an aspirin® metabolite which has been shown to react with hydroxyl free radicals (Fig 1) generated under conditions of cardiac oxidative stress to form stable reaction products that can be measured quantitatively using HPLC.

In our studies, SA was initially used to study the generation of hydroxyl free radical formation during global ischemia-reperfusion in isolated mouse hearts; however, we observed that inosine (a previously proposed biomarker of initial cardiac ischemia) levels were elevated following ischemic conditions in hearts perfused with modified Krebs buffered solution (1 mM SA) relative to non-SA Krebs buffered solution (KBS).

Figure 1

Table 1

Sample Number	1 mM Salicylic Acid	Inosine AUC@ 20 min (ng/ml•hr)
1285	No	655
1331	No	932
1333	No	954
1288	No	1,003
874	No	2,483
875	No	2,593
1291	Yes	3,648
1290	Yes	4,501
857	Yes	7,344
858	Yes	17,964

Table 2

Sample Number	1 mM Salicylic Acid	Tmx (min)	Inosine peak height	Hypoxanthine peak height	Inosine/Hypoxanthine peak height ratio
8841	Yes	0	61	3.3	1.8
8842	"	1	940	91.9	10.5
8843	"	3	222	30.5	7.3
8844	"	5	84.8	17.8	4.8
8845	"	10	12.1	5.7	2.1
8846	"	20	4.7	3.5	1.3
8841	No	0	0.0	1.5	NA
8842	"	1	7.4	2.2	3.1
8843	"	3	2.6	7.3	2.8
8844	"	5	11.5	5.3	2.2
8845	"	10	11.5	5.3	2.2
8856	"	20	0.0	1.5	NA

METHODS

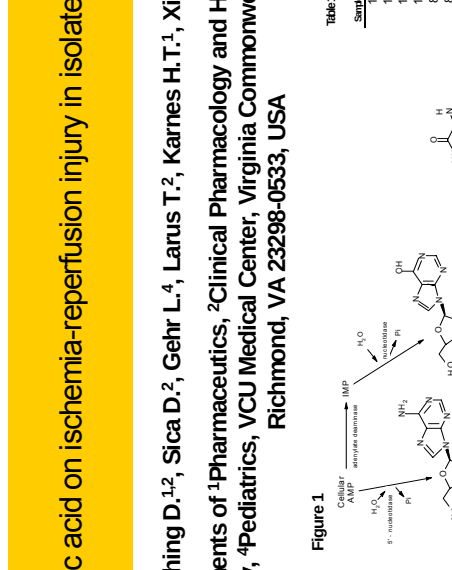
Further investigations were conducted to measure inosine levels from perfusate of ICR outbred mouse hearts to which global ischemia had been induced. The mice were anesthetized with sodium pentobarbital, hearts were surgically removed and isolated for experiments using a Langendorff system. The hearts were perfused using either KBS (non-SA) or a modified KBS (1 mM SA) purged with 95%O_2; 5%CO_2 (pH 7.4). Global cardiac ischemia was initiated by adjusting the KBS to a zero flow rate for 20 min.

Upon heart reperfusion, samples of the KBS perfusate were collected at 1, 3, 5, 10 and 20 min and frozen prior to analysis by HPLC. 50 µL of the collected samples were injected for HPLC analysis using chromatography conditions consisting of a Onyx™ monolithic C_{18} column, HPLC mobile phase of 0.05% TFA in deionized water/methanol (5% to 90% gradient) and diode array detection. Area under the curve (AUC) computations were performed using the trapezoidal rule.

RESULTS

- Inosine and hypoxanthine levels were quantified using DAD absorbance at 250 nm (Fig 2) and LC/MS was used to confirm hypoxanthine (RT 4.9 min) and inosine (RT 6.9 min) identity in the mouse perfusate test (ischemic, 0401.D) versus control (0301.D) samples.
- Inosine levels (Table 1) found in the perfusate samples from ischemic hearts subjected to 1 mM SA (n=4) were significantly higher ($p<0.05$) as compared to non-SA (n=6) ischemic hearts (mean AUC inosine 8514 ± 3284 SEM versus 1437 ± 348 SEM, respectively).

Figure 2



CONCLUSION

These results suggest that 1 mM SA negatively affects ischemic myocytes undergoing nucleotide purine catabolism and potentiates (~3x) ATP degradative products (e.g. inosine, hypoxanthine). However, evaluation of the enzymatic conversion of inosine to hypoxanthine ratio (Table 2) indicates that SA does not inhibit cardiac muscle enzyme purine nucleotide phosphorylase but instead affects an upstream event in the cellular respiration pathway.

Our results indirectly support a recently published report demonstrating salicylic acid's role in decoupling cardiac mitochondrial respiration (Nulton-Persson et al., J. Cardiovasc. Pharmacol. 44(5) 2004). Further research will be performed to determine if a dose response relationship exist between SA concentration and resulting inosine levels during cardiac ischemia-reperfusion.

APPENDIX C

Virginia Commonwealth University (VCU) IRB Application and Approval Forms.

VCU

Virginia Commonwealth University

MCV Campus

Office of Research Subjects Protection

Sanger Hall, 1-023
1101 East Marshall Street
P.O. Box 980568
Richmond, Virginia 23298-0568

804 828-0868
Fax: 804 827-1448
TDD: 1-800-828-1120

DATE: April 6, 2005

TO: Domenic Sica, MD
Internal Medicine
Box 980160

FROM: Lea Ann Hansen, PharmD
Chairperson, VCU IRB Panel D
Box 980568

RE: **VCU IRB #: 4336**
Title: Evaluation of Potential Biomarker(s) in Human Plasma for Initial Cardiac Ischemia

On March 29, 2005, the following research study was ***approved*** by expedited review according to 45 CFR 46.110 Category 5. This approval includes the following items reviewed by this Panel:

RESEARCH APPLICATION/PROPOSAL: None

PROTOCOL: Evaluation of Potential Biomarker(s) in Human Plasma for Initial Cardiac Ischemia

CONSENT/ASSENT:
- All four conditions for waiver of consent have been met. See §45 CFR 46.116(d). The IRB Panel has waived all elements of consent.

ADDITIONAL DOCUMENTS: None

This approval expires on February 28, 2006. Federal Regulations/VCU Policy and Procedures require continuing review prior to continuation of approval past that date. Continuing Review report forms will be mailed to you prior to the scheduled review.

This Institutional Review Board is in compliance with good clinical practices (GCP) as defined under the U.S. Food and Drug Administration (FDA) regulations and the International Conference on Harmonization (ICH) guidelines. Virginia Commonwealth University is approved by DHHS to conduct human subjects research under a Federal Wide Assurance #FWA00005287. **All correspondence related to this research study must include the IRB protocol number and the investigator's name(s) to assist us in locating your file.**

The Primary Reviewer assigned to your research study is Vimal Chadha, MD. If you have any questions, please contact Dr. Chadha at vchadha@vcu.edu or 828-9614; or you may contact Susan Kimbrough, IRB Coordinator, VCU Office of Research Subjects Protection, at sdkimbrough@vcu.edu or 827-1445.

Attachment – Terms of Approval

TERMS OF APPROVAL

In order to comply with federal regulations, industry standards, and the terms of this approval, the investigator must *(as applicable)*:

1) Conduct the research as described in and required by the approved protocol.

2) Obtain informed consent from all subjects without coercion or undue influence, and provide the potential subject sufficient opportunity to consider whether or not to participate (unless Waiver of Consent is specifically approved).

3) Document informed consent using only the most recently dated consent form bearing the VCU IRB "APPROVED" stamp (unless Waiver of Consent Documentation is specifically approved).

4) Provide non-English speaking subjects with a translation of the approved consent form in the subject's first language. The panel must approve the translated version.

5) Obtain prior approval from the VCU IRB before implementing any changes whatsoever in the approved protocol or consent form, unless such changes are necessary to protect the safety of human research subjects. Any departure from these approved documents must be reported to the VCU IRB immediately.

6) Adverse Event/Unanticipated Problem Reporting Timeline: Please refer to the VCU IRB Written Policies and Procedures (specifically WPP #: VIII-7) available at http://www.research.vcu.edu/oeco/fedreg-info/vcuirbwpp.doc

7) Other Reporting Timelines:

   - Report in writing to the VCU IRB within 10 days of any such changes made to protect the safety of human subjects enrolled on this study.
   - Report to the VCU IRB within 10 days the receipt of any new information that may adversely affect the safety of the subjects or the conduct of the trial.

8) Obtain prior approval from the VCU IRB before use of any advertisement or other material for recruitment of study subjects.

9) Promptly report and/or respond to all inquiries by the VCU IRB concerning the conduct of the approved research when so requested.

10) All protocols that administer acute medical treatment to human research subjects must have an emergency preparedness plan. For additional information, please refer to guidance on Emergency Preparedness Plans at http://www.research.vcu.edu/oeco/guidance_epp.html

11) VCU IRB approval is limited to review under 45 CFR 46 and guidance offered by both the Office of Human Research Protections and the Food and Drug Administration. Additional approvals or documentation may be required by the VCU Health System or certain VCU Departments/Divisions under the Health Insurance Portability and Accountability Act (HIPAA).

12) If you plan to involve subjects in this study at a site under the jurisdiction of an institution other than VCU or the VCU Health System, you must refer to the guidance on the Use of a Non-VCU Site/Facility in the VCU IRB Written Policies and Procedures (specifically WPP #: XVII-6) available at http://www.research.vcu.edu/oeco/fedreg-info/vcuirbwpp.doc . This guidance includes the requirements to: (1) request permission to do so in writing from the office of the person at that institution who has responsibility for protecting the rights and well being of human research subjects; and (2) determine that adequate resources are available at the site to conduct your study safely and effectively in full accordance with the approved protocol. You may proceed to conduct your study at the site only if that office or that person provides you with written permission to do so.

OFFICE OF RESEARCH SUBJECT PROTECTION SUBMISSION IRB NUMBER: _____

INITIAL REVIEW SUBMISSION FORM

SECTION 1: REVIEW TYPE REQUEST

1. Protocol Type (check one):
   - ☑ Biomedical **(Research involving medical interventions and/or FDA-regulated products)**
   - ☐ Socio-Behavioral **(Social or behavioral research which does NOT involve medical procedures or FDA-regulated products)**
2. Review Type Requested (check one):
   - ☐ **Full Board Review** (*Note*: Industry-sponsored Clinical Trials **MUST** use the WIRB submission form and process as explained on the VCU IRB web page at http://www.orsp.vcu.edu/irb/irb_forms/index.html)
   - ☑ **Expedited Review - Expedited Request Form must be attached** (*Note*: Expedited Review Guidance & Form Supplement are located at http://www.orsp.vcu.edu/irb/irb_forms/vcu_project.html)
   - ☐ **Exempt Review – Exempt Request Form must be attached** (*Note:* Exempt Request Guidance & Form Supplement are located at http://www.orsp.vcu.edu/irb/irb_forms/vcu_project.html)

SECTION 2: INVESTIGATOR/PROJECT INFORMATION

1. Principal Investigator: Sica, Domenic Department: Internal Medicine
   (Last, First, Middle Initial ——must be a VCU faculty/staff member)
   PI Title and Degrees: Dr. Domenic Sica, M.D.
   Mailing Address: Dept. of Int Med, Div of Nephrology, Clin Pharm and Hypertension, Sanger Hall 8-060, Richmond, Va.
   Phone: 828-2223 Fax: 828-7567 Pager: 3333 E-Mail: dsica@hsc.vcu.edu
2. Research Coordinator: N/A
   Phone: N/A Fax: N/A Pager: N/A E-Mail: N/A
3. Is this a Student Project (where research activities will be carried out by a student under your supervision)? ✓ Yes _____ No
   If yes: Student Name: Don Farthing
   Phone: 274-2640 Fax: 828-7567 E-Mail: defarthi@hsc.vcu.edu
4. Title of Protocol Submission: Evaluation of Potential Biomarker(s) in Human Plasma for Initial Cardiac Ischemia
5. Are there any other IRB approved associated with this submission? _____ Yes ✓ No
   If yes, please list the associated VCU IRB Protocol #'s: _____

SECTION 3: SPONSOR DATA

Please check one of the categories below:

A. ✓ The research specified in this protocol will definitely not be funded.
B. _____ The research specified in this protocol may be funded, but no research application/proposal has been submitted to the VCU Office of Sponsored Programs Administration (OSPA) or the VCU Office of Industry Partnerships (OIP).
C. _____ The research specified in this protocol may be funded, and a research application/proposal has been submitted to the VCU Office of Sponsored Programs Administration (OSPA) or the VCU Office of Industry Partnerships (OIP).

If you checked Category C, you must provide the OSPA # for each associated research application/proposal:

Name of Sponsor	OSPA #
_____	____-____
_____	____-____

5-27-03

Please Note: Federal regulations require IRB approval of new or competing continuation federal research applications/proposals. If there is a new or competing continuation VCU federal research application/proposal associated with this protocol, you must include a copy of your entire application/proposal (exclusive of appendices) with this submission. Failure to do so may delay your research award start date. Other sponsors also may require IRB approval of research applications/proposals. It is the investigator's responsibility to determine whether this review is needed.

PRINCIPAL INVESTIGATOR STATEMENT OF COMPLIANCE:

I understand and accept responsibility for ensuring the safety and welfare of all human subjects who participate in the proposed research study. I certify that all key study personnel, including myself, co-investigators and study coordinators, have completed required training on human subjects protection at VCU. I agree to a continuing exchange of information with the VCU IRB including the requirements to (i) obtain IRB approval before making non-emergency changes/revisions to the protocol, (ii) provide progress reports to the VCU IRB at their request (and at least annually), and (iii) report promptly to the IRB all unanticipated problems or serious adverse events involving risk to human subjects.

Signature of Investigator: _____ ***(Date of Signature)*:** 1/31/05

MEDICALLY RESPONSIBLE INVESTIGATOR (if applicable):

If research procedures involve medical interventions and the PI is not a qualified physician, an M.D. or D.O. must be named as a medically responsible investigator to oversee the medical monitoring program.

Signature of Medically Responsible Investigator: _____ ***(Date of Signature)*:** _____

STUDENT INVESTIGATOR STATEMENT OF COMPLIANCE (if applicable):

This is a student study, which will potentially be presented outside the classroom and/or published. I understand that I may not proceed with the research without first receiving a formal written letter from the VCU Office of Research Subjects Protection.

Signature of Student: _____ ***(Date of Signature)*:** 1-28-05

DEPARTMENT/DIVISION CHAIRPERSON OR DEAN STATEMENT OF COMPLIANCE:

I certify that the research protocol referenced in this document has been reviewed by this department. It is judged to be well designed, scientifically sound, and likely to yield new knowledge that will be useful to society.

Print Name of: Todd W.B. Gehr, MD
Department/Division Chairperson Or Dean

Signature of: _____ ***(Date of Signature)*:** 1/31/05
Department/Division Chairperson or Dean

CONTINUE TO NEXT PAGE FOR
SECTION 4: STUDY PERSONNEL AND LOCATIONS

SECTION 4: STUDY PERSONNEL AND LOCATIONS

LEAD VCU STUDY PERSONNEL:

Provide the names of the most responsible personnel first. If the PI cannot be contacted, these persons may be contacted by the IRB. Within Section 7 of this application you will have the opportunity to list all study personnel.

Sub/Co-Investigators:

(1) Name (Last,First,MI), Degrees: Farthing, Don E., BS Chemistry (Graduate Student) Department: Pharmaceutics

(2) Name (Last,First,MI), Degrees: N/A Department:

Research Coordinators:

(1) Name (Last,First,MI), Degrees: N/A Department:

(2) Name (Last,First,MI), Degrees: N/A Department:

NON-VCU OR VCU HEALTH SYSTEM STUDY SITES:

Please Note: If you plan to involve subjects in this study at a site under the jurisdiction of an institution other than VCU or the VCU Health System, you must: (1) request permission to do so in writing from the office of the person at that institution who has responsibility for protecting the rights and well being of human research subjects; and (2) determine that adequate resources are available at the site to conduct your study safely and effectively in full accordance with the approved protocol. You may proceed to conduct your study at the site only if that office or that person provides you with written permission to do so.

(1) Name of Site: N/A

(2) Name of Site: N/A

(3) Name of Site: N/A

(4) Name of Site: N/A

(5) Name of Site: N/A

(6) Name of Site: N/A

(7) Name of Site: N/A

(8) Name of Site: N/A

(9) Name of Site: N/A

(10) Name of Site: N/A

CONTINUE TO NEXT PAGE FOR SECTION 5: PROJECT DETAIL

SECTION 5: PROJECT DETAIL

ANSWER ALL OF THE FOLLOWING QUESTIONS (by marking the appropriate box to the right): **YES** ↓ **NO** ↓

		YES	NO
1.	**Will drug(s) be administered in this study?** IF YES, supply the following information (attach a separate sheet if necessary): **Drug Name(s):** _____ _____ If drug is investigational or involves an IND, please complete the following: **IND #:**_____held by (check one): ☐ Sponsor ☐ Investigator ☐ N/A •If IND is held by the Sponsor, provide a copy of the Investigator's Brochure and the sponsor's protocol. •If IND is held by the Investigator, provide a copy of the IND application submitted to the FDA and safety information •Attach copy of FDA Form 1572	☐	☑
2.	**Will biologic(s) be used in this study?** IF YES, supply the following information: Biologic Name(s):_____	☐	☑
3.	**Are you evaluating investigational or marketed medical device(s) in this study?** IF YES, supply the following information: **Device Name(s):** _____ **IDE #:**_____held by (check one): ☐ Sponsor ☐ Investigator •If IDE is held by the Sponsor, provide a copy of the Investigator's Brochure and the sponsor's protocol. •If IDE is held by the Investigator, provide a copy of the IDE application submitted to the FDA. **For a device with an IDE, check one of the appropriate categories listed below:** ☐ 510K device (*Provide a copy of the FDA letter confirming the 510K status*) ☐ Non-significant risk device study. (*Provide justification of the non-significant risk determination.*) ☐ Marketed device	☐	☑
4.	A. Does this protocol involve the use of any procedure(s) that will expose the research volunteer to ionizing radiation? ☐ YES (if yes, go to item B) ☑ NO (if no, proceed to question 5) B. Are the procedures for the **direct clinical benefit** of the research volunteer? ☐ YES (if yes, no further information required) ☐ NO (If No, go to item C) C. Radiation Safety Committee (RSC) approval is required if you answered "No" to Question B. Do you have RSC approval for this study? ☐ YES (attach a copy of RSC approval letter) ☐ NO (if no, please contact the Radiation Safety Section at 828-9131 for approval information) *See also* http://views.vcu.edu/oehs/radiation/humanuseguide.pdf		
5.	**Does this project involve the use of Recombinant DNA, Bio-Hazardous Substances (Adenovirus, HIV, Hepatitis B) and/or Carcinogens?** **IF YES,** *please see* http://views.vcu.edu/oehs/chemical/ *for guidance in submitting to the Institutional Biosafety Committee, or call the Office of Chemical and Biological Safety (828-4866).*	☐	☑
6.	**Does this project involve Gene Therapy?**	☐	☑
7.	**Is this a cancer treatment research protocol?** **IF YES,** the research protocol must be reviewed and approved by the Massey Cancer Center Protocol Review and Monitoring System **prior** to IRB Review, and a copy of the approval letter provided? **For information, see http://www.vcu.edu/mcc/research_info/massey_protocol_review.htm or call the MCC Office for Clinical Research, Martha Wellons, at 828-0450.**	☐	☑
8.	**Will the VCU/VCUHS Investigational Drug Pharmacy be utilized (required for all in-patient protocols)?** For information regarding the Investigational Drug Pharmacy, call 828-7901. IF NO, **your research synopsis must describe detailed, appropriate drug storage and dispensing plans.**	☐	☑

5-27-03

9.	**Has each investigator on your protocol completed and signed a Conflict of Interest Disclosure Statement for this project?** (This form is available on-line at http://www.research.vcu.edu/forms/coi_disclosure.pdf). Please attach a copy of the completed and signed form for each investigator to each copy of your protocol submitted (see Section 8 below). As of September 1, 2002, protocols will not be accepted without the forms.	☐	☑

CONTINUE TO NEXT PAGE FOR
SECTION 6: RESEARCH SUBJECT INFORMATION

SECTION 6: RESEARCH SUBJECT INFORMATION

PRIMARY SUBJECT POPULATION(S) *TO BE TARGETED* (Check all that apply): Age range : N/A

1. **General Populations:** ☐ Males ☐ Females ☐ Adults ☐ Outpatients ☐ Healthy Volunteers ☐ Other (cell lines, etc.)

2. **Special Populations:** Check all of the following categories of subjects ***targeted*** where additional protections may apply:
   ☐ Nursing Home Resident ☐ Institutionalized/Hospitalized ☐ Chronic Disease or Condition ☐ Terminally Ill
   ☐ VCU/VCUHS Employees ☐ Limited or Non-Reader ☐ VCU Student ☐ Poor/Uninsured

3. **Vulnerable Populations:** Check all of the following categories of subjects ***targeted***, noting that your research synopsis should justify inclusion of these 'vulnerable' populations, including details on additional protections or consent processes that would be implemented to address the rights and welfare of these groups.
   ☐ Children* *(Note: You must identify the research category in BOX 1, below)* ***(In Virginia, less than 18 years of age. For statistical purposes, NIH considers anyone less than 21 years of age to be a child)***
   ☐ Prisoners** *(Note: You must identify the research category in BOX 2, below)*
   ☐ Mentally Disabled ☐ Pregnant Women ☐ Disabled ☐ Fetuses (or fetal tissue)

***BOX 1: CHILDREN:** Research involving children as subjects must be classified into one of the following categories. Information should be included in the application to support a risk category and include a statement to support that the benefit to children outweighs the risks of the research procedures (use a mark to suggest appropriate category):*

___Research not involving greater than minimal risk (*45 CFR 46.404*) – [see below for definition of minimal risk]
___Research involving greater than minimal risk but presenting the prospect of direct benefit to individual subjects (*45.CFR 46.405*)
___Research involving greater than minimal risk and no prospect of direct benefit to individual subjects, but likely to yield generalizable knowledge about the subject's disorder or condition. (*45.CFR 46.406*)
___Research not otherwise approvable which presents an opportunity to understand, prevent, or alleviate a serious problem affecting the health or welfare of children. (*45.CFR 46.407*)

Minimal Risk means that the probability and magnitude of harm or discomfort anticipated in the research are not greater in and of themselves than those ordinarily encountered in daily life or during the performance of routine physical or psychological examinations or tests.

***BOX 2: PRISONERS:** Research involving prisoners as subjects must be classified into one of the following categories*
___Research not involving greater than minimal risk (*45 CFR 46.404*) – [see below for definition of minimal risk]
___Research involving greater than minimal risk but presenting the prospect of direct benefit to individual subjects (*45.CFR 46.405*)

Minimal risk means that the probability and magnitude of physical or psychological harm that is normally encountered in the daily lives or in the routine medical, dental, or psychological examination of healthy persons.

SUBJECT ENROLLMENT PLAN:

Anticipated # of subjects (if this is a multi-center study, list only subjects under this IRB approval): Total Study #:_____
Is this a multi-center study? ___ Yes ✓ No
If YES, please provide: ___ # of sites ___# of subjects across all sites

5-27-03

CONSENT DOCUMENTATION: *(Mark the type of consent process/documentation planned):*

- ✓ This study is submitted with a request for exemption and does not include documentation of informed consent.
- ___ **Standard Consent Form:** A copy of the proposed consent form(s) to be signed by all subjects is attached to this application.
- ___ **Waiver of Documentation of Consent:** A request is being made to use a verbal consent statement and a copy of the proposed consent script is attached to this application.
- ___ **Assent Form:** A copy of the assent form for children is attached to the application.
- ___ **Assent Waiver Request:** A request is being made to waive the requirement to obtain prospective assent from children. Your research synopsis should explain why the capability of some or all of the children is so limited that they cannot reasonably be consulted or that the intervention or procedure involved in the research holds out a prospect of direct benefit that is important to the health or well-being of the children and is available only in the context of the research.
- ___ **Consent Waiver Request:** A request is being made to waive the requirement to obtain prospective informed consent from subjects. Your research synopsis should explain (i) why the project could not be practicably done if written consent were required, (ii) whether consent is normally obtained for the treatment/procedure, (iii) why the waiver will not adversely affect the rights and welfare of subjects who participate, and (iv) whether or not subjects will be debriefed after their participation.
- ___ **Emergency Research Consent Waiver**

CONTINUE TO NEXT PAGE FOR
SECTION 7: RESEARCH SYNOPSIS

SECTION 7: RESEARCH SYNOPSIS

Use this template (outline) to provide a synopsis of your research to the IRB. Your responses should be in terms for the non-specialist to understand and be ***no more than 6 pages (single-spaced)***. Please also note that the VCU IRB uses a primary reviewer system. As such, not all reviewers receive a full copy of the protocol. Please make sure to address each area noted below. When not applicable, DO list the HEADING and simply indicate "N/A".

I. **Title**

II. **Investigators:** Include a list of key study personnel including brief responsibilities.

III. **Hypothesis:** Briefly state the problem, background, importance of the research, and goals of the proposed study.

IV. **Specific Aims**

V. **Background and Significance:** Include information regarding pre-clinical and early human studies. List appropriate references.

VI. **Preliminary Progress/Data Report:** if available

VII. **Research Method & Design:** Include a brief description of the study design and procedures.

VIII. **Statistical Analysis:** for investigator–initiated studies

IX. **Data and Safety Monitoring: [FOR BIOMEDICAL RESEARCH STUDIES ONLY]** Provide a Data and Safety Monitoring Plan or proof of a Data and Safety Monitoring Board. Include a description of anticipated adverse events and how they will be monitored. For more information on Data and Safety Monitoring Plans, please see http://views.vcu.edu/crc/proi.htm.

X. **Human Subjects Instructions (Be sure to use the sub-headings under A-H):**

- A. **Description:** Provide a detailed description of the proposed involvement of human subjects in the work. Describe the characteristics of the subject population. Include the anticipated number to be enrolled at VCU and a breakdown by age, gender, ethnic makeup and populations requiring additional protections (if applicable). Identify the criteria for inclusion or exclusion of any subpopulation. Explain the rationale for the involvement of special cases of subjects, such as fetuses, pregnant women, children, prisoners, institutionalized individuals, or others who are likely to be vulnerable.
- B. **Research Material:** Identify the sources of research material obtained from individually identifiable living human subjects in the form of specimens, records, or data. Indicate whether the material or data will be obtained specifically for research purposes or whether use will be made of existing specimens, records, or data.
- C. **Recruitment Plan:** Describe plans for the recruitment of subjects and the consent procedures to be followed. Include the circumstances under which consent will be sought and obtained, who will seek it, the nature of the information to be provided to prospective subjects and the method documenting consent.
- D. **Potential Risks:** Describe potential risks physical, psychological, social, legal, or other and assess their likelihood and seriousness. Where appropriate, describe alternative treatments and procedures that might be advantageous to the subjects.
- E. **Risk Reduction:** Describe the procedures for protecting against or minimizing potential risks, including risks to confidentially, and assess their likely effectiveness. Where appropriate, discuss provisions for ensuring necessary medical or professional intervention in the event of adverse events to the subjects. Also, where appropriate, describe the provisions for monitoring the data collected to ensure the safety of subjects.
- F. **Risk/Benefit:** Discuss why the risks to subjects are reasonable in relation to the anticipated benefits to subjects and in relation to the importance of the knowledge that may reasonably be expected to result. If a test article (investigational new drug, device, or biologic) is involved, name the test article and supply the FDA approval letter.

G. **Compensation Plan:** Compensation for subjects (*if applicable*) should be described, including possible total compensation, any proposed bonus, and any proposed reductions or penalties for not completing the protocol.

H. **Consent Issues:**

1. Consent setting (Who will obtain consent, where and when will consent be obtained, and how much time will subjects be afforded to make a decision to participate?)
2. Comprehension (Will an assessment of consent material be conducted to assure the subjects [guardians] understand the information.)
3. Special Consent Provisions (If some or all subjects will be cognitively impaired, or have language/hearing difficulties, describe how capacity for consent will be determined.)
4. If applicable, explain the assent process for children.
5. If request is being made to waive the requirement to obtain prospective assent from children, explain why the capability of some or all of the children is so limited that they cannot reasonably be consulted or that the intervention or procedure involved in the research holds out a prospect of direct benefit that is important to the health or well-being of the children and is available only in the context of the research.
6. If request is being made to waive the requirement to obtain prospective informed consent from subjects, explain (i) why the project could not be practically done if written consent were required, (ii) whether consent is normally obtained for the treatment/procedure, (iii) why the waiver will not adversely affect the rights and welfare of subjects who participate, and (iv) whether or not subjects will be debriefed after their participation.

CONTINUE TO NEXT PAGE FOR
SECTION 8: SUBMISSION CHECKLIST

SECTION 8: SUBMISSION CHECKLIST

The following elements are reminders of steps and documentation that must be included with your submission package. If required elements are missing or provided in an order other than noted below, your review may be delayed.

This checklist must be included as the last page of the IRB Application with your submission packet

✓ 1. **VCU IRB Application Enclosed.**

✓ 2. **Exempt or Expedited Review Request Forms (if applicable).**

✓ 3. **Research Synopsis Enclosed:** must follow the outline in Section 7 of this form and be attached at submission.

____ 4. **Protocol Enclosed:** Sponsor's protocol or investigator's full research plan must be enclosed (if available). ***(Note: if a full protocol exists, it must be submitted with the research synopsis. If no full protocol exists, the research synopsis must be in sufficient detail, or it will be returned without review.)***

____ 5. **Advertisements/Subject Recruitment Materials Enclosed:** if approval is sought for advertisement/subject recruitment materials at this time.

____ 6. **Informed Consent/Assent Document(s) Enclosed (if applicable):** Informed consent document(s) should follow a version of the VCU IRB Consent Template **(date of consent document and page number must appear on each page).**

____ 7. **FDA Form 1572 Enclosed:** if investigational drugs are involved in the research.

____ 8. **IND or IDE Application Enclosed:** if a drug or device is used in the research and IND or IDE is held by the investigator [see Section 5(1) or 5(3) of this form].

____ 9. **Investigator's Brochure Enclosed:** if a drug or device is used in the study and the IND or IDE is held by the sponsor [see Section 5(1) or 5(3) of this form].

____10. **Radiation Safety Committee Approval Enclosed:** if required [see Section 5(4) of this form].

____11. **Institutional Biosafety Committee Review:** requested, if required [see Section 5(5) of this form].

____12. **Massey Cancer Center Protocol Review and Monitoring System Approval Enclosed:** if the project is a cancer treatment research protocol [see Section 5(7) of this form].

✓ 13. **Conflict of Interest Disclosure Statement(s) Enclosed:** [see Section 5(9) of this form].

____14. **Research Application/Proposal Enclosed:** if required [see Section 3 of this form].

✓ 15. **Investigator CV or 2-3 page Biosketch Enclosed:** Principal Investigator CV or a 2-3 page biosketch must be enclosed. **NOTE**: If submitting a biosketch, the NIH biosketch form (398) must be used. The biosketch form is located at the following website http://grants.nih.gov/grants/funding/phs398/biosketch.pdf. A sample biosketch is located at http://grants.nih.gov/grants/funding/phs398/biosketchsample.pdf. Adobe Acrobat 4.0 or higher is required for the biosketch form to be fillable.

____16. **Other**

5-27-03

In addition, please ensure the following:

✓ 17. All key study personnel, including the principal investigator, co-investigators and study coordinators, have completed required training on human subjects protection at VCU. The exam can be accessed from the following website http://www.research.vcu.edu/oeco_citi.htm

✓ 18. Principal Investigator, Medically Responsible Investigator (if applicable), Student (if applicable) and Department/Division Chairperson or Dean have signed the appropriate Statements of Compliance on page 2 of this form.

✓ 19. The review type requested [see Section 1 of this form] has been checked.

CONTINUE TO NEXT PAGE FOR NUMBER OF COPIES REQUIRED

5-27-03

NUMBER OF COPIES REQUIRED

PLEASE NOTE: If required documents are missing, provided in an order other than noted below, or documents are not properly collated, your review may be delayed.

A. If review type requested is **EXEMPT**, submit **(4) collated sets** containing the following documents in the order noted:

- VCU IRB Application
- Protocol (if available)
- Research Synopsis
- Advertisements/Subject Recruitment Materials (if applicable)
- Informed Consent/Assent Documents(s) (if applicable)
- Conflict of Interest Disclosure Statement(s)
- Research Application/Proposal (if applicable)
- Principal Investigator CV or 2-3 page Biosketch

B. If review type requested is **EXPEDITED**, submit **(4) collated sets** containing the following documents in the order noted:

- VCU IRB Application
- Protocol (if available)
- Research Synopsis
- Advertisements/Subject Recruitment Materials (if applicable)
- Informed Consent/Assent Documents(s) (if applicable)
- FDA Form 1572 (if applicable)
- IND or IDE Application (if applicable)
- Investigator's Brochure (if applicable)
- Radiation Safety Committee Approval Letter (if applicable)
- Massey Cancer Center Protocol Review and Monitoring System Approval Letter (if applicable)
- Conflict of Interest Disclosure Statement(s)
- Research Application/Proposal (if applicable)
- Principal Investigator CV or 2-3 page Biosketch

C. If review type requested is **FULL BOARD**, submit **(20) sets in total** as follows:

1. **(4) collated sets** containing the following documents in the order noted:
   - VCU IRB Application
   - Protocol (if available)
   - Research Synopsis
   - Advertisements/Subject Recruitment Materials (if applicable)
   - Informed Consent/Assent Documents(s) (if applicable)
   - FDA Form 1572 (if applicable)

5-27-03

- IND or IDE Application (if applicable)
- Investigator's Brochure (if applicable)
- Radiation Safety Committee Approval Letter (if applicable)
- Massey Cancer Center Protocol Review and Monitoring System Approval Letter (if applicable)
- Conflict of Interest Disclosure Statement(s)
- Research Application (if applicable)
- Principal Investigator CV or 2-3 page Biosketch

AND

2. **(16) collated sets** containing the following documents in the order noted:
   - VCU IRB Application
   - Research Synopsis
   - Advertisements/Subject Recruitment Materials (if applicable)
   - Informed Consent/Assent Document(s) (if applicable)
   - Conflict of Interest Disclosure Statement(s)

ATTACHMENT A/EXPEDITED: REQUEST FOR EXPEDITED FORM
(SUPPLEMENT SECTION TO THE VCU IRB APPLICATION)

Please circle the **number** and/or **letter** next to the categories that are involved in the research. (See the List of Categories from the Federal Register for a complete description and examples).

1. Clinical studies of drugs and medical devices only when condition (a) or (b) is met.
   (a) Research on drugs for which an investigational new drug application (21 CFR Part 312) is not required.
   (b) Research on medical devices for which (1) an investigational device exemption application (21 CFR Part 812) is not required; or (2) the medical device is cleared/approved for marketing and the medical device is being used in accordance with its cleared/approved labeling.

2. Collection of blood samples by finger stick, heel stick, ear stick, or venipuncture as follows:
   (a) from healthy, nonpregnant adults who weigh at least 110 pounds. For these subjects, the amounts drawn may not exceed 550 ml in an 8 week period and collection may not occur more frequently than 2 times per week; or
   (b) from other adults and children, considering the age, weight, and health of the subjects, the collection procedure, the amount of blood to be collected, and the frequency with which it will be collected. For these subjects, the amount drawn may not exceed the lesser of 50 ml or 3 ml per kg in an 8 week period and collection may not occur more frequently than 2 times per week.

3. Prospective collection of biological specimens for research purposes by noninvasive means.

4. Collection of data through noninvasive procedures (not involving general anesthesia or sedation) routinely employed in clinical practice, excluding procedures involving x-rays or microwaves. Where medical devices are employed, they must be cleared/approved for marketing. (Studies intended to evaluate the safety and effectiveness of the medical device are not generally eligible for expedited review, including studies of cleared medical devices for new indications.)

(5.) Research involving materials (data, documents, records, or **specimens**) that have been collected, or will be collected solely for nonresearch purposes (such as medical treatment or diagnosis).

6. Collection of data from voice, video, digital, or image recordings made for research purposes.

7. Research on individual or group characteristics or behavior (including, but not limited to, research on perception, cognition, motivation, identity, language, communication, cultural beliefs or practices, and social behavior) or research employing survey, interview, oral history, focus group, program evaluation, human factors evaluation, or quality assurance methodologies.

8. Continuing review of research previously approved by the convened IRB as follows:
   (a) where (1) the research is permanently closed to the enrollment of new subjects; (2) all subjects have completed all research-related interventions; and (3) the research remains active only for long-term follow-up of subjects; or
   (b) where no subjects have been enrolled and no additional risks have been identified; or
   (c) where the remaining research activities are limited to data analysis.

9. Continuing review of research, not conducted under an investigational new drug application or investigational device exemption where categories two (2) through eight (8) do not apply but the IRB has determined and documented at a convened meeting that the research involves no greater than minimal risk and no additional risks have been identified.

I certify that the research activities present no more than minimal risk to human subjects, and that all of the research activities involve only procedures listed in one or more of the above research categories.

Category #: #5

Procedures: None

Comments or additional information: _____

Was this submission previously sent to WIRB for full board review? Yes _____ No ✓
If yes, please provide the previous VCU submission number _____

Dominic Sisti
P. I. Signature

11/30/05
Date

OR COI FORM V 2.0

VIRGINIA COMMONWEALTH UNIVERSITY
OFFICE OF RESEARCH
CONFLICT OF INTEREST DISCLOSURE STATEMENT
For Sponsored Program Proposals, IRB Protocols, and IACUC Protocols

This form must be submitted with each new and continuing sponsored program proposal or research subjects protocol submission. A copy must be completed by each **investigator** on the proposal or protocol. Terms in **bold** are defined on page 2, and should be clearly understood.

Name: Don Farthing
(Please print or type)
Department: Pharmaceutics

School: Pharmacy

Proposal/protocol title and applicable number (if already assigned):

Proposal/protocol title Evaluation of Potential Biomarker(s) in Human Plasma for Initial Cardiac Ischemia

Sponsored Program Proposal Number N/A
IRB Protocol Number
IACUC Protocol Number N/A
Is this a sponsored activity? Yes ___ No ✓
If so, name of sponsor?
Are you the named PI on this activity? Yes ___ No ✓
If not, who is? Dr. Domenic Sica

1. Do you or any **family member** have a **financial interest** (including ownership, equity or otherwise) in, or participate in, or have a consultantship with any activities or businesses outside of VCU that might or might appear to *affect the design, conduct or reporting* of this activity?
Yes ___ No ✓

If "yes," a) is the **financial interest** expected to exceed $10,000 in a twelve month period?
Yes ____ No ____

b) does the ownership interest exceed 5%? Yes ____ No ____

c) is this research for the purpose of **regulatory approval,** or does it involve **Human Subjects**?
Yes ____ No ____

2. Do you or a **family member** have a financial, managerial, or ownership (equity or otherwise) interest in the sponsoring entity of this activity which is expected to exceed $10,000 in a twelve month period, and/or ownership in excess of 3%? (This question is for VA COI Act purposes only, and it does not apply to governmental or non-profit sponsors.) Yes ____ No ✓

3. Are you providing privileged access to information from this activity, to an entity in which you or a member of your immediate family has a financial interest?
Yes ____ No ✓

4. Do you have direct supervisory authority over a faculty member, student, or employee who receives funds for this activity from a business in which you or a member of your immediate family has a management or financial interest? Yes ____ No ✓

5. Are you purchasing equipment, instruments, or supplies for this activity from a firm in which you or a member of your immediate family has a financial or other interest?

Yes ____ No ✓

Important:
If you have answered "yes" to any of the above questions, a potential financial conflict of interest exists in the conduct of this activity. This potential conflict must be reviewed and resolved by the Conflict of Interest Review Committee (COIRC). To assist the COIRC in a timely review of the potential conflict, please attach a full and complete explanation of the nature of the relationship you (or your family member) has with the sponsoring entity or other company in which you have a financial or ownership interest. Suggested strategies for managing, reducing, or eliminating the conflict may also be included in the explanation. To preserve the objectivity of research at VCU, this new research or other activity may not begin until all financial conflicts of interests have been satisfactorily addressed.

I certify that my responses above are complete and accurate, and that during the life of this project, if circumstances occur which change my answer to any of these questions, I will immediately submit a revised Conflict of Interest Disclosure Statement

Signature: [signature]

Current Date: 1/31/05

Received at OSPA/ORSP (date) _____ by _____

"Family member" means (i) a spouse and (ii) any other person residing in the same household as yourself, who is a dependent of yourself or of whom you are a dependent.

"Financial interest" means anything of monetary value, including, but not limited to, salary or other payments for services (e.g., consulting fees or honoraria); equity interests (e.g., stocks, stock options, or other ownership interests); and intellectual property rights (e.g., patents, copyrights, and royalties from such rights). Does not include income from seminars, lectures, or teaching engagements sponsored by public or nonprofit entities; or income from service on advisory committees or review panels for public or nonprofit entities. ***It also does not include salary, royalties, or other remuneration from VCU or the VCU Health Systems.***

"Human Subjects" means a living individual about whom an investigator (whether professional or student) conducting research obtains: (a) information, specimens, or other data through intervention or interaction with the individual, or (b) identifiable private information.

"Investigator" means the principal investigator and any other person who is responsible for decision making and directing the design, conduct or reporting of this sponsored program/protocol.

"Regulatory approval" means a study known to be intended for use by the Federal or other government in developing an agency action that has the force of law.

OR COI FORM V 2.0

VIRGINIA COMMONWEALTH UNIVERSITY
OFFICE OF RESEARCH
CONFLICT OF INTEREST DISCLOSURE STATEMENT
For Sponsored Program Proposals, IRB Protocols, and IACUC Protocols

This form must be submitted with each new and continuing sponsored program proposal or research subjects protocol submission. A copy must be completed by each **investigator** on the proposal or protocol. Terms in **bold** are defined on page 2, and should be clearly understood.

Name: Don Farthing
(Please print or type)
Department: Pharmaceutics

School: Pharmacy

Proposal/protocol title and applicable number (if already assigned):

Proposal/protocol title Evaluation of Potential Biomarker(s) in Human Plasma for Initial Cardiac Ischemia

Sponsored Program Proposal Number N/A
IRB Protocol Number
IACUC Protocol Number N/A
Is this a sponsored activity? Yes ___ No ✓
If so, name of sponsor?
Are you the named PI on this activity? Yes ___ No ✓
If not, who is? Dr. Domenic Sica

1. Do you or any **family member** have a **financial interest** (including ownership, equity or otherwise) in, or participate in, or have a consultantship with any activities or businesses outside of VCU that might or might appear to *affect the design, conduct or reporting* of this activity?
Yes ___ No ✓

If "yes," a) is the **financial interest** expected to exceed $10,000 in a twelve month period?
Yes ____ No ____

b) does the ownership interest exceed 5%? Yes ____ No ____

c) is this research for the purpose of **regulatory approval,** or does it involve **Human Subjects**?
Yes ____ No ____

2. Do you or a **family member** have a financial, managerial, or ownership (equity or otherwise) interest in the sponsoring entity of this activity which is expected to exceed $10,000 in a twelve month period, and/or ownership in excess of 3%? (This question is for VA COI Act purposes only, and it does not apply to governmental or non-profit sponsors.) Yes ____ No ✓

3. Are you providing privileged access to information from this activity, to an entity in which you or a member of your immediate family has a financial interest?
Yes ____ No ✓

4. Do you have direct supervisory authority over a faculty member, student, or employee who receives funds for this activity from a business in which you or a member of your immediate family has a management or financial interest? Yes ____ No ✓

5. Are you purchasing equipment, instruments, or supplies for this activity from a firm in which you or a member of your immediate family has a financial or other interest?

Yes ____ No ✓

Important:
If you have answered "yes" to any of the above questions, a potential financial conflict of interest exists in the conduct of this activity. This potential conflict must be reviewed and resolved by the Conflict of Interest Review Committee (COIRC). To assist the COIRC in a timely review of the potential conflict, please attach a full and complete explanation of the nature of the relationship you (or your family member) has with the sponsoring entity or other company in which you have a financial or ownership interest. Suggested strategies for managing, reducing, or eliminating the conflict may also be included in the explanation. To preserve the objectivity of research at VCU, this new research or other activity may not begin until all financial conflicts of interests have been satisfactorily addressed.

I certify that my responses above are complete and accurate, and that during the life of this project, if circumstances occur which change my answer to any of these questions, I will immediately submit a revised Conflict of Interest Disclosure Statement

Signature: _____

Current Date: 1-28-05

Received at OSPA/ORSP (date) _____ by _____

"Family member" means (i) a spouse and (ii) any other person residing in the same household as yourself, who is a dependent of yourself or of whom you are a dependent.

"Financial interest" means anything of monetary value, including, but not limited to, salary or other payments for services (e.g., consulting fees or honoraria); equity interests (e.g., stocks, stock options, or other ownership interests); and intellectual property rights (e.g., patents, copyrights, and royalties from such rights). Does not include income from seminars, lectures, or teaching engagements sponsored by public or nonprofit entities; or income from service on advisory committees or review panels for public or nonprofit entities. ***It also does not include salary, royalties, or other remuneration from VCU or the VCU Health Systems.***

"Human Subjects" means a living individual about whom an investigator (whether professional or student) conducting research obtains: (a) information, specimens, or other data through intervention or interaction with the individual, or (b) identifiable private information.

"Investigator" means the principal investigator and any other person who is responsible for decision making and directing the design, conduct or reporting of this sponsored program/protocol.

"Regulatory approval" means a study known to be intended for use by the Federal or other government in developing an agency action that has the force of law.

Farthing IRB #4336 Research Synopsis

I. Title: Evaluation of Potential Biomarker(s) in Human Plasma for Initial Cardiac Ischemia

II. Investigators: Dr. Domenic Sica M.D., Don Farthing (Graduate Student)

III. Hypothesis: Our hypothesis is that endogenous inosine (purine) may be present in human plasma from the catabolism of ATP in ischemic heart tissue. Therefore, inosine may be a potential biomarker of initial human cardiac ischemia prior to heart tissue necrosis (protein biomarkers released).

IV. Specific Aims: Our specific aim of this research is to determine if inosine is present in plasma samples from patients that were admitted for treatment of myocardial infarction (MI). If inosine is present in patients diagnosed and treated for MI (test) and is not present in plasma samples from patients without MI (control), we will pursue developing a possible point-of-care technique to determine inosine levels in plasma.

V. Background and Significance: Each year in the US, approximately 7-8 million patients arrive with non-traumatic chest pain to hospital emergency rooms. Medical personnel are challenged to properly diagnose and treat these patients. It is estimated that approximately 2-5% of these patients are experiencing myocardial infarction, but due to misdiagnosis, they are incorrectly discharged leading to medical malpractice. Thus, emergency medical services to patients would benefit by having a biomarker to help differentiate initial cardiac ischemic conditions from other noncardiac illnesses, which also

may cause chest pain (e.g. GERD, heartburn, asthma, gallstones, pneumonia, and pancreatitis).

VI. Preliminary Progress/Data Report: Initial animal experiments were performed during 2003-2004 at VCU (Dr. Lei Xi, IACUC #0405-2957, Dept. of Internal Medicine, Div. of Cardiology) using the ICR outbred mouse strain. The mice were anaesthetised, hearts surgically removed and isolated for experiments using a Langendorff system. The hearts were perfused using Krebs buffered solution at pH 7.4 and contained $95\%O_2$:$5\%CO_2$. Global cardiac ischemia was initiated by adjusting the Krebs solution to a zero flow rate for 20 min. Upon heart reperfusion with $95\%O_2$:$5\%CO_2$, samples of Krebs solution eluant from the heart were collected at predetermined times and frozen prior to HPLC analysis. For HPLC analysis, 100 µL of the collected sample was injected neat, using chromatography conditions consisting of a C_{18} reversed phase column, gradient mobile phase and diode array detection.

VII. Preliminary animal test results from above initial research indicated that inosine may be a potential biomarker indicative of initial mouse cardiac ischemia. Inosine and xanthine-like products (e.g. hypoxanthine, xanthine and uric acid) were found at higher levels in mice subjected to global cardiac ischemia versus non-ischemic conditions. These results can be explained by ischemic myocytes undergoing nucleotide purine catabolism in the absence of oxygen, which activates normally dormant cellular enzymes and generates degradative products (e.g. adenosine, inosine, hypoxanthine and xanthine) from the breakdown of ATP.

VIII. Research Method & Design: As part of my Ph.D. thesis (D. Farthing, MCV graduate student, Dept. of Pharmaceutics), I am requesting IRB approval to obtain existing plasma samples from cardiovascular (ischemic) diseased patients that have received treatment from VCU Medical Center Hospitals. These patients were diagnosed with myocardial infarction and subsequently treated at VCU Medical Center Hospitals. All plasma samples (25 from non-ischemic patients (controls) and 25 from ischemic patients (tests)) will be obtained from patients previously admitted for medical treatment. The plasma samples (frozen and obtained from the Dept. of Clinical Chemistry) will be stripped of any patient information on the sample tubes for compliance with current patient privacy laws (HIPPA). The samples will be coded as control or test samples (e.g. numbered 1-25) by Dept. of Clinical Chemistry personnel who have been trained on HIPPA regulations. Human plasma samples obtained will be extracted and evaluated for inosine levels using a developed HPLC method at an MCV laboratory (Dr. Domenic Sica, Dept. of Internal Medicine, Division of Clinical Pharmacology). The results and interpretation of these experiments will likely be submitted for publication in a scientific journal (e.g. Clinical Chemistry) and used as part of D. Farthing's Ph.D. thesis. I am requesting an expedited review from Federal Regulations for the Protection of Human Subjects (45 CFR 46) based on the following reason. All plasma samples obtained will be existing samples that were initially used for patient diagnostic information.

IX. Statistical Analysis: Statistical analysis is not applicable as inosine from the pateint's ischemic cardiac tissue will either be present or not present in the plasma samples. The reason for this all or nothing expectation is that the biological half-life of inosine has been

published to be approximately one minute in humans so it may be completely metabolised and not detected. However, if detected, it may indicate that inosine is a potential biomarker of initial human cardiac ischemia prior to heart tissue necrosis.

X. Data and Safety Monitoring: Not applicable

XI. Human Subjects Instructions: Not applicable

APPENDIX D

Additional plasma purchased from ProMedDx and evaluated using HPLC.

Sample Type	Matrix	ProMedDx #	Gender	Age	Ino [µM]	Hypo [µM]	Comments	
Ctrl-1	Plasma (Li Heparin)	10372009	M	51	<0.9	<1.8	Normals (PromedDx)	
Ctrl-1	Serum (no gel)				<0.9	<1.8	"	
Ctrl-2	Plasma (Li Heparin)	10372011	M	50	<0.9	1.5	"	
Ctrl-2	Serum (no gel)				<0.9	1.3	"	
Ctrl-3	Plasma (Li Heparin)	10372018	M	20	<0.9	2.2	"	
Ctrl-3	Serum (no gel)				<0.9	2.1	"	
Ctrl-4	Plasma (Li Heparin)	10372020	M	34	<0.9	1.9	"	
Ctrl-4	Serum (no gel)				<0.9	1.9	"	
Ctrl-5	Plasma (Li Heparin)	10372026	M	49	<0.9	1.5	"	
Ctrl-5	Serum (no gel)				<0.9	2.0	"	
Ctrl-6	Plasma (Li Heparin)	10372027	M	18	<0.9	3.4	"	
Ctrl-6	Serum (no gel)				<0.9	4.6	"	
Ctrl-7	Plasma (Li Heparin)	10372028	M	38	<0.9	1.3	"	
Ctrl-7	Serum (no gel)				<0.9	1.3	"	
Ctrl-8	Plasma (Li Heparin)	10372030	M	38	<0.9	<1.8	"	
Ctrl-8	Serum (no gel)				<0.9	<1.8	"	
Ctrl-9	Plasma (Li Heparin)	10372032	M	43	<0.9	1.5	"	
Ctrl-9	Serum (no gel)				<0.9	1.5	"	
Ctrl-10	Plasma (Li Heparin)	10372035	M	38	<0.9	<1.8	"	
Ctrl-10	Serum (no gel)				<0.9	<1.8	"	
HT-1	Plasma (Li Heparin)	10417810	F	25	<0.9	2.2	Hypertension (PromedDx)	
HT-1	Serum SST				0.7	7.8	Patients	BP 210/105, Hct
HT-2	Plasma (Li Heparin)	10417811	M	44	<0.9	2.9	"	
HT-2	Serum SST				0.8	25.8	"	BP 150/104, Glipizide
HT-3	Plasma (Li Heparin)	10417814	F	30	<0.9	2.0	"	
HT-3	Serum SST				3.6	12.5	"	BP 180/98, Tenormin
HT-4	Plasma (Li Heparin)	10425962	F	43	<0.9	7.3	"	
HT-4	Serum SST				<0.9	25.8	"	BP 150/110, Tenormin
HT-5	Plasma (Li Heparin)	10425963	F	37	<0.9	1.1	"	
HT-5	Serum SST				<0.9	3.8	"	BP 160/102, Tenormin

HT-6	Plasma (Li Heparin)	10425964	F	52	<0.9	2.5	"	
HT-6	Serum SST				<0.9	11.4	"	BP 165/103, Hct
HT-7	Plasma (Li Heparin)	10425965	F	39	<0.9	5.9	"	
HT-7	Serum SST				<0.9	30.0	"	BP 175/82, Hct
HT-8	Plasma (Li Heparin)	10425966	M	29	<0.9	1.3	"	
HT-8	Serum SST				0.7	12.8	"	BP 160/89, Lotensin
HT-9	Plasma (Li Heparin)	10425968	F	45	<0.9	1.4	"	
HT-9	Serum SST				3.6	14.9	"	BP 148/98, Hct
HT-10	Plasma (Li Heparin)	10425971	F	25	<0.9	0.8	"	
HT-10	Serum SST				2.4	13.7	"	BP 140/98, Hct and Lisinopril
cTnI-1	Plasma (Li Heparin)	11075181	F	86	3.6	21.7	MI Patients (PromedDx)	
cTnI-1	Serum SST				0.9	9.1	"	cTnI 0.4 ug/L
cTnI-2	Plasma (Li Heparin)	11075182	M	86	5.3	3.3	"	
cTnI-2	Serum SST				4.4	6.3	"	0.5
cTnI-3	Plasma (Li Heparin)	11075183	F	71	<0.9	7.2	"	
cTnI-3	Serum SST				<0.9	7.2	"	0.8
cTnI-4	Plasma (Li Heparin)	11075186	F	74	<0.9	6.2	"	
cTnI-4	Serum SST				<0.9	7.4	"	0.4
cTnI-5	Plasma (Li Heparin)	11075187	F	90	1.0	7.3	"	
cTnI-5	Serum SST				0.8	13.7	"	0.6
cTnI-6	Plasma (Li Heparin)	11075189	F	59	<0.9	9.7	"	
cTnI-6	Serum SST				2.0	9.2	"	0.5
cTnI-7	Plasma (Li Heparin)	11075193	F	54	<0.9	4.6	"	
cTnI-7	Serum SST				1.2	19.8	"	0.9
cTnI-8	Plasma (Li Heparin)	11107700	F	49	<0.9	33.6	"	
cTnI-8	Serum SST				<0.9	10.9	"	0.6
cTnI-9	Plasma (Li Heparin)	11109491	M	90	<0.9	12.7	"	
cTnI-9	Serum SST				<0.9	4.1	"	31.9
cTnI-10	Plasma (Li Heparin)	11109492	F	54	<0.9	31.2	"	
cTnI-10	Serum SST				<0.9	14.3	"	1.1

Notes:

1. ProMedDX (Norton, MA), a FDA registered biorepository with all samples acquired following IRB approval.

2. Ino= Inosine, Hypo= hypoxanthine, HT = hypertension, cTnI = cardiac tropoinin I.

3. Plasma and serum samples evaluated on same analytical run using calibration curve (DI standards) and controls (plasma).
4. HT and cTnI samples from same hospital, using SST tubes (gel) and following package insert (clotting and spin time, centrifugal force etc). (per PromedDx).

5. Controls from normal donor collection center using red top serum tubes (no gel) per PromedDx.

Curriculum Vitae

Don E. Farthing
Born: 3-22-1958
Location: Ft. Meade, Maryland
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Education

B.S. Chemistry, 1987, VCU, Richmond, VA 23298
Ph.D. Pharmaceutics, 2008, VCU Richmond VA 23298)

Work Experience

Job Title: Research Scientist (Project Leader), Research, Development and Engineering, 1998-2007
Location: Research Center, Philip Morris U.S.A., Richmond, VA 23261
Duties: Responsible for the research and management of laboratory work activities of 6 salaried employees in RD&E. Analytical equipment used are GC/MSD (2), GC/TOF, GC (4), FT-IR (3), XRF (2), HPLC (2) and LC/MS/MS.

Job Title: Technical Superintendent, QA, 1995-1998
Location: Flavor Center, Philip Morris U.S.A., Richmond, VA 23261
Duties: Responsible for work activities of 14 salaried employees in the flavor laboratory. Analytical equipment used are GC/MSD (2), GC (4), FT-IR and HPLC (4). Responsible for hiring, performance appraisals, disciplinary action, training, capital and expense budget, job and work orders. Technical audit team member for PM flavor suppliers.

Job Title: Sr. Analyst, Quality Assurance, 1990-1995
Location: Flavor Center, Philip Morris U.S.A., Richmond, VA 23261
Duties: Evaluate, modify, and implement analytical procedures. Troubleshoot and maintain GC, HPLC, and GC/MS equipment. Responsible for export regulatory program. Train employees on analytical procedures.

Job Title: Research Bioanalytical Chemist (Consultant), 1988-2007 (part-time)
Location: Dept. of Internal Medicine, Division of Nephrology, Section of Clinical Pharmacology and Hypertension, MCV, Richmond, VA 23298

Duties: Method development, validation, and analysis of drugs in biological matrices. Maintenance and troubleshooting of GC and HPLC equipment. Prepare manuscripts for publication in scientific journals.

Job Title: Supervisor, Quality Assurance, 1988-1990
Location: Flavor Center, Philip Morris U.S.A., Richmond, VA 23261
Duties: Supervise flavor manufacturing QA lab. HPLC, GC, physical testing and sensory evaluations on raw materials and blended flavors. Quality control and data evaluation. Write internal procedures for quality assurance manuals.

Job Title: Analytical Chemist, 1987-1988
Location: Consolidated Labs, State of Virginia, Richmond, VA 23298
Duties: HPLC, GC, AA, and DCP analysis on samples from industrial hygienist. Implement and/or modify analytical OSHA methods. Train analytical technicians on procedures and data calculations. Wrote basic programs for quality control used for instrumental results.

Job Title: Lab Specialist, 1984-1987
Location: Department of Pharmaceutics, MCV, Richmond, VA 23298
Duties: Develop analytical methods for evaluating drugs in biological fluids using HPLC and GC. Computer evaluation of analytical data. Instrument maintenance and troubleshooting. Training analytical technicians and graduate students on analytical procedures.

Technical Publications and Posters

1. Farthing D., Sica D., Gehr T., Larus T., Farthing C., Karnes H.T. Rapid luminescence method for determination of inosine and hypoxanthine in human plasma. Poster Pittcon (New Orleans La, March 2008).
2. Farthing C., Farthing D., Brophy D., Larus T., Maynor L., Fakhry I. and Gehr T. W.B.. HPLC determination of cefepime and cefazolin in human plasma and dialysate. Manuscript accepted by Chromatographia (Dec 2007).
3. Maynor L.M., Carl D.E., Matzke G.R., Gehr T. W.B., Farthing C., Farthing D., Brophy D.F.. An in vivo-In vitro study of cefepime and cefazolin clearance during high flux hemodialysis. Manuscript submitted to Pharmacotherapy (Sept 2007).
4. Farthing D., Gehr L., Karnes H.T., Sica D., Gehr T., Larus T., Farthing C. and Xi L. Dose-dependent effects of salicylic acid on post-ischemic ventricular function and purine efflux in isolated mouse hearts. Biomarkers (2007) 12(6):623:634.
5. Farthing D., Sica D., Gehr T., Wilson B., Fakhry I., Larus T., Farthing C. and Karnes H.T. A simple and sensitive HPLC method for determination of inosine and hypoxanthine in human plasma from healthy volunteers and patients presenting with chest pain and potential acute cardiac ischemia. (J of Chrom B (2007) 854:158-164.
6. Farthing D., Sica D., Gehr L., Larus T. Karnes H.T., and Xi L. Effect of salicylic acid on ischemia-reperfusion injury in isolated mouse hearts. Poster Pittcon (Orlando Fl, March 2006).

7. Farthing D., Xi L., Gehr L., Sica D., Larus T., and Karnes H.T. HPLC determination of a potential biomarker of initial cardiac ischemia using isolated mouse hearts. Poster 29th International Symposium on High Performance Liquid Phase Separations and Related Techniques (Stockholm Sweden, June 2005).
8. Farthing D., Xi L., Gehr L., Sica D., Larus T., and Karnes H.T. HPLC determination of a potential biomarker of initial cardiac ischemia using isolated mouse hearts. J of Biomarkers (2006) 11(5):449-459.
9. Callicutt C.H., Cox R.H., Farthing D., Hsu F.S., Johnson L., Laffoon S.W., Lee P., Kinser R.D., Podraza K.F., Sanders E.B. and Seeman J.I. The ability of the FTC method to quantify nicotine as a function of ammonia in mainstream smoke. Contributions to Tobacco Research, (2006) 22(2):71-78.
10. Farthing D., Sica D., Fakhry I., Larus T., Ghosh S., Farthing C., Vranian M., and Gehr T. Simple HPLC-UV method for determination of iohexol, iothalamte, p-aminohippuric acid and n-acetyl-p-aminohippuric acid in human plasma and urine with ERPF, GFR and ERPF/GFR ratio determination using colorimetric analysis. Poster Pittcon (Orlando Fl, 2-05).
11. Farthing D., Sica D., Fakhry I., Larus T., Ghosh S., Farthing C., Vranian M., and Gehr T.. Simple HPLC-UV method for determination of iohexol, iothalamte, p-aminohippuric acid and n-acetyl-p-aminohippuric acid in human plasma and urine with ERPF, GFR and ERPF/GFR ratio determination using colorimetric analysis. J of Chrom B (2005) 826:267-272.
12. Meruva N.K., Penn J.M., and Farthing D. Rapid identification of microbial VOCs from tobacco molds using closed-loop stripping and GC/TOF-MS, J of Indus Micro & Biotech (2004) 31:482-488.
13. Farthing D., Larus T., Fakhry I., Gehr T., Prats J., and Sica D. Liquid chromatography method for determination of bivalirudin in human plasma and urine using automated ortho-phthalaldehyde derivatization and fluorescence detection, J of Chrom B (2004) 802:355-359.
14. Farthing D., Sica D., Abernathy C., Fakhry I., Roberts J.D., Abraham D.J., and Swerdlow P. High-performance liquid chromtographic method for determination of vanillin and vanillic acid in human plasma, red blood cells and urine. J of Chrom B (1999) 726:303-307.
15. Farthing D., Fakhry I., Ripley E.B.D., and Sica D. Simple method for determination of hydrochlorothiazide in human urine by high performance liquid chromatography utilizing narrowbore chromatography. J of Pharm and Biomedical Analysis (1998) 17:1455-1459.
16. Farthing D., Sica D., Fakhry I., Pedro A., Gehr T.W.B. Simple high-performance liquid chromatographic method for determination of losartan and E-3174 metabolite in human plasma, urine and dialysate. J of Chrom B (1997) 704:374-378.
17. Farthing D., Gehr L., Berger B., Fakhry I., and Sica D. Simple method for simultaneous determination of halothane, enflurance, and isoflurane in krebs solution using capillary gas chromatography. Biomedical Chrom (1997) 2:29-32.

18. Farthing D., Brouwer K.L.R., Fakhry I., and Sica D. Solid-phase extraction and determination of ranitidine in human plasma by a high-performance liquid chromatographic method utilizing midbore chromatography. J of Chrom B (1997) 688:350-353.
19. Farthing D., Sica D., Fakhry I., Walters D.L., Cefali E.A., and Allan G. Determination of flutamide and hydroxyflutamide in dog plasma by a sensitive high performance liquid chromatography method utilizing mid-bore chromatography. Biomed Chrom (1994) 8:251-254.
20. Farthing D., Sica D.A., Fakhry I., and Gehr T.W.B. Novel high-performance liquid chromatographic method using solid-phase on-line elution for determination of metolazone in plasma and whole blood. J of Chrom B (1994) 653:171-176.
21. Farthing D., Fakhry I., Gehr T.W.B., and Sica D.A. Quantitation of metolazone in urine by high-performance liquid chromatography with fluorescence detection. J of Chrom Biomed Appl (1990) 534:228-232.
22. Karnes H.T., Farthing D., and Besenfelder E. Solid phase extraction with automated elution and HPLC of torsemide and metabolites from plasma. J of Liquid Chrom (1989) 12(10):1809-1818.
23. March C., Farthing D., Wells B., Besenfelder E., Karnes H.T. Solid-phase extraction and liquid chromatography of torsemide and metabolites from plasma and urine. J of Pharm Sci (1990) 79(5):453-457.
24. Karnes H.T., Beightol L.A., Serafin R., and Farthing D. Improved method for the determination of diazepam and n-desmethyldiazepam in plasma using capillary gas chromatography and nitrogen-phosphorus detection. J of Chrom Biomed Appl (1988) 424:398-402.
25. Karnes H.T., Rajasekharaiah K., Small R.E., and Farthing D. Automated solid phase extraction and HPLC analysis of ibuprofen in plasma. J of Chrom (1988) 11(2):489-499.
26. Farthing D., Gehr T.W.B., Fakhry I., and Sica D.A. A direct injection method for determining furosemide and metolazone in urine using HPLC and fluorescence detection. LC/GC magazine (1988) 9(7):478-480.
27. Karnes H.T., Opong-Mensah K., Farthing D., and Beightol L.A. Automated solid-phase extraction and HPLC determination of ranitidine from urine, plasma and peritoneal dialysate. J of Chrom Biomed App (1987) 422:165-173.
28. Karnes H.T., and Farthing D. Improved method for the determination of oxazepam in plasma using capillary GC and nitrogen phosphorus detection. LC/GC magazine (1987) 5(11):978-979.
29. Karnes H.T., Beightol L.A., and Farthing D. Benzyl alcohol interference from heparin lock flush solutions in a HPLC procedure for mezlocillin. Therap Drug Monit (1987) 9(4):456-460.

Professional Training / Seminars

ACS Courses – GLP; QA/QC for the Laboratory; Troubleshooting HPLC systems; Practical LC/MS, Analysis and Interpretation of MS.

Pittcon Courses – Computer and Equipment Validation; Fast GC; ISO Guidelines for Uncertainty Calculations; Online Chemical Searching; Sampling of Solids, Gases, and Liquids; Statistics Review; Headspace Theory and Practical; Sample Preparations; HPLC Method Development for LC/MS.
ASMS Courses – LC/MS.
Thermo Nicolet – FT-IR Operation and Interpretation; XRF Operation and Interpreation.
ISSX – Toxicology.
Gerstel – Headspace; Thermal Desorption; Twister.
Agilent – LC/MS Lab on a Chip; 5973 Agilent MSD Troubleshooting; Macro Programming.
PBA Courses – Pharmacokinetics for Chemist, Metabonomics: Principles and Practices.
Biomarkers – Biomarker assay development and validation.

Professional Memberships and Activities
American Chemical Society – since 1996
ACS Chemical Toxicology – since 1996
Journal of Chromatography B – manuscript reviewer since 2000
Chromatographia – manuscript reviewer since 2006
Member of Philip Morris R&D Emergency Response Team (hazmat, fire, medical) – 1999-2006

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