DEVELOPMENT OF AN ANALYTICAL METHOD FOR VINCA ALKALOIDS USING LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY (LC/MS/MS)

By

MINH TRIEU NGO

Bachelor of Science in Biochemistry and Molecular Biology

Oklahoma State University

Stillwater, Oklahoma

2008

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
August 2010

DEVELOPMENT OF AN ANALYTICAL METHOD FOR VINCA ALKALOIDS USING LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY (LC/MS/MS)

Thesis Approved:

Dr. Jarrad Wagner

Thesis Adviser

Dr. David Wallace

Committee Member

Dr. Franklin R. Champlin

Committee Member

Dr. Mark E. Payton

Dean of the Graduate College

ACKNOWLEDGMENTS

I want to dedicate this thesis to my parents, Son Ngo and Kim-Long Trieu. This thesis would have not being possible if it was not for them. I am grateful to my parents for bringing me into this world and raising me. Through their love, patience, and support, I was able to follow my dreams and be who I am today.

I would also like to thank everyone in the Department of Forensic Sciences at Oklahoma State University Center for Health Sciences. Everyone has been very supportive and encouraging during my graduate years. Above all, I would like to thank my advisor, Dr. Jarrad R. Wagner, for all his advice, patience, support, and guidance throughout my journey. Without him, I would not be able to get to where I am today. I could not have asked for a better advisor. I would also like to thank my committee members, Dr. David Wallace and Dr. Franklin Champlin, for their valuable advice. They were greatly appreciated. A special thanks to Dr. Lara Maxwell and Satyanarayana Achanta of the OSU Center for Veterinary Health Sciences for their collaboration on this project.

I would also like to acknowledge the National Science Foundation (NSF) Bridge to Doctorate Louis Stoke Alliances for Minority Participants (BD-LSAMP) program for providing me with financial support to continue my education. I would also like to specifically thank Kay Porter and Fara Williams, both of OK-LSAMP, for providing

encouragement as I participated in the OK-LSAMP program and continued the pursuit of this graduate degree,

Last but not least, I would like to thank all my friends for being with me through the good and bad times. Their support was freely given, and it was and is greatly appreciated.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF EQUATIONS

ABBREVIATIONS

7-AAD	7-aminoactinomycin-D
ACN	Acetonitrile
ADME	Administration, Distribution, Metabolism, Elimination
APCI	Atmospheric pressure chemical ionization
APCI-LC/MS	Atmospheric pressure chemical ionization liquid chromatography mass spectrometry
APCI-LC/MS/MS	Atmospheric pressure chemical ionization liquid chromatography tandem mass spectrometry
CE	Capillary electrophoresis
CZE	Capillary zone electrophoresis
DVBL	Deacetylvinblastine
EA	Ethyl acetate
ESI	Electrospray ionization
ESI-LC/MS	Electrospray ionization liquid chromatography mass spectrometry
ESI-LC/MS/MS	Electrospray ionization liquid chromatography tandem mass spectrometry
ESI-MS	Electrospray ionization mass spectrometry
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
GC	Gas chromatography
GC/MS	Gas chromatography-mass spectrometry

H-ESI	Heated electrospray ionization
HPLC	High performance liquid chromatography
HPLC-ESI-MS	High performance liquid chromatography electrospray ionization mass spectrometry
IPA	Isopropanol
IS	Internal standard
LC	Liquid chromatography
LC/F	Liquid chromatography fluorescence
LC/MS	Liquid chromatography mass spectrometry
LC/MS/MS	Liquid chromatography tandem mass spectrometry
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantitation
LOD	Limit of Detection
ME	Matrix effect
MeOH	Methanol
MRM	Multiple reaction monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
NMR	Nuclear magnetic resonance
OSU CVHS	Oklahoma State University Center for Veterinary Health Sciences
PBPK	Physiologically-based Pharmacokinetic

PE	Process efficiency
R^2	Correlation coefficient
RE	Recovery
SD	Standard deviation
SFE	Supercritical fluid extraction
SPE	Solid Phase Extraction
TLC	Thin layer chromatography
UV	Ultraviolet
VBL	Vinblastine
VCR	Vincristine
VRB	Vinorelbine

Chapter I Introduction

Cancer is a disease that is prevalent throughout the world in all cultures. Cancer is caused by improperly regulated proliferation of cells, and cancer treatment using chemicals that are antineoplastic agents is referred to as chemotherapy. Vinca alkaloids, the main focus of this study, are a group of agents currently being utilized in chemotherapy.

Vinca alkaloids are antineoplastic agents that inhibit and combat cell proliferation. They disrupt mitosis, or cell division, by binding to beta-tubulin, thereby interrupting microtubule formation and function. They can also inhibit angiogenesis, or the formation of blood vessels. (C. W. N. Damen, Rosing, Schellens, & Beijnen, 2010) Members of the vinca alkaloid family include vinblastine (VBL, the subject of this study), vincristine (VCR), and vinorelbine (VRB). The majority of studies in the literature focus on vincristine, and analytical methodologies include gas chromatography-mass spectrometry (GC/MS) and liquid chromatography with tandem mass spectrometry (LC/MS/MS). As vinca alkaloids are important chemotherapeutic compounds, it is necessary to develop sensitive analytical methodologies for these materials for researchers, physicians, and crime laboratories.

In order for physicians to properly determine dosing regimens, it is necessary to understand the pharmacokinetics of the chemotherapeutic agent, or the concentration in the organism over the time course of treatment. This is possible only through sensitive

and selective analysis of biological fluids containing the chemotherapeutic agent and metabolites. As the detection methodology becomes more sensitive, the absorption, distribution, metabolism, and excretion of the drug becomes observable over longer periods of time, resulting in a more accurate prediction of the drug levels and dosing regimen for chemotherapeutic effect. In this study, the focus is on detection and quantitation of vinblastine and its major metabolite, deacetylvinblastine, over a specific time course.

Crime laboratories will find the analytical methodologies presented in this study valuable in postmortem cases where medical errors in vinca alkaloid administration may have resulted in death. Also, even though the alkaloids are generally available by prescription only, it is possible for these materials to be used in poisoning. This methodology will allow crime laboratories to detect vinca alkaloids in the body.

This study was initiated to support a pharmacokinetic study and employed LC/MS/MS to determine vinblastine and metabolite levels, although traditionally chromatography methods with non-specific detectors such as fluorescence detectors (LC/F) have been utilized. The LC/MS/MS method was developed, validated, and applied to canine plasma samples in order to begin development of a pharmacokinetic model of vinblastine.

The hypothesis of this research is that a sensitive and selective LC/MS/MS method can be developed to quantitate vinblastine and its metabolite at concentrations relevant to PBPK modeling studies (lower than 1.0 ng/mL). The following specific aims will be carried out in testing the hypothesis:

1) Develop LC/MS/MS Method
    a. Tune ion path for MS/MS detection of compounds
    b. Optimize source parameters
    c. Develop Chromatography
2) Validate LC/MS/MS method
    a. Sensitivity
3) Develop a sample preparation strategy to increase sensitivity
    a. LLE
    b. SPE
4) Validate most sensitive method
5) Apply validated method to canine plasma samples

Chapter II Review of Literature

I. History of Vinca Alkaloids

Vinca alkaloids have been used medicinally since the 1960s. They are important therapeutics in the battle against a variety of cancer types including breast cancer, non-small cell lung cancer, bladder cancer, lymphomas, and leukemia. Many vinca alkaloids have been used, including vinblastine, vincristine, and vinorelbine. (C. W. N. Damen et al., 2010)

1. Vinblastine

Vinblastine (VBL) is a natural alkaloid found in the periwinkle plant, *Catharanthus roseus*. It is primarily eliminated from the body in its unchanged form along with a small amount of deacetylvinblastine, its metabolite. (Baselt, 2008) The structure of vinblastine is shown in Figure 1 and deacetylvinblastine is shown in Figure 2.

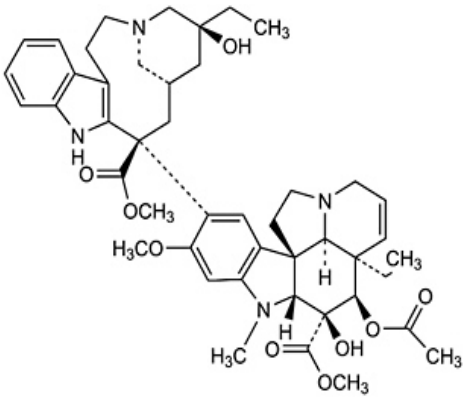

Figure 1. Chemical structure of vinblastine.
(Moffat, Osselton, Widdop, & Galichet, 2004)

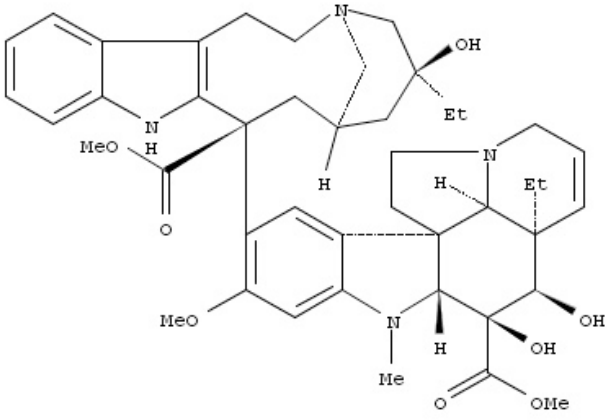

Figure 2. Chemical structure of deacetylvinblastine.
(SciFinder, 2010)

Vinblastine is administered either orally or through intravenous injection. Over a six-day period, a dose of intravenous injection is eliminated through urine and feces. The elimination product of unchanged vinblastine and deacetylvinblastine can be found in urine. Vinblastine has a half-life of 12 to 48 hours. (Baselt, 2008)

The toxicity of the drug depends on the dosage and duration of use. Side effects include nausea, vomiting, abdominal pain, muscle pain, headache, paresthesia (tingling, numbness, and pricking of the skin), malaise (discomfort), hypertension, and leucopenia (white blood cells reduction). More severe side effects include seizures, coma, epidermal necrolysis (skin loss), inappropriate secretion of antidiuretic hormone and death. (Baselt, 2008)

2. Vinorelbine

Vinorelbine (VRB) did not come into clinical use until the 1990s. It is a semi-synthetic derivative of vinblastine (Baselt, 2008). Vinorelbine has many metabolites, including desacetylvinorelbine, vinorelbine N-oxide, and 20'-OH-vinorelbine. The structure of vinorelbine is shown in Figure 3.

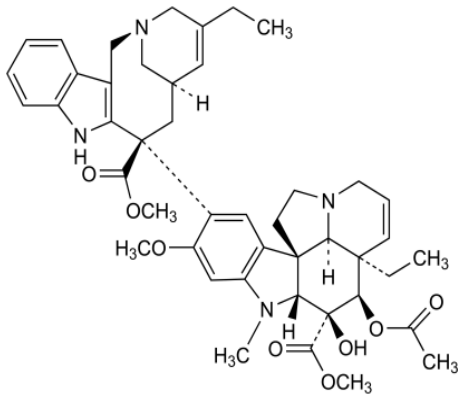

Figure 3. Chemical structure of vinorelbine.
(Moffat et al., 2004)

Vinorelbine can be absorbed rapidly when taken orally, with times ranging from 0.75 to 3.0 hours. It has a higher level of binding to platelets than to plasma proteins. One reported half-life for vinorelbine ranges from 21 to 41 hours (C. W. Damen et al.,

2008), but other sources report the half-life of vinorelbine ranging from 20 to 80 hours (Baselt, 2008).

Vinorelbine can also be taken through intravenous injection. Over a 3-week period, a dose of vinorelbine is eliminated through urine and feces. Vinorelbine, desacetylvinorelbine, vinorelbine-N-oxide, and 20`-OH-vinorelbine can all be found in urine. (Baselt, 2008)

Some side effects of vinorelbine include dose-limiting granulocytopenia, nausea, alopecia (hair loss), dyspnea (shortness of breath), paralytic ileus (intestinal blockage), thrombocytopenia (low platelets level in blood), peripheral neuropathy (nerve damage of the brain/spinal cord), fatigue, fever, tachycardia (acceleration of heart rate), myalgia, constipation, and hand-foot syndrome (leakage of blood causing tissues damage). (Baselt, 2008)

3. Methods of detection

a) Prior detection methods

Prior to LC/MS, other methods for studying vinblastine were utilized. These typically involved HPLC coupled with different detection methods, including fluorescence (Vendrig, Teeuwsen, & Holthuis, 1988) and electrochemical detection. (Volkov & Grodnitskaya, 1994)

In 1988, vinblastine was studied by using an HPLC method coupled with fluorescence. Before this method was developed, HPLC were performed on a reverse-phase column. However, this method resulted in broad and tailing peaks due to unwanted

peak absorption. The method in this paper suggested the addition of an ion-pairing reagent to the mobile phase (acetonitrile) and silica gel as the stationary phase. This alteration to the method would yield better peak shapes and a shorter retention time. Vinblastine in urine and plasma was explored with vindesine as the internal standard. Because the mobile phase had a high concentration of organic solvent, fluorescence detection was used. The calibration curve ranged from 0.5-100 ng/mL. The LOD for this method was 0.5 ng/mL for vinblastine with a noise-to-signal ratio of three. DVBL was also explored in this method; however, the HPLC method could not detect low concentrations of DVBL since DVBL co-eluted with an unknown peak. The peak was present in all samples and the two peaks could not be resolved. The overall results showed that HPLC with ion-exchange chromatography coupled with fluorescence gave a lower LOD than previous HPLC method with reverse-phase chromatography coupled with electrochemical detection. (Vendrig, Teeuwsen, & Holthuis, 1988)

In 1994, another study was done with vinblastine using an HPLC method and thin layer chromatography (TLC). For this experiment, the interest was not in plasma or urine but in the leaves of the plant *C. roseus*. Previous methods used were time consuming and involved complicated preparation steps. Therefore, TLC was used as a purification method, followed by analysis of the purified materials with HPLC. The retention time for this method ranged from 20-25 minutes, it had a very high recovery rate (greater than 96.7%), and the LOD was 2.5 µg/mL with a signal-to-noise ratio of seven. (Volkov & Grodnitskaya, 1994)

A different method of detection of vinblastine was introduced in 1996. Vinblastine was extracted from *C. roseus* plants and analyzed by capillary zone

electrophoresis (CZE) with mass spectrometry as the detection method. In this study, both vinblastine and vincristine were tested, but since both compounds are similar in structure and pKa, this caused a great challenge for method development. Voltage, buffer composition, pH and sample concentration were all explored to determine their effect on the compounds. The overall results showed that 10kV, 75 µm I.D. column, buffer pH of 6.2, 0.2 *M* ammonium acetate, and addition of an organic modifier gave the best limit of detection, resolution, and retention time. The LOD was estimated to be 1.0 ppm (or 1000 ng/mL). (Chu, Bodnar, White, & Bowman, 1996)

In 2002, another method of vinblastine analysis was developed. The method used supercritical fluid extraction (SFE) and HPLC-ESI-MS. This development is an alternative way of extracting vinblastine from plants without using any organic solvents. SFE was used to extract alkaloids from plants, which increases the solubility and desorption from the matrix. HPLC-ESI-MS was used as a simple and rapid analytical method because it does not involve any clean-up steps that could cause the loss of the analytes. For SFE, a basified supercritical carbon dioxide (CO_2) was used. The standards ranged from 0.4 – 30 µg/mL, and the assumption from this method was that the LOD was 0.4 ng/mL because no LOD or LLOQ was reported. (Choi, Yoo, & Kim, 2002)

All of the methods mentioned above were presented in peer-reviewed literature, yet the methods were not sensitive or selective enough for use in the PBPK modeling application presented here. While LC/MS results are presented above, tandem mass spectrometry (LC/MS/MS) can offer greater selectivity and is therefore the method of

choice if instrumentation is available. Therefore, the peer-reviewed literature related to vinca alkaloids analysis via LC/MS/MS is presented below.

b) LC/MS/MS methods

Many labs have developed LC/MS or LC/MS/MS methods for vinca alkaloid detection over the years. An article by Corona et al. in 2008 reported using atmospheric pressure chemical ionization liquid chromatography/tandem mass spectrometry (APCI-LC/MS/MS) to detect vincristine (VCR) in human plasma with vinblastine (VBL) as an internal standard. An APCI source was used in positive ion and multiple reaction monitoring (MRM) modes. The parent ions $[M-H]^+$ were 825.3 *m/z* and 811.3 *m/z* and daughter ions were 765.3 *m/z* and 751.3 *m/z* for VCR and VBL, respectively. (Corona, Casetta, Sandron, Vaccher, & Toffoli, 2008)

The calibrators consisted of 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, 100.0, 200.0, and 500.0 ng/mL of vincristine. Plasma sample preparation started with mixing plasma with 200 μL of methanol/0.2 M $ZnSO_4$ mixture and 20 ng/mL of VBL. The samples were then vortexed for 15 seconds and centrifuged for 10 minutes at 6000xg at 4°C. A 200μL aliquot was transferred into a vial for injection. The samples were passed through an online solid phase extraction (SPE) and liquid chromatography step before entering the MS/MS. (Corona et al., 2008)

The data underwent statistical analysis to determine the lower limit of quantitation (LLOQ), precision, and accuracy. The result for the LLOQ was 0.1 ng/mL with 1.2 to 6.8% for intra-day precision, and 3.0 to 5.1% for inter-day precision. For accuracy, the

mean relative error was from 0.1 to 6.1% for intra-day and 0.01 to 3.2% for inter-day. (Corona et al., 2008)

In the past three years, scientists have worked extensively with vincristine and vinorelbine detection using high performance liquid chromatography tandem mass spectrometry coupled with electrospray ionization (ESI-LC/MS/MS). The earliest work was the analysis of vinorelbine in mouse and human plasma in 2008. The optimized compounds have masses of 779 *m/z* and 122 *m/z* for vinorelbine, 811 *m/z* and 224 *m/z* for vinblastine, and 970 *m/z* and 355 *m/z* for vintriptol. Offline SPE was used to isolate the drugs from plasma. (C. W. Damen et al., 2008)

After spiking the plasma, the samples were agitated for ~10 s and centrifuged for 10 minutes at 10500xg. Bond-Elut C_2 cartridges were used for the extractions. The extraction started with adding 1.0 mL of methanol two times and 1.0 mL of water two times. A 200 μL aliquot of the sample was added to the column and washed twice with 1.0 mL of water. Negative pressure was applied for one minute until the column is dry. Analytes were eluted using 1.0 mL of methanol and evaporated under nitrogen gas at 40°C. The residue was resuspended using100 μL of vintripol (IS) working solution. After vortexing for one minute, the samples were centrifuged at 10900xg for 10 minute and transferred to vials. The result LLOQ for vinorelbine in mouse plasma was 0.80 ng/mL. (C. W. Damen et al., 2008)

Another article in 2009 accomplished simultaneous quantification of vincristine and actinomycin-D in human plasma using LC/MS/MS. The standards in plasma were 0.25, 1.0, 5.0, 10, 25, 50,75, and 100 ng/mL for vincristine and 0.50, 2.5, 10, 25, 50, 100,

150, and 250 ng/mL for actinomycin-D. The samples were extracted from plasma using protein precipitation and the supernatant was transferred to a vial with inserts. Vinorelbine and 7-aminoactinomycin-D (7-AAD) were used as the internal standards at a concentration of 50 ng/mL. The masses used for Q1 and Q3 detection were as follows: 825 *m/z* and 765 *m/z* for vincristine, 1256 *m/z* and 875 *m/z* for actinomycin-D, 779 *m/z* and 122 *m/z* for vinorelbine, and 1217 *m/z* and 872 *m/z* for 7-AAD. ESI, APCI, and heated ESI (H-ESI) were all tested to determine which one yielded the greatest sensitivity. The results showed that H-ESI produced the highest signal-to-noise (S/N) ratios. However, the precision was very poor; therefore, ESI was used for the remainder of the research. 7-AAD LLOQ accuracies for inter- and intra-assay met the pre-defined criteria; therefore vinorelbine was employed as the internal standard. The calculated LLOQ for vincristine was 0.25 ng/mL and the LLOQ for actinomycin-D was 0.50 ng/mL. (C. W. N. Damen, Israels, et al., 2009)

The most recent article in 2009 by this group talked about the analysis of vinorelbine and 4-O-deacetylvinorelbine in plasma using LC/MS/MS coupled with H-ESI. The calibration curve ranged from 0.10 to 100 ng/mL for both drugs. Deuterated vinorelbine, or vinorelbine-d3, was the internal standard at 50 ng/mL. The samples were extracted from human or mouse plasma by protein precipitation. The precursor ions for the vinorelbine, 4-O-deacetylvinorelbine and vinorelbine-d3 were 779 *m/z*, 737 *m/z* and 782 *m/z*, respectively. The product ion for all three drugs was 122 *m/z*. The calculated LLOQ for both vinorelbine and 4-O-deacetylvinorelbine was 0.10 ng/mL. (C. W. N. Damen, Lagas, Rosing, Schellens, & Beijnen, 2009)

A study in 1997 by Ramirez, Ogan, & Ratain used APCI-LC/MS to detect vinblastine, deacetylvinblastine, and vincristine in human plasma. The goal was to develop a method that can detect vinblastine, deacetylvinblastine, and vincristine from 0.30 to 4.00 ng/mL. The internal standard chosen for this study was vinorelbine. The samples went through liquid-liquid extractions with chloroform as the solvent and the organic phase was dried all the way down under nitrogen gas. Then, the pellet was resuspended in 100 μL of acetonitrile and transferred into vials. The calibration curve results for vinblastine ranged from 0.51 to 4.00 ng/mL, deacetylvinblastine ranged from 0.74 to 3.93 ng/mL, and for vincristine from 0.30 to 3.95 ng/mL. (Ramirez, Ogan, & Ratain, 1997)

Zhou, Tai, Sun, & Pan's research goal was to develop a method that could identify Vinca alkaloids by using direct injection ESI-MS and MS/MS, and also by ESI-LC/MS and ESI-LC/MS/MS. The sample preparation procedures included reverse phase silica gel chromatography and confirmation of compound by ultraviolet (UV), MS, and nuclear magnetic resonance (NMR) spectral data. The interest of this research was to identify vinca alkaloids and metabolites. Vinblastine precursor and product ions reported in the Zhou paper were used to compare to the ions of interest in the present research as part of method development. (Zhou, Tai, Sun, & Pan, 2005)

In summary, all the LC/MS methods reviewed above showed that LC/MS/MS is a sensitive method and vinca alkaloid, specifically vincristine, was detected at 0.050 ng/mL. The main focus of mentioned LC/MS/MS methods was on vincristine and vinorelbine, not vinblastine. Literature on the quantification of vinblastine using the

LC/MS/MS was not found; therefore, this research will focus on developing a method to analyze vinblastine using the LC/MS/MS.

II. LC/MS/MS

Liquid chromatography tandem mass spectrometry is a newer technique that is becoming more commonly used in toxicology labs. LC/MS/MS is sometimes preferred over gas chromatography due to its efficiency and sensitivity. Unlike GC, samples that are going into the LC/MS/MS for analysis do not need to go through as much preparation, and compounds that are not stable in the GC/MS are more stable in the LC/MS/MS. (*Cody*, 2006)

1. Liquid Chromatography

Liquid chromatography is defined as the separation of one or more compounds in a process involving a mobile and stationary phase. Factors that can influence compound separation are temperature, column packing, column size, solvent polarity, and flow rate. The polarity of the mobile phase depends on the solvents or the mixture of solvents. The column in the LC contains porous packing that is known as the stationary phase, which contain either silica or alumina adsorbents. LC has two types of separation, known as normal and reverse phase. (*Stafford*, 2006)

a) Normal phase chromatography

Normal phase chromatography has a nonpolar mobile phase and polar stationary phase. It is ideal to use normal phase chromatography for separation of nonpolar molecules. The idea of chromatography is to separate analytes of interest from other

molecules based on differential partitioning. Molecules will tend to stay longer with the phase that has matching polarity. As the mobile phase increase in polarity, the analytes tendency to stay in the stationary phase decreases. The retention time will be shorter because the mobile phase will have a higher polarity, causing the analytes to elute more quickly. (*Stafford*, 2006)

b) Reverse phase chromatography

Reverse phase chromatography is the opposite of normal phase. The mobile phase has high polarity and the stationary phase has low polarity. The solvents for the mobile phase usually consist of water and methanol, acetonitrile, or tetrahydrofuran. Unlike normal phase chromatography, reverse phase has better selectivity and an aqueous mobile phase, which is much preferred to organic solvents. (*Stafford*, 2006)

2. Mass Spectrometry

Mass spectrometry identifies ions traveling through the mass spectrometer and is ideal for qualitative identification. Samples can be introduced into the MS either by GC, LC, or capillary electrophoresis (CE). Once the sample enters the mass spectrometer, it goes into the ion source, which will ionize the analytes. Typically in LC/MS/MS, either electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) is used.

a) Electrospray Ionization

ESI is the most frequently used mode of ionization in the analytical toxicology field. The voltage applied determines if the nebulised droplets trapping the ionized analyte will be positively or negatively charged. ESI can produce ions in two different

conditions, positive mode and negative mode. Positive mode is best when working with basic drugs that form a stable HCl salt. In this mode, $[M+H]^+$ is the primary ion formed along with $[M+nH]^{n+}$ and $[M+nNa^+]^+$. For negative mode, it is best suited to acidic drugs that form stable Na salts. Three ions are formed from negative mode, $[M-H]^-$, $[M-nH]^{n+}$, and $[M+I^-]^-$. (*Politi, Groppi, & Polettini*, 2006)

The process for ESI involves three major components: production of charged droplets, droplet reduction and fission, and gas phase ion formation. To produce charged droplets, a large voltage is applied to the end of the capillary before the solvent reaches the MS entrance. This voltage causes the excess charge at the tip of the capillary to overcome surface tension and form a droplet. The droplets then go through desolvation and fission, which is caused by electric repulsion between like charges. The final step is to get the droplets into the gas phase, which involves several different mechanisms that play a role. (*Politi, Groppi, & Polettini*, 2006)

ESI is the ideal method to use when working with large molecules like proteins and with compounds that are not thermally stable. The ESI method can be used in a wide range of analytes, is highly efficient, and results in very little decomposition of labile analytes. However, it must be used at lower flow rates (concentration dependent), the analyte must form solution phase ions, and ion suppression is prevalent. (*Politi, Groppi, & Polettini*, 2006)

b) Atmospheric Pressure Chemical Ionization

The process for APCI is very different from ESI. For APCI, the solvent is sprayed into a heated chamber by the capillary and a coaxial flow of nebulizer gas. The

high temperature causes the solvent to evaporate leaving the analyte in the gas phase to be ionized. A corona discharge electrode is placed near the tip of the capillary to act as a source of electrons. The electrons cause the ionization of the atmosphere surrounding the tip, which causes the excess reagent ions and the analyte to interact. APCI has two different modes, negative and positive. Positive-ion mode occurs when the proton affinity of the analyte is higher than that of the reagent ions. Negative mode is when the analyte donates a proton to the reagent ions. Ionization by charge transfer can happen producing M^+ and M^- ions. (*Politi, Groppi, & Polettini*, 2006)

In cases where the polarity and molecular weight are low, APCI is the best method to use. The background level for APCI is lower than ESI; however, compounds must be thermally stable to use this method. Other APCI advantages also include: very high ionization efficiency (approaching 100%) and low susceptibility to chemical interferences. (*Politi, Groppi, & Polettini*, 2006)

c) Ion separation and detection

There are several types of mass spectrometry, but in this case a quadrupole mass spectrometer was used. Essentially, this acts as a mass filter and only allows ions of a specific mass to charge ratio to reach the detector. If a mass spectrometer has one quadrupole, it is termed a single quadrupole instrument (MS). Multiple quadrupoles coupled together with additional fragmentation allows for tandem mass spectrometry (MS/MS). In tandem mass spectrometry, there are typically three quadrupoles that allow it to function: Q1, Q2, and Q3. After ionization in the instrument source, the ions enter Q1 where precursor ions are selected specifically by their mass to charge ratio (*m/z*). The

selected precursor ions would then enter the collision cell, or Q2, where they get broken into fragments, which are known as product ions. The product ions then continue into Q3 where they are specifically selected by the mass to charge ratio (*m/z*) for detection. (*Politi, Groppi, & Polettini*, 2006)

III. Method Development

To successfully develop a method, the following steps must be followed. First, the problem must be identified, which includes reviewing the literature and selecting the analytes and internal standard. The second step is to optimize the mass spectrometry so the analyte can be detected. This includes selecting the best ionization mode (APCI or ESI), optimization of source parameters, and tuning the ion path. Next, the chromatography is optimized. After the LC/MS method is essentially developed, sample preparation, which may includes extraction processes, is optimized.

1. Tune ion path

To tune the ion path, samples are injected directly into the MS/MS to determine if the analyte of interest can be seen. It may be necessary to vary the concentration of analyte being injected, as well as modify the mode of ionization, whether it be APCI or ESI. Once it is certain that the analytes can be detected, source parameters are optimized.

2. Optimize source parameters

Like tuning the ion path, optimizing the source parameters involves direct infusion of the drug into the tandem mass spectrometer, without going through liquid chromatography. When optimizing the source parameters, it is sometimes necessary to run as much solvent into the MS/MS system as would be expected when chromatography

is developed. The goal of optimizing the source parameters is to increase the sensitivity of the analysis. This is done through modification of source parameters including voltage, gas flow and temperatures. Since ESI and APCI utilize different mechanisms of ionization, the optimized parameters for each mode are different.

3. Optimize chromatography

Once the source parameters are optimized, chromatography can be optimized. This involves selection for the appropriate chromatography column for separation. Besides selecting the correct column, flow rate and mobile phase have to be adjusted for optimal resolution and detection.

4. Optimize extraction

Optimizing extraction is part of sample preparation that involves testing many different solvents and methods of extraction that would yield the best recovery. The objectives of an extraction are to isolate the compound of interest from any interfering components and to concentrate the analyte for detection. For optimal recovery, the method must produce good yield of the analytes with the least sample loss, successful removal of other interfering components, little to no matrix effect, and the conversion of the analytes must be suitable to be detected. (Taylor, 2006) Liquid-liquid extraction (LLE) and solid phase extraction (SPE) will be focused on because they are the most common methods of extraction for vinca alkaloids.

a) Liquid-liquid extraction

LLE is the most commonly used method of extraction because drugs can be extracted directly from bodily fluids (blood, urine, etc.). LLE involves two phases, both

being liquid. To determine the best organic solvent to be used, the solvent polarity (solubility and dielectric constant) and hydrogen bonding ability are important factors to take into consideration. The drugs are usually converted to non-ionic form with a buffered solution. The non-ionic form of the drugs will partition readily in the organic solvent. The drugs are divided into 6 classes: strong acids (pK_a=1 to 5), weak acids (pK_a=5 to 9), neutrals, weak bases (neutral below pH 5), bases (neutral at 7 or above), and amphoteric bases. The pH is usually adjusted two pH units above (2 pH below for amines) the pKa of the drugs to produce 100% un-ionized forms. pH adjustment is important when doing back-extractions. Other factors that are used in consideration for solvents selection include cost, safety, and odor. (*Siek*, 2006)

b) Solid phase extraction

Solid phase extraction also involves two phases, but one is liquid and the other is solid. Typical sorbent beds include chemical functional groups that are also common to chromatography systems, like silica gel. The idea of SPE is to selectively bind drugs from an aqueous matrix, allow proteins, lipids and carbohydrates to pass through, and then allow the drugs to elute. The major goal of the process is to highly concentrate the drugs without the unwanted components. Unlike LLE where the extraction process is direct and quick, SPE involves more time, solvent, and materials. The usual steps of SPE are: wet the column with methanol, rinse with water, load samples, wash with water, rinse with methanol, dry the column, and elute the drugs with an organic solvent. (*Siek*, 2006)

IV. Method Validation

1. Sensitivity

Sensitivity refers to the smallest quantity that a method can detect (limit of detection, LOD) and quantitate (lower limit of quantitation, LLOQ).

a) Limit of detection

The limit of detection (LOD) is the calculation of the lowest concentration that the MS can detect of the ions. To calculate the LOD, the slope and the y-intercept of the line must be known. The equation of the best-fit line can be determined by plotting the quantitative ratios of each run on a scattered plot graph. The y-intercept and slope of each line will be used to calculate the standard deviation and mean of the slopes. The standard deviation will then be used to calculate the LOD. (*ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Methodology*, 1996)

Equation 1. LOD
LOD = (3.3 * SD of the y-intercept) / mean of the slope

(*ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Methodology*, 1996)

b) Lower limit of quantitation

The lower limit of quantitation (LLOQ) is the lowest concentration of the analytes that the MS can accurately and precisely measure. The calculation for LLOQ is similar to the LOD but it differs in the multiplication factor.

Equation 2. LLOQ
LLOQ = (10 * SD of y-intercept) / mean of the slope

(*ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Methodology*, 1996)

2. Precision

Precision is the reproducibility of the same results. It is determined by looking at the variability in the calibrators within a single day or between days. To determine the reproducibility of the method, the same sample must be run at least three times for comparison. The follow equation is used to determine the precision percentage:

Equation 3. Precision
% Precision = calculated concentration mean/SD of calculated concentration * 100

(Stout, Bynum, Mitchell, Baylor, & Ropero-Miller, 2009)

To calculate for precision, the concentration of each calibrator must be calculated from the standard curve. The average of the calculated concentration from each run (n=3) is taken and the standard deviation for all three calculated concentrations is used to calculate precision. To determine precision, the mean is divided by the standard deviation and multiplied by 100. (Stout et al., 2009)

3. Accuracy

Accuracy is the first step in method validation. Accuracy, also known as percent error, is calculated by determining the closeness between the experimental value and the true value. The following equation is used to calculate accuracy:

Equation 4. Accuracy
% error = (experimental value – true value)/true value x 100

(Stout et al., 2009)

For the values to be accepted, the experimental value must be within 15% of the true value, except for LLOQ where the value must be within 20%. (Stout et al., 2009)

4. Selectivity

Selectivity is used to calculate for any interference that could effect the detection of the analytes. To determine the selectivity of the method, matrix effects must be explored.

a) Matrix effects

To test for matrix effects, the samples are prepared by three different methods: no extraction (neat solution, A), spiked plasma after extraction (B), and plasma spiked before extraction (C). Matrix effect (ME) is calculated by dividing the peak area of the standards spiked after extraction (B) by the neat solution (A) and multiplying by 100 (Equation 5). The ME value takes into account any ion suppression or enhancement. The recovery percentage of the extraction is calculated by dividing plasma spiked before extraction (C) by the standards spiked after extraction (B) and multiplying by 100 (Equation 6). Precision efficiency (PE) is calculated by dividing the peak area of the pre-extraction (C) samples by neat solutions (A) and multiplying by 100 (Equation 7). The three equations are used to determine if any interference was present during the extraction process. (Matuszewski, Constanzer, & Chavez-Eng, 2003)

The following equations are used to determine matrix effects:

Equation 5. Matrix Effect
ME (%) = B/A x 100

Equation 6. Recovery Efficiency
RE (%) = C/B x 100

Equation 7. Process Efficiency
PE (%) = C/A x 100

(Matuszewski et al., 2003)

V. Case study

In 2007, an article reported a medical error case that resulted in the death of a patient. An 83-year-old woman died from an overdose of vinblastine due to mistakes made by three parties: the physician, pharmacist, and nurse. The story started when the patient was in an accident, which resulted in a contusion on her lower leg. The bruising of the leg continued for a long time, so she went to the doctor. Because the patient was diabetic and the wound would not heal, her doctor suggested that she start on therapy with a new drug that he prescribed as "vinplastin." The drug that the physician had in mind was "vasolastine," a drug that modifies vasoreactivity. The patient's daughter took the prescription to the pharmacy. The staff at the pharmacy could not read what the physician wrote and assumed that the physician wrote "vinblastin," since vinblastin and vinplastin are similar. The drug was given to the nurse who administered 5 mg of vinblastine to the patient every day without noticing that she was giving the patient an anti-cancerous drug. This caused the patient's health to deteriorate over time. The patient was admitted to the hospital after doctors realized that she was being administered a high dose of an anti-cancerous drug without reason. The patient was administered 3

different antibiotics, a granulocyte growth-inducing agent and an anti- fungal medication. The medications did not help the patient and her health continued to deteriorate. Her leukocytes dropped significantly and she was starting to have symptoms like bloody vomit, fever, pneumonia, and decrease in body pH and arterial blood pressure. The patient suffered cardiac arrest, was resuscitated, but went into a coma after four days of treatment. She died a few hours later. (Klys, Konopka, Scislowski, & Kowalski, 2007)

Postmortem examination showed that she had hemorrhage in her uterus, tubal, and ovary, petechiae (small red/purple spot caused by bleeding) in the intestinal mucous membrane and pulmonary edema (fluid in lungs). Toxicologists used APCI-LC/MS/MS method to determine the concentration of vinblastine in the patient. The analysis showed that the patient had 29 ng/g of vinblastine in her blood and 52.2 ng/g in the liver, which is much higher than what is found in a patient going through chemotherapy. (Klys et al., 2007)

Chapter III Methodology

I. Introduction

The following vinca-alkaloid studies were first carried out in methanol and water for simplicity. Once an initial method was developed, vinca alkaloids were diluted in plasma to create a finalized method that was optimized and validated. As stated previously, the following specific aims were addressed:

1) Develop LC/MS/MS Method
    a. Tune ion path for MS/MS detection of compounds
    b. Optimize source parameters
    c. Develop Chromatography
2) Validate LC/MS/MS method
    a. Sensitivity
3) Develop a sample preparation strategy to increase sensitivity
    a. LLE
    b. SPE
4) Validate most sensitive method
5) Apply validated method to canine plasma samples

1. Instrument and materials

A Shimadzu HPLC system (Shimadzu Corporation, Kyoto, Japan) consisting of a

system controller (CBM-20A), solvent delivery unit (LC-20AD), an auto-sampler (SIL-20AC) and a column oven (CTO-20AC) with a Restek Allure Pentafluorophenyl (PFP) Propyl 5µm 50x2.1mm column (Restek, Bellefonte, PA) was utilized. An Applied Biosystems 4000 Q- Trap MS/MS System (Applied Biosystems, Foster City, CA) equipped with a Turbo V™ electrospray ionization source, a Harvard Apparatus syringe pump (Holliston, MA) and a NitroGen N300DR nitrogen generator was used (Peak Scientific Instruments Ltd, Paisley, United Kingdom). Analyst® 1.5 Software was used to control the instrument and for data analysis. For SPE, an Oasis HLB Water 1 mL cartridge was used.

Chemicals used include: ACS grade methanol (VWR International, West Chester, PA) and 98% formic acid (EMD Chemicals, Gibbstown, NJ); HPLC grade acetonitrile (OmniSolv, EM Science, Gibbstown, NJ) and methanol (OmniSolv, EMD, Gibbstown, NJ); Ammonium formate, 99% (Alfa Aesar, Ward Hill, MA); Ammonium acetate crystal (Mallinckrodt, Phillipsburg, NJ); glacial acetic acid (EMD, Gibbstown, NJ); isopropyl alcohol (Mallinckrodt, Paris, KY); HPLC grade hexane (Acros, NJ); ethyl acetate (Acros, NJ), ethyl ether (VWR, BDH, West Chester, PA); chloroform (BDH, West Chester, PA). Plasma, vinblastine, deacetylvinblastine, and vinorelbine in methanol solution were provided by collaborators at the Oklahoma State University Center for Veterinary Health Sciences (OSU CVHS).

II. Specific Aim 1: Develop LC/MS/MS method

1. Tuning ion path for MS/MS detection of compounds

A mixture of all three chemicals was infused into the tandem mass spectrometry at a concentration of 100 ng/mL for vinblastine, vinorelbine, and deacetylvinblastine. The flow rate was 20 μL/min. For this experiment, APCI-LC/MS/MS was be utilized for detection, based on a comparison of signal to noise ratios. Table 1 shows the parent ions (Q1) and daughter ions (Q3) that the MS/MS was detecting for each compound.

Table 1. Q1 and Q3 masses for VRB, DVBL, and VBL.

	VRB	**DVBL**	**VBL**
Q1	**(779.4 m/z)**	**(769.4 m/z)**	**(811.5 m/z)**
Q3	122.2	124.2	144.1
	457.2	144.1	224.1
	658.2	355.1	355.1

Mobile phase:

Eluent A – (0.20% Formic, 0.20% Ammonium formate in water) 2.0 mL 1.0 M ammonium formate, 2.0 mL formic acid, 996 mL DI H_2O

Eluent B – 2.0 mL 1.0 M-ammonium formate, 2.0 mL formic acid, 996 mL Acetonitrile (ACN)

2. Optimize source parameters

To optimize the source parameters, standards were injected directly into the MS/MS. Direct infusion was used to determine the optimal parameters (gas 1, gas 2, temperature, etc.) for detection. Table 2 shows the parameters that were set for the method that was used to run the calibrators.

Table 2. LC/MS/MS Source parameters

Gas 1	Gas 2	Temp	CE	CXP
40	20	400	49	8

3. Chromatography optimization

The parameters for chromatography optimization are shown in Table 3.

Table 3. Injection and flow rate parameter

Time (min)	Eluent A	Eluent B	Injection Volume (µL)
13	30% (0.15 mL/min)	70% (0.35 mL/min)	20

The flow rate is at a constant (isocratic) ratio of 3:7 for Eluent A and Eluent B, respectively. The retention for each drugs are as followed:

DVBL: ~6.0 min.

VBL: ~ 7.2 min.

VRB: ~10.9 min.

Vinorelbine was used as the internal standard. Calibrators were made in methanol at the concentration of 100 ng/mL, 10 ng/mL, 5.0 ng/mL, 1.0 ng/mL, and 0.10 ng/mL DVBL and VBL together with 50 ng/mL of internal standard in each. The blanks were made up of only 50 ng/mL of internal standard in methanol.

III. Specific Aim 2: Validate LC/MS/MS Method

1. Sample preparation

A stock solution of each drug was provided at the following concentrations:100 μg/mL VRB , 50 μg/mL DVBL, and 50 μg/mL VBL. The calibrators were prepared at the following concentrations: 100 ng/mL, 10 ng/mL, 1.0 ng/mL, and 0.10 ng/mL. To reach the desired concentration for the standard curve, the calibrators underwent dilutions.

a) Dilutions

The first dilution was done by pipetting 100 μL of 50 μg/mL DVBL and 50μL of 100 μg/mL VBL into a 1.0 mL volumetric; deionized water was used to bring to volume. This was the standard mixture used for dilutions. 50 μg/mL of VRB was diluted down to 500 ng/mL with deionized (DI) water.

200 μL of 5.0 μg/mL of standard mix were diluted with water to make 100 ng/mL. 1.0 mL of 100 ng/mL standards was diluted down to 10 ng/mL by adding 9.0 mL

of water. The last step was repeated for 1.0 ng/mL and 0.10 ng/mL standards. After each dilution step, the samples were vortex for 10 seconds and centrifuged at 3000 rpm for 10 seconds. 100 L of internal standard was added to 1.0 mL of standards. All dilutions were done at room temperature, ~25°C.

Table 4. Dilutions of stock solution

	Concentration	Amount of drug added	Final Volume
VRB	50 μg/mL		
VRB 1	500 ng/mL	10 μL [VRB]	1.0 mL
DVBL	250 μg/mL		
DVBL 1	5.0 μg/mL	20 μL [DVBL]	1.0 mL
DVBL 2	500 ng/mL	100 μL [DVBL 1]	1.0 mL
DVBL 3	200 ng/mL	40 μL [DVBL 1]	1.0 mL
DVBL 4	50 ng/mL	100 μL [DVBL 2]	1.0 mL
DVBL 5	20 ng/mL	100 μL [DVBL 3]	1.0 mL
DVBL 6	5.0 ng/mL	100 μL [DVBL 4]	1.0 mL
VBL	50 μg/mL		
VBL 1	500 ng/mL	10 μL [VBL]	1.0 mL
VBL 2	200 ng/mL	400 μL [VBL 1]	1.0 mL
VBL 3	50 ng/mL	100 μL [VBL 1]	1.0 mL
VBL 4	20 ng/mL	100 μL [VBL 2]	1.0 mL
VBL 5	5.0 ng/mL	100 μL [VBL 3]	1.0 mL

Table 4 shows the dilution of the stock solutions that were used to make the calibrators. For example, to make 1.0 mL of 200 ng/mL VBL, 400 μL of 500 ng/mL VBL ([VBL 1]) was added to a 1.0 mL volumetric test tube and filled up to the line with

methanol. All stock solutions were made up in methanol at room temperature and stored in the fridge.

2. Sensitivity determination

The stock solutions were then analyzed using the optimized LC/MS/MS method from Specific aims 1 and 2. Following data collection, the data were analyzed to determine sensitivity according to the methodology described in Chapter II, section IV.

IV. Specific Aim 3: Develop a sample preparation strategy to increase sensitivity

The method mentioned above was used to optimize the MS/MS and chromatography. Samples used for optimization was made up in MeOH. Once the sensitivity was determined, the samples underwent LLE or SPE to determine if sensitivity had been enhanced.

1. LLE protocol

After adding the internal standard to each dilution, 500 μL of organic solvents were added for extraction. The extractions were performed twice for better recovery. Three different mixtures of organic solvents were used during this research to see which one gave the best recovery.

Extraction 1 – Hexane: Ethyl acetate [9:1]

Extraction 2 – Chloroform: Isopropanol [10:1]

Extraction 3 – Ethyl ether

Because the drying state for each extraction can affect the recovery, the samples were either dried all the way or partially dried to compare the recovery rate. Extraction 1 and Extraction 2 were left to air dry for one to two minutes. Since ether is a volatile organic, the samples from Extraction 3 were left to air dry after extraction. 100 μL of Eluent A and Eluent B [3:7] was used to resuspend the samples after drying.

2. Solid Phase Extraction

a) Parameters for SPE samples

Some parameters for LLE stayed the same for SPE with some alterations. Below are modifications of the LC/MS/MS method for SPE samples.

Eluent A2: 5.0 mM ammonium acetate at pH 3.5

Eluent B2: 100% HPLC Methanol

The parameters for chromatography optimization are shown in Table 5.

Table 5. Injection and flow rate parameter

Time (min)	Eluent A2	Eluent B2	Injection Volume (μL)
15	10% (0.075 mL/min)	90% (0.675 mL/min)	100

The flow rate was set at a constant (isocratic) ratio of 1:9 for Eluent A2 and Eluent B2, respectively. The retention time for each drugs were:

DVBL: ~5.0 min.

VBL: ~ 3.8 min.

VRB: ~4.5 min.

Vinorelbine was used as the internal standard. Calibrators were made in methanol at concentrations of 5.0 ng/mL, 1.0 ng/mL, 0.50 ng/mL, and 0.10 ng/mL of both DVBL and VBL with 10 ng/mL of internal standard in each. The blanks were made up of 10 ng/mL of internal standard in methanol.

b) Dilutions

For plasma samples, the lower range of the calibrators was used. The plasma calibrators' concentrations were 5.0 ng/mL, 1.0 ng/mL, 0.50 ng/mL, and 0.10 ng/mL. The internal standard concentration was 10 ng/mL. Because plasma clogged up the SPE column, the samples underwent dilutions prior to solid phase extraction (SPE). The resuspending buffer was 5.0 mM ammonium acetate (pH=3.5), acetonitrile, and methanol [50:25:25]. Below is the dilution scheme for plasma solid phase extraction samples:

Table 6. Sample dilutions for plasma calibrators.

Concentration (ng/mL)	**VBL**	**DVBL**	**VRB**	**Plasma (µL]**
10	10 µL [VBL 1]	10 µL [DVBL 2]	10 µL [VRB 1]	480
5	12.5 µL [VBL 2]	12.5 µL [DVBL 3]	10 µL [VRB 1]	475
1	10 µL [VBL 3]	10 µL [DVBL 4]	10 µL [VRB 1]	480
0.5	12.5 µL [VBL 4]	12.5 µL [DVBL 5]	10 µL [VRB 1]	475
0.1	10 µL [VBL 5]	10 µL [DVBL 6]	10 µL [VRB 1]	480
Blank	0	0	10 µL [VRB 1]	480 (+ 20 µL MeOH)

Table 6 shows the dilution for each calibrator using the corresponding stock solutions. Plasma was aliquoted into microcentrifuge tubes in the amount listed in Table 6. 10 μL of 500 ng/mL VRB was added to each calibrator as the internal standard. The number in the brackets corresponds to the stock solution number in Table 4. For example, 10 μL of 500 ng/mL VBL ([VBL 1]), 500 ng/mL DVBL ([DVBL 2]) and 500 ng/mL VRB ([VRB 1]) was added to 480 μL of plasma to make up the 10 ng/mL calibrator. After the addition of the drugs into the microcentrifuge tubes containing plasma, the samples were vortexed and centrifuged (3000 rpm) for 10 seconds. To dilute the plasma, 490 mL of deionized (DI) water was added to each tube. The samples were mixed by drawing the solution up and down inside a pipettor. All dilutions were done under a vacuum hood and at room temperature.

c) SPE protocol

For solid phase extraction (SPE), an Oasis HLB Cartridge (1.0 mL) (Waters Corporation, Milford, MA) was used. The column was conditioned with 1.0 mL of methanol, then 1.0 mL of DI water. The samples were loaded onto the column. After the samples were drawn through the column, 2.0 mL of DI water was used to wash the cartridge. The column was dried under vacuum for 30 minutes. The waste and test tube were discarded. The samples were eluted by adding 2.0 mL of methanol and collected in new test tubes. The cartridges were dried down completely (~5.0 minutes). 125 μL of 5.0 mM ammonium acetate (pH=3.5), acetonitrile, and methanol [50:25:25] was used as the resuspending buffer.

V. Specific Aim 4: Validate most sensitive method

Method validation included four calculations: sensitivity, selectivity, precision, and accuracy. The goal of this research was to reach an LLOQ of less than 1.0 ng/mL; therefore, sensitivity was the deciding factor if a method was sensitive enough for analysis. Until the LLOQ reaches a concentration lower than 1.0 ng/mL, selectivity, precision and accuracy will not be analyzed. The calculations for method validation were determined in the following order: sensitivity, selectivity, precision, and accuracy.

1. Sensitivity

The sensitivity was determined by the LOD and LLOQ values. A standard curve was generated and was used in calculating the LOD and LLOQ. Equation 1 and Equation 2 were used to determine the LOD and LLOQ for VBL and DVBL.

2. Selectivity

Selectivity was determined by calculating for matrix effects. For the interest of this research, three different equations were used to calculate for matrix effects. Equation 5, Equation 6, and Equation 7 were the three main equations used to calculate for matrix effects.

3. Precision

Precision was calculated by using the mean and standard deviation of the calculated concentration from the plasma samples. Using the calculated quantitation ratio, three calibration curves were generated from the three runs within a single day. The average y-intercept and slope was determined and used in calculating for the

concentrations of each calibrator based on the curve. The three new calculated values for each calibrator were used in calculating for the average concentration and standard deviation. Equation 3 was used to determine the method precision.

4. Accuracy

Accuracy was calculated by using the experimental and true value to determine the variability between each run. Using the calculated quantitation ratio, a calibration curve was generated. Three curves were generated for each drug and the average y-intercept and slope was calculated. The two values were used to back calculate for the concentration of each calibrator based on the curve. Those calculated (experimental) values were used to determine the variability between each run. Equation 4 was used to analyze the accuracy of the method.

VI. Specific Aim 5: Application of validated method to canine plasma

Once the developed method was finalized and validated, it was applied to canine plasma. The extraction protocol was transferred to the collaborating party for application to canine plasma, then the extracted canine samples were transferred to this laboratory for LC/MS/MS analysis.

Chapter IV Results

I. Specific Aim 1: Developed LC/MS/MS Method

An LC/MS/MS method was developed as described in the Methods section. Figure 4 shows a representative chromatogram from a calibrator generated with this method.

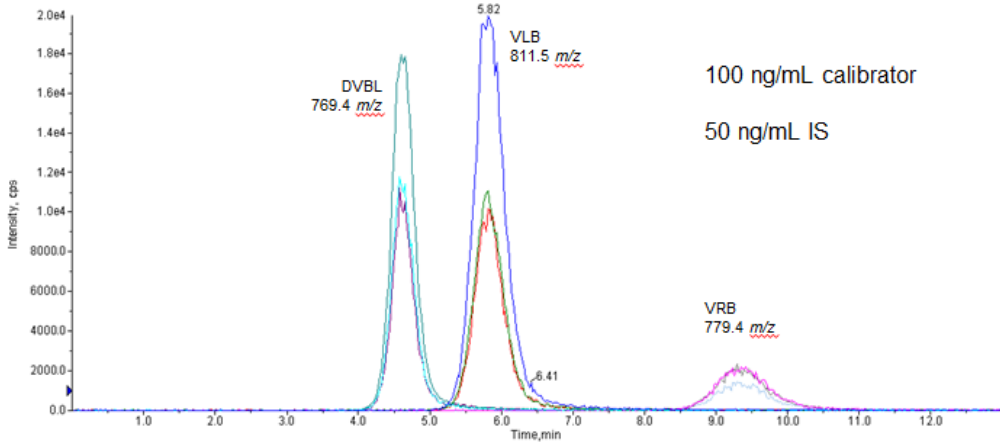

Figure 4. Sample chromatogram of VBL, DVBL, and VRB.

II. Specific Aim 2: Validation of LC/MS/MS Method

1. LOD and LLOQ for calibrators in methanol only

Table 7 shows the quantitative ratios for VBL and DVBL on the three runs that were done on the same day. The calibrators were in methanol only, no extractions.

These quantitative ratios were used to generate the standard curve, shown in Figure 5 and Figure 6.

Table 7. Quantitative ratios for VBL and DVBL

VBL	**a**	**b**	**c**
10 ng/mL	0.642	0.698	0.678
5 ng/mL	0.222	0.213	0.213
1 ng/mL	0.0688	0.0711	0.0698
0.1 ng/mL	0.00888	0.00725	0.00690
DVBL	**a**	**b**	**c**
10 ng/mL	0.586	0.658	0.634
5 ng/mL	0.210	0.198	0.178
1 ng/mL	0.0563	0.0652	0.0565
0.1 ng/mL	0.00551	0.00704	

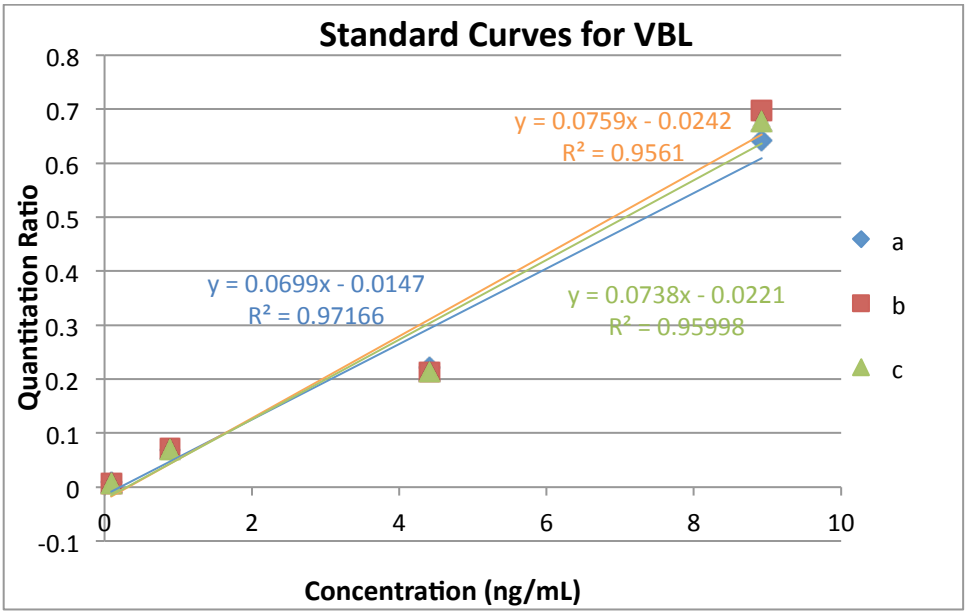

Figure 5. Calibration curve for VBL in methanol of three different runs.

Figure 5 shows the standard curve for VBL on three different runs. The samples were all done on the same day. The R^2 value determines how well the best-fit line matches the data. As show on the graph, the R^2 value for all three lines are greater than 0.950. The mean slope for all three curves is 0.0677 and the SD for the y-intercepts is 0.00522.

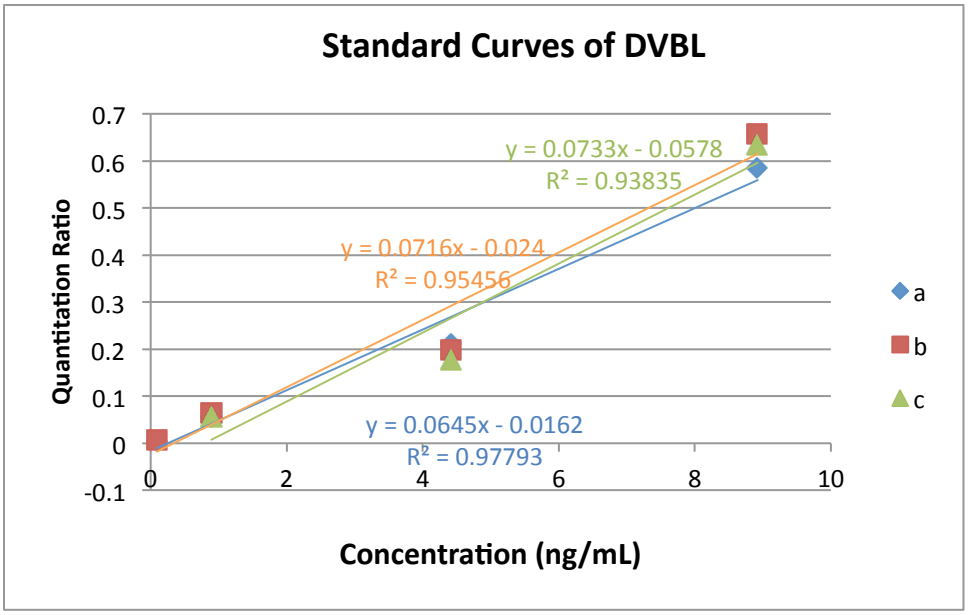

Figure 6. Calibration curve for DVBL in methanol of three different runs.

Figure 6 showed the calibration curve for DVBL only. The runs were all done on the same day. All R^2 are higher than 0.938. The average of all three slopes is 0.0621 and the SD for the y-intercepts is 0.0223.

Table 8. LLOQ and LOD for vinblastine and deacetylvinblastine in MeOH

	Vinblastine (ng/mL)	**Deacetylvinblastine (ng/mL)**
LOD	≈0.25	≈1.2
LLOQ	≈0.77	≈3.6

Table 8 shows the limit of detection and limit of quantitation for both VBL and DVBL. To calculate for the LOD, the mean of the slope and the SD of the y-intercept

must be calculated from the standard curve generated from the quantitative ratios. The values mentioned above were used to calculate the LOD and LLOQ of VBL and DVBL. The LOD and LLOQ for VBL are 0.254 ng/mL and 0.770 ng/mL, and for DVBL are 1.186 ng/mL and 3.595 ng/mL, respectively. The results show that both LLOQ and LOD for VBL are lower than DVBL.

III. Specific Aim 3: Developed sample preparation strategy to increase sensitivity

1. Liquid-liquid extraction results

a) LLE solvents

As mentioned above, three different LLE solvents were tested. The first solvent was a mixture of hexane and ethyl acetate. The chromatogram (Figure 7) showed that the solvent did not work in extracting the drugs. The next solvent was a mixture of chloroform and isopropanol. The extraction of VBL and DVBL was successful, however, the internal peak was not present (Figure 8). Note that the intensity of VBL is at 100,000 cps. The last solvent used was ethyl ether. Figure 9 shows the chromatogram of the extraction; notice that all three peaks are present. However, the intensity of VBL is only 19,000 cps.

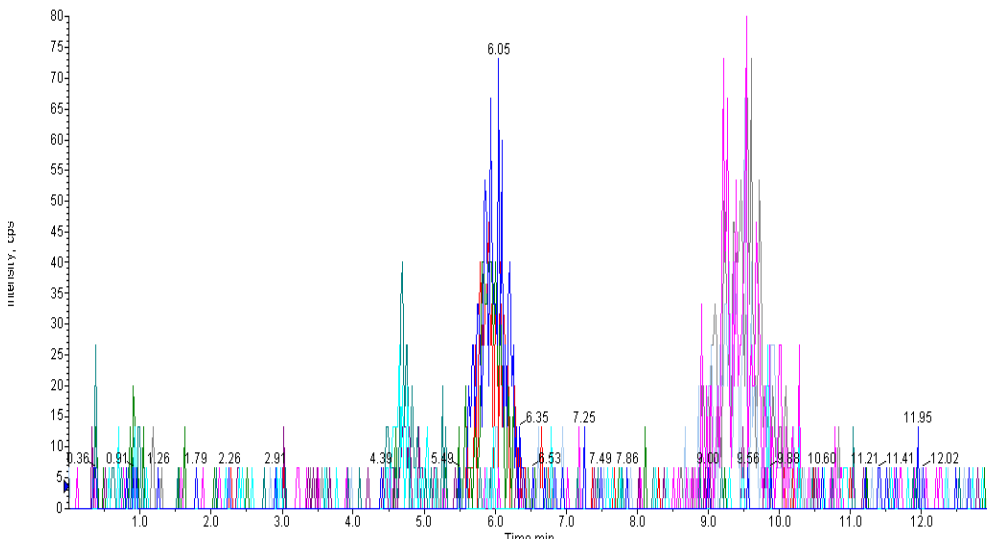

Figure 7. Intensity versus time chromatogram of hexane: ethyl acetate LLE. The standards are at 100 ng/mL with the IS at 50 ng/mL. No peaks detected.

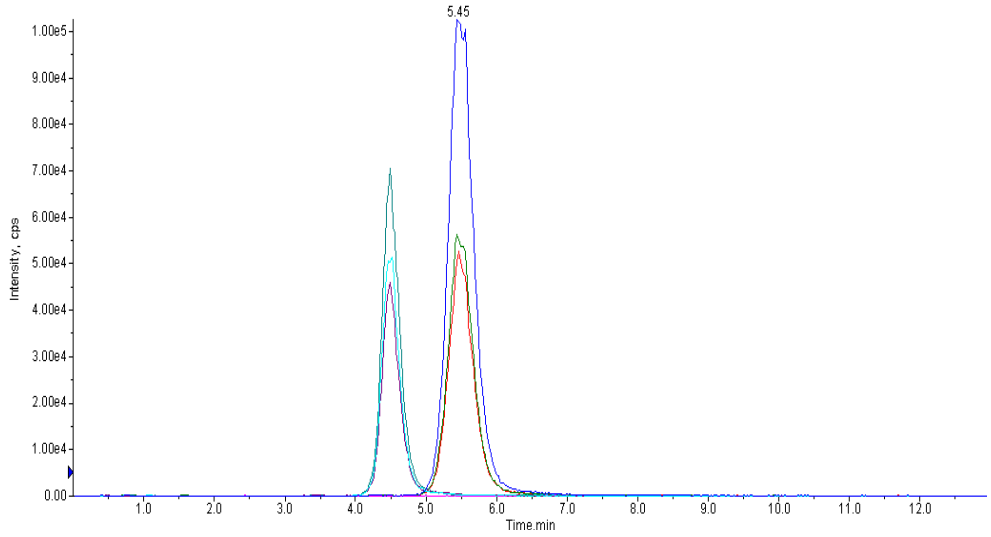

Figure 8. Chromatogram of LLE with chloroform: isopropanol mixture. The standards are at 100 ng/mL with the IS at 50 ng/mL. DVBL is at the RT 4.5 min (first peak), VBL RT is 5.5 min (second peak), and no VRB peak.

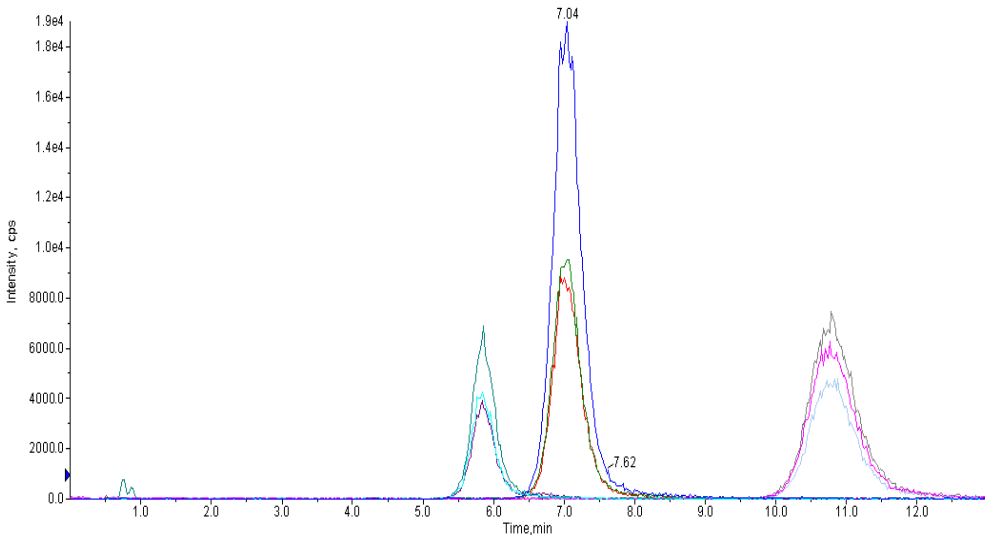

Figure 9. Chromatogram of ether LLE. The standards are at 100 ng/mL with the IS at 50 ng/mL. DVBL is at the RT 5.9 min (first peak), VBL RT is 7.0 min (second peak), and VRB RT is at 10.8 min (third peak).

b) Drying methods

From the results above, chloroform and isopropanol gave the best VBL and DVBL recovery. However, the internal standard peak was missing. To determine what caused the peak disappearance, different methods of drying were tested. Initially, the drying method was to let the samples air dry, which took 1.5 to 2.0 hours. However, an alternative method of drying was used to reduce the drying time. Nitrogen gas was the alternative method and reduced the drying time to about 30 min. Below are sample chromatograms of air drying (Figure 10) versus drying with nitrogen gas (Figure 11). Notice that the internal standard peak is not present in Figure 10, but reappears in Figure 11.

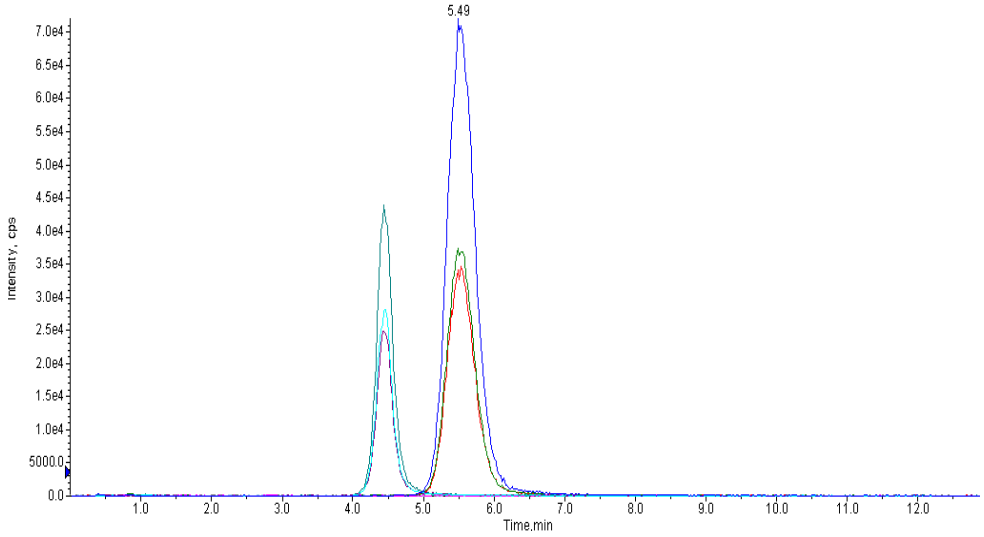

Figure 10. Chromatogram of air-dried sample.

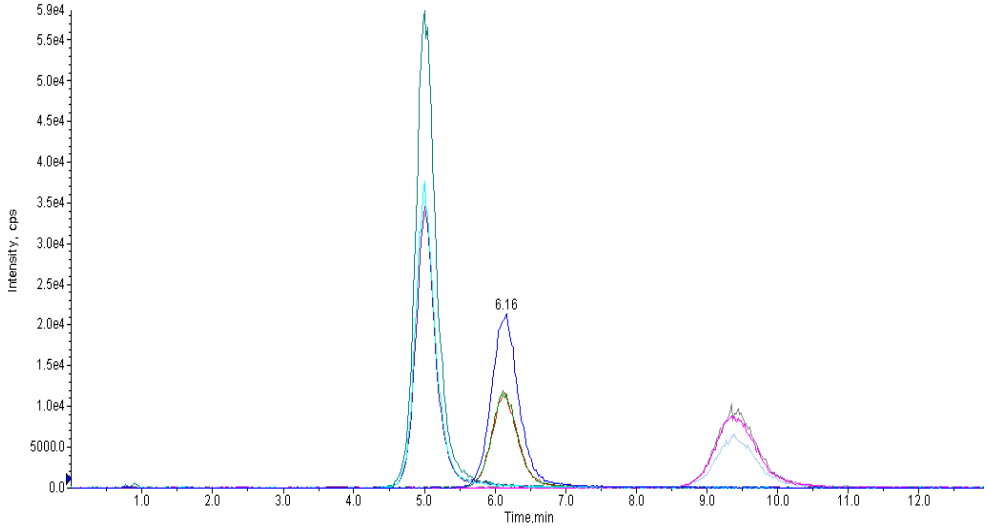

Figure 11. Chromatogram of drying method with nitrogen gas.

Overall, the best method of LLE was using ether as a solvent because all peaks were recovered without any problem of byproducts. Different drying times were tested

for ether also, but the drying time did not affect the overall recovery, presumably due to its volatile state.

c) LLE with plasma

Since the best LLE method utilized ether, it was applied to plasma samples. The results for ether extraction with plasma were not the same as those obtained with methanolic standards. Ether was not able to extract all the proteins in the samples, therefore, caused the column in the LC to become clogged and leak. An alternative way to remove the proteins was by using acetonitrile (ACN). An ACN crash method was used and all proteins were successfully removed. However, along with the proteins, the drugs were also removed. Figure 12 shows the chromatogram of an ACN crash. Notice that nothing was detected, which means that all the drugs were lost during the protein crash. Because LLE did not work with plasma, the second option was to use SPE to clean up the samples.

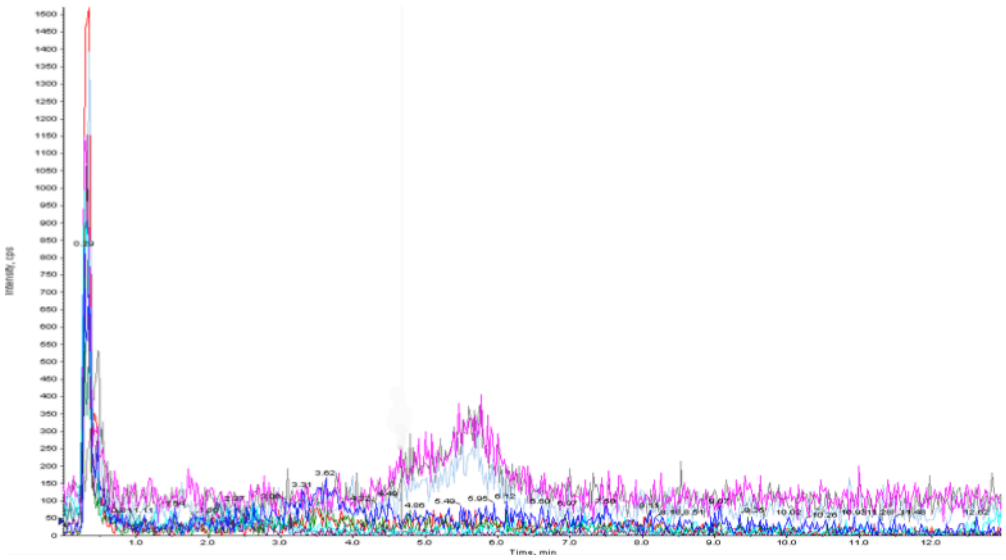

Figure 12. Chromatogram of ACN crash.

2. Solid phase extraction results

Both LLE and SPE methods were test in sample clean up and preparation and the results were compared. Overall, SPE was the best method for the vinca alkaloids because proteins were successfully removed from the samples and no drugs were lost during the process. SPE samples did not clog up the system and cause leaking like LLE samples. Because SPE successfully recovered all drugs and removed unwanted compounds, the next step is to determine the LOD and LLOQ. Below are method validation results for SPE.

IV. Specific Aim 4: Validation of the most sensitive method

1. Sensitivity: Calibration curve, LOD, and LLOQ for calibrators in plasma

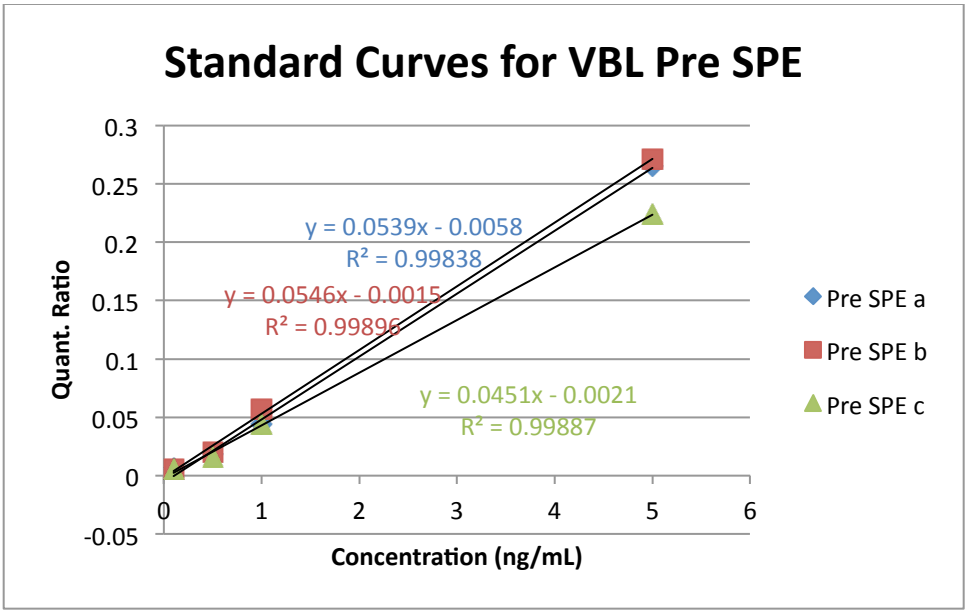

Figure 13. Calibration curves of pre-SPE VBL.

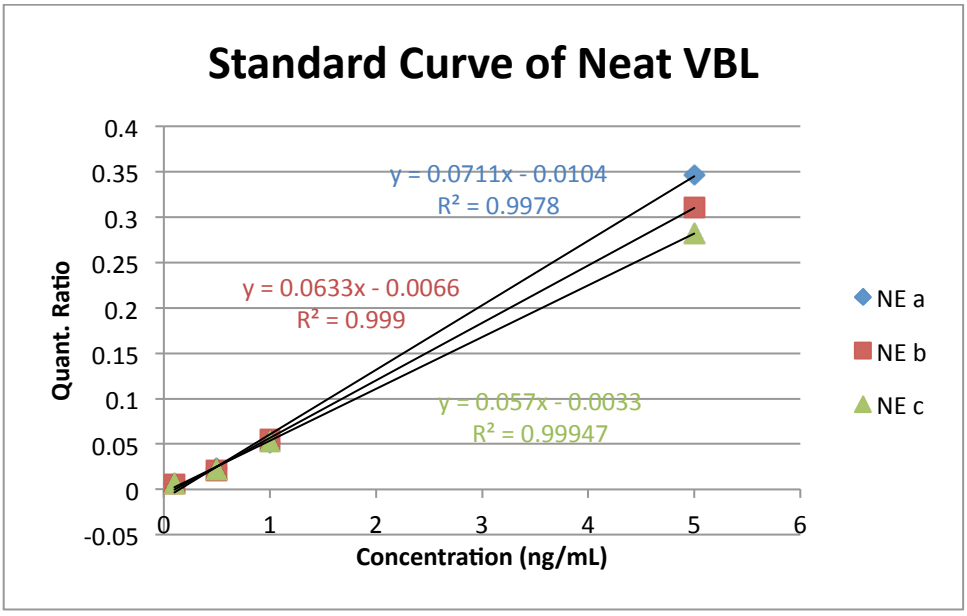

Figure 14. Calibration curves for neat VBL.

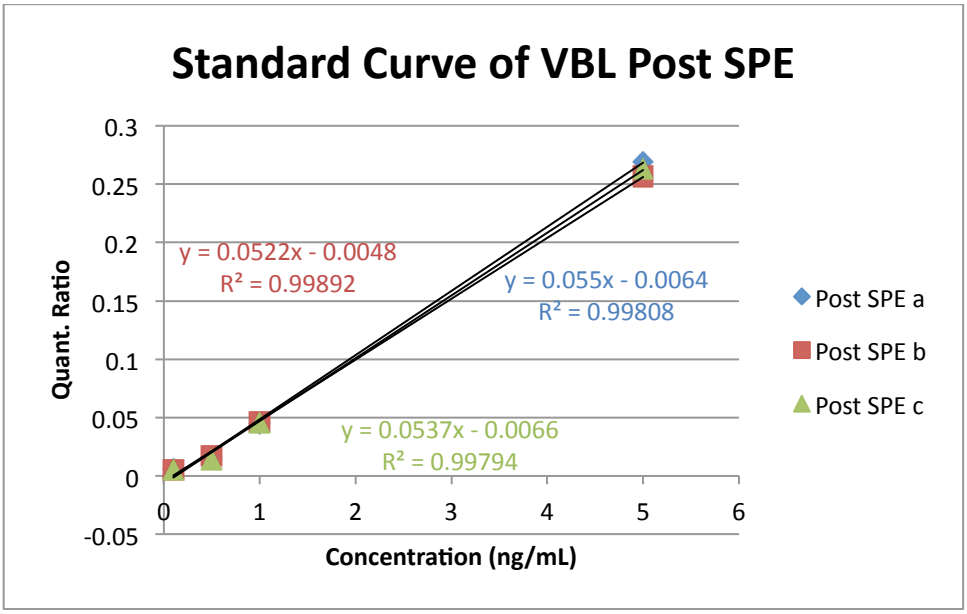

Figure 15. Calibration curves for post-SPE VBL.

Figure 13, Figure 14, and Figure 15 are standard curves for VBL of pre-SPE, neat, and post-SPE. The figures show the linearity of all three curves for each set of

calibrators. Notice that all three curves within a set of samples are very close to each other, if not overlapping. This shows that each sample was replicated successfully with little variation. The correlation coefficients, R^2, for every curve in all three figures were above 0.997. The calculated mean slope for all three curves in Figure 13 was 0.0512 and the SD of the y-intercepts was 0.00233. In Figure 14, the calculated mean slope is 0.0638 and the SD is 0.00355. In Figure 15, the calculated mean slope is 0.0536 and the SD is 0.000987. The SD value and the mean slope will be used in determining the LOD and LLOQ of VBL.

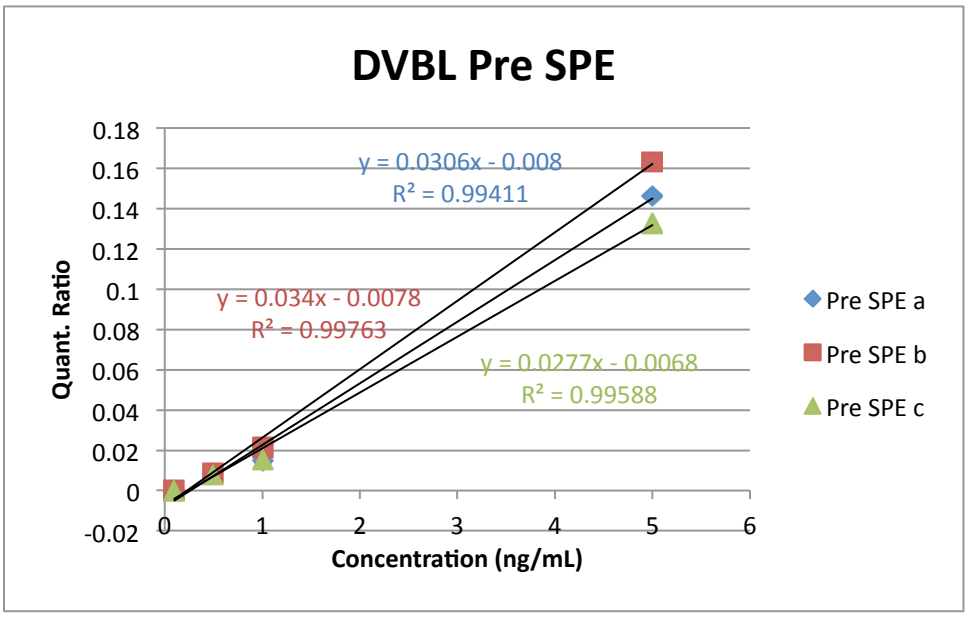

Figure 16. Calibration curves of pre-SPE DVBL.

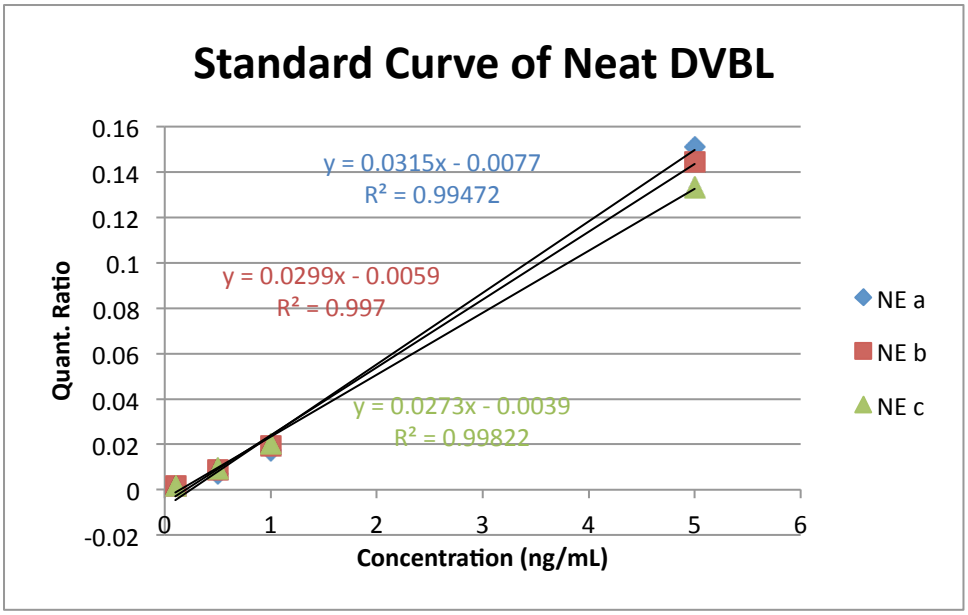

Figure 17. Calibrations curves for neat DVBL.

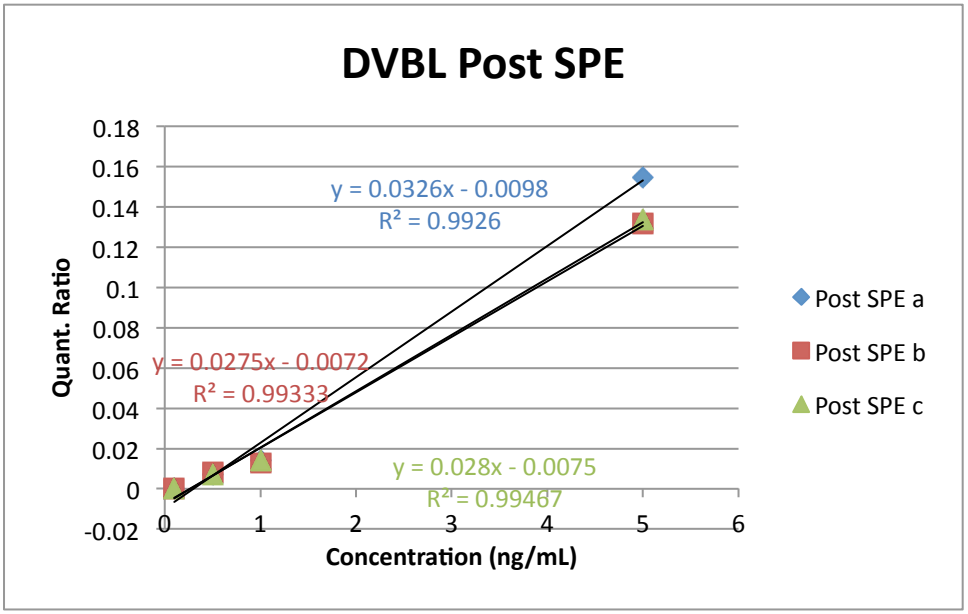

Figure 18. Calibration curves for post-SPE DVBL.

Figure 16, Figure 17, and Figure 18 are standard curves for DVBL of pre-SPE, neat, and post-SPE. The figures show the linearity of all three curves for each set of

calibrators. Again, notice that all three curves within a set of samples are very close to each other, if not overlapping. This shows that each sample was replicated successfully with little variation. The correlation coefficients, R^2, for every curve in all three figures are above 0.994. The mean slope for Figure 16, Figure 17, and Figure 18 is 0.0308, 0.0296, and 0.160, respectively. The SD of the y-intercepts for Figure 16, Figure 17, and Figure 18 is 0.000643, 0.00190, and 0.484, respectively. The SD value and the mean slope will be used in determining the LOD and LLOQ of DVBL.

Table 9. LOD and LLOQ for VBL and DVBL for neat, pre-SPE, and post-SPE

	Neat (ng/mL)		**Pre SPE (ng/mL)**		**Post SPE (ng/mL)**	
	LOD	LLOQ	LOD	LLOQ	LOD	LLOQ
VBL	0.184	0.557	0.150±0.016	0.455±0.047	0.061	0.184
DVBL	0.212	0.643	0.069±0.003	0.209±0.01	0.160	0.484

Table 9 shows the LOD and LLOQ for VBL and DVBL for all three different methods. LODs for VBL were 0.184, 0.150, and 0.061 ng/mL for neat, pre-SPE, and post-SPE, respectively. The estimated LODs for DVBL were 0.212, 0.069, and 0.160 ng/mL for neat, pre-SPE, and post-SPE, respectively. Estimated VBL LLOQs for the three methods were about 0.557, 0.455, and 0.184 ng/mL. Estimated DVBL LLOQs were around 0.643, 0.209, and 0.484 ng/mL. Since the goal of this research was to obtain a LLOQ lower than 1.0 ng/mL, the next steps were to calculate for recovery, matrix effects, precision, and accuracy of the method.

2. Selectivity

a) Matrix effect, recovery, and process efficiency

The calculations for matrix effects were done for only the 5.0 ng/mL calibrators. The numbers shown in columns A, B, and C are the peak area of each drug. As shown in the table, each column was assigned a letter corresponding to the letters in Equation 5, Equation 6, and Equation 7. To calculate for ME(%), the mean value for column B was divided by the mean value in column A for each drug. For RE(%), the mean value in column C was divided by the mean value in column B for each drug. For PE(%), the mean value in column C was divided by the mean value in column A for each drug. The results for ME(%) were 65.7%, 76.5%, and 78% for VBL, DVBL, and VRB, respectively. RE(%) results were 65.3%, 72%, and 68.7 for VBL, DVBL, and VRB, respectively. Lastly, the values for PE(%) were 42.9%, 55.1%, and 53.6% for VBL, DVBL, and VRB, respectively. The calculations and results are shown on Table 10.

Table 10. ME, RE, and PE calculations for 5.0 ng/mL VBL, DVBL, and VRB in neat, post-SPE, and pre-SPE

	A	B	C		
	VBL Neat	VBL Post	VBL Pre		
1	8.91E+05	5.47E+05	2.86E+05	ME (%)	65.7
2	8.39E+05	5.34E+05	4.04E+05	RE (%)	65.3
3	7.99E+05	5.81E+05	3.96E+05	PE (%)	42.9
Mean	8.43E+05	5.54E+05	3.62E+05		
% Avg. recovery	42.9				
	DVBL Neat	DVBL Post	DVBL Pre		
1	3.88E+05	3.14E+05	1.58E+05	ME (%)	76.5
2	3.90E+05	2.74E+05	2.43E+05	RE (%)	72.0
3	3.77E+05	2.95E+05	2.35E+05	PE (%)	55.1
Mean	3.85E+05	2.94E+05	2.12E+05		
% Avg. recovery	55.1				
	VRB Neat	VRB Post	VRB Pre		
1	2.57E+06	2.03E+06	1.08E+06	ME (%)	78.0
2	2.70E+06	2.08E+06	1.49E+06	RE (%)	68.7
3	2.83E+06	2.21E+06	1.77E+06	PE (%)	53.6
Mean	2.70E+06	2.11E+06	1.45E+06		
% Avg. recovery	53.6				

3. Precision

Table 11. Precision calculations for VBL and DVBL from pre-SPE samples

VBL						
	Pre SPE a	Pre SPE b	Pre SPE c	Mean	SD	Precision
5 ng/mL	5.021	4.993	5.007	5.007	0.014	0.27%
1 ng/mL	0.922	1.076	1.037	1.011	0.080	7.91%
0.5 ng/mL	0.440	0.402	0.394	0.412	0.024	5.85%
0.1 ng/mL	0.221	0.127	0.170	0.173	0.047	27.41%
Average					0.041	10.36%
DVBL						
	Pre SPE a	Pre SPE b	Pre SPE c	Mean	SD	Precision
5	5.042	5.026	5.039	5.036	0.009	0.17%
1	0.753	0.863	0.799	0.805	0.055	6.89%
0.5	0.545	0.484	0.526	0.518	0.031	5.99%
0.1	0.261	0.229	0.245	0.245	0.016	6.52%
Average					0.028	4.89%

Table 11 shows the precision results for VBL and DVBL. Precision was calculated by taking the calculated SD and dividing by the mean of the three pre-SPE runs for each calibrator. As shown on the table, from 5.0 ng/mL to 0.50 ng/mL VBL the precision percentages were under 10%, but at 0.10 ng/mL the precision was 27.41%. The precision percentage for DVBL ranges from 6.89% - 0.17%.

4. Accuracy

Table 12. Vinblastine and Deacetylvinblastine Accuracy Calculations

	Calculated concentration (ng/mL)				
VBL	Pre-SPE a	Pre-SPE b	Pre-SPE c	Mean calc. conc.	Accuracy (% error)
5 ng/mL	5.021	4.993	5.007	5.007	0.14
1 ng/mL	0.922	1.076	1.038	1.011	1.14
0.5 ng/mL	0.440	0.402	0.394	0.412	-17.58
0.1 ng/mL	0.221	0.127	0.170	0.173	72.70
	Calculated concentration (ng/mL)				
DVBL	Pre-SPE a	Pre-SPE b	Pre-SPE c	Mean calc. conc.	Accuracy (% error)
5 ng/mL	5.042	5.026	5.039	5.036	0.71
1 ng/mL	0.753	0.863	0.799	0.805	-19.50
0.5 ng/mL	0.545	0.484	0.526	0.518	3.65
0.1 ng/mL	0.261	0.229	0.245	0.245	145.45

Table 12 shows the calculation for the accuracy of the data. Accuracies were calculated by dividing the calculated mean concentration (n=3) by the true value of each calibrator in the pre-SPE batch. As shown on the table, the 0.10 ng/mL VBL calibrator has the highest % error at 72.70%. Both 5.0 ng/mL and 1.0 ng/mL have the lowest accuracy error at 0.14% and 1.14%, respectively. The accuracy of the 0.50 ng/mL VBL was -17.58%. For DVBL, 5.0 ng/mL and 0.50 ng/mL have the lowest

accuracy percentage at 0.71% and 3.65%, respectively. The 1.0 ng/mL accuracy value is at -19.50%, while the 0.10 ng/mL DVBL have the highest percent error at 145.45%.

V. Specific Aim 5: Application of validated method to canine plasma samples

Clinical application was made to samples previously collected from a canine named Tyson. Tyson had been previously treated with vinblastine and plasma samples were taken from him in intervals from 0 to 24 hours. The calibrators ranged from 0.125 ng/mL to 20 ng/mL. The calibrators and canine samples for this analysis were prepared by collaborators at the OSU CVHS.

Table 13. VBL calibrators quantitation ratios of low and high end.

Low End		High End	
Sample (ng/mL)	**Quant. Ratio**	**Sample (ng/mL)**	**Quant. Ratio**
0.125	0.0022	2	0.0483
0.25	0.0033	5	0.1732
0.5	0.0083	10	0.4048
1	0.0250	20	0.8604
2	0.0483		

Table 14. DVBL calibrators concentration and quantitation ratios.

Sample (ng/mL)	Quant. Ratio
0.125	0.0014
0.25	0.0023
0.5	0.0049
1	0.0168
2	0.0271

Table 13 and Table 14 show the calibrator concentrations for VBL and DVBL, respectively, that were used in generating the standard curve. The ID ratios were calculated for each calibrator and all were within 20% of expected values for both VBL and DVBL, which mean none of the calibrators were excluded. Two standard curves were generated for VBL, low and high end, because a combined curve caused skewed data points. Both tables show the calculated quantitation ratios.

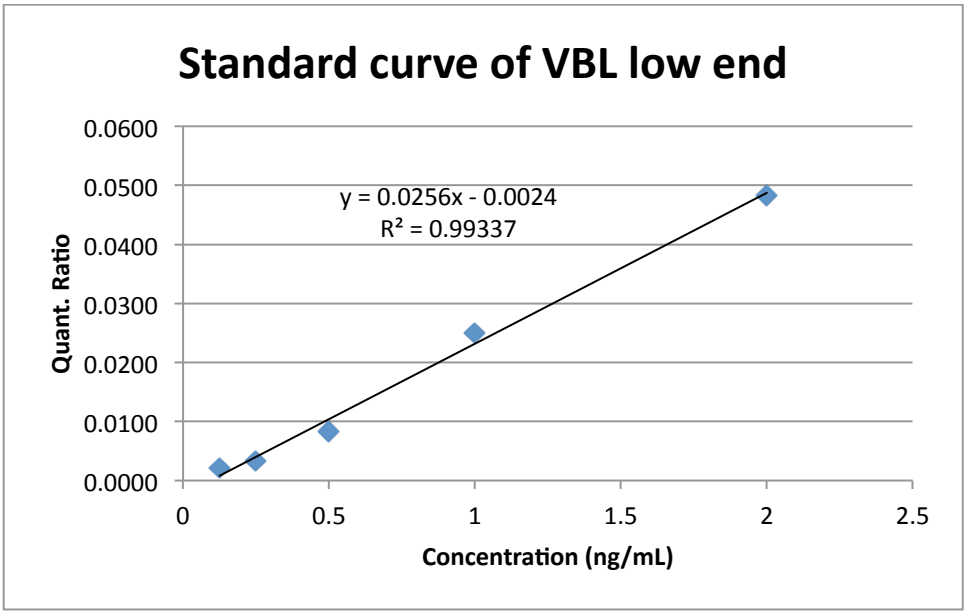

Figure 19. Low end of VBL standard curve.

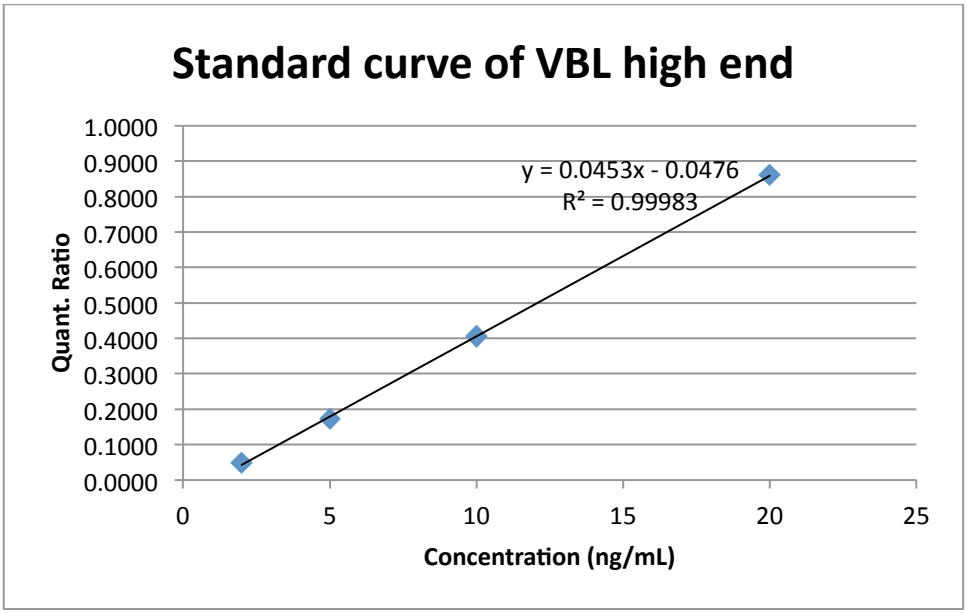

Figure 20. High end of VBL standard curve

Figure 19 and Figure 20 shown above is the lower and higher end of VBL calibrators, respectively. The axis of the graph was quantitative ratio versus

concentration. As shown in the figures, both curves were linear and the R^2 values were above 0.990.

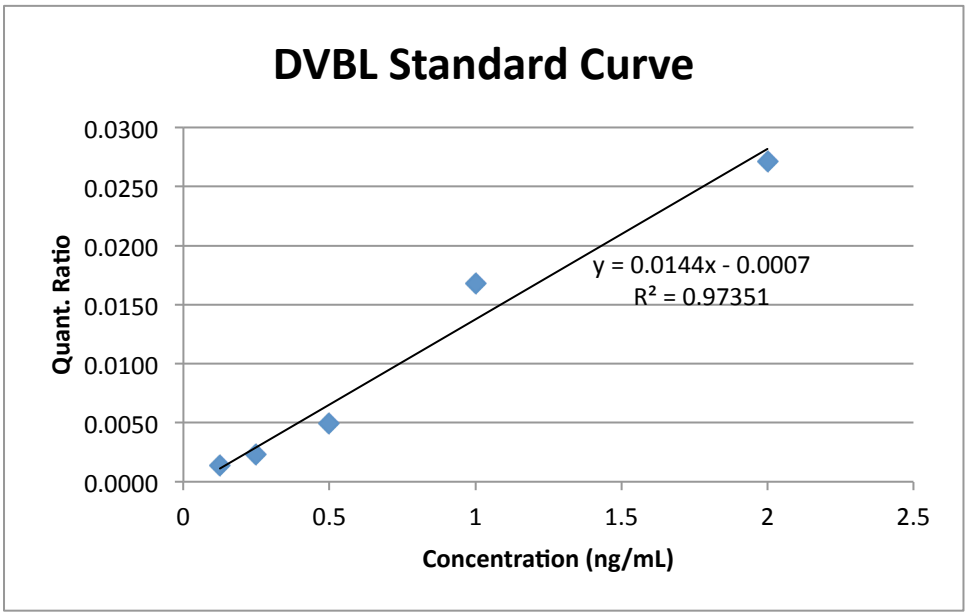

Figure 21. Standard curve of DVBL.

Figure 21 shows the standard curve for DVBL. This curve was not divided into high and low end since the calibrators for DVBL only range from 0.125 ng/mL to 2 ng/mL and there was no high end. The R^2 value was above 0.970 was not quite as good as the VBL standard curve but sufficient for quality control purposes and valid in determining the unknown concentrations.

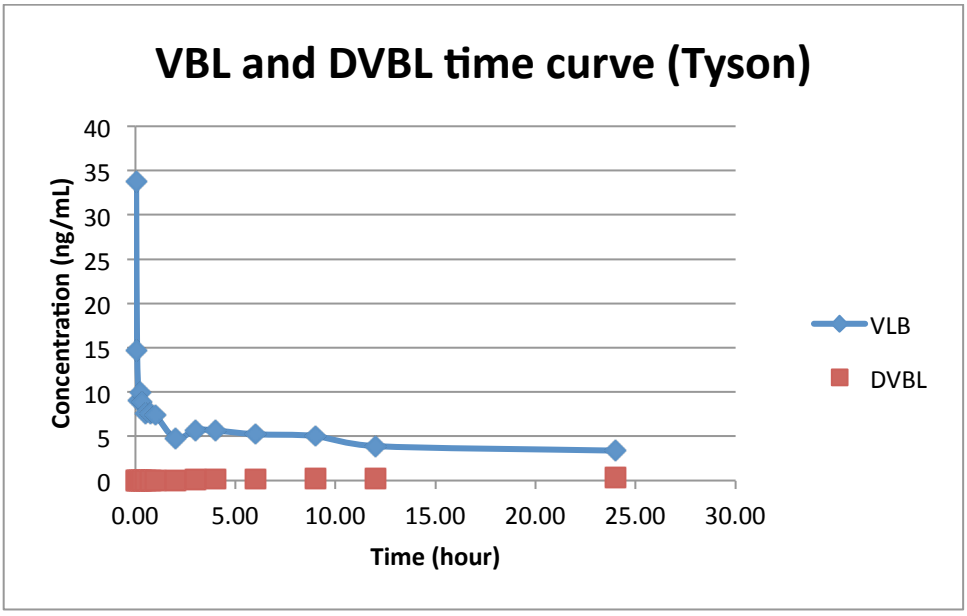

Figure 22. Time curve of Tyson VBL and DVBL level over a 24-hour period.

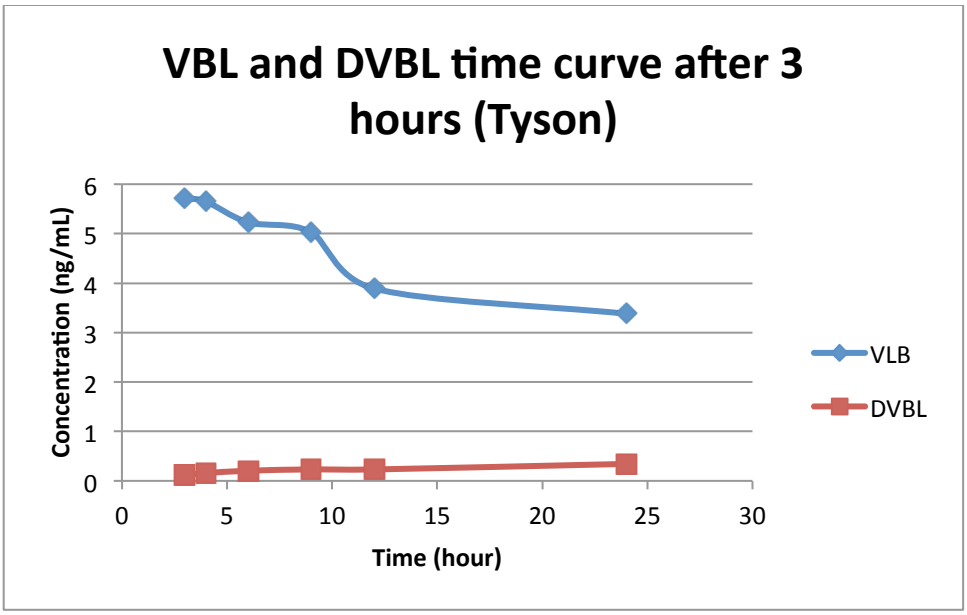

Figure 23. Time curve for Tyson's plasma samples after 3 hours.

Figure 22 is a concentration versus time graph that shows the concentration of VBL decreasing over time. The calculated concentration for each unknown was determined by using the standard curves above (Figure 19, Figure 20, and Figure 21). As shown on the graph, VBL decreased very rapidly within an hour. Figure 23 is a close up look at the relation between VBL and DVBL level after 3 hours. The graph showed that VBL was slowly decreasing as DVBL was increasing. The graphs showed that as VBL was breaking down, DVBL was being produced.

Chapter V Discussion

I. Specific Aim 1 & 2: Development and validation of LC/MS/MS method

1. ESI vs. APCI

As part of method development, both ESI and APCI were used to determine which ionization method was the best for vinblastine detection. Both ESI and APCI detected VBL, DVBL, and VRB, but APCI seem to have higher sensitivity than ESI. Table 15 shows the LOD and LLOQ comparison between ESI and APCI mode. As shown on Table 15, the LODs and LLOQs for VBL are lower when APCI mode was used. VBL LOD and LLOQ were about 0.0246 ng/mL and 0.0821 ng/mL in APCI mode versus 0.0309 ng/mL and 0.103 ng/mL for ESI mode, respectively. For DVBL, ESI mode seems to be the better choice with a LOD of 0.202 ng/mL and LLOQ of 0.672 ng/mL. DVBL in APCI mode has a higher LOD and LLOQ of 0.307 ng/mL and 1.02 ng/mL, respectively. When comparing the APCI and ESI chromatograms, the noise level was lower and the peaks had better resolution when using APCI.

Table 15. VBL and DVBL LOD and LLOQ comparison between ESI and APCI

	ESI		APCI	
	LOD (ng/mL)	LLOQ (ng/mL)	LOD (ng/mL)	LLOQ (ng/mL)
VBL	≈0.0309	≈0.103	≈0.0246	≈0.0821
DVBL	≈0.202	≈0.672	≈0.307	≈1.02

The results above were only used to determine which mode of ionization would be optimal for the compound of interest. Even though the LLOQ under APCI was very low, the value will change depending on the specimen. In reality, unknown samples will come in specimens (plasma, urine, etc.) that will need to be purified, which can affect sample recovery. This research was developed for samples in plasma; therefore, preparation procedures for other matrices may need to be generated. In this case, the LC/MS/MS was tuned and optimized successfully for vinca alkaloids in APCI mode.

II. Specific Aim 3: Develop a sample preparation strategy to increase sensitivity

1. Liquid-liquid extraction

During the whole method development for this project, LC/MS/MS and extraction methods were changed many times in order to reach the desired results. The first attempted extractions were liquid-liquid extraction using three different solvents. The first solvent used was a mixture hexane and ethyl acetate, and none of the drugs were

recovered (Figure 7). The second solvent was a mixture of chloroform and isopropanol, which showed higher recovery, however, the internal standard peak disappeared (Figure 8). Chloroform was the most commonly used solvent for LLE involving vinca alkaloids, and none of the studies reported any problems with chloroform. Therefore, the hypothesis was that VRB interacted with isopropanol, which caused byproducts. A full scan of the samples were done, which showed other related materials by *m/z* in the solution. However, the identity of the byproducts was not determined due to time constraints. Because of the byproducts associated with isopropanol, ether was attempted as a third solvent (Figure 9). The LLOQ for this method was 0.682 ng/mL and 0.990 ng/mL for VBL and DVBL, respectively. Since ether gave the best overall detection, the solvent was tried with plasma samples.

When ether was used on plasma as opposed to methanolic standards, a new problem arose. The HPLC column got clogged, which caused leaking due to pressure build up on the system. Even though ether successfully extracted the drugs, enough proteins were still present in the samples to cause LC failure. Therefore, another method was developed using acetonitrile to crash out the proteins before LLE. Even though the ACN crash eliminated the clogging problem by removing the proteins, all of the drugs were lost during this step (Figure 12). As available references indicate that the fraction of drug that is protein bound is 75% (Baselt, 2008), the acetonitrile crash likely caused the vinca alkaloids to be lost from the extraction along with precipitated proteins.

a) Drying methods

Two different drying methods were tested to determine which one gave the best recovery. When the VRB peak disappeared in the chloroform:isopropanol extraction, a factor that was taken into consideration was the drying time. The initial hypothesis was that isopropanol was reacting with VRB, which formed byproducts. Therefore, it was felt that if the time of exposure to the isopropanol was minimized, there would be less time for reactions to occur and this would result in better recovery. The original drying time at room temperature was greater than two hours, so another method of drying was used, which used nitrogen gas to speed up the drying time. By using nitrogen, the drying time was decreased to less than one hour and the IS peak returned with the nitrogen gas modification (Figure 11).

Even though nitrogen gas worked in eliminating the problem of byproducts, to totally eliminate the problem in the future, a new method was created. Since LLE caused many issues and did not give optimal results, SPE was developed to test for sensitivity. To eliminate any drying variables that could affect the results, both drying at room temperature and with nitrogen gas was used after SPE. The overall results showed that the drying methods did not affect sample recovery in the SPE method. This result confirms the hypothesis of vinca alkaloid loss through reactivity with isopropanol during LLE.

2. Solid phase extraction

Because LLE was unsuccessful due to column clogging, low recovery, and peak disappearance, SPE was attempted. Two different SPE methods were developed,

although results from the first SPE method did not yield optimal recovery. The LOD and LLOQ for VBL were 0.348 ng/mL and 1.05 ng/mL, respectively. The second SPE method was a modification of the first method to increase sensitivity. The change from a low percentage methanol wash to a water wash served to leave more vinca alkaloids on the SPE column prior to elution, preventing unnecessary losses and therefore increasing sensitivity. The increase in column drying time served to ensure that there were no voids due to immiscible liquids when the vinca alkaloids were eluted from the SPE column, increasing efficiency of extraction. Combined, the changes to the method did increase the recovery rate and the LOD and LLOQ of VBL for the new method were 0.119 ng/mL and 0.361 ng/mL, respectively.

III. Specific Aim 4: Validation of the most sensitive method

1. Precision

The results for VBL showed that the precision is within 10% from 0.50 ng/mL to 5.0 ng/mL, but at 0.10 ng/mL the error was over 20%, while all DVBL precision results were under 10%. In general, working at lower concentrations causes the method to be more sensitive to variations in the amount of analyte injected on the LC column, since any changes are a larger proportion of the instrument signal. Variations could be caused by pipetting variables with internal standard or sample volumes. In any case, the concentrations that exhibited precision outside the normally accepted value of 20% were actually below the calculated method lower limit of quantitation. Therefore, the lack of precision at the low concentration is actually an encouraging result since it validates the approach used to calculate the LLOQ and the LOD.

2. Accuracy

The accuracy for DVBL and VBL were close to 100%±20% from 0.50 ng/mL to 5.0 ng/mL. However, at 0.10 ng/mL the accuracy error was over 20%. Accuracy is like precision in that at lower concentrations small changes can result in a large variation in the result. As in the case with precision, the only accuracy value that was out of the normally accepted 20% was below the calculated LLOQ. Therefore all values obtained with the method were within normally accepted quality control parameters.

3. Sensitivity

The lowest LOD and LLOQ that could be detected with the new method were 0.150 ng/mL and 0.455 ng/mL for VBL, and 0.069 ng/mL and 0.209 ng/mL for DVBL, respectively. As discussed above, the precision and accuracy at these calculated values were within the normally acceptable quality assurance criteria. These values compare favorably with prior LC/MS/MS studies on vincristine. (Corona et al., 2008) While certain LC/F methods are capable of producing similar results for VBL, they are not as selective as LC/MS/MS and their sensitivities to DVBL are low. (Vendrig, Teeuwsen, & Holthuis, 1988)

4. Selectivity

a) Matrix effects and recoveries

The matrix effects for the developed method for VBL, DVBL, and VRB were 65.7%, 76.5%, and 78%, respectively. The equation for calculating ME was to divide post-SPE by neat samples. ME was calculating for the matrix effect of the process starting from when the analytes were eluted from the column. The matrix effect for VBL

was 65.7%, which meant that 34.3% of the analytes were suppressed. 23.5% of DVBL were being suppressed and 22% for VRB. There are several possible mechanisms of suppression. One mode purports that there is a limited amount of material that can be ionized as it travels into the source. Therefore, if multiple compounds emit simultaneously, compounds that ionize easily might be more likely to ionize than those that do not ionize with high efficiency. Another mode might be through a co-eluting compound binding to the analytes, which then prevent them from ionizing and entering the MS. While the mechanism of suppression in this case is not known, it is apparent that suppression is occurring. As the suppression is directly related to the matrix being extracted, plasma, this method is only valid for use in plasma and the experiments would need to be performed again in a different matrix to determine if suppression was still occurring. While minimal suppression would be ideal, there is no current value at which a method is disqualified due to its presence. (Matuszewski et al., 2003)

The equation for RE was calculated by taking the pre-SPE results and dividing by the post-SPE results. RE was calculating for the recovery of the analytes between the extractions to the drying process. PE was calculating the overall yield from the first step to the last step of the whole process. From the results above, 65.3% (RE) of what was initially there was recovered after extractions, which means 34.7% was lost on the column. The end results gave the total yield of 42.9% (PE), which means the process was not very efficient. However, even with the loss of efficiency, the method was more sensitive and selective than previous methods.

IV. Specific Aim 5: Application of validated method to canine plasma samples

The developed LC/MS/MS and extraction method was successfully applied to canine plasma. The graphs for Tyson showed that after three hours, as VBL was decreasing, DVBL was increasing. This meant that after 3 hours of injecting the drugs into the canine, VBL was broken down into DVBL, the metabolite. The goal of a pharmacokinetic study is to determine the absorption, distribution, metabolism and elimination of the drug in the system after administration, also known as ADME. The results suggest that VBL decreases rapidly within the first hour after administration, and the metabolite, DVBL, can be detected after three hours. If the only mode of metabolism and excretion were through the production and elimination of DVBL, then the amount of VBL lost should equal the amount of DVBL being made. However, VBL can be broken down into different metabolites or eliminated from the body in its original form, so the amount of DVBL produced is less than the decrease in VBL concentration. This research is only looking at canine plasma and not urine, so the amount of parent VBL being eliminated through urine is not known.

This method was applied to one canine subject, but the collaborator goals in the OSU CVHS are to apply it to samples from animals of various sizes, determine the appropriate pharmacokinetic model for the animals, and use the developed pharmacokinetic model for more accurate chemotherapy dosing. This method is the first step towards these greater goals.

V. Conclusions

A sensitive and selective SPE LC/MS/MS method was develop that can detect and quantitate vinblastine and its metabolite, deacetylvinblastine, at a concentration lower than 1.0 ng/mL. LLE, protein precipitation, and SPE were explored and the end results showed that SPE was the best extraction method. The accuracy and precision of the calibrators were acceptable in the linear range of the method. This method was successfully applied to samples from a canine subject, and will be used on more subjects in the future.

VI. Future Work

Due to time constraints, this newly developed method was only applied to one dog, but there are several samples from canine subjects awaiting analysis. Even though the newly developed SPE LC/MS/MS method was successful in detecting and quantitating low concentrations of VBL and DVBL, more improvements need to be explored to increase process efficiency. Since more than half of the samples were lost due to matrix and column effects, an alternative solvent and/or column could be used to increase recovery and decrease matrix effects. Another area that needs to be explored is the unknown peak eluting at 2.0 minutes on the chromatogram from extracted calibrators and samples. Again, because of the time constraints, the unknown peak was not identified, but future work might attempt to identify the unknown peak.

Chapter VI References

Baselt, R. (2008). *Disposition of toxic drugs and chemicals in man* (8th ed.): Biomedical Publication.

Choi, Y. H., Yoo, K. P., & Kim, J. (2002). Supercritical fluid extraction and liquid chromatography-electrospray mass analysis of vinblastine from Catharanthus roseus. *Chemical & Pharmaceutical Bulletin, 50*(9), 1294-1296.

Chu, I. H., Bodnar, J. A., White, E. L., & Bowman, R. N. (1996). Quantification of vincristine and vinblastine in Catharanthus roseus plants by capillary zone electrophoresis. *Journal of Chromatography A, 755*(2), 281-288.

Cody, J. (2006). Mass spectrometry. In B. Levine (Ed.), *Principles of Forensic Toxicology* (2nd ed.). Washington D.C.: AACC Press.

Corona, G., Casetta, B., Sandron, S., Vaccher, E., & Toffoli, G. (2008). Rapid and sensitive analysis of vincristine in human plasma using on-line extraction combined with liquid chromatography/tandem mass spectrometry. *Rapid Communications in Mass Spectrometry, 22*(4), 519-525.

Damen, C. W., Rosing, H., Tibben, M. M., van Maanen, M. J., Lagas, J. S., Schinkel, A. H., . . . Beijnen, J. H. (2008). A sensitive assay for the quantitative analysis of vinorelbine in mouse and human EDTA plasma by high-performance liquid chromatography coupled with electrospray tandem mass spectrometry. *J*

Chromatogr B Analyt Technol Biomed Life Sci, 868(1-2), 102-109.

Damen, C. W. N., Israels, T., Caron, H. N., Schellens, J. H. M., Rosing, H., & Beijnen, J. H. (2009). Validated assay for the simultaneous quantification of total vincristine and actinomycin-D concentrations in human EDTA plasma and of vincristine concentrations in human plasma ultrafiltrate by high-performance liquid chromatography coupled with tandem mass spectrometry. *Rapid Communications in Mass Spectrometry, 23*(6), 763-774.

Damen, C. W. N., Lagas, J. S., Rosing, H., Schellens, J. H. M., & Beijnen, J. H. (2009). The bioanalysis of vinorelbine and 4-O-deacetylvinorelbine in human and mouse plasma using high-performance liquid chromatography coupled with heated electrospray ionization tandem mass-spectrometry. *Biomedical Chromatography, 23*(12), 1316-1325.

Damen, C. W. N., Rosing, H., Schellens, J. H. M., & Beijnen, J. H. (2010). High-performance liquid chromatography coupled with mass spectrometry for the quantitative analysis of vinca alkaloidsVinca alkaloidsvinca alkaloids in biological matrices: a concise survey from the literature. *Biomedical Chromatography, 24*(1), 83-90.

ICH Harmonised Tripartite Guideline, Validation of Analytical Procedures: Methodology. (1996). Paper presented at the International Conference on Harmonisation of Technical requirements for Registration of Pharmaceuticals for Human Use.

Matuszewski, B. K., Constanzer, M. L., & Chavez-Eng, C. M. (2003). Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on

HPLC/MS/MS. *Anal Chem, 75*(13), 3019-3030.

Moffat, A. C., Osselton, M. D., Widdop, B., & Galichet, L. Y. (2004). *Clarke's analysis of drugs and poisons* (3rd ed.): Pharmaceutical Press.

Politi, L., Groppi, A., & Polettini, A. (2006). Ionisation, ion separation, and ion detection in LC-MS. In A. Polettini (Ed.)*, Applications of LC-MS in Toxicology (2nd ed.): Pharmaceutical Press.*

Klys, M., Konopka, T., Scislowski, M., & Kowalski, P. (2007). Fatality involving vinblastine overdose as a result of a complex medical error. *Cancer Chemotherapy and Pharmacology, 59*(1), 89-95.

Ragot, S., Sauvage, F. L., Rousseau, A., Genet, D., Dupuy, J. L., Tubiana-Mathieu, N., & Marquet, P. (2001). Sensitive determination of vinorelbine and its metabolites in human serum using liquid chromatography-electrospray mass spectrometry. *Journal of Chromatography B, 753*(2), 167-178.

Ramirez, J., Ogan, K., & Ratain, M. J. (1997). Determination of vinca alkaloids in human plasma by liquid chromatography atmospheric pressure chemical ionization mass spectrometry. *Cancer Chemotherapy and Pharmacology, 39*(4), 286-290.

SciFinder. (2010). Deacetylvinblastine substance detail, CAS 3352-69-0. Retrieved 11/2010, from American Chemical Society https://scifinder.cas.org/

Siek, T. J. (2006). Specimen preparation. In B. Levine (Ed.), *Principles of Forensic Toxicology* (2nd ed.). Washington D.C.: AACC Press.

Stafford, D. T. (2006). Chromatography. In B. Levine (Ed.), *Principles of Forensic Toxicology* (2nd ed.). Washington D.C.: AACC Press.

Stout, P. R., Bynum, N. D., Mitchell, J. M., Baylor, M. R., & Ropero-Miller, J. D. (2009).

A comparison of the validity of gas chromatography- mass spectrometry and liquid chromatography- tandem mass spectrometry analysis of urine samples for morphine, codeine, 6-acetylmorphine, and benzoylecgonine. *Journal of analytical toxicology, 33*(8), 398-408.

Taylor, P. J. (2006). *Method development and optimisation of LC-MS.*: Pharmaceutical Press.

Vendrig, D. E., Teeuwsen, J., & Holthuis, J. J. (1988). Determination of vinca alkaloids in plasma and urine using ion-exchange chromatography on silica gel and fluorescence detection. *Journal of chromatography, 434*(1), 145-155.

Volkov, S. K., & Grodnitskaya, E. I. (1994). Application of High-Performance Liquid-Chromatography to the Determination of Vinblastine in Catharanthus-Roseus. *Journal of Chromatography B-Biomedical Applications, 660*(2), 405-408.

Zhou, H., Tai, Y. P., Sun, C. R., & Pan, Y. J. (2005). Rapid identification of Vinca alkaloids by direct-injection electrospray ionisation tandem mass spectrometry and confirmation by high-performance liquid chromatography-mass spectrometry. *Phytochemical Analysis, 16*(5), 328-333.

VITA

Minh Trieu Ngo

Candidate for the Degree of

Master of Science

Thesis: DEVELOPMENT OF AN ANALYTICAL METHOD FOR VINCA ALKALOIDS USING LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY (LC/MS/MS)

Major Field: Forensic Sciences

Education:

Completed the requirements for the Master of Science in Forensic Sciences at Oklahoma State University Center for Health Sciences, Tulsa, Oklahoma in December 2010.

Completed the requirements for the Bachelor of Science in Forensic Science at Oklahoma State University, Stillwater, Oklahoma in 2008.

Name: Minh Trieu Ngo Date of Degree: December, 2010

Institution: Oklahoma State University CHS Location: Tulsa, Oklahoma

Title of Study: DEVELOPMENT OF AN ANALYTICAL METHOD FOR VINCA ALKALOIDS USING LIQUID CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY (LC/MS/MS)

Pages in Study: 75 Candidate for the Degree of Master of Science

Major Field: Forensic Sciences

Scope and Method of Study: - The purpose of this study was to develop and validate an analytical method for vinblastine (VBL) and deacetylvinblastine (DVBL) using liquid chromatography tandem mass spectrometry (LC/MS/MS). The goal of the study was to develop a method that is sensitive enough to quantitate less than 1.0 ng/mL in plasma. The first step in method development was to optimize the LC/MS/MS by tuning the ion path and optimizing source parameters and chromatography. After development of the LC/MS/MS method, a solid phase extraction sample preparation method was developed and optimized to increase sensitivity. Method validation was carried out through determination of the sensitivity, selectivity, precision, and accuracy of the method. This method was then applied to canine plasma samples.

Findings and Conclusions: The LC/MS/MS method was successfully developed for detection of vinblastine and desacetylvinblastine with vinorelbine as the internal standard. The LC method utilized isocratic flow rate with atmospheric chemical ionization (APCI) and three ions per analyte in multiple reaction monitoring (MRM) mode. Calibrators in methanol resulted in a LLOQ for vinblastine of approximately 0.77 ng/mL and of deacetylvinblastine at approximately 3.6 ng/mL. The LLOQ for plasma samples after SPE was 0.445±0.047 for VBL and 0.209±0.01 ng/mL for DVBL. The developed method for plasma extracted with SPE and analyzed via LC/MS/MS was validated and successfully applied to canine plasma samples.

ADVISER'S APPROVAL: Jarrad R. Wagner, Ph.D.