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Analysis of soy natural products and esterase enzymes for drug development

Crystal A. Jurkiewicz
The University of Toledo

A Thesis

entitled

Analysis of soy natural products and esterase enzymes for drug development

by

Crystal A. Jurkiewicz

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the Master of Science degree in Pharmacology and Toxicology

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An Abstract of

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Two broad subjects of the drug development process are covered in this thesis, because of this the first chapter gives needed background information in order to fully understand the projects presented in the next two chapters. The first chapter deals with identification of a source for scale-up production of an active pharmaceutical ingredient (API) for possible future investigational new drug submission. Glyceollin's (GLY's) or phytoestrogens that are made in soy plants in response to stress, have been implicated in the treatment for breast and prostate cancer. In order to determine relative amounts of the GLY's in the plants, we have undertaken several studies. Firstly, we have performed preliminary tests on extracts from various parts of the plant (roots, stems, leaf, seed and seed pod) to determine the amounts of GLY produced. It has been demonstrated that most of the GLY is generally found in the roots as this is where the cyst root nematode (CRN) attacks the plant. Decreasing amounts of GLY are found further away from the roots>>stem>>leaf>>seed pod>>seeds. Extraction of the GLY's from the roots of CRN infected soybean plants is a viable source for the anti-estrogenic GLY I (-).

Lastly, we investigated the possibility that the soybean plant stores glycosylated

forms of the GLY's for use when the plant is attacked or stressed. Extractions taken from the roots of the soybean plant were incubated with the enzyme β-glucosidase. The results showed no evidance of glycosylated forms of the GLY's present in the roots of the plant.

The second chapter shows the development of a screening assay that could be used to help identify or narrow the list of lead candidates for pre-clinical drug testing. Carboxylesterase (CES) is a drug metabolizing enzyme present predominatly in the liver. It is involved in not only the biotransformation of API's for purposes of excretion, but also largly associated with the activation of prodrugs. A spectrophotometric assay was designed to measure the kinetic parameters of CES with the intensily studied and characterized drug procaine. This assay is intended to be used to identify activity of this enzyme with new drug products, along with characterizing it's pharmacokinetic behavior using comparisons of Km and Vmax. Verification of the most ideal conditions to test the rate of conversion were conducted, proving a pH of 7.4 and a temperature of 37°C to be optimal. These conditions were used to measure the Km and Vmax of procaine with the CES enzyme.

Acknowledgments

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Chapter 1-General Comments

Drug design and development are key aspects of the overall process of pharmaceutical research during which possible therapeutic agents are identified and characterized by their pharmacodynamic behavior. Designing a new drug entity falls under the discipline of medicinal chemistry, which includes developing novel chemical structures that show biological function, or studying existing drugs for a new therapeutic use. This stage of the initial process produces a large list of possible drug candidates, all of which need to be screened for an intended use. Screening assays are developed in order to narrow this large list to one or two lead compounds for further development. Targeted, robust screening assays are important at this stage in order to make the most informed decision on which drug candidate has the most potential for successfully fulfilling the intended therapeutic need and making its way through the regulatory drug approval process. This thesis investigates two aspects of the drug development process: the first being characterization of a lead drug candidate and its potential natural sources for agent production; and the second in developing a screening assay that could be used to better understand the pharmacokinetic behavior of a new drug candidate.

The medicinal value of plants has been recognized by nearly every culture on every continent around the world. Since the beginning of recorded history and into the nineteenth century, bioactive natural products, particularly those originating from a plant species, provided the main source of medicines [Sneader, 2001]. Synthetic chemistry

developed rapidly in the second half of the nineteenth century giving scientists a new approach to developing and understanding the medicinal properties of compounds constructed entirely in the lab. In recent years, however, drug discovery work has been combining these two efforts by identifying lead agents in plants and then synthetically making changes to the compound to boost its therapeutic effect [Historical Background, (n.d.)]. Natural products still play a major role in drug treatment, as over 50% of the most-prescribed drugs in the US had a natural product either as the drug, or as the model in the synthesis or design of the drug [Grifo, et al., 1997]. For example Taxol, an anti-cancer drug, was derived from the Pacific Yew tree. Cocaine from coca plants served as a model for a vast number of local anaesthetics, such as procaine, with the marketed name Novacain [Fakim, 2006].

In the first chapter of this thesis an investigation into a soy-derived natural product called glyceollin is presented. Glyceollin is part of a family of compounds known as isoflavones. Soy isoflavones first started being studied when an epidemiological study of diet and disease showed that women in Japan, who have a diet high in soy, had a lower incidence of cardiovascular disease, hormone dependent cancers, and menopausal symptoms than women in Western societies [Schmid, 2002]. This study sparked interest in identifying which components of the soy plant were responsible for these findings. Many of the isoflavones are phytoestrogens due to the similarity in their chemical structure to that of the human hormone estrogen. Because of this relationship, the isoflavones have been thought to be the reason for the findings in the epidemiological study related to hormone dependent cancers. Glyceollins are different from other soy isoflavones in that they are produced in the soybean plant in response to plant pathogens

and related stress factors. In 2006 Salvo et al. identified the glyceollins as having activity against breast and ovarian cancer, and in 2009 Payton-Stewart showed some promising results with prostate cancer [Salvo et al., 2006; Payton-Stewart, Schoene, et al., 2009]. For the past few years a considerable amount of work has been done in finding a source for this material. An investigation into the amount of the glyceollins produced in the soybean plant is considered in Chapter 1.

Billions of dollars are spent in the US each year on drugs that never make it to the market because of the lack of ability to identify potential issues with the drug once it is given to the human population. If there were a way to better understand these possible issues in the early stages of development, the quality of the drugs would improve. There are many different questions that need to be answered about the disposition of the compound as it travels through the body to its intended target. The FDA uses the acronym ADMET, which stands for Absorption, Distribution, Metabolism, Excretion, and Toxicity to describe how a drug interacts with the body. Absorption describes how a drug gets into the blood stream, such as through the intestines from swallowing a pill, through the mucus membranes in the mouth, or through the skin from topical application. Once a drug is in the blood it has the potential to distribute into tissues or organs, get metabolized by biotransformation enzymes, and eventually excreted in the urine or feces. Toxicity can be associated with the drug itself or its metabolites. A clear understanding of all of these properties is important in the development of a new drug.

During drug design and development, identifying a compound to investigate for possible disease fighting properties is only the first step towards marketing that compound for human use. The road a compound has to take before even being able to

begin testing within human subjects is long, expensive, and nine-out-of-ten times a dead-end. The second chapter of this thesis addresses the need for assays that could help streamline early drug development. Because most of all marketed drugs undergo some form of metabolism once they are in the body, understanding which enzyme or enzymes are responsible for this is an important part of early drug development. An *in vitro* assay was designed in order to determine and characterize the possible contribution of one of the drug metabolizing enzymes that is common for ester-containing drug molecules, namely carboxylesterase (CES). This enzyme is responsible for the hydrolysis of short chain aliphatic or aromatic esters. It plays an important role in the overall metabolism of many marketed pharmaceuticals. Identifying this enzyme's function in the metabolism of a new drug entity would be useful not only in ascertaining possible metabolites of the drug, but also as a tool in designing *in vivo* studies. For example if it were possible to relate the enzymatic activity of CES with a new drug compound and compare it to a drug with a known profile, this could help to establish how to design the initial *in vivo* testing protocol.

Chapter 2-Isolation of Glyceollin in Soybean Plant Extracts

2.1 Introduction

Phytochemicals are natural chemicals derived from plants. Many of these natural products have been extensively studied because of their purported protective and disease preventative properties. Examples of beneficial phytochemicals are lycopene found in foods such as tomatoes and carrots, isoflavones from soy and red clover plants, and flavanoids found most commonly in citrus fruit such as grapefruit [Home, (n.d.)]. Isoflavones are often referred to as phytoestrogens or plant hormones because they commonly resemble the human hormone estrogen. This structural similarity enables them to mimic the behavior of estrogen in certain tissues in the body, which is thought to contribute to the health benefits of these compounds [Tilghmas, 1997]. Isoflavones are found at the highest concentration in the soybean plant, including two of the most common of these compounds, daidzein and genistein. Many studies have shown that isoflavones have significant potential to fight disease. Some of these health benefits arise from the fact that these compounds exhibit both estrogenic and anti-estrogenic effects, with the type of effect dependant on relative estrogen and isoflavone levels [Mueller, 2004]. The benefits seen from the estrogenic effects of these compounds include relief from menopausal symptoms and protection from osteoporosis [Isoflavones, 2010]. The anti-estrogenic effects of these compounds are related to their therapeutic activity against breast, ovarian and prostate cancers [Isoflavones, 2010].

Existing in the same biosynthetic pathway in the soybean plant as daidzein is another group of phytoestrogens, known as the glyceollins (GLYs) [Oliver, 2003]. The GLYs are produced when soybean plants become stressed by environmental factors or infections thought to represent part of the soybean plant's defense mechanism to stress, these types of compounds are also called „phytoalexins'. The GLYs were studied in the early 1970's in relation to their anti-fungal properties, which led to the identification of three isoforms of this compound, GLY I-III [Bhattacharyya and Ward, 1986, Burden and Baily, 1975]. The structures of GLY I-III are shown in figure 2.1. Reported relative amounts of these isoforms found in stressed soy plant tissue vary greatly, but most papers agree that the GLY I isoform predominates over the other two forms in the seeds and roots of the soy plant [Huang and Baker, 1991, Bhattacharyya and Ward, 1986].

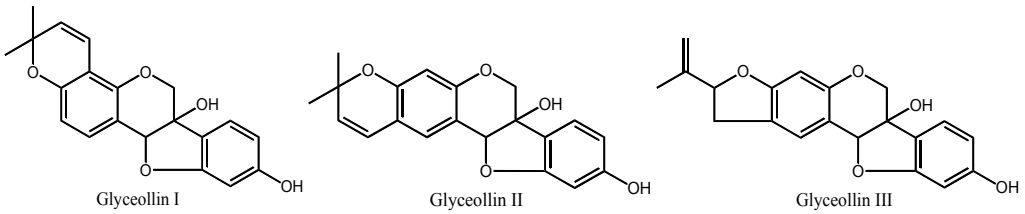

Figure 2.1. Chemical structures of glyceollin isoforms GLY I, II, and III.

While daidzein has been shown to exhibit estrogenic effects, the GLYs became compounds of interest due to their anti-estrogenic effects in relation to cancer [Burow, et al., 2001]. Studies by Wood et al. showed preliminary findings that the GLYs had possible estrogen modulating properties [Wood , et al., 2006]. Later that year Salvo et al. published a paper that not only backed-up those preliminary findings, but also showed that the GLYs inhibited estrogen-induced tumor progression in human breast and ovarian cancer cells [Salvo et al., 2006]. The estrogen modulating properties demonstrated by Wood et al., and later by Salvo et al. are important because they show that when these

compounds were tested with hormone-dependant cancer cell lines they did not display agonistic behavior with the estrogen receptor, which would cause tumor growth [Wood et al., 2006,Oliver et al., 2003]. However, when they were tested with these cell lines in the presence of 17β-estriodol, they displayed antagonistic behavior with the estrogen receptor, blocking the 17β-estridol from binding to the receptor and inducing tumor growth. These studies identified GLYs as a novel class of natural anti-estrogenic compounds, which could be used not only for the treatment but also potentially in the prevention of these types of cancers. All of these studies were conducted with a mixture of the three glyceollin isoforms, extracted from soybean seeds stressed by exposure to *Aspergillus sojae*, a fungal infection that serves as an established elicitor in the production of GLYs [Boue et al., 2000]. Studies conducted later identified GLY I as having the most potent anti-estrogenic effects of these three isoforms, and it has further been shown that the GLY I(-) enantiomer is the only chiral form expressed in the soybean plant [Payton-Stewart and Khupse, et al., 2010].

Breast cancer is the second most common form of cancer and the second leading cause of cancer deaths in American woman, while ovarian cancer is the ninth most common form, but the fifth leading cause of death [Breast-Ovarian, (n.d.)]. The high mortality rate associated with these cancers demonstrates a need for improvement in the way these diseases are being treated. The studies conducted with GLYs show that these compounds could be a step forward in the treatment options available for these types of cancers.

In establishing an interest in developing these compounds for further testing, it became necessary to identify a more plentiful source of this material to allow broadened experimental evaluation and as a means of scaling-up production for eventual use in humans.

The cyst root nematode (CRN) is a soil born parasite that attacks the roots of soybean plants. Infection of the soybean plant by CRNs is an environmental stress very common to soy plants grown in the Midwest United States. As a response to this environmental stress, the soy plant produces GLYs to fight off the infection [Gram, et al., 1991]. This presented a unique opportunity for identifying a natural source of these compounds from soy plants grown in Ohio fields, as described in the first section of this chapter. The North Central Agricultural Research Station (NCARS) located in Fremont, Ohio and operated by Ohio State University has been working on developing soy plant strains that are resistant to infection by the CRN. In order to investigate these plants as a source of the GLYs, our labs have collected several samples. Soy plants grown in CRN infected NCARS test fields from several different seed types, or cultivars, were collected and separated into their component parts, including roots, stems, leaves, pods, and seeds. After drying, samples from each of these plant parts were extracted into methanol and analyzed for total GLY levels. The results of these studies show the relative levels of total GLY in different plant tissues and indicate which plant parts would be most useful as a natural source for GLYs.

An important part of this chapter describes further testing performed to characterize the level of glycosylation of GLYs derived from soy plant materials. Many plant products, including the isoflavones daidzein, genistein and glycitein in soy plants, are

present in plant tissue as both glycosides and aglycones (figure 2.2). Glycolysis is directly involved in many biochemical adaptations of plant and non-plant species due to environmental stresses [Plaxton, et al., 1996]. It is thought that glycolysis of certain compounds in the plant is done for the purposes of storage of these compounds for use at a later time. For example, GLYs may be produced and stored for later release when the plant is exposed to stress conditions in which a chemical response is needed. The direct relationship between daidzein and the GLYs suggests a possibility that the GLYs are present in both of these forms in the plant. Without proper evaluation, the true amount of GLYs available in the soy plant materials may be underestimated. In order to determine whether glycosylated GLYs are present at significant levels in the soy plant, incubation studies with the enzyme β-glucosidase were performed [Matsuura and Obata, 1993, EC.3.1.1.1, (n.d.)]. β-Glucosidase is an enzyme that hydrolyzes the terminal, non-reducing β-D-glucosyl residue of a glycosylated plant molecule, releasing β-D-glucose and the aglycone form of the compound [Gram et al., 1991]. These incubation samples were run using a method developed to identify the total GLY content in the plant parts. If significant amounts of the glycosylated forms of these compounds are present in the extraction samples, an increased amount of total GLY will be measured after the enzyme treatment.

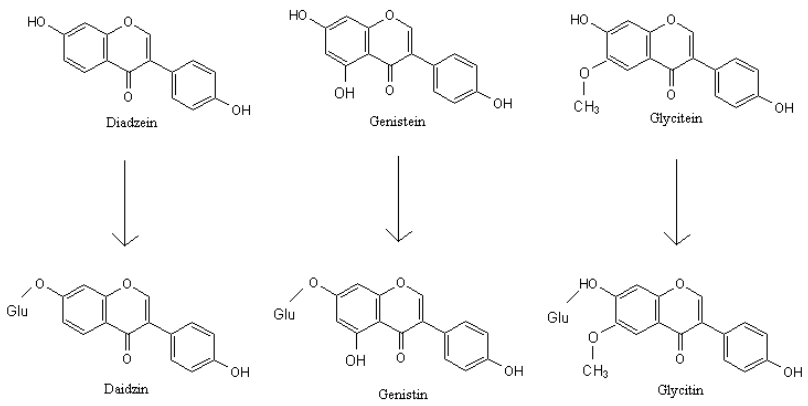

Figure 2.2. Common isoflavones present in the soybean plant as both glycosides and aglycones. Glu signifies incorporation (glycosylation) of a glucose molecule.

2.2 Materials and Methods

2.2.1 Analytical Instrumentation

An Alliance® HT HPLC (model 2795) equipped with a quaternary pump, a degasser, an autosampler, and a column oven from Waters Corporation (Milford, MA, USA) were used. Mass spectrometric analysis was performed using a Quattro Micro™ triple–quadrupole instrument from Micromass (Manchester,UK) equipped with an ESCi™ multi mode ionization source. MassLynx (version 4.01) software from Micromass was used for data acquisition and processing. All weighings were done on an XS205 analytical balance from Mettler Toledo (Columbus, OH, USA). Buffer pH was adjusted using an Accumet Excel XL15 pH meter from Cole-Parmer (Vernon Hills, IL, USA).

2.2.2 Reagents

HPLC grade methanol, formic acid, glacial acetic acid, sodium acetate and the enzyme β-glucosidase were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The water used in these experiments was purified using a Barnstead International

(Dubuque, IA, USA) water purification unit. The argon and nitrogen gas used on the mass spectrometer was supplied by Airgas (Ann Arbor, MI, USA). Glyceollin I (-), glyceollin I (racemic) and glyceollin II were received from our center's synthetic chemistry group at the University of Toledo (Toledo, OH, USA). Glyceollin I, II, III mixture isolated from natural sources was kindly provided by Steven Boue of the USDA Southern Regional Research Center (New Orleans, LA, USA).

2.2.3 HPLC Conditions

An LC-MS/MS isocratic method was used at a flow rate of 200 µl/min with an injection volume of 10 µl and a total run time of 3 minutes. The samples that were analyzed for the detection of total glyceollin from the extraction method were run through a Gemini C_{18} analytical column (2.00 mm×100 mm, 5µm) and a guard column (2.0 mm×10 mm, 5µm) purchased from Phenomenex (Torrance, CA, USA). The samples that were analyzed for the detection of total glyceollin, diadzein and diadzin from the enzyme assay were run through a Gemini C_{18} analytical column (2.00 mm×50 mm, 5µm) and a guard column (2.0 mm×10 mm, 5µm) purchased from Phenomenex (Torrance, CA, USA). The mobile phase was 20%-0.1% formic acid in water and 80%-0.1% formic acid in methanol. A 0.2% formic acid in methanol/water mixture (50:50, v/v) was used as the needle wash solvent; 0.1% formic acid in water was used as the purge solvent and 100% methanol was used as the seal wash solvent. The column and samples were kept at 35±5°C and 4±5°C, respectively.

2.2.4 Mass Spectrometric Conditions

MS detection was used with the electro-spray ionization (ESI) source in the positive mode using the following source settings. Nitrogen was used as the desolvation

and cone gas, and was set at a flow rate of 700 L/h and 25 L/h, respectively; the source and desolvation gas temperatures were 120°C and 350°C, respectively. The ESI source tip (capillary) voltage was 3.3 kV, extractor was 2V, ion energy for MS1 was 1.0V and for MS2 was 0.5V. The MS was operated in the MRM mode with argon gas used as the collision gas. The argon gas cell pressure was approximately 3.1×10−3 mbar. The dwell time for detection of each compound was 200 ms. Full-scan mass spectra of each compound was obtained by direct infusion into the ESI source at a flow rate of 5 µl/min. Protonated molecular ions (parent-ions) were produced and optimized using the first quadrupole analyzer. Collision-induced fragmentation was achieved using suitable collision energy to produce product-ions within the second quadrupole. The specific parameters for each compound are shown in Table 2.1.

Table 2.1. Compound Specific MS Conditions.

Compound	Detected ions	Parent-ions (m/z)	Product-ions (m/z)	Cone Voltage (V)	Collision Energy (V)
Glyceollins	$[M\text{-}H_2O]^+$	321	305	45	32
Daidzin	$[M+H]^+$	417	255	22	15
Daidzein	$[M+H]^+$	255	137	42	30

2.2.5 Processing Soybean Plants From the Field For Analysis

Soybean plants were collected from the North Central Agricultural Research Station research field located in Fremont, Ohio. Five different soybean cultivars were collected, all having a different cyst root nematode resistance package. The seed types with the resistance package in parentheses included Peking (SC9288), Gries (PI88788), L2620RX (CystX), S32-E2 (PI 188788), and S30-D4 (Susceptible). These plants were harvested 120 days post planting. All plants were immediately separated into their

component parts, including seeds, pods, stems, leaves, and roots, and placed in open containers. All samples were then allowed to air-dry at room temperature in a dark, low humidity environment for two weeks.

The component samples of each plant part were then cut into smaller pieces using scissors and/or a sharp blade. Roots and stems were sliced into 1-2 cm long segments, with thicker segments halved or quartered lengthwise. Leaves and seed pod casings were cut into pieces approximately 1cm x 1cm. Small seeds were sliced in half, while larger seeds were quartered. All samples were then air-dried at room temperature for another 2 weeks.

At the end of this second drying period, each component plant part was processed in a Krups model F203 grinder for 2-3 minutes to give a relatively homogeneous particle size. All samples were then air-dried at room temperature for another 2 weeks. The materials were then screened through a #25 sieve into Petri dishes. Coarse materials collected on the sieve for each sample were processed for an additional 2-3 min with the Krups grinder, and re-sieved into the corresponding Petri dish. Each of these samples was then dried a final time for 3 days at 50°C.

At the end of this final drying process, the dried materials from each sample were screened through a #25 sieve onto a clean sheet of paper. The resulting powder was transferred into an amber glass vial, and sealed tightly with a Teflon-lined lid. Each sample was then assigned a unique sample ID number and stored at room temperature for subsequent extraction and chromatographic analysis. Once all of the samples were died, a total of 25 samples were ready for analysis. This included 5 different plant parts, from 5 different cultivars.

2.2.6 Total GLY Extraction From Soy Plant Parts

Approximately 100 mg of dry plant powder was placed into a 25 ml round-bottom flask. The exact amount weighed into each flask was recorded. Approximately 10 ml of HPLC grade methanol was added to the dry plant powder in the 25 ml round-bottom flask. The flask was then placed in a heating mantle with a water-cooled glass condenser attached to the opening of the flask to stop methanol evaporation. The sample was then refluxed at 78°C for 2 hours [Boue et al., 2000].

The methanol was collected and an additional 10 ml of fresh methanol was added to the powder and again heated to reflux for 2 hours. This process was repeated a third time and all three extractions were then combined. The vials that these three extractions were combined in were weighed before the samples were combined and then again after mixing. These weighings were used to calculate the exact volume of methanol used for extraction, by dividing the mass difference by the density of methanol, which is 0.7918 g/ml.

The sample was then filtered with a Titan syringe filter consisting of a glass wool prefilter and a 0.45 μm final filter. 200 μl of each sample was put into a 0.3 ml HPLC micro vial for LC-MS-MS analysis using the LC-MS/MS conditions described above to measure total glyceollins. The remaining extraction sample was stored at 4°C for future testing.

A calibration curve with two quality control (QC) samples of GLY I were made and run with each set of samples obtained from the extraction process. Two stock solutions of GLY I were prepared in methanol at 1.00 mg/ml, one stock used to prepare the standards while the other was used to prepare the QC samples. The calibration curve

prepared for GLY I was from 1-10,000 ng/ml, and the three QC samples were 2, 300 and 7000 ng/ml.

2.2.7 Incubation Studies With β-Glucosidase

The positive control for the experiment with β-glucosidase was daidzin, a known substrate for β-glucosidase which converts daidzin to daidzein [Matsuura and Obata, 1993]. Diadzin is known to be produced in the same stress-induced biosynthetic pathway as glyceollin in the soy plant [Matsuura and Obata, 1993]. Preliminary tests on extracts from soy plant roots showed the presence of both daidzin and daidzein at concentrations sufficient to serve as an endogenous positive control to monitor the activity of the β-glucosidase enzyme.

Four milliliters of the methanolic extract described in the previous section was dried completely on a rotary evaporator and then resuspended in 8.1 ml of 100 mM sodium acetate buffer (pH 5) and 180 µl was aliquoted into microcentrifuge tubes. These tubes were incubated for 10 min at 37°C in a water bath shaking incubator. 20 µl of enzyme was added to the reaction samples while 20 µl of water was added to the control samples. Enzyme activity was stopped at various time points by addition of 800 µl of methanol. The samples were then centrifuged at 10,000 rpm for 5 minutes and 200 µl of the sample was transferred to a 0.3 ml HPLC micro vial for LC-MS/MS analysis using the instrument conditions described for glyceollin, daidzein and daidzin. Preliminary testing showed that the conversion of daidzein to daidzin was very quick and completed in less than 15 min. Therefore sample time points for this assay were chosen at 0, 2, 4, 6, 10, and 15 minutes.

2.3 Results

2.3.1 Total GLY Extraction From Soy Plant Parts

A representative chromatogram using the detection method described for total glyceollin is shown in figure 2.3.

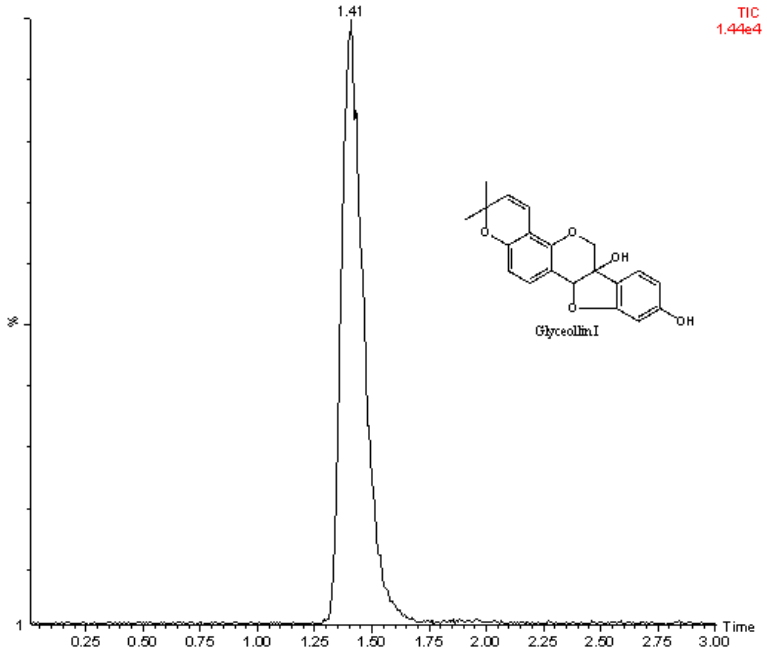

Figure 2.3. A representative chromatogram for the detection of total glyceollin content.

A representative chromatogram for the detection of total glyceollin, daidzein, and daidzin is shown in figure 2.4. All samples were analyzed by averaging triplicate injections.

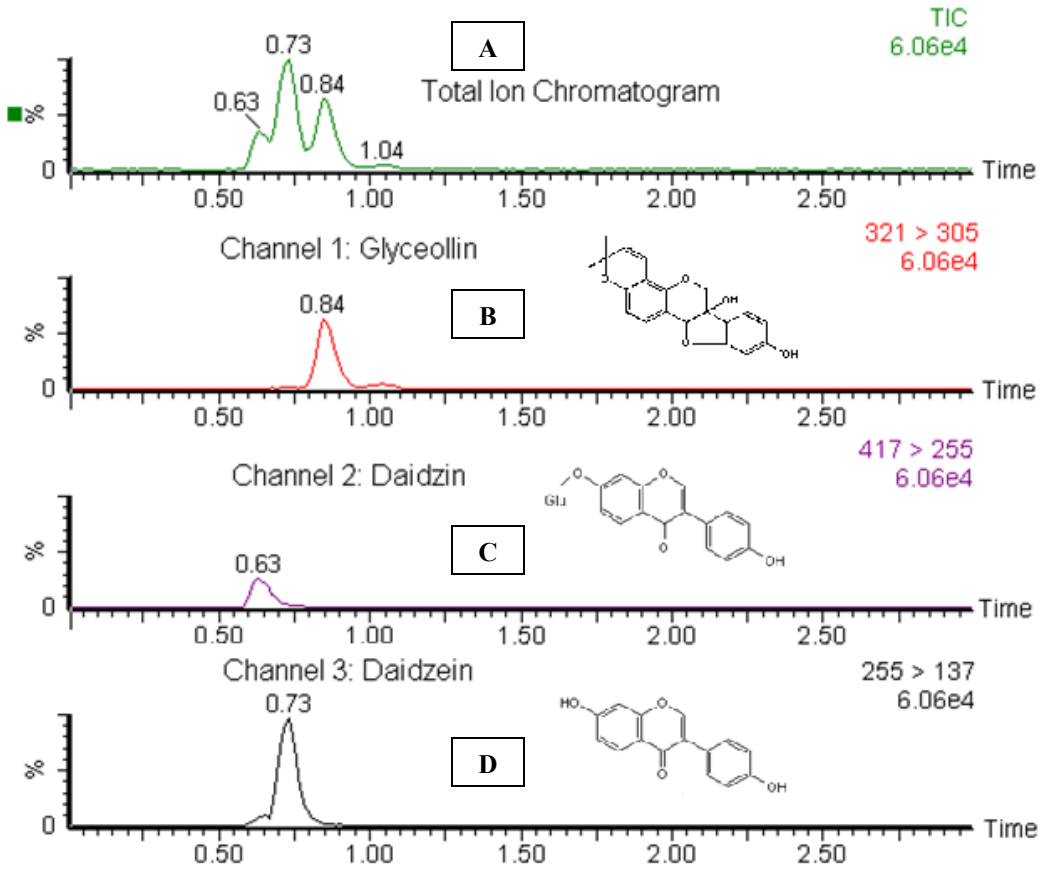

Figure 2.4. A. The total ion chromatogram which represents the sum of channels 1, 2 and 3.
B. Channel 1 represents the transition from m/z 321 to 305 for glyceollin.
C. Channel 2 represents the transition from m/z 417 to 255 for daidzin.
D. Channel 3 represents the transition from m/z 255 to 137 for daidzein.

As GLY I, II and III all share the same molecular weight, along with having a very similar chemical structure, chromatographic separation of these three compounds proved to be difficult. Since it has been shown previously that the extraction method will completely extract all three of these compounds from the plant, identifying what ratio of these compounds was being seen in the LC-MS/MS detection method also needed to be further addressed. The method that is described in this chapter for the detection of GLY was developed using pure GLY I. A sample of pure GLY II was obtained and was infused on the mass spectrometer. GLY II proved to behave the same as GLY I by

sharing the same ionization fingerprint. They not only both lose a water molecule during the ionization process and pick up hydrogen, but they also fragment to an ion with m/z of 305 in the collision cell. Calibration curves of these two compounds were made to compare how similar their detection was using the same method with LC-MS/MS to detect both. The slope for the curve for GLY II was greater than for GLY I. This is shown in figure 2.5. The differences in the calibration curves were attributed to the efficiency with which the two different compounds ionize when injected into the mass spectrometer. It was theorized that GLY III would exhibit similar behavior. Because of differences in ionization efficiencies for GLY I, II, and III, the detection of total GLY in this method should be considered semi-quantitative.

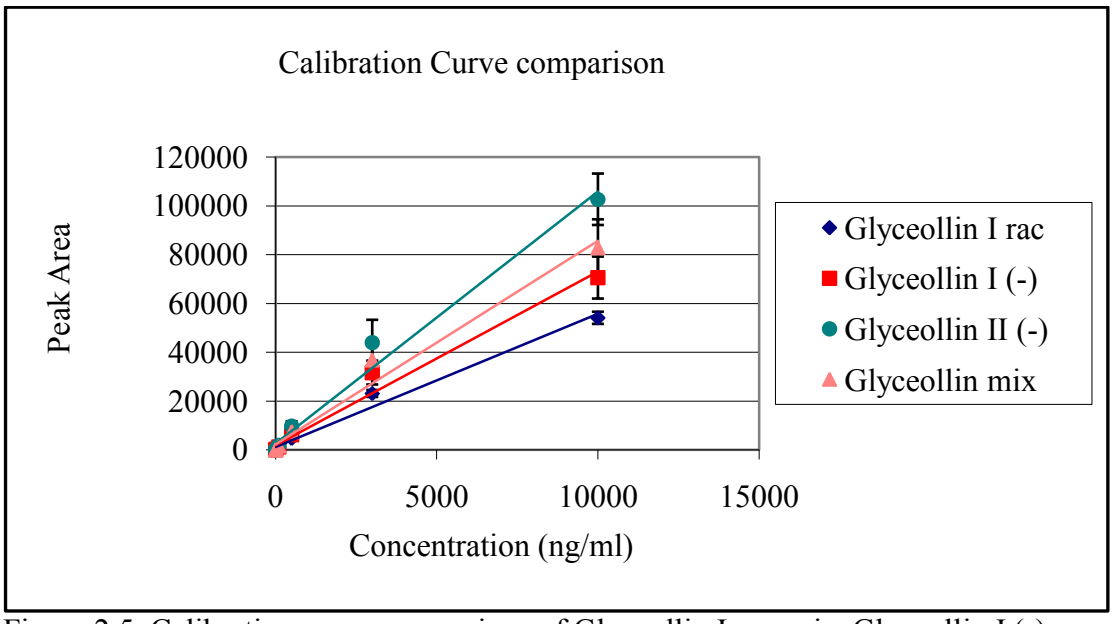

Figure 2.5. Calibration curve comparison of Glyceollin I racemic, Glyceollin I (-), Glyceollin II (-), and a mixture of Glyceollin I, II, and III.

The response for the total GLYs was found to be linear from 1-10,000 ng/ml with a correlation greater than 0.99. Amounts of GLY found in each plant part were calculated using log-log (Power equation) regression of the calibration curve. Figure 2.6 shows a

representative calibration curve for total glyceollin and Table 2.2 shows the statistical results for the calibration curve and the QC samples.

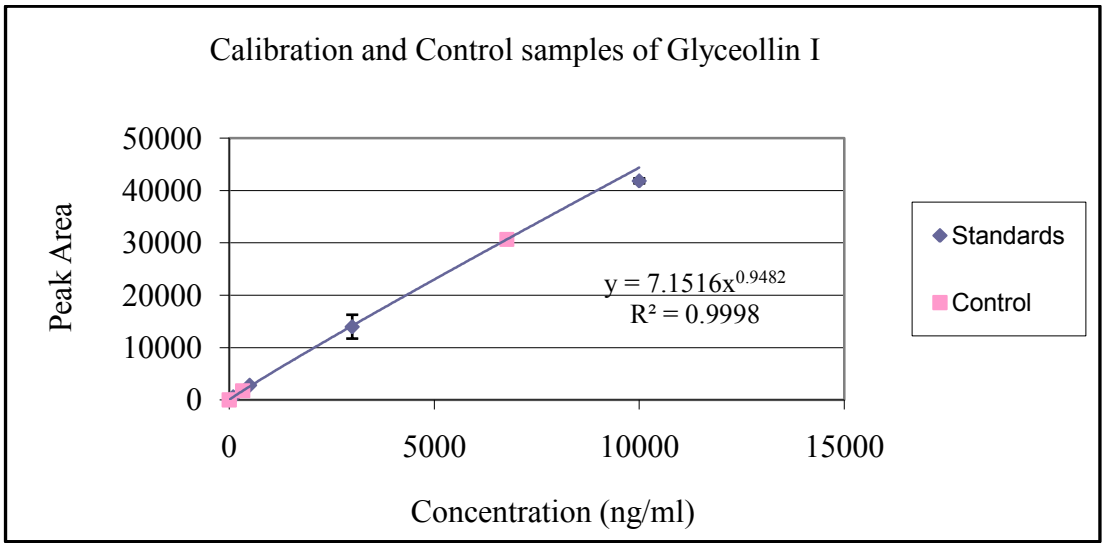

Figure 2.6. Representative calibration curve for total glyceollin.

Table 2.2. Calibration Curve and QC Results.

	Concentration (ng/ml)	Peak Area	std dev	calculated conc. (ng/ml)	% CV	% RE
Standards	1	6.97	0.73	0.97	10.45%	-2.68%
	5	32.29	0.83	4.90	2.58%	-1.96%
	25	149.61	3.06	24.70	2.05%	-1.20%
	100	591.45	23.55	105.26	3.98%	5.26%
	500	2808.85	68.18	544.32	2.43%	8.86%
	3,000	13973.26	2277.04	2955.93	16.30%	-1.47%
	10,000	41828.66	470.67	9394.79	1.13%	-6.05%
QC	2	17.01	1.88	2.49	11.03%	24.71%
	300	1743.78	51.11	329.24	2.93%	9.75%
	7,000	30656.21	281.52	6769.52	0.92%	-3.29%

Using a Power equation calibration curve to predict the concentrations in the standards produces a relative error of less than 10%. The calibration curve predicts the concentrations of the QC samples with less than 10% error at the mid and high level, but the low QC has a relative error larger than 20%. This shows that there would be a large

amount of error associated with the extraction samples that had a concentration of 2 ng/ml or less. For the purposes of this study, this larger error at the low end of the curve will not significantly affect the determination of relative GLY levels in different plant parts.

Using the calibration curve from the same run, the concentration of total GLY was calculated in each sample. Equations 2.1 and 2.2 were used to calculate the amount of total GLY found per 100 mg of each plant part. A summary of these results are shown in Table 2.3.

$$ng\ of\ total\ GLY = (\text{Calc. Conc. of total gly}) \times (\text{Calc volume of MeOH}) \quad \text{(eq. 2.1)}$$

$$\frac{ng\ of\ total\ gly}{100\ mg\ of\ plant\ material} = ng\ of\ total\ gly \times \frac{100}{recorded\ weight\ of\ plant\ material} \quad \text{(eq. 2.2)}$$

Table 2.3. Amount of Total Glyceollin Found in 100 mg of Dry Plant Material.

Seed Type (resistance package)	Seeds (ng)	Stems (ng)	Leaves (ng)	Pods (ng)	Roots (ng)
Peking (SC9288)	207 ± 22	14,084 ± 1,695	7,057 ± 1,069	8,739 ± 2,167	73,557 ± 3,426
Gries (PI88788)	178 ± 5	5,969 ± 492	10,729 ± 1,361	13,125 ± 1,900	57,734 ± 5,802
L2620RX (CystX)	21 ± 4	21,277 ± 1,909	9,380 ± 50	4,714 ± 516	56,233 ± 7,764
S32-E2 (PI88788)	29 ± 2	17,245 ± 471	3,806 ± 174	12,970 ± 1,330	44,481 ± 6,443
S30-D4 (Susceptible)	165 ± 15	10,309 ± 509	7,676 ± 400	6,175 ± 913	64,394 ± 7,775

Figure 2.7 shows a comparison of the total amount of the GLYs found in each of the different seed types. This graph shows that the amount of total GLY produced by the plant is generally the same regardless of the resistance of the strain to the CRN infection.

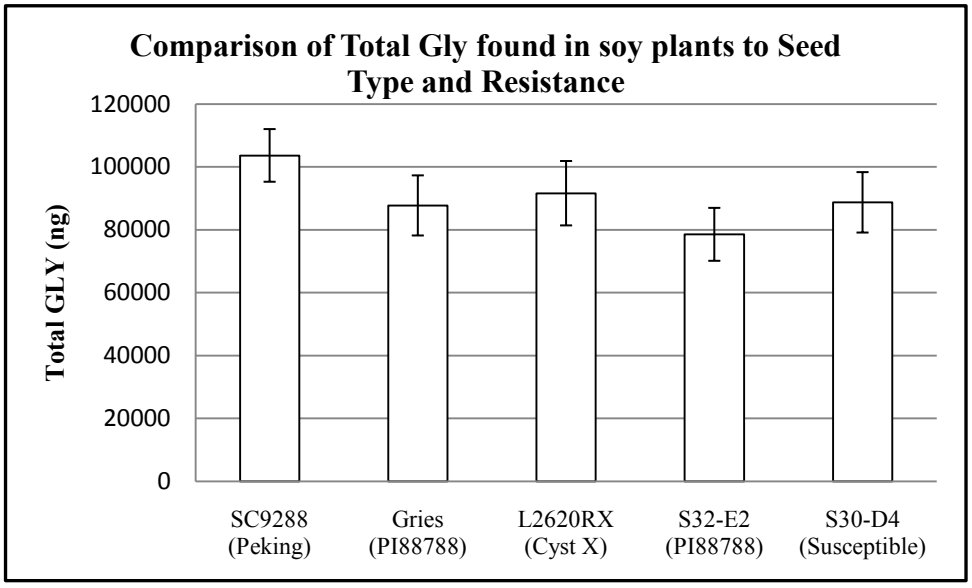

Figure 2.7. Total GLY accumulation in ng/100 mg of soybean plant material from several different cultivars harvested from CRN infected research fields.

A paper published in 1991 by Huang and Baker reported the amount of GLY I found in the roots of soy plants infected with CRN under the controlled conditions of a laboratory [Huang and Baker, 1991]. Extraction of total GLY was conducted using a method similar to the one described in this Chapter, and detection of the three GLY isomers was achieved using an HPLC based separation method. Total GLY was extracted from the roots of the soy plants 2, 4, 6, and 8 days post penetration of the CRN. The seed types used for these experiments were a resistant cultivar, Centennial and a susceptible cultivar, Ransom. The amount of GLY I found in these plants is shown in Table 2.4. GLY II was not found to be present in any of the extracts, and GLY III was found at low levels only in the Centennial seed type, 8 days post penetration.

Table 2.4. Amount of GLY I Extracted From the Roots of Soy Plants on Different Days Post CRN Penetration [Haung and Baker, 1991].

	2 days	4 days	6 days	8 days
Ransom	5.5	7.5	7	6.5
Centennial	12	19	23	16

Amount is μg of GLY I/gram root material

What these findings seem to indicate is that once the CRN penetrates the roots, GLY I production increases for a period of time and then begins to decrease. This could mean that there is a period of time post CRN penetration during which the plant is producing the maximum amount of GLY I. The studies conducted in this Chapter harvested plants 120 days post planting, or well after CRN penetration, which means there may be a potential for extracting a larger amount of GLY I from plants harvested earlier in the growing season. A new set of studies are being planned to look at the amount of GLY I present in plants harvested in the early, mid and late parts of the growing season.

2.3.2 Incubation Studies With β-Glucosidase

Table 2.5 shows the initial peak areas for all three compounds during the enzyme incubation study. These peak areas were calculated with a 95% confidence interval by averaging the peak areas of the three individual tests that were performed on the root extract samples.

Table 2.5. Initial Peak Areas of Glyceollins, Daidzin, and Daidzein For Each of the Samples Used For Incubation Testing, Represented By Mean ± 95% CI.

Sample	Glyceollins Peak Area	Diadzin Peak Area	Daidzein Peak Area
1	2658 ± 93	5479 ± 234	3175 ± 83
2	2658 ± 227	7811 ± 460	5986 ± 144
3	3363 ± 97	3509 ± 415	2989 ± 321

Statistical analyses were performed by two-way ANOVA, with $p < 0.05$ used as the criteria for significant differences. As seen in the graphs in figure 2.8, none of the incubations exhibited statistically significant differences versus initial peak area without added enzyme. Daidzin and daidzein peak areas were significantly different than initial peak areas for all incubation times with added enzyme, with daidzin peak areas decreasing and daidzein peak areas increasing as the enzyme converted glycosylated daidzin to aglycone daidzein. Daidzin and daidzein peak areas with enzyme were also significantly different than the corresponding incubation samples without enzyme at all tested incubation times. Incubation of glyceollin with enzyme exhibited no significant differences in peak areas versus the initial peak area or versus the corresponding incubation without enzyme.

Based on a POWER analysis using the measured average glyceollin peak area coefficient of variation (CV%) of 2.3%, these tests should have been able to detect an enzyme-induced change in glyceollin peak area with $p < 0.05$ at a POWER of 0.800 if the level of glycosylated glyceollins was 4.8% or higher. Thus, these results strongly indicate that, if present at all, the levels of glycosylated glyceollins are less than 5% in these soy extract samples.

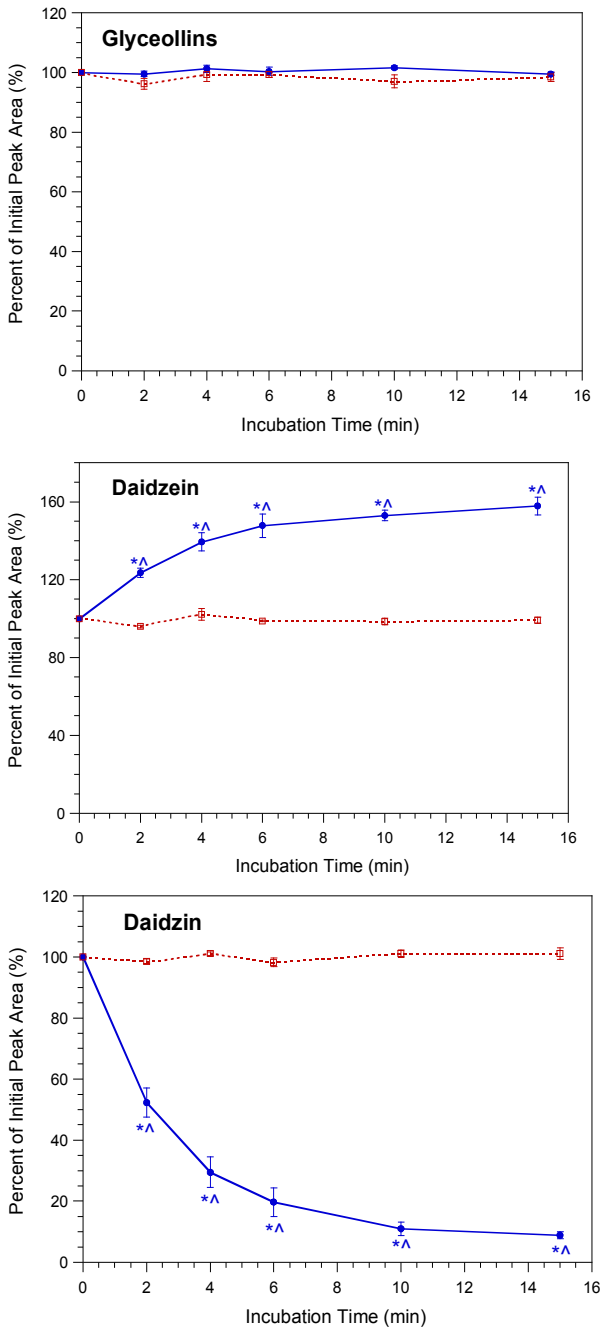

Figure 2.8. Percent of initial peak areas for the glyceollins in soy extract samples incubated at 37°C with β-glycosidase (solid circles, solid line) and without enzyme (open squares, dashed line). * indicates a significant difference ($p < 0.05$) versus corresponding initial peak area, while ^ indicates significant difference ($p < 0.005$) versus corresponding peak area without enzyme.

2.4 Conclusion

Based on the results of the extraction of GLY from the different plant parts, it is clear that the roots of the plant after attack by CRNs provide the most concentrated source of GLY I. From an agricultural stand point this finding is most ideal in that the roots of the soybean plant are left in the field after harvest by the farmer. If a use for this part of the plant is identified, it would make the soybean plant a much more lucrative crop, especially for farm fields in the Midwest United States which are plagued by the presence of the cyst root nematode found in the soil in this part of the country.

While these experiments provide some early indication about the possibility of attaining GLY I (-) after harvest of soybean plant parts, additional work is needed to more fully define the usefulness of this source for future scale-up production of GLYs. Efforts are underway to develop a better detection method in order to distinguish between GLY I, II and III. Once that method is developed, it will provide a way of distinguishing how much of the total GLY detected is actually GLY I (-).

A study of the amount of GLY I (-) present in plants harvested at different times during the growing season could indicate the potential for extracting a larger amount of total GLY than what was presented here.

The next step would then be to identify a method of isolating and purifying these three compounds from the mixture of molecules present in the source material. Once that is accomplished, larger-scale production and further testing the anti-cancer effects of GLY I (-) could begin.

The results of the β-hydrolase incubation studies done with the soy extract samples answer an important question. They show that it is unlikely that the GLYs are

stored in a glycosylated form in the plant. This makes our measurement of the amount of total GLY that could be extracted from the roots of the plant that much more meaningful. The potential for finding glycosylated GLY still exists in the extractions taken from other parts of the plant. In order verify completely that no glycosylated forms of the glyceollins are present, the extractions taken from the other parts of the plant would need to be testing using the incubation study conducted here.

Chapter 3-Development of a Screening Assay For Analyzing Carboxylesterase Activity

3.1 Introduction

According to the FDA draft guidance on drug interaction studies, an adequate assessment of the safety and effectiveness of a drug includes a description of its metabolism and the contribution of metabolism to overall elimination [Food and Drug, 2006]. The function of the metabolism (or biotransformation) of a xenobiotic agent such as a drug is to change the chemical structure of the compound so it can be more readily excreted from the body. Metabolites are typically less active than their precursor forms. For some drugs, however, metabolites can be the primary therapeutic form, with the starting drug having little or no beneficial activity without biotransformation [Sanghani, et al., 2003]. Drug agents that utilize this type of bioactivation are known as prodrugs. Medicinal chemists design some molecules to utilize this prodrug bioactivation process in order to overcome problems associated with direct delivery of the active form, such as making a prodrug that is more readily absorbed from the intestines to improve the bioavailability [Erhardt, et al., 2010]. Once the drug is in the body, the prodrug is converted into the therapeutic chemical structure by drug metabolizing enzymes. Note that for both drugs and prodrugs, some biotransformation processes can also change the chemical structure of the compound into an undesired inactive or even toxic metabolite.

It is common for a doctor to prescribe more than one drug for a patient at the same time while in treatment. Because of this frequent practice, an understanding of how

two or more drugs will interact in the body is needed to avoid loss of efficacy or toxic exposure for one or the other drug. If two drugs are given concomitantly and share the same elimination pathway, one might compete with the other during the elimination process causing one or both of the drugs to deviate from their normal pharmacokinetic profile [Quinney, et al., 2005]. This could result in the drug concentration reaching toxic levels that cause undesired side effects including possible death of the patient, or the drug not achieving the concentration needed to elicit its therapeutic effect. Identifying the enzyme or enzymes involved in the metabolism of a drug can help to not only characterize the elimination pathway of that drug, but also in identifying metabolites that have the potential for causing toxic effects or additional drug interactions.

Drug metabolizing enzymes are a broad group of proteins that are typically expressed at the highest levels in the liver, and are involved in the clearance of not only xenobiotics including drugs and environmental pollutants, but also endogenous compounds such as steroids and fatty acids [Guengerich, 1995]. The enzymes involved in the metabolism of drugs belong to one of two groups, phase I or phase II enzymes. These categories describe the type of biotransformation reaction the enzyme performs on substrates. Phase I enzymes use functionalization reactions, which include oxidation, reduction, or hydrolysis [EC.3.1.1.1, (n.d.)]. Phase II enzymes use biosynthetic reactions which are conjugation reactions [EC.3.1.1.1, (n.d.)].

This chapter focuses on the phase I enzyme carboxylesterase (CES), which is a hydrolytic enzyme present in many tissues throughout the body, but predominately in the liver [Satoh and Hosokawa, 1998]. It is responsible for the metabolism of a vast number of structurally diverse drugs that contain an ester or amide bond [Ross and Boraziani,

2007]. Although five different forms of this enzyme are known to be expressed in human liver, drug metabolism is largely associated with only two of these isozymes, namely carboxlyesterase I (CES1) and carboxylesterase II (CES2).

The conditions in which the activity of an enzyme is measured directly affect the performance of the enzyme. During *in vitro* assays these conditions include the temperature at which the reaction is maintained and the pH of the reaction mixture.

Enzymes typically work best within a pH range between 6 to 8 [Campbell, 1996]. If the environment is too acidic or too alkaline, the enzyme can shift to a less active conformation or it might be less able to produce intermediates. It could also begin to denature. The pH can also have an effect on the compounds present in the reaction mixture that is used to measure the activity of the enzyme. A balance between enzyme activity and compound stability is important when measuring the activity of the enzyme.

Temperature is another important factor to consider. In general as the temperature of the reaction mixture increases the activity of the enzyme increases. However if the temperature becomes too high, the enzyme may change conformation or begin to denature, at which point the enzymatic reaction rate can drop sharply. Biological enzymes generally have an optimal temperature at which the reaction rate is the fastest, typically between 35°C and 40°C for mammalian enzymes [Campbell, 1996].

The intention of the studies conducted in this chapter was to identify an optimized, simple, robust and reproducible *in vitro* assay for characterizing the activity of the CES enzyme with new drug products in the non-clinical phase of development.

To begin selecting the study conditions for the assay, some preliminary work was conducted in order to identify known substrates of the CES enzyme and to determine

proper storage conditions for the enzyme to prevent or minimize loss of activity. The substrates chosen for the studies conducted here included 4-methylumbelliferyl acetate (4-MBA) for the optimization of the buffer pH. It was discovered during preliminary testing that this substrate was the only one soluble across the range of pH tested. For assays in which the temperature of the reaction was optimized, p-nitrophenyl acetate was chosen based on its solubility at both room temperature and at 37°C. Once these assay conditions were optimized the substrate procaine was used in order to characterize the enzyme kinetics of CES.

Procaine is a local anesthetic drug with the trade name Novocain ®, which has been on the market for many years. Because this drug has been around for so long, a vast number of studies have been conducted identifying many aspects of its pharmacokinetic profile. The amount of information available about procaine made it the drug of choice for this assay in an attempt to add another important level of standardized utility, namely direct comparison to actual clinical results Once the kinetic properties of procaine were measured using this assay, they could then be used as a basis of comparison when this assay is performed with a new drug product and, in turn potentially better extrapolated to predicted clinical behavior.

The kinetic parameters measured when trying to characterize the activity of an enzyme with a substrate are Km and Vmax. Km is a measure of the affinity of a substrate for an enzyme and Vmax refers to the maximum rate at which the enzyme converts the substrate to its product. These two parameters define the kinetic behavior of an enzyme as a function of substrate concentration [S]. This relationship is defined by Michaelis-Menten kinetics shown in equation 3.1.

$$V = \frac{Vmax \times [S]}{Km + [S]} \quad \text{(eq. 3.1)}$$

Measurements of these values are calculated by incubating different concentrations of substrate with enzyme and measuring the rate at which this reaction takes place. The results are then plotted as reaction rate versus substrate concentration. This plot takes the form of a hyperbolic curve. The mathematics involved in trying to define a relationship between points presented in this way become difficult. Several different ways of linearizing this equation have long been used to estimate the values of Km and Vmax, each method having its advantages and disadvantages. The most accurate measurement of Km and Vmax is done using non-linear regression. This method does the best job of reducing the overall error associated with defining a relationship between all of the data points. Non-linear regression works by making systematic changes to the estimates of the values given for Km and Vmax in order to produce a curve that gives the best prediction of the reaction rate at any given substrate concentration. It is important to have the most accurate measurement of these two parameters if this assay will be used to help define a relationship between a new drug product and this enzyme.

In order to determine the length of time the enzyme could be stored without losing significant activity, a stability test was conducted. The assay developed was based on the one published on the Sigma-Aldrich website [EC.3.1.1.1, (n.d.)]. Sigma uses this assay to determine and define the activity of the rabbit CES that they sell, which is greater than or equal to 75 units per milligram protein. The unit definition based on this reaction is one unit of enzyme will hydrolyze 1 μmole of ethyl butyrate to butyric acid and ethanol per minute at pH 8.0 at 25°C. Stability of the enzyme was determined by verifying that our

assay could measure the same activity that was defined on the label of rabbit CES purchased from Sigma. Once that was established, the enzyme was stored at 4°C and the activity was measured at various time points up to 6 months. This conversion is shown in figure 3.1.

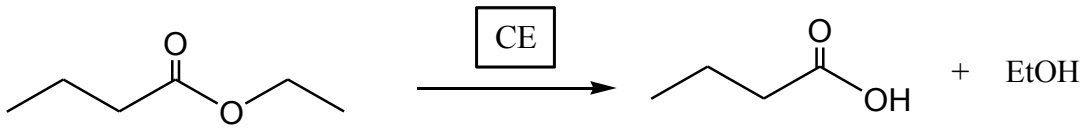

Figure 3.1. Conversion of ethyl butyrate to butyric acid and ethanol by the CES enzyme.

When developing an assay that is intended to be used for screening purposes, the more readily it can be accomplished, the better chance it has at being useful. Several different types of methods are used to monitor enzyme activity, including titrametric, spectrophotometric (SPEC), and HPLC [Zhou, et al., 1995]. All of these methods are useful and necessary when considering the large number of possibilities the structure of a drug could possess. Titrametric methods are useful but are limited to neutral substrates that produce an acidic or basic metabolite. SPEC assays are inherently more simplistic than HPLC based assays because of the need for chromatographic method development for HPLC drug detection. For this reason, the assay we chose to design to screen drugs for enzyme activity was spectrophotometric-based. One consideration that would need to be addressed before using this assay to determine enzyme activity would be if the new drug product being tested has an absorbance or if a derivitization step would be needed in order for detection to be possible.

In order to determine the optimum pH for testing activity of this enzyme, 4-methylumbelliferyl acetate (4-MBA) was used as a substrate. Carboxylesterase converts

4-MBA to 4-methylumbelliferone (4-MBO) by cleaving the ester linkage of the acetate group from the compound, this conversion is shown in figure 3.2.

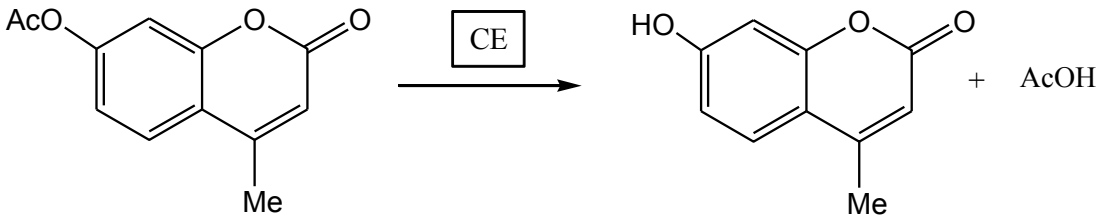

Figure 3.2. Conversion of 4-MBA to 4-MBO by the CES enzyme.

The last assay condition tested was the optimum temperature to use for testing the activity of this enzyme. To test this parameter, p-nitrophenyl acetate (pNPA) was used as a substrate. The latter is converted to p-nitrophenol (pNP) by carboxylesterase. This conversion is shown in figure 3.3.

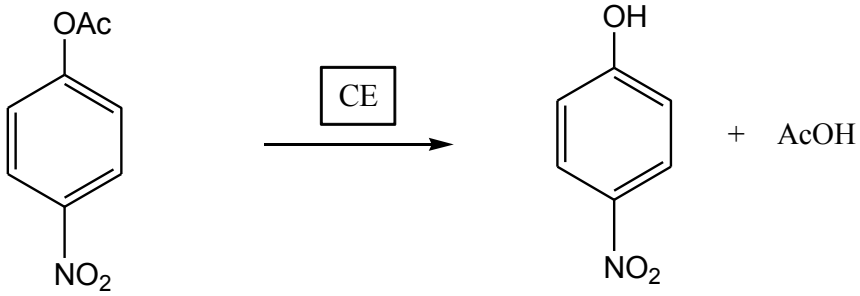

Figure 3.3. Conversion of 4-nitrophenyl acetate to 4-nitophenol by the carboxylesterase enzyme.

Once these assay conditions were determined, procaine was used to measure Km and Vmax for the carboxylesterase enzyme. The conversion of procaine to para-amino benzoic acid (PABA) by carboxylesterase is shown in figure 3.4.

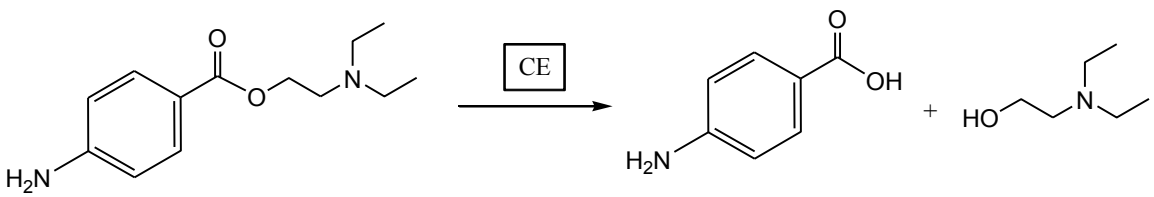

Figure 3.4. Conversion of Procaine to PABA by the carboxylesterase enzyme.

3.2 Materials and Methods

3.2.1 Reagents and Equipment

All of the reagents used in these experiments were purchased from Sigma-Aldrich (St.Louis, MO, USA). Rabbit carboxylesterase (CES) was purchased as a lyophilized powder containing 12.5 mg of protein or 1,000 units. The enzyme was reconstituted in cold deionized water at a concentration of 1,000 U/ml and was kept at 4°C for no more than two months. A fresh dilution from this stock was made for each experiment or set of experiments.

Stock solutions were prepared for 4-MBA and 4-MBO in DMSO at 50 mM. Stocks of pNPA were prepared in methanol while pNP was prepared in water both at 50 mM. Procaine and PABA were both prepared in water at 10 mM. Working solutions were prepared by appropriate dilution of the stock just before use. All weighings were done on an XS205 analytical balance from Mettler Toledo (Columbus, OH, USA).

A 10 mM Borate buffer was made while citrate and tris buffer was prepared at 100 mM. The pH for the borate buffer was adjusted to 8, citrate buffer pH was 4,5 and 6 and TRIS buffer pH was 6,7,7.4 and 8. All pH adjustments were made using 1N hydrochloric acid or 1N sodium hydroxide. Buffer pH was measured using an Accumet Excel XL15 pH meter from Cole-Parmer (Vernon Hills, IL, USA).

3.2.2 Spectrophotometric Readings

The enzymatic conversion of 4-MBA was monitored at a wavelength of 350 nm and the absorbance was measured every minute for thirty minutes. For the substrate pNPA, an absorbance reading was taken every fifteen seconds for five minutes at 450 nm. Two wavelengths were used to monitor for procaine and PABA at 265 nm and 290 nm, respectively. Measurement were taken every minute for thirty minutes. All SPEC assays were performed on a Molecular Devices (Sunnyvale, CA, USA) Spectramax M5 spectrophotometer. Softmax Pro v 5.3 software supplied by Molecular Devices was used for data collection.

3.2.3 Enzyme Stability Tests

200 ml of a 0.1% (v/v) ethyl butyrate solution was made by adding 200 µl of ethyl butyrate to 199.8 ml of 10 mM borate buffer. On the first day of testing the rabbit CES was taken up in 1 ml of cold water to make a concentration of enzyme at 1,000 U/ml. This stock was kept at 4°C for the duration of the stability testing. The activity was measured on day one and again 2, 4, 8, 16 and 26 weeks after stock preparation. A dilution of this stock was made on each day of testing at 50 U/ml by adding 5 µl of the 1,000 U/ml stock to 995 µl of cold 10 mM borate buffer. The stability tests were performed on a Radiometer Analytical (Lyon, France) TitraLab TIM856 titration manager.

A total of 25 ml of the 0.1% ethyl butyrate solution (containing 25 µl of ethyl butyrate) was added to a titration vessel and the pH was adjusted to 8.1 using 10 mM sodium hydroxide. 100 µl of the 50 U/ml enzyme solution was then added and the pH was monitored until it reached a pH of 8.0 at which time the pH Stat titrator was started.

The instrument was set to maintain the pH at 8.0 by small additions of 10 mM sodium hydroxide for 2 minutes. The instrument recorded the amount of sodium hydroxide it added over this time period in order to maintain the set pH value at 8.0. The activity of the enzyme was calculated using equations 3.2 and 3.3 and reported in units/mg protein.

$$\frac{units}{ml\ of\ enzyme} = \frac{(Molarity\ of\ NaOH) * (Volume\ (milliters)\ of\ NaOH\ used\ in\ assay)}{(Time\ (minutes)\ of\ assay) * (Volume\ (milliters)\ of\ enzyme)}$$

(eq. 3.2)

$$\frac{Units}{mg\ protein} = \frac{Units/ml\ of\ enzyme}{mg\ protein/ml\ of\ enzyme}$$

(eq. 3.3)

3.2.4 Calibration Curve Validation

Calibration curves of both the substrates and the products were developed on the spectrophotometer to determine linearity of these compounds in each buffer and at each pH. Standards were prepared for each compound by making dilutions from the stocks described earlier. The standards prepared for 4-MBA covered the range of 0.2-1 mM and for 4-MBO from 0.02-0.4 mM. The buffers loaded with these two compounds were 100 mM citrate with pH 4, 5 and 6 and TRIS buffer with pH 6, 7, 7.4 and 8. Standards for pNPA were prepared from 0.4-2 mM and pNP from 0.010-0.2 mM. The buffers loaded with these compounds were 100 mM TRIS at pH 6, 7, 7.4 and 8. Standards for procaine were from 10-600 µM and PABA from 2-50 µM. The buffer loaded with these compounds was 200 mM TRIS at pH 7.4. Blank solutions or "vehicle" solutions were

made for each substrate and product based on the diluents used for stock preparation. The vehicle solution for 4-MBA and 4-MBO was 2% DMSO in water, for pNPA it was 2% ethanol in water and the vehicle solution for pNP, procaine, and PABA was water. The vehicle solutions were used for background subtraction. A 96-well plate was loaded with 100 µl of blank solution or the calibration standards and 100 µl of buffer and the plant was read on the SPEC.

2.2.5 Enzyme Concentration Test

In order to insure linear kinetics, an enzyme concentration test was conducted. Several different concentrations of enzyme were incubated with the substrate, and the reaction rates were measured. All of the concentrations described for substrate, product, enzyme and buffer represent the incubation concentration once loaded on the plate. Substrate and product concentrations were 4-MBA at 0.5 mM, 4-MBO at 0.2 mM, pNPA at 1 mM, pNP at 0.1 mM, procaine at 100 µM and PABA at 25 µM. TRIS buffer at pH 7.4 was used at a concentration of 50 mM. The enzyme concentrations in the reaction mixture with 4-MBA and pNPA were 0.1, 0.05, 0.025 and 0.01 U/ml and for procaine were 12.5, 6.25 and 3.125 U/ml.

Vehicle solutions for each substrate and product were prepared as described in Section 2.2.4. A blank solution for wells containing enzyme were prepared by adding the appropriate amount of enzyme to each vehicle solution. Control wells were prepared without enzyme to monitor spontaneous hydrolysis of either substrate or product in buffer. Once the plate was loaded with buffer and substrate, enzyme was quickly added and the SPEC was started.

3.2.6 Selecting Assay Buffer pH

In order to determine the optimum buffer pH, a set of tests were conducted from a pH of 4 to a pH of 8. The enzyme activity was measured at each pH using 4-MBA as a substrate for rabbit CES. The incubation concentrations for substrate, product, enzyme and buffer were as follows; 4-MBA at 0.5 mM, 4-MBO at 0.1 mM, CES at 0.05 U/ml and citrate buffer (pH 4, 5, 6) and TRIS buffer (pH 6, 7, 7.4, 8) at 50 mM. Vehicle solutions for both substrate and product were prepared as described in section 2.2.4. A blank solution for wells containing enzyme were prepared by adding the appropriate amount of enzyme to each vehicle solution. Control wells were prepared without enzyme to monitor spontaneous hydrolysis of either substrate or product in buffer. Once the plate was loaded with buffer and substrate, enzyme was quickly added and the spectrophotometer readings were started.

3.2.7 Selecting Assay Temperature

In order to determine the effect of temperature on the enzyme activity tests were performed at room temperature and 37°C. The enzyme activity was measured at each temperature using pNPA as a substrate for rabbit CES. The concentration of substrate, product, enzyme and buffer in the reaction mixture were pNPA at 1 mM, pNP at 0.1 mM, CES at 0.05 U/ml and TRIS buffer (pH 6, 7, 7.4, 8) at 50 mM. Vehicle, blank and control solutions for both substrate and product were prepared as described in section 2.2.4. Once the plate was loaded with buffer and substrate, enzyme was quickly added and the SPEC data collection was started. For the testing that was performed at 37°C, after the plate was loaded with buffer, substrate and product it was placed in the plate reader for 15 min so the reaction mixture could equilibrate to 37°C before the enzyme was added.

3.2.8 Assay For Measuring Km and Vmax

In order to measure the Km and Vmax of the enzyme the optimized assay conditions were used. Procaine was used as a substrate with TRIS buffer at pH 7.4 at a temperature of 37°C. The incubation concentrations of substrate, product, enzyme and buffer were procaine from 3.125-300 μM, PABA at 25 μM, CES at 6.25 U/ml and TRIS buffer (pH 7.4) at 50 mM. Vehicle, blank and control solutions for both substrate and product were prepared as described in section 2.2.4. Once the plate was loaded with buffer, substrate and product it was equilibrated to 37°C in the plate reader, enzyme was quickly added and the SPEC data collection was started.

2.2.9 Method For Calculating Compound Concentration

If the absorption of the compound being measured by the spectrophotometer is proportional to the concentration as described by Beer's law, then equation 3.4 can be applied where: "A " is the absorbance, "a" is the molar absorptivity, "b" is the path length of the cell in cm, and "c" is the concentration of the compound.

$$A = a*b*c \quad \text{(eq. 3.4)}$$

For our experiments the pathlength was constant, and since the molar absorptivity was also constant, the relationship between absorbance at a given wavelength and the concentration of the compound could be represented by equation 3.5. The slope of a plot of c vs. A is represented as M.

$$c = A*M \quad \text{(eq. 3.5)}$$

Equation 3.5 can be expanded upon in order to calculate the concentration of two compounds occurring in the same mixture. This is shown in equations 3.6 and 3.7, where A_1 is the absorbance at one of the wavelengths that you are measuring and A_2 is the

absorbance at the other wavelength. Ms_1 is the slope of compound 1 at the first wavelength and Ms_2 is the slope of compound 1 at the second wavelength. Mp_1 is the slope of compound 2 at the first wavelength and Mp_2 is the slope of compound 2 at the second wavelength. The concentration of the first compound is represented as c_1 and c_2 represents the concentration of the second compound.

$$A_1 = Ms_1 * C_1 + Mp_1 * C_2 \quad \text{(eq. 3.6)}$$

$$A_2 = Ms_2 * C_1 + Mp_2 * C_2 \quad \text{(eq. 3.7)}$$

Solving these two equations for the two unknowns, C_1 and C_2, give you equations 3.8 and 3.9.

$$C_1 = \frac{A1 * Ms2 - A2 * Mp2}{Ms1 * Ms2 - Mp2 * Ms1} \quad \text{(eq. 3.8)}$$

$$C_2 = \frac{A1 * Mp2 - A2 * Mp1}{Ms1 * Mp2 - Ms2 - Mp1} \quad \text{(eq. 3.9)}$$

3.3 Results

3.3.1 Enzyme Stability Tests

Figure 3.5 is a graphical representation of the stability of rabbit CES stored at 1,000 U/ml in water for six months at 4°C. Each point represents the mean of three experiments with the standard deviation.

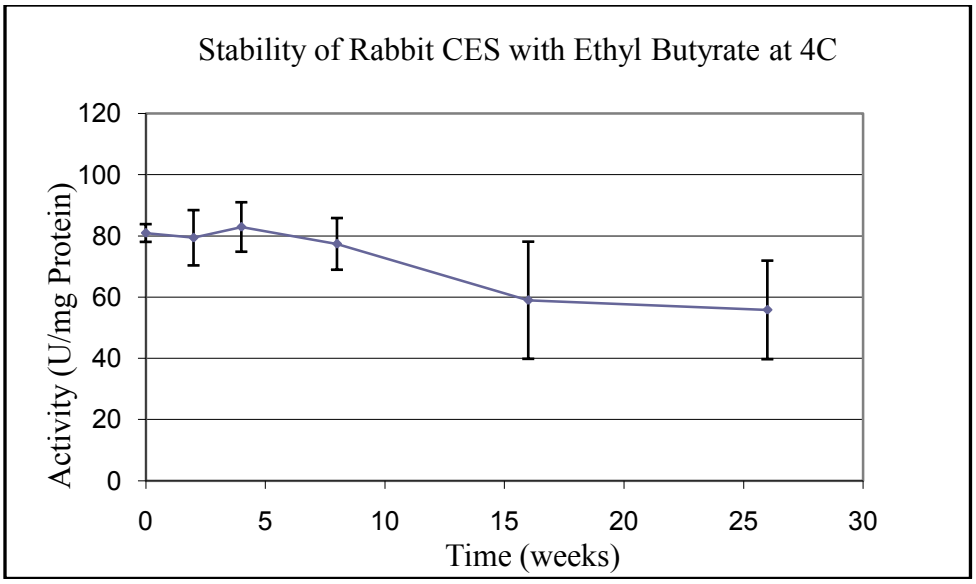

Figure 3.5. Results of stability testing done with rabbit CES stored at 1,000 U/ml in water for six months at 4°C.

Table 3.1 is a summary of the measured activity of the enzyme at each time point of the stability study. The activity of the enzyme is specified by Sigma to be ≥75 U/mg protein at the time of purchase, and stable when stored at -20°C as a lyophilized powder. The activity measurement at the beginning of the study was 80.95 U/mg protein which fits with this definition. For this testing, the enzyme was considered to be stable if the activity measured was ≥ 90% of its original activity. At the conditions tested, the enzyme is stable for up to eight weeks. The stability was tested before any of the studies in this chapter were started, such that with this information the enzyme stock was kept at 4°C for no longer than two months once it was reconstituted.

Table 3.1. Summary of Activity of Enzyme in Water Stored at 4°C.

Time (weeks)	Activity (U/mg protein)	Std dev	% CV
0	80.95	2.90	3.58%
2	79.39	9.03	11.38%
4	82.91	8.08	9.75%
8	77.39	8.45	10.91%
16	58.97	19.15	32.47%
26	55.81	16.10	28.86%

3.3.2 Calibration Curve Validation

Calibration curves for both the substrate and the product were made in each buffer and at each pH used for testing. Figure 3.6 represents the calibration curves made for 4-MBA. The curves do not appear to be linear and the absorbance changes dramatically for pH 4-6. The low amount of absorbance at these pH levels could indicate that the absorbance of 4-MBA is not strong enough to overcome interference by the buffer components. All absorbance readings for all concentrations are less than 1 absorbance unit (AU).

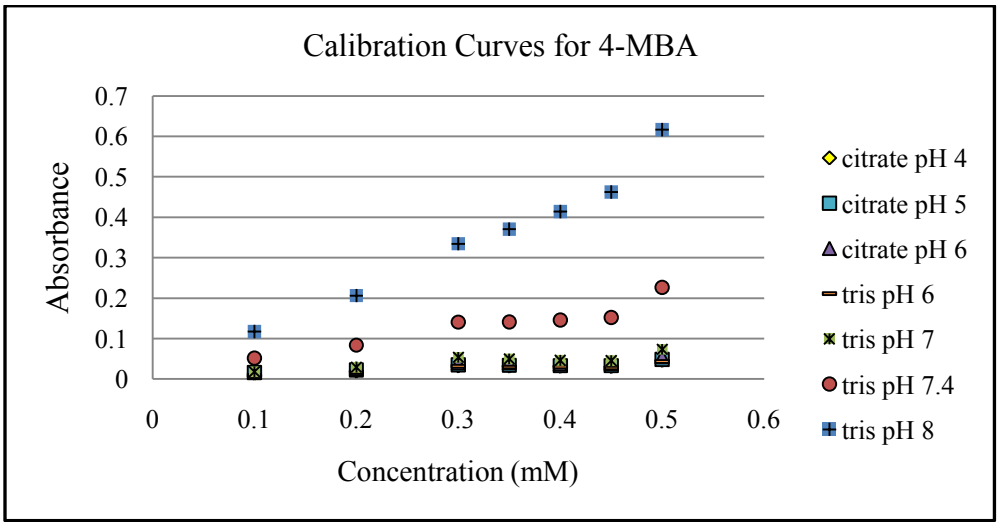

Figure 3.6. Representative calibration curves in citrate buffer (pH 4-6) and TRIS buffer (pH 6-8) for the substrate 4-MBA. Curves at low pH are superimposed on this graph.

Figure 3.7 represents the calibration curves made for 4-MBO. These curves appear to be linear for all pH levels. The absorbances at all pH levels are close to 1 AU.

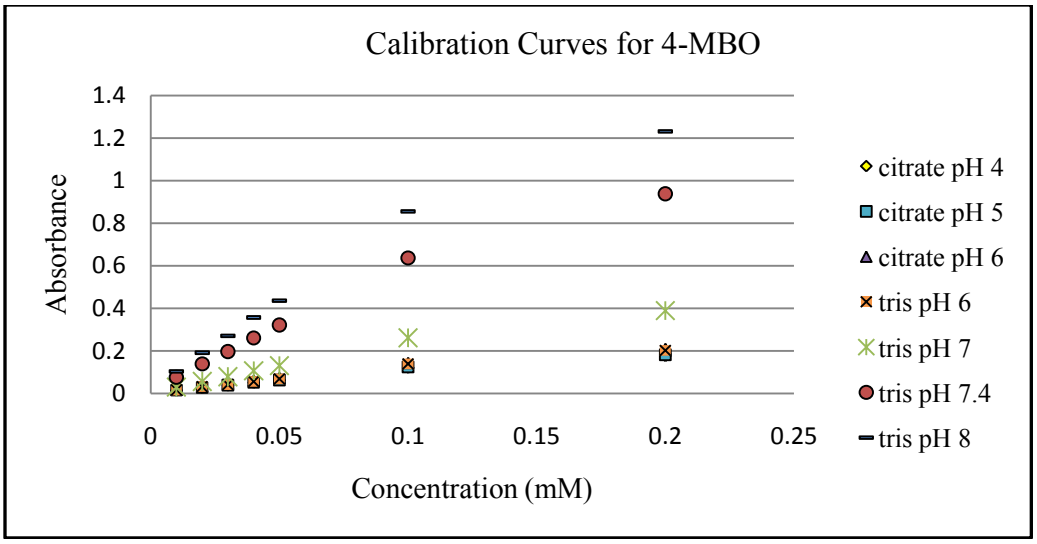

Figure 3.7. Representative calibration curves in citrate buffer (pH 4-6) and TRIS (pH 6-8) for the product 4-MBO. Curves at low pH are superimposed on this graph.

Table 3.2 summarizes the correlation coefficients for both 4-MBA and 4-MBO at each pH. A correlation coefficient of 0.9 or greater indicates an acceptable level of linearity in the data. The curves for 4-MBA at the low pH range had a correlation of 0.8961, 0.8563 and 0.8741 which was caused by the very low absorbance readings measured at those levels. The linearity of the calibration data for 4-MBO on-the-other-hand, had a correlation of at least 0.9 across the entire pH range. For this reason it was decided to just measure the formation of this product during the testing for selecting the buffer pH. This decision eliminated the problem of monitoring the substrate at the low pH levels. Since the absorbance of 4-MBO was proportional to the concentration, a one-point standardization was justified in place of calibration curves for the buffer pH assay.

Table 3.2. Correlation Coefficient Values for the Calibration Curves.

	Citrate Buffer			Tris Buffer			
Compound	pH 4	pH 5	pH 6	pH 6	pH 7	pH 7.4	pH 8
4-MBA	0.8961	0.8563	0.9172	0.8741	0.9337	0.9263	0.9816
4-MBO	0.9708	0.9713	0.9739	0.9600	0.9657	0.9665	0.9602

Figure 3.8 represents the calibration curves made for pNPA. The curves do not appear to be linear especially at pH 6 and 7. The absorbance readings at the higher pH are well above 1AU. It is typical for linearity of curves to change when the absorbance readings go beyond this measurement. This could be corrected for by changing the wavelength that is being used to one in which the compound has less absorbance.

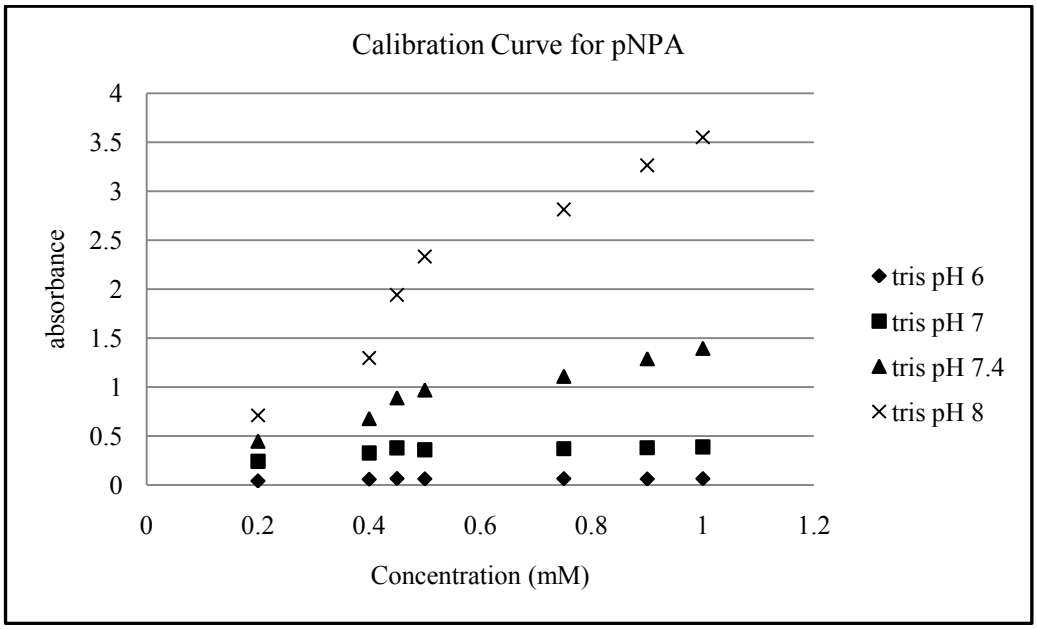

Figure 3.8. Representative calibration curves in TRIS buffer (pH 6-8) for the substrate pNPA.

Figure 3.9 represents the calibration curves made for pNP. These curves appear to be very linear for all pH. The absorbances at all pH are less than 1 AU.

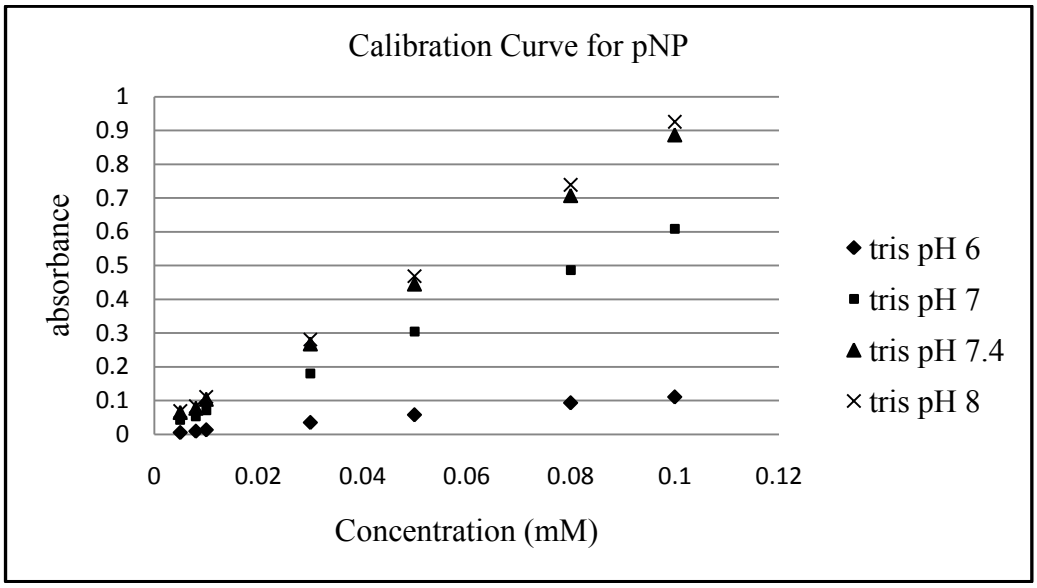

Figure 3.9. Representative calibration curves in TRIS buffer (pH 6-8) for the product pNP.

Table 3.3 summarizes the correlation coefficients for both pNPA and pNP at each pH. The correlation coefficients for pNPA at pH 7.4 and 8 are 0.613 and 0.4124, which do not show good correlation in these measurements. The curves for pNP showed excellent linearity across the entire pH range. For this reason it was decided to just measure the formation of this product during the testing for the selection of optimal temperature. Measuring the reaction rate based on product formation eliminates the need to adjust the wavelength to one that is better for substrate detection. Since the absorbance of pNP was proportional to the concentration, a one-point standardization was justified in place of calibration curves to calculate product formation during the assay.

Table 3.3. Correlation Coefficient Values For the Calibration Curves.

Compound	pH 6	pH 7	pH 7.4	pH 8
pNPA	0.9493	0.952	0.613	0.4124
pNP	0.9997	0.9997	0.9997	0.9984

Wavescans of procaine and PABA were performed in order to decide what wavelength would give the best detection of these compounds. A wavelength of 265 nm

was optimum for the detection of PABA over procaine, while a wavelength of 290 nm was optimum for the detection of procaine over PABA. Figure 3.10 shows the calibration curves for both of these compounds at the 265 nm wavelength. Both of these compounds prove to be linear at these conditions. The absorbance of procaine does go slightly over 1 AU but the curves for both compounds appear to remain linear, so this was not seen as a problem.

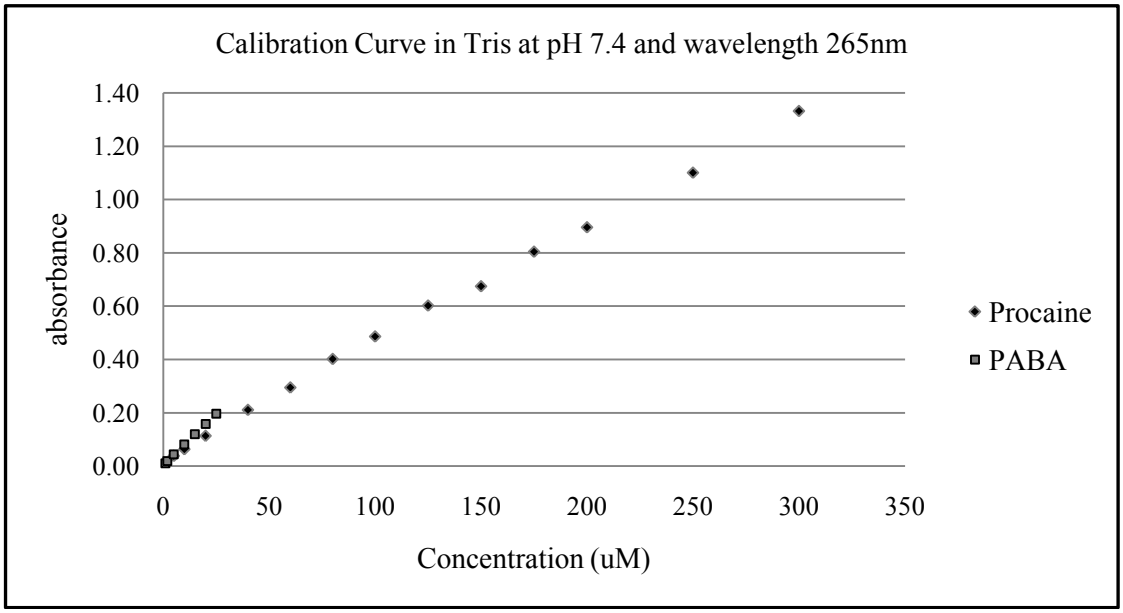

Figure 3.10. Representative calibration curve for the substrate procaine and the product PABA in TRIS buffer pH 7.4 at 265 nm.

Figure 3.11 shows the calibration curves for both procaine and PABA at the 290 nm wavelength. The absorbance for procaine at this wavelength goes slightly further above 1 AU, but again the readings appear to remain linear.

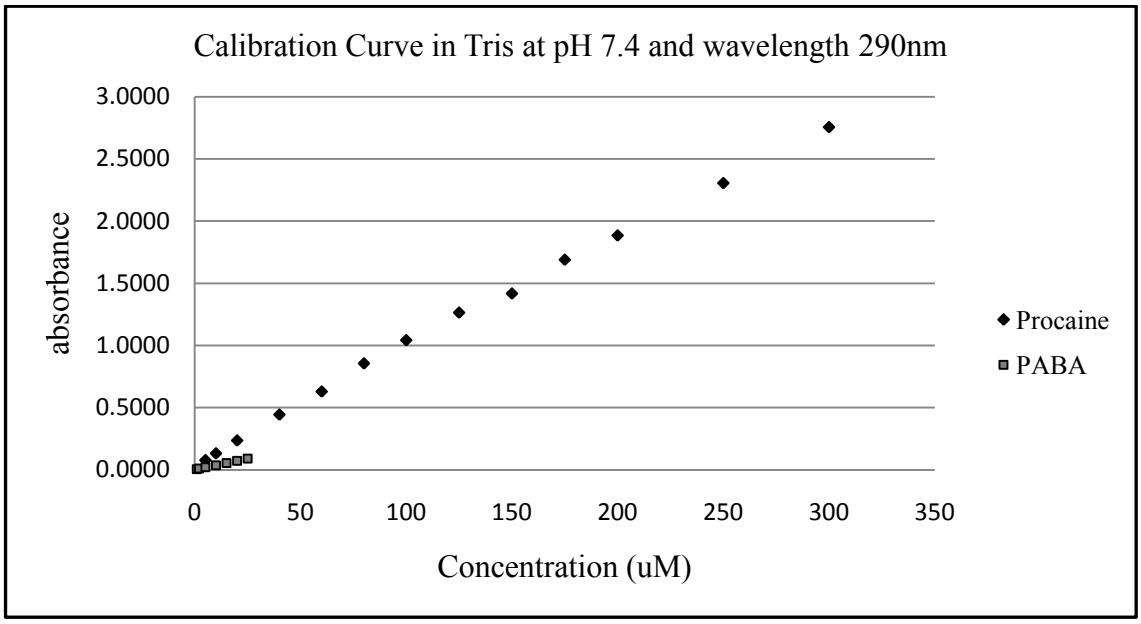

Figure 3.11. Representative calibration curve for the substrate procaine and the product PABA in TRIS buffer pH 7.4 at 290 nm.

Table 3.4 is a summary of the correlation curves for both compounds at both wavelengths. Since the concentration of both compounds remained proportional to the absorbance, a simplified method of calculating the concentrations of each compound in the reaction mixture was used. It required that only one concentration of each compound be loaded on the plate during the enzyme assay.

Table 3.4. Correlation Coefficient Values for the Calibration Curves.

Compound	Wavelength	
	265 nm	290 nm
procaine	0.9987	0.998
PABA	0.9998	0.9997

3.3.3 Enzyme concentration tests

Figure 3.12 shows the results of the enzyme concentration test performed with different concentrations of rabbit CES using 4-MBA as the substrate. The kinetics appeared to be linear across the time period tested, so it was decided to use an enzyme concentration of 0.05 U/ml for the assay used to select buffer pH.

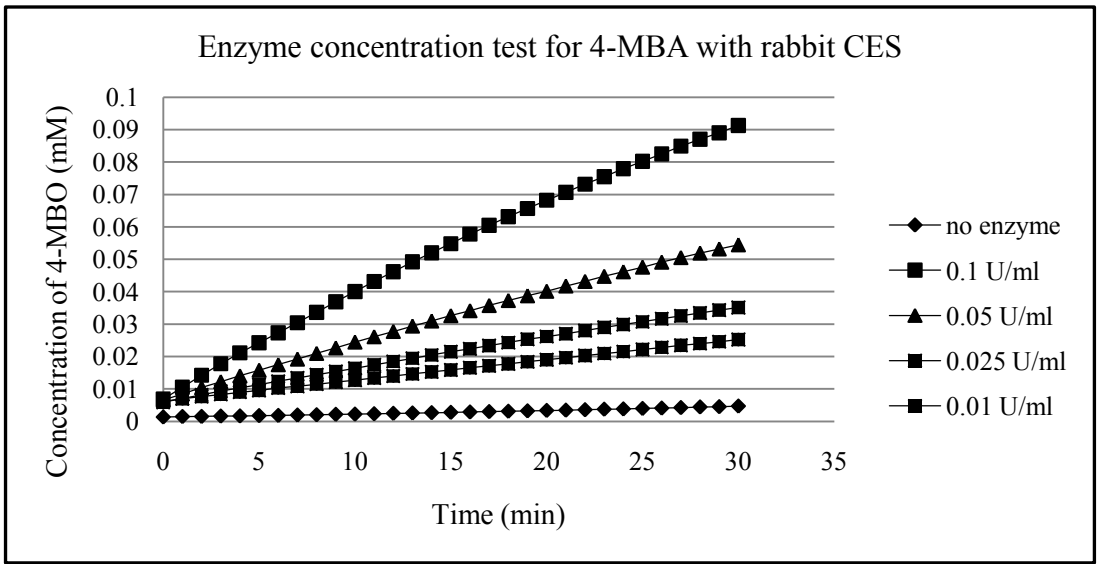

Figure 3.12. CES enzyme concentration testing using 4-MBA as a substrate.

The enzyme concentration test with rabbit CES using pNPA as a substrate is shown in figure 3.13. The kinetics remained linear at each enzyme concentration tested. It was decided to use an enzyme concentration of 0.05 U/ml for the assay in which the effects of temperature on enzyme activity were measured.

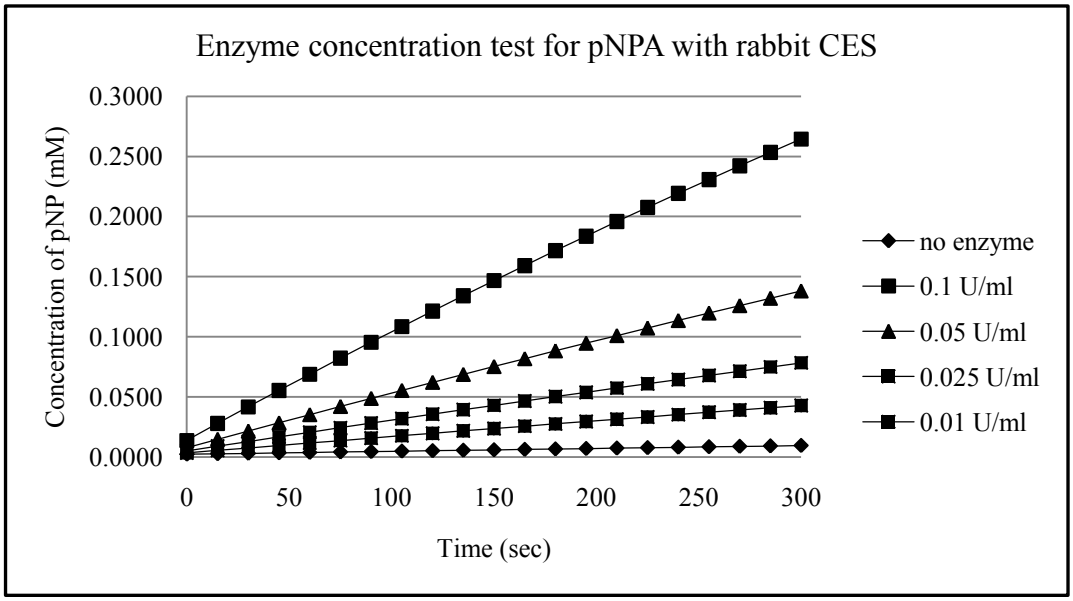

Figure 3.13. CES enzyme concentration testing using pNPA as a substrate.

The results of the enzyme concentration test performed with rabbit CES using procaine as a substrate is shown in figure 3.14. At the highest enzyme concentration tested the concentration of the product, PABA at the start of the test was already 2 mM. The test was repeated with the same result. Initially we thought that the delay between loading the enzyme and starting the SPEC program was long enough and the reaction rate was fast enough, then this could explain the starting concentration of PABA. Once the reaction rate was calculated the delay would have to have been almost 10 minutes. A rough estimate of the delay is closer to 1 minute so this theory does not seem to explain the observation. Because of this starting condition issue, the higher enzyme concentration was not used and an enzyme concentration of 6.25 U/ml was selected for the Km and Vmax measurement.

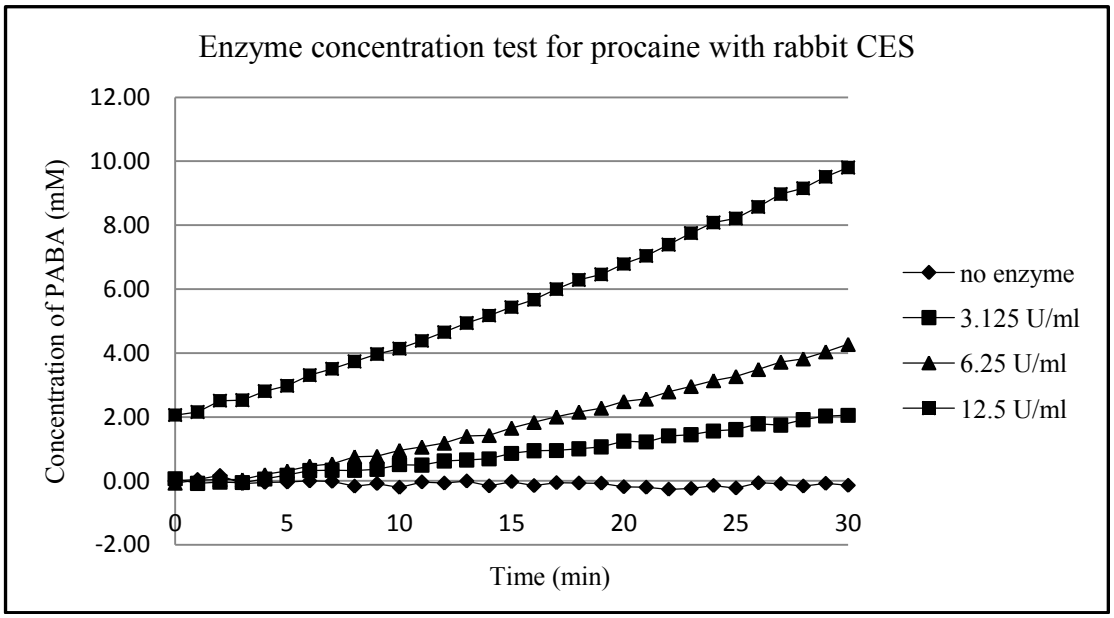

Figure 3.14. CES enzyme concentration testing using procaine as a substrate.

3.3.4 Effect of Buffer pH

Figure 3.15 represents the results of the testing done with rabbit CES using 4-MBA as a substrate. The points represent the mean of three experiments with the standard deviation.

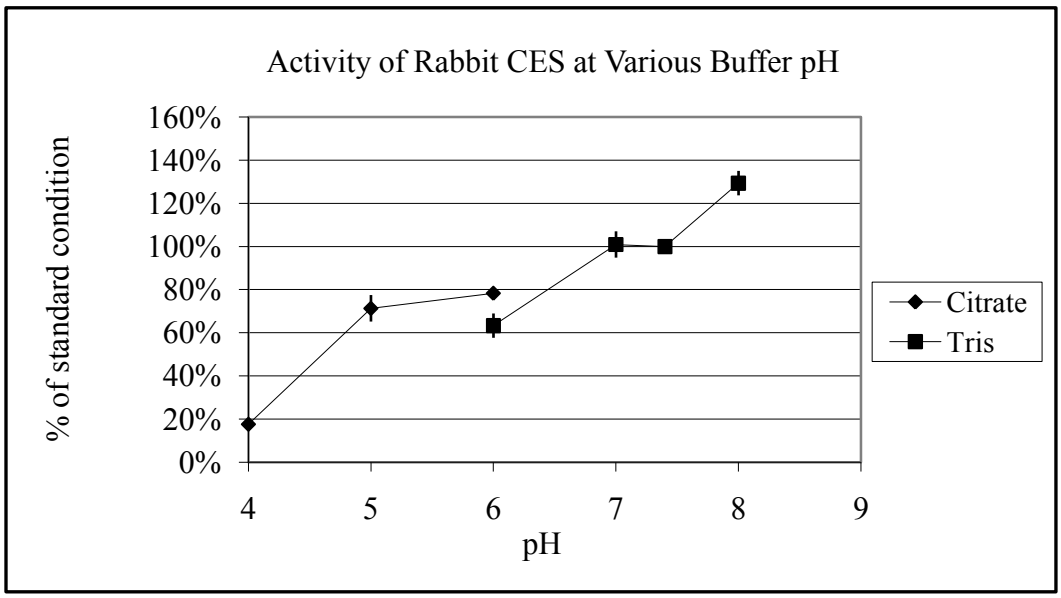

Figure 3.15. Results of the testing done to study the effects of buffer pH on enzyme activity using 4-MBA as a substrate.

In order to compare the activity of the enzyme at each condition, the rate of conversion at each pH was calculated. The rate was based on the mM of 4-MBO created per minute of reaction time. The standard condition was chosen to be pH 7.4 and the rate at each pH was divided by the rate calculated at the standard condition. Table 3.5 summarizes these results.

Table 3.5. Summary of Assay pH Testing With 4-MBA.

	Buffer pH	% of Std condition	Std dev
Citrate	4	17.57%	0.027
	5	71.30%	0.062
	6	78.33%	0.029
Tris	6	63.26%	0.056
	7	100.90%	0.061
	7.4	100.00%	0.000
	8	129.35%	0.057

The rate of enzyme conversion increases as pH increases, with pH 8 having the largest conversion rate. During testing, control wells were made at each pH that contained substrate without enzyme. The rate of substrate conversion was measured in these wells, and from pH 4 to 7.4. This conversion rate was relatively insignificant. At pH 8 the conversion rate was much higher, which indicated that the substrate was unstable at that pH. For this reason the buffer pH chosen for the Km and Vmax measurement assay was 7.4.

3.3.5 Effect of Temperature

Figure 3.16 represents the results of the testing done with using pNPA as a substrate for rabbit CES. Each point on the graph represents the mean of three tests with the standard deviations.

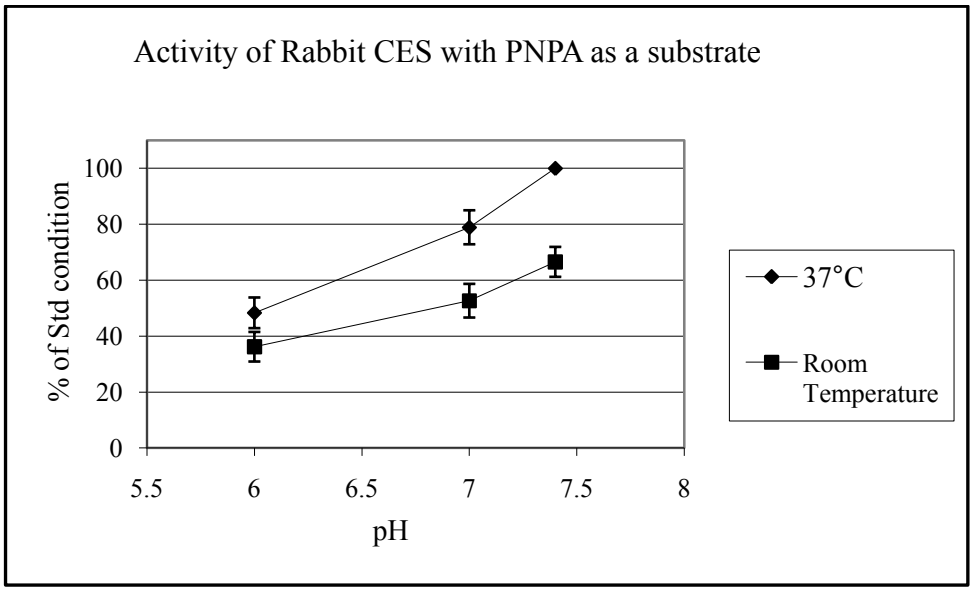

Figure 3.16. Results of the temperature optimization assay using pNPA as a substrate in tris buffer.

The rate of enzyme activity was measured in mM of pNP created per second at each pH at both room temperature and at 37°C. The comparison in this assay was done the same as in the buffer optimization assay. The hydrolysis rates measured at each pH were divided by the rates at the standard condition, which is summarized in Table 3.6. All of the rates measured at room temperature fell below those measured at 37°C. The highest measured rate of CES activity at both temperatures was at a pH of 7.4, which is in agreement with the assay in which buffer pH effects were studied. Based on the measured temperature and pH effects on CES, the selected conditions to measure the rate of rabbit CES activity are a buffer pH of 7.4 and a temperature of 37°C.

Table 3.6. Summary of Assay Temperature Testing With pNPA.

37°C			Room Temperature		
Buffer pH	Ave % Std	SD	Buffer pH	Ave % Std	SD
7.4	100	0	7.4	66.52	5.36
7	78.89	6.08	7.00	52.63	6.01
6	48.31	2.98	6.00	36.18	0.44

3.3.6 Assay For Measuring Km and Vmax

The results of the assay in which the concentration of procaine was varied with rabbit CES, and the reaction rate was measured by monitoring the appearance of product and the disappearance of substrate, are shown in figure 3.17. Each point represents the mean of three experiments with the standard deviation. This graph shows that our enzyme followed Michaelis-Menten kinetics with procaine as a substrate.

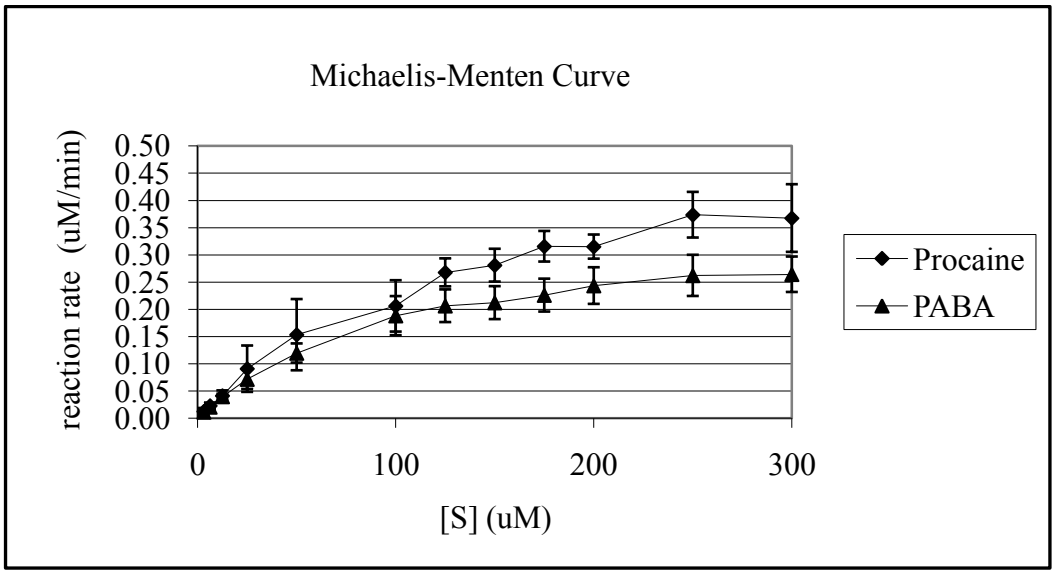

Figure 3.17. Saturation curve for rabbit CES showing the relationship between substrate concentration and reaction rate.

In order to use non-linear regression to estimate the value of Km and Vmax, an initial guess of these values needed to be made. To do this the Eadie-Hofstee method, of calculating these values was used. To use the Eadie-Hofstee method a plot of reaction rate (v) versus the reaction rate divided by the substrate concentration (v/[S]) was made, as shown in Figure 3.18.

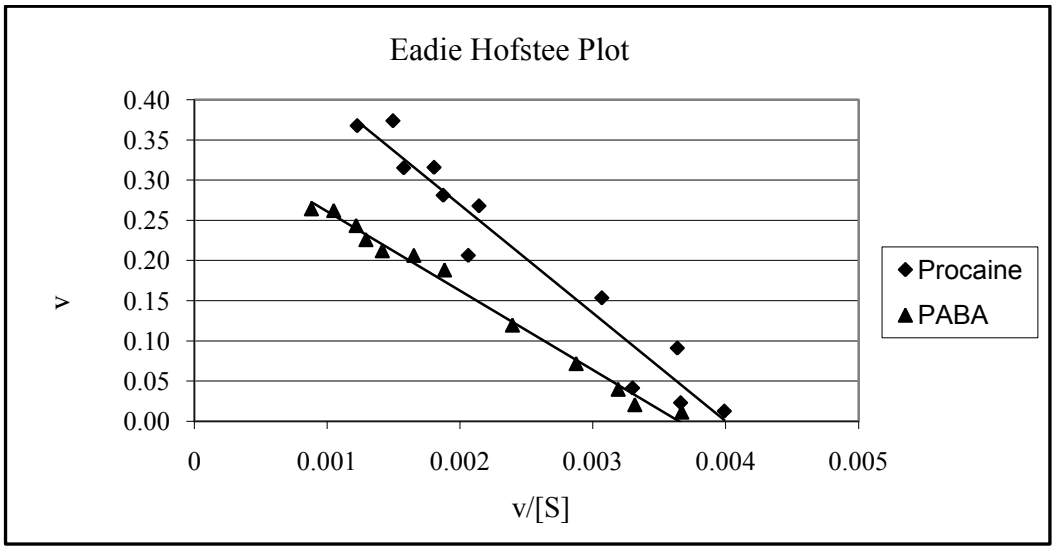

Figure 3.18. Eadie-Hofstee plot used to calculate the initial guess of Km and Vmax.

From this plot the initial guess of Km and Vmax for both procaine and PABA were made by performing a linear regression on the data points and relating the slope and intercept to these parameters.

$$Km = -slope$$

$$Vmax = y\text{-intercept}$$

Kaleidagraph Synergy Software (Reading, PA, USA) was used to perform non-linear regression of the data. A plot of the results is shown in figure 3.19. The triangles in the plot represent the consumption of procaine and the open squares represent the formation of PABA. The error bars represent the standard deviation associated with the reaction rate measurements made at each substrate concentration.

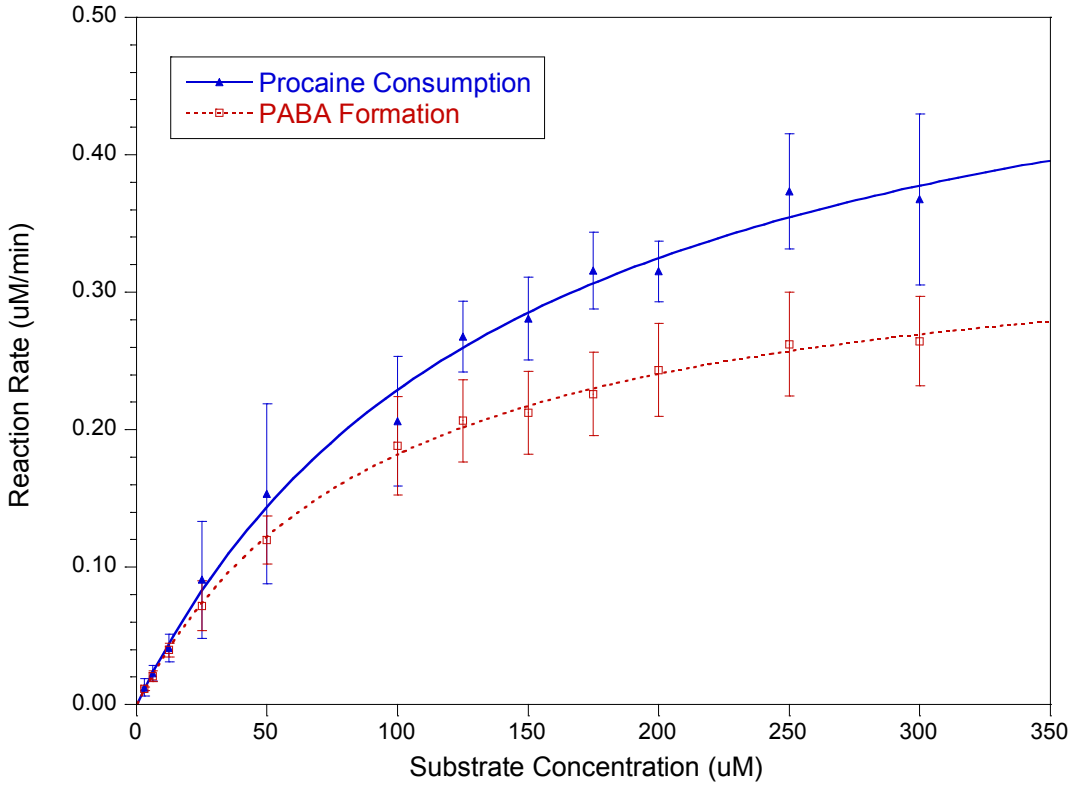

Figure 3.19. Reaction rate vs. substrate concentration plot for both procaine and PABA showing the non-linear regression fit.

Table 3.7 shows the initial guess for Km and Vmax from the Eadie-Hofstee method, and the optimized values for these parameters made using non-linear regression. The values measured for Km and Vmax using procaine vary somewhat from the values measured using PABA. However, the difference is small enough that these differences are most likely related to experimental error.

Table 3.7. Measurements of Km and Vmax.

	Eadie Hofstee		Non-linear Regression	
	Procaine	PABA	Procaine	PABA
Km †	134.64	98.61	143.82 ± 18.71	94.97 ± 5.50
Vmax ‡	0.0115	0.0077	0.0120 ± 0.0007	0.0072 ± 0.0002

† Km was measured in µM of substrate or product respectively
‡ Vmax was measured in µmol/min/mg protein of substrate or product respectively

These Km and Vmax measurements seem to be in line with what is published in

the literature about the relationship between procaine and the CES enzyme. Arndt and Krisch and later Mentlein and Heymann measured the Vmax of procaine with purified rat liver CES as having a value 20x larger than the one found here [Breast-Ovarian, (n.d.), Arndt and Krisch, 1973]. In the literature, hydrolysis rates for rabbit CES with procaine have been measured to be 2-5x faster than the hydrolysis rates measured for rat CES [Lee and Livett, 1967; Short, et al., 1988]. The reason the rates measured here were smaller than what was expected can be explained by the preparation of the enzyme being used. Vmax is directly affected by the amount of enzyme present in the reaction mixture, the rabbit CES used for these experiments was a crude mixture of protein, while the rat liver enzyme used was purified enzyme. This meant that all of the protein used to measure the activity in rat liver was all or mostly all enzyme, where the preparation of rabbit CES was known to contain at least 15 % of protein identified as not being the CES enzyme.

The Km values measured with rat liver CES were similar to what were measured here. Arndt and Kirsch measured a value of 0.132 mM, Mentlein and Heymann measured the Km value to be 0.070 mM [Arndt and Krisch, 1973; Mentlein and Heymann, 1984]. This means that the binding affinity of procaine to rat CES is comparable to rabbit CES.

In 1997 Takai et al. measured the Km and Vmax of procaine with purified human CES reporting values of 3.33 mM and 0.029 μmol/min/mg protein, respectively [Gram, et al., 1991]. While a direct comparison of the Vmax of rabbit CES measured here cannot be made with the purified human enzyme used in those experiements, the Km value shows that procaine has a 20 to 30 times greater binding affinity to rabbit CES than to human CES.

3.4 Conclusion

By conducting the stock stability test of rabbit CES, we were able to determine the length of time this enzyme could be stored without any significant changes in its activity. This was important not only for the studies conducted here but also in establishing appropriate storage conditions to be used during future work. Determining the effects of temperature and pH allowed selection of the conditions that provided the most accurate and reproducible measurement of the kinetic properties of this enzyme with procaine. CES being a mammalian enzyme present in the body, the conclusion that the optimal pH is 7.4 and the optimal temperature is 37°C is fitting with what would be expected from this type of enzyme.

The usefulness of this assay lies in the ability of the researcher in making correlations between the results found here and the results of this assay performed on a candidate drug still having an unknown profile. For example, the literature has established a list of drug interactions procaine has with other marketed pharmaceuticals, some relating to procaine's metabolite PABA, but others are indicated because of the common elimination pathway that they share, specifically metabolism by CES enzymes [EC.3.1.1.1, (n.d.)]. The results of using this assay to measure the Km provides an indication of the extent of possible drug-drug interactions the unknown compound might exhibit.

The other parameter measured in this assay Vmax, also could be used for insights into a new drug's behavior clinically. The half-life when procaine is injected intravenously into humans is less than 10 minutes, which is a result of its rapid metabolism by the esterase enzymes [Casarett, et al., 2008].Measuring Vmax for other

test agents by this same assay method would provide an early indication of whether the agent's half-life will be longer or shorter than procaine. A good initial study design prior to in vivo testing helps to minimize the amount of experiments needed to obtain a good understanding of the pharmacokinetic profile of the drug.

Another valuable piece of information that can be gained from the use of this assay would be to establish whether an unknown compound is a substrate for the esterase enzymes. Since the way in which the esterase enzymes metabolize substrates is well defined, an educated guess can then be made as to the metabolites this compound will produce. This would be useful in ascertaining any potential metabolite-related side effects.

The usefulness of this assay could be expanded upon by characterizing other marketed pharmaceuticals with this method. Comparison between a range of compounds all having well understood drug profiles would be much more informative and helpful in designing studies for a new drug candidate in the beginning stages of development. With the optimized conditions described here, a standard HPLC method could also be designed for the analysis of compounds that do not lend themselves to this spectrophotometric-based assay.

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