Investigations into the therapeutic potential of a bacterial cocaine esterase for the treatment of cocaine toxicity and cocaine abuse

by

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Dedication

This thesis is to my parents, Barry and Claudia Brim, for their 25 years of love support and sacrifice that made this work, and all my dreams, possible.

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5-HT: 5-hydroxy-tryptamine (serotonin)

BChE: human butyrylcholinesterase

CocE: Bacterial Cocaine Esterase

DA: dopamine

DAT: dopamine transporter

FDA: United States Food and Drug Administration

Hr: Hour

IP: intraperitoneal

IPTG: isopropyl β-D-1-thiogalactopyranoside

IV: intravenous

K_m: concentration of substrate needed to achieve an enzyme velocity one-half the maximum

Min: minute

NE: norepinephrine

NET: norepinephrine transporter

PBS: Phospho-buffered Saline, pH 7.4

KQ-CocE: L169K/G173Q Cocaine Esterase

RQ-CocE: T172R/G173Q Cocaine Esterase

SERT: serotonin transporter

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

$t_{1/2}$: half-life

V_{max}: maximum enzyme velocity

WT: wild-type

Abstract

Cocaine use is a widespread problem in the United States with 2 million current users who make about half a million emergency department visits each year. Although use is prevalent, there is currently no FDA-approved pharmacotherapy to specifically treat cocaine abuse or cocaine toxicity. Approaches toward developing therapies for these indications include small molecule inhibitors of cocaine binding and cocaine vaccines. These methods have proven to block the strong reinforcing properties of the drug; however, they do not prevent nor reverse cocaine's serious physiological effects. Our approach to treating cocaine toxicity and abuse is to rapidly hydrolyze the cocaine molecule into inactive metabolites using a bacterial cocaine esterase (CocE), thus eliminating cocaine's harmful and strong reinforcing effects.

This work has taken major steps toward developing CocE into a viable pharmacotherapy for both cocaine addiction and toxicity. Initially two thermostabilizing mutations were combined to improve the half-life of CocE. This mutant was pharmacologically characterized *in vitro* as well as *in vivo* using rodent models of cocaine lethality and reinforcement. The pharmacodynamic and pharmacokinetic properties of CocE, including *in vivo* rates of cocaine hydrolysis across species, circulating half-life, and mechanisms of elimination, were assessed. CocE's capacity to hydrolyze cocaine in the presence of commonly co-abused drugs, and its capacity to hydrolyze active cocaine metabolites were investigated. The results from these studies support the notion that cocaine esterase displays strong therapeutic potential, and that it

may proceed towards clinical development. Moreover, it is a comprehensive analysis of how protein biologics may be used as effective pharmacotherapeutics.

Chapter 1

Introduction

History of Cocaine, Use, and Abuse

Cocaine is a natural alkaloid derived from the leaves of the South American shrub *Erythroxylon coca*. Members of the Inca Empire, who originally populated South and Central America, chewed the leaves of the coca plant to induce feelings of well-being and to alleviate hunger [1]. The uses of coca leaves, along with the first accounts of tobacco use, were originally documented in 1569 by Nicolas Monardes, a Spanish physician who published a book on plants of "the New World" [1-3]. Coca was not used prevalently because it could not be smoked, and its leaves lost potency during shipment from South America [3].

The interesting properties of coca leaves were rediscovered in 1845 by the French physician Charles Fauvel after he used an alcohol solution containing soaked coca leaves to perform throat surgery without pain to the patient [1]. The active ingredient of the coca leaf, "cocaine", was extracted in 1860 by Albert Niemann [1, 4] who drew comparisons to other plant-based stimulants like coffee and tobacco. Ten years after this, Karl Koller discovered that drops containing cocaine could be used as an effective local anesthetic for the eye [1, 4] which acted as a catalyst for the use of cocaine medically. Cocaine demand soared over the last part of the century, exemplified by the increase in Merck's production from 1.4 kg in 1883-4 to 30 kg in 1884-5 [4].

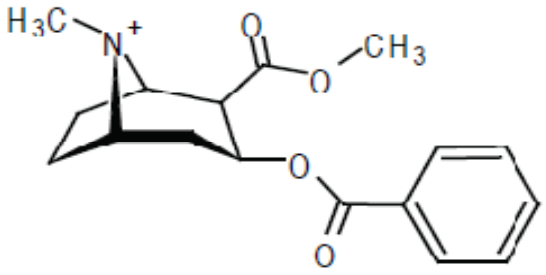

Figure 1.1 Chemical structure of cocaine

As cocaine became widely available, physicians began administering cocaine as a local anesthetic and experimenting with the drug on themselves. They quickly encountered instances of cocaine overdose and addiction. For example, in 1884, Dr. William Halsted invented nerve-block anesthesia by injecting cocaine directly into major nerve fibers. However, by 1886, Halsted himself had developed a cocaine habit that was reportedly up to 2 grams per day, and he disappeared mysteriously [5].

The French pharmacist Angelo Mariani popularized coca to the European public. Mariani prepared “wine tonics” in the late 1860’s, which were mixtures of chemicals and wine that made chemicals soluble and more palatable to consume. His tonic containing coca and wine sold as Vin Mariani by 1870 and contained about 150-300 grams of cocaine per liter. American coca wines contained more cocaine than Vin Mariani, close to 400 grams per liter in the case of H. Casewell & Company’s wine. The well-known soft-drink Coca-Cola contained only 5.3 mg per liter. The problems of cocaine abuse and addiction were really never a consequence of these very popular coca wines because oral bioavailability of cocaine is low, and even drinkers of coca wines with high cocaine concentrations could never achieve the high blood concentrations of cocaine observed in modern cocaine abusers.

By the turn of the 20th century, the licit and illicit markets were flooded with cocaine and the psychoactive properties of the drug were discovered. Between 1910 and 1920 the earliest cases of perforated septum from the repeated nasal insufflation of cocaine were reported [1]. This route of administration creates much more rapid and complete absorption into the blood stream and significantly contributed to cocaine's abuse potential. Descriptions of delirium and crazed behavior were being reported, particularly among the poor and especially during the prohibition era [1].

By the late 1920s, the initial wave of cocaine use tapered, most likely due to high-profile overdoses and increased publicity about the dangers of cocaine through literature, the government, and the press [1]. In addition, the availability of cocaine diminished from the disruption of trade routes during World War I. By the time these routes re-opened, the drug had become de-popularized and was too expensive for the general public.

It was not until the 1980s when the popularity of cocaine resurfaced. David Musto, a drug policy expert and policy advisor to Jimmy Carter, speculates that cycles of drug abuse are produced when a new generation forgets the lessons learned by a previous generation [7]. This is most likely the case for cocaine, in combination with the increase in supply and decrease in price due to more efficient distribution and new production technologies. The introduction of free-base or "crack" cocaine at this time decreased price, increased accessibility, and popularized the drug because it could be smoked. Women were especially susceptible to the lure of crack, as smoking behavior was being glamorized by the cigarette industry during the same period.

Through government drug control, especially the criminalization of cocaine possession (nearly 1/3 of the prisoners in the United States are drug offenders [1]), the

current rate of cocaine use is much lower than in the 1980s. It is estimated that there are still approximately 2 million Americans that self-report being cocaine users [8], although the actual number is most likely higher according to recent studies. Fifteen percent of Americans have tried cocaine [8] and 6% of high school seniors have used the drug [9]. Close to half a million emergency room visits occur each year due to cocaine related toxicities [10], making cocaine the leading cause of illicit drug-related emergency department visits, nearly double the visits for opioids like heroin [11]. The economic cost to the United States government from drug abuse has grown to a staggering $180.8 billion due to a combination of the costs associated with health care, criminal justice, social welfare, incarceration and lost productivity [12]. However, there is currently no FDA-approved pharmacotherapy designed to treat cocaine addiction or cocaine toxicity.

Mechanism of Action of Cocaine

Outside of medical use, cocaine is generally administered via nasal insufflation (snorting) or intravenously (shooting). "Crack" cocaine (cocaine in it's free-base form) is smoked, producing peak effects nearly as quickly as intravenous cocaine. Cocaine elicits its powerful effects through a myriad of mechanisms in the human body.

Cocaine blocks sodium channels on non-myelinated C-type nerve fibers, blocking the ascending pain pathway and giving cocaine the anesthetic properties for which it was first known [13]. Cocaine also blocks voltage-gated ion channels elsewhere in the body. Blockade of cardiac sodium and calcium channels alters proper cardiac conductivity [14, 15] and blockade of neuronal sodium and potassium channels increases excitability of hippocampal neurons [16, 17] and decreases GABAergic

inhibition of dopaminergic neurons in the ventral tegmental area [18]. Cocaine also blocks potassium channels. The alteration of membrane potentials throughout the body by cocaine's promiscuous binding results in massive alterations in neurotransmission as well as paracrine and endocrine signaling.

The sites of action responsible for cocaine's behavioral consequences are the monoamine transporters for dopamine (DA), norepinephrine (NE), and serotonin (5-HT) [19-21]. Using DA as the representative monoamine, under normal physiological conditions, DA is released from pre-synaptic nerve terminals and acts on post-synaptic dopamine receptors, as well as pre-synaptic autoreceptors. The duration and intensity of DA signaling in the brain is regulated by the concentration of DA in the synapse. Once DA is released it is quickly removed from the synapse by reuptake through the dopamine transporter (DAT) and by degradation by monoamine oxidases. When cocaine binds with high affinity to the DAT, it blocks the transport of DA back into the pre-synaptic terminal. As a result, high concentrations of DA accumulate in the synapse causing a flood of signaling through the post-synaptic dopamine receptors. This, along with increases in 5HT signaling, are the basis for the feelings of euphoria and well-being associated with the drug [19-21, 106-107]. Elevated noradrenergic signaling is responsible for increased heart rate, blood pressure and vasoconstriction, accounting for the feelings of agitation and restlessness documented by cocaine users.

Cocaine-elicited increased dopaminergic and serotonergic signaling are primarily responsible for the powerful addictive effect of the drug and leads to physiological cocaine dependence. In the presence of cocaine, the synaptic DA concentration is elevated, increasing dopaminergic signaling. When cocaine is used over a long period of time, post-synaptic dopamine receptors become desensitized and down-regulate. This decrease in total receptor sensitivity reduces the brain's response to basal

dopamine levels and to natural increases in dopamine levels caused by pleasurable activities such as eating and sex. Thus, a cocaine abuser must continue to take cocaine to achieve a “normal” state of dopaminergic function; this is known as dependence [3, 105]. This physiological phenomenon also occurs in the serotonergic and noradrenergic systems [3, 105-107].

Cocaine is readily and extensively metabolized with a half-life of only 60-90 minutes in humans, degrading into 40-45% benzoylecgonine (by liver human carboxylesterase-1, hCE-1) [22], 40-45% ecgonine methyl ester and benzoic acid (by serum butyrylcholinesterase, BChE), and 5% norcocaine (by P450 CYP 3A4) [23, 24]. If alcohol is present, hCE-1 catalyzes the transesterification reaction of ~10% of cocaine to cocaethylene, and consequently reduces the production of benzoylecgonine [22, 25]. Norcocaine and cocaethylene are both equipotent to the parent molecule at blocking cardiac sodium channels [26-28], and cocaethylene is more potent than cocaine at monoamine transporter blockade [29, 30]. Metabolites of cocaine will be discussed further in Chapter 4.

Presentation and treatments for cocaine toxicity

High doses of cocaine have toxic effects on the body. Clinically, cocaine intoxication presents with cardiovascular, respiratory and neurologic complications, hyperthermia, and psychosis [31]. Although cocaine overdose victims do not exhibit all of these toxic symptoms at once, there are specific drugs used by emergency personnel to manage and treat these patients. Patients presenting with altered mental status receive dextrose (to rule out hypoglycemia), thiamine (to prevent Korsakoff's syndrome, a deficiency in vitamin B1 in the brain from poor nutrition or alcoholism) and naloxone (to reverse potential opioid overdose) [3]. Patients are also given a benzodiazepine for

sedation in the cases of agitation or delirium. Beyond the foregoing, only symptomatic treatment of the effects of cocaine can be employed.

Cocaine can cause a myriad of arrhythmias and cardiac arrest [3, 31]. These cardiac effects are caused by action at several of cocaine's targets. In addition to producing ischemia and myocardial depression, cocaine can promote the release of excess of catecholamines, which can elicit ventricular fibrillation and ventricular tachycardia. This makes it difficult to determine the proper pharmacotherapy. For example, epinephrine is indicated in the case of ischemia and myocardial depression, but epinephrine may exacerbate toxicity if an excess of catecholamines is already maximally stimulating cardiac alpha-adrenergic receptors. There is no clear consensus on which drug is most effective in these scenarios. Lidocaine and other Type 1 antiarrythmics are sometimes used however these can exacerbate cocaine's depressive effects [31]. Beta-adrenergic receptor antagonists (β-blockers) are theoretically effective in these instances, but studies performed in the early 1990s demonstrated that use of β-blockers increased vasoconstriction due to uninhibited alpha-adrenergic activity [3, 32, 33], again exacerbating cocaine's effects. β-blockers have been contraindicated for the treatment of cocaine-induced arrhythmia since that time. Recently, a new study suggested that β-blockers may indeed be a suitable option for a large percentage of these patients, as no harm was seen after β-blockers were administered for cocaine-related chest pain in a modern clinical setting [34]. The types of arrhythmias seen with cocaine intoxication are most commonly short-lived and can be closely monitored until cocaine concentrations decline; however, their consequences can be lethal.

Cocaine causes ischemic chest pain, often seen up to 24 hours after cocaine use. New literature suggests that this latent chest pain may be caused by the cocaine metabolite benzoylecgonine (see Chapter 4). The management of this chest pain can

be treated similarly to that of non-drug-users, by administering nitrates plus either a beta- or calcium channel-blocker. A recent study suggests that patients presenting with cocaine-related chest pain are not at risk for further cardiac events if a short observation period (9-12 hours) elapses without ischemic or cardiac changes [35].

Hypertension associated with the strong vasoconstrictive effects of cocaine is often accompanied with agitation and both can be treated with benzodiazepine sedation. Often this hypertension will fall into normal levels within a short observation period and additional pharmacological intervention is not needed. The vasoconstrictive effects can also present as abdominal cramping from intestinal ischemia [31].

High-dose cocaine causes tonic-clonic seizures in both animal models and in humans. Diazepam is commonly given to reduce seizures and protects against cocaine lethality [3]. Cocaine-induced neurotoxicity can also present as hemorrhage, intracranial bleeding and ischemic strokes. The rapid increase in blood pressure caused by systemic vasoconstriction can cause hemorrhage and usually occurs in patients with underlying conditions [31]. In addition to neurotoxicity, cocaine also causes neuropsychiatric states including violent agitation, delusions, paranoia, and psychosis. Benzodiazepines such as diazepam are used to treat these symptoms and protect against possible seizures if they have not yet occurred. Patients on benzodiazepines must be carefully monitored due to their possible concurrent states of hypothermia, trauma, and acute renal dysfunction.

Hyperthermia, which is a result of an excess of sympathomimetic signaling, excessive ignition, vasoconstriction and seizures is treated with rapid cooling with ice packs and IV fluids. Drugs for malignant hyperthermia, like dantrolene, are not effective

in cocaine-induced hyperthermia as the mechanisms producing the hyperthermia differ [31].

It is thus apparent that with cocaine's myriad of physiological effects, it is difficult to tailor a therapy and, although most symptoms can be managed with benzodiazepines, the cocaine molecule is still able to damage the liver, kidneys, brain and heart.

Current and developing treatments for cocaine addiction

The most logical way to prevent cocaine toxicities is to prevent cocaine use among the most susceptible population for toxicity: cocaine abusers. The only treatments available for cocaine abuse are behavioral therapies. Even if a pharmacological treatment is developed, addicts will need extensive therapy, as with any addiction. Thus, a drug that can block the reinforcing effects of cocaine will always be used in conjunction with behavioral therapies.

Cognitive behavioral therapy (CBT) is successful in the treatment of cocaine abuse for some users. CBT is made up of two components: functional analysis, which helps the patient identify their state of mind and environment before and after they use cocaine, and skills training, which teaches patients how to use coping strategies and healthier life skills that do not involve drug use [36]. CBT is normally short in duration (about 12 weeks) and, in many cases, does not reduce cocaine use during the time of treatment [37]. However, in the long-term, individuals undergoing CBT showed a great improvement over those who were not exposed to CBT [36, 37].

Contingency management or motivational incentives (CM/MI) is another successful treatment option for some substance abuse patients that offers rewards as an incentive for positive behavior [38]. Generally, this behavior consists of producing drug-free urine tests and allows the patient to earn an incentive, such as money, the

opportunity to work for money, or tokens that can be exchanged for a reward. This approach produces a rapid decrease in cocaine-taking behavior compared to no therapy, but there is some debate as to whether these effects are sustained long-term after incentives stop [39, 40]. There are differences between successful CM/MI programs, as the choice of behavior rewarded, incentive, frequency of incentive and duration of program are all variable, and all affect responses [38]. Overall, CM/MI has been effective in many clinical studies used alone or in combination with other behavioral [41] or pharmacological therapies. However, this evidence-based method is currently not well-accepted by substance abuse treatment counselors or integrated into practice due to a negative perception of giving rewards for abstinence and a lack of education and training about CM/MI [42].

Developing a pharmacotherapy to treat cocaine addiction has been a difficult task, in part because of a lack of good pre-clinical models. The reinstatement of cocaine self-administration behavior in rats induced by cues, context, stress, or cocaine is currently the model used most to test drugs in development. A myriad of both monoaminergic and non-monoaminergic drugs have been tested in these models with varying success. There are vast differences between studies using the same drug, or same class of drug, due to the variability in route, timing, and regimen of drug dosing [43].

Although this abundant body of pre-clinical animal research has given mixed results, several clinical trials have been conducted. Drugs that increase GABAergic activity (baclofen, topiramate) have reduced cocaine intake and increased cocaine negative urine screens [44-46]. Vigabatrin, a GABA transaminase inhibitor, also increased cocaine negative urine screens [47]. The negative side-effects of these GABAergic drugs include drowsiness and depression, which are not conducive to re-

entering a sober society. Interestingly, tiagabine, an inhibitor of pre-synaptic GABA release that reduces GABAergic activity, also decreased cocaine use [48, 49]. The discrepancy between the mechanisms of action of the drugs that reduced cocaine intake points to the complications in understanding synaptic pathways and the potential off-target effects of these drugs.

Dopaminergic drugs have been extensively tested as potential treatments for cocaine abuse in human subjects. Buproprion, which, like cocaine, blocks the reuptake of dopamine but with slower kinetics, has shown both variable [50] and negative [51] results in human trials. Disulfiram has been shown to reduce cocaine use [52, 53], possibly through the inhibition of dopamine β-hydroxylase, effectively reducing the concentration of norepinephrine in the brain. The overall reduction of this monoamine in the brain of a cocaine user changes the physiological effects of the drug and thus disconnects cocaine use from the normal internal cues it elictis [54]. When tested in a large placebo-controlled study, low doses of disulfiram increased cocaine use but high doses were inconclusive [55]. Risperidone and olanzapine (antagonists at both the DA D_2 receptor and the 5-HT_{2A} receptor) are antipsychotic drugs currently used to treat schizophrenia and showed promising results in schizophrenics with co-morbid cocaine addiction [56, 57]. However, trials examining these drugs in non-schizophrenic cocaine-dependant individuals showed no significant reduction in cocaine use [58-61]. Aripiprazole, a partial agonist at both the DA D_2 receptor and the $5HT_{1a}$ receptor, has shown promise in blocking self-administration in mice and blunting the effects of methamphetamine in human laboratory studies [62, 63]. A Phase II clinical trial is currently being conducted with aripiprazole to evaluate whether it decreases cocaine self-administration, subjective effects and cravings compared to placebo [64]. The D1

agonist DAS-431 (adrogolide HCl, ABT-431), decreases subjective scores of cocaine "high", but lacks desirable pharmacokinetic properties [65].

It is apparent that small molecule drugs have shortcomings in efficacy for treating cocaine abuse. To this end, several groups have started examining alternative approaches to treating this complex health problem, including active immunization, catalytic antibodies, and cocaine hydrolyzing enzymes.

Active immunization for the treatment of cocaine abuse was initiated by the development of an anti-cocaine vaccine, TA-CD, by Celtic Pharmaceuticals. This vaccine was created by the fusion of succinylnorcocaine to a non-toxic region of recombinant cholera toxin and is administered with aluminum hydroxide as an adjuvant [66, 67]. Theoretically, a vaccine would elicit the production of antibodies against cocaine, and upon cocaine use, cocaine would be sequestered away from the central nervous system and away from its peripheral sites of action. Thus the vaccine would blunt the cocaine "rush" upon use of the drug and help to disconnect the behavior of cocaine-seeking with reward. The vaccine is meant to be used in conjunction with behavioral therapies, as the blockade of cocaine's effects is not expected to be complete.

These principles were confirmed by an early study that used cocaine antibodies to block cocaine self-administration behavior in rats and elicited an immune response in mice sufficient to reduce both brain and plasma levels of cocaine compared to controls [68]. Phase I trials of TA-CD demonstrated the safety and efficacy of the vaccine to produce anti-cocaine antibodies in humans, but there was also the loss of immune response over time [67]. TA-CD was efficacious at reducing relapse rates in a Phase IIa clinical trial and its safety profile was further validated. For those individuals who did

relapse over the course of the study, cocaine's effects were blunted [69]. This blunting was further evaluated and is positively correlated to the number of antibodies the individual produced after vaccination [70]. The study also found that individuals with higher antibody titers had increased cocaine-induced cardiovascular disturbances, most likely due to the sequestration of cocaine in the periphery. Preclinical studies demonstrate increased blood cocaine concentrations in the periphery of vaccinated animals, and thus cocaine could more readily bind adrenergic receptors. The full Phase II placebo-controlled trial is currently recruiting patients [71].

Catalytic antibodies have been generated that not only bind to cocaine producing the same effects as the vaccine, but also hydrolyze cocaine itself. Since the original publication [72] describing cocaine hydrolysis by a catalytic antibody, successful results were seen in rats and mice with mAb 15A10 which blocked both the toxic and reinforcing effects of cocaine [73-77]. However, when mAb 15A10 was tested for efficacy in non-human primate models of cocaine use, similar results were not observed [78].

Although the catalytic antibody could not achieve results in primate models, the concept of rapid cocaine hydrolysis to treat cocaine toxicity and abuse was being used to concurrently develop cocaine hydrolyzing enzymes. It is well known that cocaine is hydrolyzed by serum butyrylcholinesterase (BChE) into the inactive metabolites ecgonine methyl ester and benzoic acid. Studies using large doses of wild-type BChE have shown reversal of cocaine-induced toxicity and rapid removal of cocaine from the serum [79-84]. These studies were the basis for several research groups to begin engineering BChE mutants with an increased catalytic efficiency for cocaine [85-92]. These enzymes show robust protection against cocaine toxicity, as well as the reinforcing effects of cocaine. The main hindrance to the development of BChE is the inability to produce it in large quantities as it must be expressed in low-yield mammalian

cell cultures due to complex post-translational modifications. Strategies under investigation to increase yield include production in yeast, alteration of post-translational modifications to allow expression in bacteria, or producing the enzyme in goats' milk. To circumvent this production problem, researchers at the University of Michigan, in collaboration with Columbia University and the University of Kentucky, began work on a bacterial cocaine esterase that was known to rapidly hydrolyze cocaine [93-95].

History of Bacterial Cocaine Esterase

Cocaine esterase (CocE) is a serine hydrolase originally isolated from the MB1 strain of *Rhodococcus* bacteria that grows in the soil surrounding the roots of the coca plant [93]. These Rhodococci can live in minimal media supplemented with cocaine as their sole carbon and nitrogen source. The presence of cocaine in the media induces expression of CocE. This group previously isolated a heroin esterase from *Rhodococcus* sp H1 [96] and a morphine dehydrogenase from *Pseudomonas putidamio* [97]. They used these two bacterial enzymes, in conjunction with luciferase from *Vibrio harveyi*, to develop a biosensor that could rapidly and easily detect low concentrations of both heroin (89 ng/mL) and morphine (2 ng/mL) [97]. Cocaine esterase was to be used in a similar manner, creating an easy way for customs officials to detect cocaine.

Two years later, the biochemical properties and crystal structure of CocE were published (V_{max}= 468 and K_m=640 nM [95]). At the time of this report, CocE was the fastest cocaine hydrolyzing enzyme (it remains the fastest naturally occurring enzyme), and could also hydrolyze cocaethylene. The authors speculated that CocE could be useful as a detoxifying agent in emergency room settings. The structure revealed an α/β serine hydrolase with three distinct domains [94]. Domain 1 has a prototypical α/β sandwich fold and contains the active site residues His287, Asp259, and Ser117, in a pocket formed at the interface of all three domains [94]. Domain 2 is a large 96 amino

acid insertion into Domain 1 between residues 145 and 241. It contains 7 α-helices, with helix 2 and 3 packing in an anti-parallel fashion and forming a lid-like structure over the active site. Insertions like Domain 2 are common in the α/β hydrolase family, however this is the largest insertion currently documented. Domain 3 is primarily structural in nature and is composed of β-sheets packed into a jelly-roll-like topology. Cocaine binding to the active site pocket of CocE is likely due to extensive van der Walls contacts between the benzoyl moiety of cocaine and F261 and W151. The molecular basis for the specificity of cocaine binding has not been elucidated, but is likely due to other active site pocket residues, as other tropane alkaloids like atropine are not hydrolyzed [93].

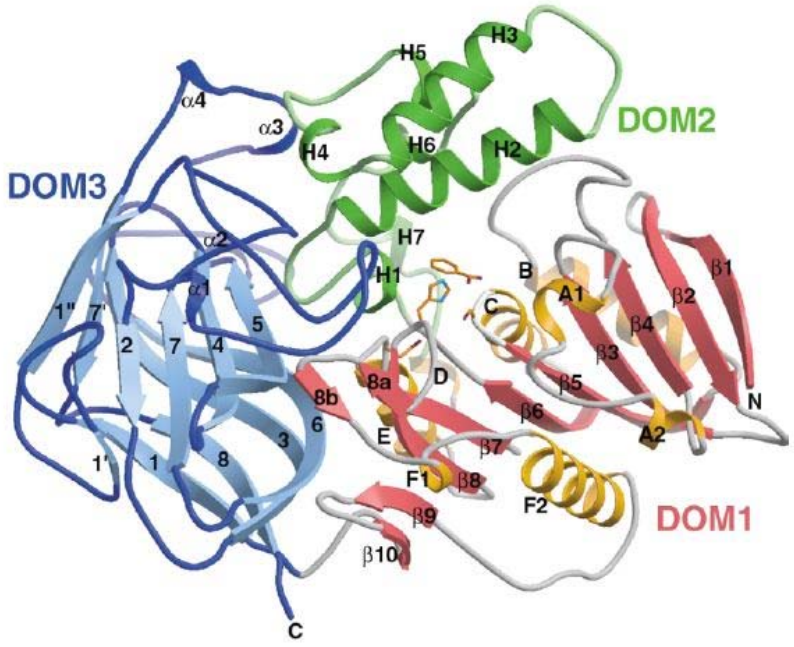

Figure 1.2: Crystal structure of bacterial Cocaine Esterase. Domain 1 (red) contains a prototypical α/β sandwich fold, and has a large insertion (Domain 2 shown in green) that acts as a lid over the active site. Domain 3 is composed primarily of β-sheets and primarily plays a structural role. Benzoic acid is shown bound in the active site pocket located at the interface of all three domains next to histidine 287. Figure from original crystallization work by Larsen et al. [94], reprinted by permission from Macmillan Publishers Ltd: Nature Structural Biology Jan; 9 (1): 17-21, copyright 2002.

The authors of the crystallization and characterization reports were unable to see an effect of CocE in animals treated with cocaine [98]. The first account of CocE working as a detoxifying agent was not reported until 2006, after collaboration led to alternative purification approaches [79]. Very small doses of this purified CocE could protect animals from cocaine-induced lethality better than large doses of recombinant human BChE [79].

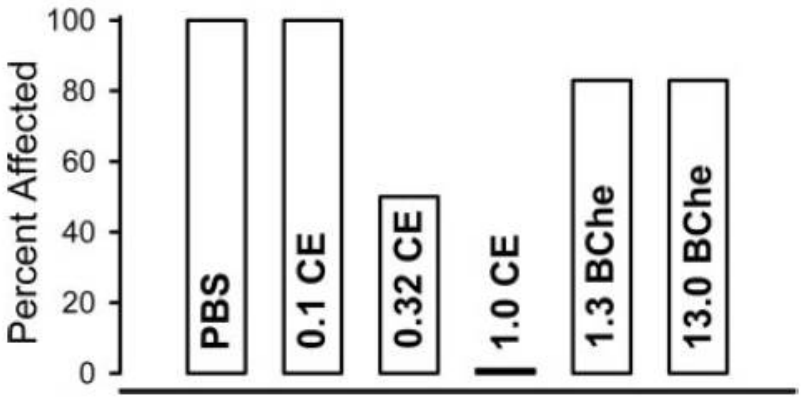

Figure 1.3: Comparison of bacterial Cocaine Esterase to human butyrylcholinesterase. Male Sprague Dawley rats were administered the given dose (in mg) of either CocE (CE), recombinant human butyrylcholinesterase (BChe), or phosphate buffered saline (PBS) intravenously 1 minute before a 180 mg/kg dose (LD_{100}) of cocaine (IP). No rats receiving only 1 mg of CocE succumb to cocaine-induced lethality, while 80% of animals receiving 13 times the dose of human butyrylcholinesterase were susceptible to the lethal effects of cocaine. Figure from Cooper et al. [79] reprinted by permission (see Appendix 2).

Mutations of the active site residues of CocE eliminated the protective effects of the enzyme against cocaine. Although this study illustrated the robust effect and potential of CocE, it also revealed that CocE has an unusually short half-life at 37°C (~13 minutes) [79]. Even with this short half-life, several more studies were performed that further confirmed the efficacy of this enzyme against cocaine toxicities.

CocE was shown to block a myriad of physiological cocaine-induced effects such as seizures, convulsions and cardiovascular disturbances [99-100]. Ko et al.

demonstrated the effects of repeat and time-dependant dosing of CocE in mice [101]. CocE (0.32 mg/mouse), given 1 minute before an LD_{100} dose of cocaine, protected 100% of mice from lethality. Longer pre-treatment times resulted in a loss of protection (67% protection at 5 minutes, 0% protection at 20 minutes), demonstrating the instability of CocE *in vivo*. CocE given 1 minute after an LD_{50} dose of cocaine reversed convulsions in all animals and rescued all animals from lethality. The dosing regimens used in this initial study (CocE once per week for 3 weeks or CocE once every 2 weeks for 8 weeks) highlighted an increase in anti-CocE antibodies after CocE administration, but that this effect was not dose-dependent, nor near the level generated when CocE was given with Freund's adjuvant. Animals were not adversely affected by CocE, but, after repeat dosing, the protection against cocaine-induced lethality and convulsions by CocE was reduced. Current unpublished studies suggest that advanced purification techniques (removing bacterial endotoxin to levels determined by the FDA as acceptable for humans) eliminates the immune response seen in the early studies (personal communication, M. C. Ko)

Although CocE showed robust effects protecting animals against cocaine toxicities, the short half-life needed to be corrected, if possible, for several reasons. First, the instability of the molecule made it difficult to purify (purifications were conducted at 4°C) and transport. Second, the short half-life of CocE made it unattractive candidate for a cocaine abuse therapy. Studies with the cocaine vaccine demonstrate that if cocaine can be kept out of the brain, it loses its reinforcing effects and people are less likely to use the drug [69, 70, 73]. If the half-life of CocE could be extended, it would not only keep cocaine out of the brain, but also eliminate the molecule and prevent its physiological effects.

To circumvent the short half-life of CocE, molecular dynamics simulations on CocE were performed at the University of Kentucky. These simulations revealed that Domain 2, the large, lid-like insertion in the α/β fold of Domain 1, was the least thermostable portion of the enzyme. About fifty possible thermostabilizing mutations that could increase the half-life of the enzyme were predicted. Cloning revealed three mutations (L169K, T172R, and G173Q) that all individually increased the half-life of CocE [102, 103]. The combination of T172R and G173Q had an additive effect on the half-life ($t_{1/2}$=4.5 hours) and no effect on the catalytic efficiency of the molecule. This double mutant (T172R/G173Q or RQ-CocE) was tested *in vivo* and was found to increase the duration of protection (the time of CocE pretreatment that produces 50% protection from the a LD_{100} dose of cocaine in mice) from 14 minutes to 4.5 hours [103], closely comparable to the *in vitro* half-life data. These studies led to the hypothesis that if the thermostability of the enzyme could be improved, so would its duration of action *in vivo*.

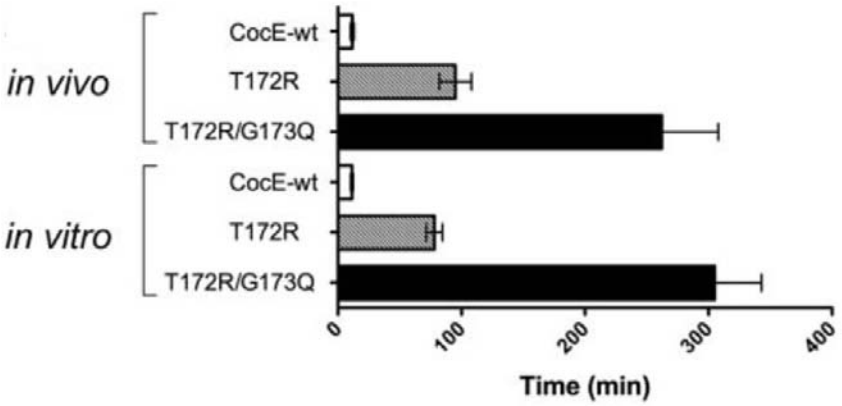

Figure 1.4: *In vitro* half-life of CocE compared to *in vivo* duration of action in mice. *In vitro*, wt, T172R and T172R/G173Q CocE (RQ-CocE) were incubated in phosphate-buffered saline (PBS) at 37°C and time to 50% activity loss was assessed and recorded as the *in vitro* half-time. *In vivo*, NIH Swiss mice were pre-treated intravenously with 1 mg of either wt, T172R or RQ-CocE and challenged with an intraperitoneal injection of 180 mg/kg cocaine (LD_{100}) at times afterwards. The time to challenge that resulted in 50% of animals succumbing to cocaine-induced lethality was recorded as the *in vivo* duration time. For wt, T172R and RQ-CocE, the *in vitro* half-times and in the *in vivo* duration times are equivalent. These experiments led to the hypothesis that an increased half-life *in vitro*, would lead to a longer *in vivo* duration of action. Figure from Gao et al. [102], reprinted by permission (see Appendix 2).

Through the production of a stabilized enzyme, the potential for CocE to be a therapy for cocaine abuse was demonstrated in a rat cocaine self-administration model [104]. RQ-CocE, which did not suppress responding in a food-reinforced paradigm, could block cocaine-reinforced responding when given as a pre-treatment before sessions. This protection against the reinforcing effects of cocaine was directly related to the thermostability of the enzyme, as even wild-type CocE was able to block responding for the first 10 minutes of the cocaine self-administration session.

Although many studies have been performed with CocE and its efficacy against cocaine toxicity is well established, there are still many unknowns. Foremost, how much can the enzyme be stabilized, and will this increased stabilization translate into an increased duration of action *in vivo*? There are currently no reports of the pharmacodynamics or pharmacokinetics of this enzyme and its efficacy has not been assessed in any other species besides rodents. It is also unknown if CocE can be inhibited with small molecule drugs, or if it is promiscuous enough to eliminate the toxic metabolites of cocaine. The specific aims of this thesis seek to answer these questions.

Specific Aims

Specific Aim I: The aim of the first set of studies (presented in Chapter 2) is to determine whether the stability of CocE can be increased by the combination of single point mutations. This aim includes testing several combinations of mutations predicted to be thermostabilizing and assessing their activity *in vitro*. The combination mutant that exhibited a synergistic increase in thermostability (L169K/G173Q-CocE, KQ-CocE) is further characterized *in vivo* using both mouse lethality assays and cocaine-reinforced operant responding in rats. Finally, the *in vivo* half-life of both the parent wt-CocE and KQ-CocE is assessed.

Specific Aim II: The second aim of this thesis (presented in Chapter 3) is to determine the pharmacokinetic and pharmacodynamic properties of CocE *in vivo*. Using [^{35}S]-RQ-CocE, the half-life, volume of distribution and clearance of CocE is determined in male rats. The time course and mechanism of elimination of CocE *in vivo* is also discussed. To assess the pharmacodynamics of CocE, the *in vivo* profiles of cocaine hydrolysis are determined and compared to *in vitro* experiments of the same experimental design. The efficacy of CocE is also assessed across species between rats and non-human primates, and between sexes.

Specific Aim III: The final set of experiments (presented in Chapter 4) is designed to determine if there are any pharmacologically and physiologically relevant drug-CocE interactions, or cocaine metabolite-CocE interactions. The effect of CocE on the metabolic profile of cocaine *in vivo* in rats is also quantified using mass spectrometry.

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Chapter 2

A Thermally Stable Form of Bacterial Cocaine Esterase: A Potential Therapeutic Agent for Treatment of Cocaine Abuse

Introduction

Cocaine abuse affects over 1.7 million Americans [1]. Chronic use causes multiple health problems including heart arrhythmias, high blood pressure, liver necrosis, mental illness, and death [2]. The economic cost of health care and lost wages due to cocaine use accounts for a large portion of the $180.7 billion spent on drug abuse treatment in the United States [3]. Despite these tragic personal and financial costs, there is no FDA-approved pharmacotherapy for cocaine abuse.

Cocaine blocks monoamine transporters [2, 4, 5] accounting for the drug's psychotropic effects. The enhanced adrenergic stimulation and direct blockade of sodium channels also leads to profound cardiac disturbances [6, 7]. Inhibitors that block cocaine's action at dopamine and serotonin transporters [8-11] cannot eliminate the cardiovascular effects of cocaine or enhance *in vivo* degradation.

An alternate pharmacological approach is to enhance cocaine metabolism. The endogenous enzyme butyrylcholinesterase (BchE) cleaves cocaine to physiologically inert products; ecgonine methyl ester and benzoic acid (V_{max} = 3.9 min^{-1}). Large doses of BChE can protect animals from cocaine-induced lethality [12-16]. Albumin-fused engineered mutants of BChE displaying enhanced kinetics against cocaine (V_{max}=2,700 min^{-1}) [17-19] increased the plasma half-life of BChE [20-23] and blocked both the lethal

effects of cocaine, and the reinstatement of cocaine-seeking behavior in a rat cocaine self-administration model [20]. Commercial production of this enzyme, however, may be challenging due to low yields from mammalian expression systems and complex post-translational processing.

Our approach to treating cocaine abuse and toxicity has been the use of a bacterial cocaine esterase (CocE). Isolated from the MB1 strain of *Rhodococcus* found in the Rhizosphere soil surrounding the coca plant, CocE has a high V_{max} towards cocaine (V_{max} =2,300 min^{-1}) [24] and produces the same products as BChE [25-27]. CocE has previously been shown to block cocaine-induced cardiac disturbance, neurological changes, and lethality in rodents when administered before or after cocaine [28-31].

Wild-type (wt) CocE cannot be used as a pharmacotherapy for cocaine abuse due to its 13.7 min half-life at 37°C [24, 28, 30]. We have previously identified a double mutant of CocE (T172R/G173Q) that extends the half-life of CocE to ~4.5 hours, as assessed by *in vitro* kinetic assays (at 37°C) or *in vivo* by protection against cocaine-induced lethality in mice [24]. These mutations are thought to improve stability by burying additional surface area and/or through the formation of additional hydrogen bonds between domains I and II of CocE [24, 32].

The increased stability of RQ-CocE allowed the enzyme to block the reinforcing effects of cocaine during one-hour cocaine self-administration sessions following a pretreatment with RQ-CocE [33]. This behavioral effect was specific to cocaine-reinforced behavior, and did not block responding for food or WIN-35065-2, a cocaine analog not hydrolyzed by CocE. These data support the hypothesis that CocE hydrolyzes cocaine rapidly enough to prevent reinforcing effects. Although RQ-CocE is

capable of blocking the reinforcing effects of cocaine after an immediate pre-treatment and can protect against cocaine toxicity for up to 4.5 hours, its actions are still too short in duration to make the enzyme a feasible candidate to treat cocaine abuse.

Given that rapid cocaine hydrolysis is sufficient to block the reinforcing properties of cocaine [33] and the reinstatement of cocaine-reinforced responding [20], we set out to derive a mutant of CocE that would be more appropriate for cocaine abuse therapy. Here we describe L169K/G173Q CocE (KQ-CocE), the most thermostable CocE variant characterized to date ($t_{1/2}$ at 37°C=2.9 days). KQ-CocE was characterized in both *in vitro* stability assays and *in vivo* duration of action studies. We also provide structural evidence as to why this enzyme is the most stable variant to date. Initial pharmacokinetic data from this long-acting mutant compared to wt CocE is also presented.

Methods:

Site directed mutagenesis: Point mutations were introduced into the CocE sequence present in the bacterial expression vector pET-22b (+) using a modified QuickChange (Stratagene) mutagenesis protocol and confirmed by sequencing in both directions over the entire coding region. Wt and CocE mutants were expressed and purified as described previously [24, 32].

Spectrophotometric cocaine assay: Cocaine hydrolysis was measured spectrophotometrically using a protocol adapted from [27]. Varying concentrations of cocaine (0.5, 2.5, 5, 12.5, 25, 50, 100, and 150 μM) in PBS Buffer (50 mM Tris HCl, pH

8.0, 150 mM NaCl) were added to a UV-permeable 96 well plate (100 µl). CocE was added to the 96-well plate to give a final concentration of 10 ng/mL and a final volume of 200 µL. Cocaine hydrolysis was followed by the change in absorbance at 240 nm at 10 second time points for 20 min using a SpectraMax Plus 384 UV plate reader (Molecular Devices, Sunnyvale, CA) using SOFTmax Pro software (Version 3.1.2). The V_{max} and K_m of the enzyme were determined using Prism (GraphPad, San Diego).

In vitro measurements of thermostability: Mutants were incubated at 37˚C in human plasma (obtained from the University of Michigan Hospital blood bank) at a concentration of 60 µg/mL. All samples were prepared immediately before incubation for the indicated times, and cocaine hydrolyzing activity was assayed as described above.

Crystallization and structure determination: KQ-CocE was purified as previously described [24]. CocE crystals were grown by hanging drop vapor diffusion in VDX plates on siliconized glass cover-slips (Hampton Research). One µL of CocE at 5 mg/mL was combined with one µL well solution (1.7 M ammonium sulfate, 10 mM Tris HCl, pH 7.3, 25 mM NaCl) and incubated at 293 K over 1 mL of well solution. Crystals reached their maximum size within two days and were harvested within one week of tray setup. Five µL of cryo-protectant (1.5 M Ammonium sulfate, 5 mM Tris HCl pH 7.3, 10 mM HEPES pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA, 825 mM NaCl, 25% glycerol) were added to the drop during harvest to prevent ammonium sulfate crystal formation. CocE crystals were transferred to a 20 µL drop of cryo-protectant for one to five minutes then flash-frozen in liquid nitrogen. X-ray diffraction data were collected at the Advanced Photon Source, LS-CAT, beamline 21-ID-D using one second exposures at a wavelength of 1.02Å. Data were integrated and scaled using HKL2000 [34] and refined using the structure of wt

CocE as the standard model [26]. Modeling was performed by alternating rounds of refinement using REFMAC5 [35] and manual density fitting using Coot [36]. Coordinates were validated by MolProbity [37] and deposited into the Protein Data Bank under accession code 3IDA.

Intravenous enzyme administration: Mice were placed in small restraint chambers (outer tube diameter: 30 mm, inner tube diameter: 24 mm, model no. BS4-34-0012; Harvard Apparatus Inc., Holliston,MA) that left the tail exposed. The tail was then placed under a heat lamp for 7 seconds to increase blood flow and bring the tail vein to the surface. The tail was wiped with an alcohol pad and a 30.5 gauge precision glide needle was inserted into one of the lateral veins of the tail. After injection of 0.2 mL, the needle was removed and the bleeding was stanched using sterile gauze and pressure on the injection site.

Behavioral toxicity: Cocaine-induced toxicity was evaluated by observing the occurrence of convulsions and/or lethality in male NIH Swiss mice (25-30 g) (Harlan Inc. Indianapolis, IN). Mice were housed in groups of nine mice per cage (16x28x20 cm) and allowed *ad libitum* access to food and water. Animals were maintained on a 12 hour light-dark cycle with lights on at 7:00 AM. Experiments were performed according to guidelines established by the National Institutes of Health in the Guide of the Care and Use of Laboratory Animals. Experimental protocols were approved by the University Committee on the Use and Care of Animals (UCUCA) at the University of Michigan.

Mice were given intravenous tail vein injections of KQ-CocE (1 mg, 0.3 mg, 0.1 mg, 0.032 mg, and 0.01 mg) or saline 1 min prior to an intraperitoneal challenge dose of

cocaine (100, 180, 320, 560, or 1000 mg/kg). The mice were observed for behavior in a Plexiglas container (16x28x20cm) for 45 minutes or until death. Assessment of the *in vivo* duration of action used the above protocol except that CocE (1 mg) was given at longer times pre-treatment to a challenge dose of 180 mg/kg cocaine. Convulsions were defined as loss of righting for more than 5 seconds. Lethality was defined as a loss of respiration and heart beat.

Serum collection: Blood was collected via (cheek pouch blood sampling) using a mouse bleeding lancet (GoldenRod 4.0 mm animal lancet; MEDIpoint Inc., Mineola, NY). Blood was collected in tubes (BD Microtainer) and placed on ice. Bleeding was stopped using a sterile gauze pad applied with direct pressure. The animal was then returned to its home cage and allowed to recover. Samples were spun at 4000 rpm for 5 minutes to separate the serum fraction. Each mouse gave approximately 50 µL serum.

Western blot analysis: Plasma samples (20 µg total plasma protein) were resolved by SDS-PAGE on 10% polyacrylamide gels. Protein was transferred to a nitrocellulose membrane and probed with a rabbit anti-CocE polyclonal primary antibody (a kind gift from Dr. Donald Landry at Columbia University) and HRP-linked anti-rabbit secondary antibody (BioRad). The membranes were stripped and re-probed with rabbit anti-mouse apolipoprotein (ApoA1) antibody (Affinity BioReagents PA1-23059) and the same secondary antibody for loading control. Western blots for CocE using these conditions can detect CocE to about 10 ng. Analysis of band densities from scanned films was performed with ImageJ Software (NIH). Area under curve analysis was done to determine densities of both CocE and ApoA1 bands. To normalize CocE densities, the fraction of the mean ApoA1 density was calculated for each ApoA1 band and divided from each CocE density.

Cocaine self-administration: Male Sprague Dawley rats (300-350 g) (Harlan Inc., Indianapolis, IN) were housed in groups of 3 animals per cage with *ad libitum* access to food and water on a 12 hour light-dark cycle (lights on at 7:00 AM). After catheter implantation, rats were singly housed. All experimental protocols were approved by UCUCA at the University of Michigan.

Rats were implanted with an indwelling femoral vein catheter under ketamine (90 mg/kg) and xylazine (10 mg/kg) anesthesia. The catheter is run under the skin and fixed to a metal plate that is sewn into the muscle of the back under the skin. This plate can then be attached to an IV catheter line present in the self administration box. After a 5 day recovery period post-surgery, rats were initiated into the self-administration procedure. Training and testing occurred at the same time each day at the middle of the light cycle. Rats were placed for one hour sessions into a standard operant chamber equipped with a "nose-poke" apparatus, in which an entry into a hole was recorded with a photo-beam break and counted as one response. Rats were required to respond with a nose poke for an injection of 0.56 mg/kg cocaine (0.1 mL/kg injection volume) on a fixed ratio (FR) 1 schedule (one nose poke delivers one cocaine injection). Once responding stabilized in this phase of training, rats were gradually moved up to an FR 5 and then switched to a dose of 0.1 mg/kg/injection cocaine in order to maintain the most reliable behavior throughout the session. Once animals responded consistently ($\leq$ 20% variation in responding and no upward or downward trend) for three days, they were given either 1 mg KQ-CocE or saline at the indicated times prior to the session. After the test day, rats continued the training schedule until responding recovered to baseline. Animals were also tested by substituting saline for cocaine. Immediate CocE pretreatment and saline substitution conditions were compared to cocaine-reinforced

responding behavior maintained by 0.1 mg/kg/injection by one-way ANOVA with Bonferroni post-tests. CocE pretreatments at 1 and 2 hours before the session were compared to baseline responding by student's t-test.

Drugs: Cocaine hydrochloride was obtained from the National Institute on Drug Abuse (Bethesda, MA).

Results

Stability of mutants: Previous studies showed that the L169K mutant of CocE (CocE-L169K) had a 33-fold longer half-life at 37 °C than wt CocE (measured by time to 50% loss of V_{max}) [32]. However, CocE-L169K had an increased K_m that decreased the catalytic efficiency of the enzyme 12-fold. To compensate for this deficiency, L169K was paired with single mutations to test if the elevated K_m could be corrected, or, if the half-life could be extended in a synergistic manner. Mutants were assessed for initial activity using the spectrophotometric cocaine hydrolysis assay, and (if initial kinetics were better than or comparable to wt CocE) were subjected to incubation at 37 °C for 24 hrs to test for their thermostability (Table 1). The L169K mutant retained 55% of its catalytic efficiency after a 24 hr incubation period at 37°C . The combination of L169K and T172R ($t_{1/2}$@ 37°C CocE-T172R= 46.8 min [32]) produced an enzyme with a lower V_{max} and a higher K_m than wt (5.6-fold loss in catalytic efficiency). Combination of L169K and N197K (predicted to create novel contacts with the dimer partner of CocE) had similar kinetics to wt CocE, but did not retain any measurable activity after 24 hours. The addition of other point mutations that we predicted to either increase the stabilization of Domain 2 by creating additional buried surface area between Domain 1 and Domain 2 (N42V), or create a new hydrogen bond between the subunits of the CocE dimer (A193D), failed to increase thermostability, increase V_{max}, or further reduce the K_m.

However, combining L169K and G173Q conferred a prolonged half-life at 37°C, and retained nearly 75% activity after 24 hours. Although the K_m was 4-fold elevated above that of the wt K_m (30 μM compared to 7 μM) it was half that of CocE-L169K. Importantly, KQ-CocE exhibited a higher V_{max} than any CocE variant characterized to date. The higher V_{max} of KQ-CocE compensates for its slightly increased K_m, such that KQ-CocE had a catalytic efficiency of 220 $min^{-1}\mu M^{-1}$, similar to that of wt (350 $min^{-1}\mu M^{-1}$), which represented a 7-fold improvement over CocE-L169K alone (30 $min^{-1}\mu M^{-1}$). Impressively, the half-life of KQ-CocE at 37 °C (Figure 2.1A) was 340 fold longer than wt CocE, corresponding to 2.9 days. After a 10 day incubation at 37°C, KQ-CocE still retained approximately 15% activity.

Temperature of inactivation experiments were conducted by incubating KQ-CocE for 10 minutes at a variety of temperatures (Figure 2.1B). The V_{max} of KQ-CocE decreased as the temperature of incubation increased above 42°C, with the temperature of inactivation between 42°C and 48°C. This is a significant improvement over wt CocE, which has a temperature of inactivation of 37° [32], and closer to that of butyrylcholinesterase, which inactivates between 54-57°C [38]. This indicates that the increased stability of KQ-CocE derives, at least in part, from increased thermostability.

Crystal structure of KQ-CocE: CocE consists of three domains; Domain I has an α/β hydrolase fold and contains the catalytic residues, Domain II consists primarily of a pair of large helices, and Domain III has a jelly roll-like topology. The KQ-CocE structure was determined to a resolution of 1.6 Å (Table 2), and can be superimposed onto the four previously determined structures of wt CocE with an RMSD of < 0.58 Å for all Cα

atoms (PDB ID: 1JU3, 1JU4, 1L7R, 1L7Q). The crystal packing of KQ-CocE is the same as in previous structures, and is consistent with CocE existing as a homodimer.

The L169K and G173Q mutations are both located in helix 2 of Domain II. The mutated side chains are easily distinguished in the electron density maps (Figure 2.2B) and each creates new interactions between Domain I and II. The Gln173 creates a hydrogen bond to the backbone oxygen of Pro43 in Domain I, whereas Lys169 reaches over a portion of the entrance to the active site to create Van der Waals interactions with Tyr44 of Domain I. Unlike for a leucine side chain, the ζ-nitrogen of Lys169, is able to favorably interact with solvent, and in the structure stabilizes a glycerol molecule that was used as a cryoprotectant. Notably, the loop between helices 2 and 3 of Domain II, which exhibit multiple conformations in other CocE structures determined, adopt a single conformation in the KQ-CocE structure, suggesting greater stability in this region of the protein. As reported previously, DTT and CO_2 present during protein purification was observed to form a tetrahedral 1,4-dithio-2,3-butylene carbonate (DBC) adduct with Ser117 in the active site [32].

Duration of action against cocaine-induced lethality: Cocaine administration at a dose of 180 mg/kg into the intraperitoneal cavity of NIH Swiss mice produced lethality in 100% of animals. Pretreatment with KQ-CocE produced dose-dependent, rightward shifts in the cocaine dose response curve (Figure 2.3A). KQ-CocE exhibited a subtle degree of protection even with a very low dose of 0.01 mg, and increased the LD_{100} of cocaine to 1000 mg/kg at a dose of 0.1 mg KQ-CocE, one quarter log unit lower than what is needed for the same effect with wt CocE (0.32 mg). The time-to-death after cocaine injection was also increased by KQ-CocE. With saline pretreatment, animals

died within 2-3 minutes after administration of 180 mg/kg cocaine. However, with a pretreatment of 0.032 mg KQ-CocE, it took a dose of 560 mg/kg cocaine to produce near 100% lethality and produced a slight increase in the time to death of some animals (animals died within 2-7 minutes after 560 mg/kg cocaine, data not shown).

KQ-CocE pretreatments of longer duration were able to protect animals from cocaine induced lethality (Figure 2.3B). We have previously demonstrated that wt CocE is rendered ineffective at preventing lethality from a challenge dose of cocaine after a 30 minute pretreatment [30]. RQ-CocE (1 mg) protected 50% of animals from a LD_{100} dose (180 mg/kg) of cocaine up to 4 hours after CocE administration [24]. KQ-CocE (1 mg), given 7-8 hours before administration of 180 mg/kg cocaine, protected 50% of the animals, representing a nearly 16-fold improvement over the wt enzyme.

In vivo *protection against the reinforcing effects of cocaine:* Sprague Dawley rats were trained to respond for cocaine (0.1 mg/kg/injection) on a fixed ratio (FR) 5 schedule. After saline pretreatments, animals responded for 75-100 injections of 0.1 mg/kg cocaine per one-hour session. When saline was substituted as the reinforcer, total responses were reduced and clustered at the beginning of the sessions (data not shown). Immediate pretreatment with KQ-CocE produced a reduction in the number of reinforcers earned to a level not significantly different than saline-reinforced responding (Figure 2.4). Both saline substitution and immediate pretreatment with KQ-CocE only produced about 25% of the responding of baseline sessions, similar to the behavior seen in the initial session of responding extinction. One hour pretreatment times also resulted in a reduction in responding, the degree to which was smaller than that with the immediate pretreatment and not statistically significant (three of 5 animals reduced

responding to under 50% of control levels). Two hour pretreatments with KQ-CocE resulted in a cumulative average of responses that was not significantly different than saline pretreatment.

In vivo *serum half-life:* The presence of wt and KQ-CocE remaining in the serum of NIH Swiss mice was analyzed using Western blot analysis on serum samples taken at increasing times after CocE administration (Figure 2.5A and 2.5B). Wt and KQ-CocE appear to be eliminated from serum following a similar time course. The elimination time course yields a serum half-life of 2.3 hours for KQ-CocE and 2.2 hours for wt CocE (one-phase decay model, r^2=0.8 for both enzymes tested). Although the *in vitro* half lives of these enzymes at 37°C is 340-fold different, the *in vivo* serum half-life is virtually identical and follows the same pattern of elimination. The increased duration of action of KQ-CocE compared to wt-CocE is thus due to its increased thermostability and not differential serum elimination

Discussion

This study demonstrates the superior stability of KQ-CocE *in vitro* and its extended duration of action *in* vivo and provides the first description of CocE pharmacokinetics in rodents. Wt CocE has previously been shown to prevent the acute toxic effects of cocaine [28-31], but its instability at 37°C, and its short duration of action *in vivo* limit its therapeutic potential. However, KQ-CocE appears to be the first attractive CocE candidate for treatment of cocaine abuse (should its half-time in serum be extended), due to its long duration of action and ability to block the reinforcing properties of cocaine.

KQ-CocE displays a significantly higher V_{max} than the wt or T172R/G173Q mutant [32]. The increase in catalytic turnover is accompanied by a small increase in the K_m of the enzyme. Crystallographic evidence suggests that the elevation in K_m is most likely due to the extended side chain of Lys169, which may partially occlude access to the active site pocket. This increase in K_m, however, does not affect enzyme function at the very high cocaine concentrations which cause toxicity *in vivo*. Our data also suggests that this defect does not seem to hinder the efficacy of KQ-CocE at rapidly clearing low concentrations of cocaine as seen in rats in self-administration models. The increased K_m does not hinder KQ-CocEs ability to hydrolyze cocaine at a faster rate than RQ-CocE even at the very low cocaine concentrations seen in these studies (0.1 mg/kg/injection or approximately 0.49 μM when dispersed in total body water of the rat), due to KQ-CocE's much higher V_{max}.

The L169K/G173Q mutations dramatically increased the thermostability of the bacterial enzyme from 37°C for wt to 48-55°C for KQ-CocE. Butyrylcholinesterase has been reported to have a temperature of inactivation of 54-57°C [20]. Furthermore, these two mutations slow aggregation of CocE, as seen by size exclusion chromatography (data not shown). After incubation at 37°C, wt CocE is 100% aggregated within one hour [32] while KQ-CocE is only 30-40% aggregated after 24 hours.

Analysis of the crystal structure of KQ-CocE reveals a complex molecular mechanism for its improved thermal stability. Lys169 and Gln173 provide additional anchor points between Domain I and the conformationally flexible region of Domain II. The newly introduced hydrogen bond between Gln173 (Domain II) and Pro43 (Domain I), the novel Van der Waals interaction between Lys169 (Domain II) and Tyr44 (Domain I), and more favorable interactions with solvent by the Lys169 side chain also likely contribute to the thermostability of KQ-CocE.

In vivo studies in mice confirm that KQ-CocE displays an increased potency and duration of action over wt CocE. The higher potency of KQ-CocE is most likely due to the combination of its higher V_{max} and increased stability at 37°C, allowing it to quickly degrade high concentrations of cocaine and remain active over a longer period of time. It is reasonable to expect that at concentrations of cocaine resembling a human overdose situation, cocaine would be hydrolyzed more rapidly by KQ-CocE than by wt CocE, although less of a difference in turnover rate would be seen at lower cocaine concentration, due to the elevated K_m of KQ-CocE.

The rat self-administration study presented here examining the effects KQ-CocE expands on the work previously conducted by Collins et al. [33]. They demonstrated that RQ-CocE did not suppress food- or WIN-35065-2 (a non-hydrolyzable cocaine analog)-reinforced operant responding at doses that suppressed cocaine-reinforced responding. These results demonstrated that CocE was specifically eliminates the reinforcing properties of cocaine, and does not simply suppress operant responding. The Collins study also highlighted a dose-response relationship between RQ-CocE and cocaine-reinforced responding. No change in responding was seen with the lowest dose of 0.032 mg CocE given to rats responding for 0.1 mg/kg/injection cocaine. A dose of 0.1 mg CocE increased the rate of responding for 0.1 mg/kg/injection (as would be expected to surmount a low dose of “antagonist”). At the higher doses of RQ-CocE, 0.32 and 1.0 mg, responding for cocaine decreased by half, and down to saline levels, respectively. The time course data presented here on KQ-CocE (Figure 2.5) demonstrates that after a 2-hour pretreatment, there is still enough circulating CocE to reduce operant responding slightly. It is likely that with longer pre-treatment times, operant responding for cocaine would increase due to very low levels of CocE remaining in the serum.

The duration of action of KQ-CocE *in vitro* greatly surpasses wt and the previously reported thermostable mutant RQ-CocE. However, the *in vivo* duration of action is shorter than the *in vitro* half-life at 37°C. In measures of protection against cocaine-induced lethality, wt CocE, CocE-L169K and RQ-CocE both lose their protective effects over a time course similar to their respective *in vitro* half-lives at 37°C [24, 32]. The duration of the KQ-CocE protective effect *in vivo,* although longer than RQ-CocE and wt CocE, is much shorter than its *in vitro* half-life at 37°C (7-8 hrs to 50% protection against cocaine-induced lethality versus 2.9 days to 50% activity at 37°C). This discrepancy is also seen in the protection against the reinforcing properties of cocaine measured in the rat self-administration model. Some reduction in responding can be seen after a 1-hour pretreatment of KQ-CocE; however this protection is completely eliminated after 2 hours. CocE dose response analysis by Collins et al. [33] illustrate that plasma levels of RQ-CocE need to be sufficiently high or else the effect of CocE-T172R/G173Q is surmountable. This leads us to hypothesize that the discrepancy between the *in vitro* stability and *in vivo* protection against lethality and self-administration data of KQ-CocE, may be due to a reduction in plasma levels of the enzyme.

Indeed, Western blot analysis on serum from mice injected with KQ-CocE shows that the enzyme is eliminated from the serum with a $t_{1/2}$ of 2.3 hrs. Processes unrelated to thermostability most likely explain the relatively short pharmacokinetic properties of the enzyme. One possibility is protease degradation, which may remove or destroy the regions of CocE recognized by the polyclonal anti-body and yield results that mimic the disappearance of CocE. Glomerular filtration is unlikely, as CocE is about 134 kDa as a dimer [32]. However, it is possible that the dimer interface may be disrupted *in vivo* and the monomer or smaller proteolysed products are filtered from the blood before forming

aggregates (CocE aggregates can be observed in mouse serum with size exclusion chromatography, data not shown).

It is of great interest that both the thermally stable KQ-CocE and the wt CocE disappear from the plasma with the same kinetics. Active CocE is a dimer and a monomeric form has never been isolated *in vitro*. As CocE is heat-inactivated, the protein forms aggregates both *in vitro* and *in vivo* (determined by size exclusion chromatography discussed extensively in Narasimhan et al. [32]). Wt CocE aggregates *in vitro* within one hour at 37°C [32], consistent with its thermal inactivation, KQ-CocE maintains enzyme in the dimer form for at least 120 hours (data not shown). The observation that both dimers and aggregates are eliminated with the same half-time illustrates that the state in which the esterase exists does not affect the CocE elimination process *in vivo,* despite corresponding to the activity of the enzyme.

The long *in vitro* half-life of CocE-KQ-CocE at 37°C greatly surpasses any combination of mutations to date and is a promising step towards development of a therapy against cocaine addiction. After ten days at 37°C, this enzyme still retains 10-15% of its original activity and displays intact Michaelis-Menten kinetics. The concentrations of cocaine typically seen in cocaine abusers (0-1 mg/L or 0.6-3 μM, [39]) should be cleared in approximately the same time by both the wt and L169K/G173Q enzyme. The extended half-life and improved Michaelis-Menten parameters of KQ-CocE suggest that this enzyme represents a strong candidate for cocaine abuse therapy indication.

Creation of the thermally stable KQ-CocE was a necessary advancement toward treatment of cocaine addiction by CocE in that it demonstrates that the instability of wt CocE at 37ºC can be surmounted. KQ-CocE has also demonstrated that the

thermostability of the CocE enzyme does not affect the *in vivo* rate of elimination from the serum. The short plasma half-life of KQ-CocE presents an obstacle that must be overcome if CocE is to be used as a treatment for cocaine abuse. Strategies that have shown success at increasing circulating protein half-life *in vivo*, including PEGylation, encapsulation in red blood cells and sugar modification, are currently under investigation.

CocE Variant	V_{max} (min^{-1})	K_m (μM)	Catalytic Efficiency (min^{-1} μM^{-1})	V_{max} 24 hours @ 37°C (min^{-1})	K_m 24 hours @ 37°C (μM)	Catalytic Efficiency Remaining at 24 hrs (min^{-1} μM^{-1})	Percent Catalytic Efficiency Remaining at 24 hrs
Wild Type	2510	7.2	348.6	270	7.3	37	10.8
L169K	3100[a]	105[a]	29.5[a]	950[b]	79.8[b]	159	55.0
L169K/T172R	1510	24.2	62.4	N/D[c]			
L169K/N197K	3120	58.3	53.5	N/M[d]			
L169K/G173Q	6670	30.3	220.1	3890	27.9	170	77.5
L169K/G173Q/ N42V	3180	56.4	56.4	N/M[d]			
L169K/G173Q/ A193D	2350	21.4	110	340	30.6	11.1	10.0

Table 2.1: Cocaine hydrolysis by CocE variants V_{max} and K_m were measured spectrophotometrically immediately after thaw from -80°C and after 24 hr incubation at 37°C. Catalytic efficiency was calculated from the V_{max} and K_m values and is expressed as min^{-1} μM^{-1}. CocE variants that lost all activity, or had negligible activity, are reported here as no Michaelis-Menten Kinetics remaining.

[a] Indicates data cited from [1]
[b] Personal communication (D. Narasimhan)
[c] Not Determined
[d] No Michaels-Menten kinetics remaining at 24 hours

X-ray Source:	APS LS-CAT 21-ID-D
Wavelength (Å)	1.02
Resolution (Å)	25.0 - 1.60 Å
Space group	$P6_522$
Cell constants (Å, °)	*a=b=108.3, c=227.2,* $\alpha=\beta=90.0^{\circ}$, $\gamma=120.0^{\circ}$
Unique reflections	104,076
Average redundancy	9.5 (8.3)
R_{sym} **(%)**[a]	9.4 (61.3)
Completeness (%)	99.1 (98.3)
$\langle I \rangle / \langle \sigma_I \rangle$	22.4 (3.1)
Refinement resolution (Å)	24.6-1.60 Å
Total reflections used	97,839
Protein atoms	4,799
Non-protein atoms	761
RMSD bond lengths (Å)	0.012
RMSD bond angles (°)	1.19
Estimated coordinate error (Å)	0.08
Ramachandran plot statistics:	
Most favored, disallowed (%)	96.7, 0.2
R_{work}[b]	17.7 (22.5)
R_{free}[c]	19.4 (25.5)

[a] $R_{sym} = \Sigma_{hkl}\Sigma_i |I(hkl)_i - I(hkl)| / \Sigma_{hkl} I(hkl)_i$, where I(hkl) is the mean intensity of *i* reflections after rejections.
[b] $R_{work} = \Sigma_{hkl}||F_{obs}(hkl)| - |F_{calc}(hkl)|| / \Sigma_{hkl} |F_{obs}(hkl)|$; no I/σ cutoff was used during refinement.
[c] 5% of the truncated data set was excluded from refinement

Table 2.2: Crystallographic data and refinement statistics

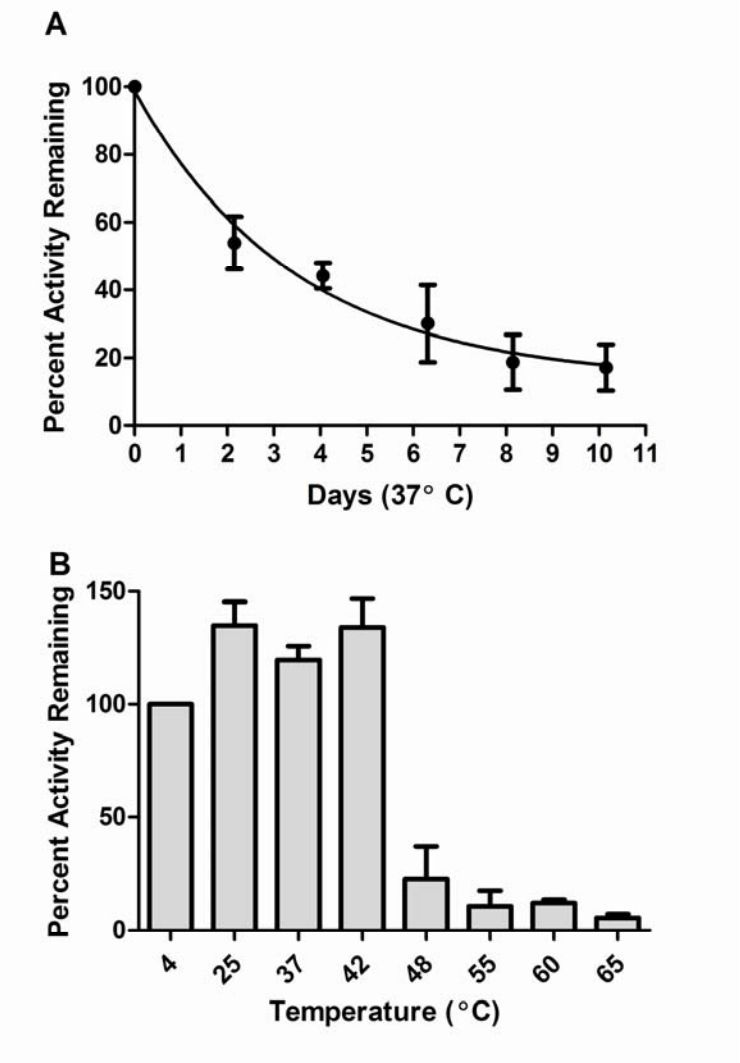

Figure 2.1: ***In vitro*** **stability of KQ-CocE** **A** Ten day time course of CocE activity at 37 ˚C. CocE was incubated at 60 µg/mL for the time indicated. Activity was assessed using the spectrophotometric cocaine hydrolysis assay. V_{max} and k_{cat} were determined and the catalytic efficiency was plotted as a percentage of the catalytic efficiency of non-incubated enzyme. A single phase exponential decay model shows that 50% activity is retained until approximately 2.9 days. **B** Heat inactivation of KQ-CocE. CocE was incubated at 60 µg/mL at the temperatures indicated for 10 min. Incubations were stopped on ice, and V_{max} was determined by the spectrophotometric assay. V_{max} activity was plotted as a percentage of the un-incubated CocE V_{max} kept at 4˚C. KQ-CocE retains over 50% activity up to 42˚C. At higher temperatures, the activity rapidly drops, but residual activity is observed up to 65˚C.

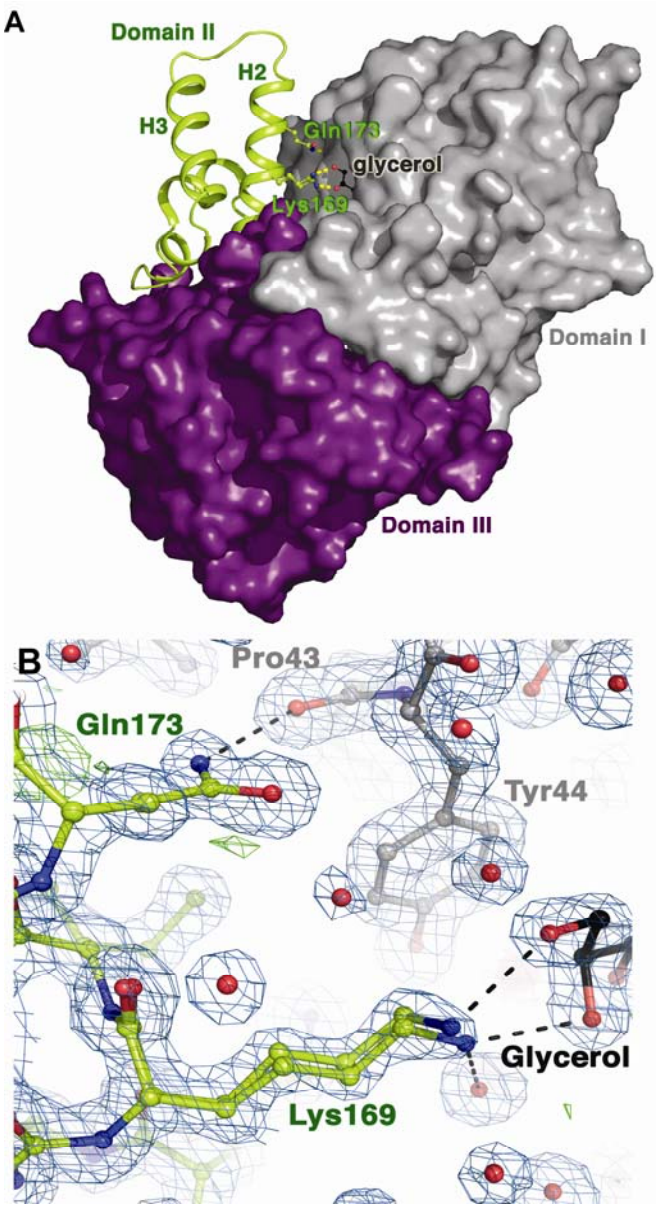

Figure 2.2: Crystal structure of KQ-CocE A Domain structure of CocE. Domain I (grey) contains the catalytic residues, Domain II (green) is helical with two pronounced anti-parallel helices (H2 and H3). Domain III is shown in purple. The active site is formed at their intersection. Domains I and III are shown as their solvent-excluded surfaces. The L169K and G173Q mutations (ball and stick side chains) are found in H2 of Domain II, close to the entrance to the active site, partially occupied here by a molecule of glycerol. **B** Refined model of KQ-CocE. 2|*Fo*|-|*Fc*| electron density is shown as a blue wire cage contoured at the 1σ. Lys169 is observed in two distinct conformations that interact with a bound molecule of glycerol from the harvesting solution. Carbon atoms are shown as their respective Domain colors (see panel A), oxygens are red, and nitrogens blue.

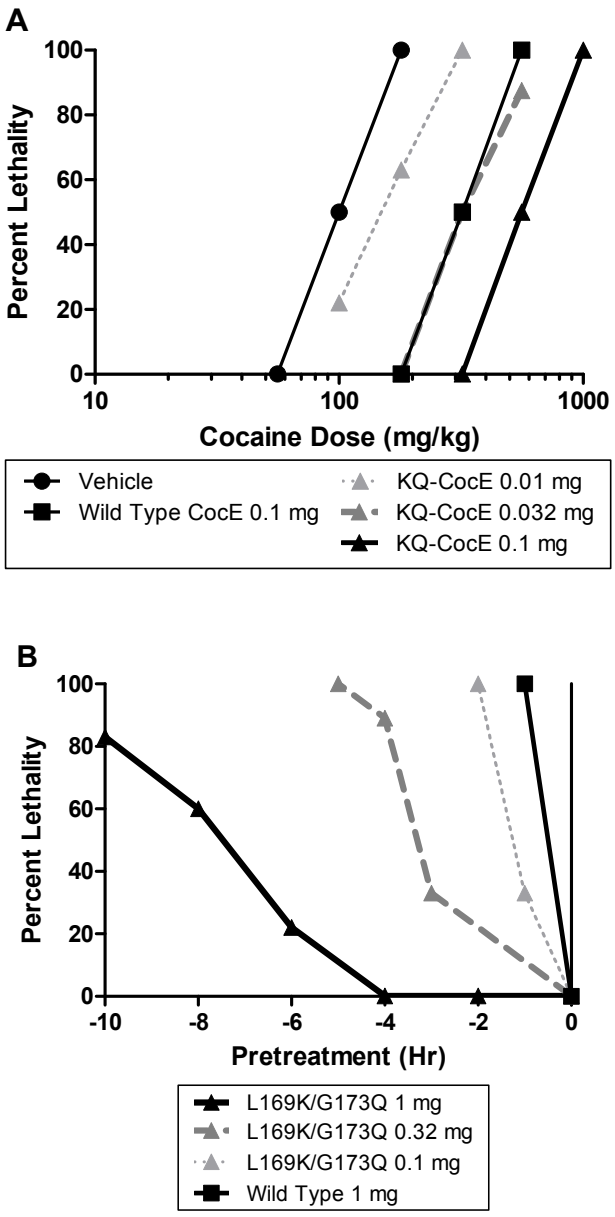

Figure 2.3: ***In vivo*** **potency and duration of action of KQ-CocE** **A** Potency of KQ-CocE against cocaine induced lethality. KQ-CocE was administered to NIH Swiss mice intravenously one minute prior to a cocaine challenge given intraperitoneally. Increasing doses of KQ-CocE cause significant rightward shifts in the cocaine dose-response curve. **B** KQ-CocE pretreatment protects against cocaine lethality more than wt CocE. CocE is administered intravenously at time of pretreatment. A lethal dose of 180 mg/kg is used as a challenge dose at time 0. The percentage of mice experiencing lethality after a one hour post-cocaine period is plotted.

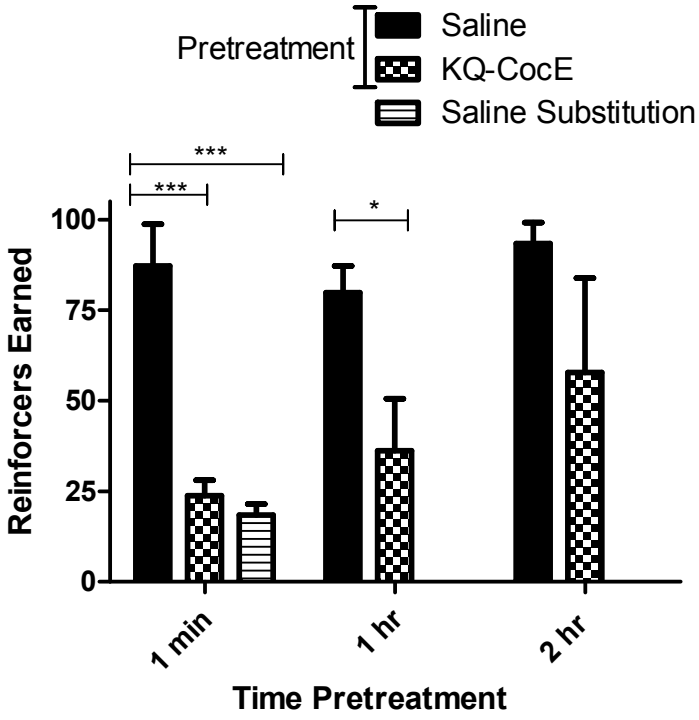

Figure 2.4: KQ-CocE protection against cocaine-reinforced operant responding in Sprague Dawley rats KQ-CocE (1 mg) was given as a pretreatment to cocaine self-administration sessions at the times indicated. Rats in the saline substitution condition received no cocaine from nose-pokes during the session.

*** One-way ANOVA $F(2, 12)=27.4$, Bonferroni post test $p<0.001$

* Students t-test $p<0.05$

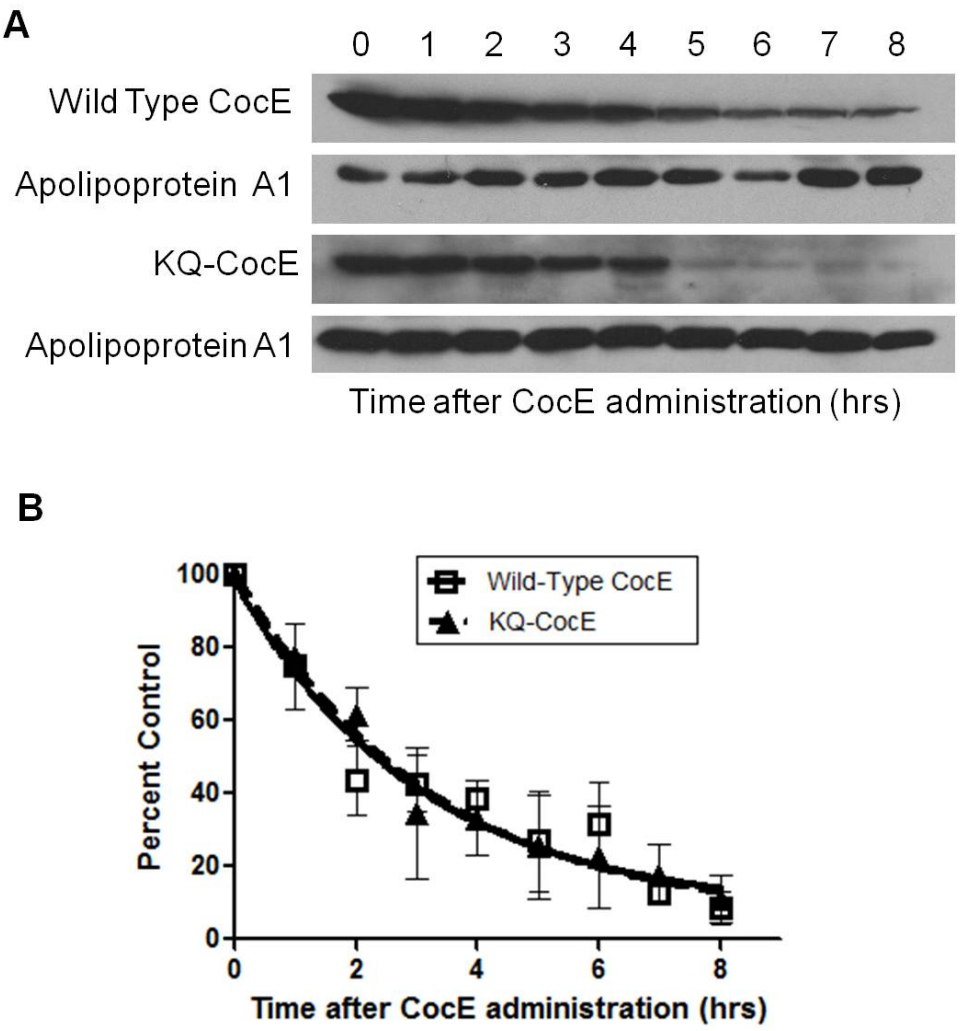

Figure 2.5: ***In vivo*** **CocE plasma half-life A** Representative Western blots of CocE from mouse serum over time. CocE (L169K/G173Q or wt) was administered to mice intravenously via the lateral tail vein. Blood samples were taken at the times indicated by submandibular sampling. Serum was collected and 20 µg total serum protein was run on a 10% SDS PAGE gel. Blotting was performed with rabbit anti-CocE antibody and rabbit anti-Apolipoprotein A1 antibody. Wt and KQ-CocE were both tested in 3 independent groups of animals followed by serum analysis. **B** Quantification of wt and KQ-CocE Western blot densities analyzed with Image J software (NIH). All time points are adjusted as a fraction of the apoA1 loading control. Fit to a one phase exponential decay model, the half-life of KQ-CocE was determined to be 2.3 hours post administration (wt=2.2 hrs).

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Chapter 3

The Fate of Bacterial Cocaine Esterase in Sprague Dawley Rats: An *in vivo* Study of CocE-Mediated Cocaine Hydrolysis, CocE Pharmacokinetics, and CocE Elimination

Introduction

Cocaine abuse is a widespread problem in the United States, with over 2 million people currently abusing the drug. This natural alkaloid, derived from the South American shrub *Erythroxylon coca*, elicits its powerful euphoric and addicting effects by blocking monoamine transporters in the brain and increasing the levels of endogenous catecholamines. Cocaine abuse has a detrimental effect on the life of the user, ranging from health issues to loss of income and family. It is estimated that the US economy losses $180.8 billion a year from decreased productivity and medical expenses related to drug use [1].

In addition to causing its euphoric effects, cocaine is toxic at high doses, causing convulsions, severe hypertension, ventricular fibrillation, hyperthermia and even death. Each year, over half of a million emergency department visits are caused by cocaine toxicity [2]. Toxicities can be caused by cocaine's blockade of cardiac sodium channels in addition to the high levels of norepinephrine that accumulate during to monoamine transporter blockade.

There is currently no Food and Drug Administration approved pharmacotherapy to treat either cocaine abuse or cocaine toxicity. To this end, we have been working to develop a bacterial cocaine esterase (CocE) into a suitable human therapy for cocaine

toxicity. CocE is an α/β serine hydrolase originally isolated from the soil bacterium *Rhodococcus* strain MB1 [3] and is the most efficient natural cocaine hydrolase, metabolizing cocaine into the inactive products ecgonine methyl ester and benzoic acid [3-5]. Wild-type CocE has been extensively classified in rodent models as being able to reverse the cardiovascular, neurological, and toxic effects of cocaine [6-9].

Through the addition of thermostabilizing mutations (T172R, G173Q, L169K, T172R/G173Q-CocE [10, 11], KQ-CocE [12]) the duration of action of CocE *in vivo* was improved. These improvements allowed proof of concept studies that revealed that CocE could block the reinforcing effects of cocaine while preserving CocE's ability to block the toxic effects of the drug [13]. We now possess a potential therapeutic for cocaine toxicity, and one that has potential as a therapeutic for cocaine abuse that is worthy of pharmacodynamic and pharmacokinetic studies.

The rapid rate of cocaine hydrolysis by CocE *in vitro* has been extensively documented [4, 9-12], and *in vivo* studies using toxic doses of cocaine and large doses of CocE have demonstrated the rapid elimination of cocaine-related behaviors and toxicities. However, a study directly measuring cocaine elimination *in vivo* has not been performed. Herein we describe the rate of cocaine hydrolysis by both low dose and pharmacologically relevant doses of RQ-CocE in both male and female Sprague Dawley rats and Rhesus monkeys using mass spectrometry. To complement these data, the pharmacokinetics of CocE are determined using [^{35}S] labeling and the distribution and elimination are assessed by immunologic and radiologic techniques. This work represents a significant contribution to the understanding of the way that live biological systems handle foreign biological proteins, as many of the FDA-approved biologic drug products currently on the market have unknown mechanisms of metabolism and elimination.

Methods

Production and purification of RQ-CocE: A BioFlow 3000 bioreactor (New Brunswick, NJ) was prepared with 10 L of Terrific Broth (TB, Maniatis, BentonDickinson) with kanamycin (50 µg/mL). For the inoculum, BL21 cells transformed with pET24b plasmid containing RQ-CocE were grown in 250 mL TB containing kanamycin (50 µg/mL) at 37°C while shaking until the culture reached log phase growth (about 8 hours). In this growth phase, the bioreactor was inoculated with the starter culture and allowed to grow at 37°C until the culture reached an OD_{600} of 5. Once the target turbidity was reached, the bioreactor was cooled to 18°C and then induced by adding IPTG for a final concentration of 1 mM. After 16 hours of induction, the culture was harvested by spinning down the broth at 4500 x *g* for 20 minutes. The resulting pellet was stored at -80°C.

Cell paste from 5 liters of a fermentor run was resuspended in PBS pH 7.4. The resuspended paste was passed through a French Press twice or three times to lyse the cells. The maximum pressure for lysis is approximately 1100 psi. The lysate was clarified by spinning at 100,000 x g in an ultracentrifuge (Beckman Coulter XL-100K ultracentrifuge) using the rotor type Ti45. Clarified lysate was diluted by adding an equal volume of Q-buffer A (20mM Hepes pH 8.0) to a final volume of 1000 mL.

The clarified lysate (500 mL) was passed onto a 450 mL Q Sepharose HP column attached to Pharmacia P-500 pumps and a Pharmacia LCC 501 FPLC controller at room temperature. The column was washed with 1000 mL Q-buffer A followed by a wash gradient set to reach 50% Q-buffer B (20mM Hepes pH 8.0 + 1M NaCl) within 1000 mL. Flow-through and washes were saved for analysis. Protein was eluted from the column using a gradient from 50 to 100% Q-buffer B over 5 column volumes (2250 mL). Fraction collection began after the washes were completed using a Pharmacia LKB

Frac 100 fraction collector. The entire run was carried out at a flow rate of 10 mL/min. The RQ-CocE protein was monitored by running 5 μl of each fraction on an 8% SDS PAGE Gel and staining with Coomassie Brilliant Blue stain according to established protocols.

Fractions from the first Q Sepharose HP separation were pooled and adjusted to 1M ammonium sulfate (final volume 525 mL). A 450 mL Phenyl-Sepharose column was washed with two column volumes (1000 mL) of PS-Buffer A (Buffer A: 50mM Na PO4, pH 7 + 1M AS). An additional wash step was performed to increase PS-Buffer B (Buffer B: 50mM NaPO4, pH 7) concentration to 30% within two column volumes (1000 mL). A gradient with PS buffers was run from 30% to 100% PS Buffer-B over 5 column volumes (2250 mL). Again, 25 mL fractions were collected over the elution gradient. The presence and purity of RQ-CocE were checked by running 5 μl of each fraction on an 8% SDS PAGE gel.

Fractions from the Phenyl-Sepharose column were pooled and extracted twice with triton X 114. The twice-extracted protein was then diluted with Q-buffer A and this was loaded on to a second Q-Sepharose HP column (450mL). Chromatography was performed as described above. Figure A1.1 illustrates this purification process.

Production and purification of [^{35}S]-RQ-CocE: B834 (DE3) methionine-autotrophic competent cells (Novagen®) were transfected with a pET24b plasmid containing cDNA for histidine-tagged RQ-CocE and ampicillin resistance. Cells containing the plasmid were selected for on agar plates with Luria-Bertani (LB) media supplemented with ampicillin. A transfected clone was selected and confirmed by DNA sequencing.

RQ-CocE expression was induced by innoculating 5 mL LB medium containing ampicillin with B834-pET24b cells and incubated for 12 hours at 37°C. The 5 mL culture

was then used to inoculate 25 mL LB medium containing ampicillin and culture was grown to at 37°C to OD_{600}=0.8. The culture was spun at 4000 g for 5 minutes and resuspended in 10 mL M9 minimal media containing ampicillin two times to remove LB media. The final resuspension was added to 15 mL M9 media containing ampicillin and grown at 37°C for 30 minutes. RQ-CocE expression was induced with final concentration of 1 mM IPTG, 10 µg/mL methionine and 1 mCi of [^{35}S]-methionine and grown at 18°C overnight.

After induction cells spun and resuspended in 3 mL Tris HCl buffered saline pH 7.4 (TBS) containing 3 µg/mL of both leupeptin and lima bean trypsin inhibitor (LS) as well as and lysozyme 50 µg/mL. To initiate cell lysis, cells were incubated for 20 minutes at room temperature and snap frozen with liquid N_2. Lysate was thawed and added to 4 mL B-PER® Bacterial Protein Extraction Reagent (Thermo Scientific) per gram cell pellet containing 8 µg/mL DNase 1 and 1 mM $MgCl_2$. Lysate was incubated 20 minutes at room temperature and centrifuged at 13,000 rpm for 30 minutes to remove cellular debris.

[^{35}S]-RQ-CocE was purified away from cellular lysate using a 3 mL nickel-nitriloacetic acid (Ni-NTA) column and a 1 mL Q-Sepharose Column. Lysate was loaded onto the Ni-NTA column and washed with 15 mL TBS+LS, 15 mL TBS+LS + 300 mM NaCl and 15 mL TBS+LS+ 10 mM imidizole. RQ-CocE was eluted with 1.5 mL fractions of 100 mM imidizole. Fractions were resolved by SDS-PAGE and visualized using Coomassie brilliant blue stain.

Fractions containing [^{35}S]-RQ-CocE were combined, diluted 4x in 50 mM Tris HCl, pH 8.0, and loaded onto the Q-Sepharose Column. The column was washed with 5 mL 50 mM Tris HCl, pH 8.0, 5 mL 50 mM Tris + 300 mM NaCl and eluted with 6 0.5 mL fractions of 50 mM Tris HCl, pH 8.0 + each 325-475mM NaCl in 25 mM increments. Fractions were resolved by SDS-PAGE.

Gels from both Ni-NTA and Q-Sepharose columns were dried under vacuum onto 3mm blotting paper using a Bio-Rad model 483 slab dryer for 4 hours at 80°C. Dried gels were exposed to film for 8-12 hours at -4 ° C to assess purity of samples (Figure A1.2). Fractions from the Q-Sepharose column containing [^{35}S]-RQ-CocE were combined, dialyzed into sterile saline to remove excess salt and stored at 4°C until injection.

Animal housing and welfare: Male and female Sprague Dawley rats (normal and unilaterally nephrectomized, 250-350g, obtained from Harlan Sprague Dawley, Indianapolis) were maintained on a temperature controlled environment with a 12-hour light cycle, on at 7am. Rats were housed in Plexiglas cages (49 cm long x 23 cm wide x 21 cm high) with cob bedding and had *ad libitum* access to food and water. Rats were allowed to acclimate to the room for at least 5 days before any surgical procedures were performed.

One adult male and one adult female rhesus monkey (*Macaca mulatta*) both fitted with indwelling catheters in the jugular or femoral vein, were used for this study. Monkeys were singly house in stainless steel caging in a temperature controlled environment with a 12 hour light cycle beginning at 7am. Monkeys were fed 20-50 Lab Fiber Plus Monkey Diet Chows (Lab Diet; PMI Nutrition International, LLC; Brentwood, MO) per day, fresh fruit, and had free access to water.

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health, and all experiments were additionally approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA).

Animal preparation for determination of* in vivo *rates of cocaine hydrolysis:

For the dose dependence study (rats): Animals were administered 3.5% isoflurane anesthesia and 100% oxygen through a rat anesthesia mask. Animals were placed on a heating pad set to maintain the animal's body temperature at approximately 37ºC. Body temperature was monitored throughout the experiment via a thermometer placed in the rectum. The region of the animals left and right legs were shaved and sterilized with alcohol and betadine. The femoral veins were exposed by incision and by peeling away connective tissue. A catheter was inserted and tied in place with sterile silk suture. After the catheter was in place it was flushed with saline. Anesthesia was continued and doses of RQ-CocE (13.6 or 50.5 µg/kg) or vehicle control were administered intravenously via one catheter. Two minutes post RQ-CocE or vehicle administration, rats were given a dose of cocaine (4 mg/kg) intravenously through the second catheter. Blood samples (approximately 200 µL/sample) were collected from the first catheter at 30 seconds, 1, 2, and 5 minutes post cocaine administration. Blood samples were immediately placed into 70 µL of EDTA solution containing 50 µL 50mM EDTA and 20 µL 1M NaF. Samples were stored on ice and spun at 1200 rpm in a bench-top microcentrifuge to separate plasma. Plasma was transferred to a clean low retention tube and prepared for LC/MS (see Cocaine Quantification).

For the species comparison study (rats): Rats were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) for catheter implantation into both femoral veins as described above. Catheters were threaded under the skin and attached to stainless steel tubing that runs through a metal tether plate sutured to the back muscle. Animals were allowed 5 days after surgery to recover from the procedure.

On the day of the experiment, rats were placed into 49 cm long x 23 cm wide x 21 cm high Plexiglas cages containing cob bedding. A long piece of catheter tubing was run out of the cage from the stainless steel tubing of the indwelling catheter so that rats

could be infused, and blood could be drawn without handling. At time t=0, rats were given a 10 second bolus of 5.6 mg/kg cocaine followed later by bolus of either RQ-CocE (0.32 mg/kg) or PBS at t=10 minutes. Blood was sampled (200 µL) at t= 2, 5, 9, 10:45, 12, 15, 20, 40 and 60 minutes and immediately added to 5 µL of 500 mM EDTA and 20 µL of 1M NaF to prevent coagulation and further cocaine hydrolysis respectively. Plasma was collected by centrifugation at 4,000 rpm in a bench top microcentrifuge, flash frozen in liquid nitrogen and stored at -80°C until preparation for mass spectrometry.

For the species comparison study (monkeys): (Animal work conducted by M.C. Ko) A male and female rhesus monkey trained for arm-restraint chairs were used for this study. Cocaine (3.2 mg/kg at time t=0) and RQ-CocE or PBS (0.32 mg/kg at time t=10 minutes) were administered through intravenous catheters. Blood samples were taken at time t=0, 8, 15, 30, 60, 90 and 120 minutes from the saphenous vein. Blood was immediately collected into tubes containing EDTA (BD Vacutainer K2EDTA Plus Blood Collection, 5 mL) and 1/10 volume 1M NaF to preventing clotting and eliminate further cocaine metabolism.

***Sample preparation and mass spectral analysis of RQ-CocE-mediated cocaine hydrolysis* in vitro *and* in vivo**

In vitro Rates of Cocaine Hydrolysis by RQ-CocE: One mL human plasma samples obtained from the University of Michigan Hospital Blood Bank were spiked with 8 µM cocaine. Plasma samples containing cocaine were incubated at 37°C for 5 minutes. After warming the plasma, RQ-CocE was added. Twenty µL aliquots of plasma were taken from the 1 mL sample at time 20 and 40 seconds, 1, 2, and 5 minutes after RQ-CocE addition. These aliquots were immediately placed into 80 µL of acetonitrile solution (containing 4 µl of saturated (1M) NaF) to prevent further cocaine metabolism

and 10 µL of internal standard (deuterated cocaine) and then immediately prepared for LC-MS/MS.

Quantification of Cocaine: Plasma collected from animals (as described above) was extracted 3x volume of 100% acetonitrile containing NaF and 10µL internal standard then immediately prepared for LC-MS/MS. Samples were then centrifuged at 13,000 rpm for 30 minutes and the supernatant collected into a clean low-retention centrifuge tube. Extracts were dried on a Savant Speed Vac to remove acetonitrile and frozen at -80°C for one week or less until analysis. Samples were then reconstituted in water and further diluted 10-100 times depending on the sample time point. LC/MS was performed by Dr. Kathleen Noon at the University of Michigan Biomedical Mass Spectrometry Facility.

LC-MS/MS analysis was performed on a Prominence HPLC system (Shimadzu, Kyoto, Japan) interfaced directly to the Turbo Ionspray source of an API 3000 triple quadrupole mass spectrometer (PE Sciex, Toronto, ON, Canada). Separation was achieved with a Phenomenex Synergi Hydro RP column (50 x 2.0mm i.d., 4 µm packing) maintained at 40ºC using a binary gradient and a flow rate of 0.15 mL/min. Solvent A was water and Solvent B was acetonitrile; both solvents were modified with 0.1% formic acid (v/v). The gradient program was as follows: 20% B at 0 min, 100% B at 3 min, hold 100% B for 1 min, return to initial conditions in 2 min and re-equilibrate at 20% B for 9 min. Each assay was completed in 15 minutes. Ten µL aliquots were injected onto the column and the sample tray was cooled to 10ºC to prevent sample degradation.

Positive ions were generated in the source using purified air for the source gases under the following conditions and settings: Turbo ESI gas, 7.0 L/min; nebulizer (NEB) setting, 6; ion spray voltage (IS), 4200 V; source gas temperature (TEM), 300ºC; declustering potential (DP), 75 V; focusing potential (FP), 160 V; entrance potential (EP), 10 V. Nitrogen was used as the curtain gas (CUR) at a setting of 8. Mass analysis was performed by single reaction monitoring (SRM) with 300 ms dwell times. Nitrogen

served as the collision gas (CAD=8). The collision energy (CE) was set to 32 eV (lab frame) with a collision cell exit potential (CXP) of 12 V. Precursor/product-ion pairs for the SRM transitions were *m/z* 304.2 → 182.2 for cocaine and *m/z* 307.2 → 185.2 for d3-cocaine. Analyst software (version 1.4.2; MDS Sciex, Toronto, ON, Canada) was used for instrument control, data acquisition, and quantitative analysis. Calibration curves were constructed from the standard samples using the ratio of cocaine to the deuterium-labeled internal standard (d3-cocaine) and a least squares linear regression analysis with *1/x* weighting.

Determination of cocaine concentrations across species by mass spectrometry: Plasma fractions from each blood sample (50-200 µL) was added to 570 µL ACN, 20 µL 1M NaF, and 2µL of internal standard solution containing 750 nM deuterium-labeled norcocaine, cocaine, benzoylecgonine and ecgonine methyl ester. Samples were vortexed for 30 seconds and centrifuged at 25,000 rcf at 20°C for 30 minutes. The supernatant was removed and added to a clean microcentrifuge tube. Samples were centrifuged a second time using the same conditions, and the supernatants were again transferred to clean tubes. Samples were evaporated to dryness in a vacuum centrifuge and stored at -80°C. Samples were analyzed within a week of preparation.

Mass spectral analysis was performed by Dr. Kathleen Noon at the University of Michigan Biomedical Mass Spectrometry Facility. For mass spectral analysis, the dried samples were reconstituted with 30 µL of 10 mM ammonium formate, pH 4.6: acetonitrile (97:3; v/v) to yield a 50 nM final concentration of each internal standard. To achieve concentrations of cocaine, norcocaine, benzoylecgonine, and ecgonine methyl ester within the limits of quantification, further dilutions (varying along the time course) were done with 10 mM ammonium formate pH 4.6:acetonitrile (97:3; v/v) and 50 nM internal standards. Samples were vortexed for 30 seconds, then centrifuged at 13,600 rcf for 20

minutes. Aliquots of the supernatants were transferred to polypropylene autosampler vials for analysis within 12 hours.

Analysis was performed on a Prominence HPLC system (Shimadzu, Kyoto, Japan) interfaced directly to the Turbo Ionspray source of an API 3000 triple quadrupole mass spectrometer (PE Sciex, Toronto, ON, Canada). Separation was achieved with a Thermo Hypersil Gold column (50 x 2.1mm i.d., 1.9 µm packing) maintained at 45ºC using a binary gradient and a flow rate of 0.45 mL/min. The flow was split approximately 1 to 3.5 so that 0.13 mL/min was directed into the ionization source. Solvent A was 10 mM ammonium formate pH 4.6 and Solvent B was acetonitrile. The gradient program was as follows: 2% B at 0 min, hold 2% B for 1 min, 18% B at 2 min, 40% B at 10 min, 100% B at 11 min, 2% B at 12 min, and re-equilibrate at 2% B for 3 min. Each analysis was completed in 15 minutes. Four µL aliquots were injected onto the column and the sample tray was cooled to 10 ºC to prevent sample degradation.

Positive ions were generated in the source using purified air for the source gases under the following conditions and settings: Turbo ESI gas, 7.0 L/min; nebulizer setting, 15; ion spray voltage, 3000 V; source gas temperature 400 ºC. Compound-specific ionization parameters were optimized for cocaine, each metabolite, and the internal standards as shown in Table A1.1. Nitrogen was used as the curtain gas at a setting of 12. Mass analysis was performed by single reaction monitoring with 100 ms dwell times. Nitrogen served as the collision gas (CAD=12). The chromatogram was divided into three time segments so that individual periods contained a subset of one or two target compounds. The corresponding SRM transitions were scanned only during the time segments in which they eluted. Period 1 spanned the first 2 minutes, Period 2 covered the next 1.7 minutes, and Period 3 extended for the remainder of the chromatogram. Precursor/product-ion pairs for the SRM transitions and their respective scan periods are listed in Table A1.2. Analyst software (version 1.4.2; MDS Sciex, Toronto, ON, Canada)

was used for instrument control, data acquisition, and quantitative analysis. Calibration curves were constructed from the standard samples. The ratios of the peak areas of the target compounds to the corresponding deuterium-labeled internal standards were plotted as a function of the analyte concentration normalized to the internal standard concentration. Calibration curves were generated using a least squares linear regression analysis with *1/x* weighting.

Preparation of calibration standards for mass spectrometry: For the *in vitro* and *in vivo* comparison, calibration standards of cocaine (3.16 μM-0.0043 μM) were prepared in human plasma (obtained from the University of Michigan Hospital Blood Bank) and commercial rat plasma from untreated Sprague Dawley rats (Valley Biomedical, Winchester VA). For the species comparison, calibration standards of cocaine, benzoylecgonine, ecgonine methyl ester (4.0-0.0313 μM), and norcocaine (0.4-0.00313 μM) were prepared in plasma from untreated Sprague Dawley rats (Valley Biomedical, Winchester VA). All standards were stored at -80°C until sample preparation. The respective calibration standards were prepared with every set of experimental samples. Twenty μL each calibration stock was extracted with 68 μL ACN, 4 μL 1M NaF, and 8 μL of internal standard as described above. Calibration standards were reconstituted to 100 μL resulting in final internal standard concentrations of 50 nM.

Serum and urine collection from conscious rats: Male Sprague Dawley rats (300-350g) (Harlan Sprague Dawley, Indianapolis) were implanted with indwelling jugular catheters. Briefly, the rats were anesthetized with 90 mg/kg ketamine and 10 mg/kg xylazine. Tubing was inserted into the jugular vein of the neck. Catheters were threaded under the skin and out the back.

On test day, rats were placed into cages fitted with a wire mesh bottom that

allowed the passage of urine and feces through to the floor of the cage and did not permit animal access to excrements. After administration of either RQ-CocE or [^{35}S]-RQ-CocE, urine was collected from the cage once each hour after RQ-CocE administration for a total of 12 hours. Blood samples (100 µL) were taken from the jugular catheter at the same time as urine collection and the volume was replaced with 100 µL saline. Serum was collected from blood samples using BD Microtainer centrifuge tubes. Both serum samples and urine samples were aliquoted and flash frozen with liquid nitrogen. Samples were stored at -80°C until analysis.

Western blotting: The presence of RQ- or [^{35}S]-RQ- CocE in urine and serum was determined by Western blotting. Urine (120 µL) was de-salted using Protein Desalting Spin Columns (Thermo Scientific). De-salted urines were concentrated to a volume of 30 µL. A portion of the desalted sample (15 µL) was loaded onto a 10% SDS-PAGE gel in loading buffer containing β-mercaptoethanol.

Serum samples from animals administrated RQ- or [^{35}S]-RQ-CocE were assessed for total protein concentration using a Bradford assay and 25 µg of total protein was loaded onto a 10% SDS-PAGE gel in loading buffer containing β-mercaptoethanol.

The same Western blotting procedure was used on both urine and serum samples from animals receiving RQ- or [^{35}S]-RQ-CocE. Gels were run at 200 volts for 40 minutes. Protein was transferred into a PVDF membrane at 60 volts for 1.5 hours. Membranes were blocked in Blotto for 1 hour before the addition of rabbit anti-CocE primary antibody (generously produced and supplied by New England Peptide) at a concentration of 1:4,000 diluted in Blotto. Primary antibody was incubated overnight shaking at 4°C. Membranes were washed 3 times for 15 minutes in PBS containing 0.1% Tween (w/v) (PBS-T) before the addition of goat anti-rabbit-HRP secondary

antibody diluted 1:30,000 in Blotto. Secondary antibody was incubated for 1 hour at room temperature. Blots were washed 3x for 15 minutes in PBS-T and exposed to ECL reagent for one minute. Blots were exposed on Kodak Film.

Blotting membranes containing samples from [^{35}S]-RQ-CocE treated animals were allowed to stand for 6 hours after ECL exposure to ensure total completion of the light emitting enzymatic reaction. These membranes were then exposed to film for 2 weeks at room temperature to assess the radioactive contents of the membranes.

Urinalysis: Urinalysis was performed with 10SG Urine Reagent Strips (Fisherbrand) according to package instructions. Urine measures from normal rats was compared to urine measures from RQ-CocE treated animals to ensure that readings outside the specified normal range were due to a species difference between rats and humans, not an effect of RQ-CocE.

Determination of glomerular filtration: Glomerular filtration rates of normal and unilaterally nephrecotmized animals were determined using an adapted method [14]. Briefly, a 5% FITC-Inulin (Sigma Chemical) solution was made in 0.9% sodium chloride (saline, Hospira) by boiling for 2 minutes. Solution was dialyzed overnight in 1000 mL Saline using a dialysis membrane (1000 Da cutoff, Spectra/Por® 6, Spectrum Laboratories). Solution was sterilized by filtration before injection. Forty mg/kg FITC-Inulin solution was injected intravenously through indwelling jugular catheters. Serum samples were taken at 2, 5, 8, 11, 15, 20, 40, 60, 20, 120, 180 and 240 minutes after injection. Samples were buffered by diluting 1:25 in 500mM HEPES pH 7.4. Fifty µL of the buffered sample was added in duplicate to a 96 well, and fluorescence was determined using a SpectraMax M5 (Molecular Devices) with 485 nm excitation and 538 nm emission.

Fluorescence per µL of serum was plotted and fitted to a 2-phase decay model using GraphPad Prism Software. Glomerular filtration rate was calculated using equation (1) where I is the amount of total fluorescence delivered by the bolus injection, A and B are the y-intercept values of the fast and slow decay rates and α and β are the decay constants for the fast and slow decay phases.

(1) $$GFR = \frac{I}{(\frac{A}{\alpha}+\frac{B}{\beta})}$$

Calculation of pharmacokinetic parameters: To assess the radioactive content in the blood, 3 µL of each sample was taken at t=3, 16, 20, 30, 45, 60,and 90 minutes, as well as 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 18, 19 and 29 hours. Urine samples were collected after every urination. Samples were analyzed by a Packard 1900 TR liquid scintillation analyzer in 4 mL of CryoScint ES (MP Biomedicals). From the radioactive content, the concentration of CocE (determined using equation 2) in the blood was plotted against time with Prism software.

(2) $$\frac{\mu g}{mL}\ CocE = \frac{cpm}{\mu L} \div \frac{total\ cpm\ injected}{total\ ug\ CocE\ injected}$$

Area under the curve (AUC) analysis was carried out by the Prism software and clearance (Cl) was determined using equation 3 [15]. C_{max} was determined from the y-intercept of a log plot of the concentration data, and volume of distribution (V_d) was subsequently calculated using equation 4 [15]. Half-life ($t_{1/2}$) in the blood was then determined using equation 5 [15].

(3) $$Cl = \frac{Dose\ (\mu g)}{AUC\ (\mu g/mL/min)}$$

(4) $$V_d\ (mL) = \frac{Dose\ (\mu g)}{Cmax\ (\mu g/mL)}$$

(5) $$t_{1/2}\ (min)\ = \frac{0.693}{Cl\ (mL/min)} \times V_d\ (mL)$$

Estimations of these parameters were initially made using densitometry from

Western blots. The C_{max} was assumed to be the total amount of CocE injected divided by the blood volume of each animal (7% total body weight). Density from the serum sample taken 5 minutes after CocE injection was assumed to be 100% of this C_{max}. Each subsequent density was expressed as a percentage of the 5 minute time point and multiplied by the C_{max}. Half-life was determined by a one-phase decay model fit of the estimated concentrations by GraphPad Prisim Software.

Collection of organs: Male Sprague Dawley rats were deeply anesthetized with sodium pentobarbital (75 mg/kg). After animals no longer responded to paw pressure, the abdomen was opened and the sternum cut to reveal the heart and lungs. The right atrium was cut and an 18.5 gauge needle was inserted into the left ventricle. Sixty mL of saline was delivered through the left ventricle. Complete perfusion was determined by no red color remaining in the heart or any lobe of the liver. Organs were removed and placed into 10% buffered formalin (100 mL 37% Formaldehyde, 4g sodium phosphate monobasic, 6.5 g sodium phosphate dibasic, and volume to 1 liter) for immunohistochemistry.

Preparation of organs and histology:

Immunoperoxidase: Organs were fixed in 10% buffered formalin overnight at room temperature. Dehydration was completed by 3x 10 minute washes in 30% ethanol, followed sequentially by 3x 10 minute washes in both 50%, and 70%. Slide preparation and staining was performed at the University of Michigan Comprehensive Cancer Center Tissue Core. Organs were paraffinized and stored at room temperature until sections were cut from the blocks. Paraffin sections were cut on a microtome to 5 microns and heated for 20 minutes at 65 degrees Celsius. Slides were deparaffinized in xylenes, 3

changes of 2:00 minutes each. Slides were then rehydrated through graduated washes of 2:00 minutes each, ending with tap water (100% alcohol, 95% alcohol, 70% alcohol, water). Epitope retrieval was completed by incubating slides for 30 minutes in 80°C citrate buffer (pH 6.0). Slides were cooled for 10 minutes followed by a 10 minute running water wash.

Staining was performed on the DAKO AutoStainer at room temperature. Slides were blocked with peroxidase before the addition of primary rabbit anti-CocE anti-body at a dilution of 1:1000 for 30 minutes. After 3x PBS buffer rinse, EnVision Plus Rabbit detection kit was used as the secondary antibody for 30 minutes. Slides were exposed to chromagen for five minutes before hematoxylin counter-stain for two minutes. Slides were rinsed with water and dehydrated in gradual alcohol rinses. Three changes of xylenes for two minutes each were followed by addition of the coverslip. Slides were stored at room temperature until imaging using a Nokia slide scanner at the Microscopy and Imaging Laboratory at the University of Michigan. Images were adjusted for contrast and brightness, as well as background removed in Adobe Photoshop CS2.

Fluorescence: Organ sections were prepared as described above. Non-specific binding was reduced by blocking slides with 10% goat serum for 1 hour. Primary rabbit anti-CocE anti-body (generously produced and supplied by New England Peptide) was added onto slides at a dilution of 1:1000 in 10% goat serum overnight at 4°C. Slides were washed 3x for 10 minutes in PBS. Slides were exposed to secondary goat anti-rabbit Dylight-594 antibody (Jackson ImmunoResearch Laboratories) at a 1:1000 dilution in 10% goat serum for 1 hour. Coverslips were placed onto slides using ProLong Gold (Invitrogen). Slides were stored in the dark at room temperature until imaging with a Leica DMI6000 B microscope. Exposures were adjusted so that the maximal pixel intensities of each time course set were at least half saturation. Fluorescence was

quantified using Image J (NIH) software and statistics performed with GraphPad Prisim software. Images were adjusted equally for contrast and brightness using Adobe Photoshop CS2.

Radiography: Organs sections were prepared for freezing by dehydration in 10% sucrose for 6 hours and 30% sucrose overnight. Organs were frozen on dry ice in Optimal Cutting Temperature Compound (O.C.T, Tissue Tek® Sakura) and stored at -80° until sections were cut from the blocks. Frozen section were cut on a microtome at -20°C to 30 µM and fixed to glass slides. To image, slides were exposed to Kodak film at room temperature for 3 weeks.

Results

RQ-CocE-mediated cocaine hydrolysis* in vitro *and* in vivo*: Cocaine hydrolysis by RQ-CocE *in vitro* in plasma was compared to equivalent concentrations *in vivo*. To establish a baseline for the *in vivo* experiments, human plasma was spiked with 8 µM cocaine, the peak plasma concentration achieved in an anesthetized Sprague Dawley rat after a 4 mg/kg bolus intravenous injection (data not shown). Low concentrations of RQ-CocE (0.3-10 nM) were chosen in order to ensure slow cocaine hydrolysis that would allow differentiation between RQ-CocE concentrations.

Plasma spiked with cocaine followed by the addition of PBS (vehicle for RQ-CocE) did not show any reduction in cocaine concentration over the time course, demonstrating that the plasma was void of any rapid cocaine metabolizing enzymes. RQ-CocE was able to dose dependently decrease the cocaine concentration over time, with 0.3 nM RQ-CocE creating a slight reduction in the cocaine concentration and 10 nM eliminating cocaine to levels below the limit of quantification within 2 minutes (Figure

3.1A). Three nM RQ-CocE was able to eliminate approximately 50% of the cocaine within 1 minute after addition.

To determine the *in vivo* effect of these low doses of RQ-CocE and to explore any effect of sex on RQ-CocE-mediated cocaine hydrolysis, male and female Sprague Dawley rats were dosed with 13.6 or 50.5 μg/kg RQ-CocE, corresponding to *in vitro* concentrations of 0.003 μM and 0.01 μM, respectively (calculated based on a 350 gram rat with a blood volume 7% that of the body weight). RQ-CocE was administered as an IV bolus 2 minutes before an IV bolus of cocaine. RQ-CocE (13.6 μg/kg) significantly reduced cocaine plasma concentrations at the 30 second ($p<0.05$) and 2 minute ($p<0.01$) time points (Figure 3.1B). This dose was able to eliminate the initial spike in cocaine concentration seen in the untreated animals, however it was not able to completely clear all cocaine, as seen *in vitro*. RQ-CocE at a dose of 50.5 μg/kg (0.01 μM) significantly reduced the cocaine concentrations at all points ($p<0.001$), eliminated the initial cocaine concentration spike and eliminated cocaine to below detectable levels.

Analysis of the data collected from these male and female rats used in this paradigm revealed that the sex of the animals tested did not have a statistically significant impact on overall cocaine concentration (Figure A1.3A). The effectiveness of 3 nM RQ-CocE on a high concentration of cocaine near what has been seen in post-mortem studies of cocaine overdose victims was also assessed. Even 3 nM RQ-CocE diminished the cocaine concentration in human plasma originally spiked with 100 μM by 50% over the short 5 minute time course (Figure A1.3B).

Cocaine hydrolysis by RQ-CocE across species: To determine if cocaine hydrolysis by RQ-CocE is affected by the plasma matrix of individual species, RQ-CocE's ability to

eliminate convulsant levels of intravenous cocaine was assessed by mass spectrometry in both rats and monkeys.

The doses of cocaine used in this experiment (5.6 mg/kg in the rat and 3.2 mg/kg in the monkey) were physiologically equivalent by cardiovascular (Figure A1.4) and behavioral parameters. Rats receiving 5.6 mg/kg cocaine experienced a change in mean arterial pressure (MAP) of 40-50 mmHg. CocE (0.32 mg/kg) or PBS was administered 10 minutes following the cocaine injection. The MAP of rats treated with CocE rapidly returned to baseline by 15 minutes, however, the MAP of rats treated with PBS did not return to baseline within 30 minutes. Monkeys receiving 3.2 mg/kg cocaine also experienced an increase of about 40-50 mm Hg. RQ-CocE (0.32 mg/kg) 10 minutes after cocaine administration caused a rapid decrease in MAP to baseline levels by 50 minutes after RQ-CocE injection. The respective doses of cocaine also cause tonic-clonic convulsions in both species.

Figure 3.2 outlines the elimination of cocaine over time in rats and monkeys. In the rat, 5.6 mg/kg cocaine produced transient tonic-clinic convulsions and initial plasma cocaine levels in a range from 7-27 μM (Figure 3.2A). The cocaine concentration in rats receiving only the PBS control 10 minutes after cocaine fell to 0.1-1.7 μM over the 1 hour time period, due to endogenous metabolism of cocaine. In contrast, animals receiving RQ-CocE after 10 minutes had unquantifiable cocaine concentrations after only 45 seconds. Interestingly, between the 20 and 60 minute time points, the cocaine concentrations began to rise into the lower limits of quantification.

In the female monkey, administration of 3.2 mg/kg cocaine yielded a plasma concentration between 4.2-30.8 μM, a similar range as the rats (Figure 3.2B). After administration of RQ-CocE at t=10 minutes, all cocaine was hydrolyzed to below

quantifiable levels within 5 minutes. Although, due to technical limitations, this blood sample was taken longer after RQ-CocE administration in the monkey, it demonstrates the rapid hydrolysis of cocaine in the blood. Like the rat, the cocaine concentrations begin to slowly rise after the 30 minute time point, suggesting that this rise is not a species specific phenomenon.

The male monkey was tested on a different dosing regimen, as RQ-CocE was given only 1 minute after cocaine (Figure 3.2C). Even at this short time point when not all cocaine has equally distributed, RQ-CocE still eliminates blood cocaine concentrations to below quantifiable levels by 8 minutes (the first sample taken after CocE injection). The male monkey also showed results consistent with the rats and female monkey, in that after 30 minutes, blood concentrations of cocaine begin to slowly rise.

Elimination of RQ-CocE from serum: In both the rat and the monkey, very small amounts of cocaine reappeared in the plasma by 30 minutes after RQ-CocE administration that initially appeared to clear all cocaine. Previous work demonstrated that a high dose of both wild-type and a thermostable CocE mutant (KQ-CocE, KQ-CocE, $t_{1/2}$ @37° *in vitro* =2.9 days) were rapidly eliminated from the serum of mice with an estimated half-life of 2.1 and 2.2 hours from Western blot analysis [12]. If this elimination seen in mice also occurs in rats and monkeys, it would cause a reduction in the rate of cocaine hydrolysis in the circulation and therefore allow very low concentrations of cocaine in the periphery to diffuse into the circulation. We thus examined if this elimination was occurring in the rat test system.

The presence of RQ-CocE in the serum of rats at times after intravenous 8 mg/kg RQ-CocE injection was assessed by Western blot. The initial concentration after bolus

injection was assessed by serum taken 5 minutes after RQ-CocE injection, and followed by serum taken at 1, 2, 4, 6, 8, and 12 hours. Only full length protein is seen in these Western blots (Figure 3.3A).

RQ-CocE is rapidly cleared from the rat with an estimated half-life of 2.3 hours from this method similar to that found for KQ-CocE and wt-CocE in the mouse [12]. This further confirms that thermostability of the protein does not play a role in the elimination of the protein. Estimated pharmacokinetic parameters from the Western blot study are shown in Table 1. Estimations are based on total dose of CocE, total blood volume, and percentage of CocE remaining over time assessed by densitometry.

To confirm that smaller fragments of CocE are not present in the serum samples and not that the antibody cannot recognize smaller fragments of CocE, serum samples were boiled under denaturing and reducing conditions to create chemical cleavages. Western blot analysis on these samples revealed 5 chemical cleavage products of CocE (Figure A1.5), confirming that the antibody used can recognize fragments of CocE. These data support the hypothesis that either CocE is not undergoing proteolytic cleavage *in vivo* or that proteolyzed products are rapidly removed from the serum.

Urine samples were also collected from these animals and desalted, to allow for analysis by Western blot. Urine samples taken one and two hours after CocE injection contained full length and some smaller molecular weight fragments (Figure 3.3B). Urinalysis by dip-stick was also conducted on these samples and all samples had glucose, bilirubin, ketone, specific gravity, blood, pH, protein, urobilinogen, nitrate and leukocytes readings that did not differ from animals not receiving RQ-CocE (data not shown).

Pharmacokinetics of RQ-CocE: To accurately determine the pharmacokinetics of CocE, and determine the mechanism of observed elimination, [^{35}S]-methionine was used to metabolically label the protein with a traceable marker. RQ-CocE contains 11 methionines (Met1, 13, 16, 113, 141, 323, 350, 483, 507, 521, 550), all well distributed throughout the molecule to assure that large fragments of RQ-CocE would retain a radioactive residue. [^{35}S]-labeled RQ-CocE ([^{35}S]-RQ-CocE) was purified by nickel affinity and ion exchange chromatography (Figure A1.2).

Before injection of 8 mg/kg (~18 μCi/kg CocE), the total counts per minute (cpm) were assessed to determine the specific activity of each individual radioactive dose. Serum and urine samples were collected from each animal over a 29 hour period and assessed for radioactive content (Figure 3.4A and 3.4B). The pharmacokinetic properties of RQ-CocE determined from monitoring [^{35}S] in the blood with scintillation counting are presented in Table 1. These parameters were determined using curve fit and AUC analysis by Prism software over the 10 hours post-CocE injection. The half-life of CocE determined by [^{35}S] labeling (125 minutes) is not significantly different than the estimation from Western blotting studies (144 minutes). The similar half-lives, RQ- and [^{35}S]-RQ CocE do not behave differently *in vivo,* validating the [^{35}S] labeling test system. The volume of distribution (V_d) was calculated using the C_{max} and total dose of CocE (equation 4). The V_d is close to the total blood volume of a rat, confirming assumptions based on the molecular weight (65 kDa monomer, 130 kDa dimer) that CocE does not reside outside the bloodstream. Although the V_d is slightly higher than the estimated blood volume, it is not due to protein binding of RQ-CocE as the cpm of each sample were determined using a whole blood sample, and is most likely due to underestimation of the blood volume using percentage body weight. .

The concentration of RQ-CocE in the blood over time in the rats (calculated using equation 2, methods) is illustrated in Figure 3.4A. The concentration of CocE peaks immediately after injection, due to the 100% bioavailability of intravenous delivery. Over the first four hours after injection, there is a slow and steady decrease in concentration to approximately 40 µg/mL/kg RQ-CocE (see Figure 3.4A insert). For the remainder of the time course, the concentration of CocE (as calculated using counts per minute, cpm) remains constant. To determine whether the measured radioactivity represented full length CocE, or smaller fragments in the circulation, further analysis was conducted.

Serum was separated from whole blood and Western blots were performed on the samples to determine whether the radioactivity in the blood was from full length [^{35}S]-RQ-CocE. Radiography was performed on the same membranes after Western blotting to determine whether epitope-free fragments of CocE exist that were not visualized by the immunological method. The comparison between radiograph and Western blot also revealed that these methods of analysis were highly correlated, and that the cpm analyzed in the blood, at least for the initial time points, corresponds to full length RQ-CocE (Figure 3.5A). Full length (65 kDa) RQ-CocE is strongly visible in the radiograph (Figure 3.5B) at 0.5, 1 and 2 hours after injection. However at later time points, only a small amount of full length RQ-CocE is visible, and more radioactive labeling of higher and lower molecular weight species is seen. This suggests that the radioactive label is being incorporated into other proteins, suggesting that amino acids from RQ-CocE may be recycled.

Urine samples were analyzed by scintillation counting. A significant percentage (~10%) of the total cpm injected was recovered in the urine over 29 hours. This cumulative elimination is illustrated in Figure 3.4B and reveals elimination occuers in two phases. The first encompasses the first four hours and was rapid, with 25% of the total

cpm eliminated within this time frame (1.24 x 10^6 cpm/hr). The second phase of elimination over the last 25 hours is only half the rate of the first (0.6 x 10^6 cpm/hr).

Presence of RQ-CocE in major organs: Analyzing serum samples by Western blot and radiography, we determined that counts per minute were a good indicator of full length CocE for the first 4 hours after adminstration. However, after this time, more diffuse radioactivity across a wide range of molecular weights was seen on radiographs. Additionally after the amount of radioactivity in the serum stabilized to a constant level, the cumulative amount of radioactivity eliminated in the urine continued to rise. We hypothesized that CocE could either undergo specific or non-specific receptor mediated endocytosis, uptake into major organs, or rapid degradation to produce recycled amino acids for cellular pathways. To this end, we performed immunohistochemistry on the heart, lung, liver, and kidney of male Sprague Dawley rats.

Rats were given RQ-CocE IV into the tail vein 8 mg/kg every day for 14 days or 24 mg/kg every 4 days for 14 days. Six hours after the final RQ-CocE injection, rats were sacrificed and organs collected for immunohistochemistry and pathological analysis. No significant pathological difference was found between the organs of saline and RQ-CocE treated rats (data not shown). Upon immunohistological analysis, no RQ-CocE was detected in the heart, liver, or lung. However, significant RQ-CocE was detected in the kidney, specifically localized to the renal papilla (Figure 3.6). Dose-dependent papillar accumulation was seen, as the kidneys from the 8 mg/kg treated animals showed much less immunoreactivity than the kidneys from the 24 mg/kg treated animals (Figure 3.6). This finding is consistent with the [^{35}S]-RQ-CocE labeling data that revealed significant amounts of radioactivity in the urine.

Time course of renal accumulation and clearance: To assess the hypothesis that kidney plays a significant role in RQ-CocE elimination, a time course of RQ-CocE in the renal papilla was performed by immunohistochemistry on kidney slices. Sprague Dawley rats were given either saline or 8 mg/kg RQ-CocE intravenously through an indwelling jugular catheter. At each time point after RQ-CocE injection (0.5, 1, 2, 4, 8, 12, 24 hours) three rats were sacrificed and kidneys prepared for immunohistochemistry (Figure 3.7A). RQ-CocE reaches maximum accumulation levels in the papilla at 2 hours after RQ-CocE injection and reaches near baseline levels by 4 hours. By 12 to 24 hours after RQ-CocE injection, there are no longer significant amounts of RQ-CocE reactivity compared to saline (Figure 3.7B).

Effect of unilateral nephrectomy: To test whether the kidneys play a major role in the elimination of full or fragmented CocE from the circulation, or whether renal elimination of CocE is a secondary to another means of elimination, [^{35}S]-RQ-CocE was given to unilaterally nephrectomized rats with a reduced glomerular filtration rate compared to normal animals (2619 µL/min compared to 6030 µL/min respectively). Assessment of the pharmacokinetics of ^{35}S-RQ-CocE as described above in normal animals, yielded parameters that were not statistically significantly different from the normal animals (Table 1). Figure 3.4A shows that the patterns of elimination from the blood are not significantly different in the nephrectomized animals. Although no statistically significant differences between groups were seen, the nephrectomized animals clearance rate that was lower (0.16 compared to 0.21 mL/min) and a $t_{1/2}$ of CocE that was higher (156 compared to 125 minutes) than normal animals. These differences suggest that the kidneys are most likely assisting with the clearance of CocE, but that glomerular filtration, of either full length or fragmented CocE is not the predominant mechanism of elimination. Although this could be predicted by the finding

that only 10% of the total cpm injected were found in the urine, the results that nephrectomy had no significant effect on even the 10% of RQ-CocE eliminated in the urine definitively demonstrates the very minor role of renal clearance.

Uptake of radioactivity into organs: To evaluate the fate of the unrecovered 90% of radioactivity administered, animals receiving [^{35}S]-RQ-CocE were analyzed post-mortem. [^{35}S]-RQ-CocE treated animals were sacrificed after the 29 hour observation period and thoroughly perfused to remove all radioactivity in the circulatory system so that the major organs could be analyzed by radiography. Slices (30 μM) of heart, lung, liver, kidney, spleen, stomach, and small intestine were exposed to film for 3 weeks. All tissues from rats receiving [^{35}S]-RQ-CocE were non-specifically labeled with [^{35}S], as demonstrated in Figure 3.7C. Immunohistochemistry on the kidney and liver from rats treated with RQ-CocE 24 hours prior reveals no CocE immunoreactivity demonstrating that the [^{35}S] in the organs is due to amino acid recycling and uptake into cellular pathways.

Discussion

This study explored the biologic fate of bacterial cocaine esterase, both its ability to hydrolyze cocaine once injected, and its elimination, using Sprague Dawley rats and Rhesus monkeys. The pharmacodynamics of CocE were assessed using the disappearance of cocaine as a marker for CocE activity. The *in vivo* cocaine hydrolysis by RQ-CocE was measurable and was similar to the *in vitro* time course of degradation. The *in vitro* assessment was done under very controlled conditions, eliciting constant and steady degradation of cocaine from a constant 8 μM of cocaine. Eight μM cocaine was chosen as a starting concentration for the *in vitro* study based on a preliminary study that demonstrated that the average peak concentration of a 4 mg/kg intravenous

bolus dose in an anesthetized rat was 8 μM. The *in vivo* data reflect not only a reduction in cocaine concentration due to RQ-CocE, but also the slow reduction in cocaine concentration due to the initial phase of cocaine distribution after IV administration. Therefore, in the animals receiving RQ-CocE vehicle only, there is still a small decrease in the cocaine concentration over the 5 minute period examined.

The *in vivo* experiment is novel to the cocaine metabolism literature in that it took place over a very short time course (5 minutes). This study also used very low doses of RQ-CocE (nanomolar concentrations, 13.6 and 50.5 μg/kg) demonstrating the efficacy of the enzyme. Additionally, this dose selection gives the minimally effective dose of RQ-CocE that would be active *in vivo* and can be used as a guide to determine appropriate doses for first-in-man clinical trials. These results demonstrate that very low doses can disrupt the normal metabolism of cocaine and that the minimally effective dose *in vivo* is slightly higher than it is *in vitro*. This is expected, due to the constant redistribution of cocaine that must take place across biological membranes to reach the enzyme in the bloodstream, a barrier not present in controlled *in vitro* experiments.

This is also the first study to test CocE across sex and species. Both female rats and monkeys were compared to males, with no significant difference seen in RQ-CocE's ability to hydrolyze cocaine. Testing CocE across species was done to confirm that CocE's ability to hydrolyze cocaine would not be affected by different plasma matrices. Rats and monkeys were subjected to a similar regimen, a convulsant dose of cocaine followed by 0.32 mg/kg CocE. Monkeys were challenged to this regimen once every 2 weeks, to evaluate whether repeat dosing would produce neutralizing antibodies against CocE (full immunological study to be reported elsewhere by M.C. Ko); however, it is clear from Figure 3.8A and 3.8B, that CocE's efficacy against cocaine does not diminish with multiple doses. This agrees with recent findings by Collins et al. (2009) that

although there is an increase in anti-CocE antibody titer after repeat dosing in rhesus monkeys, this titer does not affect CocE's ability to reverse cocaine-induced cardiovascular changes [16]. Interestingly, later doses of CocE seemed to be cleared more slowly than earlier doses. However, it is not clear whether this is a true correlation, or whether this is simply variation between administrations. When individual rats were put through this dosing regimen, a similar variation was seen between animals, suggesting that the variation between doses in monkeys may simply be variation and not a trend due physiological changes.

We have previously shown that CocE has a short serum half-life in mice and that a short serum half-life limits CocE's duration of action *in vivo* [12]. Here we have significantly expanded on this work to describe the pharmacokinetics of CocE and the mechanism of CocE's rapid elimination. Using a lower dose of RQ-CocE (8 mg/kg) in male Sprague Dawley rats, we found a similar half-life for full length RQ-CocE; 2.4 hrs for full length RQ -CocE by Western blot analysis and 2.1 hrs in radiometric studies, compared to an estimated 2.3 and 2.2 hours by Western blot for KQ-CocE and wild-type CocE respectively. This short half-life fits with several pieces of data. First, Brim et al. (2010) demonstrated that KQ-CocE could block cocaine self-administration behavior in Sprague Dawley rats after a 1 hour pretreatment. However, a two hour pretreatment did not elicit a significant reduction in responding and led to an increase in responding in some animals (as would be expected with a surmountable type of antagonist). Second, in the current study, CocE administration after high dose cocaine in both rats and monkeys resulted in rapid cocaine elimination followed by a small and slow reappearance of cocaine after 30 minutes. This reappearance of cocaine is most likely due to the very low concentrations of cocaine left in the body being able to diffuse back into the bloodstream as the elimination rate of cocaine is slowed as CocE is eliminated.

These data demonstrating short half-lives *in vivo* are consistent with the clinical half-life of several protein theraputics with similar size to RQ-CocE currently on the US drug market such as agalsidase (Fabrazyme®, Genzyme, 100 kDa, $t_{1/2}$=45-102 minutes [17]), denileukin difitox, (Ontak®, Eisai, 58 kDa, $t_{1/2}$=70-80 minutes [18]) and laronidase (Aldurazyme®, Genzyme, 83kDa, $t_{1/2}$=1.5-3.6 hrs [19]). These proteins are subject to rapid elimination, presumably through receptor mediated endocytosis and rapid breakdown in the liver [20]. Interestingly, these biologic drug products, unlike small molecules, do not have a mechanisms of elimination that are well understood and do not require metabolism or mechanism of elimination information to be included in the full prescribing information mandated by the Food and Drug Administration.

We sought to understand the metabolism and elimination of CocE from the circulation. Studies using metabolically-labeled RQ-CocE (with [^{35}S] methionine) confirmed data that CocE is present in the urine as a full length protein, as well as demonstrated that elimination of RQ-CocE (or its fragments) continued to be eliminated renally over the 29 hour period after injection, accounting for about 10% of the total radioactivity administered. An additional 10% of the total radioactivity remained in the blood but 80% of the radioactivity was unaccounted for after 29 hours. To investigate where the full length or fragmented RQ-CocE was accumulating, we examined the distribution of RQ-CocE to determine if receptor mediated uptake was occurring..

Immunohistochemical analysis revealed that RQ-CocE was not found in the tissues of the heart, lung, or liver. However, there was extensive RQ-CocE reactivity in the renal papilla, the last portion of the kidney the renal filtrate flows through before entering the collecting duct and leaving the body through the ureters and bladder. This reactivity was time dependant and reversible, as no significant reactivity was seen 24 hours after RQ-CocE administration.

It is possible that the RQ-CocE detected in the renal papilla could either be full length, or fragments, due to the polyclonal antibody's recognition of both and the visualization of both by Western blotting of the urine. These findings combined are consistent with renal elimination for at least a percentage of the RQ-CocE administered (estimated 10% from [^{35}S] labeling). However, the hypothesis that renal elimination is a significant contributor to the loss of CocE from the circulation is discounted, as the kinetics after unilateral nephrectomy (reported to alter the kinetics of renal eliminated drugs in humans and animals [21, 22]) are not altered.

Since the kidneys appear not to play a major role in the elimination of CocE, and no epitope-containing RQ-CocE was detected in the major organs at 6 hours or 24 hours after injection, the organs from [^{35}S]-RQ-CocE treated animals were analyzed using radiography. Animals were fully perfused with saline (3 times the blood volume) to fully remove any radioactive contribution from the blood. Exposure of these organs to film resulted in dark, dense shadows of the organs themselves. Though it is possible that in highly vascularized organs like the liver and heart that some residual blood may have been left behind after perfusion, organs with less vascular integration like the intestine and stomach displayed the same strong radioactive signal. This implies that CocE's amino acid are being recycled and used in cellular processes in all tissues.

Uptake of our radioactively labeled methionine is most likely occurring as uptake of all circulating amino acids occurs. Amino acids circulate at concentrations from 0-650 µmol/L (16-30 µmol/L methionine) in adult humans depending on the amino acid [23]. These concentrations are maintained by consent uptake and efflux from cells (especially liver and muscle) through various high affinity transporters. Neutral, cationic and anionic amino acids each have their own carrier systems and transporters to move them throughout the body. Becuase our label is on methionine, the movement of methionine

into and out of cells by the L-type transport system is briefly described (the A-type, sodium dependent symport [24-27], ASC-type, sodium dependent antiport [24, 25, 28] and B^o-type, sodium or calcium dependent symport [24] systems also play roles in this phenomenon).

L-type transporters (LAT1-4) are sodium-independent and transport large neutral amino acids with high affinity [29]. LAT2 is an antiporter from the solute carrier gene family (SLC7) with a broader substrate specificity the closely homologus LAT1 [24, 30]. LAT2 is widely distributed throughout the body but concentrated in organs with epithelial barriers such as kidney, intestine, placenta, brain, lung, and skeletal muscle. Its primary function is amino acid exchange across epithelial barriers, but its expression in tissues without these barriers indicates its broader function [29, 31]. LAT1 also functions to transport amino acids across barriers but is also much more highly expressed in proliferating cells [31]. LAT3 and LAT4 are uniporters from the SLC43 family and are less homologous to LAT1 and 2 as they are to each other [24, 32, 33]. LAT3 and 4 have much narrower carrier specificity and LAT3 has a very narrow tissue distribution, only being expressed in the pancreas and liver. However, LAT4 is widely distributed in kidney, intestine, heart, brain, adipose, and lymphocytes [24]. The broad tissue distribution of the L-type transporters agrees with the radiograph data from [^{35}S]-methionine treated rats that demonstrated complete and ubiquitous labeling of the major organs.

As a treatment for cocaine toxicity, the rapid breakdown of the enzyme into recyclable amino acids is advantageous, as it could be given to a patient, act rapidly on cocaine, but be eliminated within hours. This should reduce the likelihood of a large immune response in humans. In fact, very little immune response is seen with CocE in mice [7, 34] or in monkey [16, 35], and rapid degradation and elimination may contribute

to this. RQ-CocE is also not accumulating as large aggregates, which may cause damage to capillary beds or organs like the lung and kidney, supported by our data that shows no evidence of full-length protein in the major organs. The advantage to RQ-CocE is that, like other biologic drugs, they are simply metabolized by the body to amino acids, and not to potential toxins like many metabolites of small molecule drugs.

If CocE is to be used as a treatment for cocaine abuse (for which proof of concept was established by Collins et al. [13]), the rapid elimination needs to be prevented to ensure a long half-life. This is feasible using established techniques. PEGylation (the chemical modification of proteins with polyethylene gycol chains) has successfully been used to extend the half-life of FDA-approved biologics such as L-asparaginase (pegaspargase, Oncaspar®) and filgrastim (pegfilgrastrim, Neulasta®). Pegasparagase and pegfilgrastim both have greatly extended half-lives compared to their parent drugs: extension from 8-30 hours [36] to 3.2-5.7 days [37] after PEGylation of L-asparaginase and 3.5 hours [38] to 15-80 hours [39] for filgrastim. These increases are due to an increase in hydrodynamic volume protecting the proteins from proteolysis and glomerular filtration [20]. PEGylation of CocE prevents proteolysis by trypsin *in vitro* [40], and *in vivo* studies with PEGylated CocE are currently under investigation in our laboratory.

Overall, this study is the first account of the pharmacodynamics and kinetics of CocE and gives insight into the ability of bacterial enzymes to become feasible drug products.

	Western Blot Estimation	Normal (+/- SD)	Unilateral Nephrectomy (+/- SD)
GFR (µL/min)		6030 (+/- 106.4)	2610 (+/- 53.4) ***
Urine Output (total 29 hrs, mL)		13.0 (+/- 1.8)	15.2 (+/- 6.3) n/s
Weight (g)		343.3 (+/- 31.75)	302.0 (+/- 10.82) n/s
AUC (µg/mL/min)		14,461 (+/- 7445)	14,932 (+/- 606.8) n/s
Clearance (mL/min)	0.12	0.21 (+/- 0.07)	0.16 (+/- 0.01) n/s
V_d (mL)	24.6 mL	38.1 (+/- 11.4)	36.6 (+/- 5.4) n/s
$T_{1/2}$ (min)	144	125 (+/- 6.8)	156 (+/- 19.8) n/s

Table 3.1: Pharmacokinetic parameters of RQ-CocE. ***p<0.001 Students T-test compared to normal. n/s p>0.05 Students T-test compared to normal.

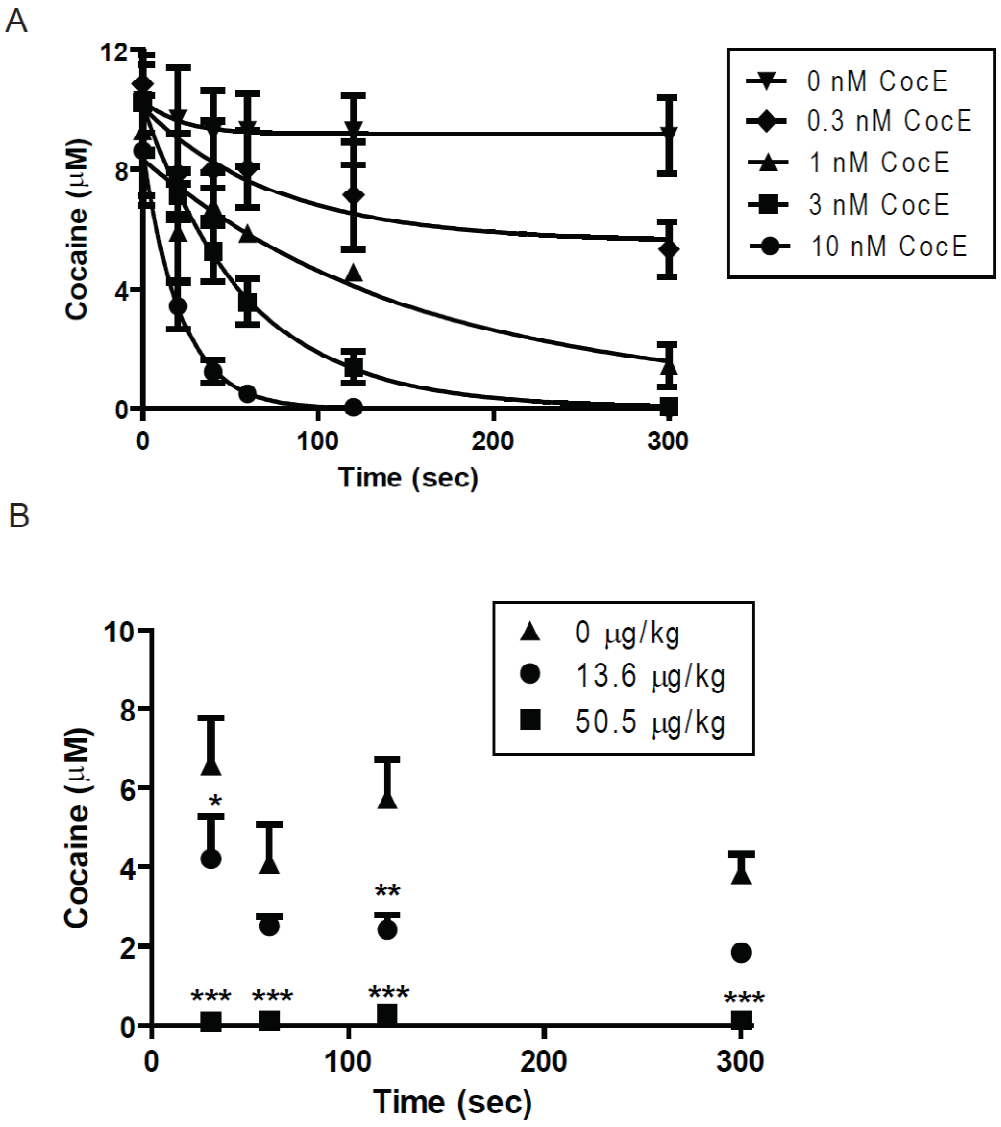

Figure 3.1: *In vitro* and *in vivo* cocaine hydrolysis by RQ-CocE **A** *In vitro* RQ-CocE dose response analysis. Human plasma samples were spiked with 8 μM cocaine at time=0. After 5 minutes at 37°C, RQ-CocE was added at the given concentrations to the plasma after which aliquots were taken and all hydrolysis stopped at the given points. Aliquots were assessed for remaining cocaine concentration by LC/MS analysis. **B** *In vivo* assessment of cocaine hydrolysis by RQ-CocE. Sprague Dawley rats were intravenously administered 0, 13.6 or 50.5 μg/kg RQ-CocE followed 2 minutes later by an intravenous administration of 4 mg/kg cocaine. Blood samples were taken at the times after cocaine injection shown, hydrolysis activity was stopped and samples were evaluated by LC/MS for cocaine concentration.

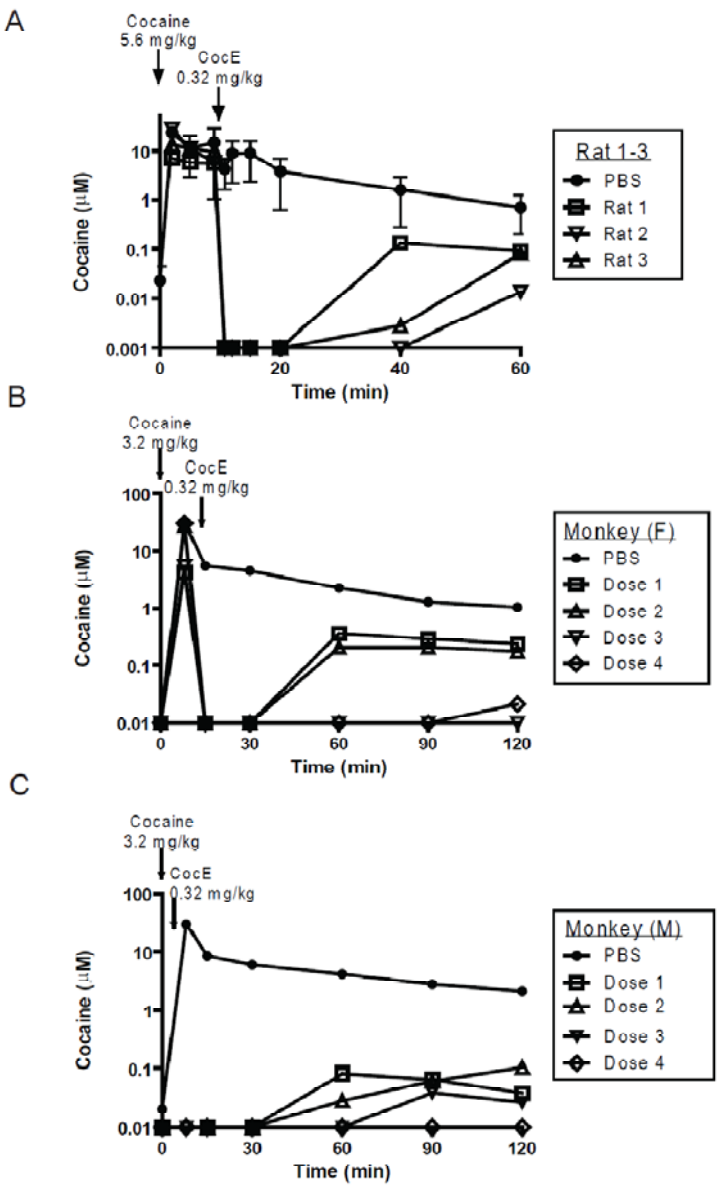

Figure 3.2: RQ-CocE hydrolysis of high-dose cocaine across species **A** Three male Sprague Dawley rats were administered 5.6 mg/kg cocaine (a physiologically equivalent dose to the Rhesus monkey) at time 0 and RQ-CocE or PBS at time 10 minutes. PBS data are the average data from all three rats. RQ-CocE data are separated by individual rat. RQ-CocE rapidly removes cocaine to below the limit of quantification within 45 seconds **B** A female rhesus monkey was administered 3.2 mg/kg cocaine intravenously at time 0 and RQ-CocE or PBS at time 10 minutes. Plasma cocaine concentrations were assessed at the given time points by mass spectrometry. This regimen was repeated every 2 weeks for a total of 4 doses administered. After RQ-CocE administration, cocaine levels fall to below the 30 nM limit of quantification, At times later than 30 minutes, low concentrations of cocaine appear in the plasma, suggesting that the elimination of RQ-CocE from the serum allows very low amounts of cocaine to diffuse back into the blood from other body compartments. Monkey F exhibits the same pattern of cocaine elimination as the rat, suggesting no effect of species or repeat dosing of RQ-CocE in the non-human primate. **C**. A male rhesus monkey 1 was administered 3.2 mg/kg cocaine intravenously at time 0 and RQ-CocE or PBS at time 1 minute. Samples were analyzed as described above for the female monkey. Like the rat and the female, cocaine reappears at higher concentrations by 40 minutes after cocaine injection.

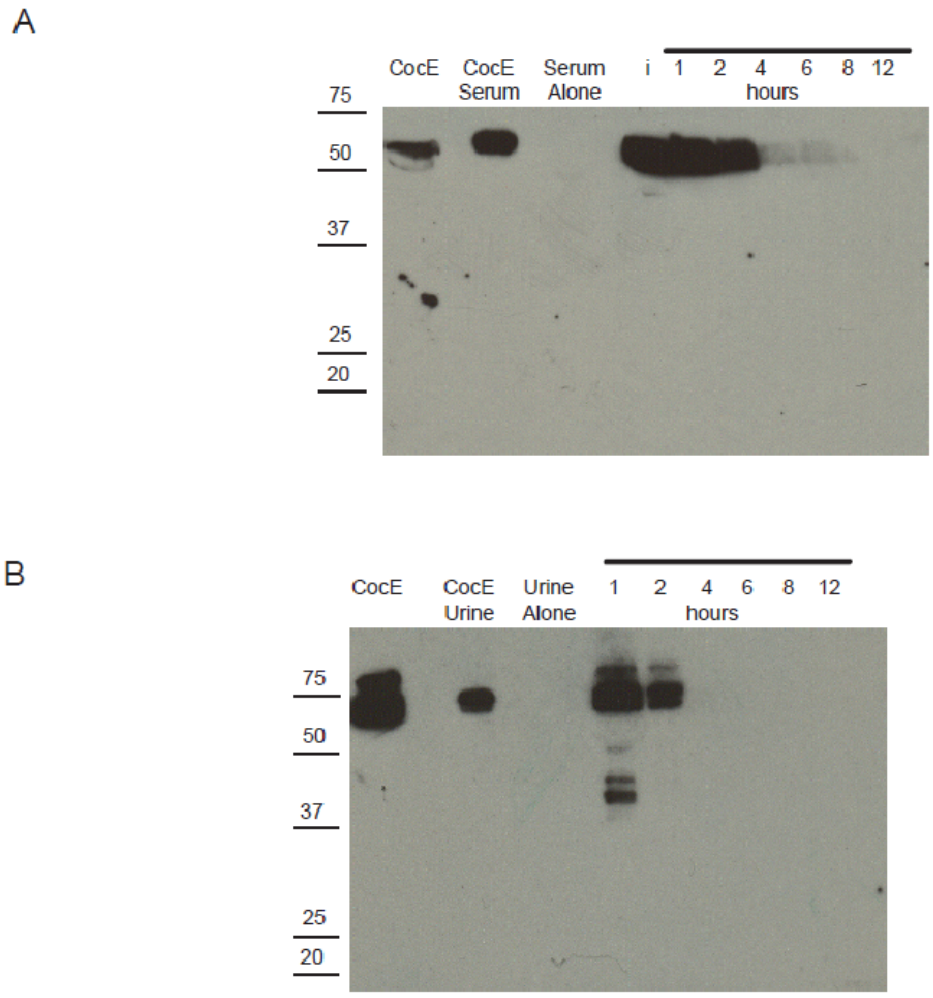

Figure 3.3: Elimination of RQ-CocE in the serum of Sprague Dawley rats **A** Western blot analysis of RQ-CocE in the serum of Sprague Dawley rats administered 8 mg/kg RQ-CocE. Blood samples were taken from rats at t= 5min "i", 1, 2, 4, 6, 8, and 12 hours after RQ-CocE administration. Samples were added to SDS and BME loading buffer and immediately loaded onto 10 % SDS-PAGE gels. RQ-CocE (25 ng) alone was loaded in lane 1 as a positive control. Rat serum (20 µg) alone was loaded in lane 3 as a negative control. RQ-CocE (25 ng) was spiked into 20 µg of serum protein to control for any alteration in migration due to the presence of large quantities of protein. Animal samples were loaded in a time-dependant manner. **B** Western blot analysis of the presence of RQ-CocE in urine. RQ-CocE (40 ng) was loaded as a positive control, de-salted urine alone as a negative control and 40 ng RQ-CocE spiked into urine to control for protein recovery from urine desalting. Urine collected at the times shown after CocE injection was loaded in a time-dependant order. Full length and fragmented CocE can be seen.

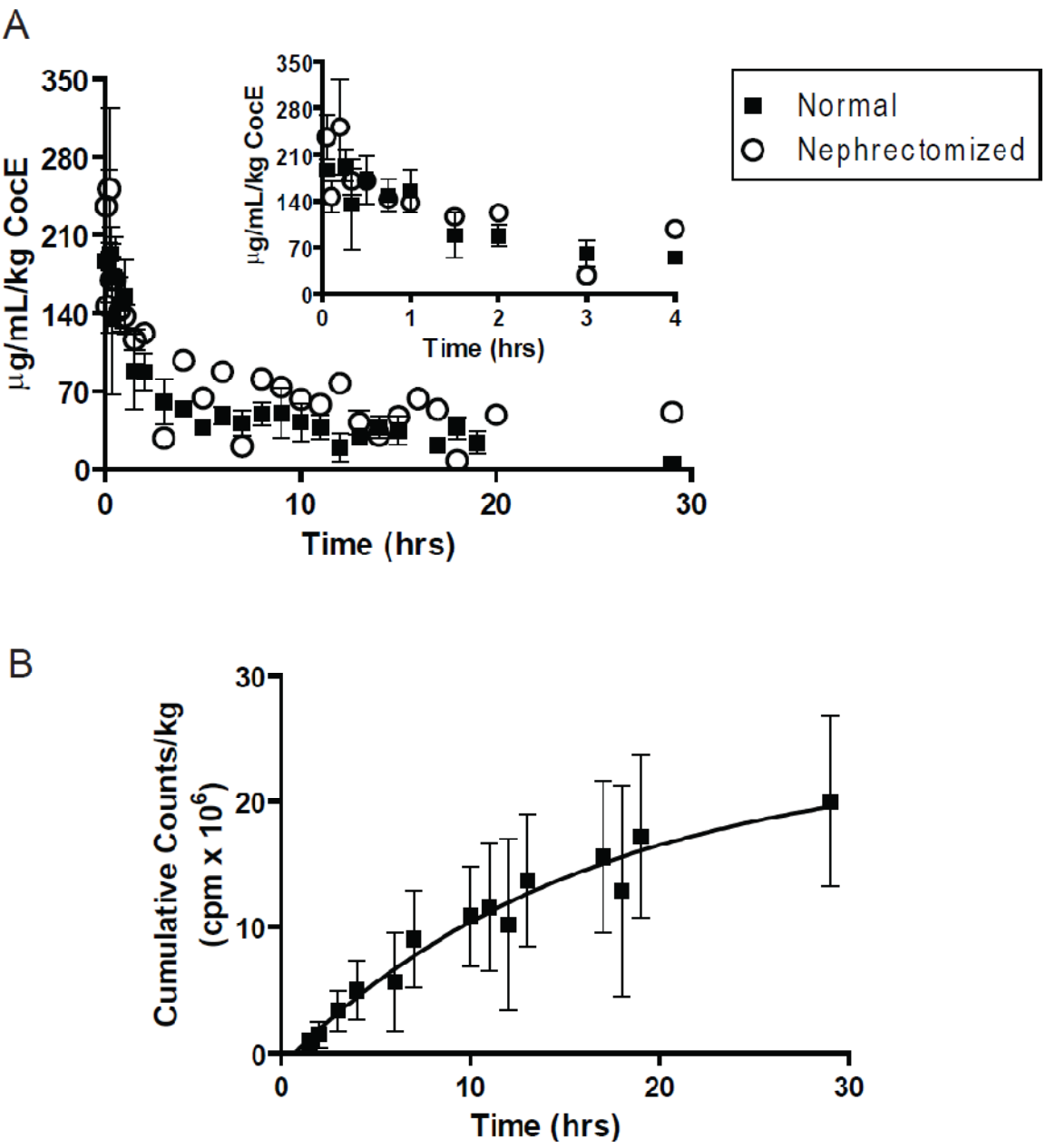

Figure 3.4: Kinetics of CocE in serum and urine of Sprague Dawley rats. **A** Concentration of RQ-CocE in the serum of rats as measured by 35S methionine labeling over time. Eight mg/kg [^{35}S]-RQ-CocE (18 μCi/kg) was administered intravenously through an indwelling jugular catheter at time 0. Blood samples (3 μL) were taken and assessed for radioactive content by scintillation counting. The concentration of CocE was calculated using the specific activity of each radioactive dose and plotted against time. CocE is eliminated rapidly over the first 4 hours after injection, until reaching a plateau state (insert). Both normal and unilaterally nephrectomized animals exhibit the same profile of serum elimination. **B** Cumulative urine accumulation of [^{35}S] radioactivity. After [^{35}S]-RQ-CocE administration, urine was collected and assessed for radioactive content by scintillation counting. Cumulative counts per minute per kilogram are shown against time and fit to a one-phase association model (Y=Y0 + (Plateau-Y0)*(1-exp(-K*x))).

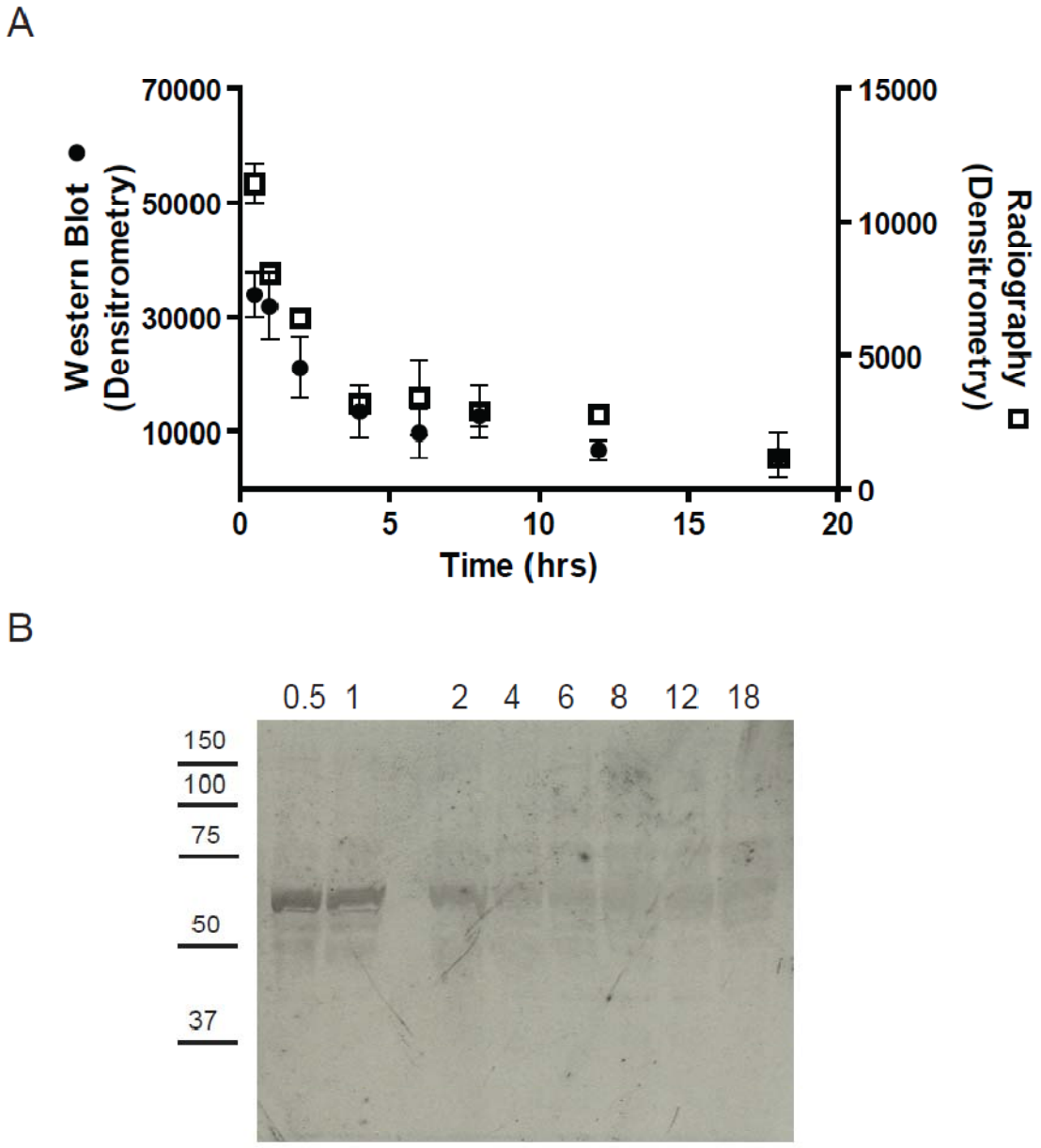

Figure 3.5: Comparison of Western Blot and radiography assessments of [^{35}S]-RQ-CocE. **A** Male Sprague Dawley rats were administered 8 mg/kg [^{35}S]-RQ-CocE at time 0. At 0.5, 1, 2, 4, 6, 8, 12, and 18 hours post CocE injection, serum samples were taken. Total serum protein (25 µg) from each time point was loaded onto a 10% SDS-PAGE gel. Gel was transferred onto a PVDF membrane after running to completion and Western blotting was performed. After visualization of the immunolabled protein with chemiluminescence and film, the membrane was allowed to sit for 6 hours. The membrane was then exposed to film for 2 weeks to visualize the radioactivity contained on the membrane. Both the chemiluminescent and radiographic data were assessed using densitometry of the 65 kDa band. Raw values are plotted against time. **B** Representative radiograph of [^{35}S]-RQ-CocE in serum over time using the method described above.

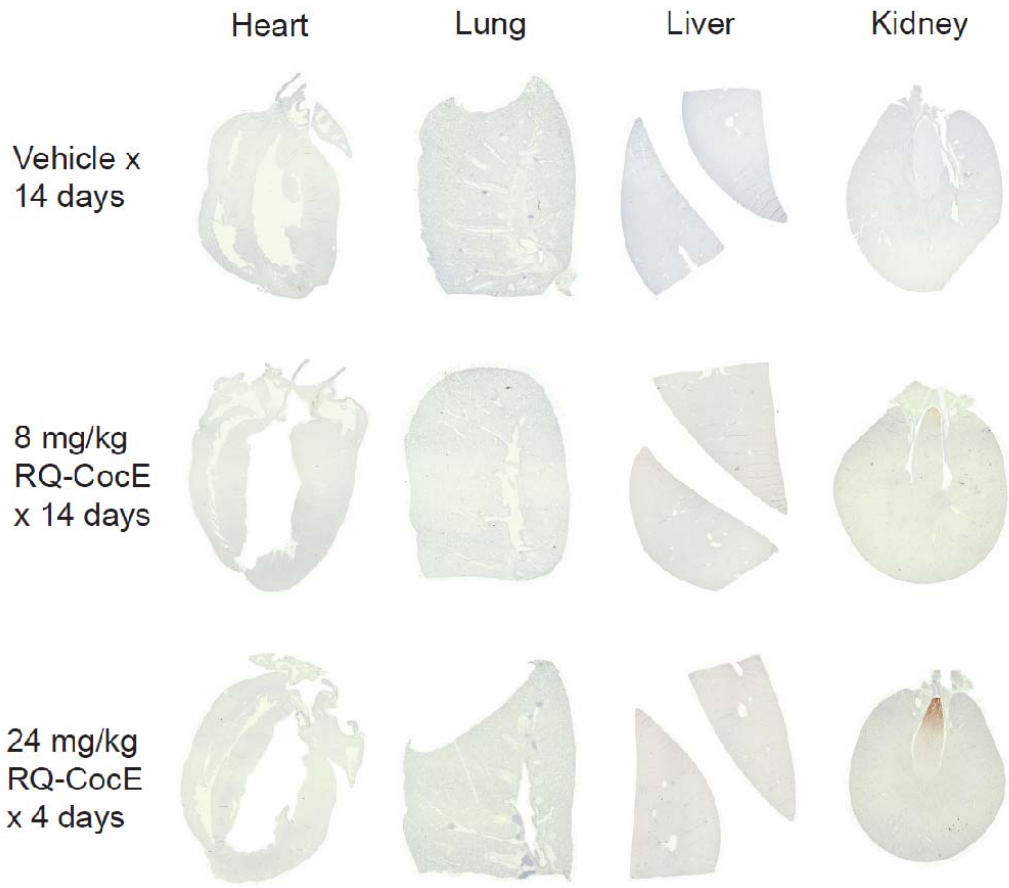

Figure 3.6: Immunohistochemical analysis of RQ-CocE distribution in perfused organs from Sprague Dawley rats Sprague Dawley rats received intravenous administration of the RQ-CocE or vehicle once daily for 4 or 14 days. Six hours after the final dose of RQ-CocE, rats were sacrificed and perfused, with organs fixed and embedded in paraffin for immunohistochemical analysis. Sections were counter-stained with hematoxylin (blue) and RQ-CocE reactivity indicated by brown precipitate formed by diaminobenzadine. Positive RQ-CocE reactivity is dose dependently seen at the tip of the renal papilla.

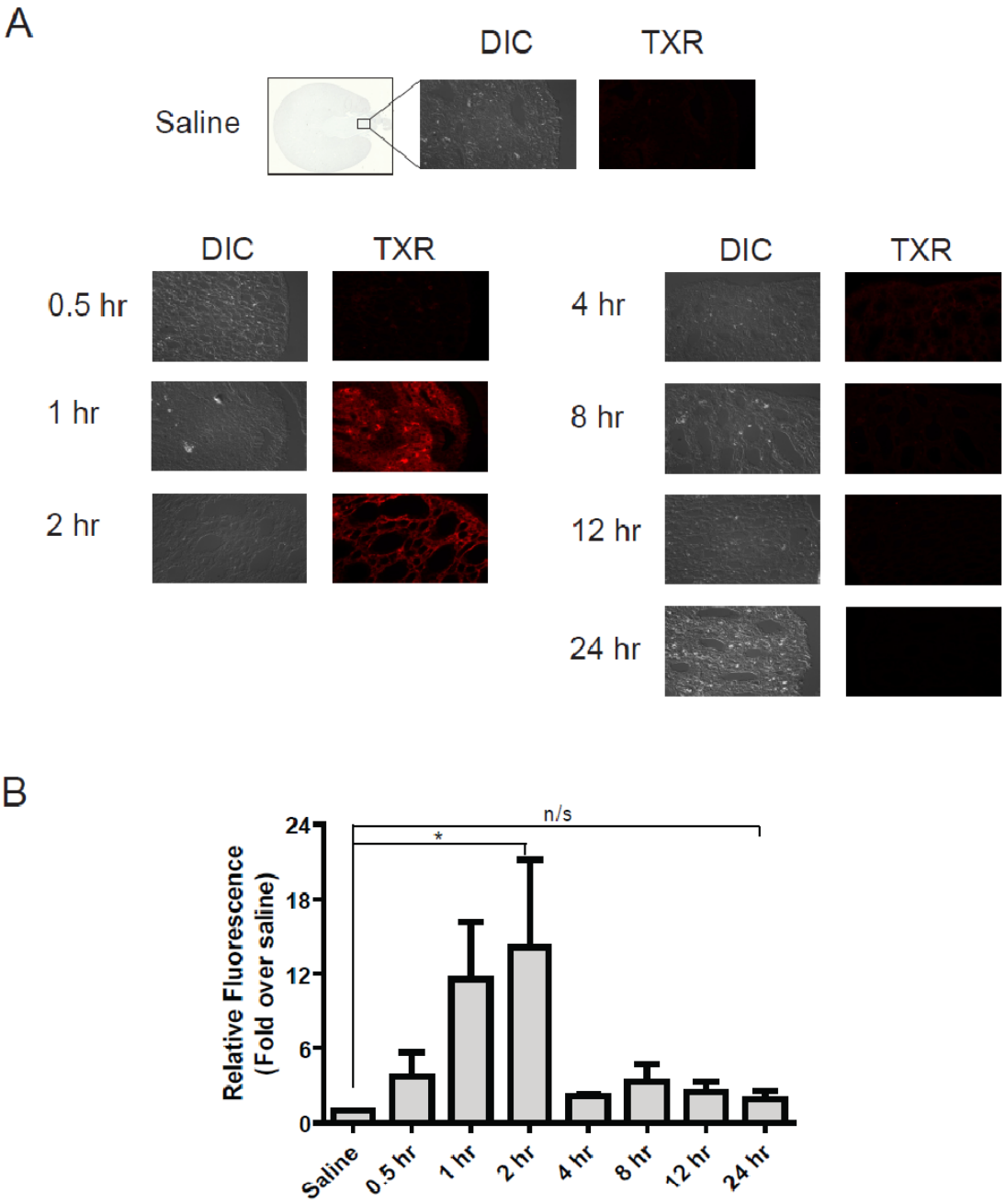

Figure 3.7: Immunohistochemical analysis of RQ-CocE accumulation the renal papilla. A Sprague Dawley rats were administered 8 mg/kg RQ-CocE intravenously. At the times shown after injection, rats were sacrificed and perfused with saline and kidneys were fixed and embedded in paraffin. Immunohistochemistry was performed on kidney sections. Representative images from each time point are shown. Each differential interference contrast image (DIC) shows the area of the papilla that the florescence image highlights (TXR). The overall area of the papilla that the images are taken from is exemplified by the saline sample. **B** Analysis of kidneys from three animals at each time point reveals peak RQ-CocE accumulation at 2 hours, after which there is a rapid decline. At 24 hours, RQ-CocE reactivity is no longer seen. (* One-way ANOVA $F(7, 16)=2.47$; Dunnett's Multiple Comparison Test, $p<0.05$.)

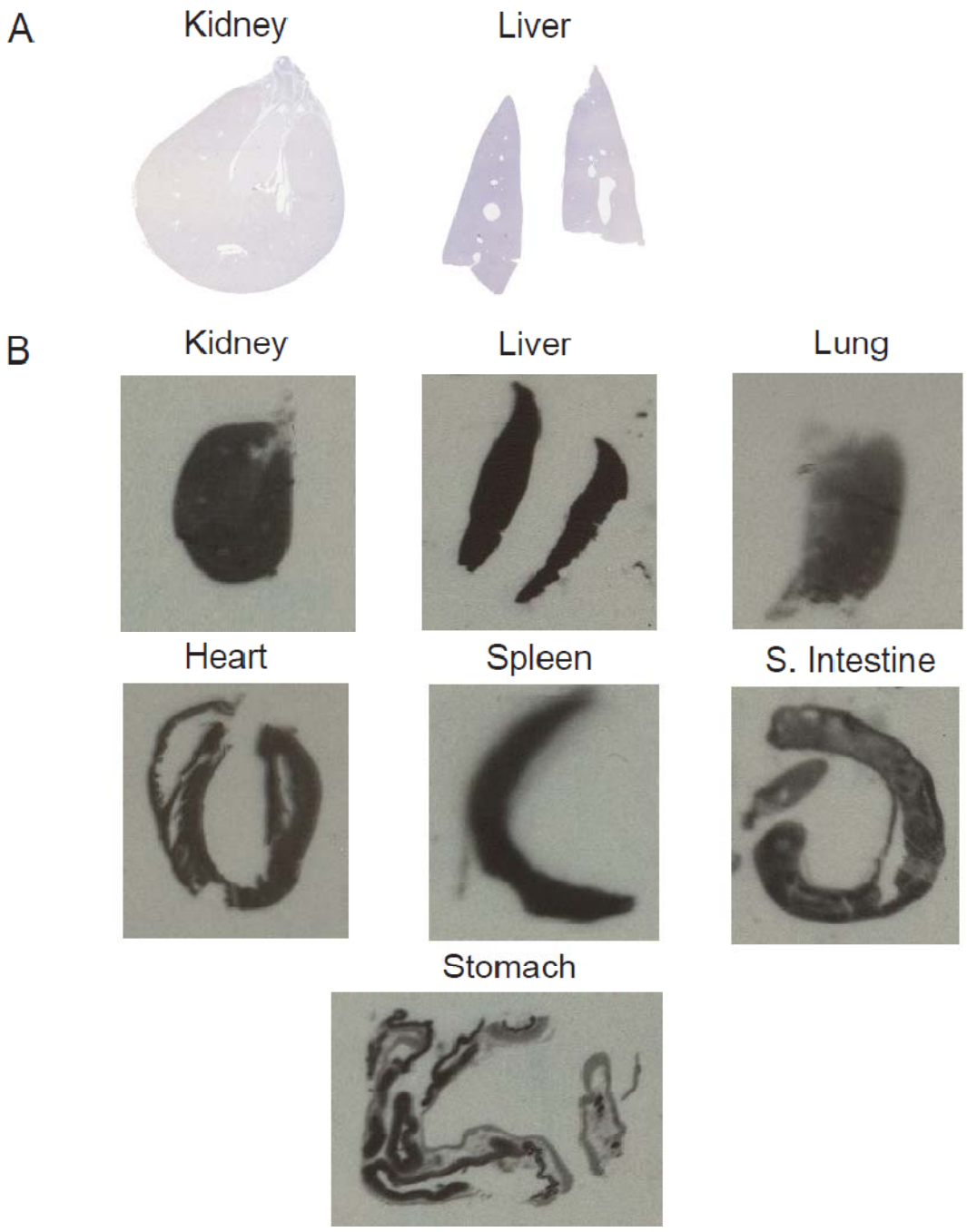

Figure 3.8: Radiography and imunohistochemistry of organs one day post-CocE injection. Rats administered either RQ-CocE or [^{35}S]-RQ-CocE were sacrificed and perfused with saline 29 or 24 hours after administration, respectively. Organ slices were subjected to immunohistochemistry (**A**) or radiography (**B**), respectively, and shown here. No CocE reactivity is detected with the anti-CocE antibody; however, evenly distributed and very dense radioactivity was detected in all organ slices.

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Chapter 4

The Capacity of Bacterial Cocaine Esterase to Hydrolyze Cocaine's Metabolites and the Alteration of the Esterase's Hydrolytic Activity by Commonly Co-Administered Drugs

Introduction

Cocaine is a psychotropic and addictive natural alkaloid derived from leaves of the South American shrub *Erythroxylon coca*. In the United States, over 2 million people use cocaine [1], resulting in more than 500,000 emergency room visits each year related to cocaine toxicity [2]. Although the addictive and pleasurable effects of cocaine are caused by blockade of DA transporters in the brain, cocaine toxicity is primarily due to the blockade of NE transporters, effectively increasing both central and peripheral norepinephrine levels. The blockade of peripheral sodium channels leads to the cardiovascular effects of cocaine including coronary vasospasm, increased heart rate, hypertension and ventricular arrhythmia. In addition to cardiac symptoms, the hallmarks of cocaine toxicity also include hyperthermia, convulsions, seizures, and psychosis.

Because cocaine blocks several targets and causes a wide range of symptoms, it has been difficult to develop a pharmacotherapy to treat cocaine toxicity, and there is currently no FDA-approved therapy for this indication. Emergency personnel are limited to treating only the symptoms of cocaine toxicity, but this does not alter the root cause which is simply a high circulating cocaine concentration.

Although cocaine causes much of the observed toxicities, it is quickly metabolized by the body, with a half-life of about 2 hours in humans. However,

cocaine's metabolites have strong physiological effects as well. The most abundant metabolite of cocaine is benzoylecgonine, produced by hydrolysis of the methyl ester of cocaine by human liver carboxylesterase-1 [3]. Benzoylecgonine is inactive at monoamine transporters, but has been found to have some vasoconstrictive effects unrelated to the adrenergic pathway by which cocaine acts. These may be mediated through interactions with Ca^{2+} channels [4]. This long acting metabolite ($t_{1/2}$=7 hours) may be the cause of latent chest pain associated with cocaine use that develops long after cocaine has been metabolized [4]. Benzoylecgonine is also the urinary metabolite measured in drug screens testing for cocaine use.

Cocaethylene is also formed by human liver carboxylesterase-1 when alcohol is co-abused with cocaine through a transesterification reaction with alcohol. This reaction decreases the hydrolysis of cocaine into the inactive metabolite, benzoylecgonine. Cocaethylene has been shown to be equally or more potent than the parent cocaine molecule. It is known to increase striatal DA concentrations and inhibit dopamine uptake in *ex vivo* assays [5]. This observation has also been validated *in vivo* by microdialysis assays in primates [6]. Cocaethylene, like cocaine, can also block cardiac sodium channels [7]. This blockade is stronger than that of cocaine and causes larger changes in blood pressure, oxygen saturation, cardiac output, and QT interval [8].

Norcocaine is formed from the demethylation of cocaine by CYP3A4 in liver microsomes [9, 10] and represents only a small fraction of cocaine metabolites. However, norcocaine and its downstream product *n*-hydroxynorcocaine, are hepatotoxic and have been shown to cause liver damage [11]. Furthermore, norcocaine blocks cardiac sodium channels *in vitro* [12] causing the same hemodynamic changes as the parent molecule [13], making it both a dangerous and destructive metabolite.

The inactive metabolites, ecgonine methyl ester and benzoic acid, account for approximately 40% of cocaine metabolism and are formed naturally by the serum enzyme butyrylcholinesterase. The bacterial enzyme cocaine esterase (CocE) catalyzes this same reaction but with a rate nearly 1000 times faster than the endogenous enzyme (V_{max}=2300 min^{-1}) [14-18]. CocE is an α/β serine hydrolase that was originally isolated from the *Rhoddococcus sp.* MB1 soil bacterium [14, 16]. We have added thermostabilizing mutations to CocE (T172R and G173Q, RQ-CocE) that preserved the hydrolytic function of the enzyme [17, 19]. CocE has been well-classified as a cocaine hydrolyzing molecule in both buffer [17] and human plasma [18, 20]. However, these studies examined changes only in cocaine levels after CocE addition. Because the active cocaine metabolites described above contain the same CocE hydrolyzable ester linkage as cocaine, we investigate here if CocE can hydrolyze these metabolites as well. We have employed both *in vitro* experiments and *in vivo* studies with Sprague Dawley rats. A high performance liquid chromatography-tandem mass spectrometry method (LC-MS/MS) was developed to enable simultaneous quantification of cocaine, ecgonine methyl ester, benzoylecgonine and norcocaine. By investigating these topics, we will gain insight into the specificity of the stable RQ-CocE mutant as a potential clinical therapy for cocaine toxicity.

Methods

Production and purification of RQ-CocE: A BioFlow 3000 bioreactor (New Brunswick, NJ) was prepared with 10 L of Terrific Broth (TB, Maniatis, BentonDickinson) with kanamycin (50 μg/mL). For the inoculum, BL21 cells transformed with pET24b plasmid containing RQ-CocE were grown in 250 mL TB containing kanamycin (50 μg/mL) at 37°C while shaking until the culture reached log phase growth (about 8 hours). In this growth phase, the bioreactor was inoculated with the starter culture and allowed to grow

at 37°C until the culture reached an OD_{600} of 5. Once the target turbidity was reached, the bioreactor was cooled to 18°C and then induced by adding IPTG for a final concentration of 1 mM. After 16 hours of induction, the culture was harvested by spinning down the broth at 4500 x *g* for 20 minutes. The resulting pellet was stored at -80°C.

Cell paste from 5 liters of a fermentor run was resuspended in PBS pH 7.4. The resuspended paste was passed through a French Press twice or three times to lyse the cells. The maximum pressure for lysis is approximately 1100 psi. The lysate was clarified by spinning at 100,000 x g in an ultracentrifuge (Beckman Coulter XL-100K ultracentrifuge) using the rotor type Ti45. Clarified lysate was diluted by adding an equal volume of Q-buffer A (20mM Hepes pH 8.0) to a final volume of 1000 mL.

The clarified lysate (500 mL) was passed onto a 450 mL Q Sepharose HP column attached to Pharmacia P-500 pumps and a Pharmacia LCC 501 FPLC controller at room temperature. The column was washed with 1000 mL Q-buffer A followed by a wash gradient set to reach 50% Q-buffer B (20mM Hepes pH 8.0 + 1M NaCl) within 1000 mL. Flow-through and washes were saved for analysis. Protein was eluted from the column using a gradient from 50 to 100% Q-buffer B over 5 column volumes (2250 mL). Fraction collection began after the washes were completed using a Pharmacia LKB Frac 100 fraction collector. The entire run was carried out at a flow rate of 10 mL/min. The RQ-CocE protein was monitored by running 5 μl of each fraction on an 8% SDS PAGE Gel and staining with Coomassie Brilliant Blue stain according to established protocols.

Fractions from the first Q Sepharose HP separation were pooled and adjusted to 1M ammonium sulfate (final volume 525 mL). A 450 mL Phenyl-Sepharose column was washed with two column volumes (1000 mL) of PS-Buffer A (Buffer A: 50mM Na PO4,

pH 7 + 1M AS). An additional wash step was performed to increase PS-Buffer B (Buffer B: 50mM NaPO4, pH 7) concentration to 30% within two column volumes (1000 mL). A gradient with PS buffers was run from 30% to 100% PS Buffer-B over 5 column volumes (2250 mL). Again, 25 mL fractions were collected over the elution gradient. The presence and purity of RQ-CocE were checked by running 5 μl of each fraction on an 8% SDS PAGE gel.

Fractions from the Phenyl-Sepharose column were pooled and extracted twice with triton X 114. The twice-extracted protein was then diluted with Q-buffer A and this was loaded on to a second Q-Sepharose HP column (450mL). Chromatography was performed as described above. Figure A1.1 illustrates this purification process.

Spectrophotometric cocaine and cocaine metabolite assay: RQ-CocE (25 ng/mL final concentration) was added to a 96-well UV-permeable plate containing cocaine or a single cocaine metabolite (0.25, 0.5, 2.5, 5, 12.5, 25, 50, and 100 μM final concentrations) in phosphate-buffered saline (50mM Tris HCl, pH 8.0, 150mM NaCl, pH 7.4) to give a final volume of 200 μL. The absorbance of cocaine at 240 nm was monitored over 15 minutes with readings every 10 sec by a SpectraMax Plus 384 UV plate reader (Molecular Devices, Sunnyvale, CA) using SOFTmax Pro software (Version 3.1.2). The specific extinction coefficient was used to convert the change in absorbance over time to the change in concentration over time. Extinction coefficients (ε) for the metabolites were determined using the absorbance of these metabolites at 240 nm and Beer's Law ($A=\varepsilon bc$). Our laboratory has determined these to be: norcocaine=8.7 L/mmol/cm, benzoylecgonine=7.5 L/mmol/cm, cocaethylene=11.6 L/mmol/cm. The rate of cocaine hydrolysis per mole of enzyme (V_{max}) and the K_m were determined using Prism software (GraphPad, San Diego).

Metabolite formation and elimination* in vivo*: Male Sprague Dawley rats (300-350g) (Harlan Sprague Dawley, Indianapolis) were maintained in a temperature controlled environment with a 12-hour light cycle, beginning at 7am. Rats were given *ad libitum* access to food and water, and were allowed to acclimate to the room for at least 5 days before surgery. Rats were implanted with 2 indwelling femoral catheters. Briefly, the rats were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg). Catheters were inserted into the femoral vein of both legs after the vein was manually separated from the femoral artery and nerve. Catheters were threaded under the skin and attached to stainless steel tubing that runs through a metal tether plate sutured to the back muscle. Animals were allowed to recover from the procedure for 5 days before use.

On the day of the experiment, rats were placed in 49 cm long x 23 cm wide x 21 cm high Plexiglas cages containing cob bedding. A long piece of catheter tubing was extended from the stainless steel tubing of the metal tether plate out of the cage so that rats could be infused and blood could be drawn without direct handling. At time t=0, rats were given a 10 second bolus of 5.6 mg/kg cocaine (5.6 mg/mL) followed by a bolus of either RQ-CocE (0.32 mg/kg, 0.32 mg/mL) or PBS at t=10 minutes. Blood was sampled (200 µL) at t= 2, 5, 9, 10:45, 12, 15, 20, 40, and 60 minutes, and immediately added to 5 µL of 500 mM EDTA plus 20 µL of 1M NaF to prevent coagulation and further cocaine hydrolysis, respectively. Plasma was separated from blood cells by centrifugation at 1500 rcf in a bench top microcentrifuge, flash frozen in liquid nitrogen, and stored at -80°C until preparation for mass spectrometry.

Experiments conformed to the Guide for the Care and Use of Laboratory Animals from the National Institutes of Health, and all experiments were additionally approved by the University of Michigan Committee on the Use and Care of Animals (UCUCA).

Preparation of plasma samples for mass spectrometry: The plasma fraction from each 200 µL blood sample was added to 570 µL ACN, 20 µL 1M NaF, and 2µL of internal standard solution containing 750 nM deuterium-labeled norcocaine, cocaine, benzoylecgonine and ecgonine methyl ester. Samples were vortexed for 30 seconds and centrifuged at 25,000 rcf at 20°C for 30 minutes. The supernatant was removed and added to a clean microcentrifuge tube. Samples were centrifuged a second time using the same conditions, and the supernatants were again transferred to clean tubes. Samples were evaporated to dryness in a vacuum centrifuge and stored at -80°C. Samples were analyzed within a week of preparation.

For mass spectral analysis, the dried samples were reconstituted with 30 µL of 10 mM ammonium formate, pH 4.6:acetonitrile (97:3; v/v) to yield a 50 nM final concentration of each internal standard. To achieve concentrations of cocaine, norcocaine, benzoylecgonine, and ecgonine methyl ester within the limits of quantification, further dilutions (varying along the time course) were done with 10 mM ammonium formate pH 4.6:acetonitrile (97:3; v/v) and 50 nM internal standards. Samples were vortexed for 30 seconds, then centrifuged at 13,600 rcf for 20 minutes. Aliquots of the supernatants were transferred to polypropylene autosampler vials for analysis within 12 hours.

Preparation of calibration standards for metabolite mass spectrometry: Calibration standards of cocaine, benzoylecgonine, ecgonine methyl ester (4.0-0.0313 µM), and norcocaine (0.4-0.00313 µM) were prepared in plasma from untreated Sprague Dawley rats (Valley Biomedical, Winchester VA). All standards were stored at -80°C until sample preparation. Calibration standards were prepared with every set of experimental samples. Each calibration stock (20 µL) was extracted with 68 µL ACN, 4 µL 1M NaF, and 2 µL of 2.5 µM of each deuterium-labeled norcocaine, cocaine, benzoylecgonine

and ecgonine methyl ester as described above. Calibration standards were reconstituted to 100 µL resulting in final internal standard concentrations of 50 nM.

Quantification of cocaine metabolites by LC-MS/MS: Analysis was performed on a Prominence HPLC system (Shimadzu, Kyoto, Japan) interfaced directly to the Turbo Ionspray source of an API 3000 triple quadrupole mass spectrometer (PE Sciex, Toronto, ON, Canada). Separation was achieved with a Thermo Hypersil Gold column (50 x 2.1mm i.d., 1.9 µm packing) maintained at 45ºC using a binary gradient and a flow rate of 0.45 mL/min. The flow was split approximately 1 to 3.5 so that 0.13 mL/min was directed into the ionization source. Solvent A was 10 mM ammonium formate pH 4.6 and Solvent B was acetonitrile. The gradient program was as follows: 2% B at 0 min, hold 2% B for 1 min, 18% B at 2 min, 40% B at 10 min, 100% B at 11 min, 2% B at 12 min, and re-equilibrate at 2% B for 3 min. Each analysis was completed in 15 minutes. Four µL aliquots were injected onto the column and the sample tray was cooled to 10ºC to prevent sample degradation.

Positive ions were generated in the source using purified air for the source gases under the following conditions and settings: Turbo ESI gas, 7.0 L/min; nebulizer (NEB) setting, 15; ion spray voltage (IS), 3000 V; source gas temperature (TEM), 400ºC. Compound-specific ionization parameters were optimized for cocaine, each metabolite, and the internal standards as shown in Table A.1. Nitrogen was used as the curtain gas (CUR) at a setting of 12. Mass analysis was performed by single reaction monitoring (SRM) with 100 ms dwell times. Nitrogen served as the collision gas (CAD=12). The chromatogram was divided into three time segments so that individual periods contained a subset of one or two target compounds. The corresponding SRM transitions were scanned only during the time segments in which they eluted. Period 1 spanned the first 2 minutes, Period 2 covered the next 1.7 minutes, and Period 3 extended for the

remainder of the chromatogram. Precursor/product-ion pairs for the SRM transitions and their respective scan periods are listed in Table A.2. Analyst software (version 1.4.2; MDS Sciex, Toronto, ON, Canada) was used for instrument control, data acquisition, and quantitative analysis. Calibration curves were constructed from the standard samples. The ratios of the peak areas of the target compounds to the corresponding deuterium-labeled internal standards were plotted as a function of the analyte concentration normalized to the internal standard concentration. Calibration curves were generated using a least squares linear regression analysis with *1/x* weighting.

Drug interaction studies:

Spectrophotometric: Drugs chosen for testing were screened for absorbance at 240nm, to ensure no overlap between the drug tested and cocaine. Only diazepam had an absorbance spectra overlapping cocaine and was therefore tested using mass spectrometry. Drugs were tested for interaction in the spectrophotometric cocaine hydrolysis assay originally described by Turner et al. [16].

RQ-CocE (50 ng/mL)was incubated with 2x the concentration of drug tested in Table 1 in PBS 10 minutes at room temperature. The RQ-CocE solution (100 µL) was then added to each well of the spectrophotometric assay containing increasing concentrations of cocaine, to achieve a final concentration of 25 ng/mL RQ-CocE and the concentration of interacting drug listed in the table. Each plate contained a no-drug vehicle + RQ-CocE control as well as three different drug concentrations, each in duplicate. Michaelis-Menten kinetic parameters were obtained from curve fit by Graph Pad Prism software. Significance of a drug interaction was determined by Students T-test comparing the Michaelis-Menten kinetic parameters to those obtained with cocaine alone.

Mass Spectrometry: To test the interaction between diazepam and RQ-CocE, diazepam (concentrations listed in Table 4.1) was added to human plasma samples (obtained from the University of Michigan Hospital Blood Bank) at 37°C containing 8 μM cocaine. Midazolam was used as a control to test the equivalence of the assays. Diazepam vehicle was tested at the volume used for the "3x" interaction point. No RQ-CocE and no drug controls were also obtained.

RQ-CocE (0.003 μM final concentration) was added to the samples at time t=0. Aliquots of plasma (20 μL) were taken from the one mL sample at time t=20 sec, 40 sec, 1 min, 2 min, and 5 min. These aliquots were immediately placed into a solution of 66 μL of acetonitrile, 4 μL of saturated (1M) sodium fluoride, and 10 μL deuterated cocaine (as an internal standard) to prevent further cocaine metabolism and to prepare samples for LC/MSMS. Samples were vortexed 15 seconds on high. Samples were centrifuged at 13,000 rpm for 45 minutes and the supernatant was collected into a clean low retention centrifuge tube. The extract was concentrated on a Savant Speed Vac to remove acetonitrile. Extracted dried samples were reconstituted and further diluted 1-20 times depending on the time point.

Preparation of calibration standards for drug-interaction metabolite mass spectrometry: Calibration standards of cocaine (3.16 μM-0.0043 μM) were prepared in human plasma (obtained from the University of Michigan Hospital Blood Bank). All standards were stored at -80°C until sample preparation. Calibration standards were prepared with every set of experimental samples. Calibration stocks (20 μL) were extracted with 68 μL ACN, 4 μL 1M NaF, and 2 μL of 2.5 μM of deuterium-labeled cocaine as described above. Calibration standards were reconstituted to 100 μL

resulting in final internal standard concentrations of 50 nM.

Mass spectrometry of cocaine to test for CocE drug interactions: LC-MS/MS analysis was performed on a Prominence HPLC system (Shimadzu, Kyoto, Japan) interfaced directly to the Turbo Ionspray source of an API 3000 triple quadrupole mass spectrometer (PE Sciex, Toronto, ON, Canada). Separation was achieved with a Phenomenex Synergi Hydro RP column (50 x 2.0mm i.d., 4 µm packing) maintained at 40ºC using a binary gradient and a flow rate of 0.15 mL/min. Solvent A was water and Solvent B was acetonitrile; both solvents were modified with 0.1% formic acid (v/v). The gradient program was as follows: 20% B at 0 min, 100% B at 3 min, hold 100% B for 1 min, return to initial conditions in 2 min and re-equilibrate at 20% B for 9 min. Each assay was completed in 15 minutes. Ten µL aliquots were injected onto the column and the sample tray was cooled to 10ºC to prevent sample degradation.

Positive ions were generated in the source using purified air for the source gases under the following conditions and settings: Turbo ESI gas, 7.0 L/min; nebulizer setting, 6; ion spray voltage, 4200 V; source gas temperature, 300ºC; declustering potential (DP), 75 V; focusing potential (FP), 160 V; entrance potential (EP), 10 V. Nitrogen was used as the curtain gas at a setting of 8. Mass analysis was performed by single reaction monitoring with 300 ms dwell times. Nitrogen served as the collision gas (CAD=8). The collision energy (CE) was set to 32 eV (lab frame) with a collision cell exit potential (CXP) of 12 V. Precursor/product-ion pairs for the SRM transitions were *m/z* 304.2 → 182.2 for cocaine and *m/z* 307.2 → 185.2 for d3-cocaine. Analyst software (version 1.4.2; MDS Sciex, Toronto, ON, Canada) was used for instrument control, data acquisition, and quantitative analysis. Calibration curves were constructed from the standard samples using the ratio of cocaine to the deuterium-labeled internal standard (d3-cocaine) and a least squares linear regression analysis with *1/x* weighting.

Results

Effect of RQ-CocE on cocaine metabolites* in vitro*: Cocaine esterase hydrolyzes cocaine at the ester linkage between the tropane and benzoyl rings, producing the inactive metabolites ecgonine methyl ester and benzoic acid, the same reaction catalyzed by human serum butyrylcholinesterase (Figure 4.1). Norcocaine (formed by CYP3A4) and cocaethylene and benzoylecgonine (formed by human liver carboxyesterase-1), all retain the ester linkage found in the parent molecule. The ability of RQ-CocE to hydrolyze these metabolites was assessed using the spectrophotometric assay. The extinction coefficient for each metabolite and cocaine was determined experimentally (data not shown) and used to convert the loss of absorbance at 240 nm over time to the loss of metabolite. Cocaethylene, the cocaine metabolite formed by human liver carboxyesterase-1 in the presence of ethanol, was hydrolyzed by RQ-CocE with a statistically significant lower V_{max} and a statistically significant higher K_m than cocaine (18.9 μM for cocaethylene compared to 14.19 μM for cocaine), resulting in an approximately 75% reduction in catalytic efficiency (Figure 4.2). RQ-CocE hydrolysis of norcocaine did not display statistically significantly different V_{max} but did have a significantly lower V_{max}/K_m than cocaine (Figure 4.2). This was due to the higher K_m (29.0 μM for norcocaine compared to 14.19 μM for cocaine), which resulted in the approximately 50% reduction in catalytic efficiency. Interestingly, benzoylecgonine was not hydrolyzed by RQ-CocE. Even when higher concentrations of RQ-CocE were assessed (100 ng/mL compared to 25 ng/mL), benzoylecgonine was not hydrolyzed according to Michaelis-Menten kinetics to any measurable level.

***Effect of RQ-CocE on cocaine metabolites* in vivo:** To expand on the spectrophotometric assay and to validate these findings in a physiological setting, the elimination of cocaine and its metabolites were measured in male Sprague Dawley rats.

Rats were given a dose of 5.6 mg/kg cocaine at time t=0 and a dose of 0.32 mg/kg RQ-CocE at time t=10 minutes. Blood samples were taken at t=2, 5, 9, 10:45, 12, 15, 20, 40, and 60 minutes for analysis by LC-MS/MS. Cocaine, benzoylecgonine, ecgonine methyl ester and norcocaine were quantified simultaneously in each serum sample using the method developed for this study.

Cocaine (5.6 mg/kg) produced an initial concentration of 7.1-27.1 μM in the rats. In the animals receiving only PBS vehicle at the 10 minute time point, the concentration of cocaine slowly fell over the 60 minute period due to slow natural hydrolysis. The majority of cocaine was converted into benzoylecgonine and ecgonine methyl ester, with norcocaine produced as a minor metabolite in the rats, as expected (Figure 4.3). The natural metabolism of cocaine was significantly and predictably altered by the administration of RQ-CocE 10 minutes after cocaine dosing. Cocaine was rapidly metabolized by RQ-CocE to levels below the limit of quantification within 2 minutes after administration. The cocaine was immediately converted into ecgonine methyl ester and peak concentrations ranged from 9.1-11.4 μM at t=12 min. Consistent with the *in vitro* results, RQ-CocE also eliminated the norcocaine that was formed before esterase administration. The concentration of norcocaine decreased from 10 nM at t=9 min to levels below the quantification limit of 3 nM at t=10:45, only seconds after the administration of RQ-CocE. Although RQ-CocE did not hydrolyze benzoylecgonine *in vitro*, a rapid reduction then stabilization of benzoylecgonine concentration was observed in rats after RQ-CocE administration.

Drug-CocE interactions: Cocaine hydrolysis by CocE was measured by a spectrophotometric assay originally described by Turner et al. [16]. Commonly co-administered drugs were tested for inhibition of CocE at the concentrations shown in Table 1. These concentrations represent 1, 3 and 10x the pharmacologically relevant

serum concentrations in humans [21, 22]. The 1x level for drugs of abuse represent typical concentrations seen in drug abusers, while the 1x levels for drugs administered by medical professions are the serum concentrations found after typical dosing regimens. All drugs in this assay were first tested for absorbance at 240 nm to assure that they would not interfere with the absorbance spectrum of cocaine. No drug tested in this assay was found to interfere with the exception of diazepam, which was subsequently analyzed using LC-MS/MS. Cocaine hydrolysis by RQ-CocE was not inhibited by any drug tested in the spectrophotometric assay (Table 4.1). Figure 4.4 illustrates typical results for the drugs tested using morphine (an active metabolite of heroin) and CocE.

Inhibition of CocE by diazepam: Diazepam and midazolam were tested for inhibition of CocE-mediated cocaine hydrolysis using LC-MS/MS (Figure 4.5A). Cocaine concentrations were followed over time after the addition of CocE and one of the benzodiazepines. Midazolam, at 10x the normal serum concentrations achieved in medical settings, did not interfere with cocaine hydrolysis by CocE, confirming the results of the *in vitro* assay. In contrast, diazepam, at concentrations 3 and 10x serum levels achieved with patient sedation, did significantly slow the hydrolysis of cocaine by CocE. The addition of fluorine on the benzoyl ring of midazolam may explain the difference in effect on CocE between these two structurally similar benzodiazepines (Figure 4.5B).

Discussion

This work describes not only the ability of cocaine esterase to eliminate cocaine metabolites, but also demonstrates the utility of simultaneous detection of the major cocaine metabolites. Our data show that RQ-CocE efficiently hydrolyzes the active

cocaine metabolites norcocaine and cocaethylene but not benzoylecgonine. These data suggest several structure-function relationships between RQ-CocE and its substrates. Compared to the parent molecule, norcocaine lacks the N-methyl group of the tropane ring. This leads to a loss of positive charge on the nitrogen, but does not affect the maximum potential of RQ-CocE to mediate catalysis which is demonstrated by the equivalent V_{max} of cocaine and norcocaine. This loss of charge does, however, lead to a decreased ability for norcocaine to bind RQ-CocE, as reflected by the higher K_m of this reaction. It is possible that norcocaine, with a slightly more negative charge than cocaine, is not properly oriented in the enzyme active site pocket containing the developing negatively changed hydroxyl group of serine 117 that initiates the nucleophilic attack on the benzoyl ester linkage of cocaine . Cocaethylene retains the same charge as the parent molecule and contains an additional methyl group. The larger molecular size of cocaethylene probably decreases its ability to bind to the active site of RQ-CocE, most likely due to simple steric hindrance, and slightly decreases the ability of RQ-CocE to hydrolyze the molecule once bound.

No activity against benzoylecgonine was detected in our *in vitro* assay. This result was surprising, considering that compared to cocaine, benzoylecgonine is only slightly smaller and retains most of the charge properties. Loss of the methyl group exposes a hydroxyl group, which could create a small, negative charge hindrance that reduces binding, similar to norcocaine. However, these *in vivo* data suggest that RQ-CocE may be binding to benzoylecgonine, becuase there is a significant and rapid decrease in the concentration of benzoylecgonine after the addition of RQ-CocE that persists for about 2 minutes, at which point the concentration stabilizes. This suggests that the lack of kinetics observed with RQ-CocE and benzoylecgonine is most likely due to an inability to hydrolyze the molecule, not an inability to bind.

To further validate our *in vitro* results, we developed a LC-MS/MS method to simultaneously detect and quantify cocaine and its major metabolites. Most drug screening assays include cocaine and at least one major metabolite, usually benzoylecgonine, and an increasing number of these screens are now performed using LC-MS/MS technology [23-30]. While the methods have been well-documented [23-30], the goal of a screen is to incorporate as many compounds as possible into a single assay. Comprehensive screens may even require the use of expensive ultra performance liquid chromatography instrumentation [23, 27, 28] to achieve sufficient resolution of all components. The study described in this report centers on the metabolism of a single drug (cocaine), using a simple, targeted method for the analysis of cocaine, norcocaine, benzoylecgonine, and ecgonine methyl ester. Because of the structural similarities of these compounds, the product ion spectra generated in MS/MS are also very similar. Many overlapping fragment ions were observed, though the relative abundances varied from compound to compound. As a result, good chromatographic separation is required for accurate quantitative analysis becuase these compounds could not be separated entirely by the mass spectrometer. Traditional microbore HPLC columns (3 µm, 150mm x 2.0mm i.d.) containing stationary phases such as C18 and phenyl hexyl in combination with water/acetonitrile/formic acid solvent gradients did not resolve cocaine and norcocaine. Furthermore, ecgonine methyl ester, the most polar metabolite, eluted in or very near to the void volume of these columns, and was often obscured by non-retained salts and low molecular weight contaminants. Ecgonine methyl ester was also not strongly retained on columns that combine polar end capping with hydrophobic alkyl chains to provide mixed-mode retention mechanisms (e.g., Phenomenex Synergi Hydro RP). Peak shapes were significantly improved by the substitution of 10 mM ammonium formate pH 4.6 for 0.1% formic acid in water as the

aqueous solvent but cocaine and norcocaine were still unresolved, despite the ion pairing capabilities of ammonium formate.

Since cocaine and norcocaine could not be separated on the basis of stationary phase selectivity, it was necessary to utilize a column with smaller particle size and thus, higher separation efficiency and improved resolution. There have been reports in the literature of the use of ultra performance liquid chromatography (UPLC) columns in conventional HPLC systems [31-34] to generate large improvements in chromatographic resolution. The Thermo Hypersil Gold column provided the best combination of retention and resolution of cocaine and the three metabolites of interest (Figure 4.6). Ecgonine methyl ester was slightly retained on this column, and cocaine and norcocaine were nearly baseline-resolved. The LINAC™ technology [33] incorporated into the collision cell prevents any cross talk from the small amount of overlap between the cocaine and norcocaine peaks thus allowing accurate quantification of these two compounds. Chromatographic peaks were narrow and symmetrical with the exception of ecgonine methyl ester which displayed a slight amount of tailing. As a result of all these adaptations, the final method was accurate and precise, with good linearity for all the target compounds (Figure 4.7).

In vivo results obtained by analyzing plasma samples with this method supported the *in vitro* data; both demonstrating that RQ-CocE was effective at hydrolyzing cocaine and norcocaine, but not benzoylecgonine. The *in vivo* concentrations of cocaine and norcocaine well illustrate the loss of RQ-CocE activity over time. After rapid elimination of cocaine to levels below the quantification limit, the plasma concentrations of both these molecules begin to increase after 20 to 40 minutes post-cocaine injection. We have previously shown that the concentration of CocE in the serum of mice and rats decreases rapidly over time with a half-life of about 1-2 hours [20, 35]. The data

presented here demonstrate that this previously reported loss of RQ-CocE from the circulation likely leads to the reduction in the rate of cocaine removal. At the reduced rate, more cocaine can be found in this compartment, as the return to equilibrium is established by redistribution throughout the body. This demonstrates the need for a CocE with a longer circulating half-life if all cocaine is to be eliminated at the low RQ-CocE doses tested here. However, the levels of cocaine that redistribute into the plasma 50 minutes after CocE administration only range from 13.7-92.4 nM, a level that is no longer toxic and far lower than that observed in recreational cocaine users [21].

We have demonstrated that RQ-CocE is able to hydrolyze the active and dangerous cocaine metabolites norcocaine and cocaethylene. Using an LC-MS/MS method to simultaneously quantify cocaine and three of its major metabolites, we have additionally demonstrated the alteration of natural cocaine metabolism in the rat by RQ-CocE. We have also shown that CocE does not interact with any commonly co-administered drug with the exception of diazepam. This interaction is something that would be easily avoided in the clinic by using another benzodiazepine like midazolam for sedation. Reduction in physiologically active metabolites and lack of drug interaction are both properties for CocE that have not been reported with engineered human butyrylcholinesterases [36-41]. Wt-BChE metabolizes several drugs besides cocaine such as succinylcholine, procaine, and mivacurium, which suggests that potential drug interactions with mutant BChEs warrant serious investigation. These data further strengthen the pre-clinical evidence that CocE may be an effective treatment for cocaine toxicity in humans.

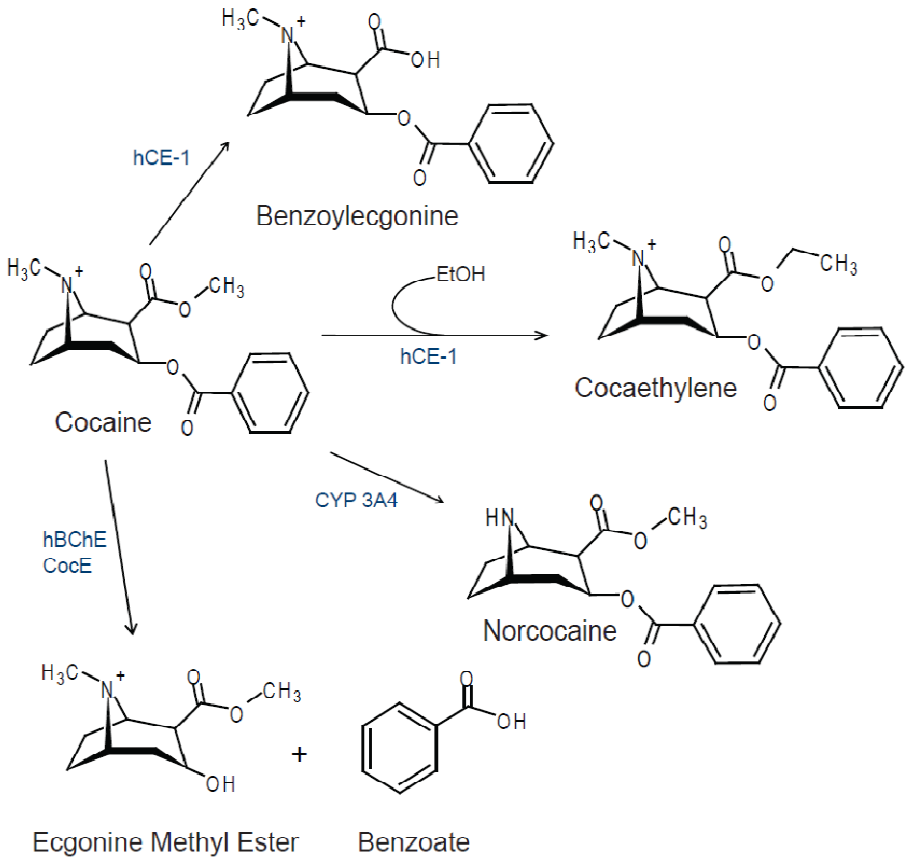


Figure 4.1: Major cocaine metabolites. Cocaine is rapidly converted into several metabolites. Human liver carboxylesterase-1 demethylates 40-45% of cocaine to benzoylecgonine. hCE-1 also catalyzes the conversion of cocaine into the more potent metabolite cocaethylene when alcohol is present. Liver P450 CYP3A4 n-demethylates about 5-10% of cocaine to norcocaine. Serum butyrylcholinesterase (K_m=14 μM, k_{cat}=3.9 min^{-1} [42]) hydrolyzes 40-45% of cocaine into ecgonine methyl ester and benzoic acid. RQ-CocE also catalyzes this reaction with a faster rate (K_m=14 μM, k_{cat}=1432 min^{-1}).

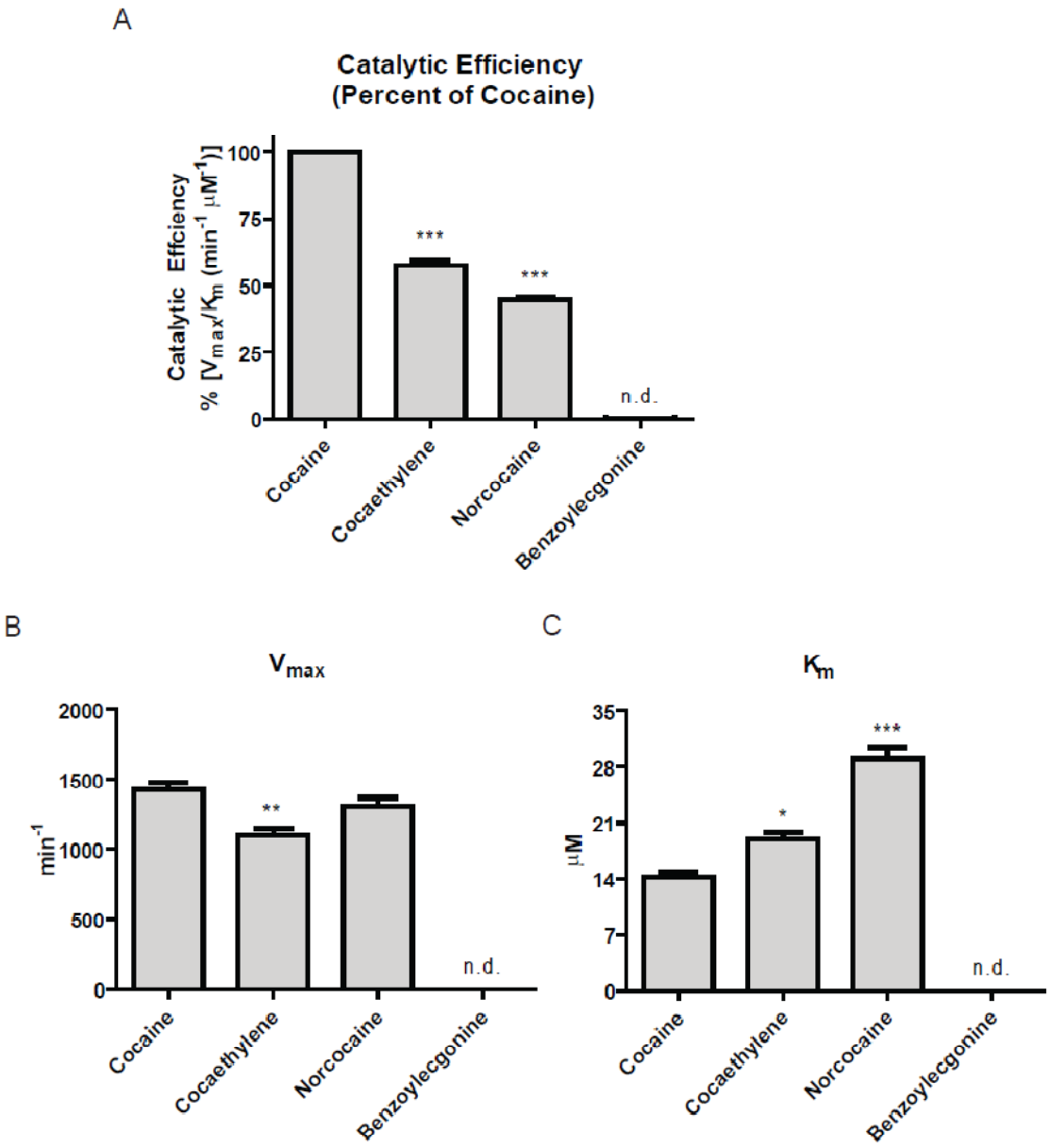


Figure 4.2: Hydrolysis of cocaine metabolites by RQ-CocE. RQ-CocE can hydrolyze the active cocaine metabolites cocaethylene and norcocaine but cannot hydrolyze benzoylecgonine, as determined by the spectrophotometric cocaine hydrolysis assay. Benzoylecgonine was not hydrolyzed, therefore data are not reported **A** The catalytic efficiency of RQ-CocE for cocaethylene and norcocaine is 58 and 45% respectively that of cocaine ($p<0.001$, Students T-test). **B** The V_{max} of RQ-CocE for norcocaine was unchanged from the V_{max} for cocaine. Cocaethylene had a significantly reduced V_{max} as determined by students T-test ($p<0.01$). **C** The K_m of RQ-CocE for both cocaethylene and norcocaine was significantly elevated above that for cocaine ($p<0.05$ and $p<0.001$ respectively).

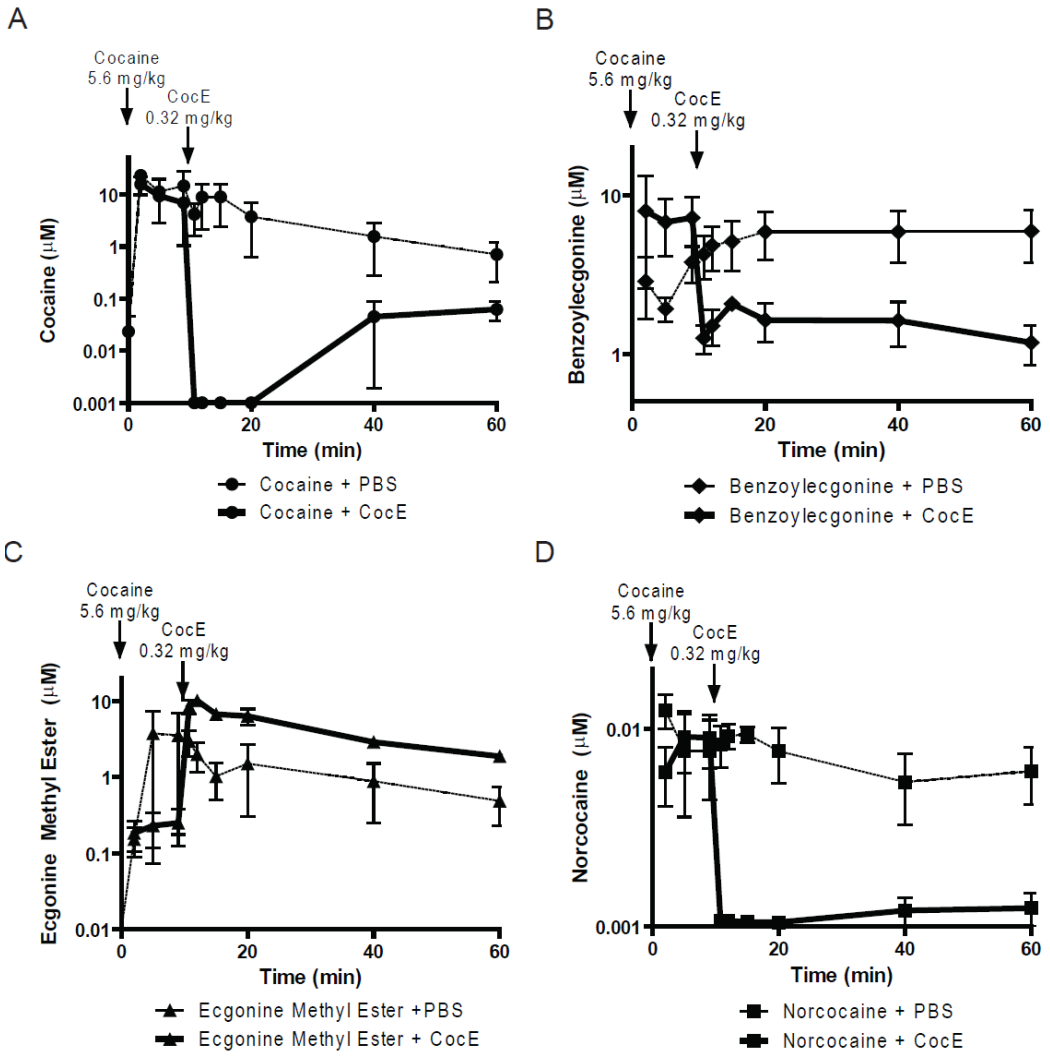
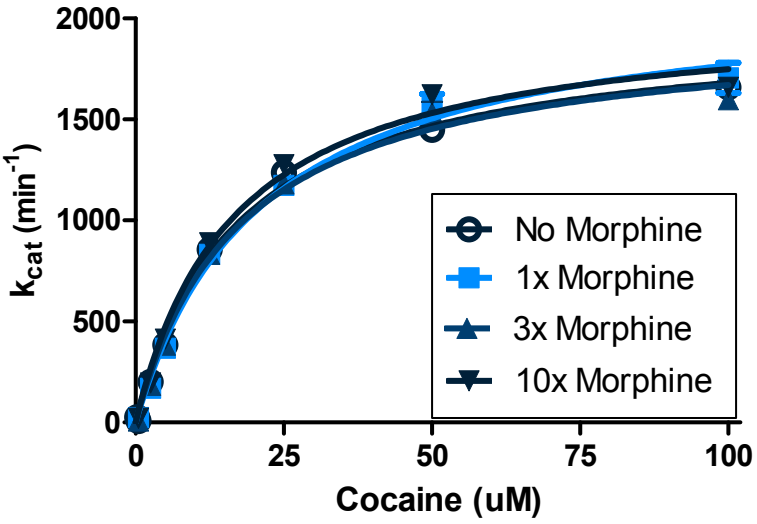


Figure 4.3: Hydrolysis of cocaine metabolites by RQ-CocE in Sprague Dawley rats. Cocaine (5.6 mg/kg) was injected IV at time 0, followed 10 minutes later by either PBS or 0.32 mg/kg RQ-CocE. Blood samples were taken throughout the time course and analyzed by mass spectrometry for cocaine metabolites simultaneously. **A** Cocaine is rapidly eliminated by RQ-CocE. The concentration of cocaine rises again at t=40 minutes due to the elimination of CocE and redistribution of cocaine. **B** Benzoylecgonine concentrations are affected by CocE, most likely due to binding and sequestration by CocE. Also, the absence of high concentrations of cocaine after 10:45 reduces the substrate for hCE-1 production of benzoylecgonine. **C** Ecgonine methyl ester concentrations are higher in RQ-CocE treated animals (equal to that of the original cocaine concentrations) demonstrating the production of EME by RQ-CocE. **D** Norcocaine is rapidly eliminated by RQ-CocE.

Drug	Physiological/Pharmacological Blood Concentrations			Significant Interaction
	1x	3x	10x	
Alcohol	80 mg/dL	240 mg/dL	800 mg/dL	None
Nicotine	0.12 μM	0.36 μM	1.2 μM	None
Morphine	0.18 μM	0.54 μM	1.8 μM	None
Phencyclidine	0.41 μM	1.23 μM	4.1 μM	None
Ketamine	0.35 μM	1.05 μM	3.5 μM	None
Meth-amphetamine	6.7 μM	20.1 μM	67 μM	None
Naltrexone	25 nM	75 nM	250 nM	None
Naloxone	1 μM	3 μM	10 μM	None
Diazepam	1 μM	3 μM	10 μM	***
Midazolam	0.64 μM	1.9 μM	6.4 μM	None

Table 4.1 Drug concentrations and tested interactions with RQ-CocE. ***Tested with mass spectrometry, None=No significant interaction was tested by one-way ANOVA with Dunnet's post-test between the control and various drug conditions for V_{max}, K_m and catalytic efficiency.



	V_{max}	K_m	V_{max}/K_m
No Morphine	1971	17.2	114.6
1x Morphine	2142	21.2	101.3
3x Morphine	1965	17.6	111.5
10x Morphine	2041	16.6	123.0
Significant?	No	No	No

Figure 4.4: Morphine does not affect cocaine hydrolysis by CocE. Using the spectrophotometric assay, all drugs were assessed for interaction with CocE by adding a physiological (or higher) concentration of drug to a 50 ng/mL CocE solution for 10 minutes at room temperature. The hydrolysis assay was also run in the presence of drug. Morphine does not inhibit cocaine hydrolysis by cocaine at any concentration tested. The Michaelis-Menten kinetic parameters are listed in the Table. The effect of each concentration of morphine on each Michaelis-Menten parameter of CocE was assessed by one-way ANOVA with Dunnet's post-test.

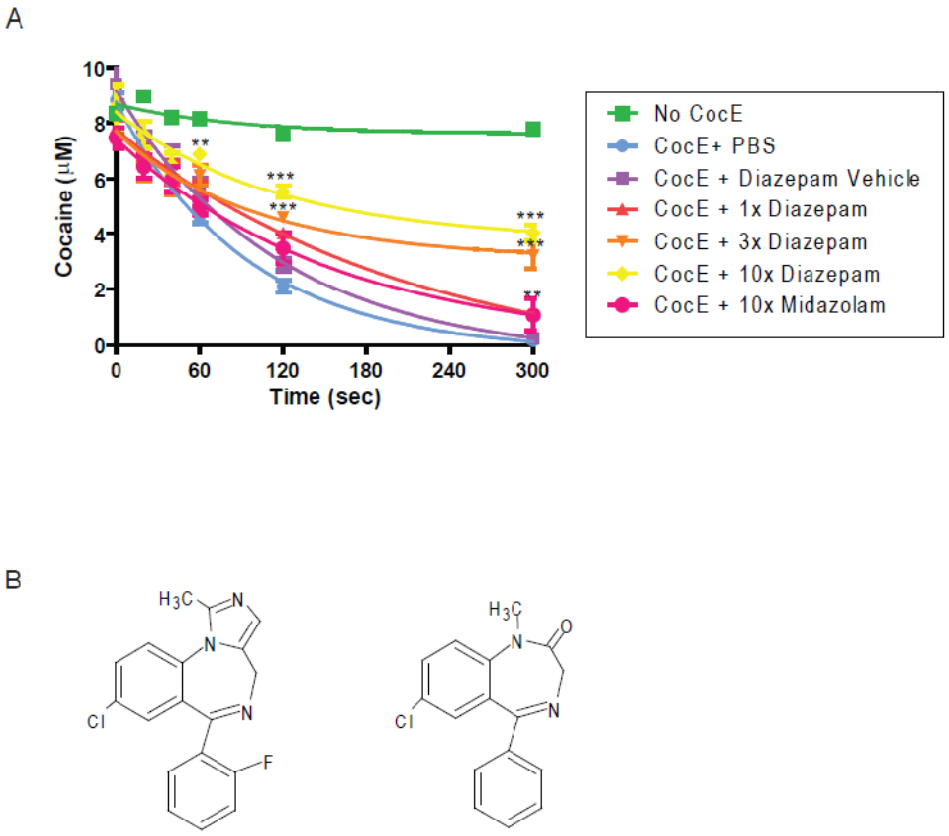


Figure 4.5: Diazepam, but not midazolam, inhibits cocaine hydrolysis by RQ-CocE. Hydrolysis of 8 µM cocaine by 3 nM CocE in human plasma in the presence or absence of interacting drug was assessed by mass spectrometry. The significance of the benzodiazepine interactions was assessed by 2-way ANOVA using Bonferroni posttests ($F(6, 60)=7.37$) **A** The addition of 3 nM CocE to human plasma spiked with 8 µM cocaine caused rapid cocaine hydrolysis (blue squares). Neither diazepam vehicle (purple) or midazolam (pink) had an effect on the rate of cocaine hydrolysis by CocE ($p>0.05$ at all time points). Significant interactions were seen with 1x ($p<0.01$ at 5 minutes), 3x ($p<0.001$ at 2 and 5 minutes), and 10x diazepam ($p<0.01$ at 1 minute, $p<0.001$ at 2 and 5 minutes). **B** The chemical structures of the benzodiazepines reveal the larger size and fluorination of midazolam (left) as compared to diazepam (right).

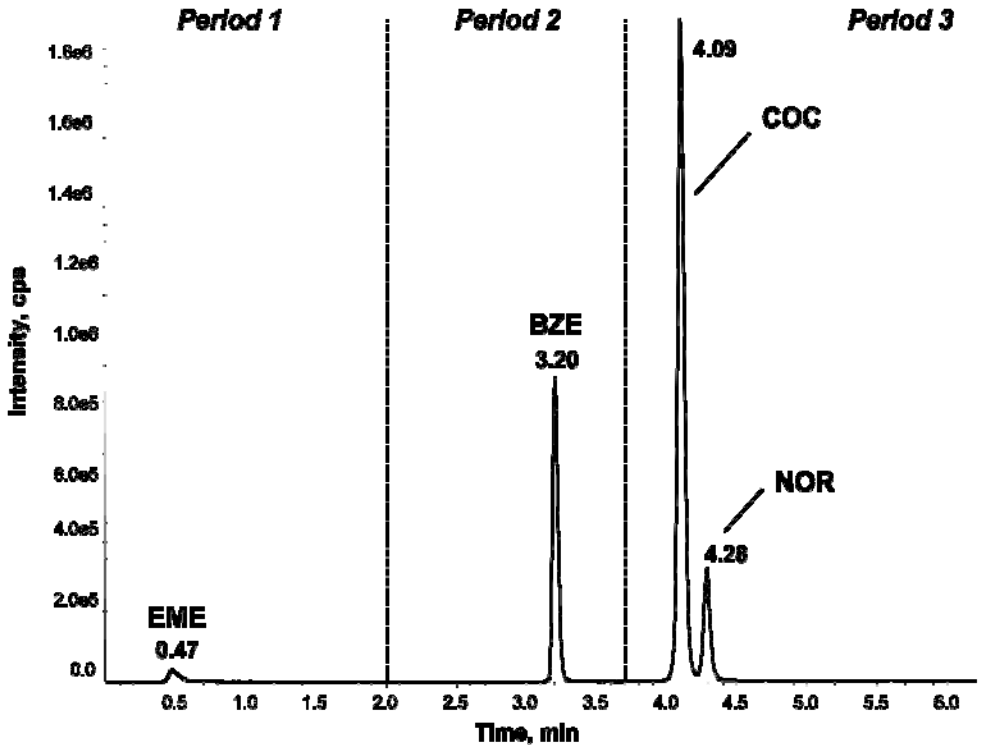


Figure 4.6: LC-MS/MS analysis of cocaine and cocaine metabolites. Total ion chromatogram of a 4.00 µM calibration standard showing separation of ecgonine methyl ester (EME), benzoylecgonine (BZE), cocaine (COC), and norcocaine (NOR) on a Hypersil Gold UPLC column (50mm x 2.1mm i.d., 1.9 µm particles) using 10 mM ammonium formate pH 4.6 as Solvent A and acetonitrile as Solvent B at a flow rate of 0.45 mL/min. Four µL aliquots were injected onto the column and compounds were eluted over an 11 minute gradient that began with an initial hold at 2% B for 1 minute, increased to 18% B in the next minute, gradually ramped to 40% B over 8 minutes, and finally increased to 100% B in 1 minute. Selected reaction monitoring was used for compound detection and quantification. The chromatogram was divided into 3 time segments during which only the relevant SRM transitions were scanned. This allowed for more accurate characterization of chromatographic peaks and thus, more precise quantification.

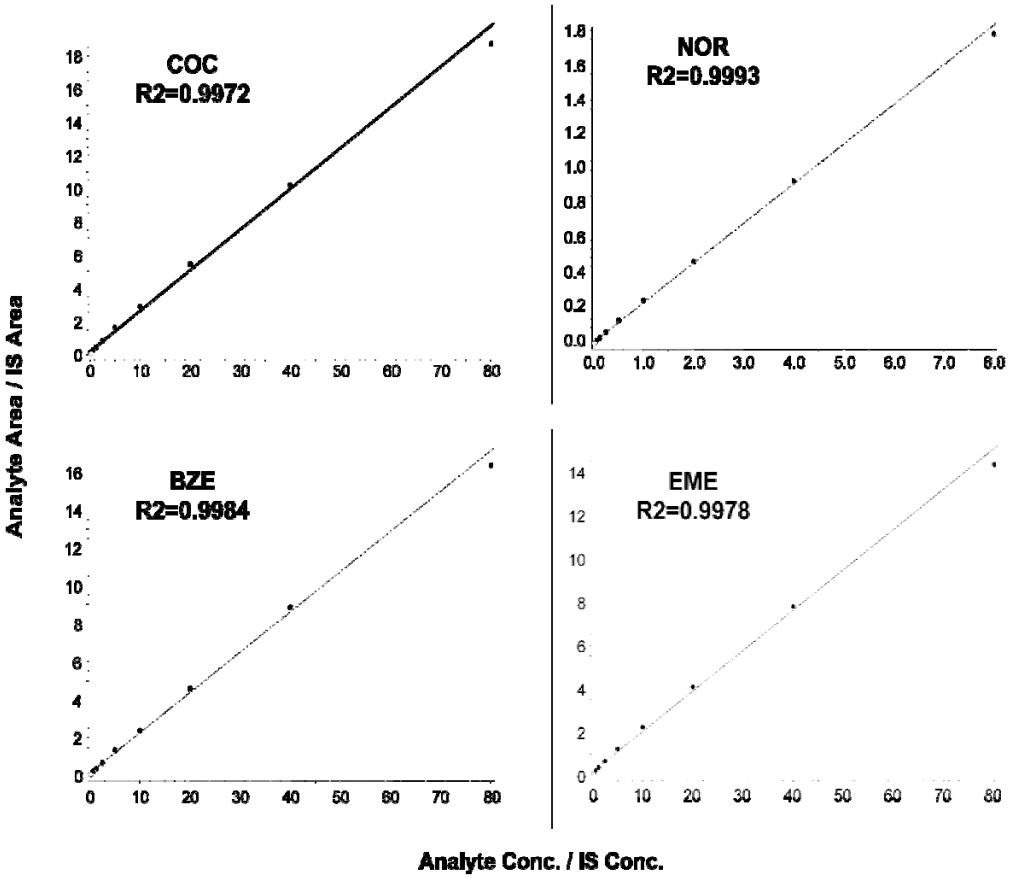


Figure 4.7: LC-MS/MS calibration curves for cocaine and cocaine metabolites. Calibration curves were constructed for each compound by calculating the ratios of the LC-MS/MS peak areas of the target compounds to the corresponding deuterium-labeled internal standards, and plotting the ratios as a function of analyte concentration normalized to the concentration of internal standard. Data were fitted to a curve using least squares linear regression analysis with *1/x* weighting. The linear range for cocaine (COC), benzoylecgonine (BZE), and ecgonine methyl ester (EME) was 0.0313 µM to 4.00 µM. Due to the extremely low concentrations of norcocaine (NOR) observed in the *in vivo* studies, the calibration curve was adjusted correspondingly and ranged from 0.00313 µM to 0.400 µM. Regression co-efficients (R^2) were all ≥ 0.995 and accuracies typically ranged from 67-130%.

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Chapter 5

General Discussion and Conclusions

Summary and Importance of Findings

The work presented here is a significant contribution to the development of CocE as a biological pharmacotherapy for cocaine toxicity and addiction. In addition, it has set the groundwork for other bacterially-derived biological drug products by enhancing our understanding of the pharmacokinetics and metabolism of CocE.

Enzyme kinetics

Although the *in vitro* kinetics had been extensively described, the *in vivo* time course of hydrolysis was unknown at the time this work began, and simply assumed to be "rapid", based on animal assays with physiological endpoints (cardiovascular [1, 2], neurological [3], lethal [4-8]). To this end the *in vivo* enzyme activity of RQ-CocE was assessed using cocaine concentration as an endpoint. This information would allow better approximation of the minimal effective dose as CocE moves towards clinical studies. Using the mass spectrometry techniques described in Chapter 3, pharmacologically relevant concentrations of cocaine (4 mg/kg, 8 μM) and very low concentrations of RQ-CocE (0.3-10 nM) were assessed *in vivo* in Sprague Dawley rats (*in vitro* experiments were also performed with the mass spectrometry methodology for comparison). Only one half-log higher dose is required *in vivo* to produce the same rapid elimination of cocaine seen *in vitro*. There was no effect of sex on elimination of cocaine by RQ-CocE.

The finding that *in vitro* assays can accurately predict the rates of substrate elimination for exogenous enzyme behavior *in vivo* is significant because it indicated that plasma matrices may not be required in initial screens for enzymes that may be used as effective biologic pharmacotherapies. This is advantageous since assays using plasma are much more difficult and time consuming to conduct. Additionally, the similarity between CocE's *in vitro* and *in vivo* behavior speaks to the advantage of using a biological product to treat cocaine toxicity or abuse. Small molecules targeting receptors often have off-target or incomplete effects due to specificity issues (see introduction).

In order to describe cocaine elimination in a more clinically relevant model, Sprague Dawley rats were given a convulsant dose of cocaine (5.6 mg/kg), followed 10 minutes later by RQ-CocE. Cocaine was rapidly eliminated by RQ-CocE to levels below the quantification limit of the mass spectrometry assay within 45 seconds. This rapid hydrolysis was also seen in Rhesus monkeys with no major species difference observed, as would be predicted by RQ-CocE's mechanism of action.

In addition to the examination RQ-CocE's capacity to hydrolyze cocaine, RQ-CocE's kinetics hydrolyzing major cocaine metabolites was also assessed to yield information about the enzyme's specificity and ability to further prevent toxicity. This is important as the major metabolites norcocaine and cocaethylene retain the physiological activities of the parent molecule as detailed in Chapter 4. It has been suspected that these active metabolites contribute to cocaine toxicity. A recent retrospective study found that patients presenting to the emergency room with detectable cocaethylene in their urine (a metabolite only formed when alcohol and cocaine are abused together) are admitted to the intensive care unit of hospitals with greater frequency than those with no cocaethylene (43% compared to 24%) [9]. Thus, the finding presented in Chapter 4 that CocE can hydrolyze both norcocaine and cocaethylene about 50% as well as cocaine is

extremely significant. The hydrolysis of norcocaine was confirmed *in vivo*. Although cocaethylene was not tested *in vivo* for logistical reasons, the finding that our *in vitro* activity assay predicts the *in vivo* activity of RQ-CocE within one log unit suggests that CocE would easily clear cocaethylene *in vivo*. Interestingly, RQ-CocE cannot hydrolyze benzoylecgonine *in vitro* or *in vivo*. From a drug treatment and law enforcement standpoint, this is a positive finding. Benzoylecgonine is used in urinalyses to test for cocaine use, and RQ-CocE, if introduced to the drug market, could not be used to pass drug screens after cocaine use.

The enzyme kinetics of CocE were also tested in the presence of commonly co-administered drugs. CocE activity against cocaine was not altered by the presence of pharmacologically relevant concentrations of alcohol, nicotine, morphine, phencyclidine (PCP), ketamine, methamphetamine, naltrexone, naloxone, or midazolam. However, cocaine hydrolysis by CocE was slowed by diazepam (30 and 80% by 1x and 10x pharmacologically relevant concentrations, respectively), a benzodiazepine often given to sedate patients. The phenyl ring structure on diazepam may cause active site competition between diazepam and cocaine. This assay was performed with very low RQ-CocE concentrations, and the results show dose-dependence, suggesting that with a higher dose of CocE this interaction may not be relevant. However, from a clinical perspective, another benzodiazepine, like midazolam, may be a better choice for patient sedation if RQ-CocE is to be administered.

Pharmacokinetics

This thesis describes in detail the pharmacokinetics of CocE. The question of CocE's pharmacokinetics first came to light after *in vivo* experiments with KQ-CocE. KQ-CocE is the most rapid cocaine hydrolyzing CocE produced to date (determined *in*

vitro and validated *in vivo* by KQ-CocE's greater potency protecting mice from cocaine-induced lethality when directly compared to wild-type CocE). In addition, KQ-CocE was the most thermostable CocE to date at the time of publication, with a half-life of 2.9 days at 37°C *in vitro*. We hypothesized that this mutant would therefore have the longest *in vivo* duration of action based on work by Narasimhan and Gao that described other thermally stable mutants of CocE [6]. L169K-CocE, G173Q-CocE, T172R-CocE and RQ-CocE were all described *in vitro* and *in vivo* and had equivalent half-lives and durations of action. However, upon testing, KQ-CocE had an *in vivo* duration of action of only 7.5 hours. Although this was the longest duration of action seen yet, it was not on the order of 2.9 days.

The reason for this discrepancy was elucidated by Western blot analysis (a method validated as being a good predictor of true circulation half-life in Chapter 3) on serum samples from NIH Swiss mice given 1 mg of either the thermally unstable wild-type CocE or stable KQ-CocE. Both rapidly disappear from the serum rapidly with half-lives of 2.2 and 2.3 hours respectively.

To further confirm that the half-life of CocE was not related to thermostability, the species being examined, or the dose of CocE administered, the Western blot studies were repeated in Sprague Dawley rats with RQ-CocE at a lower dose (8 mg/kg CocE). This experiment yielded a similar half-life of 2.4 hours, indicating that the thermostability of CocE, dose of CocE, and species tested (at least among rodents) do not affect the circulating half-life of CocE.

To extend and validate the Western blot studies, and to quantifiably describe the pharmacokinetic parameters of CocE, RQ-CocE was metabolically labeled with [^{35}S]-methionine and dosed to Sprague Dawley rats at 8 mg/kg (18 μCi/kg). This study

confirmed the accuracy of the estimations derived from the Western blot densitometry studies. The pharmacokinetic parameters (Table 3.1) were similar to those estimated with only total blood volume and the qualitative serum half-life. RQ-CocE's volume of distribution is close to the blood volume of the respective animals, demonstrating that it is not leaving the bloodstream, nor is it binding serum proteins. Immunohistochemical data also demonstrate that epitope-containing RQ-CocE is not leaving the vasculature and entering the heart, liver, or lungs.

The 2 hour half-life is relatively short but comparable to other clinically used protein drugs of similar size (see Chapter 3, discussion). It is interesting to note that the mechanism of elimination and metabolism is currently unknown for many widely used biological drugs including proteins and antibodies. Mechanisms of clearance were investigated here purely to gain scientific knowledge of how biological drugs as a class may be eliminated, and not as a safety concern (no long term effects of toxicity have been seen in multiple dosing studies in both rodents and non-human primates [2, 7, 8, 10]). Ten percent of the [^{35}S]-label was excreted in the urine of rats given [^{35}S]-RQ-CocE over a 29 hour period. Full length and some small fragments of CocE were found in the urine 1 and 2 hours after CocE administration. However, the kidneys were ruled out as an important mechanism of elimination after [^{35}S]-CocE pharmacokinetic studies were repeated in nephrectomized Sprague Dawley rats and no significant change in any pharmacokinetic parameter was found.

To investigate where the remaining 90% of radioactive label deposited, animals administered [^{35}S]-RQ-CocE were perfused and dissected after a 29 hour observation period, and organ slices were exposed to film. Radiographs showed clear shadows of each organ demonstrating that the radioactive label had undergone non-specific uptake into all tissues. Although the predominant mechanism of elimination of CocE is most

likely proteolysis by liver and serum proteases, these products are either rapidly eliminated by the kidney (~10%) or rapidly recycled in other cellular processes (~90%) as no major fragments of CocE are seen in the serum either by Western blot or radiographic analysis.

Protection against cocaine-induced toxicity and reinforcing effects

Although not the focus of this thesis, important data have been collected here on CocE's ability to protect against the toxic and reinforcing effects of cocaine. Work with KQ-CocE expanded on the study conducted by Collins et al. that originally demonstrated the ability of CocE to block the reinforcing effects of cocaine [4]. Wild-type CocE administered immediately before the operant session caused a transient suppression of cocaine-reinforced responding, with RQ-CocE eliciting full suppression. Experiments with thermostable KQ-CocE sought to use a more clinically relevant paradigm by giving longer pretreatments of CocE before operant sessions. Pre-treatments one hour before the self-administration session significantly suppressed responding; however, a 2 hour pre-treatment either increased or decreased responding, varying between individual animals to elicit an average that was unchanged from saline pre-treatment.

Using the RQ-CocE dose response analysis performed by Collins et al. and the pharmacokinetic data presented in Chapter 3, it is reasonable to conclude that rapid clearance of CocE to about 50% after 2 hours would lower the CocE dose enough to become surmountable with increased cocaine administration behavior. Issues with surmountability are also seen with the cocaine vaccine TA-CD in development for abuse [11], and speak to the necessity for a cocaine abuse therapy to have a very stable, predictable and long-lasting kinetic profile (see Ongoing and Future Work).

CocE elicited equivalent protection against cocaine toxicity across species. Both rats and monkeys were given a convulsant dose of cocaine (5.6 mg/kg and 3.2 mg/kg respectively), followed 10 minutes later by 0.32 mg/kg RQ-CocE. The effect of CocE was the same between species; cocaine was rapidly eliminated by RQ-CocE to levels below the quantification limit of the mass spectrometry assay. This is significant because the effective transition to higher species is essential for drug development. The catalytic antibody mAb15A10 that hydrolyzes cocaine into inactive products is an example of a therapy that was very promising in rodent models, but was not able to produce the same robust results in non-human primates [12]. Thus the ability of CocE to work across species clears a major pre-clinical hurdle.

Ongoing and Future Work

CocE and mutants of CocE have been extensively studied in both academic and industrial laboratories since 2000. However, CocE is not the only biologic drug in development for the indications of cocaine toxicity and cocaine abuse. Here the current state of CocE and other biologics in development for each indication is discussed.

Biologics for cocaine toxicity

Pre-clinical studies with RQ-CocE for the treatment of cocaine toxicity have been completed at the University of Michigan and at contract laboratories throughout the United States and Canada. These studies have adequately addressed the efficacy and safety of RQ-CocE. Work presented in this thesis has directly contributed to the pre-clinical data that will be presented to the Food and Drug Administration. At the time of publication, large commercial production of RQ-CocE has begun in preparation for Phase 1 human clinical trials.

Potential immune response is of top concern moving RQ-CocE from pre-clinical animal models to humans. Although studies performed in NIH Swiss mice and Rhesus monkeys have found that antibodies against CocE are elicited after repeat dosing, these antibodies do not diminish CocE's effectiveness, nor have they caused toxicity to the animals tested [2, 7, 8, 10]. Additionally, the repeat dosing study in monkeys presented in Chapter 3 shows that the rate of cocaine hydrolysis was not slowed by the presence of antibodies. These data actually showed that the more CocE injections the animals had, the more complete the cocaine hydrolysis was (although the significance of this is unclear from only two animals, and is most likely natural variability).

Recombinant mutants of human serum butyrylcholinesterase (BChE) are also being developed to treat cocaine toxicity. BChE is the endogenous enzyme which hydrolyzes cocaine into the inactive metabolites ecgonine methyl ester and benzoic acid. The design and production of mutant BChE with specific activity for cocaine has been ongoing since the late 1990s. Although large-scale studies were originally hindered by difficulties expressing and purifying this tetrameric, glycoslyated protein, advances in expression (new cell systems, transgenic expression in animal milk) and purification technology have facilitated this approach.

BChE mutants specifically designed for a detoxification indication have been investigated primarily by Zhan [13-19] and Lockridge [20, 21]. These mutants were designed using virtual modeling of cocaine transition states, and the most efficient (A199S/F227A/S287G/A328W/E441D-BChE) is 1,730 more efficient at hydrolyzing cocaine than the wild-type enzyme (k_{cat}/K_m=1.6 x 10^9 M^{-1} min^{-1}) [13]. This is comparable to KQ-CocE which has a catalytic efficiency of 2.2 x 10^8 M^{-1} min^{-1} [22]. Protection of NIH Swiss mice against cocaine toxicity by 180 mg/kg cocaine was achieved with a 0.02 mg

IV pretreatment of A199S/F227A/S287G/A328W/E441D-BChE [13]. KQ-CocE achieved protection in this assay with a pretreatment of 0.032 mg IV [22].

The use of a cocaine hydrolyzing enzyme as a treatment for cocaine toxicity (either CocE or BChE) has a large advantage over the current symptomatic treatments: CocE simply clears the cocaine molecule and eliminates its physiological effects, reducing the need for emergency department physicians to make decisions about what drugs can effectively treat a patient with recent cocaine use. A current debate among physicians is whether or not to use β-blockers to treat cocaine related chest pain. Current cardiology guidelines from the American Heart Association recommend against using β-blockers to treat patients with cocaine-associated chest pain [23-25], as small studies have shown some worsening in outcome among this group [26, 27]. However, a recent retrospective study examined over 300 patients who had positive urine screens for cocaine, half of whom received β-blockers, and demonstrated that there was no significant increase in adverse cardiac events among the β-blocker group [28]. The β-blocker group actually had significant decreases in blood pressure and rates of cardiovascular related death compared to the control group. However, others in the field have expressed concern over the experimental design of this study, specifically that these patients were not suffering from acute cocaine toxicity, but were older patients most likely suffering from benzoylecgonine-induced chest pain based on the time-frame of treatment [29, 30].

Together, all of these reports do not support or refute the current recommendations but do demonstrate the disagreement in the field over the proper way to pharmacologically treat patients with cocaine-induced cardiovascular complications. A drug like CocE, that simply eliminates the cocaine without harmful side effects, would

eliminate this debate and help patients recover faster without further potential drug interactions.

Biologics for cocaine abuse

Currently two proof of concept studies have been performed with CocE, and both demonstrate that CocE can effectively block the reinforcing effects of cocaine if serum levels are adequately high [4, 22]. However, CocE's very short circulating half-life (about 2 hours) makes it an unsuitable candidate for treatment of cocaine abuse in its current form. Unpublished studies from our laboratory have used the introduction of new mutations to RQ-CocE (G4C/S10C/T172R/G173Q, CCRQ-CocE) to produce an enzyme that retains 100% activity at 37° for longer than 45 days [31]. To increase the serum half-life, PEGylation (e.g. the maleimide coupling of 40-kDa branched polyethylene-glycol chains to surface cysteines) was performed on CCRQ. PEG-CCRQ-CocE (32 mg/kg) was able to protect mice and rats from cocaine-induced toxicity with a 48 hour pretreatment, and retained some protective activity after a 72 hour pre-treatment [31]. More importantly, PEG-CCRQ-CocE fully blocked rat cocaine self-administration behavior for 3 days following IV injection [31]. Currently, different routes of administration for PEG-CCRQ-CocE and sustained release formulations of CCRQ are being investigated to further extend the duration of CocE *in vivo*.

Concurrently with the development of CocE, recombinant mutant human BChE is has also been undergoing development as a potential treatment for cocaine abuse, led by work by Brimijoin [32-40]. In addition to protein engineering, this group has pioneered work extending plasma half-life of BChE and delivering BChE with viral vectors. These advances have helped not only the cocaine abuse field, but also the field of general biologic drug development.

Wild-type BChE has a serum half-life of approximately 16 hours after IP injection (found in both 129sv mice and Sprague Dawley rats [20]). A BChE mutant designed by Gao and Brimijoin (A199S/S287G/A328W/Y332G, CocH) was fused to serum albumin (Albu-CocH) and exhibited a half-life of 8 hours in Wister rats after IV injection [32, 37]. This difference in half-life is most likely due to the route of injection, as protein injected IP is protected from proteases until it enters the circulation. Albu-CocH blocked cocaine-seeking in a relapse model: rats that had previously self-administered cocaine and had completed extinction with saline were primed for reinstatement with cocaine, saline or amphetamine. Albu-CocH was able to block cocaine-primed reinstatement when given 2 hours before the session [32].

Even with IP injection, PEGylation, or albumination, serum proteases eliminate these exogenous proteins relatively rapidly. Ideally, a therapy for cocaine abuse should be fully efficacious for one week to allow a reasonable dosing schedule. However, Brimijoin performed proof-of-concept studies that showed viral transduction of F227A/S287G/A328W/Y332A-BChE (AME-BChE) was not only possible, but resulted in long-term elevations in cocaine hydrolase activity after one IV injection [35, 36]. More importantly, adenoviral transduction of CocH blocked the reinstatement of cocaine responding in the same paradigm described above for 24 weeks (although at 24 weeks, the single injection was still fully protective, predicting a much longer duration of action) [40]. Clinically, the use for adenovirus therapy is limited as it has exhibited severe toxic effects, has unknown long-term effects, and clinical trials with adenovirus vectors have had very limited success [56-58]. Nevertheless these studies provide firm evidence that the presence of a cocaine hydrolase over a long period of time is effective at preventing relapse behavior.

Cocaine hydrolases have an advantage over small molecule therapies (described in more detail in the introduction) as the likelihood of off-target effects is greatly diminished. For example, RQ-CocE can hydrolyze cocaine, norcocaine and cocaethylene but not benzoylecgonine, the most structurally similar metabolite. No toxicity was seen in commercially performed repeat dosing studies, suggesting that no off target substrates were being hydrolyzed during the studies. BChE mutants have been engineered to have specificity for cocaine over natural substrates. CocH, A199S/F227A/S287G/A328W/E441D-BChE, and A199S/F227A/S287G/A328W/Y332G/E441D-BChE were all tested for activity against acetylcholine (ACh) and were found to have only 30-90% of the activity found with wild-type BChE but greater than 1000-fold the activity of wild-type against cocaine [13]. Although computer modeling predicted that CocE would display some acetylcholinesterase activity [41], the lack of toxicity to animals in repeat and high dose studies, the specificity for cocaine found in metabolite studies and the lack of activity from BChE mutants all suggest that this concern is unfounded in a physiological system.

One concern that is often voiced is that addicts will be unmotivated to come to a clinic for pharmacological treatment, whether this is a cocaine vaccine or a long-acting injection of a cocaine hydrolase. Although this is outside of the scope of the research presented here, it is important to note that contingency management, a procedure that reinforces behavior with desired rewards, has been extensively studied among drug users and has produced good results for achieving drug abstinence [42], and compliance to medication schedules [43-45]. A recent study by Stitzer et al. expanded on this large body of work, testing adherence by drug users to a 6-month vaccination protocol with the hepatitis B virus vaccine on a schedule similar to what would be required of the cocaine vaccine (once-per clinic visits required) [46]. Groups receiving

monetary incentives received more injections on time and, although not statistically significant, completed more total injections and had better attendance. This study supports many others that contingency management is effective at producing desired compliance and it further advances the field it by showing positive results with a rigorous schedule (24 weekly visits) that may be required of cocaine addiction therapies.

New directions for cocaine abuse therapy

As the development of both mutant CocE's and BChE's have made great progress over the last 10 years, the fields of vaccination and small molecule treatment have also made great strides. Two very recent developments in these fields have introduced new ideas into the literature for the treatment of cocaine abuse.

Most of the focus on vaccines over the last two decades has been on the TA-CD vaccine, which is currently in Phase IIa clinical trial for efficacy [11, 47, 48]. This vaccine elicits antibody response by delivering succinylated norcocaine conjugated to a cholera toxin B vector [49, 50]. However, as mentioned in the introduction, the efficacy of this vaccine is tightly linked to antibody titer, and those subjects who do not produce a high antibody titer do not receive the protective benefits of the vaccine. Several groups have demonstrated that passive immunization with catalytic or non-catalytic antibodies can also block cocaine's effects [51-53]; however this is not a suitable strategy for long term addiction treatment.

Attempting to achieve higher antibody titers, Hicks et al. used a new vaccine strategy linking the cocaine analog GNC (6-(2R,3S)-3-(benzoyloxy)-8-methyl-8-azabicyclo [3.2.1] octane-2-carbonyloxy-hexanoic acid) to a replication incompetent adenovirus [54]. This vaccine produced high antibody titers in mice that were sustained

for 5 weeks after the 3rd dose of the vaccine (longer time points were not assessed) [54]. Three weeks after the 3rd dose, a cocaine dose to the mice resulted in a serum concentration of cocaine about seven times that of non-vaccinated mice [54], meaning that cocaine was being sequestered in the blood keeping it away from the brain and other tissues. Vaccination also reduced cocaine induced locomotor activity in these mice [54]. Although longer-term studies need to be performed to validate this vaccine strategy, it has potential to surpass the encouraging results seen with TA-CD and could be used with other small molecule drugs of abuse like nicotine.

The second new approach towards the treatment of cocaine abuse is to alter dopamine synthesis and therefore the chemistry of the brain. Disulfiram, which inhibits aldehyde dehydrogenase, has reduced cocaine intake in some studies but overall results have been variable (see Introduction). However it is hypothesized that the positive results with disulfiram treatment of cocaine abusers is due to disulfiram inhibition of dopamine β-hydroxylase (required for norepinephrine synthesis). However, a recent study by Yao et al. demonstrated that inhibition of aldehyde dehydrogenase in the presence of cocaine increased tetrahydropapaveroline (THP) (a potent inhibitor of tyrosine hydorxylase which is required for dopamine synthesis) and decreased dopamine synthesis [55]. This finding suggests that disulfiram may be eliciting its effects on cocaine users by inhibiting both norepinephrine and dopamine synthesis in the brain, effectively disconnecting cocaine from its rewarding and stimulating physiological effects. There are several key experiments that need to be performed; especially since disulfiram itself was not shown to increase THP (a specific aldehyde dehydrogenase inhibitor was used). However, the questions this study raises about possible abuse therapies are quite interesting. If a selective inhibitor of neurotransmitter synthesis was designed, could it reduce cocaine intake? Would it be safe? Are there other drugs like

the specific aldehyde dehydrogenase inhibitor that can cause the accumulation of natural chemicals that could provide negative feedback? Could any of these pathways be induced with cocaine use?

All of these questions represent new directions that can be taken in the future of this field. These new directions, as well as the advanced state of research of BChE, the cocaine vaccine and CocE mean that a treatment for cocaine toxicity and cocaine abuse is not impossible, and that brighter horizons exist for those individuals struggling to live with this debilitating addiction.

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Appendix

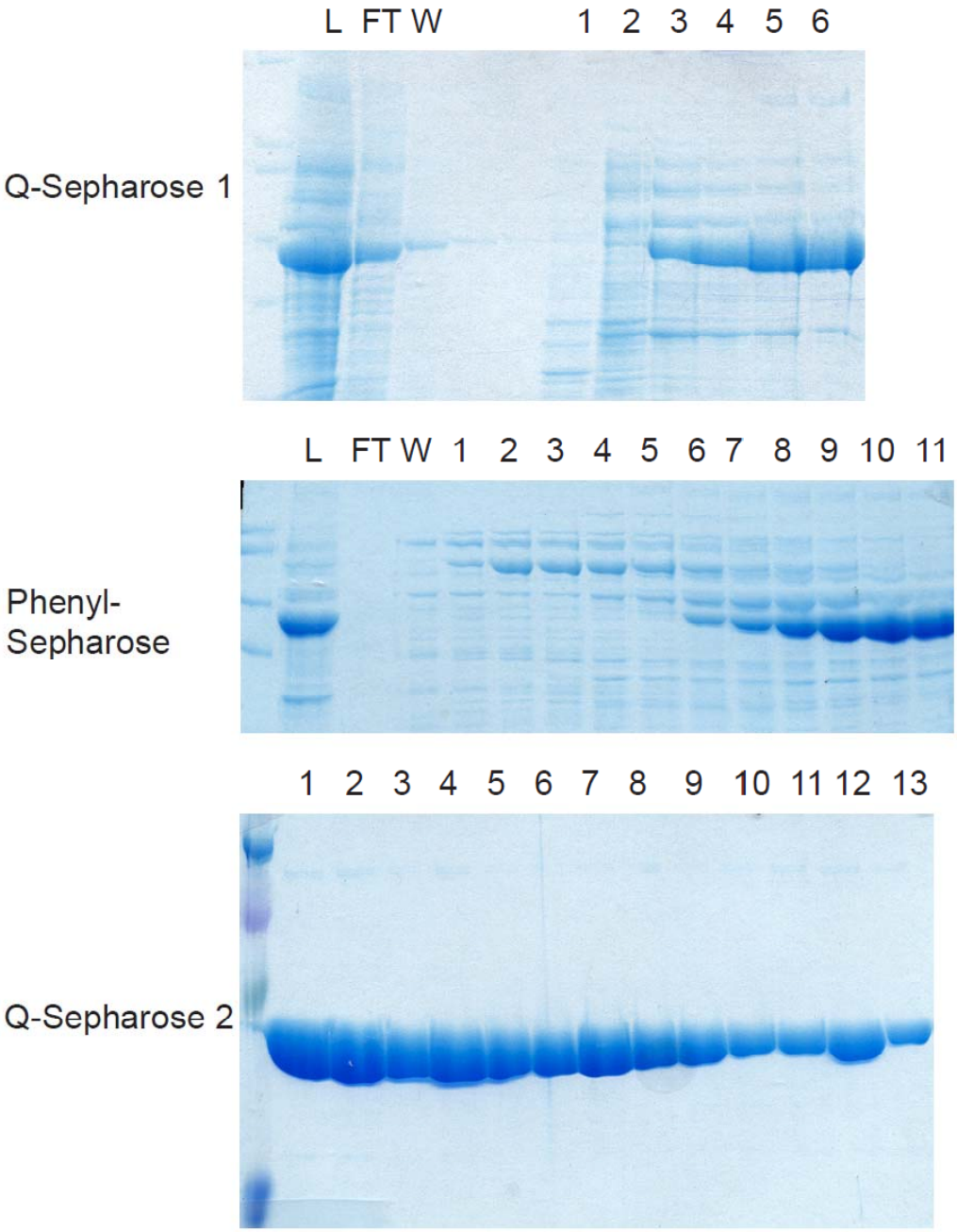


Figure A1.1: Purification of RQ-CocE. Purification of RQ-CocE protein was performed with three sequential columns, Q-Sepharose, Phenyl-Sepharose, and Q-Sepharose. Total cell lysate was added to Q-Sepharose column 1 (L) and the flow through was collected (FT). The column was washed with 20 mM Hepes Buffer pH 8.0 (W) followed by elution with 20 mM Hepes + 500-1000 mM NaCl by gradient (1-6). Fractions containing RQ-CocE were pooled and added to 1M ammonium sulfate for loading onto the phenyl-sepharose column. Column was washed with 50 mM NaPhos pH 7.0 + 1M AS (W) and eluted with 50mM NaPhos + 700-0 mM AS. After triton extraction of fractions containing RQ-CocE, a second Q-sepharose column was run as described above. Pictures reprinted with permission from Dr. Diwahar Narasimhan

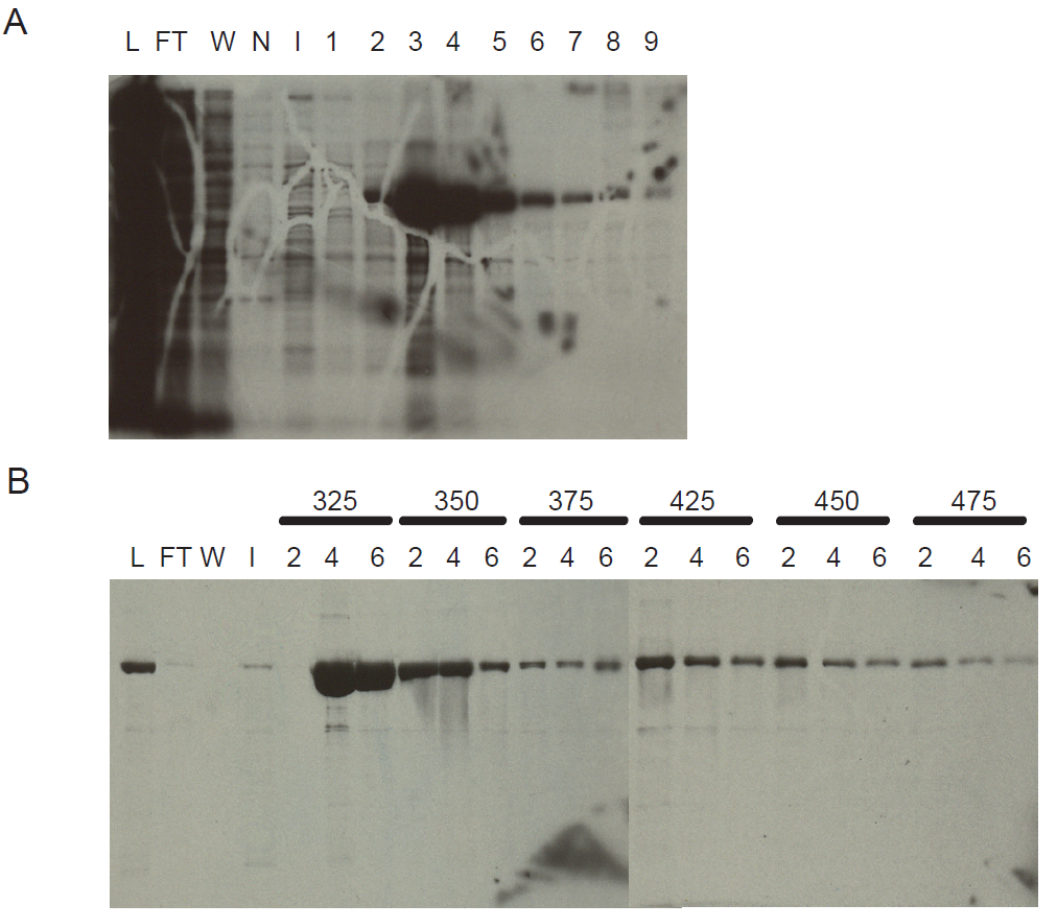


Figure A1.2: Purification of [^{35}S]-RQ-CocE **A** Purification by Nickel-NTA Column. Total cell lysate was added to a Nickel-NTA column (L) and the flow through was collected (FT). The column was washed with TBS buffer (W) followed by 300 mM NaCl (N) and 10 mM imidizole (I). The column was eluted with 9 fractions of 100 mM imidizole (1-9). Fractions were resolved by SDS-PAGE. The resolving gel was dried and exposed to film overnight. The radiograph is shown above. **B** Purification by Ion Exchange Chromotography. Fractions 2-7 from the Nickel-NTA elution were loaded onto the Q-Sepharose column for further purification (L) and the flow through was saved (FT). The column was washed with 50 mM Tris HCl, pH 8.0 (W) and 50 mM Tris HCl, pH8.0 + 300 mM NaCl (I). The column was eluted with 325-500 mM NaCl stepwise with 25 mM steps and 6 fractions per step. Fractions 2, 4, and 6 from each step were resolved with SDS-PAGE. The gel was prepared and exposed to film as described above. Fraction 4 from step 325- fraction 6 from step 375 were pooled and used for RQ-CocE pharmacokinetic analysis.

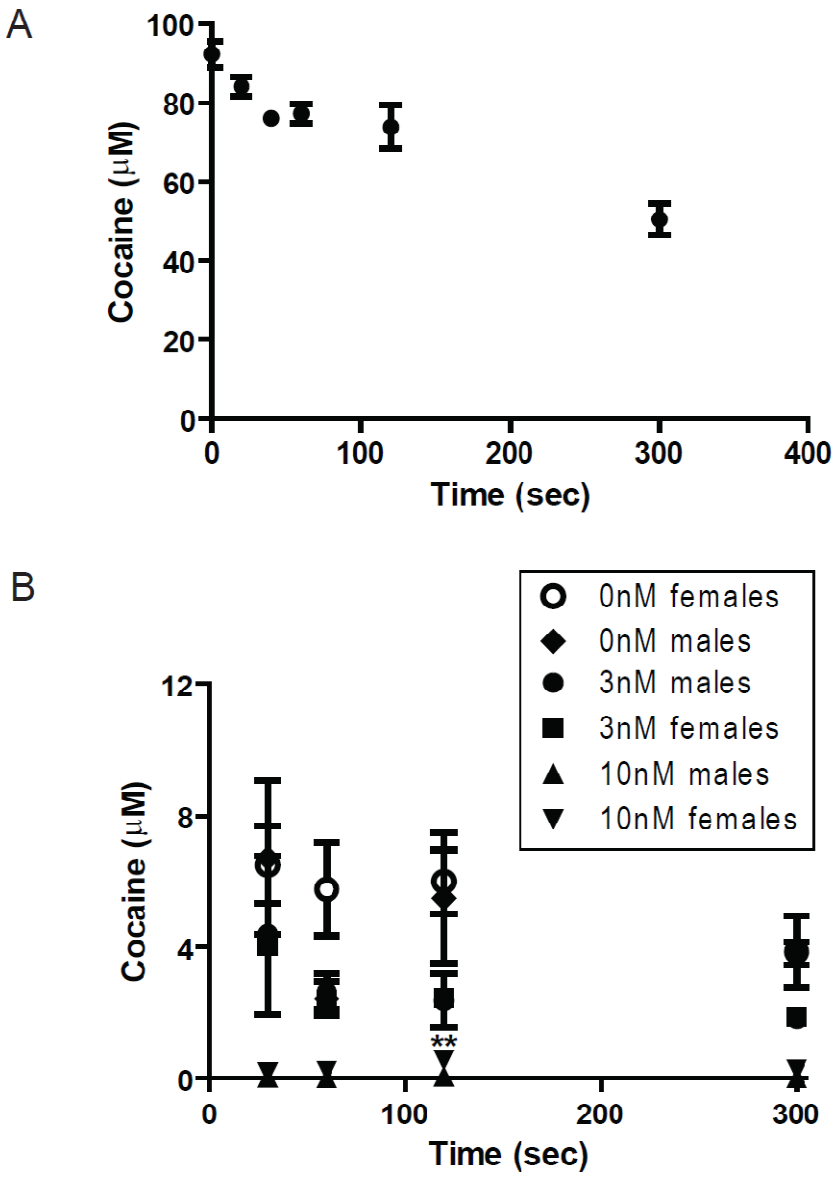
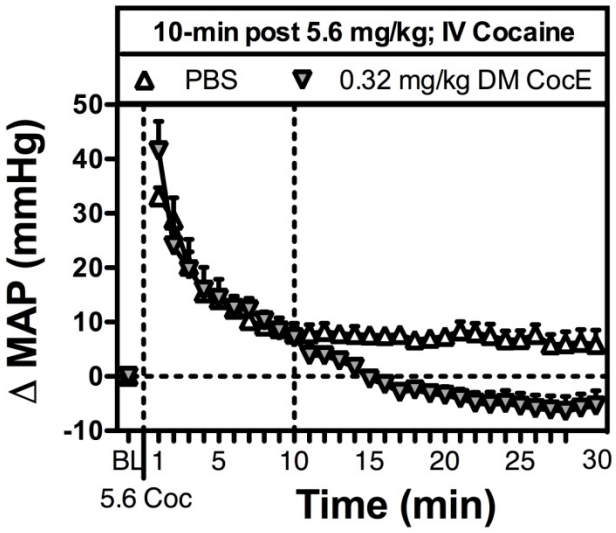


Figure A1.3: Mass spectral analysis of RQ-CocE hydrolysis of cocaine A *In vitro* analysis of highly concentrated cocaine by RQ-CocE hydrolysis. Human samples were spiked with 100 μM cocaine at time=0. After 5 minutes at 37°C, 3 nM RQ-CocE was added at plasma and aliquots were take, stopping hydrolysis at the given points. Aliquots were assessed for remaining cocaine concentration by LC/MS analysis. **B** *In vivo* assessment of cocaine hydrolysis by RQ-CocE. Sprague Dawley rats were intravenously administered 0, 13.6 or 50.5 μg/kg RQ-CocE followed 2 minutes later by an intravenous administration of 4 mg/kg cocaine. Blood samples were taken at the times after cocaine injection shown, hydrolysis activity was stopped and samples were evaluated by LC/MS for cocaine concentration. Data presented to show the individual sexes of the rats assessed. No significant difference was seen between the sexes except at the 2 minute time point in animals treated with 50.5 μg/kg CocE ($p<0.001$, mean males=0.06 μM, mean females=0.55 μM).

A



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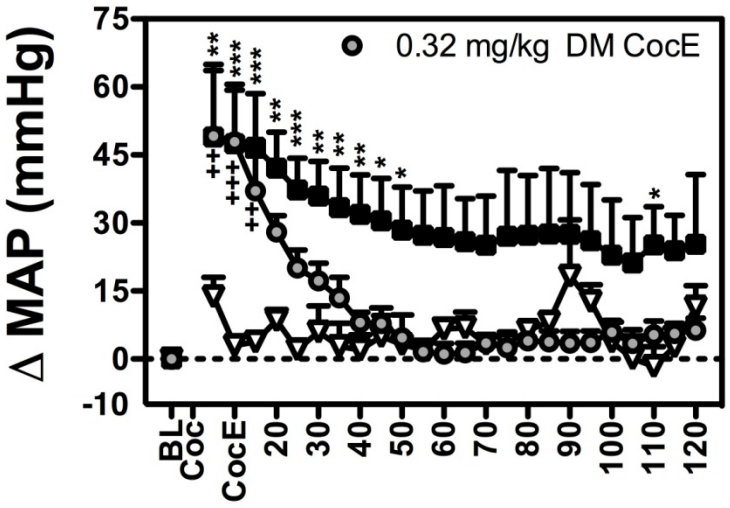

Figure A1.4: Mean arterial pressure of rats and monkeys in response to cocaine and RQ-CocE **A** Rats surgically implanted with an indwelling femoral catheter and a telemetric blood pressure transmitter (Transoma Medical, Inc.) were administered 5.6 mg/kg cocaine at time 0. Ten minutes after the cocaine injection, either PBS (open triangles) or 0.32 mg/kg CocE (filled inverse triangles) was administered. The mean arterial pressure of rats receiving RQ-CocE returned to baseline faster than the rats receiving only PBS. **B** Monkeys were surgically implanted with telemetric blood pressure probes (DSI Inc) and interdwellling jugular or femoral catheter. Monkeys received 3.2 mg/kg cocaine (circles) or saline (inverse triangles) at time 0. Ten minutes later, animals receinving cocaine were administered either 0.32 mg/kg RQ-CocE (grey circles) or PBS control (black circles). RQ-CocE dramatically decreased the high mean arterial pressure induced by cocaine administration by 60 minutes. Data produced by Dr Gregory Collins, reprinted with permission.

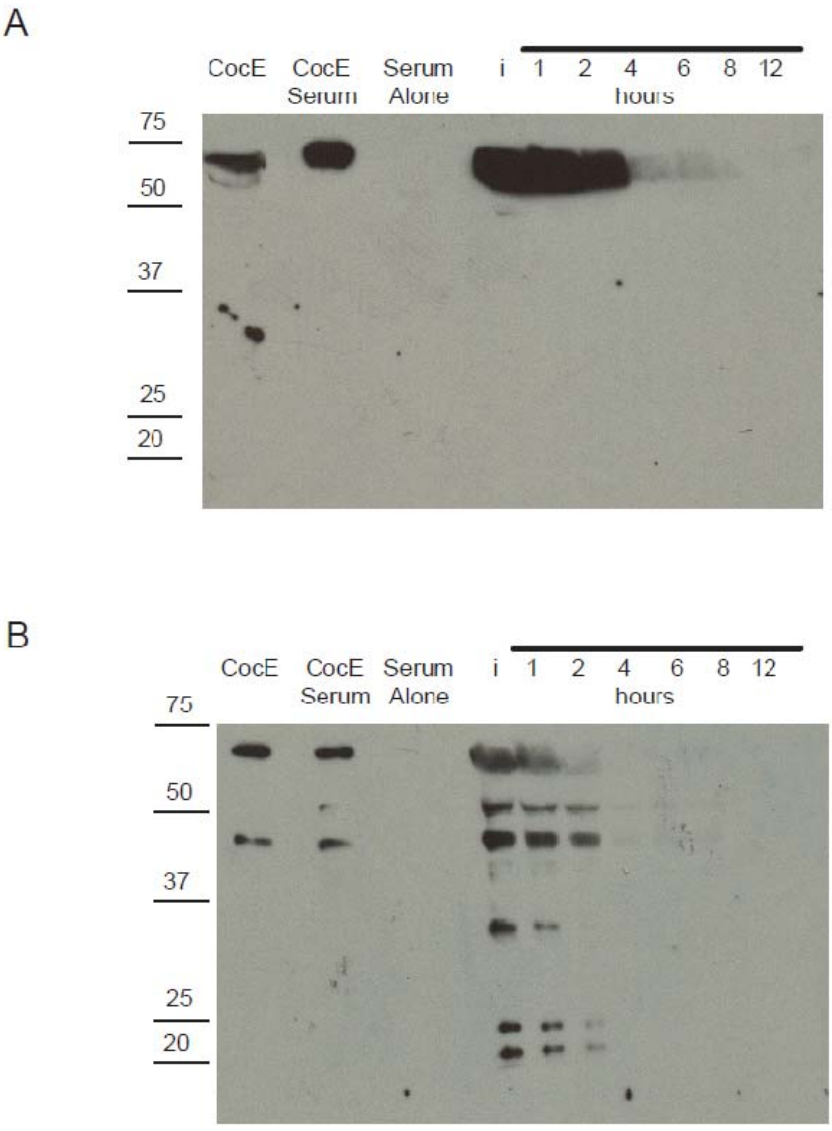

Figure A1.5: Thermal cleavage of RQ-CocE A (Reprint of Figure 3.3A) Western blot analysis of RQ-CocE in the serum of Sprague Dawley rats administered 8 mg/kg RQ-CocE. Blood samples were taken from rats at t= 5min "i", 1, 2, 4, 6, 8, and 12 hours after RQ-CocE administration. Samples were added to SDS and BME loading buffer and immediately loaded onto SDS-PAGE gels. RQ-CocE (25 ng) alone was loaded in lane 1 as a positive control. Rat serum alone (20 µg) was loaded in lane 3 as a negative control. RQ-CocE (25 ng) was spiked into 20 µg of serum protein to control for any alteration in migration due to the presence of large quantities of protein. Animal samples were loaded in a time-dependant manner. **B** Western blot analysis of RQ-CocE in the boiled serum of Sprague Dawley rats administered 8 mg/kg RQ-CocE. Samples were obtained and handled as described above, expect for a boiling procedure. After the addition of SDS and BME loading buffer to the samples the resulting solution was heated to 100°C for 10 minutes, then immediately loaded onto SDS-PAGE gels. The heating resulted in the fragmentation of CocE into 5 epitope retaining pieces.

Compound	Declustering Potential (v)	Focusing Potential (V)	Entrance Potential (V)	Collision Energy (eV)	Collision Cell Exit Potential (V)
Cocaine	34	140	10	31	12
d3-Cocaine	44	148	10	33	11
Norcocaine	34	140	10	27	10.5
d3-Norcocaine	34	158	10	27	11
Benzoylecgonine	42	192	10	32	10
d3-Benzoylecgonine	36	161	10	32	11
Ecgonine Methyl Ester	34	134	10	35	10.5
d3-Ecgonine Methyl Ester	42	145	10	35	8

Table A1.1: Optimized compound-specific source parameters for electrospray ionization
Determined by Dr. Kathleen Noon, Biomedical Mass Spectrometry Facility.

Compound	Precursor Ion (*m/z*)	Product Ion (*m/z*)	Scan Period
Cocaine	304.2	182.2	Period 3
d3-Cocaine	307.2	185.2	Period 3
Norcocaine	290.2	168.2	Period 3
d3-Norcocaine	293.3	171.3	Period 3
Benzoylecgonine	290.2	168.2	Period 2
d3-Benzoylecgonine	293.3	171.2	Period 2
Ecgonine Methyl Ester	200.1	82.0	Period 1
d3-Ecgonine Methyl Ester	203.2	85.0	Period 1

Table A1.2: Precursor-to-product ions pairs for SRM transitions Determined by Dr. Kathleen Noon, Biomedical Mass Spectrometry Facility.

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January 7, 2011

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