Advanced Analysis of Compounds of Toxicological Importance by Ice Concentration Linked with Extractive Stirrer (Icecles) GC-MS and RP-HPLC-ESI-MS-MS

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BY ICE CONCENTRATION LINKED WITH EXTRACTIVE STIRRER (ICECLES)
GC-MS AND RP-HPLC-ESI-MS-MS

BY

JOSEPH K. DZISAM

A dissertation submitted in partial fulfillment of the requirements for the
Doctor of Philosophy
Major in Chemistry
South Dakota State University
2019
ADVANCED ANALYSIS OF COMPOUNDS OF TOXICOLOGICAL IMPORTANCE
BY ICE CONCENTRATION LINKED WITH EXTRACTIVE STIRRER (ICECLES)

GC-MS AND RP-HPLC-ESI-MS-MS

JOSEPH K. DZISAM

This dissertation is approved as a creditable and independent investigation by a
candidate for the Doctor of Philosophy 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 candidates are necessarily the conclusions of the major
department.

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This dissertation is dedicated, firstly, to Almighty God for His mercies and grace upon my life, and being with me all my life, and also to my late parents, Rebecca, and Eric for their confidence in me. Also to late Nii Boye Adjetey for his numerous support and advice. I would also love to thank my family and kids, especially Maame Efua Dzisam for being patient with me during this period. Lastly, I would like to thank all extended family and friends for their continuous support, encouragement and timely motivation that kept me on and ‘alive’ during this period.
ACKNOWLEDGEMENTS

My greatest acknowledgement goes to Professor Brian A Logue for his awesome mentorship, guidance, and timely assistance during my graduate career, and making this happen is a very tremendous way. My special thanks also goes to my lab members of the ‘Logue Awesome Research Group Extraordinaire’ (LARGE) group, not forgetting Bob Oda, and Raj Bhandari (Ph.D.) for introducing me to the LARGE group initially. I would also like to thank all my colleagues, the Department of Chemistry and Biochemistry, the excellent and distinguished committee members, and all faculty and staff for their assistance, support and encouragements. Furthermore, I would like to thank all institutions and funding agencies that funded my research and contributed significantly for me to complete my degree. Funding for individual projects is acknowledged at end of each research paper.
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ABBREVIATIONS

μM: Micromolar
3- MP: 3-Mercaptopyruvate
3-MST: 3 Mercaptopyruvate sulfurtransferase
Å: Angstrom
AAALAC: Association for Assessment and Accreditation of Laboratory Animal Care
ACN: Acetonitrile
ADP: Adenosine diphosphate
APCI: Atmospheric-pressure chemical ionization
ATCA: 2-amino-2-thiazoline-4-carboxylic acid
ATP: Adenosine triphosphate
BP: Boiling point
CAD: Collision-activation dissociation
Cbi (H₂O)(OH): Aquohydroxocobinamide
Cbi (H₂O)₃⁺: Diaquocobinamide
Cbi (NO₂)₂: Dinitrocobinamide
Cbi (OH)₂: Dihydroxocobinamide
Cbi(CN)₂: Dicyanocobinamide
Cbi: Cobinamide
Cbl: Cobalamine
CDHS: California Department of Health Services
CI: Chemical Ionization
CID-MS-MS: Collision-induced dissociation tandem mass-spectrometric analysis
CI-MS: Chemical-ionization mass spectrometry
CN: CN⁻ or HCN
CSPE: Commercially available cartridge SPE
CWA: Chemical warfare agent
Cytc_{ox}: Oxidized Cytochrome oxidase a_3
Cytc_{red}: Reduced Cytochrome oxidase a_3
DBP: Disinfection byproduct
DL: Detection limits
DMTS: Dimethyl trisulfide
DNA: Deoxyribonucleic acid
DWTPs: Drinking water treatment plants
EI: Electron Ionization
EPA: Environmental Protection Agency
ESI: Electrospray ionization
ETC: Electron-transport chain
FDA: Food and Drug Administration
GC: Gas chromatography
GC-CI-MS-MS: Gas-chromatography chemical-ionization tandem mass spectrometry
GC-HR-MS: Gas-chromatography high-resolution-mass spectrometry
GC-MS: Gas-chromatography-mass spectrometry
HPLC: High-performance liquid chromatography
HR: High Resolution
HRLs: Health reference levels
IACUC: Institutional Animal Care and Use Committee
ICECLES: ICE Concentration Linked with Extractive Stirrer
IM: Intramuscular
IV: Intravenous
LCMRL: Lowest-concentration minimum-reporting levels
LC-MS-MS: Liquid chromatography-tandem mass spectrometry
LINAC: Linear-accelerator collision cell
LLE: Liquid-liquid extraction
LLME: Liquid-liquid microextraction
LLOQ: Lower-limit of quantification
LOD: Limit of detection
Log Kow: log base 10 of octanol/water partition coefficient
LTQ: Linear ion trap
MDL: Method detection limit
MP: Melting point
MRL: Minimum reporting levels
MRM: Multiple-reaction monitoring
NADH: Nicotinamide adenine dinucleotide
NAs: Nitrosoamines
NCD: Nitrogen chemiluminesence detection
NDPA: Nitrosodipropylamine
ng/L: nanogram per liter
NIH: National Institute of Health
NINDS: National Institute of Neurological Disorders and Stroke
NPD: Nitrogen–phosphorus detection
PDMS: Polydimethylsiloxane
PLRP: Polymeric reverse phase column
POTWs: Publicly owned treatment works
PRAs: Percent Residual Accuracies
QC: Quality control
Rh: Rhodanese
ROS: Reactive oxygen species
Rs: Resolution
RSD: Relative standard deviation
RT: Room temperature
S/N: Signal-to-noise
SBSE: Sorptive stir-bar extraction
SCN: Thiocyanate
SEM: Standard error of the mean
SIM: Selective ion monitoring
SNP: Sodium nitroprusside
SPE: Solid-Phase Extraction
SPME: Solid-phase microextraction
TEA: Thermal energy analyzer
TFA: Trifluoroacetic acid
UCMRL: Unregulated-Contaminant Monitoring Reporting Levels
ULOQ: Upper Limit of quantification
USAMRICD: U.S. Army Medical Research Institute of Chemical Defense
V: Volts
WWI: World War I
WWII: World War II
α-KgCN: α-Ketoglutarate cyanohydrin
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ABSTRACT
ADVANCED ANALYSIS OF COMPOUNDS OF TOXICOLOGICAL IMPORTANCE
BY ICE CONCENTRATION LINKED WITH EXTRACTIVE STIRRER (ICECLES)
GC-MS AND RP-HPLC-ESI-MS-MS

JOSEPH K. DZISAM

Cyanide and nitrosoamines (NAs) are ubiquitous compounds, found in our food and water, either by natural process or through man-made activities. The toxicity of cyanide is exerted by its ability to inhibit metalloenzymes such as cytochrome c oxidase, causing concomitant cascades of biochemical effects such as lactic acidosis, inhibition of ATP production, respiratory seizure, and potential death. Nitrosoamines, on the other hand, undergo biotransformation (metabolic activation in cytochrome P450) in the body to produce unstable intermediates that alkylate DNA, causing mutations, and leading to carcinogenesis. In order to further the advancement of a promising cyanide, cobinamide (Cbi), an LC-MS-MS method was developed to analyze cyanide-complex Cbi, while an ICECLES-GC-EI-MS procedure was developed to detect nitrosodipropylamine (NDPA) at low concentrations in drinking water via a flexible, facile, relative easy performed, and green method.

Cbi has shown promise as a therapeutic for cyanide poisoning. While current analysis techniques only measure total Cbi, methods to elucidate the behavior of cyanide-bound Cbi, total Cbi, and available Cbi (i.e., the difference between cyanoCbi and total
Cbi) would be valuable for biomedical and pharmacokinetic studies. Therefore, a method was developed for the analysis of cyanoCbi in plasma via liquid chromatography-tandem mass spectrometry (LC-MS-MS). Plasma samples were prepared by denaturing proteins with 10% ammonium hydroxide in acetonitrile. The resulting mixture was centrifuged, and the supernatant was removed, dried, and reconstituted. CyanoCbi was then analyzed via LC-MS-MS. The limit of detection was 0.2 µM, and the linear range was between 1-200 µM. The accuracy was 100±17% and the precision, measured by relative standard deviation (%RSD), was ≤18.5%. Carryover, a severe problem when analyzing Cbi via liquid chromatography, was eliminated using a polymeric-based stationary phase (PLRP-S) and a controlled washing protocol. The method allowed evaluation of the cyanide-bound and ‘available’ Cbi from treated animals and, when paired with a method for total Cbi analysis. This method allowed for estimation of Cbi utilization when treating cyanide poisoning and verified for the first time, the hypothesized mechanism of treatment of cyanide poisoning by Cbi (direct binding of cyanide).

To overcome the challenges associated with the analysis of NAs at ultratrace levels (i.e., difficult extraction protocols, laborious sample preparation techniques, and requirement for sophisticated/expensive instrumentation), an advanced sample preparation technique, ICE Concentration Linked with Extractive Stirrer (ICECLES), coupled to an inexpensive low-resolution gas-chromatography electron ionization mass-spectrometry instrument was used to analyze NDPA (MRL = 7 ng/L (ppt)). An LOD of 0.2 ng/L was obtained for NDPA, along with linear range of 2 to 50 ng/L was produced (using NDPA-d_{14} internal standard). Both inter- and intraassay precision were ≤13%RSD, while the method accuracy was 100±17.5%. The ICECLES method was applied to screen
for possible NA contamination in selected drinking water sources. The concentration of NDPA in one drinking water source was 2.38±0.34 ng/L. Moreover, NDPA was detected in the two other municipalities tested (i.e., concentrations > 0.2 ng/L), but it was not quantifiable.
CHAPTER 1. INTRODUCTION

1.1. Overall Significance and objectives

Cyanide is a toxic chemical that may be introduced into living organisms by natural process and/or anthropogenic uses [1]. Potential sources of cyanide include edible and non-edible plants (such as apricots, stone seeds, and cassava), cigarette smoke, and occupational exposure from industrial operations such as plastic processing and paint manufacturing. Over one million tons of cyanide are used for industrial purposes, such as being the lixiviant of choice in the mining industry [2]. Cyanide has been used as a chemical warfare agent for terrorist purposes, and for criminal purposes. Cyanide causes toxicity in living organisms by inhibiting cytochrome c oxidase in the mitochondria, resulting in impairment of oxidative phosphorylation, and subsequently leading to progressive lactic acidosis, histotoxic hypoxia, cytotoxic anoxia, and ultimately, death [3-5]. Cobinamide (Cbi) is a novel cyanide antidote proposed to be superior to available FDA-approved therapeutics. However, current methods for analyzing Cbi in biological systems lack the ability to measure the available Cbi, which is imperative to understand the pharmacokinetic behavior in order to approve Cbi as a cyanide antidote. Therefore, the objective of our work was to create a method for the analysis of Cyano Cbi. This method would pair with a method to detect the total Cbi previously developed in our lab to allow quantification of the available Cbi [6].

Nitrosoamines (NAs) are potent mutagens, carcinogens, and teratogens. NAs exert their toxicity by a series of metabolic activation in cytochrome P450 through hydroxylation of the α-C-atom of the N-N=O group of NAs. The α-hydroxy nitrosamine is highly unstable, and can easily degrade to form formaldehyde and methyldiazohydroxyde. The fate of these compounds leads to the production of
diazonium ion or a carbenium intermediate, which both have the propensity to alkylate DNA, RNA and proteins, leading to mutations, and ultimately carcinogenesis [7-11]. NAs are associated with food and soils, and are typically, together with their precursors, found in agro-chemicals, tanned leather products, solvents, cutting fluids, drugs, plastics, rubber additives, detergents, and ground or drinking water. Drinking water is perhaps one of the most concerning routes for NA exposure [12]. NAs are formed during the water purification process as a disinfection byproduct (DBP) during chlorination and chloramination [13-16]. Disinfectants, such as chlorine, ozone, chlorine dioxide or chloramines, react with naturally occurring organic matter or anthropogenic contaminants to produce NAs [13-19]. The presence of NAs in drinking water can also result from contamination by industrial and/or agricultural sources [13, 20], or from anion exchange resins [21] (i.e., anion exchange resins used to remove anionic contaminants can produce higher NA levels in finished products). The US EPA estimates a 10⁻⁶ cancer risk of 0.2–20 ng/L for most nitrosamines in drinking water and unregulated contaminant monitoring minimum reporting levels (UCMRL) range from 2 to 7 ng/L. Analysis of NAs at these concentrations is extremely difficult, requiring sophisticated instrumentation and laborious sample preparation techniques. Therefore, there is the need to develop a simple method that is capable of analyzing one or more NAs as a probe for indicating the presence of NAs (i.e., NAs are not typically found in isolation), to mitigate the cancer risk among the populace across the US and beyond. The specific objective of this work was to develop an ICE Concentration Linked with Extractive Stirrer (ICECLES)-gas chromatography (GC-MS) analysis method to detect nitrosodipropylamine, NDPA, (MRL = 7 ng/L) as a probe for NA contamination.
Chapter 2 reports the determination of available Cbi via liquid chromatography-tandem mass spectrometry (LC-MS-MS). Chapter 3 details the ICECLES-GC-MS, a novel method that has the ability to detect concentrations of NDPA below the EPA minimum reporting limits (MRL) levels without using expensive and sophisticated instrumentation, such as high resolution GC-MS or GC-MS-MS or LC-MS-MS. Chapter 4 details the broader impacts and benefits of this study as well as conclusions and future directions.

1.2. History, Uses and Exposure to Cyanide

Cyanide was first isolated from Prussian blue dye in 1786, and later extracted from almonds around 1800. In about 1815, Gay-Lussac identified cyanide as a colorless and poisonous gas having a characteristic almond-like smell/flavor. He named it cyanogen.

1.2.1. Criminal Uses of Cyanide

Cyanide is a common chemical for suicides and homicides. The malicious use of cyanide has been well-recorded. In November 18, 1978, about 913 members of People’s Temple in Jonestown, Guyana, were murdered by Jim Jones after giving the members a punch laced with cyanide [22]. Seven (7) people in September 29, 1982 were killed when cyanide was added into Tylenol capsules [1, 23]. This led to modern “tamper-proof” packaging ubiquitous in the pharmaceutical industry. The murder of a dry-cleaning owner, Urooj Khan, by cyanide occurred just a day after he won lottery in July 20, 2012 [24, 25]. The killing of physician Dr. Autumn Klein in April 20, 2013 by her husband, Robert Ferrante [26], was perpetrated with a cyanide-laced energy drink. Cyanide fishing, a practice whereby sub-lethal doses of cyanide are used to stun fish for easy catch is also another illegal use of cyanide [1]. Some planned but failed uses of cyanide for criminal purposes includes the purported use of
cyanide in cloud bombing at the World Trade Center by Ramzi Yousef, the Philippine Airlines Flight 434, the attempted poisoning of water supplies to the US embassy in Morocco in 2002, and the planned of dispersing of cyanide gas through indoor ventilation systems by alQaeda terrorists as reported by US Department of Homeland Security in 2003 [27, 28]. In the 1995 Tokyo subway attack that caused over 5000 injuries and 12 deaths [1, 27], cyanide gas precursors were found in the subway washrooms, where the perpetrators had intended to use both cyanide and sarin for the attack [1, 29, 30].

1.2.2. Cyanide as a Chemical Warfare Agent

Chemical warfare agents are defined as any chemical substance whose toxic properties are utilized to kill, injure or incapacitate an enemy in warfare and associated military operations [31, 32]. CWAs are mostly synthetic chemicals that are highly toxic, with fast rapid onset of action, and are generally imperceptible to the senses [27]. Cyanide is a considered blood agent because its mode of action entails prevention of normal oxygen utilization by the tissues [27, 31]. Hydrogen cyanide (HCN) and cyanogen chloride (CNCl) [inclusively referred to as cyanide] are the most common CWAs of the blood agent class, and were first used by the French as a CWA in the battle of Somme in 1916 [28, 33]. Zyklon B gas (HCN) was used by the Nazis during World War II to kill millions of innocent civilians [27, 28, 30]. Cyanide was also used as a CWA during the Iran–Iraq war from 1983-1988 [28]

1.2.3. Industrial Uses of Cyanide

Worldwide, over 1 million tons of hydrogen cyanide are produced annually for legitimate purposes and approximately 0.9 million tons of this are used in the United States [1, 2]. Cyanide is mostly used in plastic manufacturing, photographic developing, metallurgy, adhesives, computer electronics, fire retardants, cosmetics,
some dyes, some pharmaceuticals, and in ship fumigation [1, 2, 34, 35]. Cyanide is also used for metal mining (e.g., gold and silver ores), metal cleaning/polishing, production of triazines for agricultural herbicides, production of adiponitrile for nylon, and in making rocket propellant [2, 24, 34]. Other uses include the production of methionine for animal feed supplements, and the production of chelating agents such as nitrilotriacetate for water and waste water treatment [36, 37]. Some derivatives of cyanide, such as sodium ferrocyanide and ferric ferrocyanide, are used as road salts to serve as anticaking agents [36-38].

1.2.4. Potential Sources of Cyanide and Exposure

The sources of cyanide can be natural or anthropogenic [34]. Exposure of cyanide in any form can occur through inhalation (such as smoke from cigarettes or fires), ingestion of cyanogenic compounds (e.g., cassava, almonds, lima beans etc.), dermal absorption (e.g., occupational hazards, i.e., coming into contact with cyanide at the workplace and by parenteral administration (such as the administration of sodium nitroprusside for vasodilation) [34, 39, 40].

1.2.4.1. Natural Sources of Cyanide

Cyanide is ubiquitous in nature and can be produced in certain plants, bacteria, fungi, and algae [34, 41-43]. Most natural sources of cyanide emanate from organocyanide complexes (i.e, organic compounds that has part of it as cyanide functional group attached to a carbon atom by way of covalent bonding, e.g., such as acetonitrile). Under normal environmental conditions, there are over 2650 cyanophoric plant species (of about 550 genera and 130 families) (Figure 1.1) to produce cyanogenic glycosides and HCN as a natural defense mechanism (typically against herbivory and pathogenic attack). Cyanogenesis may also occur when a plant is damaged or stressed [43]. Most glucosidic cyanogens are produced naturally from
amino acids (e.g., lotaustralin from isoleucine, linamarin from valine, amygdalin and prunasin from phenylalanine, and dhurin from tyrosine) [42]. Some examples of cyanogenic plants include plants with significant economic and commercial value such as cassava, lima beans, corn, forage materials (e.g., alfalfa, sudan grasses, and sorghum), stone fruits (e.g., apricots, apples, peaches), and horticultural plants (e.g., ornamental cherry and cherry laurel). Cassava, a common tropical staple food in most African countries, contains about one-half the lethal dose of cyanide if consumed daily [42], and it has been associated with the neurological disease known as Konzo [44-51]. Cyanides are also produced by almost all fruit-bearing plants during ethylene synthesis and nitrile metabolism [34, 43, 52]. Some nitriles can also be found in shale oils, as plant growth hormones or as a product of cyanohydrins. Some species of fungi, such as Clitocybe, Marasmus, Pholiota, Polyporus, and Tricholoma are capable of undergoing fungal cyanogenesis to produce hydrogen cyanide. In fact, the first production of cyanide by microbes was reported in Marasmius oreades by von Lösecke in 1871 [42]. Other types of microbes that produce cyanide are some strains of the bacteria pseudomonads and Chromobacterium violaceum, as well as the algae Chlorella vulgaris [42, 43, 53, 54]. Also, HCN is present in the earth’s atmosphere, and has been detected in the troposphere and the stratosphere [43].
Figure 1.1. Cyanogenesis in Plants [43].

1.2.4.2. Production of Cyanide

Cyanide in its various forms is directly produced or is used as a raw material/precursor for the production of other compounds. An increasing amount of HCN has been produced in the industry over recent years, as shown in Figure 1.2 [43]. Cyanide is produced by the Andrussov process (a three-step process where ammonia, natural gas and air are reacted in the presence of platinum catalyst to form HCN) or the Blausaure Methane Anlage (BMA) process (which involves the use of ammonia and natural gas for the production of cyanide) [55]. HCN is very important in industry because it is the main precursor for the manufacturing of methyl adiponitrile, methacrylate for polymer manufacturing, cyanuric chlorides, chelating agents, and sodium/potassium cyanide for hydrometallurgy of minerals [34, 55]. Apart from the sources listed above, cyanide can also be emitted from iron and steel production, carbon black production, carbon fiber production, and petroleum refining.
The number of workers exposed to cyanide are estimated as follows: 4,005 to hydrogen cyanide, 66,493 to sodium cyanide, 64,244 to potassium cyanide, 3,215 to potassium silver cyanide, 3,606 to calcium cyanide, 22,339 to copper (I) cyanide, and 1,393 to cyanogen chloride [34]. An example of an occupational exposure was the August 12, 2015 warehouse explosion in Tianjin, China [56-58], where an explosion occurred in a warehouse containing cyanide, and 114 people were killed, 700 injured, and 69 were declared missing [58].

Figure 1.2. HCN production from 1983 to 2001 [43].

1.2.4.3. Environmental Sources of Cyanide

Potential sources of cyanide in the environment are wide-ranging, and include natural sources such as cyanogenic plants or anthropogenic sources, including
discharges from industrial processes, cyanogenic drugs, laboratories, fires, chemical warfare operations, and cigarette smoke.

It is estimated that cyanide emissions are about 44 million pounds/year. The main source of cyanide is from car/automobile exhausts. Other cyanide sources include chemical processing (e.g., methyl methacrylate, acrylonitrile, hydrogen cyanide, and municipal waste incinerators), metabolic or biogenic processes from microbes such as fungi and bacteria, and higher plants also release hydrogen cyanide into the atmosphere. In addition, biomass burning contributes about 1-3 billion pounds of cyanide/year in the form of hydrogen cyanide and acetonitrile according to field measurements and modelling estimates [43].

A significant source of cyanide in water and soil is from accidental leaks from heap leaching pads from the gold mining industry [43]. Examples include the release of over 1 billion gallons of cyanide bearing tailings due to a dam failure in Guyana in 1995, 7 million gallons of cyanide from a storage pond to the Beaver Dam Wash in Utah in 1995, and the over 250,000 gallons of cyanide solution from a heap pad in a Gold Quarry Mine in Nevada, 1997 [43]. The release of cyanide into aquatic systems may occur from discharges from point sources such as publicly owned treatment works (POTWs), the organic chemical industry, and the iron and steel industry. Nonpoint sources are mainly from agricultural, road runoff, and atmospheric fallout [34, 59].

The largest sources of cyanide in soil are from landfills and anticaking agents. Cyanide can also be released into the soil through natural biogenic processes of bacteria, fungi, and cyanogenic plants as well as by way of decay of plant tissues from the Brassica family [34].
1.2.4.4. Iatrogenic Sources of Cyanide

Cyanide exposure can occur when taking sodium nitroprusside (SNP) as antihypertensive drug for a prolonged period of time [39, 60-62]. Usually, nitroprusside is administered intravenously to reduce peripheral blood pressure by vasodilatation (by the release of nitric oxide, NO), and also to control bleeding during surgery [63-66]. However, SNP contains about 44% cyanide by molecular weight, which, can be released upon administration [39, 63, 67-69]. The liver enzyme rhodanese is capable of converting cyanide to the innocuous thiocyanate. However, toxicity may occur if the administration is prolonged or there is a defect in cyanide metabolism, such as impaired renal function, thiosulfate deficiency, or rhodanase deficiency [39]. The toxicity of sodium nitroprusside can be eliminated when other therapeutics such as a sodium thiosulfate and or sodium nitrile are co-administered [61, 63, 70]. Another Iatrogenic source of cyanide is from succinonitrile, a commonly used drug in Europe, as an antidepressant [71] and laetrile, an anticancer drug [39, 72-78].

1.3. Fate and Toxicity of Cyanide

Cyanide forms stable and reversible complexes with biologically active metal ions in metalloenzymes (e.g., cytochrome c oxidase, peroxidase, nitrogenase, etc.), and consequently inhibits the function of the enzymes. Furthermore, the cyanide can inhibit the function of some non-metalloenzymes, such as ribulose diphosphate carboxylase [71]. The main cause of cyanide toxicity is a result of binding cyanide with cytochrome c oxidase $a_3$ (the ferric iron atom), a terminal oxidase of the mitochondria electron-transport chain (ETC), and consequently blocks oxidative phosphorylation [79]. Cytochrome c oxidase is the primary source of energy production in the form of ATP (Adenosine triphosphate). It facilitates a series of
electron and proton transfer from O₂ to produce water [34, 40, 80], as shown in Figure 1.3 [81]. NADH delivers electrons and protons initially to complex I, which in turn, are transported throughout the electron-transport chain (ETC) via complex I through IV. As the electrons are transferred, protons are sequentially pumped to the cytosolic side of the inner membrane, creating an electrochemical gradient. The electrochemical gradient generates a membrane potential that is harnessed by ATP-synthase to produce ATP [40, 82]. The net reaction in the cytochrome c oxidase a₃ (complex IV) in transport of electrons and protons is shown in Equation 1.1 [82]. The transfer of electrons to O₂ is the terminal step for oxidative phosphorylation. When cyanide interacts with cytochrome c oxidase a₃, it binds with the trivalent iron in the enzyme, and inhibits the activity of the enzyme complex, leading to anaerobic respiration 33 [34, 82]. To compensate, excess pyruvate is produced, but cannot be channeled through the ETC [81-83]. This results in the production of excess lactic acid via anaerobic metabolism, leading to metabolic acidosis, and decreased pH in the body. The anaerobic metabolism may also lead to progressive histotoxic hypoxia, oxidative stress (from production of excess reactive oxygen species, ROS), cytotoxic anoxia, and cell death [5, 39, 40, 83-88].
Figure 1.3. Electron transfer mechanism and the generation of proton-motive force required for oxidative phosphorylation to take place in the mitochondria. Four protons, known as the ‘chemical protons’ (since they are ‘participants’ of the reaction) are taken up from the matrix side to oxygen. At the same time, four additional protons are transported from the matrix side into the cytosol (cytoplasm) to bring about the electrochemical gradient that is required for the production of ATP through bioenergetics-coupled reactions [82].

$$4\text{Cyt}_{\text{red}} + 8H^+_{\text{matrix}} + O_2 \rightarrow 4\text{Cyt}_{\text{ox}} + 2H_2O + 4H^+_{\text{cytoplasm}} \quad (1.1)$$

In Equation 1.1, $\text{Cyt}_{\text{red}}$ and $\text{Cyt}_{\text{ox}}$, refers to the reduced and the oxidized form of Cytochrome oxidase $a_3$, respectively. A CN-bound cytochrome $c$ oxidase cannot be reduced, and cellular oxygen utilization is impaired. Consequently, aerobic respiration is reduced or stopped [81, 84, 86].
1.3.1. Absorption of Cyanide

The major route of cyanide absorption is through the gastrointestinal tract and the respiratory system, with minimal dermal absorption [34, 40, 55, 84]. Cyanide is absorbed readily in the gastrointestinal tract but the rate of absorption across the gastrointestinal mucosa depends on a number of factors such as the pH of the gut, the solubility and the form of cyanide [40]. Usually, small, neutral and non-ionized compounds are readily absorbed by biological membranes. NaCN and KCN are easily dissolvable, and can be absorbed in the stomach-post ingestion. Also acidic conditions in the stomach enhance the absorption of hydrogen cyanide (pKₐ of 9.2 at 25 °C) [34, 40]. HCN is mostly absorbed after inhalation, where it instantly passes through the alveolar epithelium of the lungs [34, 40].

1.3.2. Distribution of Cyanide

Once cyanide enters the body and is absorbed, it is distributed throughout the body, with targeted tissues being those that have the highest oxygen demand (e.g., heart, brain, liver, kidney, stomach). The brain and the heart are the primary target organs in acute cyanide intoxication, with characteristic symptoms, such as convulsion, impaired respiratory activity, and cardiovascular difficulties. Also, elevated cyanide levels can be measured in absorptive tissues, such as lungs (when cyanide is exposed via inhaling) and stomach (when cyanide is orally ingested) [34, 40, 55]. Approximately 60% of absorbed cyanide binds to proteins, and has high affinity for transition metals, such as iron and cobalt [84].

1.3.3. Acute Effects of Cyanide

Acute and subacute effects of low-level cyanide may result in lactic acidosis, progressive anorexia, anxiety, weak and rapid pulse rate, nausea, and vertigo [34, 43, 71]. The symptoms progress to irregular heartbeats, cardiac arrhythmias, drop in body
temperature, cyanosis of lips and face, and sometimes, frothy bloody saliva from mouth, and death [34, 39, 71, 83]. For humans, inhalation of about 110 to 135 ppm of cyanide may result in death within few hours, while an inhalation of about 220 to 270 ppm causes death in minutes [71].

1.3.4. Chronic Effects of Cyanide

Chronic effects of cyanide are systemic and can include neurological, respiratory, cardiovascular, and hepatic disorders [34, 71]. Chronic cyanide exposure leads to neuropathic and thyrotoxic disorders [52, 71].

Brain areas that control skeletal muscle activity (i.e basal ganglia, cerebellum), memory (hypothalamus), and cognitive ability (cortex) are the most effected parts of the central nervous system from cyanide poisoning [43]. Human neuropathies associated with nonlethal chronic cyanide exposure of low cyanide levels includes Nigerian nutritional neuropathy (tropical ataxic neuropathy, TAN), Konzo, tobacco amblyopia, and Leber’s optical atrophy [34, 71, 89]. Neurological effects of cyanide poisoning are mainly due to the blockage of mitochondrial cytochrome c oxidase activity, leading to rapid biochemical changes in the brain, such as changes in ion flux, neurotransmitter release (both excitatory and inhibitory), peroxide formation, and decrease in the ATP/ADP ratio required for cellular calcium homeostasis [34, 43].

The cardiovascular system is an important site for cyanide toxicity [43]. Specific cardiovascular symptoms of cyanide exposures include hypotension, supraventricular tachycardia, atrioventricular blocks, ventricular fibrillation, and asystole [34, 39]. Cyanide toxicity also alters respiration due to inadequate cellular oxygen utilization, leading to deficit in traditional global oxygen consumption/oxygen saturation curve [78, 90]. Respiratory effects of cyanide toxicity include coughs, sore
throat, nasal congestion, epistaxis, hemoptysis, dyspnea, apnea, decrease in respiratory rate/hypoventilation, and asphyxia [34, 39].

1.4. Cyanide Antidotes

Cyanide has several antidotes that have different mechanisms of action/pharmacokinetics, clinical effects, and risk-benefit profiles. Cyanide antidotes include the Nithiodote (which contains sodium nitrile and sodium thiosulfate) and Cyanokit (hydroxocobalamin). Dicobalt-ethylenediamine tetraacetic acid (dicobalt Edetate) and 4-dimethylaminophenol (4-DMAP) are used to a lesser extent due to their relatively significant contra-indications [6, 39, 84, 91-93].

1.4.1. Clinical Properties of Ideal Cyanide Antidotes

A cyanide antidote that meets the need of a victim in acute exposure of cyanide should have some important properties, including 1) rapid onset of action, 2) capable of neutralizing cyanide without interfering with cellular oxygen use or transport, 3) conducive safety and tolerability profiles in pre-hospital settings and emergency situations, 4) safe for use with victims with smoke inhalation, 5) not harmful when administered to non-poisoned patients and 6) easy to administer, especially in mass casualty situations [91, 93]. Table 1.1 [91] shows some cyanide antidotes and their ability to satisfy these ideal qualities.
Table 1.1. Selected cyanide antidotes and their suitable qualities as cyanide antidotes [91].

<table>
<thead>
<tr>
<th>Quality</th>
<th>CAK</th>
<th>Na₂S₂O₃ alone</th>
<th>4-DMAP</th>
<th>Dicobalt edetate</th>
<th>Cbl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rapid onset of action</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Neutralize CN without compromising oxygen-carrying capacity</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Tolerability and safety profile conducive to prehospital use</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Safe use in smoke-inhalation victims</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Not harmful when administered to non-patients</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Easy to administer</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

1.5. 1.4.2. Mechanism and General Classes of Cyanide Antidotes

The current US FDA approved cyanide antidotes are sodium nitrite, sodium thiosulfate, and hydroxocobalamin [6, 30, 35, 39, 56, 84, 91, 92, 94-96].

1.4.2.1. Methemoglobin generators

A methemoglobin generator, such as sodium nitrite, reverses cyanide poisoning by interacting with hemoglobin (Hgb) to form the oxidized Hgb, methemoglobin (MetHgb). CN has low affinity to Hgb but has relatively high affinity to MetHgb. Consequently, CN reacts with MetHgb to form cyano-MetHgb complex (Scheme 1.1), which can be facilely excreted via urine [84, 91, 92]. Although nitrite activity is mainly attributed to the formation of MetHgb, recent studies suggest that nitrite-mediated conversion of Hgb to MetHgb is too slow to account for cyanide
antagonism [96]. It has been proposed recently that another mechanism of action accounts for the nitrite’s antidotal activity; nitrites induce the generation of nitric oxide, which subsequently displaces cyanide bound to the active site of cytochrome c oxidase [84, 95-97], which restores the activity of cytochrome c oxidase. The displaced CN may be scavenged by circulating MetHgb, or converted to SCN [84]. However, methemoglobinemia produced by nitrite reduces the oxygen saturation level and transport. This is a serious condition for victims who are exposed to cyanide through smoke inhalation, where the formation of carboxyhemoglobin occurs, in addition to methemoglobinemia. As a result, administration of sodium nitrite is followed by careful observation of victims’ oxygen level, and constant oxygenation. Another disadvantage of sodium nitrite administration is that it requires intravenous administration. Thus, the main challenges of sodium nitrite administration as a cyanide antidote are its contra-indication exhibited by compromising oxygen transport, and difficulty with mode of administration, requiring special skill for administration [91].

\[
\text{NaNO}_2 + \text{HgbO}_2 \rightarrow \text{MetHgb}
\]

\[
\text{MetHgb} + \text{CN-CytC} \rightarrow \text{CN-MetHgb} + \text{CytC}
\]

**Scheme 1.1.** Sodium nitrite (NaNO\(_2\)) helps in scavenging cyanide. Fe\(^{2+}\) in the oxyhemoglobin is oxidize to Fe\(^{3+}\) in MetHgb. Formation of large amounts of Methgb with its concomitant trivalent ferric iron has relatively high affinity for cyanide, and so binds cyanide more than *cytochrome c oxidase* (CytC) [56, 98].

### 1.4.2.2. Sulfur Donors

Sulfur donor-based antidotes, such as sodium thiosulfate (Na\(_2\)S\(_2\)O\(_3\)) detoxify cyanide by first donating sulfur (S) to Rhodanese (Rh) to form S-Rh [92, 95]. The sulfur-bound Rh then reacts with the CN to form SCN\(^-\) (Scheme 1.2) [99], which is
easily excreted in the urine. *CytC Oxidase* activity is restored in this process and aerobic respiration resumes [39, 91, 92]. However, Rh is predominantly located in the mitochondrial matrix of the liver and kidney but limited in the central nervous system (brain) and heart, leaving these two important organs relatively unprotected [40, 56, 91, 92, 95]. In addition, Na$_2$S$_2$O$_3$ has slow onset of action due to limited penetrability through the mitochondrial membranes to the enzyme Rh. Furthermore, Na$_2$S$_2$O$_3$ requires intravenous administration [6, 56, 60, 84, 92, 95].

\[
\begin{align*}
Rh & \stackrel{k_1}{\rightleftharpoons} SSO_3^- \\
& \stackrel{k_{-1}}{\rightleftharpoons} Rh(SSO_3^-) \\
& \stackrel{k_2}{\rightleftharpoons} Rh - S + SO_3^- \\
Rh - S + CN^- & \stackrel{k_3}{\rightarrow} SCN^- + Rh
\end{align*}
\]

**Scheme 1.** Rhodanese enzyme reaction with CN to produce SCN$^-$. Mechanistically, the reaction goes through a double replacement process to catalyze the detoxification of cyanide by sulfuration [40, 99, 100]. The free enzyme (Rh) reacts with a sulfane sulfur donor (e.g., SSO$_3^-$) to cleave the S-S bond to form the persulfide-substituted enzyme, Rh-SSO$_3^-$. The Rh-SSO$_3^-$ then reacts with sulfur acceptor substrate (CN$^-$) to form the innocuous thiocyanate, SCN, and regenerates the enzyme, Rh [40, 99].

**1.4.2.3. Direct Binding Agents**

The only FDA-approved direct binding cyanide antidote is hydroxocobalamin [84, 91, 92, 95, 101, 102] (Figure 1.4). Hydroxocobalmin (Cbl) is a hydroxylated form of vitamin B12 [40, 84]. Cyanide has a greater affinity to bind with Cbl than cytochrome oxidase a$_3$ [103]. Thus, Cbl directly reacts (non-enzymatically) with cyanide to form the cyanohydroxocobalamin (CN-Cbl) (Scheme 1.3), which is renally excreted [101]. Cbl is relatively safe because it does not interfere with oxygen-carrying capacity of blood, it is not harmful when administered to non-poisoned patients, and it is easy to administer for small number of casualties at the site of exposure etc. [39, 40, 102, 104]). Even though Cbl is effective, large volumes are
required via intravenous administration. Consequently, Cbl is not suitable in mass casualty situations [40, 56, 84, 95].

\[ \text{Cbl} + \text{CN}^- \rightarrow \text{CN-Cbl Complex} \]

**Figure 1.4.** Chemical structure of hydroxocobalamin, with the nucleotide moiety, dimethylbenzimidazole ribonucleotide, shown in blue color.

**Scheme 1.3.** Reaction of hydroxocobalamin with cyanide, without the aid of enzymes. Cbl binds cyanide molecule in a 1:1 mole ratio [105, 106].

**1.4.2.4. Combination Therapies**

In the US, Nithiodote is the only FDA-approved combination therapy. Nithiodote is typically administered by intravenous infusion, which is not practical when trained personnel are absent and in mass casualty situations [35]. For effective and efficient treatment during mass casualty scenarios, the best mode of administration should be via intramuscular injection of the antidote by first responders, usually from a prefilled autoinjector. For this situation to be feasible, the
antidote needs to be stable in solution, sufficiently soluble to be administered in 1-3 mL doses, needs to be highly potent so that relatively small amount is administered, and should be readily absorbed when administered intramuscularly [35, 94]. Sodium nitrite and sodium thiosulfate are quite soluble in water, and stable under anaerobic conditions, and are typically effective when administered intravenously. However, current work by Bebarta et al. [94] shows that Nithiodote has some activity against cyanide when administered by intramuscular injection.

When studying other combination therapies, Berbata et al. [107] showed that hydroxocobalamin with sodium thiosulfate led to faster return to baseline mean arterial pressure compared with administration of sodium nitrite with sodium thiosulfate. There was no significant difference in mortality, serum acidosis, or serum lactate for either of the antidote combinations. Hall and Rumack [108] reported that hydroxocobalamin with sodium thiosulfate is an efficacious cyanide antidote with little inherent toxicity.

1.4.3. Next Generation Cyanide Antidotes

Since the onset toxicity of cyanide is rapid, there is the need for antidotes that are capable of being rapidly administered by minimally trained individuals, especially in a mass exposure scenario. Therefore, the development of next generation antidotes, which possess better therapeutic indices and efficacy, have a relatively easy mode of administration (e.g., intramuscularly or orally), and are equally effective throughout the body, is an important priority going forward. Currently, the most well-studied next generation cyanide antidotes under investigation are cobinamide [6, 30, 35, 84, 93, 105, 109], sulfanegen (i.e., 3-mercaptopyruvate) [6, 84, 92, 95, 96, 105, 110], and dimethyl trisulfide [56, 111-114]. Table 1.2 outlines some of these antidotes, each of
which can be administered intramuscularly (IM) in mass casualty scenarios, and their
mode of action [84].

**Table 1.2.** Novel Cyanide therapies in development for rapid IM in mass casualty
scenarios [84].

<table>
<thead>
<tr>
<th>Novel therapy</th>
<th>Mode of action</th>
<th>Older analog</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cobinamide</td>
<td>Direct binding agent</td>
<td>Cbl</td>
</tr>
<tr>
<td>Sulfanegen</td>
<td>Sulfur donor (leverages 3 mercaptopyruvate sulfur transferase)</td>
<td>3-MP</td>
</tr>
<tr>
<td>DMTS</td>
<td>Sulfur donor (with/without Rhodanese enzyme)</td>
<td>NA</td>
</tr>
</tbody>
</table>

**1.4.3.1. Cobinamide**

Cobinamide is the penultimate precursor in the biosynthesis of cobalamin (Cbl). Structurally, Cbi is similar to Cbl but Cbi lacks dimethylbenzimidazole nucleotide tail moiety [6] that is coordinated to the cobalt atom in the lower axial position of the Corrin ring (Figure 1.5). The lack of the dimethylbenzimidazole ribonucleotide in Cbi makes it possible for it to have a second free binding site, and prevents the trans site steric hindrance associated with the dimethylbenzimidazole group [30, 79, 84, 109, 115-120]. Consequently, Cbi has relatively smaller mass [6], and a mole of Cbi binds to two moles of cyanide (1 mole Cbl binds to only 1) with an overall cyanide binding affinity of $10^{22} \text{ M}^{-2}$ (10$^{12}$ mol/L for Cbl) [117-119]. Another therapeutic advantage of Cbi includes its relative solubility (about 5 times than Cbl), and stability at room temperature, as well as its ability to be administrated intramuscularly [35, 93, 105, 109, 116]. Cbi is the generic term used for diaquocobinamide [(H$_2$O)$_2$Cbi$^{2+}$] or its conjugate base, hydroxoaquocobinamide
[(OH)(H₂O)Cbi⁺] [117, 119]. Cbi binds strongly to transmembrane proteins, placing a challenge on its distribution [121, 122]. However, the dinitrocobinamide form [(NO₂)₂Cbi] [6, 35] and the sulfite form [(SO₃)₂Cbi] [93] had been reported to be fairly distributable in biological system. Research by Broderick et al. [115] revealed that Cbi was several-fold more effective than Cbl in (i) reversing cyanide inhibition of oxidative phosphorylation in mammalian cells; (ii) rescuing mammalian cells and Drosophila melanogaster from cyanide toxicity; and (iii) reducing cyanide inhibition of Drosophila Malpighian tubule secretion. Brenner et al. [93, 116] also showed that Cbi was better at reversing cyanide toxicity than Cbl in cyanide exposed animals. Adriano et al. [105] reported that Cbi has a higher affinity for nitric oxide than Cbl, an additional advantage for Cbi’s greater efficacy as a cyanide antidote. Nitric oxide has the ability to regulate the activity of cytochrome c oxidase, the main cyanide target.

Figure 1.5. Structure of cobinamide without any ligands attached, and lacking the dimethylbenzimidazole ribonucleotide (as in Cbl in Figure 1.4).
1.4.3.2. Sulfanegen/3-Mercaptopyruvate (3-MP)

Sulfanegen, a prodrug of 3-MP (i.e., sulfanegen converts to 3-MP upon administration), is a potent cyanide antidote. 3-MP is a sulfur donor that converts cyanide to thiocyanate by utilizing the ubiquitous cyanide detoxifying enzyme, 3-mercaptopyruvate sulfur transferase (3-MPST) [6, 84, 92, 95, 96, 123], as outlined in Scheme 1.4 [92]. 3-MPST is relatively more widely distributed in tissues, including the central nervous system, and can be found both in the mitochondria and the cytosol. These features of 3-MPST make it more advantageous for efficient detoxification of cyanide than rhodanase (which is limited in distribution and found only in the mitochondria) [6, 92]. Similarly to rhodanese, the mechanism of reaction of 3-MPST involves generation of an enzyme-persulfide intermediate (3-MPST-3MP) that reacts with cyanide to produce thiocyanate, and regenerates the free enzyme, 3-MPST [96]. The sulfur donor for the 3-MPST is from the cysteine catabolite 3-MP and the site of the persulfide formation is postulated to take place at Cys248 [96]. However, 3-MP decomposes rapidly and facilely in the blood. Therefore, to ensure continuous supply, an intramuscular administration of sulfanegen is required for cyanide detoxification [84, 92]. Interestingly, current research work by Chan et al. [105] shows that combination of cobinamide and sulfanegen, when administered intramuscular, gives a great promise as a new approach to treating cyanide poisoning.
Scheme 1. Structure of sulfanegen sodium and the enzymatic transfer of sulfur from 3-MSPT to pyruvate with concomitant conversion of cyanide (CN) to the innocuous thiocyanate (-SCN) [92].

1.4.3.3. Dimethyltrisulfide (DMTS)

Dimethyl trisulfide (DMTS), is a promising next-generation cyanide antidote that functions by acting as a sulfur donor to detoxify cyanide by converting it into thiocyanate with or without rhodanese [56, 124]. The proposed mechanism for the detoxification of cyanide by DMTS via rhodanese is well enumerated, but the non-enzymatic routes are not as well-understood [56]. Compared to thiosulfates, a recent study shows that DMTS is about 40 times more effective than thiosulfates \textit{in-vivo} in the presence rhodanese, and about 80 times more efficacious than thiosulfate via non-enzymatic routes. In addition, studies by Kiss \textit{et al.} [124] revealed that DMTS has better efficacy than the Cyanokit (hydroxocobalamin) and the Nithiodote (sodium nitrite and sodium thiosulfate) therapies. The main therapeutic feature of DMTS is its relatively high lipophilicity, making it possible to facilely cross the blood brain barrier [56]. It can also be administered intramuscularly [112, 125] and provides cardiovascular protection [124]. However, recent studies by Dong \textit{et al.} [126] shows
that DMTS does react slowly with Hgb to form MetHb. They suggested that the reaction of DMTS and Hgb could explain the lower recoveries of DMTS from fresh blood in which the Hb/metHb ratio was higher than in aged blood.

1.5. Analytical Methods For Detection of Cobinamide derivatives in Biological Fluids

Several methods, including colorimetric techniques, HPLC, UV, ICP-MS, and currently HPLC-MS-MS have been used to analyze Cbi and its derivatives [6, 118, 127-131]. Absorbance-based techniques have relatively low sensitivity. The ICP-MS method produces quite good sensitivity but lacks the method efficiency and detection limit for the sample size expected, and complex sample matrix [118]. Alsberg et al. [132] developed a method for the analysis of hydroxyalkyl derivatives of cobalamin (i.e., hydroxypropyl cobalamin, OH-PrCbl), using HPLC with ESI-MS-MS and ultraviolet diode array detection (UV-DAD). One of the methods made use of two different mobile phases (eluent A and B, respectively) and two column systems: Eluent A was acetonitrile and water with trifluoroacetic acid (TFA) added to a concentration 0.4 mM (ACN/water with TFA), and Eluent B was made up of ACN/water with 1-methylpiperidine. Column I and II used were Kromasil C18 (4.6 mm x 250 mm, 5 mm), and Zorbax C18 (2.1 x 150 mm, 3.5 mm), respectively. The limit of detection obtained was 0.2 pg/mL. The analytical method by Schwenter et al. [133] involved an LC-MS-MS method for the detection of hydroxocobalamin (HOCbl) and cyanocobalamin (CNCbl) in swine plasma, obtaining an LOD of 1.0 μM each for HOCbl and CNCbl. An online SPE HLB column (2.1 mm × 20.0 mm, 25 μm) was used for extraction, while a C18 Phenomenex Luna analytical column (2.0 mm × 150 mm, 5 μm) was used for the analytical separation. Mobile phase A consisted of 2.5 mM ammonium acetate in 50:50 methanol:water, (pH 4.0), and
solvent B was 10% acetonitrile in water. Similarly, Mendes et al. [134] performed pharmacokinetic studies for HOCl in human plasma using HPLC-ESI-MS-MS. The samples were extracted from the plasma by liquid-liquid extraction using 100% ethanol, at -20°C, and separated with Prevail C8 analytical column (2.1×100 mm, 3 μm). Acetonitrile: water (50:50 v/v) was used as mobile phase A, while mobile phase B was composed of 10 mM ammonium acetate with 0.1% formic acid. The limit of quantification was 5 ng/mL. McCracken and Brittain [118] also reported an LC-ESI-MS-MS method for the analysis of total cobinamide in swine plasma, following Cbi ‘total’ conversion to dicyanoCbi with excess cyanide in swine plasma. The lower limit of quantification was 25 ng/L. The separation of the analyte was performed using a Phenomenex Kinetex C18 column (2.1 × 150 mm, 2.6 μm). The mobile phase composition was 0.01% formic acid in water (A) and 0.01% formic acid in acetonitrile (B). Stutelberg et al. [6] gave the first comprehensive method for the simultaneous analysis of cyanide antidotes Cbi and 3-MP using LC-ESI-MS-MS. The sample preparation protocol for the Cbi was similar to that reported by McCracken and Brittain. Separation was performed by using a Phenomenex Synergy Max RP column (2.0 × 50 mm, 4 μm) with the elution aided by 5-mM aqueous ammonium formate with 10% methanol (Mobile Phase A) and 5-mM ammonium formate in 90% methanol (Mobile Phase B). This analytical method produced an LOD of 0.2 μM and a linear range of 0.5 to 50 μM with excellent precision and accuracy.

1.6. History, Formation and Exposure to Nitrosoamines

Most nitrosoamines are relatively polar (i.e., very soluble in water with low log K_{ow}) and have very small Henry’s constants (Table 1.3) [16]. The low log K_{ow} of NAs makes it difficult to extract them with organic solvents, and the small Henry’s constants is an indication that they cannot be removed from water by aeration [16].
Table 1.3. Some physical and chemical properties of some NAs [135].

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical Structure</th>
<th>BP(^a) (°C)</th>
<th>MP(^b) (°C)</th>
<th>Henry’s LC(^b) (atm m(^3)/mol) (10(^{-6}))</th>
<th>Color (physical state)</th>
<th>Water Solubility (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-nitrosodibutylamine (NDBA)</td>
<td><img src="image" alt="chemical structure" /></td>
<td>116 at 14 mmHg</td>
<td>&lt;25</td>
<td>13.2 at 37 °C</td>
<td>Yellow oil</td>
<td>1.2 at 24 °C</td>
</tr>
<tr>
<td><strong>N-nitrosodipropylamine (NDPA)</strong></td>
<td><img src="image" alt="chemical structure" /></td>
<td>206</td>
<td>-12-6.6</td>
<td>5.38</td>
<td>Yellow liquid</td>
<td>13</td>
</tr>
<tr>
<td>N-nitrosodiethylamine (NDEA)</td>
<td><img src="image" alt="chemical structure" /></td>
<td>175-177</td>
<td>&lt;25</td>
<td>3.63</td>
<td>Slightly Yellow liquid</td>
<td>106 at 24 °C</td>
</tr>
<tr>
<td>N-nitrosopyrrolidine (NPyr)</td>
<td><img src="image" alt="chemical structure" /></td>
<td>214 at 760 mmHg</td>
<td>-</td>
<td>0.0489 at 37 °C</td>
<td>Yellow liquid</td>
<td>10(^3) at 24 °C</td>
</tr>
<tr>
<td>N-nitrosomethylthylamine (NMEA)</td>
<td><img src="image" alt="chemical structure" /></td>
<td>163</td>
<td>-</td>
<td>1.82 at 37 °C</td>
<td>Yellow liquid</td>
<td>300 at 20 °C</td>
</tr>
<tr>
<td>N-nitrosodimethylamine (NDMA)</td>
<td><img src="image" alt="chemical structure" /></td>
<td>151-153</td>
<td>-50</td>
<td>1.44 at 25 °C</td>
<td>Yellow liquid</td>
<td>10(^3)</td>
</tr>
</tbody>
</table>

\(^a\)Boiling point \(^b\)Melting point
As early as the 1950s, analytical methods were developed for NAs because they were suspected to be generated after adding nitrates as a meat preservative. Nitrite is added to most meat to prevent the growth of *Clostridium botulinum*, and this leads to high concentrations of NAs in bacon, sausage, and ham, but low in unprocessed meat and salted fish [136]. Nitrosamines are formed in food when organic amines or their derivatives react with nitrosating compounds. Besides food, NAs or their precursors can be found in air, water, pesticides, herbicides, cosmetics, tobacco, and packing materials [136-139]. Several studies in the US revealed that NAs can be formed at drinking water treatment plants (DWTPs) and in their distribution systems, as disinfection by-products. There are several ways by which nitrosamines are formed in water. Chloroamination is the major cause of NAs formation in drinking waters, and amine forms the major precursor during the chloramination. It is important to state that chloroamination is the low-cost option approved by the USEPA (Stage 2 DBP rule 2) for controlling trihalomethane and other disinfection by-products (DBPs) [15]. However, studies have shown that about 5% of chloramine is in dynamic equilibrium with dichloramine, which then reacts with secondary amines to form the corresponding NA, as outlined in Scheme 1.5 [15, 140]. Mechanistically, there is a nucleophilic attack on the unprotonated secondary amine on dichloramine, yielding an unsymmetrical diakylhydazine intermediate. This is then followed by oxidation of the diakylhydazine intermediate in the presence of dissolved oxygen to afford the corresponding NA [15, 140]. The reaction is a slow process and it takes days to accumulate the NA as a DBP. Another major route for NA formation in water is chlorination processes in the presence of dinitrogen tetraoxide (N$_2$O$_4$), an inorganic nitrogenous intermediate, which can lead to the nitrosation of amines to form NDMA. During ozonation of wastewater or reclaimed waters, NDMA may be formed due to
the reactions between trace amine-based organic chemicals and ozone itself. For nitrosoamines to form by ozonization and chlorination, a bromide catalyst may be required. Nitrosoamines can be formed in water from dissolved organic compound precursors, such as dimethyl amine, ranitidine (i.e., a pharmaceutical drug for antacid and antihistamine), and quaternary amines (e.g., the polymer polyDADMAC, anion exchange resins, and shampoo constituents) [12, 15, 16, 141]. Other minor routes of nitrosoamine formation in water include the formation of nitrosating species from a nitrogen gas on activated carbon surfaces under aerobic conditions, and sunlight photolysis of nitrite or UV photolysis of secondary chloramines [15].

Scheme 1.5. Showing the generalized formation of nitrosoamines in water during disinfection via chloroamination [140, 142]. (a) monochloramine, (b) dichloramine, (c) secondary amine (here, dimethylamine), (d) chlorinated unsymmetrical diakylhydrazine (chloro dimethylhydrazine), and (f) the corresponding NA from the secondary amine (c) (here, dimethyl nitrosamine, NDMA is formed).
The potential routes of most NA exposures to humans are ingestion, inhalation and dermal contact. Food and tobacco products are the major external source of human exposure to NAS, while small amount of NAs can also be produced internally in the digestive tract [143, 144].

1.7. Metabolism, Toxicity, and Heath Effects of Nitrosoamines

The metabolism of NAs is catalyzed by cytochrome P-450 or CYP-450, which is initiated by the reaction of the monoxygenase reaction [137, 139]. Cyclic nitrosoamines undergo α-hydroxylation in the presence of cytochrome P-450 or CYP-450 to yield alkylating agents such as diazohydroxide and carbonium ions. These metabolites facilely bind with DNA or may be involved in the generation of electrophiles that have the propensity to react or bind with DNA to produce DNA adducts, as outlined in Figure 1.6 [139]. The biotransformation of cyclic nitrosoamines can also start by β-hydroxylation, but the mechanism is not fully understood. In a similar fashion, acyclic or volatile nitrosoamine metabolism proceeds solely by an α-hydroxylation to give formaldehyde, which alkylates DNA to form DNA adducts or the diazohydroxide binds directly with DNA (Figure 1.7) [139].
Cyclic nitrosoamines

alpha-hydroxylation
or beta-hydroxylation

alpha-hydroxycyclic nitrosoamines

ring open

formation of the alkylation agent, alkydiazonium hydroxide

may bind directly with DNA and cause mutation

may react with surrounding water molecules to form various alkylation agents

Figure 1.6. General adduct formation mechanism and metabolism of cyclic nitrosoamines [139].
Acyclic nitrosoamines, e.g dimethylnitrosoamine

\[
\begin{align*}
&\text{dimethylnitrosoamine} \\
&\begin{array}{c}
\text{CH}_3
\end{array}
\end{align*}
\]

alpha hydroxylation

\[
\begin{align*}
&\text{monomethylnitrosoamine} \\
&\begin{array}{c}
\text{CH}_3
\end{array}
\end{align*}
\]

HCHO (formaldehyde)
alkylating agent; may bind with DNA to form DNA adducts

methylidazionium hydroxide
an alkylating agent that binds with DNA

may also form methylcarbonium ion
that binds with DNA

\[
\begin{align*}
&\text{CH}_3\text{N}=\text{N}-\text{OH} \\
&\begin{array}{c}
\text{H}
\end{array}
\end{align*}
\]

Figure 1.7. Generalized metabolic route and DNA adduct formation involving acyclic nitrosoamines, such as dimethylnitrosoamine [139].

The formation of DNA adducts, or alkyl DNAs, due to the metabolism of nitrosoamine leads to defects in the nucleotide base pairing, and consequently results in mutations that disrupts cell function, metabolism and synthesis, leading to apoptosis and cell damage [139].

1.8. Health Effects of Nitrosoamines

The USEPA classified most nitrosoamines, including NDMA, NDEA, NDPA, as likely to be carcinogenic to humans by a mutagenic mode of action under the USEPA Guidelines for Carcinogen Risk Assessment, based on evidence of carcinogenicity in animal studies [135]. The propensity of the metabolites of
nitrosoamines to bind cellular DNA leads to mutagenesis and carcinogenesis. These
carcinogenic and mutagenic metabolites alkylates several sites of DNA such as N-1,
N-3, N-7 positions of adenine N-3, N-7 and O⁶ of guanine, N-3, O² of cytosine and
N³, O⁴, O² of thymine and the oxygens of the phosphate group. The esophagus, oral
cavity, and pharynx are some of the reported sites of mortality due to the
carcinogenic effect of high levels of nitrosoamines [145]. Recent studies show that
nitrosoamines can also induce colorectal cancer in humans [146].

1.9. Current Methods for Analysis of Nitrosoamines in Drinking Water

The ‘standard’ methods for the detection and quantitation of most
nitrosamines (i.e., NDMA, NDEA, NDBA, NDPA, NMEA, and NPYR) in drinking
water are based on the EPA method 521 and the Standard Methods (SM), 6450B and
6450C [135].

The EPA Method 521 [135, 147], (Version 1.0) is the analytical method that
describes ‘Determination of Nitrosamines in Drinking Water by Solid Phase
Extraction (SPE) and Capillary Column Gas Chromatography with Large Volume
Injection and Chemical Ionization Tandem Mass Spectrometry (CI-MS-MS)’. It is
the method approved for use under the second cycle of the Unregulated Contaminant
Monitoring Rule (UCMR 2) to monitor the six nitrosamines with reference to their
health reference levels (HRLs) [135]. Essentially, the method involves the pre-
concentration of 0.5 L of a water sample containing a known concentration of a
surrogate analyte through an SPE cartridge absorbent made of coconut charcoal.
Elution of the analyte (and possible contaminants, if present) from the SPE column is
performed with methylene chloride. The sample is dried, reconstituted, and a suitable
internal standard is added. The resulting solution is analyzed via GC-CI-MS-MS. The
detection limits (DL), as well as the lowest concentration minimum reporting levels
(LCMRL) associated with this method for most common NAs, are summarized in Table 1.4 [135]. SM 6450B also makes use of an SPE absorbent cartridge made of granular carbonaceous adsorbent resin that is capable of achieving a concentration factor of 500 to 1000, and an MDL between 0.5 to 2.0 ng/L. Method 6450C is an alternative method that employs a liquid-liquid microextraction procedure, achieving a concentration factor of 200, with MDL, ranging from 2 to 4 ng/L [135].

**Table 1.4. Some Figures of Merit (Performance metrics) for NA analysis via EPA Method 521 [135].**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>DL (ng/L)</th>
<th>LCMRL (ng/L)</th>
<th>MRL (ng/L)</th>
<th>Recovery Range (%)</th>
<th>RSD Range (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDBA</td>
<td>0.36</td>
<td>1.4</td>
<td>4</td>
<td>79.7-104</td>
<td>2.9-16</td>
</tr>
<tr>
<td>NDEA</td>
<td>0.26</td>
<td>2.1</td>
<td>5</td>
<td>84.6-95.6</td>
<td>6.5-14</td>
</tr>
<tr>
<td>NDMA</td>
<td>0.28</td>
<td>1.6</td>
<td>2</td>
<td>83.7-94.7</td>
<td>3.8-12</td>
</tr>
<tr>
<td>NDPA</td>
<td>0.32</td>
<td>1.2</td>
<td>7</td>
<td>77.1-97.0</td>
<td>3.7-10.2</td>
</tr>
<tr>
<td>NMEA</td>
<td>0.28</td>
<td>1.5</td>
<td>3</td>
<td>81.4-91.0</td>
<td>4.5-9.6</td>
</tr>
<tr>
<td>NPYR</td>
<td>0.35</td>
<td>1.4</td>
<td>2</td>
<td>85.2-102</td>
<td>4.0-12</td>
</tr>
</tbody>
</table>

Similar methods developed by many researches for the determination of NA are also based on enrichment of the NA via SPE, followed by methylene chloride extraction (elution), concentrating the eluate to less than 1 mL, and analyzing via chromatographic (GC or HPLC) methods with tandem mass spectrometric (MS-MS) detection [16]. Charrois et al. [16, 148] made use of an SPE column with dual absorbent made of LiChroult EN (bottom layer) and an Ambersorb 572 (upper layer). Dichloromethane was used for the elution of the NAs from the column, and the eluate were dried with nitrogen to about 200 µL, and analyzed with GC coupled with MS detector operating in a PCI mode and ammonia as a reagent gas. A concentration
factor of 2500 was obtained. Similarly, Chen et al. [16, 149] compared three methods that basically involved the use of an SPE with Ambersorb 572, a commercially available cartridge SPE (CSPE), and a liquid-liquid microextraction system (MLLE). For the graphitized black carbon with Ambersorb 572 sorbent system, elution was carried out with dichloromethane, followed by solvent evaporation and GC-CI-MS-MS analysis, giving a concentration factor of 1000. With the MLLE, dichloromethane was also used for the extraction to a 0.5 mL and analyzed with same method, giving a concentration factor of 200. Planas et al. [150] also employed two different approaches (a) Ambersorb 572 and (b) EPA 521 cartridges with gas chromatography high-resolution mass spectrometry (GC-HR-MS) to analyze NAs in drinking water. In both sample preparation protocols, dichloromethane was used to elute the analytes, followed by solvent evaporation with air (for a) or stream of nitrogen gas (b) and subsequent analysis with GC-HR-MS. The minimum detection limits obtained with this method ranged between 0.08 and 1.7 ng/L. Recently, a group of scientists at Agilent reported a detection limit of 0.038 to 0.332 ng/L when they used the EPA 521 sample preparation protocol discussed above, and analyze their samples with low resolution GC-MS instrument (i.e., the Agilent 5977A series GCMSD) [151].

Some SPE-HPLC-MS-MS methods have been extensively used for analyzing NAs, including methods of Zhao et al. [152], Ripolles et al. [12], Krauss and Hollender [16, 153], Qian et al. [18], and Wang et al. [154]. The first LC-MS-MS method for the detection of NAs in drinking water was developed by Zhao et al. [152, 155], using an SPE cartridge packed with 350 mg of LiChrolut EN (bottom layer), 500 mg of Ambersorb 572 (middle), and glass wool (top). Dichloromethane was used to elute the analytes, and the organic eluent collected and concentrated down to 200 µL under a high purity nitrogen stream in a 40 °C water bath. NDPA-d_{14} (100 µL of
200 µg/L) was added as an internal standard and the analysis was carried out using a HPLC-ESI-MS-MS method, obtaining a detection limits of 0.1 to 10.6 ng/L. Ripolles et al. [12] used the EPA 521 SPE sample pre-concentration technique (i.e., coconut charcoal EPA 521 cartridges with a 500 mL water sample) and analyzed by LC-MS-MS with atmospheric pressure chemical ionization (APCI) in positive mode. The detection limit achieved with this method was between 1 and 8 ng/L. Krauss and Hollender [153] proposed a method for quantifying nine N-nitrosamines in drinking and wastewater samples by LC(ESI)MS/HRMS, using a linear ion trap (LTQ)–Orbitrap hybrid mass spectrometer, and combining Oasis HLB with a carbonaceous sorbent for SPE-LTQ (obtaining an LOD of 0.2–3.9 ng/L).

Qian et al. [156] developed an SPE-HPLC-MS-MS method where they used a cartridge made up of vinyl/divinylbenzene polymer that was used to pre-concentrate 100 mL of water (instead of 500 mL in other methods), yielding an LOD of 0.01–2.7 ng/L. The method developed by Wang et al. [157] involved the use of Resprep EPA 521 cartridges followed by HPLC-EI-MS-MS, a technique originally published by Planas et al. [150] to produce LOD between 0.1 and 0.7 ng/L.

Solid phase micro-extraction (SPME) techniques have been used in conjunction with either GC or HPLC to analyze NAs extensively. Grebel et al. [16, 158] developed an SPME method coupled with gas chromatography with three gas chromatography (GC) detection systems, including nitrogen chemiluminescence detection (NCD), nitrogen–phosphorus detection (NPD) and chemical ionization mass spectrometry (CI–MS). The LODs associated with this method and others discussed above, are outlined in Table 1.5 [16, 135, 148, 149, 153, 158]. Hung et al. [159] utilized SPME GC-CI-MS-MS to screen NAs in water, with LODs ranging from 3.2 to 15.2 ng/L.
### Table 1.5. Characteristics of some NA analytical methods.

<table>
<thead>
<tr>
<th>Method</th>
<th>Sample Volume (mL)</th>
<th>Sample Preparation</th>
<th>Analysis Technique</th>
<th>LOD Range (ng/L)</th>
<th>Estimated analysis time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qian et al. [160]</td>
<td>100</td>
<td>SPE</td>
<td>HPLC-ESI-MS-MS</td>
<td>0.01-0.7</td>
<td>120</td>
</tr>
<tr>
<td>You [151]</td>
<td>500</td>
<td>SPE</td>
<td>GC-CI-MS</td>
<td>0.038-0.33</td>
<td>360</td>
</tr>
<tr>
<td>Planas et al. [150]</td>
<td>1000</td>
<td>SPE</td>
<td>GC-EI-HRMS</td>
<td>0.08-1.7</td>
<td>200&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Krauss and Hollender [153]</td>
<td>500</td>
<td>SPE</td>
<td>HPLC-ESI-MS-MS</td>
<td>0.10-2.40</td>
<td>220</td>
</tr>
<tr>
<td>Zhao et al. [152]</td>
<td>500</td>
<td>SPE</td>
<td>HPLC-ESI-MS-MS</td>
<td>0.1-10.6</td>
<td>150</td>
</tr>
<tr>
<td>Munch and Basset [161]</td>
<td>500-1000</td>
<td>SPE</td>
<td>GC-CI-MS-MS</td>
<td>0.20-0.66</td>
<td>150</td>
</tr>
<tr>
<td>Charrios et al. [148]</td>
<td>500</td>
<td>SPE</td>
<td>GC-CI-MS</td>
<td>0.70-1.30</td>
<td>200</td>
</tr>
<tr>
<td>Hung et al. [159]</td>
<td>4.5</td>
<td>SPME</td>
<td>GC-CI-MS-MS</td>
<td>3.2-15.2</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ripolles et al. [162]</td>
<td>500</td>
<td>SPE</td>
<td>HPLC-CI-MS-MS</td>
<td>1.0-8.0</td>
<td>100</td>
</tr>
<tr>
<td>Cheng et al. [163]</td>
<td>500</td>
<td>SPE</td>
<td>GC-CI-MS-MS</td>
<td>0.78-1.78</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>CSPE</td>
<td>GC-CI-MS-MS</td>
<td>0.33-1.36</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>MLLE</td>
<td>GC-CI-MS-MS</td>
<td>1.80-3.90</td>
<td>94</td>
</tr>
<tr>
<td>Grebel et al. [158]</td>
<td>40</td>
<td>SPME</td>
<td>GC-NPD</td>
<td>1-890</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>SPME</td>
<td>GC-CI-MS</td>
<td>30-138</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>SPME</td>
<td>GC-NCD</td>
<td>57-193</td>
<td>75</td>
</tr>
<tr>
<td>Wang et al. [164]</td>
<td>0.1</td>
<td>LLE</td>
<td>GC-EI-MS</td>
<td>32-100.6</td>
<td>90</td>
</tr>
<tr>
<td>Current method</td>
<td>10</td>
<td>ICECLES</td>
<td>GC-EI-MS</td>
<td>0.2</td>
<td>200&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>Methods with utilizing automated sample preparation

LOD = limit of detection; SPE = solid-phase extraction; SPME = solid-phase microextraction; CSPE = cartridge solid-phase extraction; MLLE = microliquid liquid extraction; ICECLES = ice concentration linked with extractive stirrer; HPLC = high-performance liquid chromatography; MS = mass spectrometry; ESI = electrospray ionization; GC = gas chromatography; CI = chemical ionization; HR = high resolution; NPD = nitrogen phosphorous detector; NCD = nitrogen chemiluminesence detector; EI = electron ionization.
1.10. Ideal Analytical Method /Technique For Analysis of Nitrosoamines

Because of the challenges associated with current methods for analysis of NAs, it is very important to devise a method that may allow analysis of NAs at the desired sensitivity from small sample volumes and more widely available analytical instrumentation, such as low resolution GC-MS. In addition, the ideal analytical method should be simpler, “greener”, and must have ‘automated’ sample preparation protocols that should have a stark contrast to some of the sample preparation techniques listed in Table 1.5 (i.e., SPE, MLLE, and LLE). ICE Concentration Linked with Extractive Stirrer (ICECLES), a sample preparation protocol developed by Maslamani et al. [165] qualifies for most of the criteria listed above. Chapter 3 details the use of ICECLES-GC-EI-MS for the analysis of NDPA.
CHAPTER 2. DETERMINATION OF FREE CYANOCOBINAMIDE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

2.1. Abstract

In recent years, Cobinamide (Cbi) has shown promise as a therapeutic for cyanide poisoning. There are several forms of Cbi based on the identity of the ligands bound to the cobalt in Cbi and these different forms of Cbi have divergent behavior (e.g., the aquo and hydroxo forms of Cbi readily bind to proteins, limiting their distribution significantly, whereas [Cbi(CN)₂] does not). While current analysis techniques only measure total Cbi, methods to elucidate the behavior of ‘available’ Cbi versus cyanide-complexed Cbi would be valuable for biomedical and pharmacokinetic studies. Therefore, a method was developed for the analysis of cyanide-complexed Cbi in plasma via liquid chromatography tandem mass spectrometry (LC-MS-MS). Plasma samples were prepared by denaturing proteins with 10% ammonium hydroxide in acetonitrile. The resulting mixture was centrifuged, and the supernatant was removed, dried, and reconstituted. Cyanide-complexed Cbi was then analyzed via LC-MS-MS. The limit of detection was 0.2 µM, and the linear range was between 1-200 µM. The accuracy was 100±17% and the precision, measured by relative standard deviation (%RSD), was ≤18.5%. Carryover, a severe problem when analyzing Cbi via liquid chromatography was eliminated using a polymeric-based stationary phase (PLRP-S) and a controlled washing protocol. The method allowed evaluation of the cyanide-bound and ‘available’ Cbi
from treated animals and, when paired with a method for total Cbi analysis, allows for estimation of Cbi utilization when treating cyanide poisoning.

2.2. Introduction

Current treatments for cyanide exposure (CN⁻ and HCN, inclusively represented as CN) include three general classes of agents: methemoglobin generators (sodium nitrite, amyl nitrite, and dimethyl aminophenol), sulfur donors (sodium thiosulfate and glutathione), and direct binding agents (hydroxocobalamin and dicobalt edetate) [92]. While each type of treatment has been effective at countering the toxic effects of cyanide, each has major limitations, especially during mass casualty situations. For example, all cyanide approved antidotes require intravenous administration [91].

Methemoglobin generators oxidize hemoglobin in the red-blood cells to produce methemoglobin [166], a complex with high affinity for cyanide [105]. However, production of methhemoglobin leads to methemoglobinemia, which reduces oxygen transport in the erythrocytes and tissues [60, 91, 167, 168]. This is especially dangerous when smoke inhalation has occurred, causing concurrent carboxyhemoglobinemia and methemoglobinemia [92]. Nitrites, such as sodium nitrite, are also known to cause severe hypotension, cardiovascular instability and hypoxia [60]. Nitrites can also cause oxidative stress on fetal hemoglobin in pregnant patients [39]. It has been proposed recently that nitric oxide generated from nitrites displaces cyanide bound to the active site of cytochrome c oxidase [84, 95-97], restoring the activity of cytochrome c oxidase. The displaced CN may then be scavenged by circulating MetHgb, or converted to SCN⁻ in the presence of sulfur donors [84]. Sulfur-donor antidotes detoxify cyanide by converting cyanide to
thiocyanate with the help of sulfurtransferase enzymes (e.g., rhodanese and 3-mercaptopyruvate sulfurtransferase) [169]. However, sulfur donors are limited due to variable subcellular distribution of sulfurtransferase enzymes [169]. For example, rhodanese is concentrated in the mitochondrial matrix of the liver and kidney, but is not prevalent in the central nervous system [92]. Thiosulfate, the only U.S. FDA-approved sulfur donor cyanide antidote, also has a disadvantage of slow uptake into cells. Large concentrations of sodium thiosulfate produce side effects such as nausea, vomiting, local skin and muscle pain at the site of infusion, headache, and disorientation [60]. Recently, other sulfur donor compounds, including 3-mercaptopyruvate (3MP) [92, 105, 115, 170] and dimethyl trisulfide [97, 112, 114, 171, 172] have been suggested as more efficient sulfur donors for cyanide therapy. While these novel sulfur donors have many advantages, they are still under development. Direct binding agents offer an alternate mechanism for detoxifying CN by sequestering cyanide from cytochrome c oxidase to effectively reduce the toxicity of cyanide. One such binding agent is hydroxocobalamin (Cbl), which more strongly binds cyanide than cytochrome c oxidase [39]. Cbl is the only U.S. FDA-approved binding agent [39, 173] and is relatively safe and effective [92], but binds only one cyanide ion per molecule, and has a very high molecular weight. Therefore, it requires large doses (i.e., approximately 4 to 5 g) and intravenous administration to be effective, limiting its use in mass casualty situations [92, 115]. Cobinamide (Cbi), the penultimate precursor in the hydroxocobalamin biosynthetic pathway [92, 174], has been suggested as an effective cyanide binding agent. To date, studies have shown that Cbi is superior to current FDA-approved treatments, mainly due to its flexible administration (typically intramuscular) and therapeutic index advantages [30, 93]. The greater effectiveness of Cbi is due to its extremely high affinity for cyanide (K_F,
overa $= 10^{22} \text{M}^{-1}$) [30, 115, 174], its ability to directly bind two cyanide ions, and its relatively high solubility [115, 174]. Depending on the pH and ligands present, cobinamidate can exist in several forms, such as diaquocobinamide $[\text{Cbi(H}_2\text{O)}_2]^{2+}$, hydroxoaquocobinamide $[\text{Cbi(OH)(H}_2\text{O})]^{+}$, and dihydroxocobinamide $[\text{Cbi(OH)}_2]$, [120]. Cobinamidate itself exists predominantly as $[\text{Cbi(OH)(H}_2\text{O})]^{+}$ at neutral pH, as $[\text{Cbi(H}_2\text{O)}_2]^{2+}$ under acidic conditions ($\text{P}K_a=5.9$), and as $[\text{Cbi(OH)}_2]$ under basic conditions [175]. In the presence of cyanide, either one or both of the aquo or hydroxo ligand(s) are replaced to produce $[\text{Cbi(CN)(H}_2\text{O})]^{+}$, $[\text{Cbi(CN)(OH)}]$, or $[\text{Cbi(CN)}_2]$ [120, 175] depending on pH, the CN concentration, and the reaction time. Of course, a variety of other ligands may bind to the Cbi, depending on its environment.

The multiple species of Cbi exhibit disparate kinetic, thermodynamic, and biological behavior. For example, the aquo and hydroxo forms of Cbi significantly bind to transcobalamin protein (TC), haptocorrin (HC), and intrinsic factors (IF), while the dicyano form of Cbi does not. This likely results in substantially different distribution of these species [176-178]. Several methods have been developed for the determination of total Cbi by conversion to $[\text{Cbi(CN)}_2]^0$ utilizing a large excess of CN. These methods include electrochemical, spectrophotometric, and chromatographic techniques [6, 79, 115, 120, 174]. Although these methods perform well for the analysis of total Cbi, they lack the ability to measure ‘available’ and cyanide-bound Cbi. For example, we developed a method for the simultaneous analysis of total Cbi and 3-mercaptopropyruvate in swine plasma [6], while a different group reported a method for the analysis of total Cbi alone, also in swine plasma [179]. While these methods give valuable information as to the distribution and kinetics of the Cbi species in aggregate, they fail to elucidate the interplay between Cbi species and the contribution of these species to the effectiveness of Cbi. This
information is necessary for gaining a complete understanding of the distribution and pharmacokinetic profile of Cbi. Therefore, the development of a selective method for the analysis of individual Cbi species is desired. Most importantly, the selective analysis of cyanoCbi species (i.e., cyanoCbi is defined as Cbi bound to at least one CN$^-$ ion) would allow determination of the pharmacokinetic behavior of cyanide-bound Cbi. In combination with the determination of the total Cbi, factors contributing to the efficacy of Cbi may be revealed. Such information may help in the identification of more effective formulations of Cbi for treatment of cyanide.

The objective of this project was to develop a single analytical method for the determination of plasma concentrations of cyanoCbi species, (i.e., [Cbi(CN)$_2$] and [Cbi(CN)Y]$^z$, with Y representing a ligand bound to the Cbi and z representing the formal charge of the complex). A secondary objective was to significantly reduce or eliminate the well-known problem of severe carryover of Cbi during liquid chromatography analysis [118].

1.10. 2.3. Experimental

2.3.1. Reagents and standards

All reagents were HPLC grade, unless otherwise noted. Sodium cyanide (NaCN), potassium cyanide (KCN), urea, ammonium sulfate, acetonitrile, zinc chloride, zinc acetate, sodium hydroxide, perchloric acid, and ammonium hydroxide were all obtained from Fisher Scientific (Hanover Park, IL, USA). Millex® tetraflouropolyethylene syringe filters (0.22 μm, 4 mm, Billerica, MA, USA) were also purchased from Fisher Scientific. Sodium dodecylsulfate, potassium hydroxide, potassium chloride, p-toluenesulfonic acid monohydrate, and urea, were supplied by Sigma-Aldrich (St. Louis, MO, USA). Formic acid was obtained from Thermo Scientific (Rockford, IL, USA). Water was purified to 18 mΩ-cm using a Water PRO
Aquohydroxo-cobinamide was obtained from Dr. Gerry Boss, MD, Department of Medicine, University of California-San Diego, La Jolla, USA [6, 116]. A stock solution of 20 mM aquohydroxo-cobinamide was prepared by diluting the aquohydroxocbi with de-ionized water and storing at 4°C in amber vials.

Calibration and quality control (QC) standards of dicyanoCbi were prepared by adding 1 mole of aquohydroxo-cobinamide to 2 molar equivalents of NaCN (dissolved in 10 mM sodium hydroxide), allowed to stand for 5 minutes, serially diluting to the desired concentration, and spiking into plasma in a 9:1 volume ratio. Spiked standards were mixed thoroughly and allowed to stand for 10 minutes prior to sample preparation to ensure homogeneity.

**Caution.** Cyanide is toxic and hazardous to humans at blood concentrations of ~20 μM or higher. HCN is produced from aqueous cyanide containing solutions near or below a pH of 9.2. Therefore, all aqueous cyanide standards were prepared in 10 mM NaOH and handled in a well-ventilated hood with cyanide waste appropriately labeled. Cyanide-containing waste was appropriately disposed via approved processes at the institution where it was generated.

### 2.3.2. Biological fluids

Swine plasma (heparin anti-coagulated) and rabbit plasma (EDTA anti-coagulant) used for method development and validation were purchased from Pel-freeze Biological (Rogers, AR, USA), and kept frozen at -80°C until used. Plasma from rabbits exposed to cyanide and treated with 1H-tetrazole-5-acetylcobinamide was obtained from the University of California-Irvine (Beckman Laser Institute). New Zealand male rabbits (315 to 320 g, N = 3) were anesthetized with an initial intramuscular injection of Ketamine HCl 50 mg/kg (Ketaject, Phoenix Pharmaceutical
Inc., St. Joseph, MI) and Xylazine 5 mg/kg (Anased, Lloyed Laboratories, Shenandoah, IA). After administration of anesthesia, a catheter was placed in the animal’s marginal ear vein to administer continuous intravenous anesthesia with Ketamine/Xylazine at 0.17 mL/min during central line placement. The rate of anesthesia delivery was subsequently increased to 0.19 mL/min for the duration of the experiment. Rabbits were ventilated at 100% O$_2$ at 18 respirations per minute. Blood samples were drawn prior to CN exposure to establish a baseline. NaCN (20 mg in 60 mL 0.9% NaCl) was administered intravenously at 1 mL/min. Blood samples were drawn at 15 and 25 mins post-NaCN infusion start. After 30 mins of CN infusion, the ventilator rate was reduced to 16 respirations per minute, and room air was used for ventilation. Blood samples were subsequently drawn at 35 min of NaCN infusion and until blood pressure reached a target value of 40-58 mm Hg systolic. At this trigger point (designated as “time 0”), the antidote, 1H-tetrazole-5-acetylcobinamide (1 mL of 150 mM Cbi), was injected intramuscularly into the left front leg muscle. Starting at 5 minutes post-antidote administration, blood was drawn into K$_2$-EDTA vacutainers (BD, Franklin Lakes, NJ) at 2.5, 5, 7.5, 10, 15, 30, 45, 60, and 90 minutes (i.e., the post-antidote samples). The plasma was then immediately separated from blood by centrifugation at 4 °C for 20 min. The plasma was transferred to a clean microcentrifuge tube, flash-frozen in liquid nitrogen, and shipped overnight on dry ice to South Dakota State University for analysis. Upon receipt, the plasma was stored at -80 °C until analysis was performed. All rabbits were cared for in compliance with the “Principles of Laboratory Animal Care” formulated by National Society for Medical Research and the “Guide for the Care and Use of Laboratory Animals” prepared by the National Institutes of Health [6, 180]. The animal study was approved by the
University of California-Irvine Institutional Animal Care and Use Committee (IACUC).

2.3.3. Sample preparation of Cbi species

Plasma samples were denatured by adding 700 µL of 10% ammonium hydroxide in acetonitrile to 100 µL plasma. The samples were then vortexed briefly (~10 seconds), and centrifuged at 16,500 x g for 20 minutes at 8 °C. The supernatant (650 µL) was collected into a 2-mL centrifuge vial and further centrifuged for an additional 10 minutes under the same conditions. Afterwards, the supernatant (600 µL) was transferred into a 4-mL glass vial and dried with N₂. The samples were then reconstituted in 10% acetonitrile, filtered with a 0.22 µm tetrafluoroethylene syringe filter, and analyzed. Other denaturing agents were tested, including potassium chloride (1 g/mL), zinc chloride (1 g/mL), zinc acetate (1 g/mL), ammonium sulfate (1 g/mL), p-toluenesulfonic acid monohydrate (1 mM), and sodium dodecylsulfate [181]. Combinations of denaturing agents were also evaluated, including zinc chloride and urea (1 g/mL each), zinc acetate and urea (1 g/mL each), and perchloric acid (4 mM):potassium hydroxide (1 M) [181]. However, these agents produced inadequate recovery of cyanoCbi.

For total Cbi analysis, we used our previously published method [6]. Briefly, a 100 µL cyanide solution (10-mM KCN in 0.1 M NaOH) was added to 50-µL plasma and heated on a heat block at 80 °C for 15 min. Then, acetone (600 µL) was added to precipitate the proteins, followed by centrifugation, transfer of the supernatant, drying under N₂, reconstitution in 5 mM aqueous ammonium formate with 10% methanol, and filtering prior to LC-MS-MS analysis.
2.3.4. Initial MS-MS-Analysis

All mass spectrometric analysis was performed by an AB Sciex QTRAP 5500 MS (AB Sciex, Framingham, MA, USA) with electrospray ionization in positive mode. Mass spectra were initially acquired by direct infusion of aqueous Cbi solutions (10 µL/min) with scans of MS1 from 200-1200 Da over 1.6 minutes. The entrance orifice potential was 180 V, and the collision cell exit potential was 11 V. The curtain gas and ion source gases were operated at 30 and 40 psi, respectively. The ion spray voltage was 5500 V with a temperature of 500 K, and the collision activation dissociation (CAD) was set to “high”. The low energy collision-induced dissociation tandem mass spectrometric analysis (CID-MS-MS) was conducted with a linear accelerator (LINAC) collision cell of the triple quadrupole/linear ion trap instrument. The CID-MS-MS was performed in multiple-reaction monitoring (MRM) mode with optimum MRM parameters shown in Table 2.1. Analysis and data acquisition was accomplished using Analyst software (Applied Biosystems, Version 1.6.3). The transitions (m/z) monitored for cyanoCbi were 1015.5 → 930.8 and 1015.5 → 988.5. We have seen in the past, and also in the current study, that the 1064.9 m/z transitions (i.e., dicyanoCbi with sodium) produce poor sensitivity, with lower limits of detection, as compared to the 1015 transitions [6]. Consequently, the 1015.5 transitions were used for quantification and, when present above baseline noise, the 1064.9 transitions monitored for identification of dicyanoCbi.
Table 2.1. Optimized MS-MS MRM parameters for cyanoCbi species.

<table>
<thead>
<tr>
<th>Compound</th>
<th>MS1 (m/z Da)</th>
<th>MS3 (m/z Da)</th>
<th>DP (V)</th>
<th>CE (V)</th>
<th>CXP (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Cbi(CN)(_2)Na]</td>
<td>1064.9</td>
<td>930.8 (ID)(^g)</td>
<td>263.11</td>
<td>97.47</td>
<td>11</td>
</tr>
<tr>
<td>[Cbi (CN)(_Y)]</td>
<td>1015.5</td>
<td>930.8 (Quant)(^h)</td>
<td>249.42</td>
<td>75.91</td>
<td>11</td>
</tr>
<tr>
<td>[Cbi (CN)(_Z)]</td>
<td>1015.5</td>
<td>988.5 (ID)(^g)</td>
<td>237.79</td>
<td>50.71</td>
<td>11</td>
</tr>
</tbody>
</table>

\(^a\)Y represents a ligand bound to the Cbi and \(z\) represents the formal charge of the complex
\(^b\)Precursor ion
\(^c\)Product ion
\(^d\)Declustering potential
\(^e\)Collision cell exit potential
\(^f\)Identification ion

2.3.5. HPLC-MS-MS Analysis

Liquid chromatography-tandem mass spectrometry (LC-MS-MS) was conducted on a Shimadzu High Performance-LC system (HPLC, LC-20AD, Shimadzu Corp., Kyoto, Japan) coupled with the QTRAP described above. Samples were separated by a polymeric reversed phased column, PLRP-S (300A, 50 x 2.10 mm x 3 micron) from Agilent Technologies (Santa Clara, California, USA). An isocratic method of 100% acetonitrile over 5 minutes was used. The optimized transitions (Table 2.1) were monitored.

Carryover is a major and well-known problem when analyzing Cbi species [118]. Cbi can bind both column and LC-MS-MS components (e.g. the autosampler syringe). In order to reduce day-to-day carryover of Cbi, a PLRP-S stationary phase was used to avoid the strong interaction of Cbi with free silanols present in typical reversed-phase stationary phases. Moreover, a cleaning protocol was devised to eliminate residual Cbi in the LC-MS-MS components. The cleaning procedure was based on addition of anions with high affinity for Cbi to the LC components and the column to release bound Cbi as cyanide- or sulfite-complexed Cbi. The cleaning
procedure consisted of 3 steps: 1) rinsing the LC instrumentation (minus column) via injections of aqueous cyanide, 2) rinsing the column with sulfite-containing mobile phase, and 3) injecting aqueous NaCN (in basic solution) into the complete LC-MS-MS system and monitoring the detection of cyanoCbi. The flow chart in Figure 2.1 schematically represents the cleaning protocol for both the HPLC-MS-MS system and the column. Following analysis of multiple Cbi samples (e.g., analysis of calibration standards and samples from a model of treatment for cyanide exposure), the column was removed from the LC-MS-MS. The LC autosampler and connections without column were rinsed (0.25 mL/min) with water, 5% and 10% acetonitrile (ACN) for 15, 10, and 20 min, respectively, to unbound or weekly-bound residual sample components into waste using the diverter valve on the MS. The mobile phase was then changed to 100% ACN. Subsequently, injections (50 times) of aqueous cyanide (100 µM CN in 10 mM NaOH) were performed to bind residual Cbi and allow its release, as cyanoCbi, into the waste. On the last injection, the cyanoCbi signal was evaluated to determine if residual Cbi was still present in the LC system (minus column). If cyanoCbi was present, the injections of cyanide were repeated as described above and the final injection was evaluated. This process was repeated until no cyanoCbi was present in the eluant. Subsequently, 5 and 10% aqueous ACN solutions (at 0.5 mL/min) were used to flush the system of NaCN and NaOH. The column was separately washed on a different HPLC using an aqueous solution of sodium sulfite and ACN (1:10 of 1 mM Na$_2$SO$_3$:ACN) at 0.1 mL/min (i.e., sulfite strongly binds to Cbi, although not strongly as CN). After this, a 5%-ACN solution (4 hr) followed by 10% ACN (2 hr) were used to wash residual salt from the column at 0.05 mL/min. The column was then equilibrated to 100% ACN, and further cleaned by performing at least 70 autosampler injections of 100 µM cyanide in 10 mM NaOH.
(40 µL each). The column was then equilibrated with 100% ACN and stored for future use. Prior to sample analysis, the PLRP-S column was installed on the LC-MS-MS system and 100 µM cyanide in 10 mM NaOH was analyzed by the cyanoCbi method presented here to verify that residual Cbi was eliminated from the LC-MS-MS system (i.e., cyanide strongly binds to Cbi to release any residual Cbi in the autosampler or column which would appear as cyanoCbi during the LC-MS-MS analysis). The absence of a cyanoCbi peak confirmed the instrument and the column were free from residual Cbi (i.e., residual Cbi appears as a cyanoCbi peak in the chromatogram). In our work, if a cyanoCbi was detected, following the procedure in Figure 2.1, the signal was very small and repeated injections of 100 µM CN (≤10) eliminated the residual Cbi. Although we did not observe a large residual cyanoCbi peak following the procedure in Figure 2.1, if a large cyanoCbi signal is present, the overall cleaning procedure may need to be repeated multiple times to eliminate residual Cbi from the LC-MS-MS components.
Figure 2.1. Flow chart for cleaning protocol for the LC-MS-MS. ACN = acetonitrile.
2.3.6. Method Validation

FDA guidelines [182-186] were followed for bioanalytical method validation. For the determination of the limit of detection (LOD), multiple concentrations of dicyanoCbi (i.e., 100, 200, and 500 nM) were spiked in plasma, and analyzed by the method. The LOD was defined as the concentration that reproducibly produced a signal to noise ratio of 3, with the noise calculated as the peak-to-peak noise of the blank (i.e., non-spiked plasma) over the retention time of cyanoCbi. The linear range was determined by analyzing dicyanoCbi calibration standards in plasma (each in triplicate) at nine different concentrations ranging from 0.5 to 200 µM (i.e., 0.5, 1, 2, 5, 10, 20, 50, 100, and 200 µM). The criteria used for the lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) were as follows: (1) calibrator precision (measured as percent relative standard deviation, %RSD) of ≤20% and (2) an accuracy of 100±20% of the nominal calibrator concentration back-calculated from the calibration curve. The cyanoCbi concentration was back-calculated from the calibration equation using the signal of each calibrator and then averaged. Inter- and intra-assay precision and accuracy were determined by analyzing low (3.5 µM), medium (35 µM) and high (150 µM) quality control (QC) standards in quintuplicate. These QCs were not included in the calibration curve. DicyanoCbi calibration curves were prepared each day of analysis.

For stability studies, QCs (low and high) were stored at various temperatures and analyzed at multiple storage times. The short-term stability includes autosampler stability (i.e., prepared QCs stored in the autosampler at 15 °C) and bench top stability (i.e., prepared QCs stored on the bench top at room temperature (RT)). For both autosampler and bench top stability, QCs were analyzed immediately and after 2, 4, 8, 12, and 24 hours. Stability for each experiment was measured as a percentage of the
original QC signal (i.e., the QC analyzed immediately), with samples considered stable if the stored sample produced 100±20% of the original signal.

The recovery was evaluated by comparing QCs (low, medium and high) prepared in plasma to equivalent concentrations of QCs prepared in aqueous solution. The plasma samples were prepared using the sample preparation procedure described above, but the aqueous samples did not go through the sample preparation, except that they were dried, and reconstituted in 10% aqueous ACN. The standards were prepared in quintuplicate, and the recovery was calculated as a percentage of the average signal in plasma compared to the aqueous standards. Matrix effects were evaluated by comparison of the slope of an aqueous calibration curve to that of plasma. A matrix effect was said to be present if the slope of the calibration curve in water samples was significantly different from that in plasma samples.

1.11. 2.4. Results and Discussion

2.4.1. Sample Preparation and Analysis of cyanoCbi

In previous work, Cbi has been measured as total Cbi by adding excess cyanide to convert all Cbi species to dicyanoCbi [6, 118, 120, 127]. In this study, 10% ammonium hydroxide in ACN, as a denaturating solution, coupled with two-step centrifugation allowed extraction of cyanoCbi species, as opposed to total Cbi. It is important to note that other chaotropic and kosmotropic agents were tested (results not shown), but the ammonium hydroxide/acetonitrile combination was the only one which produced acceptable recoveries of cyanoCbi. The ammonium hydroxide was used to disrupt ionic interactions, likely disrupting salt bridges formed between the cobalt in the cobinamide and negatively charged moieties in the plasma protein structure. The ACN lowers the dielectric constant of the resulting solution to precipitate plasma proteins and simultaneously disrupts hydrophobic and hydrogen
bond interactions in the protein. The combination of these two denaturing agents
produced acceptable recoveries of cyanoCbi for analysis. We observed that any
further increase in the percentage of ammonium hydroxide concentration and/or
increase in the contact time during the deproteinization process produced a decrease
in the recovery of cyanoCbi. This was likely caused by a shift in the equilibrium or
kinetic stability of the Cbi species, resulting from increased hydroxo forms of Cbi
with increased pH. After denaturing the proteins, the sample was centrifuged, and the
supernatant was dried and reconstituted for analysis. The sample preparation for the
method presented here is relatively simple and easy, and the chromatographic method
is shorter as compared to other methods [6, 118]. Moreover, the stationary phase
chosen for this method is much less likely to bind Cbi under the chromatographic
conditions used. Figure 2.2 shows the chromatogram of cyanoCbi (eluted at 0.85
minutes) analyzed from spiked swine plasma. The chromatographic peak shape
($A_s=1.3$) and resolution were excellent (i.e., there were no other significant present in
the chromatogram). The chromatographic run was 5 minutes in total. In future work,
we will evaluate the necessity of the relatively long equilibration time following
elution of cyanoCbi (i.e., the method is isocratic, allowing for no equilibration time
between runs, and cyanoCbi elutes within the first minute).
Figure 2. LC-MS-MS chromatograms of dicyanoCbi-spiked and non-spiked swine plasma. CyanoCbi elutes at 0.85 min. The extra 4 minutes (after the elution of the analyte) allowed for enough equilibration and possible elimination of some of the residual Cbi in the system.
Figure 2.3. Peaks obtained after injection of 100 µM NaCN in 10 mM NaOH following one validation analysis (i.e., approximately 42 dicyanoCbi-spiked swine plasma samples were analyzed), but before performing the cleaning protocol (Figure 1). Chromatograms with the column (cyanoCbi eluted at 0.85 min) and after removing the column (cyanoCbi eluted at 0.25 min) are shown. The presence of cyanoCbi without the column shows that Cbi is bound to extra-column components of the LC-MS-MS system. The increased signal of cyanoCbi with the column attached indicates that some residual Cbi is also associated with the column. The inset presents the analysis of cyanoCbi (via injection of 100 µM CN) following the cleaning protocol, showing undetectable Cbi, which demonstrates the excellent performance of the protocol to eliminate carryover.
Cbi has an extremely high affinity for oxide surfaces. Therefore, common reversed-phase chromatography stationary phases, which have a silica-based solid support (e.g., C18), are poor choices for Cbi analysis, even though multiple methods have been published using this approach [6, 118], including one from our group. In fact, the challenges associated with residual Cbi were vividly reported by McCracken and Brittain, who used a reversed-phase C18 column (i.e., Phenomenex Kinetex C18 column) [118]. The PLRP-S stationary phase and the acetonitrile mobile phase used in this study minimized carryover that has plagued Cbi analysis. Although carryover was greatly decreased when using the PLRP-S column, carryover was still evident in the method presented in this study (Figure 2.3). This is likely caused by sorption of Cbi to a number of high affinity sites in the autosampler, LC connections and the column. Therefore, we devised a wash protocol to remove residual Cbi from the column and LC components (Figure 2.1). To evaluate the extent of residual Cbi, an aqueous cyanide solution (100 µM in 10 mM NaOH) was injected via the autosampler (40 µL) and analyzed via the method presented here. Cyanide has a high affinity for Cbi, releasing bound Cbi from the LC-MS-MS system as cyanoCbi, allowing its detection via the tandem mass spectrometry. Therefore, if bound Cbi is present in the LC-MS-MS, a cyanoCbi peak will be present in the chromatogram following injection of cyanide. Figure 2.3 shows the residual Cbi detected in the complete LC-MS-MS system, when the column is attached (elution at 0.85 minutes), and the LC-MS-MS system with the column removed (elution at 0.25 minutes) immediately after a full-day validation experiment (i.e., approximately 42 Cbi containing samples ranging from 0.5 to 200 µM). It is evident that Cbi does bind to both column and the other components of the LC-MS-MS system with approximately one-
third of the cyanoCbi signal remaining after removal of the PLRP-S column. However, with the polymeric stationary phase coupled with the cleaning procedure devised, residual Cbi was eliminated, as clearly shown in the Figure 2.3 inset, where a chromatogram of cyanoCbi (i.e., injection of 100 µM NaCN in 10 mM NaOH) after using the cleaning protocol shows cyanoCbi was not detected.

2.4.2. Limit of detection, sensitivity and linear range

The LOD of cyanoCbi from plasma was 200 nM, which is the same as that obtained in our previous total Cbi method (200 nM) [6]. It is likely that the LOD was the same for the current method, even though only cyanoCbi was measured, because binding of Cbi to the column was minimized.

The LLOQ and ULOQ were 1 and 200 µM, respectively. This represents over two orders of magnitude for the linear range (LR). It should be noted that 200 µM was the highest concentration tested, therefore, the ULOQ for the method may be higher than 200 µM. The LR is much improved from our previous work [6] and that which was reported by Boss et al. [187]. This, again, is likely because the interaction of Cbi with the chromatographic stationary phase was minimized. The LR of this method is well-suited for translational studies, including pharmacokinetic analysis of cyanoCbi [6]. The regression equations, correlation coefficients ($R^2$), and the Percent Residual Accuracies (PRAs) [188] for the 3-day validation study are outlined in Table 2.2. The PRA values for all the calibration curves generated for the 3-day validation process was ≥90% (Table 2.2), indicating a good fit over the entire linear range [188]. In addition, the precision and the accuracy of the calibration curves were acceptable (Table 2.3) [182-184, 186]. The
day-to-day inconsistency in the slope and intercept over the 3-day validation study require that a calibration curve be prepared for each day of analysis.

**Table 2.2.** Regression models, R² and PRA values for calibration curves over a 3-day period.

<table>
<thead>
<tr>
<th>Day</th>
<th>Calibration Equation a</th>
<th>R²</th>
<th>PRA b</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>y = 81397x-29620</td>
<td>0.9842</td>
<td>90.2</td>
</tr>
<tr>
<td>2</td>
<td>y = 42027x-18780</td>
<td>0.9872</td>
<td>90.2</td>
</tr>
<tr>
<td>3</td>
<td>y = 163762x-95040</td>
<td>0.9914</td>
<td>92.2</td>
</tr>
</tbody>
</table>

a Equation in the form of y = mx + b, where x = cyanoCbi concentration (µM) and y = the peak area abundance of the 1015→930 m/z transition from the LC-MS-MS chromatogram.

b Percent Residual Accuracy [188].

2.4.3. Accuracy and precision

The intra- and interassay accuracy and precision were determined by analyzing low, medium and high QCs in quintuplicate over three different days, with the results summarized in Table 2.3. The intraassay precision for the cyanoCbi species in swine plasma was <18.5% RSD, while the accuracy was 100±18%. The interassay accuracy and precision were 100±17% and <16% RSD, respectively. Even though the precision and accuracy are slightly worse as compared to the total cobinamide methods presented by Ma et al. [117], Stutelberg et al. [6], and McCracken and Brittain [118], the overall precision and accuracy for this method were acceptable [182-184]. The accuracy and
precision are likely worse for the current method because of the increased variability in the recovery of cyanoCbi using the 10% ammonium hydroxide versus a large excess of cyanide to measure the total Cbi [6, 118, 127].

**Table 2.3. Intra- and interassay accuracy and precision for the analysis of dicyanoCbi-spiked swine plasma.**

<table>
<thead>
<tr>
<th>Nominal Conc. (µM)</th>
<th>Intraassay Accuracy(^a) (%)</th>
<th>Intraassay Precision(^a) (%)</th>
<th>Interassay Accuracy(^b) (%)</th>
<th>Interassay Precision(^b) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>100±14</td>
<td>&lt;18.5</td>
<td>100±14</td>
<td>&lt;16</td>
</tr>
<tr>
<td>35</td>
<td>100±17</td>
<td>&lt;12.5</td>
<td>100±17</td>
<td>&lt;11</td>
</tr>
<tr>
<td>150</td>
<td>100±16</td>
<td>&lt;6.5</td>
<td>100±8</td>
<td>&lt;9</td>
</tr>
</tbody>
</table>

\(^a\) QC method validation for Day1 (N=5)  \(^b\) Average of QCs for the 3 different days of validation (N=15)

2.4.4. *Stability, recovery and matrix effects*

In order to reduce the high variance associated with residual Cbi from run-to-run for the stability studies, the short-term stability studies were carried out for the low QC first, followed by the high QC on separate days. The benchtop and autosampler (15°C) stability fell within 100±14% over 24 hours. The analyte was stable for at least 30 days in a standard refrigerator at 4 °C. As shown in Table 2.4, these results are consistent with what was reported earlier [6, 118].
Table 2.4. Stability profile for total Cbi for current and previous studies.

<table>
<thead>
<tr>
<th>Stability exp.</th>
<th>Ref.</th>
<th>Total Cbi stability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Long-term</td>
<td>[6]</td>
<td>≥30 days for 1.5 and 35 µM at 4, -30, and -80 ºC</td>
</tr>
<tr>
<td></td>
<td>[118]</td>
<td>≥60 days for 0.072 and 7.2 µM (reported as 75 and 7500 ng/mL) at -70 ºC</td>
</tr>
<tr>
<td></td>
<td>CS(^a)</td>
<td>≥30 days for 3.5 and 150 µM at 4 ºC</td>
</tr>
<tr>
<td>Bench-top</td>
<td>[6]</td>
<td>≥24 hours for 1.5 and 35 µM at RT</td>
</tr>
<tr>
<td></td>
<td>CS(^a)</td>
<td>≥24 hours for 3.5 and 150 µM at RT</td>
</tr>
<tr>
<td>Freeze-thaw</td>
<td>[6]</td>
<td>3 cycles; freezer at -80 ºC</td>
</tr>
<tr>
<td></td>
<td>[118]</td>
<td>3 cycles; freezer at -70 ºC</td>
</tr>
<tr>
<td>Autosampler</td>
<td>[6]</td>
<td>≥24 hours for 1.5 and 3.5 µM at 15 ºC</td>
</tr>
<tr>
<td></td>
<td>[118]</td>
<td>≥4 days for 0.072 and 7.2 µM (reported as 75 and 7500 ng/mL) at 2-8 ºC</td>
</tr>
<tr>
<td></td>
<td>CS(^a)</td>
<td>≥24 hours for 3.5 and 150 µM at 15 ºC</td>
</tr>
</tbody>
</table>

\(^a\)Current study

The matrix effect was evaluated by comparing the slopes from the calibration curves of the analyte in an aqueous matrix to that of plasma. The slope of the calibration curve in the plasma matrix was 0.4 times that of the aqueous matrix calibration curve, which indicates a considerable matrix effect. The matrix effect produced in this study is similar to what we reported previously for total Cbi analysis [6]. The recoveries of cyanoCbi for the low, medium and high QCs were 20, 40, and 50%, respectively. Stutelberg et al. [6] reported recoveries of total Cbi of 29% (for the low QC), 29% (medium QC) and 33% (high QC), while McCracken and Brittian reported 56%, 59%, and 62% for the low, medium, and high QCs, respectively [118]. The increased variability of the recovered Cbi compared to other studies is likely due to increased
variability when extracting cyanoCbi with 10% ammonium hydroxide as compared to analysis of total cyanide using excess cyanide. Since the matrix effect is a component of the signal recovery, the matrix effect is responsible for most of the signal loss seen, except for the low QC.

2.4.5. Analysis of Free CyanoCbi in Exposed Animals

The validated method was applied to analyze free cyanoCbi from the plasma of 1H-tetrazole-5-acetylcoenzyme (Cbi)-treated rabbits. Representative chromatograms of rabbit plasma obtained prior to cyanide exposure and plasma from the same rabbit following cyanide exposure and Cbi treatment (10-min post-treatment) are shown in Figure 2.4. Analogous to Figure 2.2, the cyanoCbi peaks were sharp and symmetrical. Moreover, the method was extremely selective, with no interfering peaks present in the pre-exposed rabbit plasma over the entirety of the chromatogram.

The cyanoCbi concentration, following IM-Cbi treatment of cyanide-exposed rabbits, is plotted in Figure 2.5. The cyanoCbi concentration is around 1 µM at 2.5 min following IM Cbi administration and increases to about 35 µM by 60 min. This progressive increase in cyanoCbi species is expected as the antidote distributes into the circulatory system from the muscle, binding more cyanide to produce more cyanoCbi. This data definitively shows that cyanoCbi is produced in-vivo from the treatment of cyanide poisoning via administration of Cbi (i.e., without addition of excess cyanide for analysis) and, to our knowledge, is the first study to do so. Therefore, the hypothesized mechanism of treatment of Cbi for cyanide poisoning, direct binding of cyanide, was verified for the first time by this study.
In conjunction with the cyanoCbi method, the total Cbi [6] at 7.5, 10, and 15 min post-treatment was evaluated (Figure 2.5, inset). The difference between cyanoCbi and the total Cbi was considered ‘available’ Cbi. As evident by the inset, the total Cbi concentration increased progressively from 7.5 to 15 min, while the cyanoCbi increased from 7.5 to 10 min and then decreased slightly from 10 to 15 min post-treatment. The available Cbi increased generally linearly from 7.5 to 15 min. In terms of the percentage of Cbi available, the percentage of cyanoCbi, with respect to the total Cbi, was approximately 65%, 65%, and 50% at 7.5, 10, and 15 min, respectively. Therefore, the ‘available’ Cbi was 35%, 35%, and 50%. The increasing ‘available’ Cbi from 7.5 to 15 min is due to increased availability of the Cbi in the circulatory system as it distributes from the muscle following IM injection. The relatively stable percentage of cyanoCbi versus the total Cbi is likely due to the binding of CN by Cbi being governed by equilibrium constants. Although this study is a good first step in evaluation of the availability of Cbi, a more comprehensive pharmacokinetic/pharmacodynamic study should be performed over a longer time period than presented here (i.e., allowing the elimination phase to be evaluated) with analysis of cyanoCbi and total Cbi over the entire duration of the experiment.
**Figure 2.4.** CyanoCbi (1015→930) chromatograms from rabbit plasma pre-CN-exposure and pre-treatment and 10-min post-treatment from the same animal. The peak shape was sharp, similar to what was obtained from the method developed.
Figure 2.5. CyanoCbi concentrations (circles) for a cyanide-exposed rabbit treated with 1H-tetrazole-5-acetylcobinamide. Inset: Concentrations of total, cyano and ‘available’ Cbi concentrations at 7.5, 10 and 15 min following the administration of antidote. The ‘available’ Cbi was calculated as total Cbi minus the cyanoCbi.

1.12. 2.5. Conclusion

An LC-MS-MS method for the detection of available cyanoCbi was developed. The method was applied for the detection of available cyanoCbi species in plasma and definitively show that cyanide is bound to Cbi in-vivo. The careful selection of a polymeric PLRP-S column and the cleaning protocol presented here are vital for the
analysis of Cbi and other compounds that have similar binding characteristics. The availability of this method should allow more comprehensive analysis of the behavior of Cbi for treatment of cyanide poisoning. In future, the method should be applied to a more comprehensive pharmacokinetic study.

1.13. 2.6. Acknowledgements

The research described was supported by interagency agreements (AOD16026-001-00000/A120-B.P2016-01 and AOD18015-001-00000/MRICD-IAA-18-0129-00) between the NIH Office of the Director (OD) and the U.S. Army Medical Research Institute of Chemical Defense under the oversight of the Chemical Countermeasures Research Program (CCRP) within the Office of Biodefense Research (OBRS) at the National Institute of Allergy and Infectious Diseases (NIAID/NIH). We would also like to acknowledge the tremendous support from the National Science Foundation Major Research Instrumentation Program (Grant Number CHE-0922816) for funding the AB SCIEX QTRAP 5500 LC/MS/MS (EPSCoR Grant 0091948). Finally, we would like to show our appreciation to the South Dakota State University Campus Mass Spectrometry Facility for the use of the LC–MS–MS, obtained with the support from the National Science Foundation/EPSCoR (Grant Number 0091948). Any opinions, findings and conclusions or recommendations expressed in this material are solely those of the authors and do not necessarily represent the official views of the NSF, CCRP, HHS, DoD, USAMRAA, USAMRICD.
CHAPTER 3. ULTRATRACE ANALYSIS OF NITROSODIPROPYLAMINE IN DRINKING WATER BY ICE CONCENTRATION LINKED WITH EXTRACTIVE STIRRER (ICECLES) AND LOW-RESOLUTION GAS CHROMATOGRAPHY-MASS SPECTROMETRY

3.1 Abstract

Nitrosoamines (NAs) are potent mutagens, carcinogens, and teratogens and are produced in drinking water as chlorination disinfection by-products. The US EPA estimates a $10^{-6}$ cancer risk of 0.2–20 ng/L for most NAs in drinking water and unregulated contaminant monitoring minimum reporting levels (MRL) range from 2-7 ng/L (ppt). Analysis of NAs at these ultratrace concentrations is extremely difficult, requiring sophisticated instrumentation and laborious sample preparation procedures. An advanced sample preparation technique, ICE Concentration Linked with Extractive Stirrer (ICECLES), coupled to a low-resolution gas-chromatography mass-spectrometry instrument was used to analyze NDPA (MRL = 7 ng/L (ppt)). ICECLES allowed ultratrace analysis of NDPA, producing an LOD of 0.2 ng/L, and a linear range of 2 to 50 ng/L (using an internal standard, NDPA-d$_{14}$). Both inter- and intraassay precision were ≤13%RSD, while the method accuracy was 100±17.5%. The ICECLES method was applied to screen for possible NA contamination in selected drinking water sources. The concentration of NDPA in one drinking water source was 2.38±0.34 ng/L. While NDPA was detected in the two other municipalities tested (i.e., concentrations > 0.2 ng/L), it was not quantifiable.
3.2. Introduction

Nitrosoamines (NAs) are potent mutagens, carcinogens and teratogens [20, 189-191]. They are highly toxic to human bladder tissue [192] and are associated with drinking water, food, and soil [20]. NAs have the general structure $R^1N(–R^2)N=O$, where an N-nitroso (N=N=O) functional group is typically bonded to two alkyl groups through the amine nitrogen [193]. Table 3.1 shows the structures of the most common nitrosoamines, along with some of their physical properties. As seen in Table 3.1, the NAs have a unique combination of being highly carcinogenic, and having relatively high polarity, as quantified by relatively low octanol-water partition coefficients (i.e., log $K_{ow}$s range from -0.44 to 3.13). As a result of their high polarity, most NAs are difficult to extract with organic solvents and non-polar solid sorbents [17], making the analysis of low NA concentrations very difficult.

Common sources of NAs or their precursors can be found in food, agrochemicals, tanned leather products, solvents, cutting fluids, drugs, plastics, rubber additives, detergents, and ground water/drinking water [194]. Drinking water is perhaps one of the most concerning routes for NA exposure [12]. NAs are formed during the water purification process as a disinfection byproduct (DBP) during chlorination and chloramination [13-16]. Disinfectants such as chlorine, ozone, chlorine dioxide, or chloramines react with naturally occurring organic matter or anthropogenic contaminants to produce NAs [13-19]. The presence of NAs in drinking water can also result from contamination by industrial and or agricultural sources [13, 20], or from anion exchange resins [21] (i.e., anion exchange resins used to remove anionic contaminants from drinking can produce higher NA levels in finished products).
Because of the carcinogenic nature of NAs, many established regulatory agencies have limited the concentration of NAs in drinking water (Table 3.1). For example, the U.S. Environmental Protection Agency (US EPA) estimated a lifetime $10^{-6}$ cancer risk from the consumption of drinking water containing 0.2-20 ng/L of NDMA, NMEA, NDEA, NDPA, and NDBA [13, 195, 196]. In March 2010, the US EPA also categorized NDEA, NDBA and NDPA as candidate drinking water contaminants and designated them for unregulated contaminant monitoring at 2 to 7 ng/L [16, 20]. The California EPA set the $10^{-6}$ cancer risk levels of NAs between 1 and 15 ng/L [197]. Moreover, the California Department of Health Services (CDHS) set a notification level of 10 ng/L for NDEA, NDPA, and NDMA [12, 191].

Because each of these concentration limits are considered ultratrace, and because NAs are relatively polar (Table 3.1), it is very difficult to quantify them at these levels. Therefore, quantification of NAs has required both highly sensitive analysis techniques and arduous sample preparation. Several analytical methods have been employed to detect NAs in water samples, including gas or liquid chromatography with nitrogen-phosphorous detector (NPD) [150, 190, 191, 198], thermal energy analyzer detector (TEA) [150, 190, 191], fluorescence detector (FD) [150, 191, 199], and mass spectrometry (MS) detection [12, 150, 191, 198, 200, 201]. These techniques combined with sample preparation techniques such as solid-phase microextraction (SPME), liquid–liquid (LLE) and solid-phase extraction (SPE), have produced detection limits approaching the low ng/L [191]. However, very large sample volumes (e.g. 500-1000 mL), long analysis times (e.g. 200 minutes), and sophisticated/expensive instrumentation such as GC-MS/MS, GC-HRMS, and HPLC-MS-MS are necessary to
reach the desired limits of detection [12, 191]. Therefore, methods capable of ultratrace analysis of NAs are typically too costly and time-consuming to routinely analyze NAs from drinking water sources [191]. Moreover, most institutions are not equipped with the sophisticated instrumentation necessary to perform these methods [191]. Table 3.2 compares methods of analysis of NDPA. One low resolution GC-MS method was recently developed by You using SPE was able to analyze NDPA to an LOD of 0.332 ng/L [151], but it involved a long and arduous sample preparation method involving passing 500 mL of water through an SPE column, elution with a hazardous solvent, evaporation of the solvent to a 1 mL volume (methylene chloride).

A novel sample preparation technique developed in our laboratory, coined ICE Concentration Linked with Extractive Stirrer (ICECLES), may allow analysis of NAs at the desired sensitivity from small sample volumes and more widely available analytical instrumentation [165]. ICECLES is the combination of freeze concentration and stir bar sorptive extraction. This process involves progressive freezing of an aqueous solution containing the analyte, while rapidly stirring and pre-concentrating analytes into a sorptive stir bar. After the analytes are extracted into a stir bar, they are desorbed by heating the stir bar or are back extracted into a solvent, and then analyzed. The main advantages of ICECLES are higher extraction efficiencies, the ability to extract relatively polar analytes from aqueous solutions, and greater recoveries for more volatile compounds [165].

The objective of the current study was to couple ICECLES with more readily available low resolution GC-MS instrumentation in order to detect NDPA from drinking water at relevant ultratrace concentrations (5-7 ng/L) from low sample volume and
relatively short preparation time. Because individual NAs are not typically found in isolation, NDPA may be a potential probe indicator of NA contamination. Upon finding NDPA above an acceptable limit, a more sophisticated technique to simultaneously analyze multiple NAs may be performed.
Table 3.1. Structures, physical properties, toxicity, and reporting levels of selected nitrosoamines.

<table>
<thead>
<tr>
<th>Name</th>
<th>Chemical Structure</th>
<th>Molar mass (g/mol)</th>
<th>log $K_{ow}^a$</th>
<th>$10^{-6}$ Cancer Risk (ng/L)</th>
<th>MRL (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-nitrosodiphenylamine (NDPhA)(^b)</td>
<td><img src="url" alt="Chemical Structure" /></td>
<td>198.22</td>
<td>3.13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N-nitrosodibutylamine (NDBA)</td>
<td><img src="url" alt="Chemical Structure" /></td>
<td>158.24</td>
<td>2.63</td>
<td>6(^c), 3(^d)</td>
<td>4(^e)</td>
</tr>
<tr>
<td>N-nitrosodipropylamine (NDPA)</td>
<td><img src="url" alt="Chemical Structure" /></td>
<td>130.19</td>
<td>1.36</td>
<td>5(^c,d)</td>
<td>7(^e), 10(^f)</td>
</tr>
<tr>
<td>N-nitrosomorpholine (NMorph)</td>
<td><img src="url" alt="Chemical Structure" /></td>
<td>116.12</td>
<td>-0.44</td>
<td>5(^c,d)</td>
<td>-</td>
</tr>
<tr>
<td>N-nitrosopiperidine (NPip)</td>
<td><img src="url" alt="Chemical Structure" /></td>
<td>114.15</td>
<td>0.36</td>
<td>3.5(^c)</td>
<td>-</td>
</tr>
<tr>
<td>N-nitrosodiethylamine (NDEA)</td>
<td><img src="url" alt="Chemical Structure" /></td>
<td>102.14</td>
<td>0.48</td>
<td>0.2(^c), 1(^d)</td>
<td>3(^e), 10(^f)</td>
</tr>
<tr>
<td>N-nitrosopyrrolidine (NPyr),</td>
<td><img src="url" alt="Chemical Structure" /></td>
<td>100.12</td>
<td>-0.19</td>
<td>20(^c), 15(^d)</td>
<td>2(^e)</td>
</tr>
<tr>
<td>N-nitrososomethylamine (NMEA)</td>
<td><img src="url" alt="Chemical Structure" /></td>
<td>88.11</td>
<td>0.04</td>
<td>2(^c), 1.5(^d)</td>
<td>5(^e)</td>
</tr>
<tr>
<td>N-nitrosodimethylamine (NDMA)</td>
<td><img src="url" alt="Chemical Structure" /></td>
<td>74.08</td>
<td>-0.57</td>
<td>0.7(^c), 3(^d)</td>
<td>2(^e), 10(^f)</td>
</tr>
</tbody>
</table>

\(^a\) Base 10 log of octanol-water partition coefficient \([16, 156, 195, 196]\).
\(^b\) Ph = phenyl group (C\(_6\)H\(_5\)).
\(^c\) US EPA $10^{-6}$ cancer risk level \([197, 202]\).
\(^d\) California EPA $10^{-6}$ cancer risk level \([197, 202, 203]\).
\(^e\) US EPA second Unregulated Contaminant Monitoring Rule (UCMR2) minimum reporting level \([16, 193]\).
\(^f\) California Department of Health Services notification level \([197, 202, 203]\).
<table>
<thead>
<tr>
<th>Method</th>
<th>Sample Volume (mL)</th>
<th>Sample Preparation</th>
<th>Analysis Technique</th>
<th>LOD Range (ng/L)</th>
<th>Estimated analysis time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qian et al. [160]</td>
<td>100</td>
<td>SPE</td>
<td>HPLC-ESI-MS-MS</td>
<td>0.01-0.7</td>
<td>120</td>
</tr>
<tr>
<td>You [151]</td>
<td>500</td>
<td>SPE</td>
<td>GC-CI-MS</td>
<td>0.038-0.332</td>
<td>360</td>
</tr>
<tr>
<td>Planas et al. [150]</td>
<td>1000</td>
<td>SPE</td>
<td>GC-EI-HRMS</td>
<td>0.08-1.7</td>
<td>200a</td>
</tr>
<tr>
<td>Krauss and Hollender [153]</td>
<td>500</td>
<td>SPE</td>
<td>HPLC-ESI-MS-MS</td>
<td>0.10-2.40</td>
<td>220</td>
</tr>
<tr>
<td>Zhao et al. [152]</td>
<td>500</td>
<td>SPE</td>
<td>HPLC-ESI-MS-MS</td>
<td>0.1-10.6</td>
<td>150</td>
</tr>
<tr>
<td>Munch and Basset [161]</td>
<td>500-1000</td>
<td>SPE</td>
<td>GC-CI-MS-MS</td>
<td>0.20-0.66</td>
<td>150</td>
</tr>
<tr>
<td>Charrios et al. [148]</td>
<td>500</td>
<td>SPE</td>
<td>GC-CI-MS</td>
<td>0.70-1.30</td>
<td>200</td>
</tr>
<tr>
<td>Hung et al. [159]</td>
<td>4.5</td>
<td>SPME</td>
<td>GC-CI-MS-MS</td>
<td>3.2-15.2</td>
<td>100a</td>
</tr>
<tr>
<td>Ripolles et al. [162]</td>
<td>500</td>
<td>SPE</td>
<td>HPLC-CI-MS-MS</td>
<td>1.0-8.0</td>
<td>100</td>
</tr>
<tr>
<td>Cheng et al. [163]</td>
<td>500</td>
<td>SPE</td>
<td>GC-CI-MS-MS</td>
<td>0.78-1.78</td>
<td>156</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>CSPE</td>
<td>GC-CI-MS-MS</td>
<td>0.33-1.36</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>MLLE</td>
<td>GC-CI-MS-MS</td>
<td>1.80-3.90</td>
<td>94</td>
</tr>
<tr>
<td>Grebel et al. [158]</td>
<td>40</td>
<td>SPME</td>
<td>GC-NPD</td>
<td>1-890</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>SPME</td>
<td>GC-CI-MS</td>
<td>30-138</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>40</td>
<td>SPME</td>
<td>GC-NCD</td>
<td>57-193</td>
<td>75</td>
</tr>
<tr>
<td>Wang et al. [164]</td>
<td>0.1</td>
<td>LLE</td>
<td>GC-EI-MS</td>
<td>32-100.6</td>
<td>90</td>
</tr>
<tr>
<td>Current method</td>
<td>10</td>
<td>ICECLES</td>
<td>GC-EI-MS</td>
<td>0.2</td>
<td>200a</td>
</tr>
</tbody>
</table>

*Methods with utilizing automated sample preparation

LOD = limit of detection; SPE = solid-phase extraction; SPME = solid-phase microextraction; CSPE = cartridge solid-phase extraction; MLLE = microliquid liquid extraction; ICECLES = ice concentration linked with extractive stirrer; HPLC = high-performance liquid chromatography; MS = mass spectrometry; ESI = electrospray ionization; GC = gas chromatography; CI = chemical ionization; HR = high resolution; NPD = nitrogen phosphorous detector; NCD = nitrogen chemiluminescence detector; EI = electron ionization.
3.3. Material and methods

3.3.1. Materials

All reagents were HPLC grade, unless otherwise stated. N-nitrosodipropylamine, (NDPA) standard (100 mg neat) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Isotopic labelled N-nitrosodipropylamine (NDPA-d_{14}, 98%) was obtained from Cambridge Isotope Laboratories, Inc, (Tewksbury, MA, USA). Isopropanol, (99.5%) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Water was purified using a water PRO PS polisher (Labconco, Kansas City, KS, USA) at a resistivity of 18.2 MΩ cm. Most water samples were irradiated for at least 26 hours with UV light from a xenon arc lamp (intensity 350 mW/cm²; Newport light source model 66903) to photodegrade inherent NDPA. All stock solutions were prepared in de-ionized irradiated water, while standards and blanks were prepared in irradiated tap water. A stock solution of both NDPA and NDPA-d_{14} were prepared as a 1 mM aqueous solution, placed in an amber bottles and stored at 4°C, and freezer (-30 °C) respectively, to prevent decomposition of NDPA and NDPA-d_{14}. Every three weeks, a new working stock solution of NDPA was prepared, while the working stock solution of NDPA-d_{14} was thawed immediately prior to each analysis.

3.3.2. ICECLES Sample Preparation

The apparatus and the general procedure used for ICECLES were reported by Maslamani et al. [165]. Briefly, the apparatus consisted of five main parts: 1) a circulating chiller, 2) a double-walled or “jacketed” beaker, 3) magnetic stir plate (4.25 in × 4.25 in), 4) a sorptive stir bar, and 5) an amber glass sample vial (40 mL). The ICECLES sample preparation procedure consisted of adding a water sample (10 mL,
spiked or unspiked water with 200 µL of 1 µg/L of NDPA-d_{14} internal standard) to a 40 mL glass amber vial along with a PDMS-coated stir bar. The vial was capped and placed into a double-walled beaker with coolant (-7 °C) continually circulated. Isopropanol (1 mL) was added to the bottom of the beaker to ensure adequate thermal contact between the vial and the cooled beaker. The stir bar was stirred at 1200 rpm, and the sample was allowed to freeze progressively from the bottom until the water was completely frozen. As the sample froze, the sorptive stir bar remained stirring on the top of the ice. After extraction, the PDMS stir bar containing the extracted analyte was removed from the vial using a clean teflon-coated magnet.

3.3.3. Gas Chromatography-Mass Spectrometry

All prepared stir bars were analyzed using an Agilent Technologies 7890A gas chromatograph and a 5975C inert XL electron ionization (EI)/chemical ionization (CI) mass selective detector (MSD) with Triple-Axis Detector and a Gerstel Multipurpose Sampler (MPS). The stir bars were thermally desorbed in a thermal desorption unit (TDU) in splitless mode, with NDPA trapped in a cooled injection system (CIS). The initial thermal desorption temperature was 60 °C (held for 0.20 min), increased linearly at 720 °C/min to 250 °C, and then held constant at that temperature for 1.5 min. The analyte was collected in the CIS at -100 °C, cooled with cryogenic nitrogen. The CIS was then heated to 250 °C at a rate of 12 °C/s. Subsequently, NDPA was transferred to the GC column with initial oven temperature of 35 °C. The GC oven temperature was linearly increased at rate of 20 °C/min until it reached a final temperature of 240 °C. Separation was achieved by using an HP-5MS capillary column (30 m × 250 µm x 0.25 µm) with helium carrier gas at a flow rate of 1 mL/min and a column head pressure of 6.5 psi. The
MS source and quadrupole temperatures were 280 °C and 150 °C, respectively. Electron ionization was used to fragment the NDPA with a voltage of 70 eV. Acquisition was carried out using selected ion monitoring (SIM) for ions 70 m/z (identification, [M-CH₃CH₂CH₂O]⁺), and 130 m/z (quantification, [M-H]⁺) for NDPA and 144 m/z for NDPA-d₁₄. The quantification ion was selected based on its signal to noise ratio. After confirmation of the identity of NDPA and NDPA-d₁₄, only 130 and 144 ions respectively, were used in the final method with a dwell time of 500 ms. Because the analysis of NDPA was completed at ultratrace levels, the stir bars were conditioned and cleaned with the TDU conditioner system at 300 °C for 3 hours, then cooled, and the absence of a NDPA peak in the nonspiked stir bars prior to analysis verified with the method.

3.3.4. Method Validation

For the determination of the limit of detection (LOD), multiple concentrations of NDPA below the LLOQ were spiked in irradiated drinking water in triplicate and analyzed by the analytical method. The LOD was determined as the concentration that reproducibly generated a signal to noise ratio of 3 based on the chromatograms. Noise was calculated as peak-to-peak noise in the blank (i.e., non-spiked irradiated water only) over the retention time of NDPA. The linearity of the method was determined by analyzing NDPA calibration standards in irradiated drinking water with the internal standard (200 μL of 1 μg/L NDPA-d₁₄) in triplicate at ten different concentrations ranging from 0.2 to 200 ng/L (i.e., 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200 ng/L). The average signal ratios were plotted against the corresponding nominal concentrations. The lower limit of quantification (LLOQ) and the upper limit of quantification (ULOQ) were selected based on the following inclusion criteria: precision (expressed as the relative
standard deviation) of <20% and an accuracy of 100±20% as calculated by comparing the experimental concentration to the nominal concentration of the calibrator. The analyte concentration was back-calculated from the calibration equation using the average signal ratio of each calibrator. Inter-and intraassay precision and accuracy were determined by analyzing low, medium, and high quality control (QC) standards in quintuplicate with concentrations not included in the calibration curve (i.e., 3.5, 15 and 35 ng/L, respectively) over three separate days and within ten calendar days.

The stability of NDPA was determined by storing the QC samples (low and high) at different temperatures. The internal standard was spiked immediately prior to sample preparation and analysis. In order to correct for run-to-run variability, bench top and autosampler stability were evaluated at room temperature (RT), while the long-term stability was determined at 4 °C, −30 °C and −80 °C, all at multiple storage times/durations. The samples stored at −30 °C, and −80 °C were left to thaw unassisted at room temperature for analysis. For freeze-thaw stability, three set of QC standards were stored at -80 °C. All standards were thawed, one set was analyzed, and the rest of the QC standards were re-frozen at -80 °C. The process was repeated for the remainder of the freeze-thaw QC samples. Stability of the NDPA at a particular concentration, at any given storage time and temperature, was calculated as percentage of the initial signal (QC standards freshly prepared each day of the analysis). The NDPA was considered stable if the signal from the stored sample was within 10% of the freshly prepared QC standard.

Recovery was determined by analyzing QC standards in tap drinking water (irradiated), and compared to QC standards of same concentrations in de-ionized water (irradiated), all in quintuplicate. The recovery was evaluated by dividing the average
signal of NDPA in irradiated tap water to that in irradiated de-ionized water, and expressed as a percentage.

3.3.5. Sample Collection Protocol and Analysis of Water samples

Various water samples from different locations, each with different water treatment facilities, were analyzed to determine NDPA contaminants. Tap water samples were collected from Brookings (South Dakota), Sioux Falls (South Dakota), and Lake Hendricks (Minnesota). The samples were collected in pre-cleaned amber vials with Teflon-lined polyethylene caps to prevent photodecomposition of NAs, if present. The cleaning protocol for the sample collection container glass vials was similar to that described by Maslamani et al., [165]. The amber vials were soaked in water, methanol and soap for 24 h each, rinsed several times with an NDPA free-deionized (DI) water (i.e., irradiated DI water that was previously analyzed with the method to confirm the absence of NDPA), and dried at room temperature [156] for at least 24 h. The vial was filled with NDPA-free DI water and stored in the refrigerator for 24 h. ICECLES analysis was performed (in triplicate) to ensure sampling vial cleanliness prior to sampling of the tap water at the various locations. Samples were collected as follows: 1) the valve of the water source was fully opened, and allowed to flow at full strength for about 3-5 minutes, 2) the flow was regulated to approximately 50 mL/min, and water was collected into the requisite container until overflowing, then the vial was capped immediately. The water was stored for about 5 minutes and dumped. The vial was then re-filled in the same manner and capped [165]. Samples were added to insulated containers during transportation. The samples were subsequently stored in the dark in the refrigerator
(between the temperatures of 4–6°C) until analysis. Samples collected and stored in the refrigerator were analyzed within 3 days [147, 149, 204-206].

3.4. Results and Discussion

3.4.1 ICECLES Sample Preparation and GC-MS Analysis

NAs, including NDPA, have relatively low log $K_{ow}$s, ranging from -0.57 to 3.13. Consequently, they are difficult to extract with organic solvents or common solid sorbents [16, 17]. Moreover, their Henry’s constants are very small, making it difficult to isolate them from the sample headspace [16]. Therefore, most methods for ultratrace NA analysis require very large sample volumes, arduous sample preparation, and sophisticated instrumentation (as seen in Table 3.2). The recently introduced ICECLES technique, first reported by Maslamani et al. [165], has demonstrated phenomenal extraction efficiencies of compounds with relatively low log $K_{ow}$ (<3.0). It is simpler, greener, and generally requires only a low resolution GC-MS for ultratrace analysis. In addition, the automated ICECLES procedure is a stark contrast to most SPE, MLLE, and LLE methods listed in Table 3.2, which require constant attention.

For the method presented here, a PDMS-coated stir bar was added to 10 mL of tap water and stirred as the sample was frozen. By following the experimental procedure described in the method section, the stir bar remained spinning on top of the frozen portion of the sample. After the sample was completely frozen, the stir bar was simply removed from the top of the sample. NDPA was then desorbed from the stir bar via thermal desorption and analyzed by a low-resolution GC-MS in electron ionization mode. Figure 3.1A shows the selected ion chromatograms of the quantification ion for the analyte and internal standard, both eluting at 5.1 min. The total GC-MS run time,
including desorption and equilibration, was approximately 15 minutes. The NDPA peak was sharp but did show some minor tailing ($A_s=1.6$). The method showed excellent selectivity with no interferent peaks in the boiled, irradiated tap water blank. Figure 3.1B shows the effect of UV irradiation on the tap water, where tap water was analyzed with the method before and after UV irradiation. The NDPA peak disappeared after the irradiation with UV light. Since NDPA elutes at 5.1 min (Figure 3.1A) and it is removed by UV irradiation, the peak in the non-irradiated tap water can likely be attributed to NDPA contamination of the tap water source.
Figure 3.1. Chromatograms of spiked and unspiked NDPA in irradiated tap water. A) Chromatograms showing the elution time of NDPA (130 m/z) and internal standard (NDPA-d$_{14}$, 144 m/z) at 5.1 minutes. B) The effect of irradiating the tap water with strong UV light led to photodecomposition of NDPA from the tap water. The disappearance of the peak at 5.1 minutes after UV irradiation indicates a preliminary confirmation of the presence of NDPA in the tap water (B).
3.4.2. Limit of detection (LOD), signal enhancement, dynamic range, and sensitivity

The LOD, as measured by a signal-to-noise ratio of 3, was 0.2 ng/L (i.e., 200 ppq). The excellent LOD was due to the efficient extraction of NDPA into the PDMS stir bar during ICECLES. This LOD was produced via analysis with a low resolution GC-MS, only 10 mL of sample, and automated sample preparation, in contrast to methods using more sophisticated instrumentation (i.e., GC-MS-MS or LC-MS-MS), larger sample volumes (250-1000 mL), longer sample preparation times, and sample preparation requiring constant attention (Table 3.2). The only method we found which utilizes a low resolution GC-MS, You [151] in Table 3.2, did produce an LOD similar to ICECLES (0.332 ng/L), but required 500 mL of sample, about 6 hours of sample preparation, and constant attention during addition of the sample to the SPE cartridge during sample preparation.

Theoretically, the fraction of analyte extracted (f_{extr}) via ICECLES is governed by Equation 3.1, assuming complete freezing of the sample [207]. K_{ice} represents the equilibrium constant between the liquid and solid sample matrix. The V_{ice} and V_{SB} are the final volumes of the ice and the PDMS, respectively. Using the volumes in this study, a typical K_{ice} of $10^{-3}$, and estimating K_{SB} ≈ K_{ow} of NDPA (i.e., K_{ow} is a good estimate of K_{SB}, the distribution constant of the analyte between an aqueous phase and the stir bar), the amount of NDPA expected to be extracted into the PDMS is 99.9%. Because of their similarities, the performance of ICECLES relative to SBSE was also evaluated. The combined FC and SBSE extraction of analytes via ICECLES made it possible to efficiently extract NDPA (log K_{ow} = 1.36) from drinking water, producing a signal enhancement of 198 versus room-temperature SBSE.
\[ f_{\text{extr}} = \frac{K_{SB}V_{SB}}{K_{ic}V_{ic} + K_{SB}V_{SB}} \]  

(3.1)

The linear range for the current method was 2 to 50 ng/L using a weighted \(1/x^2\) fitting linear regression. Because the MRL of NDPA is within the linear range, this method is well-suited for the evaluation of NDPA contamination in drinking water. The calibrator concentrations of 0.2, 0.5, 1, 100 and 200 ng/L were evaluated but did not fit the accuracy and precision criteria. The regression equations, correlation coefficients \((R^2)\), and the Percent Residual Accuracies (PRAs) [188] for the 3-day validation study are outlined in Table 3.3. The PRA values for all the calibration curves generated for the 3-day validation process was \(\geq 90\%\) (Table 3.3), indicating a good fit over the entire linear range [188]. In addition, the precision and the accuracy of calibration curves were acceptable (Table 3.4) [208].

**Table 3.3.** Regression models, \(R^2\) and PRA values for separate calibration curves over a 3-day period.

<table>
<thead>
<tr>
<th>Days</th>
<th>Equation for line</th>
<th>(R^2)</th>
<th>PRA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 1</td>
<td>(y = 0.0117x + 0.0139)</td>
<td>0.9887</td>
<td>90.77</td>
</tr>
<tr>
<td>Day 2</td>
<td>(y = 0.0172x + 0.0346)</td>
<td>0.9959</td>
<td>91.12</td>
</tr>
<tr>
<td>Day 3</td>
<td>(y = 0.0174x + 0.0715)</td>
<td>0.9948</td>
<td>90.25</td>
</tr>
</tbody>
</table>

\(^a\)Equation in the form of \(y = mx + b\), where \(x =\) NDPA concentration (ng/L) and \(y =\) signal ratio for the peak area abundance of the 130 /144 m/z ions from the GC-MS chromatogram.

\(^b\)Percent Residual Accuracy [188].
3.4.3. Method Validation: Inter-assay and Intra-assay Precision and Accuracy

The intra- and interassay precision and accuracy, determined by quintuplicate analysis of the QC standards (i.e., low, medium, and high) over ten calendar days, are shown in Table 3.4. The intra- and interassay precision of the NDPA in drinking water was <13% RSD, while the intra- and interassay accuracy was 100±17.5%.

<table>
<thead>
<tr>
<th>Nominal Conc. (ng/L)</th>
<th>Intraassay Accuracya (%)</th>
<th>Intraassay Precisiona (%)</th>
<th>Interassay Accuracyb (%)</th>
<th>Interassay Precisionb (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5</td>
<td>100±3</td>
<td>&lt;12</td>
<td>100±14</td>
<td>&lt;12.5</td>
</tr>
<tr>
<td>15</td>
<td>100±9</td>
<td>&lt;8</td>
<td>100±5</td>
<td>&lt;11</td>
</tr>
<tr>
<td>35</td>
<td>100±17.5</td>
<td>&lt;10</td>
<td>100±8</td>
<td>&lt;12</td>
</tr>
</tbody>
</table>

a QC method validation for Day 1 (N=5), b Average QCs for 3 different days of validation (N=15)

3.4.4. Matrix effect and recovery

Matrix effect analysis provides an estimation of signal attenuation or enhancement that may lead to loss or gain in signal of the analyte. The matrix effect was estimated by comparing the slope of a calibration curve produced in irradiated tap water to that in irradiated-deionized water (i.e., slope_{tap water} / slope_{DI water}). The slope ratio was
0.83, which is suggestive of a slight matrix effect, most likely be due to ion suppression in the irradiated tap water. The matrix effect for the internal standard corrected-calibration curve in both tap water (irradiated) and de-ionized was 1.02, indicating that the IS was effective at correcting for any variations in signals of the analyte.

The recoveries for NDPA for low, medium, and high QCs, were 93.2, 101.3, 85.3%, respectively. These recoveries were excellent. The relatively low recovery for the high QC could be due to saturation effect and/or matrix/suppression effects.

3.4.5. NDPA Stability

Bench top stability for the NDPA was evaluated for the low (3.5 ng/L) and high QC (35 ng/L) in irradiated tap water at 0, 2, 4, 8, 12 and 24 hrs. Thus, the samples were left on the bench top for the desired time, spiked with fresh IS and analyzed by ICECLES-GC-MS. NDPA (without inclusion of IS for the signal ratio) was stable up to 2 hours (105% recovered) and began to degrade after 4 hours (88% recovered). When the signal ratio was considered, the IS corrected for low and high QCs for the 24-h period (i.e., signal ratios were between 100 and 108% of the original signal ratio). It is important to note that the samples were reasonably protected from light (i.e., stored in an insulated foam container made of thick polystyrene) while standing on the benchtop and when placed on the GC-MS auto-sampler (i.e., all lights were turned off during the analysis with GC-MS).

Similarly, the autosampler stability was tested for 0, 2, 4, 8, 12, and 24 hours. The noncorrected low QC signal was not stable at any time tested within 2 hours but the high QC was stable up to 4 hours (Figure 3.2A). When using IS to correct for loss of signal, the signal ratio was stable for the 24-h period (Figure 3.2B).
The low QC NDPA was stable at 4 °C for 15 days, while high QC was stable for 30 days. With IS correction, all QC signal ratios were stable for at least 30 days. Stability at -30 and -80 °C for both low and high QCs was stable for 30 days with and without the IS correction following storage.
3.4.6. Analysis of Drinking Water for NDPA

Drinking water samples from various locations in South Dakota (Brookings and Sioux Falls) and Minnesota (Hendricks) were obtained and analyzed with the method presented here. The samples were collected in amber vials, stored in pre-cleaned 1-L amber bottles in a refrigerator, and analyzed within three days of sampling. Figure 3.3 below shows the chromatograms of NDPA spiked tap water (positive control) and
unspiked tap water sampled from Brookings. The peak at 5.1 min in the drinking water sample elutes at the same time as the spiked NDPA. Moreover, the NDPA peak disappeared when the sampled water was boiled and irradiated (similar to Figure 3.1B). The level of NDPA of the drinking water sampled from Brookings (SD) was 2.38±0.34 ng/L. This concentration of NDPA was below the USEPA MRL. NDPA in the water samples from Sioux Falls (SD) and Hendricks (MN) was above the LOD but below the LLOQ for the method (i.e., signals were above the LOD of 0.2 ng/L but not within the linear range of the calibration curve).
Figure 3.3. Chromatograms showing NDPA-spiked tap water (red), and that from unspiked tap water (blue) sampled in South Dakota on 02/07/2018. The concentration of NDPA in the unspiked tap water was between 2.04 and 2.72 ng/L at a 95% confidence limit.

3.5. Conclusion and Future Directions

A quick, flexible, simple, green, and relatively inexpensive method for NDPA analysis was developed using ICECLES sample preparation coupled with low resolution EI-GC-MS. This method could be expanded to more NAs (Table 3.1), or simply used for NDPA analysis. The use of GC-CI-MS may allow more selectivity for the NAs in Table 3.1 and should be used as a strategy for a more comprehensive method for NAs. That
said, because NAs are typically found together, NDPA may be able to act as a probe molecule for screening drinking water. Therefore, this method could potentially be used to screen drinking water for NDPA, with follow-on analysis via more comprehensive method (see Table 3.2) if NDPA concentrations in the drinking water source are concerning. The figures of merit were within USEPA guidelines, and the usefulness of the method was verified by detecting NDPA in drinking water samples.

1.14. 3.6. Acknowledgements/Disclaimer

We gratefully acknowledge the support by National Science Foundation through the Green/Environmental REU site (Grant #CHE-1461092). Furthermore, we thank the U.S. Joint Executive Office for Chem Bio Defense, Joint Program Management Protection Contract W911SR-09-0059 for funding the GC-MS instrument and the state of South Dakota (SD) for their support. Any opinions, findings and conclusions or recommendations expressed in this material are solely those of the authors and do not necessarily represent the official views of the NSF or DoD or the state of South Dakota.
CHAPTER 4. BROADER IMPACTS, CONCLUSION AND FUTURE DIRECTIONS

4.1. Broader Impacts

Currently, there are some limitations with the available cyanide antidotes, such as slow onset of action, administration requiring supportive care and trained individuals, and limited applicability to mass casualty situations. From recent studies, Cobinamide (Cbi) has shown excellent potential as a cyanide antidote. In order for the FDA to approve Cbi as an antidote, there is a need to conduct a comprehensive pharmacokinetic studies for the drug (Cbi). This includes \textit{in-vivo} determination of the “available” and “total” Cbi in biological fluids. Therefore, a bioanalytical technique to differentiate available Cbi from total Cbi is vital to help the translation towards FDA approval. Consequently, the LC-MS-MS method developed in this study should provide tremendous information for the advancement of this Cbi as cyanide antidote. Moreover, the data obtained from this study, definitely verified \textit{in-vivo} the hypothesized mechanism of treatment of Cbi for cyanide poisoning (via direct binding of cyanide).

Since nitrosamines (NAs) are produced as disinfection byproducts, and are probable cause of cancer, it is very important to test treated water prior to delivery to consumers. Current methods available for NA analysis require sophisticated and expensive instrumentation and arduous sample preparation, some requiring hazardous organic. This makes monitoring of these probable carcinogenic compounds at trace levels in drinking water quite difficult and expensive, which limits many municipalities from monitoring drinking water. Therefore, a relatively inexpensive method that utilizes a low resolution and inexpensive gas chromatographic instrumentation coupled with flexible,
automated, and greener sample preparation technique should enable monitoring of NAs at trace levels (i.e., MRLs) as stipulated by the USEPA. This would allow increased monitoring and potentially help prevent development of cancer based on NA exposure through drinking water consumption. The analytical method, ICECLES-GC-EI-MS, reported here affords a solution to the challenges associated with the current method for analysis of NAs at trace levels.

Overall, the methods presented in this dissertation should help facilitate development of Cbi as a countermeasure for cyanide (which has ubiquitous sources) and facile and prompt screening of NAs in drinking water to help decrease exposure to NAS.

4.2. Conclusions

The methods presented in this dissertation have significant impacts for the approval of Cbi as cyanide antidote, and regulation of NAs in trace amounts. We have shown systematically how to deal with a much known problem of residual Cbi by developing a comprehensive how a cleaning protocol. For the first time, we have developed a method that can be utilized to help in the classification and regulation of nitrosoamines.

The LC-MS-MS method developed was able to analyze available Cbi, and was used to verify *in vivo* for the first time, the hypothesized mechanism of Cbi as cyanide-binding agent, making it possible for more detailed pharmacokinetic studies of Cbi to be conducted, and ultimate approval by the FDA as cyanide therapeutic.

The ICECLES sample preparation coupled with low resolution GC-EI-MS method reported here allows for trace analysis of NDPA, as required by the USEPA.
This method eliminates some of the challenges of current methods for NAs analysis, and helps reduce the risk of cancer associated with NAs in drinking water.

4.3. Future Work

Ideal cyanide antidotes are very critical in reversing cyanide poisoning in a timely fashion. Consequently, the use of the presented method to evaluate CyanoCbi, total Cbi, and available Cbi for comprehensive PK studies that facilitate the determination of a detailed pharmacokinetic behavior of Cbi, which will help facilitate the approval of Cbi as cyanide antidote. The future work will address this aspect of the research.

The ICECLES-GC-EI-MS method should be extended to analyze more NAs in the future. Specifically, ICECLES sample preparation coupled with low resolution GC-CI-MS may produce very sensitive and selective method for analysis of NAs [209]. The ultratrace analysis of other NAs with ICECLES-GC-CI-MS would be explored.
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