Gender and Cocaine Use Influence the Expression of Urinary Markers of Inflammation and Oxidative Stress

Marie Meagher Bourgeois

University of South Florida

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Gender and Cocaine Use Influence the Expression of Urinary Markers of Inflammation and Oxidative Stress

by

Marie Meagher Bourgeois

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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Keywords: Autoimmune, Interleukins, Reactive Oxygen Species, Acute Phase Reaction, Cytokines

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Dedication

Without the support, understanding and unflagging faith of my husband Bobby, and my children Jake, Shelby, Maya and Logan, this would not have been possible.
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<th>Description</th>
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<tbody>
<tr>
<td>ACTS</td>
<td>Agency for Community Treatment and Services of Tampa</td>
</tr>
<tr>
<td>AKI</td>
<td>acute kidney injury</td>
</tr>
<tr>
<td>AMI</td>
<td>acute myocardial infarction</td>
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ANP</td>
<td>atrial natriuretic peptide</td>
</tr>
<tr>
<td>BE</td>
<td>benzoylecgonine</td>
</tr>
<tr>
<td>CE</td>
<td>cocaethylene</td>
</tr>
<tr>
<td>CKD</td>
<td>chronic kidney disease</td>
</tr>
<tr>
<td>CK-MB</td>
<td>creatine kinase MB isoenzyme</td>
</tr>
<tr>
<td>CRP</td>
<td>c reactive protein</td>
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<tr>
<td>CVD</td>
<td>cardiovascular disease</td>
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<tr>
<td>DAWN</td>
<td>Drug Abuse Warning Network</td>
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<tr>
<td>DM</td>
<td>diabetes mellitus</td>
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<tr>
<td>E2</td>
<td>estradiol</td>
</tr>
<tr>
<td>ECG</td>
<td>electrocardiogram</td>
</tr>
<tr>
<td>ED</td>
<td>emergency department</td>
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<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
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<tr>
<td>EME</td>
<td>ecdnine methyl ester</td>
</tr>
<tr>
<td>EMIT</td>
<td>enzyme multiplied immunoassay technique</td>
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<tr>
<td>ESRD</td>
<td>end stage renal disease</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>-----------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>HsCRP</td>
<td>high sensitivity C - reactive protein</td>
</tr>
<tr>
<td>IL1α</td>
<td>interleukin 1α</td>
</tr>
<tr>
<td>IL1β</td>
<td>interleukin 1β</td>
</tr>
<tr>
<td>IL6</td>
<td>interleukin 6</td>
</tr>
<tr>
<td>MAB</td>
<td>microalbumin</td>
</tr>
<tr>
<td>MI</td>
<td>myocardial infarction</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>N</td>
<td>normal</td>
</tr>
<tr>
<td>NGAL</td>
<td>neutrophil gelatinase-associated lipocalin</td>
</tr>
<tr>
<td>NIDA</td>
<td>National Institute on Drug Abuse</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NF-kB</td>
<td>nuclear factor-kB (NF-kB)</td>
</tr>
<tr>
<td>OS</td>
<td>oxidative stress</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>ProANP</td>
<td>pro atrial natriuretic peptide</td>
</tr>
<tr>
<td>RA</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RAS</td>
<td>renin-angiotensin system</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SAMSHA</td>
<td>Substance Abuse and Mental Health Administration</td>
</tr>
<tr>
<td>SLE</td>
<td>Systemic Lupus Erythematosus</td>
</tr>
<tr>
<td>THC</td>
<td>Tetrahydrocannabinol</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3’,5,5’-tetramethylbenzidine</td>
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Gender and Cocaine Use Influence the Expression of Urinary Markers of Inflammation and Oxidative Stress

Marie Meagher Bourgeois

Abstract

The purpose of this study was to investigate whether or not gender differences may be present in the expression of a number of urinary proteins which may serve as markers of inflammation and oxidative stress. Males and females have different patterns of illness and different life spans, suggesting basic biological traits exert significant control on the incidence of rhabdomyolysis, renal failure, atherosclerosis, myocardial ischemia, myocardial contraction band formation, autoimmune disorders and general inflammatory diseases. Men are at greater risk for cardiovascular disease; however women, particularly elderly women, have higher fatality rates due to heart failure. Renal diseases progress far more quickly in men, possibly due to testosterone. Men also have higher kidney bulk related to androgen expression. Gender disparity may be most obvious in autoimmune disorders; of the estimated 8.5 million people diagnosed with autoimmune disorders, approximately 80% are women. Hashimoto’s thyroiditis, the most common form of hypothyroidism, is up to 10 times more common in women. Systemic Lupus Erythematosus (SLE), an autoimmune disease characterized by acute and chronic inflammation, is 9 times more common in women. Rheumatoid arthritis (RA), an
autoimmune disease affecting approximately 1.3 million people in the United States, is four times more common in women. Diabetes mellitus (DM), affecting more than 17 million people – the majority of which are women, is linked to microvascular and macrovascular diseases such as kidney failure, strokes and atherosclerosis. These conditions are linked to physiological changes that may alter the expression of certain biomarkers of inflammation and oxidative stress.

Over the past several decades, it has become increasingly clear that the role of diet, smoking, and other lifestyle choices clearly influence the etiology and pathophysiology of these diseases. The use of drugs, both licit and illicit, has been clearly linked to many of these diseases. Illicit substances, particularly cocaine, have been demonstrated to produce pathophysiological changes to many systems in the body which can greatly influence the progression of existing and drug-induced disease states leading to systemic damage. A relationship between the expression of markers of inflammation, oxidative stress, cardiac damage, or other systemic injury, gender and cocaine use has not been clearly established.

Urine is an important medium for assessment of general health status. It has classically been used to monitor disease states; glucosuria as an indicator of diabetes and renal dysfunction, microorganisms signifying urinary tract or bladder infection, and biomarkers such as human chorionic gonadotropin to confirm pregnancy. Recently urine has been used to assess biomarker expression and disease states. Urine is an ideal clinical tool for toxicological screens; it is readily accessible, non invasive and typically supplied in sufficient quantity to accommodate multiple tests. In this study, urine specimens were collected and analyzed for creatinine, cocaine, total protein, aldosterone, c-reactive
protein (hsCRP), myeloperoxidase (MPO), microalbumin (MAB), neutrophil gelatinase-associated lipocalin (NGAL), heat shock protein 90α (hsp90α), vascular endothelial growth factor (VEGF), myoglobin, pro atrial natriuretic peptide (proANP) and interleukins 1α, 1β, and 6 using ELISA and colorimetric assays.

Urine specimens that tested negative for all illicit substances in the standard National Institute on Drug Abuse (NIDA) 10 panel showed differences in a number of these biomarkers which strongly suggested significant differences between males and females for aldosterone, IL1α and IL1β. In addition, significance is suggested for MPO and CRP. Although sex specific differences in serum expression have been noted for some of the markers in both animal and human models, this has not been previously demonstrated in human urine. This may have implications for what is typically referred to as ‘normal’ values. Gender specific differences were not apparent in urine specimens that tested positive for cocaine. Also, in males only, the levels of myoglobin and aldosterone significantly increased.
Chapter 1.0

Introduction

In 2001, the Institute of Medicine stated that gender is a ‘basic human variable’ that should be considered in every facet of research design [1]. Significant gender dimorphism has been documented in cardiovascular, renal and autoimmune diseases [2-8]. Higher male susceptibility to cardiovascular disease may be due to genetic, hormonal, or lifestyle factors or through a combination of mechanisms [9, 10]. Sex-based differences in the clinical presentation, diagnosis, and treatment outcomes of cardiac disease have long been recognized [11]. Gender differences in lifestyle risk factors (e.g. smoking, exercise and diet) appear to contribute to gender disparities; however, these lifestyle factors do not completely account for the dimorphism. Despite the fact that there is a higher incidence of cardiovascular diseases in men in general, the total number of deaths from cardiovascular disease has been higher for women than for men [12]. Coronary artery disease is the leading cause of death in women. More than twice as many women die from cardiovascular disease as from all forms of cancer combined. Men are more likely than women to suffer from hypertension. Multiple physiological alterations have been noted in hypertensive individuals; these include renal disturbances, neurohormonal and adrenergic disruption, endothelial dysfunction, systemic
inflammation, and increased oxidative stress. Increased creatinine, CRP, aldosterone and proANP are often seen in hypertension. CRP is an acute phase inflammatory marker considered indicative of atherosclerosis and AMI. ProANP is found in atrial myocytes and regulates blood pressure by opposing aldosterone. Proteinuria is common in renal and cardiovascular disease. Elevated urine protein concentrations are linked to increased IL 6 production and heart failure. MPO is another inflammatory marker; it is used to predict the risk of AMI in the absence of cardiac necrosis. Hsp90α, a cytosolic chaperone molecule found in cardiac tissue, is linked to oxidative stress. It is inhibited by estradiol (E2). VEGF is inducible by ischemia and anoxia; it is often elevated along with microalbumin. Microalbuminuria is associated with cardiovascular disease. It is induced by vasodilation and hypoxia-inducible factors. E2 promotes vasodilation. Myoglobin is the first marker released following myocardial necrosis and cardiac dysfunction. IL1 α and IL1β are proinflammatory cytokines stimulated by hepatic acute phase cytokines such as CRP that induce IL6. Another example of gender influence can be seen in the prevalence and progression of many renal diseases [4, 13].

The majority of chronic renal diseases occur more commonly in men and progress far more quickly. There is no discernible gender disparity in acute kidney disease rates. Chronic Kidney Disease (CKD) is one of the few chronic renal diseases that occur more commonly in women. CKD can lead to End Stage Renal Disease (ESRD) if it is not treated. The physiology behind gender differences in renal disease is unclear, but differences in kidney size and physiology, coupled with lifestyle factors and the vasoprotective effects of estrogen, have been implicated [1, 14-19]. Estrogen is thought to suppress the growth of renal scar tissue by inhibiting the production of collagen.
Interestingly, this female advantage in chronic kidney disease disappears when the women suffer from diabetes mellitus (DM). CKD is uncommon in premenopausal women. The rate of CKD increases as estrogen levels fall; however, this may be the result of the relative ratio of androgen to estrogen rather than the absolute level of estrogen. It is thought that the androgen to estrogen ratio determines the effect on the diabetic kidney. Unlike their non-diabetic counterparts, women with DM are found to have similar rates of kidney disease as males. DM is an increasingly common cause of kidney failure in developed countries. Increased renin–angiotensin system (RAS) activity is thought to play an important role in both the hemodynamic and nonhemodynamic pathways involved in organ damage, particularly for diabetes. RAS blockade has demonstrated nephroprotective properties in diabetic individuals. Two mechanisms for promoting vasoconstriction are RAS activation and nitric oxide (NO) pathway disruption. Hyperglycemia disrupts NO-mediated relaxation; despite this interaction, baseline renal vasodilation is common in diabetes [20]. Hypertension is common in diabetics; increased aldosterone concentration in urine is a risk factor for renal disease. Creatinine can be used to assess glomerular filtration rate. Increased concentrations are seen in DM, hypertension and impaired renal function. Normal urine does not have substantial protein; proteinuria is considered suggestive of renal disease. Microalbuminuria is often seen in renal dysfunction, as is elevated VEGF. Myoglobinuria is common in rhabdomyolysis and renal failure. CRP and MPO, inflammatory markers, are often elevated in renal disease. NGAL is the earliest responding marker in acute kidney injury; it is also elevated in CKD, some forms of lupus and urinary tract infections. Hsp90α, induced by heat shock, inflammation and oxidative stress, is often upregulated by renal disease. IL1α and
IL1 β, endogenous pyrogens, may be elevated in cases of renal disease. IL 6 acts synergistically with both IL1 subunits. The interleukins may also be elevated in autoimmune disorders.

A common feature of autoimmune diseases in both humans and experimental animals is that females are far more susceptible to autoimmune conditions when compared to males. In several animal models, estrogens promote B cell mediated autoimmune diseases. Androgens exert an inhibitory effect in the same models. Although the reason is unclear, females tend to secrete higher levels of interleukins such as IL1 α and IL1 β. Females display heightened immune responses not only to foreign antigens but also to self-antigens. Estrogens induce T and B cells imbalances; they appear to induce hypoactivity in subsets of T cell and hyperactivity in B cells. This may be the underlying basis for estrogen induced autoimmunity. Of the 8.5 million Americans diagnosed with autoimmune disorders, approximately 80% are women [21]. Rheumatoid arthritis (RA) is 4 times more common in women, systemic lupus erythematosus (SLE) is nine times more common in women and some forms of thyroiditis are 10 times more common in women compared with men [22, 23]. Insulin dependent DM is also more common in women than it is in men. Studies have shown that a disparity exists between male and female diabetics, particularly in the control of modifiable risk factors such as blood pressure, serum glucose and cholesterol levels [16, 24]. Some researchers believe this is why death due to heart disease has decreased among diabetic men but not in women. Creatinine is often elevated in DM. CRP and MPO may be elevated in cases of inflammatory immune diseases such as RA and SLE. CRP may be a surrogate marker of SLE activity and may be useful to monitor the course of the disease. NGAL often
increases in tandem with MPO. Hsp90α, upregulated by oxidative stress and inflammation, may increase with autoimmune disease. VEGF is another cytokine inducible by inflammation. Myoglobinuria may be seen in DM and other autoimmune disease. Subclinical inflammatory reaction has been shown to precede DM; IL1 α, IL 1 β, and IL6 are inflammatory cytokines. Studies have shown that systemic injection of IL1α and IL1 β induces the symptoms of systemic inflammation (e.g. fever, joint pain, headaches, neutrophilia and increases in circulating cytokines). Systemic and chronic inflammation are thought to contribute to the development of atherosclerosis and CHD. The plurality of these conditions has been linked to inflammation and oxidative stress and may result in the release of biomarkers into blood and other body fluids [2-4, 13, 22, 23, 25-30].

Inflammation is one of the most prominent forms of oxidative damage. Reactive oxygen species (ROS) produced by endothelial cells, neutrophils and macrophages can stimulate the activation of proinflammatory transcription factors and induce the production of inflammatory cytokines such as IL1 and IL6. Inflammation begins with hyperemia, edema, and adherence of the circulating white blood cells to endothelial cells. Local inflammatory response is typically accompanied by systemic changes that include hyperemia, leukocytosis, and induction of acute-phase reactants like CRP. Chronic inflammation is not confined to a particular tissue; it involves the endothelium and multiple organ systems. High levels of oxidative stress and inflammation can increase the probability of early incidence of multiple disease states, including atherosclerosis, Parkinson's disease, heart failure, myocardial infarction, Alzheimer's disease, fragile X syndrome and chronic fatigue syndrome (Figure 1).
Oxidative stress (OS) describes the level of oxidative damage in a cell, tissue, or organ, caused by ROS. The damage can affect everything from specific molecules to entire organisms. ROS such as free radicals and peroxides come from the metabolism of oxygen and are endogenous in all aerobic organisms. The rate at which damage occurs is primarily determined by the clearance of generated ROS by antioxidants. The rate of damage depends on the level of repair enzyme. Most are by-products of essential metabolic reactions such as mitochondrial energy generation and hepatic cytochrome P-450 detoxification reactions. Exogenous sources of ROS include lifestyle choices.
(cigarette smoking, alcohol consumption, dietary, etc.), environmental exposures (automotive emissions, industrial pollutants, asbestos, etc.), bacterial, fungal or viral infections and radiation. Exercise has the potential to induce free radical formation leading to OS [31]. Much of the damage associated with cocaine toxicity stems from OS [32]. In addition to environmental and lifestyle exposures, the determinants of oxidative stress are regulated by hereditary factors. Oxidative stress is also implicated in the ischemic cascade in reperfusion injuries. Myoglobin from myolysis, common with syndromes like rhabdomyolysis, may cause OS [33]. ROS are not uniformly destructive; they are used by the immune system as a way to attack and kill pathogens and in redox signaling.

Cocaine (benzoylmethylecgonine) is an alkaloid derived from the leaves of *Erthroxylon coca*, a shrub indigenous to South America [34]. Despite restrictions on importation and distribution, cocaine has become one of the most commonly used illicit drugs [35]. According to the 2007 National Survey on Drug Use and Health, nearly 1.6 million Americans met Diagnostic and Statistical Manual of Mental Disorders criteria for dependence or abuse of cocaine (in any form) in the past 12 months. The 2005 Drug Abuse Warning Network (DAWN) report stated that cocaine was involved in 448,481 of the total 1,449,154 (31%) visits to emergency departments (ED) for drug misuse or abuse. Cocaine is the most frequently reported illicit drug associated with ED admissions [36]. Cocaine is a powerful sympathomimetic capable of vasoconstriction and increasing heart rate, blood pressure, contractility, respiration, myocardial oxygen demand and body temperature [37]. Hyperpyrexia can lead to rhabdomyolysis, myoglobinuria, renal failure, liver damage and disseminated intravascular coagulation [38]. Cocaine use is
linked to atherosclerosis, myocardial ischemia, myocardial contraction band formation and sudden death [36, 39]. Cocaine-induced chest pain is the most common symptom reported by patients; an estimated 64,000 patients were evaluated for myocardial ischemia in 1995 subsequent to cocaine-related chest pain [40, 41]. Some of these patients exhibited elevated CK-MB levels or electrocardiogram (ECG) changes consistent with acute myocardial infarction (AMI); however, the majority reported recurrent chest pain subsequent to cocaine use following discharge [41, 42].

The metabolism of cocaine is primarily hepatic, with only about 1% excreted unchanged in the urine [43]. Cocaine has two primary metabolic pathways in humans, deesterification and demethylation. Benzoylecgonine (BE) is the primary metabolite and is not considered an important source of ROS. Ecgonine methyl ester (EME) is the second most common metabolite. Neither BE nor EME are biologically active. Transmethylation of cocaine is a minor pathway; metabolites of this pathway include norcocaine, norcocaine nitroxide, n-hydroxynorcocaine and formaldehyde. Microsomes in the brain and liver oxidize norcocaine to nitroxide. Microsomal reduction of norcocaine nitroxide in the brain generates superoxide (SO) [32]. Hepatic microsomes incubated with nitroxide or the N-hydroxy derivative results in lipid peroxidation [27]. Transesterification of cocaine occurs in the presence of alcohol and produces the biologically active intermediate cocaethylene. Various cocaine related hepatotoxicity studies indicate the generation of ROS, including lipid peroxides [44, 45]. Cocaine has been shown to induce the release of SO which has been implicated in cardiac and cerebrovascular dysfunction [32].
The vasoconstriction associated with cocaine usage is thought to result from its blockade of norepinephrine and dopamine reuptake at preganglionic sympathetic nerve endings; the extended catecholamine presence increases heart rate and blood pressure [40, 46]. Cocaine also disrupts catecholamine metabolism by inhibiting monoamine oxidase (MAO). By blocking the neuronal plasma membrane transporter, cocaine increases extracellular levels of monoamines [47]. Hypertension is associated with disruptions in endothelial cell function and oxidative stress. MPO promotes endothelial dysfunction [48]. Microalbumin is inducible by endothelial dysfunction and oxidative stress; cocaine use may impact expression of this marker [49]. IL1α and IL1β are induced by increased body temperature [50]. Cocaine is a known hyperpyrexic so it is possible that usage could result in increases in the concentrations of these markers [51]. Oxidative stress has also been implicated as an early triggering event of cocaine-induced cardiomyopathy and atherosclerosis [52]. IL6, CRP and MPO are being considered for validation as cardiac biomarkers [48, 53]. MPO has also been linked to the pathogenesis of renal injury [54, 55]. Increased expression of these cytokines may result from cocaine-induced atherosclerosis and other cardiac disruptions. Fluctuations in blood pressure activate the renin-angiotensin system RAS which may increase proANP expression along with aldosterone production. RAS activation contributes to OS and vascular inflammation; it can also change the baseline filtering activity of the kidneys. Aldosterone stimulates inflammation through ROS generation. Cocaine use is linked to both renal dysfunction and rhabdomyolysis [34, 56]. NGAL is a validated marker for acute kidney damage and inflammatory processes [57]. Myoglobinuria, proteinuria and
increased expression of creatinine are associated with rhabdomyolysis and cardiac dysfunction; both conditions may result from cocaine use [53, 58].

The link between biomarker expression in urine and gender or cocaine-based inflammation, cardiac damage, or other systemic injury has not been elucidated. Urine samples were assayed for benzoylecgonine (BE), total protein, aldosterone, c-reactive protein (CRP), myeloperoxidase (MPO), microalbumin (MAB), neutrophil gelatinase-associated lipocalin (NGAL), heat shock protein 90α (hsp90α), vascular endothelial growth factor (VEGF), myoglobin, pro atrial natriuretic peptide (proANP) and interleukins 1α, 1β, and 6 using ELISA and colorimetric assays; these markers were selected for their biological plausibility and assay kit availability. Urine is a non invasive specimen typically provided in sufficient quantity to allow multiple tests. As most toxicological screens primarily utilize urine specimens in clinical settings, we examined the possibility of establishing relationships between the expression of several urinary biomarkers associated with oxidative stress and inflammation and gender and cocaine use.

The following hypotheses were tested in this study:

1. The expression of some urinary markers of inflammation and/or oxidative stress is influenced by gender. Creatinine and myoglobin are related to lean muscle mass; therefore, males should have higher urinary mean concentrations. Total protein, microalbumin and NGAL may be elevated in females as they suffer far more urinary tract infections than males. IL1α, IL1β, IL6, MPO, VEGF and CRP may be higher in females as they suffer disproportionately from autoimmune/inflammatory diseases.
2. The expression of these markers is influenced by the use of cocaine. Creatinine and myoglobin are increased by hyperthermia and rhabdomyolysis; therefore, cocaine positive specimens should have a higher mean values than control urine specimens. Cocaine induces oxidative stress and inflammation; aldosterone, myoglobin, VEGF, NGAL, IL1α, IL1β, IL6, MPO and CRP may be higher in cocaine positive specimens. Hsp90α and proANP are associated with cardiac dysfunction; they may be higher in cocaine positive specimens.
Chapter 2.0

Literature Review

2.1 Gender

There are significant gender based differences in the etiology of disease states including cardiac and vascular disease, metabolic disorders, autoimmune disorders and drug abuse toxicology. The basis of the gender dimorphism is unclear; however, cardiac muscle and vascular tissue are influenced by hormones such as estrogen and testosterone [1, 9, 10, 12, 59-61]. Premenopausal women appear to be partially protected to some extent from cardiovascular and kidney diseases; however, this protection weakens after menopause [62]. Gender differences may exist not only in atherogenesis but also in post ischemic/infarction cardiovascular repair. Angiogenesis is essential to cardiovascular repair and regeneration. The effect of estrogen on angiogenesis has been extensively studied, but the role of androgens remains unexplored [63]. Estrogens induce vasodilation by increasing the activity of endothelial nitric oxide synthase (eNOS).

Estrogens and androgens regulate the RAS which in turn regulates the cardiovascular system and the kidneys [64]. In general, estrogen decreases renin
production thereby decreasing aldosterone production. Progesterone competes with aldosterone for mineralocorticoid receptor. Aldosterone is linked to inflammation via ROS generated oxidative stress. Aldosterone is a renal hormone in the RAS secreted in response to hypotension. Aldosterone has been implicated in renal disease progression; the majority of renal diseases are far more common in men. Increased cardiac concentrations have been linked to heart failure. Creatinine is also a general marker of muscle and kidney disease [18, 19]. Because creatinine is related to lean muscle mass, men tend to have slightly higher serum creatinine levels than do women. Increased vascular oxidative stress leads to endothelial dysfunction and hypertension. Inhibition of the RAS results in a decline in ROS production [65, 66]. Estrogen also activates natriuretic peptides such as proANP; these peptides are a counterpart of the RAS. Testosterone seems to exert the opposite effect on renin levels. These effects of sex hormones on the RAS may explain some of the gender differences in cardiovascular and kidney diseases [67].

Exposure to sex hormones modulates many endocrine factors involved in atherosclerosis. Increased vascular oxidative stress leads to endothelial dysfunction and hypertension. It has been noted that inhibition of the RAS results in a decline in ROS production [65, 66]. Studies suggest females are at lower risk of developing cardiovascular disease (CVD) as compared to males [68]. Striking sex differences exist not only in the incidence of cardiovascular disease, but also in the clinical outcomes. Cardiovascular events occur earlier in men than in women; however, it appears women have poorer short-term and long-term outcomes following these events compared to men.
Autoimmune disorders may contribute to the gender-based differences in cardiac outcomes; for example, RA, far more common in women, is associated with a higher prevalence and higher severity of atherosclerosis [69].

Autoimmune diseases are the third most common category of disease in the United States after cancer and heart disease. Male and female immune response differs; women mount more vigorous immune responses with increased antibody production [70]. Women also have far higher autoimmune disease rates [71, 72]. When men develop autoimmune diseases, they are often more severe. Many animal models of autoimmune disease have shown a similar sex bias, with a higher incidence of disease in females [73-77]. Estrogen, testosterone, and progesterone are thought to mediate most of the sex-biased differences in the immune response [78, 79]. Cytokine receptors such as interleukin 1 receptor (IL-1R) have been discovered on hormone-producing tissues. Proinflammatory cytokines such as IL-1α and IL-1β stimulate the release of glucocorticoids which regulates the inflammatory process along with androgens and estrogens. Estrogen significantly increases proinflammatory cytokine production of IL-1α, IL-1β and IL-6. [6, 27, 29, 30, 80-82]. Interleukins act specifically as mediators between leukocytes. Activated leukocytes can generate ROS, which contributes to hypertension and atherosclerosis [83]. Many autoimmune disorders are strongly associated with cardiac and metabolic disorders.

Women tend to have slightly higher serum CRP than men with the same body mass index (BMI) and age; however, these differences are not pronounced enough to warrant gender-dependent reference ranges [84, 85]. The role of gender in predisposition
to oxidative stress has yet to be determined. CRP is an acute phase inflammatory marker considered to be a reliable prognostic indicator of atherosclerosis and acute myocardial infarction (AMI) [61, 82, 86]. Urinary levels peak approximately 24 hours following the inflammatory event and are undetectable within 13 to 16 hours. As a measure of leukocyte infiltration, MPO is an inflammatory marker used to predict the risk of AMI in the absence of cardiac necrosis [54, 87]. The predictive value of this marker has not been fully characterized; however, it is associated with long term adverse cardiac events. E2 has been suggested as a potential endogenous substrate for MPO in plasma [88]. Recognition and elaboration of the innate differences of gender physiology may result in better treatment adaptations for women and better outcomes [89].

2.2 Inflammation

Inflammation can be classified as either acute or chronic. Acute inflammation is the initial response to harmful stimuli and is achieved by mobilization of plasma and leukocytes, particularly granulocytes, from the blood into the injured tissues. It is characterized by vasodilation, increased permeability and reduced blood flow; these changes are induced by multiple inflammatory mediators [90]. Trauma, inflammation, or infection leads to the activation of the inflammatory cascade. A progression of biochemical events, including components of the local vasculature, the immune system, and various cells triggers the inflammatory response. Unlike acute inflammation, chronic
inflammation is mediated by cells such as monocytes and lymphocytes. Chronic inflammation leads to a progressive shift in the type of cells present at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process [91].

Tissue macrophages, monocytes, mast cells, platelets, and endothelial cells are able to produce a multitude of cytokines. The release of as IL-1α and IL-1β leads to cleavage of the nuclear factor-kB (NF-kB) inhibitor. Removal of the inhibitor allows NF-kB to initiate mRNA production, thereby inducing production of other proinflammatory cytokines like IL-6. IL-1 is responsible for fever and the release of stress hormones (norepinephrine, vasopressin, activation of the renin-angiotensin-aldosterone system). Cytokines like IL-6 stimulate the release of acute-phase reactants such as CRP. Infection has been shown to elicit a stronger response than trauma, which translates to a greater release of IL-6. Estrogen has been shown to directly inhibit IL-6 production [92].

Inflammation is a highly regulated process that recruits the immune system to sites of infection and injury and to facilitate tissue repair processes. Prolonged inflammation produces local and systemic damage associated with a loss of normal physiological functions. Activation of proinflammatory cytokines results in vasodilatation, release of cytotoxic compounds like MPO, generation of ROS and damage to the vasculature. Proinflammatory cytokines like IL-1α, IL-1β and IL-6 upregulate endothelial enzyme expression which plays an important role in atherogenesis. Some autoimmune diseases, such as RA, SLE and Sjogren's syndrome, are characterized by chronic inflammation.
Chronic inflammation is a crucial element in the development of multiple disease processes, including renal dysfunction, cardiovascular disease, autoimmune disorders, cancer and DM [53, 91]. Assessment of inflammatory markers like CRP, MPO and the interleukins can lead to improved risk stratification in disease. Estrogen and testosterone can affect the expression of proinflammatory markers in macrophages. Testosterone may reduce the expression and secretion of IL-1β, but it does not appear to affect the expression of IL-6 or CRP. Estrogen elicits a variable response in CRP expression [93].

Aldosterone causes tissue inflammation; this may result in fibrosis and remodeling in the heart, vasculature, and kidney [94]. Aldosterone triggers endothelial cell exocytosis, which is a crucial step in leukocyte mobilization [95]. During active inflammatory disease such as Crohn’s disease or RA, metabolic shifts may cause in hypoxia in affected membranes. Activation of hypoxia-inducible factor (HIF) subsequent to hypoxia has been shown to upregulate production of hsp90α and VEGF [96]. VEGF increases endothelial permeability and is associated with microalbuminuria and myoglobinuria [97].

2.3 Oxidative stress

Oxidative stress is determined by the balance between the rate at which oxidative damage in a cell, tissue, or organ is induced and the rate at which it is repaired or removed. Inactivation of ROS by endogenous antioxidants like superoxide dismutase and
glutathione peroxidase determines the rate at which damage is induced [67, 98]. Repair enzymes determine the rate at which damage is repaired. Most ROS come from the endogenous sources as by-products of normal and essential metabolic reactions. Lifestyle and environmental exposures also contribute to ROS. While there are many biomarkers of inflammation, oxidative stress is difficult to measure in vivo. Traditional indices of oxidative stress include markers of oxidative damage to lipids, proteins and DNA. Oxidative stress is also indirectly assessed by measuring markers of inflammation such as MPO[99].

ROS include free radicals and peroxides such as hydrogen peroxide, hypochlorous acid and peroxynitrite. When converted by oxidoreduction reactions, superoxide can be transformed into more aggressive radical species capable of causing extensive cellular damage. Long term effects of ROS exposure can include damage to DNA. Oxidative stress is implicated in diseases such as atherosclerosis, Parkinson's disease, heart failure, myocardial infarction, Alzheimer's disease, fragile X syndrome and chronic fatigue syndrome. Oxidative stress is linked to certain cardiovascular diseases; oxidation of low density lipoprotein in the vascular endothelium is a precursor of atherosclerotic plaque formation. Oxidative stress plays a role in the ischemic cascade due to oxygen reperfusion injury following hypoxia. It is also associated with the detrimental effects of aging.

Aging induces a pro-inflammatory state characterized by increasing levels of inflammatory cytokines such as IL-1α, IL-1β and IL-6 [100, 101]. Aging is associated with a declining serum testosterone levels. Studies suggest a close relationship exists
between the development of a pro-inflammatory state and the decline in testosterone levels, two trends that are often observed in aging men. Additionally, E2 is weakly associated with IL-6 in older men, independent of testosterone.

Myoglobin is a molecular radical scavenger activated by ischemia and myolysis; serum and urine myoglobin concentrations increase with OS [33, 102]. Creatinine concentrations are associated with muscle mass. Activity of creatinine and myoglobin correlate to stress induced neutrophil response common in exertional and injury induced rhabdomyolysis [103]. Aldosterone is linked to generation of ROS and the induction of oxidative stress [104, 105]. Atrial natriuretic peptide (ANP) is a hormone, primarily produced by cardiomyocytes, which regulates blood pressure. Studies have linked ANP with the generation of ROS. ANP may produce either antioxidant or prooxidant effects, depending on experimental conditions and cell context [106].

ROS generation occurs in hepatocytes and in the respiratory burst of Kupffer cells, triggering a proinflammatory cascade upregulating multiple interleukins. Specific receptors in hepatocytes stimulate the expression of antioxidant enzymes such as manganese superoxide dismutase and acute-phase proteins. These responses help protect the liver against ischemia-reperfusion injury[107]. OS plays an important role in pathogenesis of hepatic diseases like alcoholic liver injury. Cardiac surgery-associated acute kidney injury (AKI) is common and is associated with increased expression of NGAL [105]. Increased OS is considered a causative factor of DM, particularly diabetic cardiovascular and renal sequelae. Hyperglycemia is thought to induce the overproduction of ROS. Oxidative stress is also a factor in the pathogenesis of
hypertension and target organ damage, largely due to its effects on the vasculature. Oxidative metabolites of cocaine generate significant ROS; much of the pathophysiological damage associated with cocaine use stems from oxidative stress and electron transfer[32].

2.4 Cocaine

Cocaine, C_{17}H_{21}NO_4, is a potent cardiac and central nervous system stimulant. It is fat soluble and freely crosses the blood-brain barrier. Cocaine is rapidly hydrolyzed by serum cholinesterase in plasma and carboxylesterases in the liver following use; the primary metabolites are BE and EME (Figure 2). Lesser metabolites include cocaethylene (CE), norcocaine and anhydrous ecgonine methyl ester (only seen in crack cocaine use). Cocaine and ethanol are independently cardiotoxic; together they exhibit synergistic cardiotoxicity. In the presence of alcohol, a nonspecific carboxylesterase catalyzes ethyl transesterification of cocaine to CE. Animal studies have demonstrated that co-administration results in prolonged cardiac toxicity and dysrhythmias [108]. It has also been demonstrated that ED patients with detectable CE concentrations are more likely to be admitted to intensive care units than those patients testing negative for CE [109]. The half-lives of cocaine, CE and BE are 40 minutes, 2.5 hours and 5 to 8 hours respectively. This may explain why cocaine-related symptoms can continue for some time after cocaine is last used. Cocaine has only one biologically active metabolite,
norcocaine. The oxidative metabolism of cocaine to norcocaine nitrooxide is postulated to be essential for cocaine hepatotoxicity [110].

Cocaine induces local vasoconstriction and an anesthetic effect by inhibiting fast sodium channels. Cocaine is a strong sympathomimetic that blocks catecholamine reuptake, effectively flooding the synaptic space with norepinephrine thereby stimulating the central and peripheral nervous systems [56]. The effect of cocaine is especially pronounced in the limbic system where it potentiates dopaminergic transmission in the

![Metabolites of Cocaine]

Figure 2. Metabolites of Cocaine
ventral basal nuclei. The potentiation of dopamine is responsible for the pleasurable behavioral effects that make cocaine a popular drug of abuse. Cocaine causes moderate release and reuptake-blockade of serotonin and dopamine [111-113]. Cocaine-induced hyperpyrexia, a potentially fatal elevation in body temperature exceeding 41° C, is thought to be associated with dopamine receptor blockade in the temperature regulation region of the hypothalamus. The sodium channel blockade decreases the resting membrane potential and action potential amplitude while simultaneously prolonging the duration of the action potential. Cocaine also blocks potassium channels. In some cellular membranes, it may block sodium-calcium exchange.

Almost every organ system is affected by cocaine use. Myocardial infarction, arrhythmias, renal failure, hypertension, atherosclerosis, and rhabdomyolysis are all in the spectrum of acute and chronic cocaine toxicity [114, 115]. Cocaine is associated with an increased vascular risk; the brain, heart, kidney, liver and lungs are all susceptible to its vasculotoxicity. It can cause abrupt changes in blood pressure, embolism via infective endocarditis, and hemostatic and hematologic abnormalities that can result in increased blood viscosity and platelet aggregation. Most ED patients with cocaine-associated chest pain have normal cardiac profiles at the time of presentation. The negative inotropic effects of cocaine observed in animal models do not appear to be present in patients who develop chest pain after using recreational doses of cocaine [116].

Cocaine is known to have specific, dose-dependent effects on brain and body temperatures, these effects are strongly modulated by an individual's activity state and environmental conditions, and change dramatically during the development of drug self-
administration. Environmental conditions potentiate the thermal effects of cocaine which may result in pathological brain overheating. Hyperthermia can exaggerate the toxicity of cocaine; environmental conditions that impair heat loss can result in acute life-threatening complications and chronic destructive CNS changes [117]. Deleterious effects of hyperthermia include activation stress response systems, decreased immune efficiency, increased blood viscosity, vasoconstriction, renal and hepatic insufficiency and rhabdomyolysis.

Rhabdomyolysis is a disorder in which injury to muscle results in leakage of myocyte intracellular contents into the plasma. Dissolution of muscle fibers and leakage of muscle enzymes, myoglobin, potassium, calcium, and other intracellular constituents, can occur. For this reason, myoglobinuria and albuminuria are common clinical markers of rhabdomyolysis. Serum proinflammatory and inflammatory cytokines like IL-1α, IL-1β, MPO and CRP are similarly elevated. Cocaine has been shown to diminish IL-6 response to proinflammatory challenges [118]. IL-6 is a multifunctional cytokine implicated in many age-related diseases, including postmenopausal osteoporosis and Alzheimer’s disease [119]. The most likely etiology of rhabdomyolysis in patients presenting to the emergency department is ingestion of drugs of abuse such as cocaine [58]. The hemodynamic actions of cocaine contribute to renal injury. Cocaine abuse has been linked with acute renal failure and acid-base and/or electrolyte disorders and may trigger the progression of chronic renal failure to end-stage renal disease.

Cocaine use is associated with frontal cortex and glial injury in both frontal gray and white matter. Women showed equivalent responses to drug administration with the
exception of the limbic system; their perception of well-being was significantly increased [120]. These findings may have implications for differential risk for acute and chronic toxicity in women. Some animal studies suggest estradiol increases sensitivity of the brain reward system [121]. Progesterone may enhance cocaine craving and relapse susceptibility in women [122]. Research also suggests adolescents are more sensitive than adults to interactions between testosterone and cocaine [123].

There are significant gaps in the literature that make it difficult to define the relationship between gender and cocaine. Social parameters have been described; males are more likely than females to use cocaine and males tend to use cocaine at an earlier age than do females. The pathophysiological gender based differences cannot be elaborated until study populations begin to include females. The bulk of research performed in recent years has focused on psychosocial and neurobiological differences between cocaine using females and males [124-126]. Given the stark physical contrasts between the genders, particularly in immune response and autoimmunity, the focus may need to be widened to include gender based differences in the inflammatory and oxidative response to cocaine use.
Chapter 3.0

Materials and Methods

The Agency for Community Testing Services (ACTS) Laboratories provided urine specimens with all information blinded apart from gender, creatinine and the results of the NIDA 10 drug screen. 41 specimens were donated by males, 39 specimens by females. 40 specimens tested negative to all substances on the NIDA 10 panel (THC, opiates, amphetamines, barbiturates, cocaine, ethanol, benzodiazepines, propoxyphene, methadone and oxycodone) and 40 specimens tested positive for cocaine metabolites alone. Limited demographic information was available for some of the donors; for example, age was available for 66 of the donors. The donors’ ages ranged from 16 to 62; the mean donor age was 34 years old. Donor weight was available for 46 of the 80 donors. The female weights ranged from 113 to 327 lbs and the mean female donor weight was 162.5 lbs. The male weights ranged from 120 to 240 lbs and the mean male donor weight was 166.6 lbs.
Table 1. Summary of Assays Performed

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Method</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Protein</td>
<td>Colorimetric</td>
<td>Pierce</td>
</tr>
<tr>
<td>Creatinine</td>
<td>Colorimetric</td>
<td>Cayman</td>
</tr>
<tr>
<td>Cocaine/Benzoylecgonine (BE)</td>
<td>ELISA</td>
<td>Immunalysis</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>ELISA</td>
<td>ALPCO</td>
</tr>
<tr>
<td>C Reactive Protein (CRP)</td>
<td>ELISA</td>
<td>ALPCO</td>
</tr>
<tr>
<td>Myeloperoxidase (MPO)</td>
<td>ELISA</td>
<td>ALPCO</td>
</tr>
<tr>
<td>Microalbumin (MAB)</td>
<td>ELISA</td>
<td>ALPCO</td>
</tr>
<tr>
<td>Myoglobin (MGB)</td>
<td>ELISA</td>
<td>Life Diagnostics</td>
</tr>
<tr>
<td>Heat Shock Protein 90α (hsp90α)</td>
<td>ELISA</td>
<td>Assay Designs</td>
</tr>
<tr>
<td>Vascular Endothelial Growth Factor (VEGF)</td>
<td>ELISA</td>
<td>Raybiotech</td>
</tr>
<tr>
<td>Neutrophil Gelatinase Associated Lipocalin (NGAL)</td>
<td>ELISA</td>
<td>Quantikine</td>
</tr>
<tr>
<td>Pro Atrial Natriuretic Peptide (proANP)</td>
<td>ELISA</td>
<td>ALPCO</td>
</tr>
<tr>
<td>Interleukin 1α (IL 1α)</td>
<td>ELISA</td>
<td>Cayman</td>
</tr>
<tr>
<td>Interleukin 1β (IL1β)</td>
<td>ELISA</td>
<td>Cayman</td>
</tr>
<tr>
<td>Interleukin 6</td>
<td>ELISA</td>
<td>Cayman</td>
</tr>
</tbody>
</table>
3.1 EMIT

Enzyme Multiplied Immunoassay Technique (EMIT) is a common method for screening urine and blood for drugs, legal and illicit. This inexpensive technique is fast, relatively sensitive and specific, and has been adapted to various automated analyzers. The non-linear relationship between the change in absorbance and analyte/drug concentration in the specimen is the reason the assay is only useful for qualitative determinations. Results are expressed as Δ absorbance (M) units and compared with results for cutoff-value calibrators to indicate a positive or negative result. This qualitative procedure may be followed with confirmatory (quantitative) GC/MS testing as necessary.

An EMIT drug test contains antibodies that target specific drugs in the urine specimen. If an antibody does not become attached to the drug in the urine specimens, it attaches itself to the chemically tagged drug in the EMIT reagent. ACTS uses DRI/Microgenics (Thermo Fisher Scientific Corporation, Waltham, MA 02454) reagents and controls for the NIDA 10 EMIT panel. The NIDA 10 panel consists of Cocaine (COC), Amphetamine (AMP), Methamphetamine (M-AMP), Tetrahydrocannabinol (THC), Methadone (MTD), Opiates (OPI), Phencyclidine (PCP), Barbiturates (BAR), Benzodiazepines (BZD) and Oxycodone (OXY) in human urine. Chemically tagged drugs without an attached antibody (i.e. the reagent) are transformed by a reaction that changes the absorbance of the test sample. Attachment of an antibody inhibits this reaction. The Olympus AU640e Chemistry ImmunoAnalyzer (OLYMPUS America Inc.,
Center Valley, PA 18034-0610) used by ACTS Laboratory measures the changes in absorbance in the test sample; the change in absorbance is then compared to the absorbance of the calibrators. If the absorbance of the test sample is equal to or exceeds the calibrator’s, the specimen is considered positive for the drug in question. Conversely, if the absorbance of the test sample is less than that of the calibrator, the specimen is considered negative for the drug.

In 1988, the American Association for Clinical Chemistry (AACC) conducted a study of the testing accuracy of laboratories with regard to drugs of abuse. This study was blinded and used cutoff concentrations very close to the guidelines of the Substance Abuse and Mental Health Services Administration (SAMHSA). Their overall accuracy rate was 97%. There were a small number of false negative results (2–4%) on samples with drug concentrations close to the cutoff. There were no false positive results.

Components of the EMIT Assay Method (Figure 3):

1. Drug
2. Antibody
3. Substrate
4. Drug enzyme complex
3.1.1 General Procedure for EMIT Assay

1. Mix sample containing drug with fixed quantity of enzyme bound drug, and antibody
2. Add substrate
3. Measure absorbance at 15 and 45 seconds after substrate addition
4. Quantitate by measuring enzyme-substrate reaction (by UV - visible spectroscopy)
5. \( \Delta \) Absorbance from Reaction rate from Drug concentration
6. Non linear relationship between \( \Delta \) Absorbance and Concentration
7. Determine standard curve

ACTS laboratory uses the Olympus AU640e for drugs of abuse analysis (Figure 4). The analyzer screens urine specimens for the NIDA 10 panel of drugs. The cocaine test analyzes urine specimens for the presence of the primary cocaine metabolite, benzoylcgonine, rather than the parent compound (cocaine). As previously stated, EMIT does not measure the amount of the drug present; it simply detects its presence or absence as determined by an established cut-off level. The drug in the manufacturer’s reagent is labeled with an enzyme such as glucose-6-phosphate dehydrogenase (G6PDH). The enzyme-ligand-antibody complex is inactive thereby allowing determination of unlabeled ligand.
3.1.2 Cocaine/Benzoylecgonine

3.1.2.1 General Principle of the Assay

The DRI Cocaine Metabolite Assay used (DRI Cocaine Metabolite Assay #0056, Microgenics Corporation Fremont, CA 94538) by ACTS Laboratory is intended for the qualitative and semiquantitative determination of benzoylecgonine in human urine. This assay provides only a preliminary analytical test result. Quantitative determination
requires alternative methods; gas chromatography/mass spectrometry (GC/MS) is the preferred confirmatory method.

The DRI Cocaine Metabolite Assay is a homogeneous enzyme immunoassay using ready-to-use liquid reagents. The assay uses a specific antibody to detect benzoyllecgonine in urine specimens. The assay is based on the competition of an enzyme glucose-6-phosphate dehydrogenase (G6PDH) labeled BE and any BE from the urine sample for a finite number of specific antibody binding sites. In the absence of BE in the sample, the specific antibody binds to the G6PDH labeled BE and the enzyme activity is inhibited. This means there is a direct relationship between the BE concentration in the urine and the enzyme activity. The enzyme G6PDH activity is determined by measuring its ability to convert nicotinamide adenine dinucleotide (NAD) to NADH spectrophotometrically at 340nm using the lab’s Olympus AU640e Chemistry-Immuno Analyzer.

3.1.2.2 Specificity

Benzoyllecgonine, cocaine and other compounds that are concurrently present in the urine were tested for cross-reactivity in the assay. Cocaine and EME are the only compounds that demonstrated crossreactivity. Urine specimens arrive in sterile containers from a number of remote collection sites, including jails, hospitals and medical offices. Possible adulteration of urine specimens is an important consideration for drug testing facilities. Accordingly, samples undergo several tests to determine the likelihood of adulteration. Color and odor are assessed for evidence of adulteration, either by dilution
or the addition of an oxidant such as bleach. The tests used to check for adulteration include creatinine concentration, pH and specific gravity. Urine specimens with values outside normal reference ranges are discarded.

The reference ranges for:

- Urine creatinine is 25-350 mg/dL
- Urine pH is 5.0 to 6.5
- Urine specific gravity is 1.003 to 1.030

Dilution of the specimen may result in abnormal lows for one or more of the markers. In some instances the specimens are highly concentrated and dilution may make creatinine levels normal. Substitution with a liquid other than urine will be revealed by the absence of creatinine. Commercial masking agents may include components such as glutaraldehyde to interfere with the drug screen; for this reason, nitrites levels are occasionally used to establish the legitimacy of a specimen. This is not foolproof as bacterial infection and contamination may increase urine nitrite levels.

3.1.3 Creatinine

Creatinine is routinely included in urinary analysis as a control for dilute or adulterated specimens. It is a metabolic by-product of creatine degradation in muscle tissue. Most methods for determining the creatinine concentration of a specimen, in urine
or serum, rely on the Jaffe reaction in which creatinine reacts with alkaline picrate to form a red-yellow solution that is then measured photometrically (Figure 5).

NaOH

Creatinine + Picric Acid $\rightarrow$ Janovski Complex

Figure 5. Modified Jaffe Reaction

Since urine contains other substances which can react with picrate, the Olympus uses reagents from Microgenics for a modified version of the Jaffe reaction with improved specificity. The color intensity of the creatinine-picric acid complex is directly proportional to the concentration of creatinine in the urine specimen and is measured spectrophotometrically at 505 nm. Each laboratory establishes its own reference range for creatinine levels but they average from 25 to 300 mg/dL. When the value obtained exceeds 300 mg/dL, the specimen should be diluted with physiological saline and re-assayed. The established cutoff value for creatinine is 20 mg/dL and the assay is said to be linear to 400 mg/dL.

3.1.4 Specific Gravity

Specific gravity simply refers to the dissolved solutes present in urine as compared to the value set by convention, for pure water (1.000). Values obtained for
specific gravity are affected by the number, size and weight of molecules in urine. For this reason, it is considered an approximation of solute concentration. The urine specific gravity assay relies upon the use of a linear relationship between urinary chloride ion concentration and measured specific gravity. Determination of the chloride ion is colorimetric; in an aqueous medium, ferric perchlorate and chloride ion form a ferrous chloride complex that has an absorbance maximum of 340 nm (Figure 6). Absorbance at 340 nm is directly proportional to concentration of chloride ion in the specimen. Urine specific gravity is extrapolated from this value.

\[ \text{Cl}^- + \text{Fe}^{3+} \leftrightarrow \text{FeCl}^{2+} \]

Figure 6. Urine Specific Gravity Reaction Equation

3.1.5 Ethanol

The ethanol assay is based on the high specificity of alcohol dehydrogenase (ADH) for ethyl alcohol. In the presence of ADH and nicotinamide adenine dinucleotide (NAD), ethanol is readily oxidized to acetaldehyde and NADH (Figure 7). The enzymatic reaction is monitored spectrophotometrically at 340nm. The ethanol in the sample is in direct proportion to the rate of change of absorbance at 340 nm.
Figure 7. Oxidation of Ethanol to Acetaldehyde

3.2 Colorimetric Analysis

Colorimetry relies on colored solutions absorbing light of a particular wavelength; however the technique can also be used to analyze colorless substances if they react with a dye. The TECAN M200 Infinite Microplate Reader (TECAN US Inc., Durham NC 27703) can be used for simple colorimetric analyses such as the creatinine assay and total protein assessment. The absorbance of the urine specimens is determined by comparing them to a calibration curve generated from known standards.
3.2.1 Total Protein

3.2.1.1 General Principle of Assay

The Coomassie Protein Assay kit used (Coomassie Protein Assay Kit #23200, Pierce Biotechnology, Rockford, IL 61105) is a typical colorimetric assay. The Pierce Coomassie Protein reagent is a modification of the Bradford Coomassie Dye-protein binding colorimetric method of protein quantitation. When Coomassie dye binds to protein in an acidic medium, the color in the wells changes from brown to blue as the absorbance maximum shifts.

A 5 μl aliquot of standard, control urine or cocaine positive (as previously determined by EMIT) urine specimen is incubated with a 250 μl of Coomassie Reagent in micro-plate wells for 10 minutes. The plate is read at 595 nm to obtain the absorbance. 5 μl of acid is then added to the wells. The absorbance is directly proportional to the concentration of protein in the sample. A set of standards is used to plot a standard curve from which the amount of total protein in samples and controls can be directly read.

3.2.1.2 Assay Procedural Details

Kit Contents:

1. Coomassie Protein Assay Reagent ready to use

2. Albumin Standard Ampules 2mg/ml requires serial dilution
Phosphate Buffered Saline (PBS) is the diluent chosen for the assay. PBS is a commonly used isotonic buffer solution in biological research. The osmolarity and ion concentrations of the solution usually match those of the human body fluids. The 1x PBS stock solution used for the Total Protein Assay was prepared by dissolving 8.00 g of NaCl, 0.20 g of KCl, 1.44 g of Na2HPO4 and 0.24 g of KH2PO4 in 800 ml of Barnstead Nanopure Water. If necessary, the pH can be calibrated to 7.4 with the addition of HCl or NaOH. The final volume is adjusted to 1 liter with additional Barnstead Nanopure Water.

The albumin standards were prepared as described in the table below (Table 2):

Table 2. Concentration of Albumin Standards

<table>
<thead>
<tr>
<th>Tube</th>
<th>Volume of Diluent (µl)</th>
<th>Volume and Source of BSA (µl)</th>
<th>Final Concentration (µl/ml protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>300 of stock</td>
<td>2000</td>
</tr>
<tr>
<td>B</td>
<td>125</td>
<td>375 of stock</td>
<td>1500</td>
</tr>
<tr>
<td>C</td>
<td>325</td>
<td>325 of stock</td>
<td>1000</td>
</tr>
<tr>
<td>D</td>
<td>175</td>
<td>175 of tube B</td>
<td>750</td>
</tr>
<tr>
<td>E</td>
<td>325</td>
<td>325 of tube C</td>
<td>500</td>
</tr>
<tr>
<td>F</td>
<td>325</td>
<td>325 of tube E</td>
<td>250</td>
</tr>
<tr>
<td>G</td>
<td>325</td>
<td>325 of tube F</td>
<td>125</td>
</tr>
<tr>
<td>H</td>
<td>400</td>
<td>100 of tube G</td>
<td>25</td>
</tr>
<tr>
<td>I</td>
<td>400</td>
<td>0</td>
<td>0 (blank)</td>
</tr>
</tbody>
</table>
All reagents must reach room temperature before use. Mix the Coomassie Reagent immediately prior to use by gently inverting the bottle several times. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

5 μl of each standard, control urine and cocaine positive urine were pipetted into correspondingly labeled wells in duplicate. Next, 250 μl of the Coomassie Reagent was pipetted into each well. The plate was incubated on a plate shaker (approximately 200 rpm) for 10 minutes at room temperature. The plate was read at 595 nm to calculate the absorbance. A standard curve was produced and the concentration of protein in each of the specimens was determined from the blank-adjusted absorbance.

3.2.1.3 Interfering Substances

Most ionic and nonionic detergents

3.2.2 Creatinine

3.2.2.1 General Principle of Assay

The Creatinine kit used (Creatinine Assay Kit #500701, Cayman Chemical Company, Ann Arbor, MI 48108) is a typical colorimetric assay. It is not appropriate for diagnostic use. It relies on the Jaffé’ reaction; in Jaffé’, a yellow/orange color is formed when the metabolite is exposed to alkaline picrate. The color derived from creatinine is
destroyed at acidic pH. The difference in the color before and after acidification is measured at 500 nm and is proportional to the creatinine concentration in the urine specimen.

A 15 μl aliquot of a diluted standard, control urine or cocaine positive (as previously determined by EMIT) urine specimen is incubated with a 150 μl of Alkaline Picrate Solution in micro-plate wells for 10 minutes. The plate is read at 500 nm; the value obtained is the Initial Absorbance. 5 μl of acid is then added to the wells. After incubating for 20 minutes, the Final Absorbance is obtained by reading the plate at 500 nm. The difference between the initial and final absorbance is directly proportional to the concentration of creatinine in the sample. A set of standards is used to plot a standard curve from which the amount of creatinine in samples and controls can be directly read.

The dynamic range of the Cayman Creatinine assay is 0 – 15 mg/dl.

3.2.2.2 Assay Procedural Details

Kit Contents:

1. Creatinine Standard requires dilution

2. Creatinine Color Reagent (1.2% picric acid) ready to use

3. Creatinine Sodium Hydroxide (0.1M NaOH) ready to use

4. Creatinine Acid Solution ready to use
5. Creatinine Sodium Borate ready to use

6. Creatinine Surfactant ready to use

To prepare Alkaline Picrate Solution sufficient for a 96 well plate, mix together 2 ml sodium borate, 6 ml surfactant, 10 ml color reagent and 3.6 ml NaOH. This resultant solution is stable for one week stored in the dark at room temperature.

Urine specimens were diluted 1:20 with Barnstead Nanopure water prior to use.

The creatinine standards were prepared as described in Table 3:

Table 3. Concentration of Creatinine Standards

<table>
<thead>
<tr>
<th>Tube</th>
<th>Creatinine Standard (µl)</th>
<th>Nanopure Water (µl)</th>
<th>Final Concentration (mg/dl creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>500</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>50</td>
<td>450</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>100</td>
<td>400</td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>150</td>
<td>350</td>
<td>6</td>
</tr>
<tr>
<td>E</td>
<td>200</td>
<td>300</td>
<td>8</td>
</tr>
<tr>
<td>F</td>
<td>250</td>
<td>250</td>
<td>10</td>
</tr>
<tr>
<td>G</td>
<td>300</td>
<td>200</td>
<td>12</td>
</tr>
<tr>
<td>H</td>
<td>375</td>
<td>125</td>
<td>15</td>
</tr>
</tbody>
</table>
All reagents must reach room temperature before use. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption.

15 μl of each standard, diluted control urine and diluted cocaine positive urine were pipetted into correspondingly labeled wells in duplicate. Next, 150 μl of the alkaline picrate solution was pipetted into each well to initiate the reaction. The plate was incubated on a plate shaker (approximately 200 rpm) for 10 minutes at room temperature. The plate was read at 500 nm to calculate the initial absorbance. 5 μl of acid solution was added to all wells and the plate was incubated for 20 minutes on a plate shaker at room temperature. The final absorbance was calculated by reading the plate at 500 nm. A standard curve was produced and the concentration of creatinine in each of the specimens was determined from the adjusted absorbance.

3.3 ELISA

Imunoassays are biochemical tests that measure the level of a substance in a biological liquid, in our case urine. The assay utilizes the specific binding relationship of antibody and antigen. Monoclonal antibodies are often used because they typically bind a single site of a particular molecule; this property enhances specificity and accuracy. Polyclonal antibodies have also been successfully applied for various immunoassays.
Assay success depends on antibody affinity for the antigen. It is possible to measure both the presence of antigen and antibodies.

Enzyme-linked Immunosorbent Assay (ELISA) uses antibodies (or antigens) coupled with an enzyme to produce an antibody specific assay with the sensitivity of simple enzyme assays. This allows an ELISA to provide a useful measurement of antigen or antibody concentration. ELISA plates consist of 96 or 480 polyvinyl chloride microwells containing enzymes, antigens or antibodies immobilized on a solid phase and the substance. Depending on the type of ELISA, an antibody capable of detecting an antigen or an antigen that will elicit the response of a particular antibody is allowed to bind to the immobilized protein.

There are 5 types of ELISA: direct, indirect, sandwich, competitive and multiplex:

1. Direct ELISA: This method directly labels the antibody. The target antigen is applied to the microwells of an ELISA plate; bound antigen may be quantitated using colorimetric, chemiluminescent or fluorescent measurement. This method provides relatively quick results with limited cross reactivity. There is little opportunity for signal amplification with this method.

2. Indirect ELISA: This two step method uses a second antibody for detection. The primary antibody is incubated with the antigen; the specimens in the wells then incubate with a second labeled antibody capable of recognizing the primary antibody. This method allows for considerable signal amplification.
3. Sandwich ELISA: This method measures the amount of antigen ‘sandwiched’ between two layers of antibodies. The sandwich ELISA requires the antigens to be measured contain two or more antigenic sites capable of binding the capture and detection antibodies; therefore, these assays are restricted to the quantitation of multivalent antigens such as proteins or polysaccharides. Sandwich ELISAs are excellent for quantitating antigens when the concentration of antigens is low or the concentration of contaminating protein is high.

Briefly, the capture antibody is purified and bound to a solid phase (usually attached to the bottom of a plate well). Specimens thought to contain the antigen of interest are added to the microwells and allowed to complex with the capture antibody. The plates are washed to remove unbound products and the labeled detection antibody is allowed to bind to the antigen. This completes the “sandwich”. The assay is quantitated using a colorimetric substrate to measure the amount of detection antibody bound to the matrix.

4. Competitive ELISA: This method requires one reagent be conjugated to a detection enzyme (horseradish peroxidase is one of the most commonly used). This detection enzyme may be linked to the antigen or the primary antibody. The microwells of the assay plate are coated with a purified unlabeled primary antibody. Following this, the plate is incubated with unlabeled standards and unknowns. The reaction is allowed to proceed to equilibrium; labeled conjugate antigen is then added to the microwells. The conjugate will bind to the available binding sites on the primary antibody. The plate is then developed with substrate and color change is measured. In a competitive ELISA, there is an inverse
relationship between the signal obtained and the concentration of the analyte in the sample i.e. the more unlabeled antigens in the sample or standard, the lower the amount of conjugated antigen bound.

5. Multiplex ELISA: This assay allows simultaneous detection of multiple analytes arrayed in a single well. Detection method can be direct or indirect, sandwich or competitive, labeling or non-labeling, depending upon antibody array technologies.

There are further assay variations depending upon the labeling and signal detection methodology. The basic approach stays the same: fixation of either antigen or antibody and detection of the antibody-antigen complex. The general steps in direct/competitive ELISAs can be seen in Figure 8.

Step 1: The antibody is attached to the walls of the microtiter plate.
Step 2: Urine is added to the well to test for the presence of the antigen

Step 3: If the antigen is present in the urine, it will bind to the antibodies attached to the microtiter walls.

Step 4: The plates are rinsed to remove test fluid and unbound antigen.

Step 5: A solution of modified antibodies is added. These antibodies carry a reporter enzyme designed to elicit a color change when the antigen-antibody complex forms.
Step 6: The sample is rinsed again to remove unbound antibodies. If the antigen is present, a complex consisting of antigen, plate-bound antibody and enzyme-conjugated antibody forms.

![Diagram of ELISA assay](image)

Step 7: The substrate of the enzyme is added. A color change indicates the presence of the enzyme-labeled antibody and the antigen.

Figure 8. Common Steps in Direct/Competitive ELISA Assay

ELISAs were performed using a TECAN M200 Infinite Microplate Reader (TECAN US Inc., Durham NC 27703). The TECAN M200 (Figure 9) is an automated microplate reader is capable of UV & VIS Absorbance (ABS), Fluorescence (FL) Intensity Top with Time-Resolved Fluorescence, Fluorescence Resonance Energy Transfer, Fluorescence Intensity Bottom, Spectrally-Enhanced PMT, Photon-counting Luminescence, Temperature Incubation and Cuvette Port Module, Single and Dual Injectors. The M200 can measure wavelength range of in tunable 1nm increments, Injector Module for fast kinetics in FL, Abs or Luminescent modes with one or two injector(s). Available features include heated incubation to 420C, linear and orbital shaking. The M200 accommodates 6, 12, 24, 48, 96 and 384-well microplate formats. It is packaged with i-Control and Magellan software for instrument measurement control,
spectral scanning, plate definition creation and raw data acquisition saved in a MS-Excel format.

A monochromator is an optical instrument that enables any wavelength to be selected from a defined optical spectrum. It operates like a tunable optical filter, allowing both the wavelength and bandwidth to be adjusted. A monochromator consists of an entrance slit, a dispersive element and an exit slit. The dispersive element diffracts the
light into the optical spectrum and projects it onto the exit slit. The dispersive element may be a glass prism or an optical grating. The infinite® M200 is designed with optical gratings. Rotation of the optical grating moves the spectrum across the exit slit so that only a small part of the spectrum (band pass) passes through the exit slit. This means that when the monochromator entrance slit is illuminated with white light, only light with a specific wavelength (monochromatic light) passes through the exit slit. The wavelength of this light is set by the rotation angle of the optical grating and the bandwidth is set by the width of the exit slit. The bandwidth can be defined as full width at half maximum (FWHM).

Monochromators block undesired wavelengths. This means when the monochromator is set for light with a wavelength of 500 nm and the detector detects a signal of 10,000 counts, light with different wavelengths is effectively dampened. Double monochromators setups permit a higher level of blocking; two monochromators are connected in series so that the exit slit of the first monochromator acts as the entrance slit of the second monochromator. This arrangement boosts the blocking count by a factor of $10^3$. In the infinite M200, a double monochromator is installed on both the excitation and detection side. This opens the opportunity for easy selection of excitation and fluorescence wavelengths with no limitations by cut off filters.

The i-control and Magellan software packaged with the TECAN M200 make operation of the microplate reader relatively simple (Figure 10). The user is able to select
Figure 10. I-Control/Magellan User Interface

from a variety of common microplate conformations. The injectors can be programmed to automate reagent injection at specific stages of the assay, reducing operator error such as faulty pipetting. The operator can set the absorbance wavelengths, measured and reference, and determine the read pattern in the same panel. The Magellan software is a wizard based interface; users can start a measurement wizard, evaluate results wizard, create and edit sample ID lists wizard, and create and edit methods (analysis protocols) wizard. Users can edit plate geometry to design the assay that suits their research. Results
can be displayed as absorbance or concentration (calculated from the standard curve/calibrator data) in each microwell (Figure 11). Results can also be displayed as averages when specimens are assayed in duplicate.

Figure 11. Typical Magellan Microwell Absorbance Results
Use of an automated plate washer enhances assay accuracy by ensuring complete well aspiration and washing. The laboratory used the Columbus Strip Washer (Figure 12). It is a fully automated microplate strip washer for 96- and 384-well plates. Its features include manifolds of 8, 12 or 16 channels, crosswise aspiration, overflow and bottom washing. Assay protocols can either be programmed on board or via PC interface using the WinWash software. Thirty distinct methods can be programmed and stored simplified operation. A liquid sensor system monitors the liquid level of waste, wash buffer and rinse solution. Rinsing is fully automated, ensuring easy maintenance and operation.
3.3.1 Cocaine/Benzoylecgonine

3.3.1.1 General Principle of Assay

The principle of the Cocaine/Benzoylecgonine ELISA Kit used (Cocaine/Benzoylecgonine Direct ELISA Kit #212-0480, Immunalysis Corporation, Pomona, California 91767) is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture. Unmetabolized cocaine urine concentration is far lower than that of its major metabolite BE. Cocaine is undetectable (at a 50 ng/ml cut-off) 12 hours after administration in comparison with BE which persists for up to 48 hours after use. It has been suggested that a BE/cocaine ratio of less than 100 is indicative of use within the past 10 hours. Results are expressed in BE equivalents per ml.

A 20 μl aliquot of a diluted standard, control urine or cocaine positive (as previously determined by EMIT) urine specimen is incubated with a 100 μl dilution of horseradish peroxidase labeled BE derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed 6 times thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 1 ng/ml.

The precision of the Immunalysis Cocaine/Benzoylecgonine Direct ELISA Kit has been verified by assessment of the mean, standard deviation (SD) and coefficients of variation (CV) in data resulting from repetitive assays. Assay sensitivity based on the
minimum BE concentration required to produce a four standard deviation from assay Ao is 1 ng/ml.

3.3.1.2 Assay Procedural Details

Kit contents:

1. 96 well micro-plate coated with polyclonal anti-benzoylecggonine and polyclonal anti-cocaine

2. Cocaine/Benzoylecgonine-Enzyme Conjugate solution

3. Positive Reference Standard containing 50 ng/ml of BE dissolved in a synthetic urine

4. Negative Standard

5. TMB chromogenic substrate

6. Stop Reagent containing 1 N hydrochloric acid

All reagents were brought to room temperature prior to use. Positive reference standards of 0, 10, 25 and 50 ng/ml were prepared. Dilution of the urine specimens with PBS pH 7.0 was unnecessary. 20 µl of appropriately diluted calibrators and standards were pipetted to each well in duplicate. 20 µl of the diluted specimens were pipetted in duplicate to the appropriate wells. Next, 100 µl of the Enzyme Conjugate was added to
each well. After tapping the sides of the plate holder to ensure proper mixing, the plate was incubated in the dark for 60 minutes at room temperature.

The microplate wells were washed 6 times with 350 μl Barnstead Nanopure water. Following incubation, the microplate was inverted and slapped vigorously on absorbent paper to ensure all residual moisture was removed. This step was critical to remove residual enzyme conjugate. Next, 100 μl of Substrate reagent was added to each well and the sides of plate holder tapped to ensure proper mixing. The plate was incubated in the dark for 30 minutes at room temperature. 100 μl of Stop Solution was added to each well, changing the blue color to yellow. Absorbance was measured at a dual wavelength of 450 nm and 650 nm. A standard curve was produced and the concentration of BE in each of the specimens was determined.

3.3.1.3 Cross-Reactivity

Aliquots of a human urine matrix were spiked with the following compounds at a concentration of 5000 ng/ml. None of these compounds gave values in the assay that were equal to or greater than the assay sensitivity level (<1 ng/ml).

Acetaminophen, Acetylsalicylic acid, Amphetamine, Aminopyrine, Ampicillin, Amobarbital, Ascorbic acid, Atropine, Barbital, Butabarbital, Caffeine, Carbamazepine, Codeine, Chloroquine, Chlorpromazine, Carbromal, Desipramine, Dextromethorphan, Dextropropoxyphene, 5,5-Diphenylhydantoin, 10-11-Dihydrocarbamazepine, Diazepam, Ethosuximide, Estriol, Estrone, Estradiol, Ethotoin, Glutethimide, Hexobarbital,
Ibuprofen, Imipramine, Lidocaine, LSD, MDA, MDMA, Methadone, Methadone - primary metabolite, Methaqualone, Methamphetamine, Metharbital, Mephenytoin, α-Methyl-propylsuccinimide, Mephobarbital, Methyl PEMA, Methsuximide, 4-Methylprimidone, Morphine, Meperidine, Niacinamide, Norethindrone, N-Normethsuximide, Phenobarbital, Phensuximide, PEMA, Primidone, Phencyclidine, Pentobarbital, Phenothiazine, Phenylpropanolamine, Procaine, Quinine, Secobarbital, Tetracycline, Tetrahydrozoline, THCCOOH

3.3.2 Aldosterone

3.3.2.1 General Principle of Assay

The Aldosterone EIA kit used (Aldosterone Direct EIA Kit #11-ALDHU-E01, ALPCO Diagnostics, Salem, NH 03079) follows the typical competitive binding scenario. Unlabeled antigen (present in standards, control and samples) and an enzyme-labeled antigen (conjugate) compete for a limited number of antibody binding sites on the microplate. The kit can be used with urine specimens with significant pretreatment (hydrolysis, neutralization and dilution). Approximately 1 ml of urine is required per duplicate determination.

A 50 μl aliquot of a diluted standard, control urine or cocaine positive (as previously determined by EMIT) urine specimen is incubated with a 100 μl dilution of horseradish peroxidase labeled aldosterone derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are
washed 3 times thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color formed is inversely proportional to the concentration of aldosterone in the sample. A set of standards is used to plot a standard curve from which the amount of aldosterone in samples and controls can be directly read.

The lower detection limit is calculated from the standard curve by determining the resulting concentration of the mean OD of Calibrator A (based on 10 replicate analyses) minus 2 SD. Therefore, the sensitivity of the Direct Aldosterone ELISA kit is 15pg/ml.

3.3.2.2 Assay Procedural Details

Kit Contents:

1. Rabbit Anti-Aldosterone Antibody Coated Microwell Plate

2. Aldosterone-Biotin: Avidin-Horse Radish Peroxidase (HRP) Conjugate

3. Aldosterone Calibrators

4. Control - Ready To Use.

5. Wash Buffer Concentrate

6. Assay Buffer

7. TMB Substrate

8. Stopping Solution
3.3.2.3 Urine Pretreatment

1 ml of each urine sample was pipetted into an appropriately labeled glass or polypropylene tube. 0.1 ml of 3.2 N HCl was added to every tube. 3.2 N HCl can be made by adding 1 ml of concentrated HCl (12N) to 2.75 ml distilled water. Tubes were then capped securely and heated for 1 hour at 60°C in the dark. Urine specimens were neutralized by adding 0.1 ml of 3.2 N NaOH to every tube. 3.2 N NaOH can be made by dissolving 1.28 grams of NaOH pellets into 10 ml distilled water. Tubes were then mixed gently and thoroughly. The final step in pretreatment requires the dilution of samples 1:50 with calibrator A.

All reagents must reach room temperature before use. Positive reference standards of 0, 20, 80, 300, 800 and 2000 pg/ml are provided ready to use. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption. The conjugate was prepared by diluting the aldosterone-biotin: avidin-HRP concentrate 1:50 in assay buffer (240 μl of HRP in 12ml of assay buffer). The wash buffer was prepared by diluting the wash buffer concentrate 1:10 with Barnstead Nanopure water (50 ml of the wash buffer concentrate in 450 ml of water).

50 μl of each calibrator, treated control urine and treated cocaine positive urine were pipetted into correspondingly labeled wells in duplicate. Next, 100 μl of the conjugate working solution was pipetted into each well. The plate was incubated on a plate shaker (approximately 200 rpm) for 1 hour at room temperature. The wells were washed 3 times with 300 μl of diluted wash buffer per well. The plate was tapped firmly
against absorbent paper to ensure complete drying. 150 µl of TMB substrate was pipetted into each well at timed intervals and the plate was incubated on a plate shaker for 10-15 minutes at room temperature. An alternate endpoint is to allow calibrator A to attain a dark blue color for desired OD. 50 µl of stopping solution was added to each well at the same timed intervals as in the TMB step. Finally, the plate was read at 450nm within 20 minutes after addition of the stopping solution. A standard curve was produced and the concentration of aldosterone in each of the specimens was determined.

3.3.2.4 Cross- Reactivity

The following compounds cross-reacted at less than 0.001%: Androsterone, Cortisone, 11-Deoxycortisol, 21-Deoxycortisol, Dihydrotestosterone, Estradiol, Estriol, Estrone and Testosterone.

3.3.3 C Reactive Protein

3.3.3.1 General Principle of the Assay

The high sensitivity C Reactive Protein EIA kit used (high sensitivity C Reactive Protein #30-9710s, ALPCO Diagnostics, Salem, NH 03079) was a sandwich enzyme immunoassay intended for the quantitative determination of C reactive protein in plasma, serum, stool and urine. The combination of two specific antibodies in the CRP ELISA significantly reduces the possibility of false negatives. It is for in vitro diagnostic use only. The wells of the microplate are coated with polyclonal antibodies directed against C
reactive protein. The kit may be used with plasma, serum, stool and urine with little pretreatment.

A 100 μl aliquot of a diluted standard, control urine or cocaine positive (as previously determined by EMIT) urine specimen is added to the appropriate wells in duplicate and incubated for an hour. The plate is then washed 5 times to remove all unbound substances. 100 μl of the diluted peroxidase-labeled CRP conjugate antibody is added and the plate is incubated for another hour. The plate is washed 5 times and a chromogenic substrate is added. After allowing the plate to incubate for 20 minutes, an acidic stopping solution is then added. The intensity of the color that forms is directly proportional to the concentration of CRP in the sample. A dose response curve of the absorbance (at 450 nm) unit vs. concentration is generated. CRP, present in the patient samples, is determined directly from this calibration curve. The assay is sensitive to 0.124 ng/ml.

3.3.3.2 Assay Procedural Details

Kit Contents:

1. Microplate with precoated strips

2. Wash buffer concentrate

3. Antibody, (rabbit-anti-CRP, Peroxidase-labeled)

4. Calibrators, ready to use, (0; 1.9; 5.6; 16.7; 50; 150 ng/ml)
5. Control

6. Sample buffer

7. TMB chromogenic substrate

8. Stop solution containing sulfuric acid

All reagents and samples should be at room temperature (18-26 °C) prior to use. Positive reference standards of 0, 1.9, 5.6, 16.7, 50 and 150 ng/ml are provided ready to use. Urine specimens must be diluted 1:5 with sample dilution buffer. The wash buffer concentrate was diluted 1:10 with Barnstead Nanopure Water before use (100 ml concentrated wash buffer + 900 ml Nanopure Water). Crystals formed in the wash buffer concentrate (a common occurrence due to high salt concentration in the stock solutions); this necessitated soaking the concentrate in a 37°C using a water bath before dilution. The conjugate required a 1:100 dilution in wash buffer (100 μl concentrated conjugate + 9900 μl wash buffer).

Calibrators, controls and specimen samples should be assayed in duplicate. The plate was washed 5 times prior to use by dispensing 250 μl of diluted wash buffer into each well. 100 μl of appropriately diluted calibrators, standards and specimens were pipetted to each well in duplicate. After incubating at room temperature on a plate shaker for an hour, the contents of the wells were discarded and the plate was washed 5 times with 250 μl diluted wash buffer. Next, 100 μl of the Enzyme Conjugate was added to each well. After tapping the sides of the plate holder to ensure proper mixing, the plate
was covered tightly and incubated on a plate shaker for 60 minutes at room temperature. The microplate wells were washed 5 times with 250 μl diluted wash buffer. Next, 100 μl of Substrate reagent was added to each well and the sides of plate holder tapped to ensure proper mixing. The plate was incubated in the dark for 15 minutes at room temperature. 50 μl of Stop Solution was added to each well and the absorption was read immediately at 450 nm against 620 nm as a reference.

3.3.3.3 Cross Reactivity

Alpha-1-Antitrypsin 0 %

Lysozyme 0 %

Albumin 0 %

Other acute phase proteins 0 %

No cross reactivity with CRP in mouse serum was observed.

3.3.4 Myeloperoxidase

3.3.4.1 General Principle of the Assay

The Myeloperoxidase EIA kit used (Myeloperoxidase #30-6630, ALPCO Diagnostics, Salem, NH 03079) is a sandwich enzyme immunoassay intended for the quantitative determination of MPO in plasma, serum, stool and urine. The combination of
two specific antibodies in the MPO ELISA significantly reduces the possibility of false
negatives. It is for in vitro diagnostic use only. The wells of the microplate are coated
with polyclonal antibodies directed against MPO. The kit may be used with urine with
little pretreatment aside from dilution.

This ELISA is suitable for the quantitative determination of MPO in urine and
stool. In the first incubation step, the MPO in the samples is bound to available antibodies
against MPO immobilized on the surface of the microwells. To remove all unbound
substances, a washing step is carried out. In a second incubation step, a peroxidase-
labeled antibody against MPO is added. After another washing step to remove all
unbound substances, the solid phase is incubated with the chromogenic substrate TMB.
An acidic stop solution is then added to stop the reaction. The intensity of the yellow
color is directly proportional to the concentration of MPO in the sample. A dose response
curve of the absorbance unit vs. concentration is generated, using results obtained from
the calibrators. MPO, present in the samples, is determined directly from this curve.

3.3.4.2 Assay Procedural Details

Kit Contents:

1. Microplate with precoated strips

2. Wash buffer concentrate

3. Detection Antibody (biotinylated)

4. Standards, lyophilized
5. Control, lyophilized

6. Conjugate, (streptavidin peroxidase labeled)

7. Sample Dilution buffer

8. TMB chromogenic substrate

9. Stop solution containing sulfuric acid

All reagents and samples should be at room temperature (18-26 °C) prior to use. The Control and Positive reference standards of 0, 3.6, 11, 33 and 100 ng/ml are lyophilized and must be reconstituted in 500 μl Barnstead Nanopure Water prior to use. Urine specimens must be diluted 1:10 with sample dilution buffer. The wash buffer concentrate was diluted 1:10 with Barnstead Nanopure Water before use (100 ml concentrated wash buffer + 900 ml Nanopure Water). Crystals formed in the wash buffer concentrate (a common occurrence due to high salt concentration in the stock solutions); this necessitated soaking the concentrate in a 37°C using a water bath before dilution. The conjugate required a 1: 101 dilution in wash buffer (100 μl concentrated conjugate + 10 ml wash buffer). The Antibody required a 1: 101 dilution in wash buffer (100 μl concentrated antibody + 10 ml wash buffer).

Calibrators, controls and specimen samples should be assayed in duplicate. The plate was washed 5 times prior to use by dispensing 250 μl of diluted wash buffer into each well. 100 μl of appropriately diluted calibrators, standards and specimens were
pipetted to each well in duplicate. After incubating at room temperature on a plate shaker for an hour, the contents of the wells were discarded and the plate was washed 5 times with 250 μl diluted wash buffer. Next, 100 μl of the diluted antibody was added to each well. After tapping the sides of the plate holder to ensure proper mixing, the plate was covered tightly and incubated on a plate shaker for 60 minutes at room temperature. The microplate wells were washed 5 times with 250 μl diluted wash buffer. Next, 100 μl of the diluted conjugate was added to each well. After tapping the sides of the plate holder to ensure proper mixing, the plate was covered tightly and incubated on a plate shaker for 60 minutes at room temperature. The microplate wells were washed 5 times with 250 μl diluted wash buffer. Next, 100 μl of Substrate reagent was added to each well and the sides of plate holder tapped to ensure proper mixing. The plate was incubated in the dark for 15 minutes at room temperature. 50 μl of Stop Solution was added to each well and the absorption was read immediately at 450 nm against 620 nm as a reference.

3.3.4.3 Cross Reactivity

No cross reactivity to other plasma proteins in stool.

Alpha-1-Antitrypsin, Albumin, CRP, Lysozyme, sIgA, PMN-Elastase, Calprotectin
3.3.5 Microalbumin

3.3.5.1 General Principle of the Assay

The Microalbumin EIA kit used (Microalbumin # 24-MABHU-E01, ALPCO Diagnostics, Salem, NH 03079) is a competitive enzyme immunoassay intended for the quantitative determination of MAB in urine. The microwells of the assay plate are coated with a purified unlabeled primary antibody. Following this, the plate is incubated with unlabeled standards and unknowns. The reaction is allowed to proceed to equilibrium; labeled conjugate antigen is then added to the microwells. The conjugate will bind to the available binding sites on the primary antibody. The plate is then developed with substrate and color change is measured.

After incubation for a fixed time, separation of bound albumin from free albumin is achieved by simple decantation and plate washing. The enzyme activity on the plate is measured using enzyme substrate and a chromogen. The absorbency of the color developed is read in an EIA colorimetric reader. In a competitive ELISA, there is an inverse relationship between the signal obtained and the concentration of the analyte in the sample i.e. the more unlabeled antigens in the sample or standard, the lower the amount of conjugated antigen bound. The concentration of albumin in the urine is determined from a calibration curve.

The assay literature indicates that sensitivity was calculated by processing ten zero calibrator (maximum binding) wells along with a calibration curve. Mean and standard deviation were calculated for the absorbance of the ten zero calibrator wells.
Apparent sensitivity was found to be 0.24 mg/ml. The antiserum is highly specific for human albumin.

3.3.5.2 Assay Procedural Details

Kit Contents:

1. Microplate with precoated strips

2. Albumin Calibrators (5)

3. Albumin Enzyme Conjugate

4. Controls (2)

5. TMB chromogenic substrate

6. Stop solution containing sulfuric acid

All reagents and samples should be at room temperature (18-26 °C) prior to use. The Control and Positive reference standards packaged ready to use. Urine specimens may be used without pretreatment or dilution. Calibrators, controls and specimen samples should be assayed in duplicate. 10 μl of appropriately diluted calibrators, standards and specimens were pipetted to each well in duplicate. Next 50 μl diluted enzyme conjugate was added to every well. After incubating at room temperature on a plate shaker for an hour, the contents of the wells were discarded and the plate was washed 5 times with 250 μl diluted wash buffer. Next, 100 μl of the diluted antibody was added to each well. After
tapping the sides of the plate holder to ensure proper mixing, the plate was covered tightly and incubated on a plate shaker for 60 minutes at room temperature. The microplate wells were washed 5 times with 250 μl diluted wash buffer. Next, 100 μl of the diluted conjugate was added to each well. After tapping the sides of the plate holder to ensure proper mixing, the plate was covered tightly and incubated on a plate shaker for 60 minutes at room temperature. The microplate wells were washed 5 times with 250 μl diluted wash buffer. Next, 100 μl of Substrate reagent was added to each well and the sides of plate holder tapped to ensure proper mixing. The plate was incubated in the dark for 15 minutes at room temperature. 50 μl of Stop Solution was added to each well and the absorption was read immediately at 450 nm against 620 nm as a reference.

3.3.5.3 Cross Reactivity

No cross reactivity to bovine serum albumin, myoglobin, hemoglobin, and alpha fetoprotein were noted at significantly high concentration.

3.3.6 Myoglobin

3.3.6.1 General Principle of the Assay

The Myoglobin EIA kit used (Myoglobin # 2110 Life Diagnostics, Inc., P.O. Box 5205, West Chester, PA, 19380) was a sandwich enzyme immunoassay intended for the quantitative determination of myoglobin in serum and plasma. The combination of two
specific antibodies in the myoglobin ELISA significantly reduces the possibility of false negatives. It is for in vitro diagnostic use only. The wells of the microplate are coated with polyclonal antibodies directed against myoglobin.

This ELISA is suitable for the quantitative determination of myoglobin in serum and plasma. Although not labeled for use with urine specimens, the technical support staff at Life Diagnostics (www.lifediagnostics.com 610-431-7707) asserted the assay was fully compatible with urine specimens. 20 μl aliquots of standards, control urine or cocaine positive urine specimens (as previously determined by EMIT) are incubated simultaneously with two antibodies, resulting in myoglobin molecules being ‘sandwiched’ between the solid phase and enzyme-linked antibodies. To remove all unbound substances, the plate is washed with Barnstead Nanopure Water. The chromogenic substrate TMB is added and the plate is incubated for 20 minutes. An acidic stop solution is then added to stop the reaction. The intensity of the yellow color is directly proportional to the concentration of myoglobin in the sample. A dose response curve of the absorbance unit vs. concentration is generated, using results obtained from the calibrators. Myoglobin, present in the samples, is determined directly from this curve. The lowest detectable level of myoglobin by this assay is estimated to be 5 ng/ml.

3.3.6.2 Assay Procedural Details

Kit Contents:

1. Murine Monoclonal Anti-Myoglobin-coated microtiter wells, 96 wells

2. Myoglobin Reference Standards (0, 25, 100, 250, 500 and 1000 ng/ml)
3. Sample Diluent

4. Enzyme Conjugate Reagent

5. TMB Reagent

6. Stop Solution (1N HCl)

All reagents and samples should be at room temperature (18-26 °C) prior to use. The myoglobin reference standards of 0, 25, 100, 250, 500 and 1000ng/ml are provided ready to use. Urine specimens do not require dilution. 20 μl of calibrators, standards and specimens were pipetted along with 200 μl of Enzyme Conjugate Reagent to each well in duplicate and thoroughly mixed for 30 seconds. After incubating at room temperature for 45 minutes, the contents of the wells were discarded and the plate was washed 5 times with 250 μl Barnstead Nanopure Water. Next, 100 μl of TMB Substrate reagent was added to each well and the sides of plate holder tapped for 5 seconds to ensure proper mixing. The plate was incubated for 20 minutes at room temperature. 100 μl of Stop Solution was added to each well and the plate was gently mixed for 30 seconds. It is important to make sure the blue color is completely changed to yellow before reading the absorption. Finally, the absorption was read immediately at 450 nm against 620 nm as a reference.
3.3.6.3 Cross Reactivity

No cross reactivity is noted in the assay kit literature.

3.3.7 Heat Shock Protein 90α

3.3.7.1 General Principle of Assay

The principle of Heat Shock Protein 90α ELISA Kit used (Heat Shock Protein 90α Direct ELISA Kit #EKS-895, Assay Designs, Ann Arbor, MI 48108) is a quantitative sandwich immunoassay. A 100 μl aliquot of a diluted standard, control urine or cocaine positive (as previously determined by EMIT) urine specimen is pipetted into the wells and Hsp90α present in a sample is bound to the wells by the immobilized antibody. The wells are washed and the Detection Antibody is added. After washing away unbound Detection Antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are washed again, a TMB Substrate Reagent is added to the wells and color develops in proportion to the amount of Hsp90α bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

3.3.7.2 Assay Procedural Details

Kit contents:

1. Anti-Hsp90α Microplate

2. 20X Wash Buffer Concentrate
3. Recombinant human Hsp90α Standard

4. Sample Diluent

5. HRP Conjugate

6. HRP Conjugate Diluent

7. TMB Substrate

8. Stop Solution

All reagents were brought to room temperature prior to use. Dilute 100 ml of Wash Buffer Concentrate into 1900 ml Barnstead Nanopure Water to yield 2000 ml of 1x Wash Buffer. Add 27.5 μl of HRP Conjugate into a tube with 11.0 μl 1x HRP Conjugate diluent to prepare diluted HRP Conjugate. Mix thoroughly. The standards are prepared by adding 2 μl 1 μg/ml Hsp90α stock standard to 500 μl Sample Diluent and performing serial dilutions according to Table 4.
Table 4. Hsp90α Serial Dilutions

<table>
<thead>
<tr>
<th>Tube</th>
<th>Volume and Source of Hsp90α (μl)</th>
<th>Sample Diluent (μl)</th>
<th>Final Hsp90α Concentration (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.0 from the Hsp90α standard vial</td>
<td>500</td>
<td>4</td>
</tr>
<tr>
<td>B</td>
<td>250 from Tube A</td>
<td>250</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>250 from Tube B</td>
<td>250</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>250 from Tube C</td>
<td>250</td>
<td>0.5</td>
</tr>
<tr>
<td>E</td>
<td>250 from Tube D</td>
<td>250</td>
<td>0.25</td>
</tr>
<tr>
<td>F</td>
<td>250 from Tube E</td>
<td>250</td>
<td>0.125</td>
</tr>
<tr>
<td>G</td>
<td>250 from Tube F</td>
<td>250</td>
<td>0.0625</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>250</td>
<td>0</td>
</tr>
</tbody>
</table>

Urine specimens do not require dilution. 100 μl of calibrators, standards and specimens were pipetted to each well in duplicate and thoroughly mixed for 30 seconds. After incubating at room temperature for 60 minutes, the contents of the wells were discarded and the plate was washed 6 times with 300 μl Wash Buffer. Next, 100 μl of HRP Conjugate was added to each well except H₁ and H₂. After incubating at room temperature for 60 minutes, the contents of the wells were discarded and the plate was washed 6 times with 300 μl Wash Buffer. Next, 100 μl of TMB Substrate reagent was added to each well and the sides of plate holder tapped for 5 seconds to ensure proper mixing. The plate was incubated for 20 minutes at room temperature. 100 μl of Stop Solution was added to each well and the plate was gently mixed for 30 seconds. It is important to make sure the blue color is completely changed to yellow before reading the
absorption. Finally, the absorption was read immediately at 450 nm against 620 nm as a reference.

3.3.7.3 Cross-Reactivity

Assay Designs’ Hsp90α ELISA is specific for Hsp90α and will not react with Hsp90β. The assay does not cross react with 100ng/mL of Grp94, Hsp60, Hsp70. The Hsp90α ELISA has been certified for the detection of human Hsp90α.

3.3.8 Vascular Endothelial Growth Factor

3.3.8.1 General Principle of Assay

The principle of Vascular Endothelial Growth Factor ELISA Kit used (Vascular Endothelial Growth Factor ELISA Kit #ELH-VEGF-001, RayBiotech Corporation, Norcross, GA 30092) is intended for the quantitative measurement of human VEGF in serum, plasma, cell culture supernatants and urine. The assay is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture.

A 20 μl aliquot of a diluted standard, control urine or cocaine positive (as previously determined by EMIT) urine specimen is pipetted into the wells and VEGF present in a sample is bound to the wells by the immobilized antibody. The wells are washed and the Detection Antibody is added. After washing away unbound Detection
Antibody, HRP-conjugated streptavidin is pipetted to the wells. The wells are washed again, a TMB Substrate Reagent is added to the wells and color develops in proportion to the amount of VEGF bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

3.3.8.2 Assay Procedural Details

Kit contents:

1. VEGF Microplate

2. Wash Buffer Concentrate

3. Recombinant human VEGF Standard

4. Assay Diluent A (serum/plasma)

5. Assay Diluent B (urine and other body fluids)

6. Detection Antibody VEGF (biotinylated anti-humanVEGF)

7. HRP-Streptavidin concentrate

8. TMB One-Step Substrate Reagent

9. Stop Solution (2 M sulfuric acid)

All reagents were brought to room temperature prior to use. Assay Diluent B is used for dilution of culture supernatants and urine; Assay Diluent B is prepared by
diluting the contents of the vial 5-fold with Barnstead Nanopure Water. Dilute 20 ml of Wash Buffer Concentrate into Barnstead Nanopure Water to yield 400 ml of 1x Wash Buffer. Add 100 μl of 1x Assay Diluent B into the vial to prepare a detection antibody concentrate. Add 2 μl of HRP-Streptavidin concentrate into a tube with 198.0 μl 1x Assay Diluent B to prepare a 100-fold diluted HRP-Streptavidin. Mix thoroughly and then pipette 100 μl of prepared 100-fold diluted solution into a tube with 15 ml 1x Assay Diluent B to prepare a final 15,000 fold diluted HRP-Streptavidin solution. The standards are prepared by adding 60 μl 50 ng/ml VEGF stock standard to 440 μl Assay Diluent B and performing serial dilutions according to Table 5:

Table 5. VEGF Serial Dilutions

<table>
<thead>
<tr>
<th>Tube</th>
<th>Volume and Source of VEGF (μl)</th>
<th>Assay Diluent B (μl)</th>
<th>Final VEGF Concentration (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>60 from the VEGF standard vial</td>
<td>440</td>
<td>6000</td>
</tr>
<tr>
<td>B</td>
<td>200 from Tube A</td>
<td>400</td>
<td>2000</td>
</tr>
<tr>
<td>C</td>
<td>200 from Tube B</td>
<td>400</td>
<td>666.7</td>
</tr>
<tr>
<td>D</td>
<td>200 from Tube C</td>
<td>400</td>
<td>222.2</td>
</tr>
<tr>
<td>E</td>
<td>200 from Tube D</td>
<td>400</td>
<td>74.07</td>
</tr>
<tr>
<td>F</td>
<td>200 from Tube E</td>
<td>400</td>
<td>24.69</td>
</tr>
<tr>
<td>G</td>
<td>200 from Tube F</td>
<td>400</td>
<td>8.23</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>400</td>
<td>0</td>
</tr>
</tbody>
</table>
100 μl of appropriately diluted calibrators and standards were pipetted to each well in duplicate. 100 μl of the specimens were pipetted in duplicate to the appropriate wells. The microplate was covered and incubated for 2.5 hours at room temperature or over night at 4°C with gentle shaking. Wash the plate 4 times with 1x Wash Solution. After the last wash, the plate was inverted and blotted against clean paper towels. Next, 100 μl of the diluted biotinylated antibody was added to each well. The plate was incubated for 1 hour at room temperature with gentle shaking. The wash cycle was repeated. 100 μl of prepared Streptavidin solution was added to each cell. After tapping the sides of the plate holder to ensure proper mixing, the plate was incubated for 45 minutes at room temperature on a plate shaker. The wash cycle was repeated a final time. 100 μl of TMB Substrate Reagent was added to each well and the plate was incubated for 30 minutes at room temperature in the dark with on a plate shaker. The reaction was stopped with the addition of 50 μl of Stop Solution to each well. The plate was read at 450 nm immediately. A standard curve was generated and the concentration of VEGF in each of the specimens was determined.

3.3.8.3 Cross-Reactivity

This ELISA kit shows no cross-reactivity with any of the following cytokines:

human Angiogenin, BDNF, BLC, ENA-78, FGF-4, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12 p70, IL-12 p40, IL-13, IL-15, IL-309, IP-10, G-CSF, GM-CSF, IFN-γ, Leptin, MCP-1, MCP-2, MCP-3, MDC, MIP-1α, MIP-1 β, MIP-
1δ, PARC, PDGF, RANTES, SCF, TARC, TGF-β, TIMP-1, TIMP-2, TNF-α, TNF-β, TPO

3.3.9 Human Matrix Metalloproteinase 9/Neutrophil Gelatinase Associated Lipocalin

3.3.9.1 General Principle of Assay

The principle of Human Matrix Metalloproteinase 9/Neutrophil Gelatinase Associated Lipocalin ELISA Kit used (Human Matrix Metalloproteinase 9/Neutrophil Gelatinase Associated Lipocalin Direct ELISA Kit #DM9L20, R&D Systems, Minneapolis, Minnesota 55413) a sandwich enzyme immunoassay intended for the quantitative determination of MMP-9/NGAL in cell culture supernates, serum, plasma, urine and saliva. The combination of two specific antibodies in the MMP-9/NGAL ELISA significantly reduces the possibility of false negatives. It is for research use only. The wells of the microplate are coated with polyclonal antibodies directed against MMP-9/NGAL.

A 50 μl aliquot of a standard, control urine or cocaine positive (as previously determined by EMIT) urine specimen is incubated with 100 μl Assay Diluent for 3 hours at room temperature. The wells are washed 3 times thoroughly and 200 μl of MMP-9/NGAL conjugate is added. The plate is incubated and washed again before a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely
proportional to the concentration of drug in the sample. The technique is sensitive to 0.013 ng/ml.

3.3.9.2 Assay Procedural Details

Kit contents:

1. 96 well micro-plate coated with mouse monoclonal anti-MMP-9/NGAL
2. MMP-9/NGAL Conjugate solution
3. Standard containing 20 ng/ml of MMP-9/NGAL, lyophilized
4. Assay Diluent RD1-87
5. Calibrator Diluent RD5-10
6. Color Reagent A
7. Color Reagent B
8. Wash Buffer Concentrate
9. Stop Reagent containing 2 N sulfuric acid

All reagents were brought to room temperature prior to use. The Wash Buffer was prepared by diluting 20 ml of Wash Buffer Concentrate in 480 ml Barnstead Nanopure Water. Color Reagents A and B were mixed together in equal volumes to prepare the Substrate Solution. It has to be used within 15 minutes of preparation. The lyophilized MMP-9/NGAL standard was reconstituted with 1 ml Barnstead Nanopure Water to yield a 20 ng/ml MMP-9/NGAL stock solution. Calibrators were prepared from the stock solution according to Table 6:
Table 6. NGAL Serial Dilution Table

<table>
<thead>
<tr>
<th>Tube</th>
<th>Volume and Source of MMP-9/NGAL (μl)</th>
<th>Calibrator Diluent RD5-10 (μl)</th>
<th>Final Concentration (ng/ml MMP-9/NGAL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>200 from Stock Solution</td>
<td>200</td>
<td>10</td>
</tr>
<tr>
<td>B</td>
<td>200 from Tube A</td>
<td>200</td>
<td>5</td>
</tr>
<tr>
<td>C</td>
<td>200 from Tube B</td>
<td>200</td>
<td>2.5</td>
</tr>
<tr>
<td>D</td>
<td>200 from Tube C</td>
<td>200</td>
<td>1.25</td>
</tr>
<tr>
<td>E</td>
<td>200 from Tube D</td>
<td>200</td>
<td>0.625</td>
</tr>
<tr>
<td>F</td>
<td>200 from Tube E</td>
<td>200</td>
<td>0.312</td>
</tr>
<tr>
<td>G</td>
<td>0</td>
<td>200</td>
<td>0</td>
</tr>
</tbody>
</table>

100 μl of Assay Diluent RD1-87 was added to each well. 50 μl aliquots of the standards, control urine or cocaine positive urine specimens were pipetted in duplicate to the appropriate wells. After tapping the sides of the plate holder to ensure proper mixing, the plate was incubated for 3 hours at room temperature. The microplate wells were aspirated and washed 4 times with 400 μl Barnstead Nanopure water. Following incubation, the microplate was inverted and slapped vigorously on absorbent paper to ensure all residual moisture was removed. Next, 200 μl of the Conjugate was added to each well and the plate was incubated for 1 hour at room temperature. The aspiration and wash cycle was repeated. Next, 200 μl of Substrate reagent was added to each well and the sides of plate holder tapped to ensure proper mixing. The plate was incubated in the dark for 30 minutes at room temperature. Finally, 50 μl of Stop Solution was added to each well, changing the blue color to yellow.
Absorbance was measured at a wavelength of 450 nm. A standard curve was produced and the concentration of MMP-9/NGAL in each of the specimens was determined.

No significant cross-reactivity has been noted.

### 3.3.10 Pro-atrial natriuretic peptide (1-98)

#### 3.3.10.1 General Principle of Assay

The pro-atrial natriuretic peptide (1-98) EIA kit used (proANP Direct EIA Kit # 04-BI-20892, ALPCO Diagnostics, Salem, NH 03079) follows the typical direct binding scenario. It is appropriate for research use only. The wells of the microplate are coated with polyclonal antibodies directed against proANP. The kit may be used with urine with no pretreatment.

A 10 μl aliquot of a standard, control urine or cocaine positive (as previously determined by EMIT) urine specimen is added to each well except the blank. 200 μl of Conjugate is added to all the wells, except the blanks, and incubated in the dark for 3 hours at room temperature. The wells are washed 5 times thoroughly and 200 μl of Substrate is added. The plate is incubated the reaction is stopped using a dilute acid stop solution. Finally, measure the absorbance at 450 nm with a reference of 620 nm. The intensity of the color developed is proportional to the concentration of drug in the sample.
3.3.10.2 Assay Procedural Details

Kit Contents:

1. Polyclonal sheep anti proANP Coated Microwell Plate

2. Conjugate (polyclonal anti proANP antibody –HRPO)

3. ProANP standards, lyophilized

4. Control, lyophilized

5. Wash Buffer Concentrate

6. Assay Buffer

7. TMB Substrate

8. Stop Solution (Sulfuric Acid)

All reagents must reach room temperature before use. The Control and Positive reference standards of 0, 0.63, 1.25, 2.5, 5.0 and 10.0 nmol/l are lyophilized and required reconstitution with 250 μl Barnstead Nanopure water. Calibrators, controls and specimen samples should be assayed in duplicate. Once the procedure has been started, all steps should be completed without interruption. The conjugate (polyclonal anti proANP antibody –HRPO) was provided ready to use. The wash buffer was prepared by diluting the wash buffer concentrate 1:20 with Barnstead Nanopure water (50 ml of the wash buffer concentrate in 950 ml of water).
10 μl of each calibrator, treated control urine and treated cocaine positive urine (as determined by EMIT) were pipetted into correspondingly labeled wells in duplicate. Next, 200 μl of the conjugate working solution was pipetted into each well, except the blanks. The plate was incubated for 3 hours at room temperature in the dark. The wells were aspirated and washed 5 times with 300 μl of diluted wash buffer per well. The plate was tapped firmly against absorbent paper to ensure complete drying. 200 μl of TMB substrate was pipetted into each well and the plate was incubated on a plate shaker for 30 minutes at room temperature in the dark. 50 μl of stopping solution was added to each well and the plate was read at 450nm with a reference of 620 nm. A standard curve was produced and the concentration of proANP in each of the specimens was determined.

3.3.10.3 Cross-Reactivity

proANP (1-30) < 1%

proANP (31-67) <1%

proANP (79-98) <1%

alpha ANP (99-126) <1%

proBNP (8-29) <1%

proBNP (32-57) <1%

proCNP (1-19) <1%

proCNP (30-50) <1%
The assay also detects mouse and rat proANP (1-98).

3.3.11 Interleukin 1α

3.3.11.1 General Principle of the Assay

The Interleukin 1α EIA kit used (Interleukin 1α #583301, Cayman Chemicals, Ann Arbor, MI 48108) is a sandwich enzyme immunoassay intended for the quantitative determination of Interleukin 1α in serum and plasma. Cayman Chemical Technical Support verified the assay was compatible with urine specimens so long as the standards are diluted with an appropriate sample matrix blank (SMB). Synthetic Urine from Immunalysis (Synthetic Urine with Creatinine Dry Powder #SUP-5, Immunalysis Corporation, Pomona, CA 91767) was used for the sample matrix blank. The combination of two specific antibodies in the Interleukin 1α ELISA significantly reduces the possibility of false negatives. It is for research use only. The wells of the microplate are coated with polyclonal antibodies directed against Interleukin 1α.

A 100 μl aliquot of a diluted standard, control urine or cocaine positive (as previously determined by EMIT) urine specimen is added to the appropriate wells in duplicate. Next, 100 μl of Acetylcholinesterase: Interleukin-1 α Fab’ Conjugate is added to all wells except the blanks and incubated for overnight at 4 °C. The plate is then washed 5 times with Wash Buffer to remove all unbound substances. 200 μl of Ellman’s Reagent is added to each well and the plate is incubated in the dark. The reaction is
monitored for several hours until the S1 wells are visibly yellow. The intensity of the color that forms is directly proportional to the concentration of Interleukin-1 α in the sample. A dose response curve of the absorbance (at 405 nm) unit vs. concentration is generated. Interleukin-1 α, present in the patient samples, is determined directly from this calibration curve. The assay is sensitive to 1.5 pg/ml Interleukin-1 α.

3.3.11.2 Assay Procedural Details

Kit Contents:

1. Microplate with precoated strips
2. Interleukin-1 α standard
3. Non-specific Mouse Serum
4. Sample Matrix Blank for plasma, serum (cannot be used for this assay)
5. Acetylcholinesterase: Interleukin-1 α Fab’ Conjugate
6. EIA Buffer Concentrate
7. Wash Buffer Concentrate
8. Tween
9. Ellman’s Reagent
All reagents and samples should be at room temperature (18-26 °C) prior to use. The EIA Buffer concentrate was reconstituted with 90 ml of Barnstead Nanopure water. The Wash Buffer was prepared by bringing 5 ml of wash buffer concentrate to a total volume of 2 L with Barnstead Nanopure water and then adding 1 ml Tween. The Acetylcholinesterase: Interleukin-1 α Fab’ Conjugate was reconstituted with 10 ml of EIA buffer. The urine specimens were used without any dilution. Interleukin-1 α standards required reconstitution with 2 ml EIA Buffer prior to dilution with SMB. This initial dilution yielded a stock solution of 5 ng/ml Interleukin-1 α. The Immunalysis Synthetic Urine served as the SMB; the lyophilized urine was reconstituted with Barnstead Nanopure water prior to use. 4.5 L of Nanopure water were added to the contents of the pouch. The solution was stirred until the lyophilized urine dissolved completely. The total volume was adjusted to 5 L with Nanopure water. The synthetic urine is stable for 1 year when stored at 4°C.

The Interleukin-1 α standards were prepared as described in Table 7:
Table 7. Concentration of Interleukin-1 α Standards

<table>
<thead>
<tr>
<th>Tube</th>
<th>Volume and Source of Interleukin-1 α (μl)</th>
<th>Sample Matrix Buffer (μl)</th>
<th>Final Concentration (pg/ml Interleukin-1α)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50 from Stock Solution</td>
<td>Dilute to 1 ml total</td>
<td>250</td>
</tr>
<tr>
<td>B</td>
<td>500 from Tube A</td>
<td>500</td>
<td>125</td>
</tr>
<tr>
<td>C</td>
<td>500 from Tube B</td>
<td>500</td>
<td>62.5</td>
</tr>
<tr>
<td>D</td>
<td>500 from Tube C</td>
<td>500</td>
<td>31.25</td>
</tr>
<tr>
<td>E</td>
<td>500 from Tube D</td>
<td>500</td>
<td>15.6</td>
</tr>
<tr>
<td>F</td>
<td>500 from Tube E</td>
<td>500</td>
<td>7.8</td>
</tr>
<tr>
<td>G</td>
<td>500 from Tube F</td>
<td>500</td>
<td>3.9</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>500</td>
<td>0</td>
</tr>
</tbody>
</table>

Calibrators, controls and specimen samples should be assayed in duplicate. 100 μl of appropriately diluted calibrators, standards and specimens were pipetted to each well in duplicate. Next, 100 μl of Acetylcholinesterase: Interleukin-1 α Fab’ Conjugate is added to all wells except the blanks and incubated for overnight at 4 °C. The plate is then washed 5 times with Wash Buffer to remove all unbound substances. 200 μl of Ellman’s Reagent is added to each well and the plate is incubated in the dark. The reaction is monitored for several hours until the S1 wells are visibly yellow. The intensity of the color that forms is directly proportional to the concentration of Interleukin-1 α in the sample. The absorption was read immediately at 405 nm.
3.3.12 Interleukin 1β

3.3.12.1 General Principle of the Assay

The Interleukin 1β EIA kit used (Interleukin 1β #583311, Cayman Chemicals, Ann Arbor, MI 48108) is a sandwich enzyme immunoassay intended for the quantitative determination of Interleukin 1β in serum and plasma. Cayman Chemical Technical Support verified the assay was compatible with urine specimens so long as the standards are diluted with an appropriate sample matrix blank (SMB). Synthetic Urine from Immunalysis (Synthetic Urine with Creatinine Dry Powder #SUP-5, Immunalysis Corporation, Pomona, CA 91767) was used for the sample matrix blank. The combination of two specific antibodies in the Interleukin 1β ELISA significantly reduces the possibility of false negatives. It is for research use only. The wells of the microplate are coated with polyclonal antibodies directed against Interleukin 1β.

A 100 μl aliquot of a diluted standard, control urine or cocaine positive (as previously determined by EMIT) urine specimen is added to the appropriate wells in duplicate. Next, 100 μl of Acetylcholinesterase: Interleukin-1 β Fab’ Conjugate is added to all wells except the blanks and incubated for overnight at 4 °C. The plate is then washed 5 times with Wash Buffer to remove all unbound substances. 200 μl of Ellman’s Reagent is added to each well and the plate is incubated in the dark. The reaction is monitored for several hours until the S1 (250 pg/ml) wells are visibly yellow. The intensity of the color that forms is directly proportional to the concentration of Interleukin-1 β in the sample. A dose response curve of the absorbance (at 405 nm) unit vs. concentration is generated. Interleukin-1 β, present in the patient samples, is
determined directly from this calibration curve. The assay is sensitive to 1.5 pg/ml Interleukin-1 β.

3.3.12.2 Assay Procedural Details

Kit Contents:

1. Microplate with precoated strips
2. Interleukin-1 β standard
3. Non-specific Mouse Serum
4. Sample Matrix Blank for plasma, serum (cannot be used for this assay)
5. Acetylcholinesterase: Interleukin-1 β Fab’ Conjugate
6. EIA Buffer Concentrate
7. Wash Buffer Concentrate
8. Tween
9. Ellman’s Reagent

All reagents and samples should be at room temperature (18-26 °C) prior to use. The EIA Buffer concentrate was reconstituted with 90 ml of Barnstead Nanopure water. The Wash Buffer was prepared by bringing 5 ml of wash buffer concentrate to a total volume of 2 L with Barnstead Nanopure water and then adding 1 ml Tween. The
Acetylcholinesterase: Interleukin-1 β Fab’ Conjugate was reconstituted with 10 ml of EIA buffer. The urine specimens were used without any dilution. Interleukin-1 β standards required reconstitution with 2 ml EIA Buffer prior to dilution with SMB. This initial dilution yielded a stock solution of 5 ng/ml Interleukin-1 β. The Immunalysis Synthetic Urine served as the SMB; the lyophilized urine was reconstituted with Barnstead Nanopure water prior to use. 4.5 L of Nanopure water were added to the contents of the pouch. The solution was stirred until the lyophilized urine dissolved completely. The total volume was adjusted to 5 L with Nanopure water. The synthetic urine is stable for 1 year when stored at 4°C.

The Interleukin-1 β standards were prepared as described in Table 8:

Table 8. Concentration of Interleukin-1 β Standards

<table>
<thead>
<tr>
<th>Tube</th>
<th>Volume and Source of Interleukin-1 β (μl)</th>
<th>Sample Matrix Buffer (μl)</th>
<th>Final Concentration (pg/ml Interleukin-1β)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50 from Stock Solution</td>
<td>Dilute to 1 ml total</td>
<td>250</td>
</tr>
<tr>
<td>B</td>
<td>500 from Tube A</td>
<td>500</td>
<td>125</td>
</tr>
<tr>
<td>C</td>
<td>500 from Tube B</td>
<td>500</td>
<td>62.5</td>
</tr>
<tr>
<td>D</td>
<td>500 from Tube C</td>
<td>500</td>
<td>31.25</td>
</tr>
<tr>
<td>E</td>
<td>500 from Tube D</td>
<td>500</td>
<td>15.6</td>
</tr>
<tr>
<td>F</td>
<td>500 from Tube E</td>
<td>500</td>
<td>7.8</td>
</tr>
<tr>
<td>G</td>
<td>500 from Tube F</td>
<td>500</td>
<td>3.9</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>500</td>
<td>0</td>
</tr>
</tbody>
</table>
Calibrators, controls and specimen samples should be assayed in duplicate. 100 μl of appropriately diluted calibrators, standards and specimens were pipetted to each well in duplicate. Next, 100 μl of Acetylcholinesterase: Interleukin-1 β Fab’ Conjugate is added to all wells except the blanks and incubated for overnight at 4 °C. The plate is then washed 5 times with Wash Buffer to remove all unbound substances. 200 μl of Ellman’s Reagent is added to each well and the plate is incubated in the dark. The reaction is monitored for several hours until the S1 wells are visibly yellow. The intensity of the color that forms is directly proportional to the concentration of Interleukin-1 β in the sample. The absorption was read immediately at 405 nm.

3.3.13 Interleukin 6

3.3.13.1 General Principle of the Assay

The Interleukin 6 EIA kit used (Interleukin 6 #583361, Cayman Chemicals, Ann Arbor, MI 48108) is a sandwich enzyme immunoassay intended for the quantitative determination of Interleukin 6 in serum and plasma. Cayman Chemical Technical Support verified the assay was compatible with urine specimens so long as the standards are diluted with an appropriate sample matrix blank (SMB). Synthetic Urine from Immunalysis (Synthetic Urine with Creatinine Dry Powder #SUP-5, Immunalysis Corporation, Pomona, CA 91767) was used for the sample matrix blank. The combination of two specific antibodies in the Interleukin 6 ELISA significantly reduces the possibility of false negatives. It is for research use only. The wells of the microplate are coated with polyclonal antibodies directed against Interleukin 6.
A 100 μl aliquot of a diluted standard, control urine or cocaine positive (as previously determined by EMIT) urine specimen is added to the appropriate wells in duplicate. Next, 100 μl of Acetylcholinesterase: Interleukin-6 Fab’ Conjugate is added to all wells except the blanks and incubated for overnight at 4 °C. The plate is then washed 5 times with Wash Buffer to remove all unbound substances. 200 μl of Ellman’s Reagent is added to each well and the plate is incubated in the dark. The reaction is monitored for several hours until the S1 (250 pg/ml) wells are visibly yellow. The intensity of the color that forms is directly proportional to the concentration of Interleukin-6 in the sample. A dose response curve of the absorbance (at 405 nm) unit vs. concentration is generated. Interleukin 6 present in the patient samples is determined directly from this calibration curve. The assay is sensitive to 7.8 pg/ml Interleukin-6.

3.3.13.2 Assay Procedural Details

Kit Contents:

1. Microplate with precoated strips
2. Interleukin-6 standard
3. Non-specific Mouse Serum
4. Sample Matrix Blank for plasma, serum (cannot be used for this assay)
5. Acetylcholinesterase: Interleukin-6 Fab’ Conjugate
6. EIA Buffer Concentrate
7. Wash Buffer Concentrate

8. Tween 20

9. Ellman’s Reagent

All reagents and samples should be at room temperature (18-26 °C) prior to use. The EIA Buffer concentrate was reconstituted with 90 ml of Barnstead Nanopure water. The Wash Buffer was prepared by bringing 5 ml of wash buffer concentrate to a total volume of 2 L with Barnstead Nanopure water and then adding 1 ml Tween. The Acetylcholinesterase: Interleukin-6 Fab’ Conjugate was reconstituted with 10 ml of EIA buffer. The urine specimens were used without any dilution. Interleukin-6 standards required reconstitution with 2 ml EIA Buffer prior to dilution with SMB. This initial dilution yielded a stock solution of 5 ng/ml Interleukin-6. The Immunalysis Synthetic Urine served as the SMB; the lyophilized urine was reconstituted with Barnstead Nanopure water prior to use. 4.5 L of Nanopure water were added to the contents of the pouch. The solution was stirred until the lyophilized urine dissolved completely. The total volume was adjusted to 5 L with Nanopure water. The synthetic urine is stable for 1 year when stored at 4°C.

The Interleukin-6 standards were prepared as described in the table below (Table 9):
Table 9. Concentration of Interleukin-6 Standards

<table>
<thead>
<tr>
<th>Tube</th>
<th>Volume and Source of Interleukin-6 (μl)</th>
<th>Sample Matrix Buffer (μl)</th>
<th>Final Concentration (pg/ml Interleukin-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50 from Stock Solution</td>
<td>Dilute to 1 ml total</td>
<td>250</td>
</tr>
<tr>
<td>B</td>
<td>500 from Tube A</td>
<td>500</td>
<td>125</td>
</tr>
<tr>
<td>C</td>
<td>500 from Tube B</td>
<td>500</td>
<td>62.5</td>
</tr>
<tr>
<td>D</td>
<td>500 from Tube C</td>
<td>500</td>
<td>31.3</td>
</tr>
<tr>
<td>E</td>
<td>500 from Tube D</td>
<td>500</td>
<td>15.6</td>
</tr>
<tr>
<td>F</td>
<td>500 from Tube E</td>
<td>500</td>
<td>7.8</td>
</tr>
<tr>
<td>G</td>
<td>500 from Tube F</td>
<td>500</td>
<td>3.9</td>
</tr>
<tr>
<td>H</td>
<td>0</td>
<td>500</td>
<td>0</td>
</tr>
</tbody>
</table>

Calibrators, controls and specimen samples should be assayed in duplicate. 100 μl of appropriately diluted calibrators, standards and specimens were pipetted to each well in duplicate. Next, 100 μl of Acetylcholinesterase: Interleukin-6 Fab’ Conjugate is added to all wells except the blanks and incubated for overnight at 4 °C. The plate is then washed 5 times with Wash Buffer to remove all unbound substances. 200 μl of Ellman’s Reagent is added to each well and the plate is incubated in the dark. The reaction is monitored for several hours until the S1 (250 pg/ml standard) wells are visibly yellow. The intensity of the color that forms is directly proportional to the concentration of Interleukin-6 in the sample. The absorption was read immediately at 405 nm. Crossreactivity with the other interleukins is limited.
Chapter 4.0

Results

Results were analyzed using a Student’s t test for significance (JMP Start Statistics, Release 4.0.4 SAS Institute, 2001) was used to compare biomarker concentration in the male and female, control and cocaine positive specimens. Because the data was not parametric, the Wilcoxon Rank Sums and Mann Whitney U tests were also used to test the means. In addition, the Tukey-Kramer HSD test was also used to analyze the means. The results were the same regardless of the analysis performed.

4.1 Gender

4.1.1 Assay results in Male and Female Control Urines

A summary of the differences in marker expression between male and female control urine specimens is presented in Table 10. The individual results are standardized and expressed per milligram creatinine. The mean aldosterone concentration was statistically significantly (p=0.0223) in female urine when compared to male urine specimens. The same was true for IL1 α (p=.0007) and IL1 β (p=.0198). The results for hsCRP (p=.0662) and MPO (p=.0562) are suggestive of statistical significance. There was no statistically significant difference in the other biomarkers.

95
Table 10. Comparison of Assay results in Male and Female Control Urines

<table>
<thead>
<tr>
<th>Assay</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>Total Protein (pg)</td>
<td>32.2 ± 12.1</td>
</tr>
<tr>
<td>Aldosterone (pg)</td>
<td>1907.7 ± 356.7</td>
</tr>
<tr>
<td>hsCRP (ng)</td>
<td>0.78± .6</td>
</tr>
<tr>
<td>Myeloperoxidase (ng)</td>
<td>20.5± 7.9</td>
</tr>
<tr>
<td>Microalbumin (ug)</td>
<td>18.4± 7.3</td>
</tr>
<tr>
<td>Hsp90 α (ng)</td>
<td>0.92± .4</td>
</tr>
<tr>
<td>VEGF (pg)</td>
<td>2453.0± 972.5</td>
</tr>
<tr>
<td>proANP (nmol)</td>
<td>0.3±1</td>
</tr>
<tr>
<td>Myoglobin (ng)</td>
<td>15.5±9.8</td>
</tr>
<tr>
<td>NGAL (ng)</td>
<td>0.08±.1</td>
</tr>
<tr>
<td>IL6 (pg)</td>
<td>0.86±.4</td>
</tr>
<tr>
<td>IL1 α (pg)</td>
<td>3.8±3.8</td>
</tr>
<tr>
<td>IL1 β (pg)</td>
<td>2.2±1.0</td>
</tr>
</tbody>
</table>

*significant at p<0.05
values expressed as mean ± standard error of the mean (SEM)

4.1.2 Assay results in Male and Female Cocaine Positive Urines

A summary of the differences in marker expression between male and female cocaine positive urine specimens is presented in Table 11. The individual results are
standardized and expressed per milligram creatinine. The results for Hsp90α (p=.0650) are suggestive of statistical significance. There was no statistically significant difference in the other biomarkers.

Table 11. Comparison of Assay results in Male and Female Cocaine Positive Urines

<table>
<thead>
<tr>
<th>Assay</th>
<th>Cocaine Positive</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein (pg)</td>
<td>66.9±22.4</td>
<td>47.4±3.2</td>
<td>.4612</td>
<td></td>
</tr>
<tr>
<td>Aldosterone (pg)</td>
<td>3331.8±399.8</td>
<td>3463.6±723.2</td>
<td>.8600</td>
<td></td>
</tr>
<tr>
<td>hsCRP (ng)</td>
<td>42.1±44.4</td>
<td>47.4±34.3</td>
<td>.9275</td>
<td></td>
</tr>
<tr>
<td>Myeloperoxidase (ng)</td>
<td>25.15±9.7</td>
<td>52.1±22.1</td>
<td>.2140</td>
<td></td>
</tr>
<tr>
<td>Microalbumin (ug)</td>
<td>32.9±9.7</td>
<td>40.5±10.2</td>
<td>.5995</td>
<td></td>
</tr>
<tr>
<td>Hsp90 α (ng)</td>
<td>0.9±.3</td>
<td>0.02±.1</td>
<td>.0650</td>
<td></td>
</tr>
<tr>
<td>VEGF (pg)</td>
<td>2900.9±1168</td>
<td>1308.4±351.9</td>
<td>.2535</td>
<td></td>
</tr>
<tr>
<td>proANP (nmol)</td>
<td>0.5±.1</td>
<td>0.3±.1</td>
<td>.3612</td>
<td></td>
</tr>
<tr>
<td>Myoglobin (ng)</td>
<td>36.9±4.8</td>
<td>45.9±9.8</td>
<td>.3586</td>
<td></td>
</tr>
<tr>
<td>NGAL (ng)</td>
<td>0.3±.12</td>
<td>20.2±12.0</td>
<td>.1534</td>
<td></td>
</tr>
<tr>
<td>IL6 (pg)</td>
<td>3.8±8.9</td>
<td>6.3±4.6</td>
<td>.6521</td>
<td></td>
</tr>
<tr>
<td>IL1 α (pg)</td>
<td>1.74±1.07</td>
<td>26.2±12.3</td>
<td>.0907</td>
<td></td>
</tr>
<tr>
<td>IL1 β (pg)</td>
<td>2.5±.5</td>
<td>9.5±3.4</td>
<td>.0846</td>
<td></td>
</tr>
</tbody>
</table>

* significant at p<0.05

values expressed as mean ± standard error of the mean (SEM)
4.2 Cocaine

4.2.1 Assay Results in Male Control and Cocaine Positive Urine Specimens

A summary of the differences in marker expression between control and cocaine positive male urine specimens is presented in Table 12. The individual assay results are standardized and expressed per milligram creatinine. The mean aldosterone concentration was significantly higher (p=0.0095) in cocaine positive urine when compared to control urine specimens. The mean myoglobin concentration was significantly higher (p=0.0332) in cocaine positive urine when compared to control urine specimens. There was no statistically significant difference in the other biomarkers.

4.2.2 Assay Results in Female Control and Cocaine Positive Urine Specimens

A summary of the differences in marker expression in control and cocaine positive female urine specimens is presented in Table 13. The individual results are standardized and expressed per milligram creatinine. There were no statistically significant differences between the control and cocaine positive means for any of the assays.
Table 12. Comparison of Assay Results in Male Control and Cocaine Positive Urine

<table>
<thead>
<tr>
<th>Assay</th>
<th>Male Control</th>
<th>Cocaine Positive</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Protein (pg)</td>
<td>32.2±12.1</td>
<td>66.9±22.4</td>
<td>0.2377</td>
</tr>
<tr>
<td>Aldosterone (pg)</td>
<td>1907.7±356.7</td>
<td>3331.8±399.8</td>
<td>0.0095*</td>
</tr>
<tr>
<td>hsCRP (ng)</td>
<td>0.78±0.6</td>
<td>42.1±44.4</td>
<td>0.3668</td>
</tr>
<tr>
<td>Myeloperoxidase (ng)</td>
<td>20.5±7.9</td>
<td>25.15±9.7</td>
<td>0.6985</td>
</tr>
<tr>
<td>Microalbumin (ug)</td>
<td>18.4±7.3</td>
<td>32.9±9.7</td>
<td>0.2474</td>
</tr>
<tr>
<td>Hsp90 α (ng)</td>
<td>0.92±0.4</td>
<td>0.9±0.3</td>
<td>0.967</td>
</tr>
<tr>
<td>VEGF (pg)</td>
<td>2453.0±972.5</td>
<td>2900.9±1168</td>
<td>0.7555</td>
</tr>
<tr>
<td>proANP (nmol)</td>
<td>0.3±0.1</td>
<td>0.5±0.1</td>
<td>0.1276</td>
</tr>
<tr>
<td>Myoglobin (ng)</td>
<td>15.5±9.8</td>
<td>36.9±4.8</td>
<td>0.0332*</td>
</tr>
<tr>
<td>NGAL (ng)</td>
<td>0.08±0.1</td>
<td>0.3±0.12</td>
<td>0.0984</td>
</tr>
<tr>
<td>IL6 (pg)</td>
<td>0.86±0.4</td>
<td>3.8±8.9</td>
<td>0.1909</td>
</tr>
<tr>
<td>IL1 α (pg)</td>
<td>3.8±3.8</td>
<td>1.74±1.07</td>
<td>0.6247</td>
</tr>
<tr>
<td>IL1 β (pg)</td>
<td>2.2±1.0</td>
<td>2.5±5.0</td>
<td>0.8124</td>
</tr>
</tbody>
</table>

* significant p < .05

values expressed as mean ± standard error of the mean (SEM)
Table 13. Comparison of Assay Results in Female Control and Cocaine Positive Urine

<table>
<thead>
<tr>
<th>Assay</th>
<th>Female</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Cocaine Positive</td>
<td>p</td>
<td></td>
</tr>
<tr>
<td>Total Protein (pg)</td>
<td>65.9±20.8</td>
<td>47.4±3.2</td>
<td>0.4443</td>
<td></td>
</tr>
<tr>
<td>Aldosterone (pg)</td>
<td>4917.96±1159.9</td>
<td>3463.6±723.2</td>
<td>0.3321</td>
<td></td>
</tr>
<tr>
<td>hsCRP (ng)</td>
<td>202.3±103.0</td>
<td>47.4±34.3</td>
<td>0.2147</td>
<td></td>
</tr>
<tr>
<td>Myeloperoxidase (ng)</td>
<td>187.1±81.3</td>
<td>52.1±22.1</td>
<td>0.1684</td>
<td></td>
</tr>
<tr>
<td>Microalbumin (ug)</td>
<td>41.2±14.4</td>
<td>40.5±10.2</td>
<td>0.9677</td>
<td></td>
</tr>
<tr>
<td>Hsp90 α (ng)</td>
<td>0.3±.2</td>
<td>0.02±.1</td>
<td>0.5478</td>
<td></td>
</tr>
<tr>
<td>VEGF (pg)</td>
<td>3286.2±1402.6</td>
<td>1308.4±351.9</td>
<td>0.2366</td>
<td></td>
</tr>
<tr>
<td>proANP (nmol)</td>
<td>0.34±.1</td>
<td>0.3±.1</td>
<td>0.9436</td>
<td></td>
</tr>
<tr>
<td>Myoglobin (ng)</td>
<td>26.5±9.1</td>
<td>45.9±9.8</td>
<td>0.1657</td>
<td></td>
</tr>
<tr>
<td>NGAL (ng)</td>
<td>12.5±9.8</td>
<td>20.2±12.0</td>
<td>0.6309</td>
<td></td>
</tr>
<tr>
<td>IL6 (pg)</td>
<td>7.52±4.4</td>
<td>6.3±4.6</td>
<td>0.8475</td>
<td></td>
</tr>
<tr>
<td>IL1 α (pg)</td>
<td>56.9±12.5</td>
<td>26.2±12.3</td>
<td>0.0963</td>
<td></td>
</tr>
<tr>
<td>IL1 β (pg)</td>
<td>6.6±1.4</td>
<td>9.5±3.4</td>
<td>0.461</td>
<td></td>
</tr>
</tbody>
</table>

* significant p < .05

values expressed as mean ± standard error of the mean (SEM)
4.3 Tables

Table 14. Male and Female Control and Cocaine Positive Urine Specimen Comparison

<table>
<thead>
<tr>
<th>Assay</th>
<th>Male</th>
<th></th>
<th>p</th>
<th>Female</th>
<th></th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Cocaine Positive</td>
<td></td>
<td>Control</td>
<td>Cocaine Positive</td>
<td></td>
</tr>
<tr>
<td>Total Protein (pg)</td>
<td>32.2±12.1</td>
<td>66.9±22.4</td>
<td>0.2377</td>
<td>65.9±20.8</td>
<td>47.4±3.2</td>
<td>0.4443</td>
</tr>
<tr>
<td>Aldosterone (pg)</td>
<td>1907.7±356.7</td>
<td>3331.8±399.8</td>
<td>0.0095*</td>
<td>4917.9±1159.9</td>
<td>3463.6±723.2</td>
<td>0.3321</td>
</tr>
<tr>
<td>hsCRP (ng)</td>
<td>0.78±.6</td>
<td>42.1±44.4</td>
<td>0.3668</td>
<td>202.3±103.0</td>
<td>47.4±34.3</td>
<td>0.2147</td>
</tr>
<tr>
<td>Myeloperoxidase (ng)</td>
<td>20.5±7.9</td>
<td>25.15±9.7</td>
<td>0.6985</td>
<td>187.1±81.3</td>
<td>52.1±22.1</td>
<td>0.1684</td>
</tr>
<tr>
<td>Microalbumin (ug)</td>
<td>18.4±7.3</td>
<td>32.9±9.7</td>
<td>0.2474</td>
<td>41.2±14.4</td>
<td>40.5±10.2</td>
<td>0.9677</td>
</tr>
<tr>
<td>Hsp90 α (ng)</td>
<td>0.92±.4</td>
<td>0.9±3</td>
<td>0.967</td>
<td>0.3±.2</td>
<td>0.02±.1</td>
<td>0.5478</td>
</tr>
<tr>
<td>VEGF (pg)</td>
<td>2453.0±972.5</td>
<td>2900.9±1168</td>
<td>0.7555</td>
<td>3286.2±1402.6</td>
<td>1308.4±351.9</td>
<td>0.2366</td>
</tr>
<tr>
<td>proANP (nmol)</td>
<td>0.3±1</td>
<td>0.5±1</td>
<td>0.1276</td>
<td>0.34±1</td>
<td>0.3±1</td>
<td>0.9436</td>
</tr>
<tr>
<td>Myoglobin (ng)</td>
<td>15.5±9.8</td>
<td>36.9±4.8</td>
<td>0.0332*</td>
<td>26.5±9.1</td>
<td>45.9±9.8</td>
<td>0.1657</td>
</tr>
<tr>
<td>NGAL (ng)</td>
<td>0.08±1</td>
<td>0.3±12</td>
<td>0.0984</td>
<td>12.5±9.8</td>
<td>20.2±12.0</td>
<td>0.6309</td>
</tr>
<tr>
<td>IL6 (pg)</td>
<td>0.86±.4</td>
<td>3.8±8.9</td>
<td>0.1909</td>
<td>7.52±4.4</td>
<td>6.3±4.6</td>
<td>0.8475</td>
</tr>
<tr>
<td>IL1 α (pg)</td>
<td>3.8±3.8</td>
<td>1.74±1.07</td>
<td>0.6247</td>
<td>56.9±12.5</td>
<td>26.2±12.3</td>
<td>0.0963</td>
</tr>
<tr>
<td>IL1 β (pg)</td>
<td>2.2±1.0</td>
<td>2.5±5</td>
<td>0.8124</td>
<td>6.6±1.4</td>
<td>9.5±3.4</td>
<td>0.461</td>
</tr>
</tbody>
</table>

* significant p < .05

values expressed as mean ± standard error of the mean (SEM)
Table 15. Control and Cocaine Positive Male and Female Urine Specimen Comparison

<table>
<thead>
<tr>
<th>Assay</th>
<th>Control</th>
<th>Cocaine Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Total Protein (pg)</td>
<td>32.2±12.1</td>
<td>65.9±20.8</td>
</tr>
<tr>
<td>Aldosterone (pg)</td>
<td>1907.7±356.7</td>
<td>4917.96±1159.9</td>
</tr>
<tr>
<td>hsCRP (ng)</td>
<td>0.78±.6</td>
<td>202.3±103.0</td>
</tr>
<tr>
<td>Myeloperoxidase (ng)</td>
<td>20.5±7.9</td>
<td>187.1±81.3</td>
</tr>
<tr>
<td>Microalbumin (ug)</td>
<td>18.4±7.3</td>
<td>41.2±14.4</td>
</tr>
<tr>
<td>Hsp90 α (ng)</td>
<td>0.92±.4</td>
<td>0.3±.2</td>
</tr>
<tr>
<td>VEGF (pg)</td>
<td>2453.0±972.5</td>
<td>3286.2±1402.6</td>
</tr>
<tr>
<td>proANP (nmol)</td>
<td>0.3±1</td>
<td>0.34±1</td>
</tr>
<tr>
<td>Myoglobin (ng)</td>
<td>15.5±9.8</td>
<td>26.5±9.1</td>
</tr>
<tr>
<td>NGAL (ng)</td>
<td>0.08±.1</td>
<td>12.5±9.8</td>
</tr>
<tr>
<td>IL6 (pg)</td>
<td>0.86±4.4</td>
<td>7.52±4.4</td>
</tr>
<tr>
<td>IL1 α (pg)</td>
<td>3.8±3.8</td>
<td>56.9±12.5</td>
</tr>
<tr>
<td>IL1 β (pg)</td>
<td>2.2±1.0</td>
<td>6.6±1.4</td>
</tr>
</tbody>
</table>

* significant p < .05

values expressed as mean ± standard error of the mean (SEM)
Chapter 5.0

Discussion

There are well-documented gender based differences in the etiology and pathophysiology in many disease states [62, 64, 67, 70, 71, 73-75, 78, 93, 127-142]. Gender differences are well known to exist for many important markers of disease including triglycerides, enzymes such as AST and SGOT, HDL, uric acid, creatine and hormones. While blood has remained the medium of clinical preference, toxicological screens however are frequently done on urine. Unlike serum and plasma, urine typically lacks interference from clotting and compounds such as proteases [143]. It is well documented that many proteins are expressed in urine, particularly during certain disease states; in fact, the human urinary proteome contains more than 1500 proteins [144]. Little is known about the differences in urinary marker expression between males and females.

Recently, the use of urine as a medium to analyze the expression of many proteins in the body has become more acceptable [145]. Urinary analysis takes advantage of protein cell and tissue specificity and their alteration over time in response to different situations [146]. The proteins and peptides in urine are referred to as the urinary proteome; variation in the levels of these protein biomarkers has been associated with
certain pathological states. Markers in the urinary proteome have been validated for inflammation, oxidative stress, cardiovascular dysfunction and renal disorders [144, 145].

Inflammation is a very common, non-specific form of pathophysiology; myeloperoxidase, C reactive protein, and IL 6 are markers of acute and systemic inflammation [147, 148]. MPO is also commonly used as an endpoint of oxidative stress [149]. IL-1α and IL-1β are immunoregulatory cytokines that favor inflammation; increased expression is associated with fever, inflammation, tissue destruction, shock and death. The difference between male and female CRP and MPO were close to statistical significance in control urine. IL-1α and IL-1β expression is also associated with autoimmune disorders. It is possible that the gender disparity seen in autoimmune disorders could reflect a chronic, low-level activation of the immune system that might be seen in elevated interleukins [150]. In control urine, female IL-1α and IL-1β were higher than male values by a statistically significant amount. Elevated concentrations of interleukins upregulate acute phase proteins such as IL 6 and CRP. Albuminuria is associated with cardiovascular disease, kidney dysfunction and DM [151-153]. Urinary NGAL expression is linked to nephritis and urinary tract infections [105, 154]. Creatinine is a general marker of myolysis and kidney disease [155, 156]. Myoglobin is another marker of myolysis; it was considered the gold standard of cardiac markers prior to the characterization of troponin. There were no significant gender based results in the analysis of IL6, microalbumin, NGAL or myoglobin. There was a statistically significant increase in myoglobin demonstrated in male cocaine positive urine specimens as compared to male control urine.
Oxidative stress has been implicated in a variety of inflammatory processes. For example, increased vascular oxidative stress leads to endothelial dysfunction and hypertension. VEGF, a marker of angiogenesis, may also be elevated by endothelial dysfunction [157]. Microalbumin expression is enhanced by many of the same pathophysiological conditions as VEGF. It is linked to increased cardiovascular and renal risk factors. Neither microalbumin nor VEGF were statistically significant in any analysis. Increased oxidative stress is implicated in the activation of the RAS and subsequent elevations in aldosterone. Aldosterone is a renal hormone in the RAS secreted in response to hypotension. Aldosterone was higher in female control urine, when compared to male control urine, by a statistically significant amount. There was also a statistically significant increase in aldosterone in male cocaine positive urine when compared to male control urine specimens. Increased cardiac concentrations have been linked to heart failure. It has been observed that inhibition of the RAS results in a decline in ROS production [65, 66]. proANP is a hypotensive peptide that counteracts the RAS by inhibiting the release of hormones such as aldosterone and renin [158]. Increased ANP levels are detected in adult congestive heart failure, chronic renal failure and in severe essential hypertension [158, 159]. No statistically significant results were seen in this study in any analysis of proANP.

While there are noted gender based differences in the etiology of cardiac and vascular disease, particularly in the area of comorbid conditions, underlying blood pressure and resultant mortality, the basis of the gender dimorphism is unclear. Clearly hormones may play an important role as cardiac muscle and vascular tissue are influenced by hormones such as estrogen and testosterone [1, 9, 10, 12, 59-61].
relationship between inflammatory cytokines and gender has not been clearly elucidated, although some differences in serum levels of risk markers, including lipids and acute phase proteins have been noted [6, 27, 29, 30, 80-82]. Studies suggest there may be gender related differences in the regulation of IL-1 so it is possible that the interleukins serve different functions depending on gender [160, 161]. Mean concentrations of IL 1α and IL 1β were significantly higher in the control urine of women than in men.

IL-6 has both pro and anti-inflammatory roles depending on the target cell. It has been dubbed a ‘myokine’, a cytokine produced by muscle and elevated during muscle contraction and has been found to act synergistically with both IL-1 subunits [162, 163]. Elevations in IL-6 frequently precede increases in CRP [164]. It may be possible that alterations in urinary IL-6 expression were transitory in our study. This could explain the failure to detect a significant difference in IL6 between control and metabolite positive urine specimens.

Women tend to have slightly higher serum CRP than men with the same BMI and age; however, these differences are not pronounced enough to warrant gender-dependent reference ranges [135, 165]. The role of gender in predisposition to oxidative stress has yet to be determined. The clinical and prognostic relevance of these differences requires further research. CRP is an acute phase inflammatory marker considered to be a reliable prognostic indicator of atherosclerosis and acute myocardial infarction (AMI) [61, 82, 86]. Urinary levels peak approximately 24 hours following the inflammatory event and are undetectable within 13 to 16 hours. The differences between male and female CRP means in control urines in this study were just outside of statistical significance
Cocaine metabolites can remain in the urine for 48 to 72 hours following acute use, whereas CRP in urine quickly wanes. The results from this study suggest that CRP levels in urine may not be a reliable prognostic indicator for these pathophysiological processes at least under the conditions of this study.

As a measure of leukocyte infiltration, the concentration of MPO may increase significantly in an acute infection such as those of the urinary tract. Elevated MPO is linked to adverse outcomes in acute coronary syndromes and can be used to identify patients at risk for cardiac events in the absence of cardiac necrosis [54, 87]. Elevated MPO levels have been shown to independently predict increased risk of major adverse cardiac events including MI at 30 days and 6 months [166]. The predictive value of this marker has not been fully characterized; however, it is associated with long term adverse cardiac events as well. The differences between male and female MPO means in control urines in this study were just outside of statistical significance (p=0.0562).

Cocaine is well absorbed mucosally and metabolized almost immediately by serum cholinesterase [56]. The serum half-life of cocaine is less than an hour. The pharmacodynamics do not change substantially for different routes of entry despite differences in onset of action, peak absorbance and duration of effect [167]. BE and EME are the primary metabolites; up to 50% of cocaine is transformed to BE and 30 to 40% is metabolized to EME. Although neither metabolite is pharmacologically active, both BE and EME been shown to elevate blood pressure [43]. The intense cardiotoxic interaction between ethanol and cocaine was the primary reason for excluding ethanol.
positive urine specimens from analysis. CE has a significantly longer half life than both cocaine and BE; inclusion of specimens that could potentially contain CE could affect the expression of biomarkers such as myoglobin, CRP and MPO.

Cocaine increases intracellular oxygen demand, elevates heart rate and arterial blood pressure [35, 168]. Cocaine use has been linked to hypertension and tachycardia, although the effects on heart rate are variable [42, 169]. Increases in myocardial oxygen demand are associated with the induction of oxidative stress. Cocaine use has been further shown to enhance endothelial permeability, which may be linked to subclinical cardiovascular disease, vascular dysfunction, kidney disease, hypertension, nephrotic syndrome, glomerulonephritis, interstitial nephritis, and rhabdomyolysis [34, 49, 58, 115].

Atherosclerosis, an endothelial cell dysfunction, is characterized by the development of abnormal vascular responses and the expression of proinflammatory and prothrombotic factors [50]. MPO is expressed as a result of atherosclerotic lesions such as those seen in chronic cocaine use. Females had a significantly higher mean concentration than did males. Estradiol has been suggested as a potential endogenous substrate for MPO in plasma [88]. As with CRP, the differences between male and female MPO means in control urines were just outside statistical significance (p=0.0562).

Examination of male and female cocaine positive urine specimens yielded no statistically significant differences. While the male mean Hsp90α was higher than the female mean (p=.0650), this result is not statistically significant. It should be noted that
several markers that exhibited significant differences in control urines were no longer significant in cocaine positive urine. In cocaine positive urine, the mean male aldosterone was significantly higher than the male control mean (p=0.0095). The female cocaine positive aldosterone mean was somewhat lower than the control mean although the difference was not significant. Mean concentrations of aldosterone were significantly higher in the control urine of women than in men.

Increased vascular oxidative stress may lead to alterations in endothelial function and may be related to hypertension [26]. The hypertensive effects of cocaine use are well established [36, 56]. Cocaine has been shown to activate the renin-angiotensin system (RAS) which contributes to its cardiotoxicity. ProANP is secreted from cardiac atria in response to myocyte strain and ischemia; increased levels of proANP have been observed in heart failure, left ventricular dysfunction, coronary artery disease, and renal failure [170]. The ANP pathway decreases blood pressure in response to hypertension [171]. Elevated proANP is considered predictive in renal failure, AMI and heart failure [172, 173]. ProANP is cleared from the circulation in 60 to 120 minutes; it seems likely that there would not be a detectable amount in urine unless the specimen was collected within 8 to 12 hours of the pathological insult. There were no significant differences in any analysis of proANP.

While it well known that cocaine affects the thermoregulating centers of the brain, it is unclear if this effect is exerted directly or via endogenous cytokines such as interleukins. IL-1α and IL-1β, acute phase response cytokines, are endogenous pyrogens capable of resetting the hypothalamus thermoregulatory center to change body
temperature [91, 174]. Our results suggest that cocaine may induce hyperexia through
the interleukins. The cellular consequences of heat and ischemic stress are quite similar
[175]. If cocaine exerted a significant thermogenic effect in our study following usage,
IL-1α and IL-1β could be anticipated to be elevated in urine specimens of users.
However, there were no significant differences between the urine of the control group
versus the cocaine positive. Unfortunately, no information was available at the time of
urine collection as to donor body temperature. Clearly, this is an important parameter that
needs to be investigated further in a prospective study.

NGAL exists primarily as a monomer in neutrophils and urine; it also occurs as a
complex with matrix metalloproteinase-9 (MMP-9). NGAL is expressed at a low level in
other tissues including the kidney, prostate and epithelia of the respiratory and alimentary
tracts. It is presently being investigated for validation as a biomarker of urinary tract
infections [176]. NGAL is upregulated in processes that include apoptosis, ischemia,
organogenesis and inflammation.

Increased levels of NGAL from acute renal injury can be detected in both urine
and blood within two hours of the insult [57]. Taken in conjunction with its presence in
urine and association with ischemia, NGAL may be a significant marker of cardiac
dysfunction stemming from cocaine use [177-179]. Most cocaine-induced chest pains do
not progress to AMI. The chest pains due to cocaine are induced by myocardial ischemia
via vasospasm. Given the role of NGAL in oxidative and inflammatory processes, we
were surprised not to see a difference in mean values of NGAL expression. The lack of
statistically significant differences did allow us to tentatively rule out UTIs as the basis
for higher mean NGAL concentrations seen in female urine specimens. Coupled with the unlikelihood that the entire female cohort suffered from UTIs, the fact that male specimens had uniformly lower NGAL concentrations than did the females appears to suggest gender based expression differences.

Myoglobin lacks cardiospecificity; elevated levels are considered strongly suggestive of AMI – particularly in the presence of other symptoms. Elevated levels of myoglobin are also associated with rhabdomyolysis, renal failure and trauma [58]. Myoglobin is released within 2 to 4 hours of significant myocardial and/or skeletal muscle injury. Levels peak 6 to 12 hours following the pathophysiological event; returning to prerenecrotic levels within 36 hours. An estimated 24% of cocaine users develop rhabdomyolysis [58]. When male control urine specimens were compared to cocaine positive specimens, there was a statistically significant difference.

The results of this study suggest there are gender-specific and cocaine-based differences in oxidative and inflammatory cytokine expression in urine. Males and females exhibit different morbidity and mortality across a wide range of conditions which suggests basic pathophysiology may control on the incidence of multiple disease processes.
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Conclusions

The purpose of this study was to investigate whether or not gender differences may be present in the expression of a number of urinary proteins which may serve as markers of inflammation and oxidative stress. It was concluded that the urinary expression of aldosterone, IL-1α and IL-1β appears to be influenced by gender. Mean aldosterone (p=0.0223), IL1 α (p=.0007) and IL1 β (p=.0198) concentrations were significantly higher in female control urine when compared to male control urine. The results for hsCRP (p=.0662) and MPO (p=.0562) were also higher in female control urine when compared to male control urine; however, they did not reach statistical significance. The pathophysiological mechanisms associated with gender based differences in marker expression will require additional prospective study with expanded demographic information.

The urinary expression of aldosterone and myoglobin in males is influenced by the use of cocaine. There were statistically significant increases in myoglobin (p=.0095) and aldosterone (p=.0332) expression in the cocaine positive urine of males. Oxidative metabolites of cocaine generate significant ROS; much of the pathophysiological damage
associated with cocaine use stems from oxidative stress and electron transfer[32].
Cocaine use affects almost every organ system; myocardial infarction, arrhythmias, renal failure, hypertension, atherosclerosis, and rhabdomyolysis are all in the spectrum of acute and chronic cocaine toxicity [114, 115].
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About the Author

Marie Bourgeois is a graduate of University of South Florida with a B.S. in Clinical Chemistry (1991) and an M.P.H. in Toxicology and Risk Assessment (2006) from the University Of South Florida College Of Public Health. In 2007, Mrs. Bourgeois was accepted to the Ph.D. program in Toxicology and Risk Assessment at the University Of South Florida College Of Public Health by the Department of Environmental and Occupational Health. Her degree focused the impact of gender and cocaine use on the urinary expression of biomarkers of oxidative stress and inflammation. She held the position of Graduate Assistant while at the College of Public Health and helped administer online courses. She has been a presenter at national conferences and is the coauthor of several publications and abstracts.