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Development of a High Performance Liquid Chromatography Method for the Analysis of Next-generation Cyanide Antidote, 3-mercaptopyruvate, in Plasma

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DEVELOPMENT OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE ANALYSIS OF NEXT-GENERATION CYANIDE ANTIDOTE, 3-MERCAPTOPYRUVATE, IN PLASMA.

BY

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This thesis is approved as a creditable and independent investigation by a candidate for Master of Science in Chemistry degree and is acceptable for meeting the dissertation requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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LIST OF ABBREVIATIONS

ATCA: 2-amino-2-thiazoline-4-carboxylic acid

ATP: Adenosine triphosphate

Cbi: Cobinamide

Cbi: Cobalamin

CN: Cyanide as CN and HCN inclusively

CWA: Chemical warfare agent

DMTS: Dimethyl trisulfide

FDA: Food and Drug Administration

HLPC: High performance liquid chromatography

IV: Intravenous

LC-MS-MS: Liquid chromatography-tandem mass spectrometry

LLQQ: Lower limit of quantification

LOD: Limit of detection

MBB: Monobromobimane

3-MP: 3-Mercaptopyruvate

3-MPB: 3-Mercaptopyruvate bimane

3-MPA: 3-Mercaptopropionic acid

3-MST: 3-Mercaptopyruvate sulfurtransferase

μM: Micromolar

QC: Quality control

Rs: Resolution

RSD: Relative standard deviation

SCN^-: Thiocyanate

S/N: Signal-to-noise

ULOQ: Upper limit of quantification

WWI: World War I

WWII: World War II

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ABSTRACT

DEVELOPMENT OF A HIGH PERFORMANCE LIQUID CHROMATOGRAPHY METHOD FOR THE ANALYSIS OF NEXT-GENERATION CYANIDE ANTIDOTE, 3-MERCAPTOPYRUVATE, IN PLASMA.

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Although the current FDA approved cyanide antidotes (i.e., sodium nitrite, sodium thiosulfate, and hydoxocoboalamine) are effective for treating cyanide poisoning, each individual antidote has major limitations, including large effective dosage, delayed onset of action, or dependence on enzymes generally confined to specific organs. To overcome these current limitations, next-generation cyanide antidotes are being investigated, including 3-mercaptopyruvate (3-MP). Analytical methods capable of detecting 3-MP from plasma are essential for the development of 3-MP as a next-generation cyanide antidote. Although 3-MP has been analyzed by LC-MS-MS, this instrument is not widely available. Therefore, a high performance liquid chromatography (HPLC) method with fluorescence detection (FLD) was developed to analyze 3-MP from swine plasma such that more labs could potentially perform the method. Sample preparation included spiking the plasma with the internal standard (3-mercaptopropionic acid) and reacting the 3-MP and IS with monobromobimane to prevent the characteristic dimerization of 3-MP. The method produced a limit of detection of 0.5 nM, a large dynamic range, and good accuracy and precision. The solid phase mixed-mode anion

exchange sample preparation protocol produced excellent selectivity for the method. The wide availability and affordability of the instrumentation and the simple of the implementation method presented should allow more labs to contribute to further investigations of 3-MP as a promising cyanide antidote.

CHAPTER 1. INTRODUCTION

1.1. Overall significance

Although cyanide is used as an important reagent for many industrial processes, such as precious metal mining, synthesis of dyes, pharmaceuticals, and plastics, it is also a highly toxic chemical that causes severe health risks which may eventually result in death. Currently, there are three next generation cyanide antidotes that are approved by the U.S Food and Drug Administration (FDA). However, all of them have major limitations. Therefore, next generation cyanide antidotes are being investigated to overcome these limitations. 3-Mercaptopyruvate is a next generation sulfur donor therapeutic that has shown promise as cyanide antidote. Although it has been analyzed successfully by LC-MS-MS, that instrumentation is only available in a few labs. Therefore, there is a critical need to develop more universal, less-costly, and simple method to analyze 3-MP from plasma.

1.2. Project objective

The objective of this project was to develop and validate an HPLC method to analyze 3-mercaptopyruvate from plasma.

1.3. Cyanide history and discovery

1.3.1. Discovery and Identification of cyanide

Cyanide is commonly known as a toxic chemical that has been used as a poison for thousands of years. Since the time of ancient Egypt, plants containing cyanide (e.g., cassava, bitter almonds, cherry laurel leaves, and peach pits) have been used as poisons.[7] Romans added cherry laurel leaves (which contain CN) into a tea and used it as a method of execution.[7-8] The first description of cyanide poisoning, was by Wepfer in 1679, from its extraction from bitter almond.[9] Even though plants containing cyanide have been used for centuries as poisons, cyanide was not identified until 1782, when Swedish pharmacist and chemist, Carl Wilhelm Scheele, isolated cyanide from Prussian blue dye.[7] In 1815, the French chemist, Joseph Louis Guy-Lussac, provided a better understanding of cyanides. Gay-Lussac, discovered cyanogen (a colorless, poisonous gas) which smelled like almonds and was considerably thermally stable.[9]

1.4. Cyanide Sources

1.4.1. Natural sources

The primary natural source of cyanide is from cyanogenic plants such as cassava, sweet potatoes, limes, peaches, and almonds.[10] The amount of HCN produced is different from one plant to another. Depending on both the biosynthesis of cyanogenic glycosides and on the existence (or absence) of its degrading enzymes.[11] The first person to isolate HCN from a plant was Scrade in 1802 (from bitter almonds and from the leaves of peachs).[11] Another natural source of cyanide is its production as a means of self-defense by many species such as algae, bacteria, and fungi.[3] Lorck was the first to show clearly that cyanide production by bacteria is dependent on the inclusion of glycine in the growth

medium.[12] The metabolic precursor of cyanide is. Relatively 1000 species of microorganisms and plants have been shown to produce cyanogenic compounds, also, there have been many reported cases of livestock and human cyanide poisoning after consumption of cyanogenic vegetation.[13-14]

1.4.2. Anthropogenic Sources

The annual production of cyanide, as HCN, is around 1.4 million tons worldwide.[15] Anthropogenic sources of cyanide include industrial operations, metal extraction, fire/smoke inhalation, chemical synthesis, paint manufacturing, plastic processing, pharmaceutical, and pesticides synthesis.[15-16] There are three major processes used to produce cyanide: 1) Andrussow, 2) Degussa, and 3) Shawinigan.[17] In the Andrussow process, HCN is produced by the reaction of ammonia and methane in the presence of oxygen and a platinum catalyst.[18] The Degussa process is similar but does not depend on the presence of oxygen.[19] In Shawinigan process, HCN is produced by the reaction of fluidized coke particles with ammonia and propane.[20]

Smoke inhalation is another source of CN^-. Cyanide is produced by combustion of nitrogen-containing compounds and inhaled as HCN.[21] HCN is produced from such common materials as plastic, wool or silk. When the combustion temperature reaches 315 ^oC (600 ^oF), HCN is released, and may be inhaled by the victim.[22] Several reports have shown that people who enter the hospital due to fire accidents may have been exposed to carbon monoxide (CO) as well as cyanide (CN), and it has been recorded that the most common source of CN poisoning in humans come from exposure to fires.[22] A related exposure is smoking, which is one of the most common sources of cyanide exposure for

people who do not work in cyanide-related industries.[3] The production of HCN from cigarettes ranges from 10-400 μg cigarette.[16]

Metal extraction, especially gold, also utilizes CN as one of the most common methods in the leaching of gold from ore. This process, called cyanidation occurs, according to Elsner's equation (Equation 1.1). [23] Gold is dissolved in gold containing ores via a dilute cyanide solution in the presence of lime and oxygen.[1]

$$4Au + 8NaCN + O_2 + 2H_2O \rightarrow 4Au[Au(CN)_2] + 4NaOH$$

(1.1)

Cyanide can be removed from solution by a number of processes, such as Merrill-Crowe Process.[1, 5] Aside from gold mining, cyanide is also used in the extraction of silver, copper, and zinc.[5] Cyanide is also used to synthesize fibers and polymers. For example, nitrile fibers are produced using hydrocyanation (Equation 1.2).[12]

$$RCH{=}CH_2 + 2HCN \rightarrow RCH(CN)_2CH_3$$

(1.2)

In addition to fibers and polymers, CN is also used in the synthesis of pharmaceuticals, pesticides, dyes, and pigments. Because of the extensive use of cyanide in industry, there is an increased risk of occupational cyanide exposure.

1.5. Cyanide as a chemical weapon

1.5.1. Illicit use of cyanide

Cyanide delivery devices are inexpensive and easy to produce, and cyanide is relatively easy to procure, so even small terrorist groups can use cyanide to create mass casualties. The first use of cyanide as chemical warfare agent (CWA) was during World War I (WWI). In late 1915 and early 1916 the French used cyanide and hydrocyanic acid (HCN gas), but they stopped its production later because HCN gas spread quickly in open areas, making cyanide ineffective.[7] In late 1916, about the same time, French and Austrians started using cyanogen chloride and cyanogen bromide because they are less volatile than HCN. In humans, both convert to CN, and have the same impact on the victims.[7, 24] Japanese used cyanide against Chinese before and during World War II. In 1980s, during the Iran-Iraq War, Iran used cyanide against Syria (city of Hama)[25], the Kurds (city of Halabja)[26] and Iraq.[25-27] Cyanide has been used in terrorist attacks or by individuals to poison enemies. For example, ancient Egyptians and Romans used cyanide from cyanogenic plants, such as cherry laurel, as a poison.[28] In 1978, Reverend Jim Jones and his 900 or more of his followers drank a grape flavored drink containing cyanide.[29] In 1982, Chicago Tylenol poisonings resulting from Extra-Strength Tylenol capsules which had been laced with potassium cyanide caused a total of seven deaths.[30] In 1995, in Tokyo subway, cyanide gas-producing devices were found in the subway and railway station restrooms during Sarin terrorist attacks.[31] Also, in 2012, a Chicago lottery winner, Urooj Khan died due to cyanide poison.[32] In 2013, it was reported that Dr. Robert Ferrante was convicted of fatally poisoning his wife with cyanide.[33]

1.6. Cyanide Toxicity

1.6.1. Mechanism of cyanide toxicity and symptoms

Cyanide is known to bind and inactivate enzymes, especially those containing iron in the ferric state and cobalt. When cyanide binds cytochrome c oxidase, which is located in the mitochondrial membrane, it prevents the transfer of electrons to molecular oxygen. Therefore, the oxygen in the blood cannot be utilized toward adenosine triphosphate (ATP) generation, which halts aerobic metabolism.[34] When this occurs, the cells first attempt to find another ATP energy source through glycolysis, leading to lactic acidosis. This source of energy cannot be sustained, especially in the metabolically active heart and brain and ultimately causes cellular necrosis.[2, 35] A diagram of the inhibition of cytochrome c oxidase in the mitochondrial membrane is shown in Figure 1.1. Cyanide can affect many functions, including visual, pulmonary, cardiac, autonomic, vascular endocrine, metabolic systems, and the central nervous system. The toxicodynamic effects can vary based on factors such as the dose, route and speed of administration, duration of exposure, and other physiological characteristics (age, sex, etc.).[9]

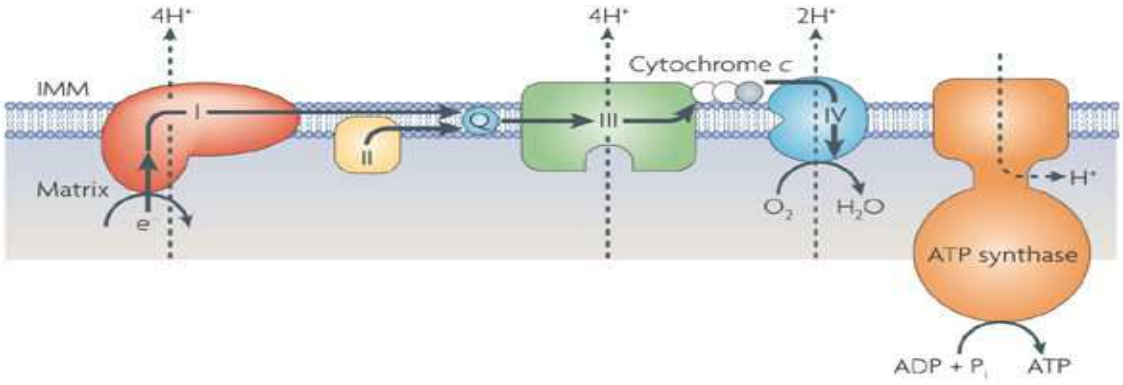

Figure 1.1 Diagram of the inhibition of cytochrome c oxidase within the electron transport chain in the mitochondrial membrane. (adapted from Ow et al.[2])

It is important to note that death may occur within 5 minutes after exposure to high doses of cyanide.[9] In short and long-term cyanide exposure cases, it is reported that some victims suffer from some chronic health problems like enlargement of the thyroid gland, myelin deterioration, nausea, and mental health symptoms such as inability to focus.[12]

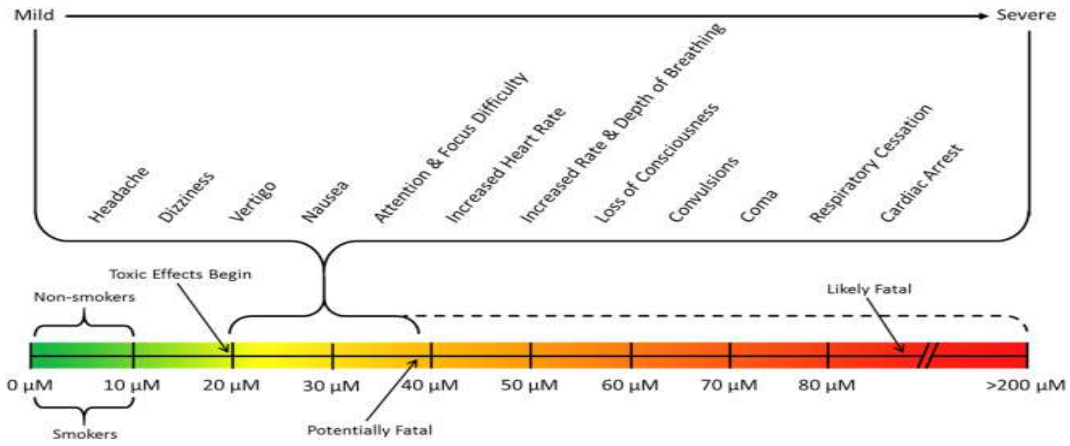

Figure 1.1 Commonly observed symptoms of CN exposure and their manifestation. (adapted from Jackson et al., 2017.[1])

1.7. Cyanide Metabolism

1.7.1. Natural cyanide metabolism pathways

Cyanide is present in human biological fluids endogenously at very low concentrations. When cyanide is absorbed into the blood stream, it's rapidly distributed throughout the body and processed by multiple metabolic pathways.[36] The major metabolic pathway of cyanide (which detoxifies about 80% of cyanide dose) is conversion of CN into thiocyanate (SCN^-) [37] using a sulfur donor and catalyzed by rhodanese enzyme this pathway is shown in Figure 1.3. Another enzyme which catalyzes conversion of CN into SCN^- is 3-mercaptopyruvate sulfurtransferase (3-MST), which yields pyruvate and SCN^- by catalyzing the sulfuration of cyanide.[4]

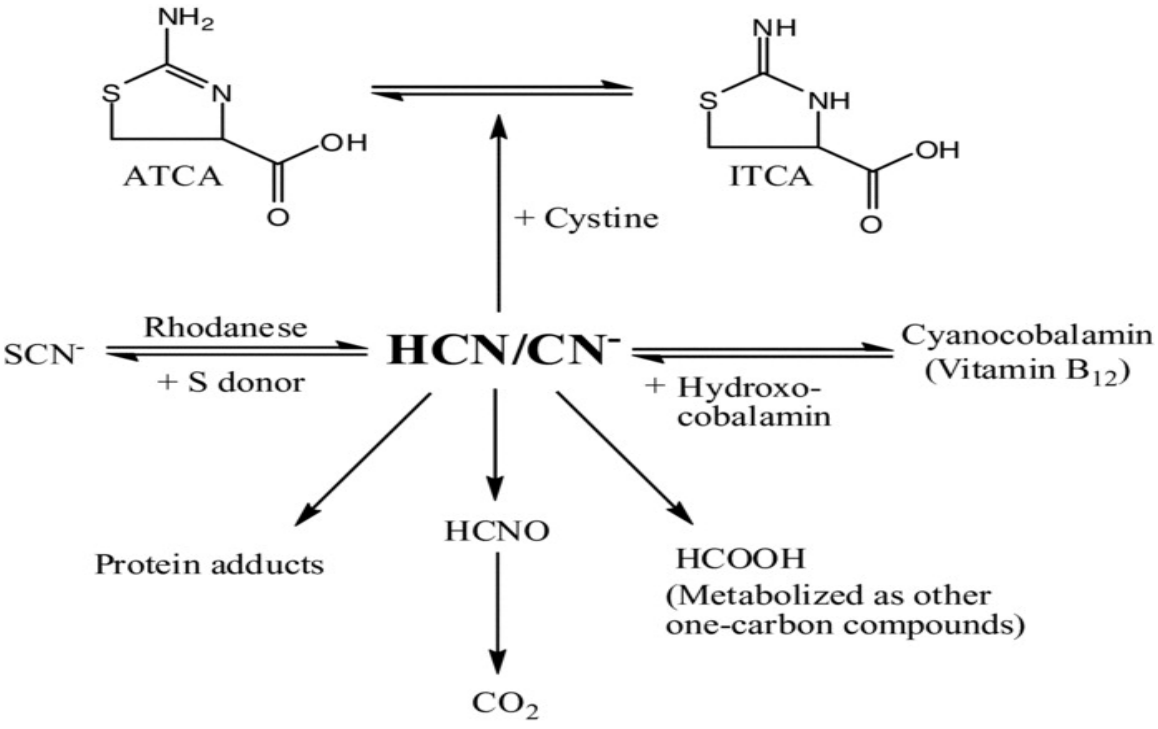

Figure 1.2 Diagram showing the metabolic pathway of cyanide and major ways of release. (adapted from Logue et al., 2010.[3])

One of the minor metabolic pathways is the production of 2-amino-2-thiazoline-4-carboxlic acid (ATCA) by the reaction of CN with L-cystine. However, this reaction only detoxifies around 0.10-9.19% of the CN.[15] There are other metabolic pathways, but their detoxification percentages are very small.

1.8. Current Cyanide Antidotes

Currently, there are three US FDA approved cyanide antidotes: hydroxocobalamin, sodium nitrite, and sodium thiosulfate. These antidotes are classified as direct sequestering, indirect sequestering, and sulfur donation, respectively.[38-39]

1.8.1. Hydroxocobalamin

Hydroxocobalamin (known as vitamin $B_{12a;}$ Cbl) is a direct sequestering antidote that detoxifies cyanide by direct binding. Cbl is a cobalt-containing molecule found in the human body.[40] The cobalt atom in Cbl strongly binds to the cyanide and produces cyanocobalamin (B_{12}) which is easily excreted from the body in the urine.[41] Although Cbl detoxifies cyanide well, it has major limitations. Cbl is a very large molecule and only binds cyanide, so a large molar ratio is needed to sequester all the free CN, leading to very large doses which must be administered by IV.[42] Mild side effects include a high blood pressure, low heart rate, allergic reaction, and red coloration of the skin, tears, sweat, and urine.[43]

1. 8.2. Sodium nitrite

Sodium nitrite indirectly sequesters cyanide from cytochrome c oxidase. Sodium nitrite is primarily classified as a methemoglobin generator, it oxidizes ferrous (2+) iron to ferric (3+) iron in hemoglobin to produce methemoglobin which has higher affinity for CN.[4, 44] Methemoglobin works as a temporary binding site for cyanide ion which decreases free cyanide in the bloodstream.[38] Recently, another alternative mechanism of action of sodium nitrite was proposed in which nitrite converts to nitric oxide which displaces cyanide bound to cytochrome c oxidase. After displacement, cyanide is converted to less harmful compounds.[38, 45] Although, sodium nitrite is an effective cyanide antidote, it has some drawbacks. Sodium nitrite produces nitric oxide and methemoglobin. Nitric oxide can cause hypotension, and methemoglobin reduces oxygen-carrying capacity of blood. The latter can cause adverse effects for smoke inhalation victims who may have high carboxyhemoglobin concentrations, which also reduces blood-oxygen-carrying capacity.[4] Additionally, if the production of methemoglobin is excessive (>30%), it can pose a health risk by itself, especially for children, causing cyanosis, fatigue, coma, and even death.[38]

1. 8.3. Sodium thiosulfate

Sodium thiosulfate is the only currently approved cyanide antidote that works as a sulfur donor for the treatment of cyanide poisoning. Sodium thiosulfate eliminates cyanide by donating a sulfur atom through sulfurtransferase enzymes as catalysts to convert cyanide to SCN^-.[46] During the conversion of cyanide to thiocyanate with sodium thiosulfate and rhodanese, a sulfur atom is transferred from the donor to the enzyme, producing a persulfide intermediate, and a sulfite is released in the transition. After that,

the persulfide is transferred from the enzyme to CN converting it to thiocyanate.[4] Although sodium thiosulfate detoxifies cyanide well, it has limitations. Its antidotal activity is limited by its short biological half-life, small volume of distribution, and its dependence on rhodanese to catalyze the sulfur transfer reaction. Rhodanese is only found in the liver and kidneys, leaving the main locations of cyanide toxicity (the heart and the brain) less protected. Additionally, sodium thiosulfate has a slow onset of action, which causes slow entry into cells and mitochondria.[4, 39] Due to its limitations, sodium thiosulfate is administered in conjunction with other antidotes.

1.9. Novel cyanide antidotes

Considering the major limitations of the current cyanide antidotes, multiple investigations have been in progress to develop the next generation antidote for the treatment of cyanide toxicity.[4] Three next-generation cyanide antidotes have been extensively studied and hold the most promise as novel CN antidotes: cobinamide (Cbi), dimethyltrsulfide (DMTS), and 3-MP.

1.9.1. Cobinamide (Cbi)

Cbi is the penultimate precursor to hydroxocobalmin and is present in human serum and bile, meaning that it is likely absorbed across intestinal epithelial cells.[4] Cbi lacks the dimethylbenzimidazole ribnucleotide tail of Cbl.[4, 47] Cbi has a great affinity towards CN binding, and it directly sequesters two CN ions.[4] Several studies have demonstrated the differences between Cbl and Cbi and the results showed that Cbi was at least two to three times more effective than Cbl at detoxifying cyanide.[4, 48] Also, Cbi has been shown to reverse the effects of cyanide faster than Cbl.[4, 8] Although Cbi detoxifies

cyanide well, it has some disadvantages as cyanide antidote, such as large molecular weight, large volume of administration, and it is costly.

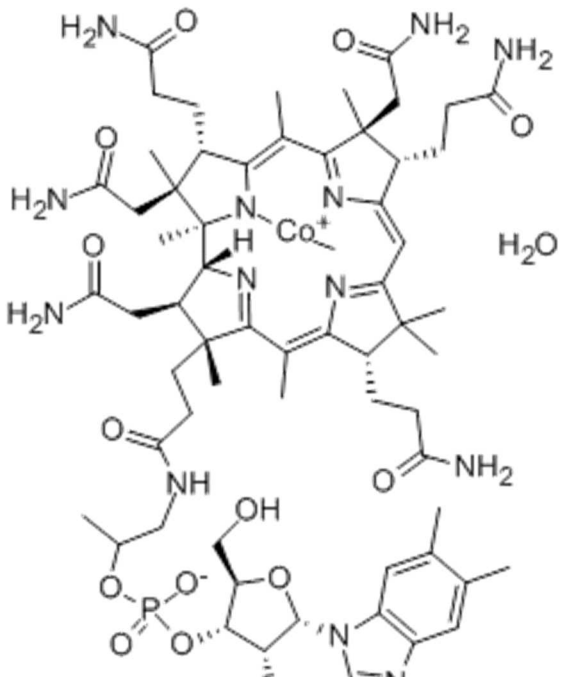

Figure 1.3 Structure of cobinamide.[4] The center cobalt atom has a great affinity toward CN binding.

1.9.2. Dimethyl trisulfide (DMTS)

DMTS is a sulfur-based molecule found in garlic, onion, broccoli, and similar plants.[49] DMTS converts cyanide into thiocyanate without the need of rhodanese and its high lipophilicity permits its effective penetration of the cell membrane and the blood brain barrier, leading to better in-vivo antidotal efficacy than thiosulfate.[38, 49] Several recent studies show that DMTS is 43 times more effective at detoxifying cyanide in the presence of rhodanese compared to sodium thiosulfate.[38, 49] Whereas, its relative efficacy is even higher in the absence of rhodanese with 79 times greater efficacy than thiosulfate.[38, 49] Although DMTS detoxifies cyanide well, it has some limitations. DMTS is unstable and it is hard to control the DMTS concentration under biological conditions. It is also highly lipophilic, which limits its solubility.

1.9.3. 3-Mercaptopyruvate (3-MP)

3-MP acts as a sulfur donor to convert cyanide to thiocyanate. This reaction is catalyzed by 3-mercaptopyruvate sulfurtransferase.[4] 3-MST is a more common enzyme than rhodanese with the most important areas of high 3-MST concentrations being the main locations of cyanide toxicity, the heart and the brain. The conversion of CN to SCN^- with 3-MP and 3-MST happens primarily in blood or tissue areas near blood. The mechanism for detoxification of cyanide with 3-MP by 3-MST is shown in Figure 1.5. It should be noted that 3-MST is distributed in both the cytosol and mitochondria.[4]

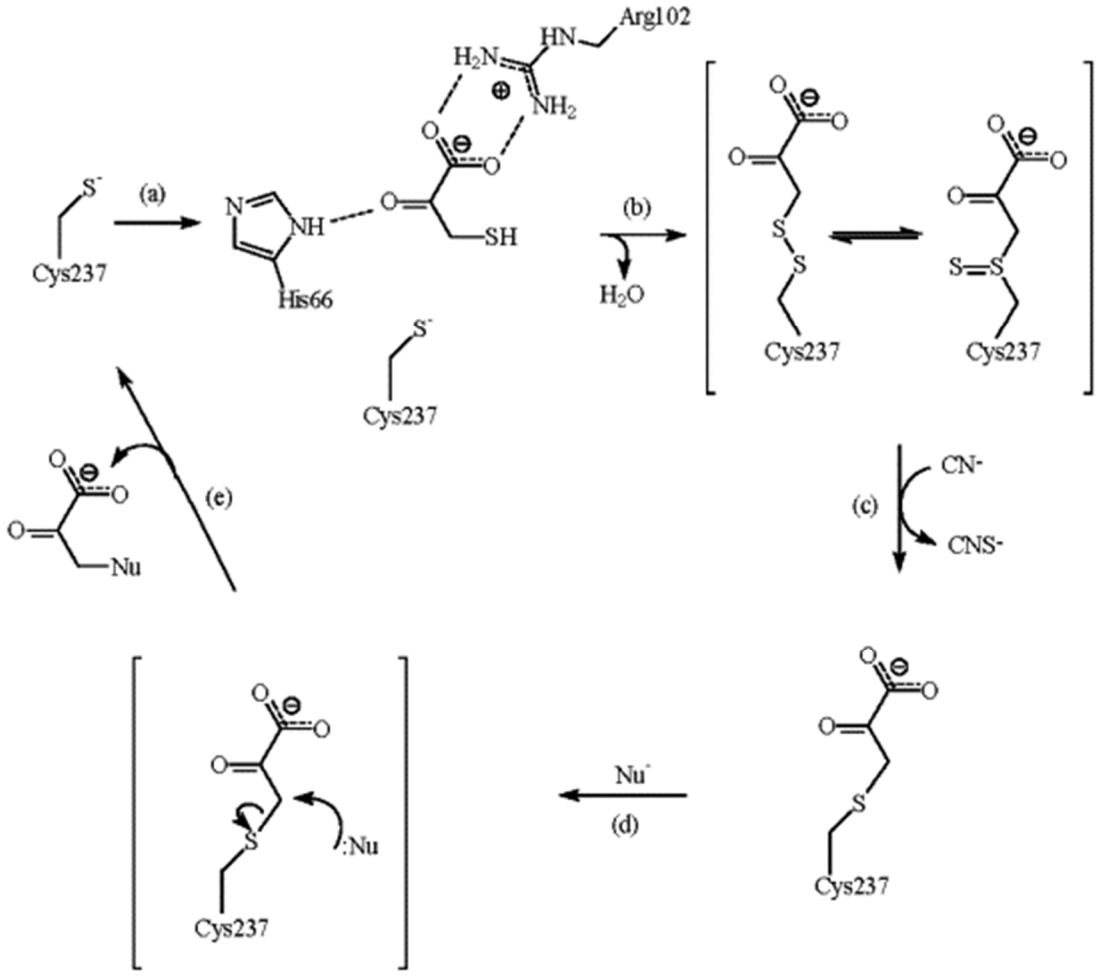

Figure 1.5 Proposed catalytic mechanism for 3-mercaptopyruvate-dependent sulfurtransfer. (a) Formation of the enzyme–substrate complex; (b) isomerization of the Cys237 covalent disulfide intermediate to the related thiosulfoxide; (c) the sulfane S atom is donated to cyanide, yielding the 3-cysteinyl-pyruvate adduct; (d) and (e) nucleophilic attack on the latter covalent intermediate releases the pyruvate analogue and frees the SseA active site. Adopted from[6]

3-MP has shown to be more effective than traditional cyanide antidotes.[12] It has good stability and it can be controlled under biological conditions. Some studies have been performed to develop a method that is able to detect 3-MP from animal plasma. However, each of these methods has specific limitations (described in chapter 2). Therefore, there is a critical need to develop simple, universal, and low-cost method without the need of mass spectral detection.

CHAPTER 2. Development of a High Performance Liquid Chromatography Method for the Analysis of Next-generation Cyanide Antidote, 3-mercaptopyruvate in Plasma.

2.1. Abstract

Cyanide is a highly toxic chemical that causes severe health risks which may eventually result in death. Although current FDA approved cyanide therapies are effective, next generation cyanide antidotes are being investigated to overcome limitations associated with these therapies. 3-Mercaptopyruvate (3-MP) is a next generation sulfur donor therapeutic that has shown promise as cyanide antidote. Analytical methods capable of detecting 3-MP in biological fluids are necessary for the development of 3-MP as a promising antidote. Although 3-MP has been analyzed by LC-MS-MS, this instrument is not widely available. Therefore, a high performance liquid chromatography (HPLC) method with fluorescence detection was developed to analyze 3-MP from swine plasma such that more labs could potentially perform the method. Sample preparation included spiking the plasma with an internal standard (3-mercaptopropionic acid), reaction with monobromobimane to prevent the characteristic dimerization of 3-MP, and mixed mode anion exchange solid phase extraction. The method produced a limit of detection of 0.5 nM and a dynamic range of 0.1-10 μM. The accuracy and precision for the method were good. The validated HPLC-FLD method was capable of detecting 3-MP in swine plasma and can be utilized for further investigations of 3-MP as promising cyanide antidote.

2.2. Introduction

Cyanide is commonly known as a toxic chemical that causes severe health risks which may eventually result in death. Exposure to cyanide is possible through a number of routes, including consumption of cyanogenic plants and fruits[38] (e.g., cassava roots, bitter almonds, cherry laurel leaves, peach pits), inhalation of smoke from cigarettes and/or fires,[7] industrial operations (e.g., electroplating and plastic processing),[15] and from use of cyanide as a chemical weapon (e.g. recently in Syria).[5, 15] The availability of cyanide, and its rapidly acting nature, makes it a threat to humanity.[38] Currently, there are three cyanide therapeutics that are approved by the U.S. Food and Drug Administration (FDA): hydroxocobalamin, sodium nitrite, and sodium thiosulfate.[4]

Hydroxocobalamin (known as vitamin $B_{12a;}$ Cbl) is direct sequestering antidote that detoxifies cyanide by directly binding. Cyanide has high affinity towards the cobalt atom in hydroxocobalamin that allows the formation of cyanocobalamin, which resides in the plasma and is excreted in urine.[8, 41] Although Cbl detoxifies cyanide well, it has some major limitations, including a high recommended dose of 5 g administered over 15 min and it is very costly. Because it requires a high dose for optimum therapeutic effect, hydroxocobalamin must be administered by IV.[4] Other mild side effects include a high blood pressure, low heart rate, allergic reaction, and red coloration of the skin, tears, sweat, and urine.[36]

Sodium nitrite indirectly sequesters cyanide from cytochrome c oxidase. Sodium nitrite is primarily classified as a methemoglobin generator, it oxidizes ferrous (2+) iron to ferric (3+) iron in hemoglobin to produce methemoglobin which has higher affinity for

CN.[38] Methemoglobin decreases free cyanide in the bloodstream by serving as a temporary binding site for cyanide ion.[39] Recently, an alternative mechanism of action of sodium nitrite was proposed in which nitrite converts to nitric oxide, which then displaces cyanide bound to cytochrome c oxidase. After displacement, cyanide is converted to less harmful compounds.[38]

The third class of cyanide therapeutic is sulfur donors. The only currently approved cyanide antidote that works as a sulfur donor is sodium thiosulfate. It eliminates cyanide by donating a sulfur atom utilizing sulfurtransferase enzymes as catalysts to convert cyanide to SCN^-.[46] During the conversion of cyanide to thiocyanate with sodium thiosulfate and rhodanese, a sulfur atom is transferred from the donor to the enzyme, producing a persulfide intermediate, and sulfite is released in the transition. After that, the persulfide is transferred from the enzyme to CN, converting it to thiocyanate.[5, 38] Although sodium thiosulfate detoxifies cyanide well, it has a few drawbacks. It has a short biological half-life, which limits its antidotal activity, a small volume of distribution, and it is dependent on rhodanese to catalyze the sulfur transfer reaction, which is only found in high concentrations in the liver and kidneys, leaving the heart and the brain which are the main locations of cyanide toxicity, less protected. Moreover, the slow onset of action of sodium thiosulfate causes slow entry into cells and mitochondria.[38] Due to its limitations, sodium thiosulfate is administered in conjunction with other antidotes.

Considering the major limitations of the current FDA approval cyanide antidotes, several investigations have been in progress to develop the next generation of cyanide therapeutics. One promising approach for novel cyanide antidotes is the development of a

sulfur-donating compound that works effectively with or without rhodanese.[38] The reaction of CN with 3-mercaptopyruvate (3-MP) utilizes 3-MST which is more common enzyme that rhodanese and it covers the heart and the brain, which are the main locations of cyanide toxicity. [4] The conversion of CN to SCN^- with 3-MP and 3-MST happens primarily in blood or tissue areas near blood. The highest concentrations of 3-MST are found in the liver and kidneys with heart, but the brain and lungs also support the enzyme.[4]

Some studies have been performed to develop a method that is able to analyze 3-MP from biological samples. However, each of them has specific limitations. Ogasawara et al.[50] reported an HPLC-fluoresence method for the analysis of 3-MP in mouse tissue. The method reported is time consuming and requires high temperatures (3-MP is highly unstable in high temperature). Other studies performed HPLC-MS-MS to analyze 3-MP from rabbit plasma, and simultaneously determine 3-MP and Cbi by LC tandem MS.[5] However, the availability of the instrumentation needed to apply this method relatively limited. Therefore, the objective of this study was to develop a relatively affordable and simple analytical method that produces similar analytical parameters to the methods available using a more universal and affordable instrument, specifically HPLC.

2.3. Experimental

2.3.1 Reagents and standards

All solvents were HPLC grade unless otherwise noted. 3-mercaptopyruvate (3-MP; $HSCH_2COCOOH$), was purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Monobromobimane (MBB) was purchased from Echelon Biosciences Inc (Salt Lake City, UT, USA). A standard solution of (MBB) (500 µM) was prepared in deionized water and stored at 4 °C. Acetone, formic acid (reagent grade, ≥95%), and 3-mercaptopropionic acid (3-MPA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Methanol was purchased from Fisher Scientific (Pittsburgh, PA, USA). SPE Mix mode coulmn were purchased from Waters corporation (Milford, MA, USA). Swine plasma (EDTA anti-coagulated) was purchased from Pel-Freeze Biological (Rogers, AR, USA) and stored at -80 °C. 3-MP calibration and QC standards were prepared from 1 mM stock solution by serial dilution with swine plasma. The internal standard solution was prepared from a stock solution of 100 µM 3-MPA and stored at 4 °C.

2.3.2. Sample preparation

Spiked or non-spiked swine plasma (100 µL) were added to 1.5 mL micro-centrifuge, along with the internal standard 3-MPA (100 µL of 50 µM). To precipitate the protein from the plasma, 300 µL of acetone was added and the tube was vortexed. The samples were cold-centrifuged (Thermo Scientific Legend Micro 21R centrifuge, Waltham, MA, USA) at 8°C for 30 min at 13,100 RPM (16,500 x g). Following centrifugation, 300 µL of the supernatant was transferred into a 4-mL glass vial and evaporated to dryness with air. Samples were then reconstituted with 100 µL of 0.1% TFA in deionized water. MBB (100 µL of 500 µM) was added to react with 3-MP and 3-

MPA to both prohibit the dimerization the reaction shown in Figure 2.1 and to add a fluorophore to 3-MP and 3-MPA. The samples were heated on a block heater (VWR International, Radnor, PA, USA) at 70 °C for 15 min to produce a 3-MP-bimane (3-MPB) and 3-MPA-bimane (3-MPAB) complex, the reaction shown in Figure 2.2. Mixed-mode anion exchange (MAX; 1 cc, 30 mg from waters) solid phase extraction (SPE) cartridges were then used to prepare the samples for the HPLC analysis. To condition the SPE columns, 1 mL of 100% methanol was added followed by 1 mL of deionized water. After 200 µL sample loading, 1 mL of deionized water was added to wash the column, followed by 1 mL of 25% methanol in water. Finally, 1 mL of 100% formic acid was added to elute 3-MPB from the column. The sample was then evaporated with air to dryness and reconstituted with 100 µL of 0.1% TFA in deionized water. The sample was filtered with a 0.22 µm tetrafluoropolyethylene membrane syringe filter into a 150 µL insert housed in glass vial.

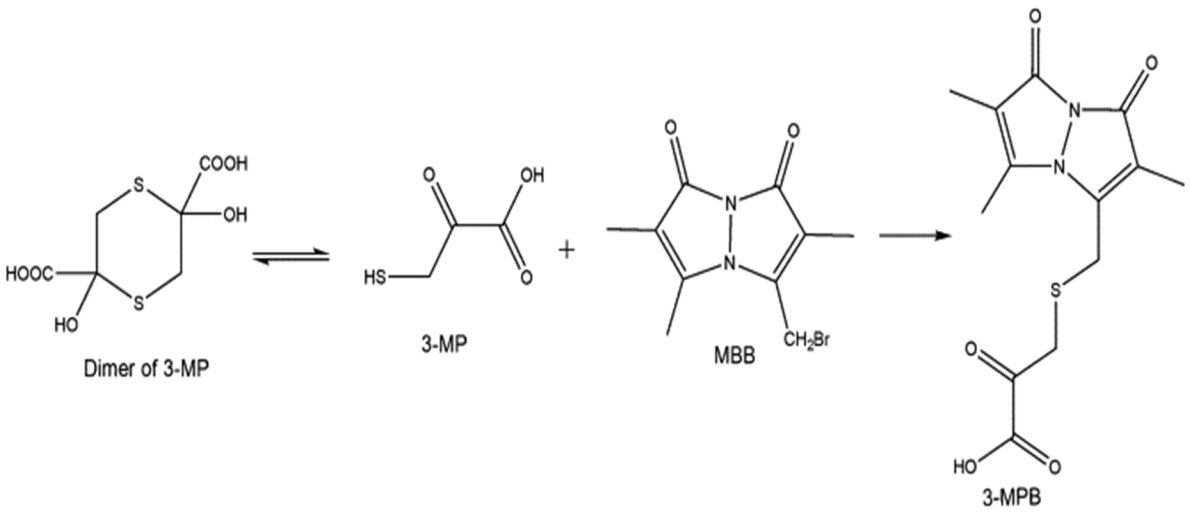

Figure 2.1 The reaction of 3-MP in equilibrium with its dimer and MBB to form a stable 3-MPB complex [5]

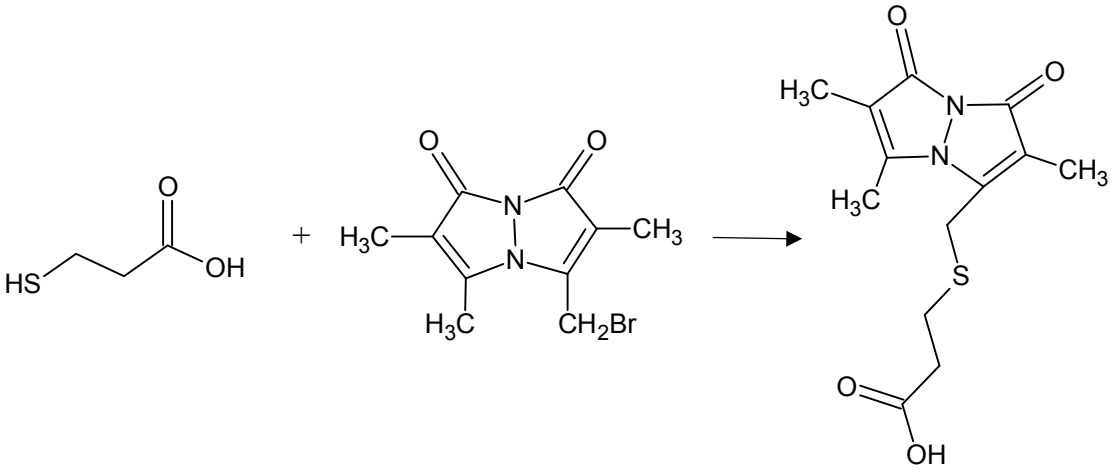

Figure 2.2 The reaction of 3-MPA with MBB to form a stable 3-MPAB complex

2.3.3. HPLC analysis

An Agilent HPLC coupled with a fluorescence detector was used for HPLC analysis of 3-MPB. Separation was performed on an Eclipse XDB C18 column, with an injection volume of 10 µL. Mobile phases were comprised of 0.1% TFA in water (Mobile Phase A) and 0.1% TFA in methanol (Mobile Phase B). A gradient of 10% B was applied over 10 minutes and increased to 100% B and held for 4 minutes then reduced to 10% B over 2 minutes. The total run-time was 16 minutes with a flow rate of 0.5 mL/min and a 3-MPB retention time of 8.5 min.

2.3.4. Calibration, quantification and limit of detection

For validation of the analytical method, we generally followed the FDA bioanalytical method validation guidelines.[51] The limit of detection (LOD) was determined by analyzing concentrations of 3-MP below the LLOQ and determining the lowest concentration that reproducibly produced a signal-to-noise ratio (S/N) of 3. The noise was estimated by evaluating the peak-to-peak noise of the blank over the retention time of 3-MP. It should be noted that 3-MP is inherently present in the plasma of mammals, making accurate assessment of the LOD difficult.[5-6] The lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) were defined using the following inclusion criteria: 1) calibrator precision of <15% RSD, and 2) accuracy of ±100%. The initial calibration curve was prepared starting with concentrations higher than the LOD and up to 10 µM to determine the dynamic range, with the range later determined 0.1–10 µM. For all experiments, calibration standards and QCs were prepared

in swine plasma. QCs (N = 5) were prepared at three different concentrations not included in the calibration curve: 0.75 μM (low QC), 2.5 μM (medium QC) and 3.5 μM (high QC). The internal standard was prepared daily and added to each sample, calibration standards and QCs during sample preparation. QCs were prepared fresh each day in quintuplicate during intra-assay (daily) and inter-assay (over three separate days, within six calendar days) analyses and were used to calculate intra-assay and inter-assay accuracy and precision.

2.4. Results and Discussion

2.4.1 HPLC-FLD analysis of 3-MP from swine plasma

3-MP reacts rapidly with itself to produce a dimer under biological conditions, which results in poor chromatography. MBB reacts with the thiol group of 3-MP to produces 3-MPB (Figure 2.1). Because the free thiol is essential for dimerization. The 3-MPB is a single species that produces good chromatographic behavior. The sample preparation scheme features reaction of 3-MP and 3-MPA with bimane, followed by mixed-mode anion exchange, SPE. Figure 2.3 shows representative chromatograms of spiked and non-spiked 3-MPB in plasma with 3-MPB eluting around 8.6 min. As shown in Figure 2.3, the method produced an excellent selectivity with only a small co-eluting peak which is presumably endogenous 3-MP. Figure 2.4 shows a chromatogram illustrates the internal standard peak with the 3-MPB peak. The main advantage of the method presented here is that it permits more affordable analysis of samples than the LC-MS-MS techniques of Stutelberg et al,[5, 52] and is more simple than the HPLC-FLD technique of Ogasawara et al.[50] The analysis of an individual sample lasted around 1 hr and 20 min, including 1 hr for sample preparation and 16 min for chromatographic analysis.

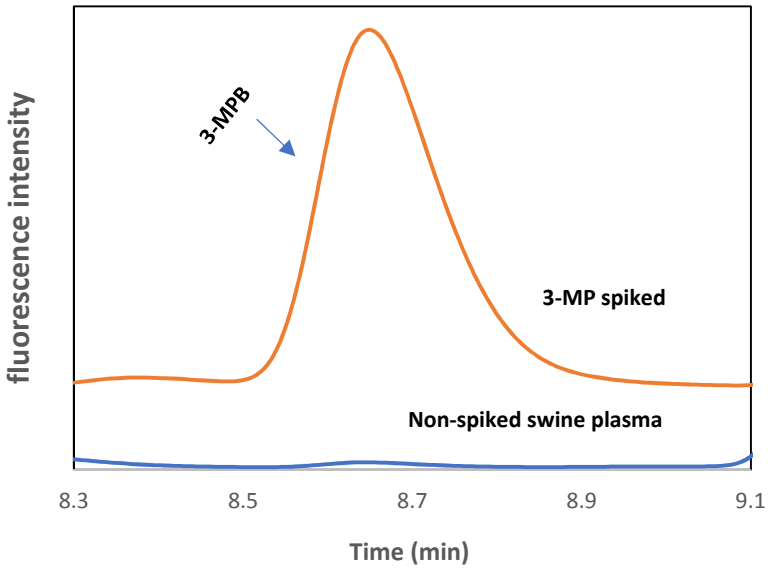

Figure 2.3. Representative chromatograms of 3-MP spiked (2 µM) and non-spiked swine plasma. The 3-MPB complex retention time is around 8.6 with only a small co-eluting peak due to endogenous 3-MP.

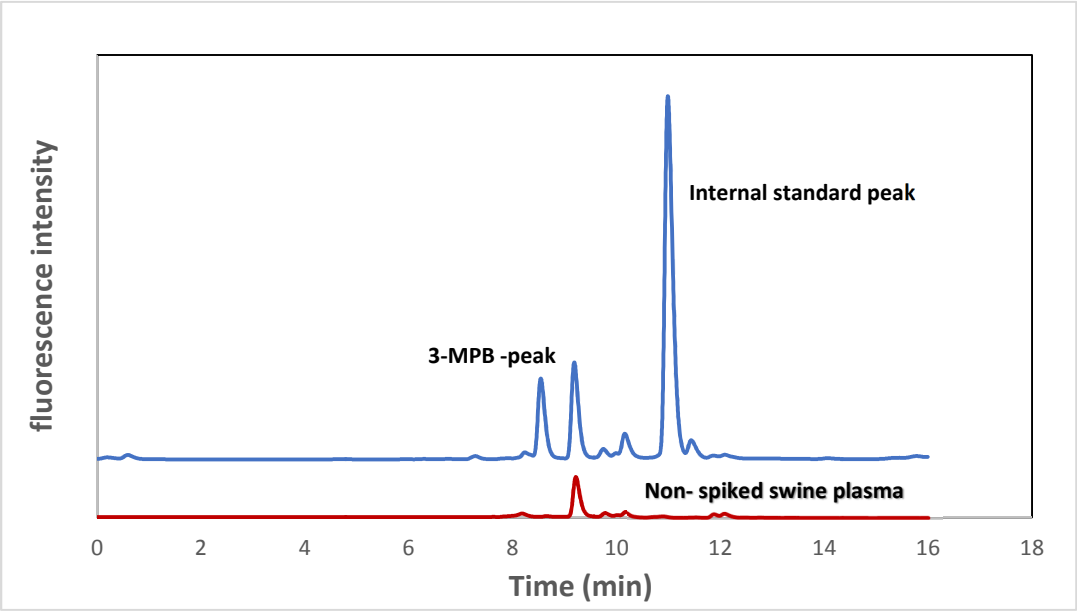

Figure 2.4. Chromatogram shows the analysis of 3-MP spiked after the internal stander addition where 3-MPB elute around 8.5 min and internal standard peak elute around 11 min.

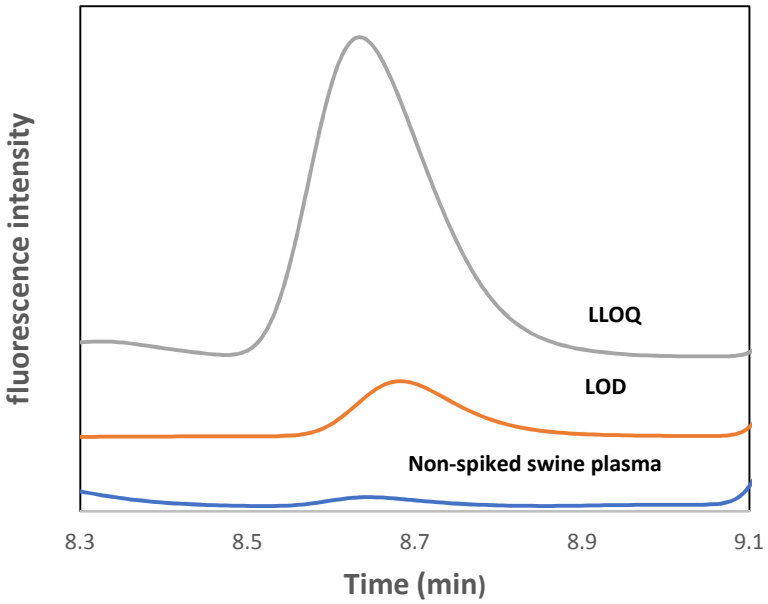

Figure 2.5. Chromatogram demonstrates the difference between the LOD analysis 0.5 nM and the LOQ analysis 100 nM.

2.4.1 Limit of detection, linear range, and sensitivity

The method produced an LOD of 0.5 nM. The resulting chromatogram is shown in Figure 2.5. In the blank, there is a small endogenous level of 3-MP coming from swine plasma. Even considering this peak as part of the noise, the LOD was excellent comparing to other methods. The LOD for the current method was approximately 2 orders of magnitude below the LOD from the HPLC-MS-MS methods of Stutelberg et al.[5] For Ogasawara el al, the minimum detectable level was 2 μM for 3-MP.[50]

The dynamic range of the method was 0.1-10 μM with a correlation coefficient (R^2) = 0.9986. The resulting plot is shown in Figure 2.6. The LLOQ and the ULOQ were 0.1 and 10 μM, respectively. It should be noted that the method did not produce a good dynamic range with lower concentrations. A chromatogram of the LOD signal comparing with the LLOQ is shown in Figure 2.5.

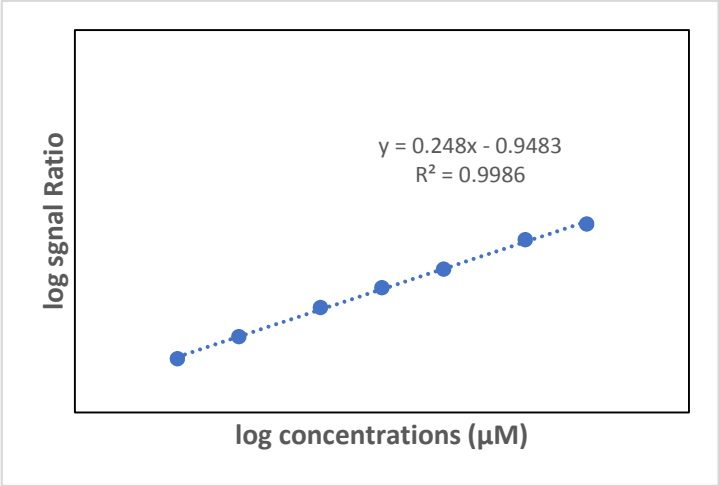

Figure 2.6. Dynamic range of 0.1-10 µM 3-MP in swine plasma µM concentrations with signal ration of 3-MP vs the internal standard.

2.4.2 Accuracy and precision

Accuracy and precision were determined by quintuplicate analysis of the low, medium, and high QCs (0.75, 2.5, and 7.5 µM, respectively) on three different days (within 6 calendar days; Table 1). The accuracy and precision for the method were good. The intra-assay accuracy ±100% and the precision was <10% RSD. The inter-assay accuracy was ±5% and precision was <9% RSD.

Table 2.1. The accuracy and precision of 3-MP analysis in spiked swine plasma by HPLC-FLD.

Concentration (µM)	Intra-assay a Accuracy (%ACC)	Intra-assay a Precision (%RSD)	Inter-assay b Accuracy (%ACC)	Inter-assay b Precision (%RSD)
0.75	±9.0	9.17	±15	7.1
2.5	±6.2	5.63	±8.2	8.6
7.5	±0.87	9.74	±5.3	6.7

a QC method validation (N = 5) for Day 1.

b QC mean from three different days of method validation (N = 15).

2.5. Conclusion

A simple and affordable HPLC-FLD method for the analysis of 3-MP was developed with a very low limit of detection, wide dynamic range, and good accuracy and precision. While Ogasawara et al[50] reported an HPLC fluorescence method for the analysis of 3-MP in mouse tissue, it was time-consuming and required high temperatures (3-MP is highly unstable at high temperatures). Other reported methods used HPLC-MS-MS, and although they produced excellent analytical parameters, the instrumentation needed to apply this method has limited availability. The availability of the method presented here will allow further investigations of 3-MP as a promising cyanide antidote.[27]

CHAPTER 3. CONCLUSIONS AND FUTURE WORK

5.1. Conclusion

Due to current limitations of cyanide antidotes, including large effective dos, delayed onset of action, or dependence on enzymes generally confined to specific organs, there is a need to develop novel cyanide antidotes. 3-MP is promising as a novel cyanide antidote, but previously reported analytical methods have limitations, including requiring sophisticated instrumentation. Therefore, the HPLC method developed in this study should aid in the development of 3-MP as next generation cyanide antidote. The HPLC-FLD method developed was effective for analyzing 3-MP from plasma. The method included simple, low-cost sample preparation, an excellent detection limit, and a large dynamic range, while producing good accuracy and precision. Also, the internal standard used in this method is inexpensive easy to prepare and store compared with previous methods. Also, the instrument used is widely available and affordable, so many labs can use this method. The extraction technique, SPE mixed-mode anion exchange, allowed excellent selectivity for the method. While the HPLC method had advantages, it still has some drawbacks that need to be overcome, including a long HPLC analysis time of 16 minutes, and a gap between the limit of detection and the dynamic range. Overall, the availability of this method will allow further investigations of 3-MP as a promising cyanide antidote.

5.2 Future work

The method presented here has significant implications in the development of 3-MP as next-generation cyanide antidote. Follow-on work should include overcoming the drawbacks of the reported method and application of the method for pharmacokinetic studies.

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