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Comprehensive Analysis and Forensic Investigation of Cyanide, Thiocyanate, and 2-Amino-2-Thiazoline-4-Carboxylic Acid In-Ante- and Postmortem Blood Utilizing Advanced Analytical Techniques

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COMPREHENSIVE ANALYSIS AND FORENSIC INVESTIGATION OF CYANIDE, THIOCYANATE, AND 2-AMINO-2-THIAZOLINE-4-CARBOXYLIC ACID IN-ANTE- AND POSTMORTEM BLOOD UTILIZING ADVANCED ANALYTICAL TECHNIQUES

BY

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

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By the grace of Allah, I dedicate this dissertation to my father, mother, wife, and loving family. Your encouragement and support throughout my Ph.D. journey have been invaluable. Your love and guidance have illuminated my path, and I attribute my success to each of you.

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ABBREVIATIONS

As: Peak asymmetry

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

ATP: Adenosine triphosphate

CE: Capillary electrophoresis

CI: Chemical ionization

C_{max}: Maximum blood concentration

$C_{baseline}$: Endogenous concentration

CN^-: Cyanide

HCN: Hydrogen cyanide

CN: HCN/CN^-

ECD: Electron capture detector

EDTA: Ethylenediaminetetraacetic acid

ESI: Electrospray ionization

FDA: Food and Drug Administration

GC-MS: Gas chromatography-mass spectrometry

HBT: Typical body temperature

HCN: Hydrogen cyanide

HFBA: Heptafluorobutyric anhydride

HPLC: High performance liquid chromatography

IACUC: Institutional Animal Care and Use Committee

IC: Ion chromatography

K_e: Elimination constant

LLE: Liquid-liquid extraction

LLOQ: Lower limit of quantification

LOD: Limit of detection

m/z: Mass-to-charge ratio

MBB: Monobromobimane

MCX: Mixed mode cationic exchange

MRM: Multiple reaction monitoring

MS-MS: Tandem mass spectrometry

MSTFA: N-methyl-N-trimethylsilyl-trifluoroacetamide

NDA: 2,3-naphthalenedialdehyde

NPD: Nitrogen-phosphorus detector

PFB-Br: Pentafluorobenzyl bromide

PFPA: Pentafluoropropionic anhydride

PFPI: Pentafluoropropanylimidazole

ppb: parts per billion

ppm: parts per million

Q1: First quadrupole mass analyzer

Q3: Third quadrupole mass analyzer

QC: Quality control

RBC: Red blood cell

RP: Reverse-phase

RSD: Relative standard deviation

RT: Room temperature

SCN^-: Thiocyanate

SEM: Standard error of the mean

SPE: Solid-phase extraction

SPME: Solid-phase microextraction

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

TFAA: Trifluoroacetoic anhydride

TFAI: Trifluoroacetylimidazole

ULOQ: Upper limit of quantification

UV: Ultraviolet

WWI: World War I

WWII: World War II

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ABSTRACT

COMPREHENSIVE ANALYSIS AND FORENSIC INVESTIGATION OF CYANIDE, THIOCYANATE, AND 2-AMINO-2-THIAZOLINE-4-CARBOXYLIC ACID IN ANTE- AND POSTMORTEM BLOOD UTILIZING ADVANCED ANALYTICAL TECHNIQUES

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Cyanide (CN), present as cyanide anion (CN^-) or hydrogen cyanide (HCN), is a deadly poison. The main mechanism of CN toxicity is blocking terminal electron transfer, leading to cellular hypoxia, cytotoxic anoxia, and potential death. Determining blood CN concentrations along with its major metabolite, thiocyanate (SCN^-), is crucial. A sensitive method for concurrent detection of CN and SCN^- from human ante- and post-mortem blood via liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) analysis was developed. The method involved active microdiffusion with chemical modification using naphthalene-2,3-dicarboxaldehyde (NDA) and taurine, while monobromobimane (MBB) modification was used for SCN^-. The method showed good sensitivity for CN with antemortem limits-of-detection (LODs) of 219 nM and 605 nM for CN and SCN^-, respectively, and postmortem LODs of 352 nM and 509 nM. The method also produced good accuracy and precision, detecting elevated levels of CN and SCN^- in both ante- and post-mortem blood samples from CN-exposed swine compared to non-exposed swine.

Forensic verification of CN poisoning by direct CN analysis in postmortem blood is challenging due to the instability of CN in biological samples. CN metabolites, SCN^- and 2-aminothiazoline-4-carboxylic acid (ATCA), have been proposed as more stable biomarkers, but their appropriateness for verification of poisoning remains unclear. The

behavior of CN, SCN^-, and ATCA in postmortem swine stored at 4°C and postmortem blood stored at room temperature (25°C; RT) and typical human body temperature (37°C; HBT) was evaluated. After poisoning swine with CN, the blood concentration of each CN biomarker increased well above the baseline. In postmortem swine, elevated CN concentrations declined rapidly, whereas SCN^- and ATCA concentrations remained relatively stable. The decline in CN accelerated as the storage temperature increased, where CN was below baseline levels within 50, 74, and 120 h for postmortem swine at 4°C and postmortem blood at RT and HBT, respectively. The half-lifes ($t_{1/2}$) of CN were 34.3 h, 10.8 h, and 6.6 h at 4 °C (in postmortem swine), RT, and HBT, respectively. SCN^- and ATCA were more stable than CN at all storage conditions, producing $t_{1/2}$s of 15-19 days and 23-26 days for SCN^- and ATCA, respectively. ATCA exhibited the most extended $t_{1/2}$ and most significant increase above baseline levels for the CN biomarkers tested, making it the most appropriate biomarker for confirming CN poisoning via postmortem blood analysis.

1. CHAPTER 1. INTRODUCTION

1.1. Overall Significance

Cyanide (CN) is a toxic chemical that can be introduced into living organisms by a number of means, such as ingestion of edible plants (e.g., cassava, spinach, chokecherries, bitter almonds, mustard, kale), inhalation of smoke from cigarettes or fires, or accidental exposure during industrial operations (e.g., pesticide production).[1-3] The primary toxic mechanism of CN involves the interruption of cellular respiration, as it binds to the ferric ion in the heme group of the mitochondrial enzyme cytochrome c oxidase, halting the transfer of electrons to oxygen, leading to cellular hypoxia, cytotoxic anoxia, and death.[1, 4-6] A concentration of 300 mg/m^3 of CN is lethal, can kill a human within about 10 min. The time is decreased to about 1 min with 3200 mg/m^3. [7-10] Detection of CN exposure is a critical concern, particularly in occupational health and safety monitoring. Direct CN analysis in ante- and postmortem blood samples is complicated due to its high volatility and reactivity. Thiocyanate (SCN^-) is a significant metabolite of CN that has been used as a biomarker for CN poisoning in biological fluids. No technique was available for analysis of CN and SCN^- concurrently from ante- and postmortem blood. Moreover, 2-aminothiazoline-4-carboxylic acid (ATCA) is a stable and valuable CN poisoning biomarker when blood samples are not immediately obtained. SCN^- and ATCA are suggested as stable indicators for confirming CN poisoning, however, their reliability is not yet fully established. Addressing these challenges is crucial for bridging existing gaps and ensuring effective verification of acute CN exposure.

1.2. Objectives of the Current Work

The lack of an available method for concurrent detection of CN and SCN^-, the objective was to develop a sensitive and accurate approach to quantify CN and SCN^- in the whole human blood. Chapter 2 aimed to develop a rapid HPLC-MS/MS method for concurrently determining CN and SCN^- in human ante- and postmortem blood.

Because ATCA stability in postmortem decay is unestablished, another objective of this dissertation work was to determine the value of ATCA as a marker for detecting CN poisoning in situations where blood samples are not immediately analyzed. Therefore, Chapter 3 details the evaluation of the stability of CN, SCN^-, and ATCA to assess their dynamics and their advantages and disadvantages as indicators of CN poisoning.

1.3. History and Use

Hydrogen cyanide (HCN) was first discovered and isolated by C.W. Scheele, a Swedish chemist, in 1782. Scheele used Prussian blue [$Fe_4(Fe_2(CN)_6)_3$], a dark blue pigment, and vitriolic acid (sulfuric acid) mixture to produce HCN.[11, 12] HCN is a volatile and colorless liquid with a low melting and boiling point (i.e., 13.24 °C and 25.7 °C).[13, 14] It is a weak acid produced at the industrial level by the oxidation of methane and ammonia at high temperatures over a platinum catalyst (i.e., CN has a dissociation constant of 4.93×10^{-10} at 25 °C and pKa 9.31).[15-18]

1.3.1. Use as a Warfare Agent

Chemical warfare agents are toxic chemicals used to kill, injure, or debilitate a foe in military wars, operations, and terrorist attacks.[19-21] The history of their use is immemorial, but the use of these agents increased during world wars I and II (WWI and II). The Franco-Prussian War (1870–1871), in which Napoleon III ordered his forces to soak the ends of

their bayonets in CN solutions, is when CN was first used in combat.[17, 19, 21] Cherry laurel water poisoned with CN was also employed as a lethal weapon by the Roman emperor Nero (37–68).[20, 21] French and Austrian soldiers used it during WWI, and Nazi Germany killed millions of people during WWII by using the rodenticide Zyklon B.[21, 22] The Japanese group Aum Shinrikyo installed CN delivery devices in public restrooms in 1995.[20, 23]

1.3.2. Illegal uses of Cyanide

In several Southeast Asia nations, sodium cyanide (NaCN) is used covertly for fishing. CN fishing is a quick way to stun fish and harvest them, but repeated use harms coral reefs permanently. Over 1 million kg of CN is thought to have been used on Philippine corals since the 1960s. Within hours of collecting, CN caught fish have been found to have fatality rates around 5 to 75%; however, the expected fatality rate from coral to consumer is greater than 90%.[24, 25]

Since ancient times, CN has been employed as a lethal substance. For example, in ancient Egypt, plants with CN compounds were employed as deadly poisons, including bitter almonds, cherry laurel foliage, peach pits, and cassava. These plants were not native to Egypt or widely available but were introduced and cultivated for their toxins.[26-28] The Louvre Museum in Paris has peach pits from ancient Egyptian punishments on exhibit, and an Egyptian papyrus mentions the "punishment of the peach." The cherry death, also known as the Romans' execution method, involves cherry laurel leaves. In 1915-1916, CN was initially produced with the intention of being use as a chemical weapon during the WWI.[26, 28] In Africa, there is an increase in the intentional, industrial, or retribution poisoning of animals, particularly the murdering of elephants (Loxodonta africana) to provide the illicit ivory trade. There are regional variations in the kinds of poisons used by hunters. In recent

years, poachers in Zimbabwe have illicitly obtained cyanide from its industrial usage in the mining industry to poison elephants for tusk extraction without the need for firearms.[28-30]

1.3.3. Uses of CN in Industry

Many industries use CN for electroplating, metal refining, organic synthesis, and different chemical processes.[31, 32] CN is largely employed in the mining business to extract silver and gold from ores. Further, CN frequently used in small amounts as a flotation reagent to extract base metals like copper, lead, and zinc. Most of these enterprises have CN treatment facilities to handle any potential risks to the health of people, animals, waterbirds, or aquatic life.[33, 34]

Gold is recovered from limited ores using cyanidation process.[32] The McArthur-Forrest process (Equation 1.1) transforms solid gold into a water-soluble gold-coordination complex. The Merrill-Crowe Method is then used to return the gold to a solid substance (Equation 1.2).[24, 32]

$$4Au + 8NaCN + O_2 + 2H_2O \rightarrow 4Na[Au(CN)_2] + 4NaOH \quad (1.1)$$

$$2Au\ (CN^-)_2(aq) + Zn\ (s) \rightarrow Zn(CN)_2(aq) + 2Au\ (s) \quad (1.2)$$

Although the main application of CN in this context is the separation of gold, it is also possible to extract other important metals from their corresponding ores, such as copper, uranium, and nickel, by combining with CN. The particular metal can then be extracted from the metal-CN complex using a redox reaction similar to Equation 1.2.[17]

1.3.4. Sources of Cyanide: From Environmental Biosynthesis to Industrials Pollutions

CN is a chemical that people are exposed to daily through consuming cyanogenic compounds from foods like cassava roots, lima beans, almonds, and spinach, as well as by

breathing smoke from cigarettes or fires and working in industrial settings where CN is used or produced.[12, 19, 35-37]

CN is released into the atmosphere from cigarette smoking. The Environmental Protection Agency (EPA) estimates CN up to 750,000 pounds per year.[12, 38] Studies have shown that fetal blood of smoking or high-smoking mothers may be at a higher risk of CN exposure.[12, 39] For example, a study found a mean concentration of 88.6 μg/L in fetal blood of mothers who smoked or were exposed to passive smoke, compared to 24.3 μg/L in unexposed mothers.[39, 40] HCN concentrations in the U.S. have been reported in various sources, including food, biomass burning, automobile emissions, and industrial processes.[38, 41] The U.S. Department of Health and Human Services reports a concentration of 0.160-0.166 ppm in indoor air, with a maximum contaminant level of 0.2 ppm. In drinking water, the concentration ranges from 1-11 ppb, with a maximum contaminant level of 200 μg/mL or 200 ppb.[12, 42-50]

CN is released into the environment by various industrial processes, including the production of HCN, methyl methacrylate, and acrylonitrile. In California, 1.2 million pounds of HCN are released into the air. Other industrial processes like coal carbonization also contribute to HCN release.[49, 50] Agricultural pest control activities released 137,000 pounds of CN in 1981. The extraction of precious metals like gold also contributes to HCN emissions, with 48,400 pounds released in the US and 44,800,000 pounds worldwide in 1992.[12, 49, 50]

CN exposure is a significant health risk in house and building fires.[31, 45, 51, 52] Eckstein and Maniscalco[53] reported several incidents where CN were the leading cause of death, including a prison fire in Argentina, an aircraft fire in Manchester, England, and the Happy

Land Social Club fire in New York City. In the Argentinean prison fire, over 90% of inmates died from lethal CN concentrations. In Manchester, England, an aircraft fire resulted in CN exposure.[3, 12, 46, 47, 54-57]

1.4. Fate, Toxicity, and Metabolism of Cyanide

Cyanides are emitted into the atmosphere due to air pollution and can harm living things in various ways.[58] The mucosal membrane of the trachea, particularly the wet part, and the gastrointestinal tract all effectively absorb CN.[58-60] In vertebrates, HCN and methemoglobin interact in the bloodstream, although most CN metabolism occurs in tissues.[31, 51, 52, 61] The electron transport chain utilizes the energy generated by electron movement to transport protons (H^+) into the membrane space (Figure 1A). CN activity primarily affects the respiratory chain's essential enzyme, cytochrome oxidase, by interacting with its trivalent iron.[18, 31, 51, 52] CN disrupts the proton gradient during cellular respiration, reducing ATP production. When CN binds to cytochrome c oxidase, it blocks the respiratory chain's reductase and oxidase enzymes from producing the proton gradient. Decreased protons (H^+) reduce the ability of ATP synthase to synthesize ATP. Cytochrome oxidase in the presence of CN reaction is depicted in Figure 1B. Combining these two causes lactic acid production to increase and blockage of the intracellular respiratory system. It is crucial to remember that the CN ions also restrict other enzymes: glutamate decarboxylase, xanthine oxidase, superoxide dismutase, NO synthase, and nitrite reductase.[31, 62, 63]

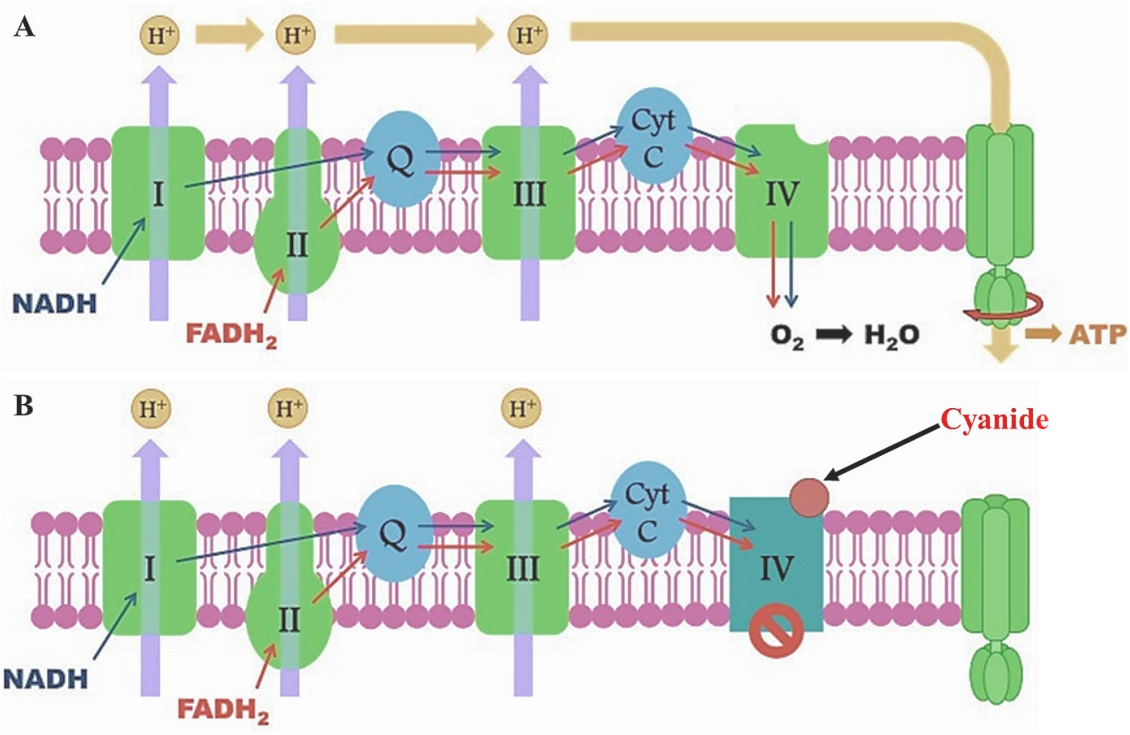

Figure 1.1. Schematic representation shows the CN inhibition on cytochrome oxidase. A) The electron transport chain uses the energy from moving electrons to move H^+ ions into the space between the membranes. This creates an electrochemical gradient. Electrons are passed from carriers to oxygen, the final electron acceptor. B) CN binds to cytochrome oxidase non-competitively, altering its active site. This blocks electron transfer to oxygen, stopping the gradient and ATP production.[64]

CN ion containing substances are poisonous right away because they stop cells from breathing.[2, 51, 63] The heart and the brain muscle, which have the highest oxygen metabolism, are the tissues most susceptible to the harmful effects of CN, but hypoxia affects how all body cells perform. The type of chemical carrying a CN ion significantly impacts the hazardous dose.[52, 61, 65] CN has toxic effects at 0.05 mg/dL and death at 0.3 mg/dL. Exposure to 546 ppm of HCN led to death after 10 min, and 110 ppm was life-threatening after 1 h. In some cases, workers exposed to HCN became unconscious and died even after receiving antidotal therapy.[35, 48, 66, 67] In other cases, exposure to 270 ppm HCN led immediately to death; 181 ppm HCN exposure was fatal after 10 min, and 135 ppm HCN was

fatal after 30 min.[68] Three deep-sea trawler men died when exposed to toxic fumes from spoiled fish, and CN exposure was confirmed in one person postmortem by a blood CN concentration of 0.05 mg/L.[69]

Numerous studies have reported acute exposure levels leading to animal deaths, with LC_{50} values (i.e., lethal concentration, 50% death) provided for various species.[35] Inhalation LC_{50} values of HCN in rats ranged from 143 ppm for 60 min to 3,417 ppm for 10 s.[70] In mice, exposure resulted in similar LC_{50} values, while in rabbits, LC_{50} values ranged from 188 ppm for 30 min to 2,200 ppm for 45 s.[70] Lethal concentrations were also reported in experiments with dogs exposed for acute and intermediate durations.[71]

The liver detoxifies CN through thiosulphate sulfurtransferase (i.e., rhodanese), an enzyme found in the mitochondria of the liver, to produce thiocyanate ions (SCN^-).[31, 52, 72] Sulfur is obtained from biological substances, like thiosulphate. SCN^- is about 200 times less hazardous than CN and is expelled mainly in the ucine.[31, 73] While SCN^- is much less toxic than CN, high concentrations of SCN^- have been linked to vertigo and unconsciousness.[5, 41, 74-78]

Other minor metabolisms of CN have been identified. When CN interacts with L-cysteine through an intermediary β-thiocyanoalanine, 2-amino-2-thiazoline-4-carboxylic acid (ATCA) is produced.[79-81] ATCA is not naturally found in biological fluids and is interested in biochemical research and forensic science. ATCA, primarily excreted in urine, has been linked to CN exposure and neurotoxic effects.[31, 82, 83] Previous studies suggest its lasting signature of CN exposure and may play a role in the neurotoxic effects of CN.[79, 82] Research on ATCA focuses on understanding its formation, role as a biomarker, and developing methods for its detection and quantification in biological samples.[31, 79] Further research is

ongoing to understand the effects of postmortem interval, preservation, and antemortem events on ATCA stability.

CN can combine with hydroxocobalamin, vitamin B12a, to form cyanocobalamin, which is vitamin B12, cyanocobalamin is excreted in urine.[18, 31, 51] CN reacts with α-ketoglutarate (α-Kg) in biological environments to form α-ketoglutarate cyanohydrin (α-KgCN), which has similar absorption and elimination kinetics to CN and ATCA.[72, 84] The interaction of CN with disulfide bonds in human plasma proteins forms cyanide-protein adducts.[4, 31, 72] CN also reacts with glutathione (i.e., a biologically abundant sulfur-donating biomolecule) to produce 2- aminothiazoline-4-oxoaminoethanioc acid (ATOEA).[51, 72]

1.5. Critical Needs in Confirming Cyanide Poisoning: The Value of Thiocyanate and ATCA Analysis

CN detection is crucial, therefore, effective CN detection allows for rapid diagnosis and treatment while enforcing safety regulations.[18, 31] Analytical techniques like chromatography, mass spectrometry, and spectroscopic methods are essential for identifying and quantifying CN in biological and environmental samples.[52, 85] The development and modification of these detection approaches are vital for forensic science, clinical toxicology, industry, and environmental monitoring applications.[51, 52, 61]

SCN^- and ATCA are promising biomarkers for CN exposure.[18, 31, 79] SCN^-, the major CN metabolite, can be measured in body fluids like blood and urine, confirming exposure. ATCA provides more specific evidence of CN metabolism.[79, 86] Although challenges remain, ATCA detection in urine or plasma shows excellent potential as a forensic marker of CN poisoning.[86-88] Combining ATCA analysis with SCN^- and CN provides specificity and sensitivity in confirming CN exposure.

1.6. Sample Preparation of Biological Fluids

Biological fluids like blood, plasma, urine, and saliva are ideal for toxicological analysis of CN due to their accessibility, short-term exposure profile, distribution data, presence of metabolites, correlation potential, stability, and cost-effectiveness.[21, 89] However, the analysis of CN and its metabolites in biological fluids can be challenging due to interfering proteins and endogenous components.[90] Sample preparation techniques like protein precipitation, liquid-liquid extraction (LLE), and solid-phase extraction (SPE) are widely used to eliminate or reduce interference components before analysis.[34, 86, 91] A summary for the analytical sample preparation techniques, advantages, and disadvantages of biological fluids are shown in Table 1.1.

1.6.1. Protein Precipitation

Protein precipitation is a crucial step in bioanalytical methods to remove proteins from complex biological fluids.[12, 31, 51] Plasma proteins, which comprise over 70% of plasma content, can hinder the analysis of small molecule like CN. Precipitation disrupts protein solubility by adding organic solvents, acids, or salts.[12] The resulting protein pellet is removed by centrifugation or filtration, preventing protein interference in downstream analysis and concentrating soluble analytes in the supernatant. This quick and cost-effective technique provides an initial crude purification essential for robust bioanalysis.[12, 52]

Protein precipitation can be achieved by adding salt, organic solvents, or varying the pH of the medium. High concentrations of salt decrease protein solubility, promoting aggregation due to increased hydrophobic interactions in the protein structure.[12, 92] Salts like ammonium or sodium acetate, ammonium sulfate, sodium or potassium chloride are commonly used for protein precipitation. Organic solvents, such as acetone or ethanol, lower

the medium's dielectric constant. This makes it easier for charged groups on the protein surface to interact.[12, 93] Because of dipole and electrostatic interactions, this moves water molecules that are attached to proteins, which leads to protein aggregation and precipitation.[12, 92] Proteins have a net positive charge at low pH due to amide groups gaining an additional proton and a net negative charge at high pHs due to carboxyl loss on the protein backbone. This minimizes electrostatic repulsion and causes hydrophobic forces to attract molecules, resulting in protein or isoelectric precipitation.[12, 92]

1.6.2. Liquid-Liquid Extraction

Liquid-liquid extraction (LLE), or solvent extraction, is partitioning an analyte into one of two phases based on differential solubility. This process involves transferring the target analyte into an organic solvent while leaving behind interferents in the aqueous matrix. Organic solvents like ethyl acetate, dichloromethane, or isobutyl acetate are commonly used for CN analysis.[12, 93] Phase transfer catalysts like tetrabutylammonium salts can assist in extracting ionic CN into the organic layer. LLE is advantageous for CN isolation due to its speed, simplicity, and compatibility with many analytical techniques.[12, 93]

1.6.3. Solid Phase Extraction

Solid-phase extraction (SPE) is a separation commonly used to extract analytes from various matrices. The process of SPE is used to isolate target constituents from a liquid sample into a solid stationary phase. It involves four steps: 1) conditioning the sorbent with a solvent to increase its surface area and effectiveness; 2) loading the solution containing the analyte; 3) washing or rinsing the sorbent with specific solvents to remove impurities; and 4) eluting the analyte from the SPE sorbent and collecting it for chemical analysis.[12, 94, 95]

Table 1.1. A summary of the analytical sample preparation techniques of biological fluids.

Technique	Principle	Applications	Advantages	Disadvantages
Protein precipitation	Precipitation of proteins by altering solution conditions (pH, salt, solvents)	Removal of plasma proteins for bioanalysis	Simple, fast, cost-effective	Less selective, further sample clean-up often needed
Liquid-liquid extraction (LLE)	Partitioning of analytes between immiscible organic and aqueous phases	Extraction of cyanide from blood, plasma, urine	Fast, simple, high recovery	Use of hazardous solvents, emulsion formation
Solid phase extraction (SPE)	Retention of analytes on solid sorbent, elution with solvent	Sample clean-up and concentration	Versatile, high selectivity, automation	Additional steps required, sorbent selection can be difficult
Derivatization for GC	Chemical modification to improve volatility and stability	Detection of polar/labile metabolites	Improves separation, sensitivity, detection	Additional reaction steps required, reagent stability issues
Derivatization for LC	Addition of chromophores/fluorophores	Detection of non-fluorescent analytes like CN	Enhances sensitivity and selectivity	Extra reaction steps, optimized conditions needed

1.6.4. Chemical Modification

Derivatization is a chemical modification of an analyte prior to analysis, allowing for the analysis of compounds that are not directly detectable due to inadequate volatility or stability. It can also improve chromatographic behavior or instrument response.

1.6.4.1. Chemical Modification for GC Analysis

Compounds with functional groups with active hydrogens, such as –COOH, -OH, -NH, and –SH, are the primary targets of derivatization. Therefore, three types of derivatizing compounds can be used to overcome this challenge: alkylation, acylation, and silylation.

Alkylation involves transferring an alkyl group from one molecule to another, reducing its polarity. Alkylating agents are widely used to modify compounds with acidic hydrogens, such as carboxylic acids and phenols. The principal reaction utilized for alkylation is nucleophilic displacement, such as PFB-Br.[12, 31]

Acylating reagents reduce the polarity of amino, hydroxyl, and thiol groups by adding halogenated functionalities, producing a derivatized analyte suitable for detection via FID and ECD. Chemical compounds derivatized with acylating reagents are typically multifunctional compounds, like carbohydrates and amino acids. Examples of acylating reagents include trifluoroacetoic anhydride (TFAA), pentafluoropropionic anhydride (PFPA), heptafluorobutyric anhydride (HFBA), trifluoroacetylimidazole (TFAI), and pentafluoropropanylimidazole (PFPI).[12, 31]

Silylating reagents produce silyl derivatives, the most widely used derivatives for GC applications. They replace active hydrogens on alcohols, thiols, and amine functional groups through nucleophilic attack. The addition of a silyl group to the analyte has several advantages, including the ability to silylate a wide variety of compounds and create very

volatile and thermally stable products. However, silylating reagents are moisture-sensitive and must be dissolved in aprotic organic solvents before use as derivatizing agents.[12, 31]

1.6.4.2. Chemical modification for LC analysis

Chemical modification in LC enhances an analyte's detection by adding UV chromophores and fluorophores to yield highly fluorescent derivatives. For example, NDA and taurine react with CN ions to form a fluorescent CBI complex, while MBB conjugates with SCN^- to form a fluorescent SCN-bimane adduct.[31] These strategies enhance detectability and selectivity, enabling sensitive and specific detection of CN markers in complex biological mixtures.[12, 31]

1.7. Detection of Cyanide and its Metabolites in Biological Fluids

The selection of a particular analytical method for identifying and characterizing CN and/or its metabolites is influenced by several variables, notably the analytical technique required, sample type, the convenience of use, and experience. This decision is also influenced by other elements, sampling timing (i.e., how soon after exposure).[96, 97] Various protocols for the individual or combined estimation of CN and its metabolites from biological fluids have been established using a variety of analytical techniques, including spectrophotometric methods, nitrogen-phosphorus (NPD), and mass chromatography (MS) detectors, ion chromatography (IC), gas chromatography (GC) coupled with electron capture (ECD) high-performance liquid chromatography-mass spectrometry (HPLC-MS), fluorometry (CE).[18, 85, 98] Methods for CN and its metabolites are summarized in Table 1.2.

1.7.1. Direct Detection of Cyanide

The extreme toxicity of CN necessitates rapid, sensitive, and selective analytical techniques for rapid poisoning diagnosis, environmental monitoring, workplace safety, and

forensic investigation.[96, 99] The high volatility of CN poses significant challenges for direct detection in biological fluids. Despite this, various analytical techniques have been employed with success, including spectrophotometry, GC-MS, HPLC-FL or HPLC-MS, and capillary electrophoresis coupled with fluorescence detection are among the methods utilized.[99-102]

The spectrophotometric approaches typically involve oxidizing CN into cyanogen, followed by a reaction with pyridine-benzidine (i.e., organic reagent) to yield a detectable product.[103-105] However, these methods can be limited by their low sensitivity and the potential for lengthy analysis times. In addition, interferences, especially from SCN^-, can affect the accuracy of CN analysis. Headspace (HS) GC-ECD has been employed to detect CN levels as low as 50 ng/mL and 0.5 ng/mL in blood[106] and various tissues in the human body[107], respectively, without interference from cyanate or SCN^-.[106, 107] GC-NPD coupled with a megabore capillary column has been utilized to determine CN in blood at 1 ng/mL.[108] HS solid-phase microextraction (SPME) coupled with GC-NPD has been used to determine CN and volatile alkyl nitriles in whole blood with an LOD of 3 ng/mL.[109] GC-MS is utilized to assess CN concentrations in cattle serum and rumen fluid, with concentrations ranging from 0.7 to 35 μM in serum and 0.7 to 28 μM in rumen fluid.[110] SPME followed with GC-MS method was used to measure CN in blood, with an LOD of 6 ng/mL.[111] An isotope-dilution GC-MS method was also developed to measure CN in blood, with an LOD of 60 ng/mL.[112]

HPLC is another popular technique for CN analysis, often requiring derivatization due to cyanide's subpar liquid chromatographic characteristics lack of a natural chromophore or fluorophore.[18, 31]

HPLC with fluorescence detection was used to determine CN concentrations from human red blood cells (RBCs), where CN was chemically modified with 2,3-naphthalenedialdehyde (NDA) and taurine to produce a fluorescent product (LOD = 100 pmol/ml).[113] An HPLC-MS/MS technique was developed using hydroxocobalamin to bind CN ions for the quantitative analysis of plasma CNCbl.[114] The LOD for plasma HOCbl and plasma CNCbl were 0.1 and 0.5 μM, respectively.[114]

CE has been used to quantify CN in biological samples, achieving low detection limits by forming complexes with specific reagents that allow for sensitive measurement. Meng et al.[115] described a technique for simultaneously determining and extracting free CN from biological materials (such as samples of human urine and saliva). The researchers used capillary electrophoresis after hollow fiber-protected headspace liquid-phase microextraction (CE). Ni (II)-NH_3 was utilized to derivatize CN. Ammonium pyromellitic and sodium carbonate were the internal standards employed. With the aid of CE, a stable $Ni(CN)_4$ complex was created and yielded an LOD 0.26 ng/mL.[115]

Human breath can also be analyzed by selective ion flow tube spectrometry (SIFT-MS) for HCN concentrations up to 81 ppb.[116] [117, 118]

1.7.2. Thiocyanite Detection

SCN^- is the major metabolite of CN with a physiologically half-life of around 5 days.[31] Since the late 19th century, SCN^- has been used as a biomarker to assess CN exposure.[119] Detection and quantification of SCN^- in biological fluids provides clues of CN exposure and metabolism.[120] SCN^- quantification has been achieved using various analytical methods, including GC-MS, spectrophotometry, HPLC, IC, CE, and novel techniques like paper-based devices and surface enhanced Raman scattering.

Jacob et al.[121] developed a GC-EC-MS method for the determination of SCN^- in plasma using an ion pair with the tributylsulfonium ion and an NPD. The method was used to analyze plasma samples from nonsmokers and smokers, producing an LOD of 9 µg/mL and 0.2 µg/mL, respectively. Youso et al.[4] developed a GC-MS method for SCN^- determination using protein-bound CN moieties from CN-exposed plasma proteins, by derivatizing SCN^- released from base hydrolysis with pentafluorobenzyl bromide (PFB-Br), to produce an LOD of 2.5 ng/mL.[4] Lundquist et al.[122] developed a spectrophotometric method for determining SCN^- in plasma and urine using an adsorbing SCN^- on a weak anion-exchange resin, chlorinated with hypochlorite and quantified using isonicotinic acid and 1,3-dimethyl-barbituric acid. The LOD was 6.2 µg/mL in plasma and 0.25 µg/mL in urine.[122] Ammazzini et al.[119] developed a GC-MS method for determining SCN^- in human saliva. Triethyloxonium tetrafluoroborate was used to convert SCN^- into ethyl thiocyanate to produce an LOD of 5 ng/g.[119]

HPLC has been utilized for determining SCN^- from human saliva and plasma, with a method developed by Chen et al.[97] that uses a fluorimetric detector to detect SCN^- anion and a Nova-Pak C18 reversed-phase column (LOD = 3.3 fmol).[97] Connolly et al.[123] developed an HPLC-UV method to determine SCN^- concentrations in urine samples of nonsmokers and smokers, with an LOD of 0.1 µg/mL.[123] Flieger et al.[124] developed an HPLC using a phosphatidylcholine immobilized artificial membrane column with a diode-array detector (HPLCE-IAM-DAD) for the determination of SCN^- in human saliva of cigarette smokers, e-cigarette smokers, and nonsmokers (LOD = 15.97 µg/L).[124]

Pena-Pereira et al.[125] developed a paper-based analytical device for instrumental-free detection of SCN^- in the saliva of tobacco smokers. This method relied on creating a colored

iron(III)-SCN complex in a paper-based sensing device and following image processing using a scanner as a sensing device (LOD = 60 μM).[125]

Casalla et al.[126] described a method using IC with electrochemical and UV detection for determining SCN^- in human urine samples (i.e., LOD = 29 ng/mL).[126] Glatz et al.[127] developed a method for analyzing SCN^- in human serum and urine samples, with an LOD of 90 ng/mL in serum and 50 ng/mL in urine.[127] Xu et al.[128] used CE for saliva SCN^-, with an LOD of 4 ng/mL.[128] Vitali et al.[129] developed a new CE method for human saliva samples (LOD = 230 ng/mL).[129] Wu et al.[130] used surface enhanced Raman scattering (SERS) to capture SCN^- in body fluids including human serum and saliva (LOD = 1 μM).[130] Zhang et al.[131] used a cystamine-modified gold nanoparticle probe (Au-NP) for the determination of SCN^- in human urine samples, based on electrostatic attraction between SCN^- and cystamine on the surface of an Au-NP (LOD = 0.2 μM).[131]

1.7.3. Simultaneous Detection of Cyanide and Thiocyanate

The simultaneous detection of CN and its major metabolite offers more information than analysis of one marker of CNN poisoning, accounting for exposure and metabolism of both CN and SCN^-.[18, 96, 98, 132] Various techniques have been devised to rapidly identify CN and SCN^- in biological fluids including GC-MS, HPLC-UV, HPLC-MS/MS, and IC.

Bhandari et al.[98] developed a method for determining CN and SCN^- in plasma using chemical ionization (CI) GC-MS. Both CN and SCN^- were derivatized using PFB-Br. The LODs were 1 μM for CN and 50 nM for SCN^-.[98] Paul and Smith[132] developed a GC-MS method for analyzing CN and SCN− in human saliva after treatment with PFB-Br. The LODs were 1 μmol/L for CN and 5 μmol/L for SCN^-.[132]

Luo et al.[96] developed an HPLC-FLD to determine CN and SCN^- in swine plasma using a fluorescent label with LOD of 0.25 μg/L CN and 0.30 μg/L for SCN^-.[96] Bhandari et al.[18] developed an HPLC-MS/MS method to determine CN and SCN^- in swine plasma using naphthalene-2,3-dicarboxaldehyde (NDA) and taurine to derivatize CN, and monobromobimane (MBB) for SCN^- modification. The LODs were 10 nM for CN and 50 nM for SCN^-.[18]

Chinaka et al.[102] described an IC method with UV detection to determine CN and SCN^- in blood samples from smokers, nonsmokers, and fire victims with LODs of 3.8 pmol/mL for CN and 86 pmol/mL for SCN^-.[102]

1.7.4. ATCA detection

Analysis of CN and SCN^- has limitations, therefore, ATCA has gained interest as a promising alternative marker of CN poisoning, mainly in a biological matrices. Several analytical techniques are available for determining CN and $SCN^-$from biological fluids, but few reports detail the analysis of ATCA from these fluids such as GC-MS and HPLC-MS/MS.

Li et al.[133] developed a dispersive microsolid phase extraction (d-μSPE) coupled with GC/MS to analyze ATCA in synthetic human urine and bovine blood. The ATCA was derivatized via N-methyl-N-trimethylsilyl-trifluoroacetoamide (MSTFA). The LODs of 15 and 25 ng/mL were obtained for synthetic urine and bovine blood, respictivelly.[133] Logue et al.[134] developed a solid-phase extraction on a mixed-mode cation exchange column followed by GC/MS to analyze ATCA in synthetic human urine and swine plasma. The ATCA was derivatized via MSTFA and the method LOD was 25 ng/mL.[134]

Rużycka et al.[86] developed an LC-MS/MS method for determining ATCA in post-mortem biological materials (blood and organs) of fire victims (LOD = 0.43 ng/mL).[86] Nishio

et al.[135] developed an HPLC-ESI-MS/MS method for quantifying ATCA in postmortem human blood samples, including those intentionally ingested CN or fire victims, with an LOD of 25 ng/mL).[135] Hisatsune et al.[136] developed a probe electrospray ionization tandem mass spectrometry (PESI/MS/MS) method to analyze ATCA in postmortem blood of fire victims (LOD = 43 ng/mL).[136]

Table 1.2. The key analytical techniques for the detection of CN and its metabolites in biological fluids.

Analyte	Technique	Sample Type	Detection Limit	References
CN	HS-GC-ECD	Blood, tissues	50 ng/mL, 0.5 ng/mL	106
	GC-NPD	Blood	1 ng/mL	108
	HS-SPME-GC-NPD	Blood	3 ng/mL	109
	GC-MS	Serum, rumen fluid	0.7 to 35 μM/ 0.7 to 28 μM	110
	SPME-GC-MS	Blood	6 ng/mL	111
	Isotope dilution GC-MS	Blood	60 ng/mL	112
	HPLC-FLD	Red blood cells	100 pmol/ml	113
SCN^-	GC-EC-MS	Plasma	0.2-9 μg/mL	86
	GC-MS	Plasma	2.5 ng/mL	96
	HPLC-FLD	Saliva and plasma	3.3 fmol	97
CN and SCN^-	CI-GC-MS	Plasma	1 μM CN, 50 nM SCN^-	98
	HPLC-FLD	Plasma	0.25 μg/L CN, 0.30 μg/L SCN^-	96
	HPLC-MS/MS	Plasma	10 nM CN, 50 nM SCN^-	4
	IC-UV	Blood	3.8 pmol/mL CN, 86 pmol/mL SCN^-	14
ATCA	GC-MS	Urine, blood	15-25 ng/mL	133
	LC-MS/MS	Post-mortem blood, organs	0.43 ng/mL	86

2. CHAPTER 2. CONCURRENT DETERMINATION OF CYANIDE AND THIOCYANATE IN HUMAN AND SWINE ANTEMORTEM AND POSTMORTEM BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY

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Abstract

Cyanide (in the form of cyanide anion (CN^-) or hydrogen cyanide (HCN), inclusively represented as CN) can be a rapidly acting and deadly poison, but it is also a common chemical component of a variety of natural and anthropogenic substances. The main mechanism of acute CN toxicity is based on blocking terminal electron transfer by inhibiting cytochrome c oxidase, resulting in cellular hypoxia, cytotoxic anoxia, and potential death. Due to the well-established link between blood CN concentrations and the manifestation of symptoms, the determination of blood concentration of CN, along with the major metabolite, thiocyanate (SCN^-), is critical. Because currently there is no method of analysis available for the simultaneous detection of CN and SCN^- from blood, a sensitive method for the simultaneous analysis of CN and SCN^- from human ante- and post-mortem blood via liquid chromatography-tandem MS analysis was developed. For this method, sample preparation for CN involved active microdiffusion with subsequent chemical modification using naphthalene-2,3-dicarboxaldehyde (NDA) and taurine (i.e., the capture solution). Preparation for SCN^- was accomplished via protein precipitation and monobromobimane (MBB) modification. The method produced good sensitivity for CN with antemortem limits-of-detection (LODs) of 219 nM and 605 nM for CN and SCN^-, respectively, and postmortem LODs of 352 nM and 509 nM. The dynamic ranges of the method were 5-500 µM and 10-500 µM in ante- and post-mortem blood, respectively. In addition, the method produced good accuracy (100 ± 15%) and precision (≤15.2% relative standard deviation). The method was able to detect elevated levels of CN and SCN^- in both antemortem (N = 5) and postmortem (N = 4) blood samples from CN-exposed swine compared to nonexposed swine.

1. Introduction

Cyanide is a highly toxic chemical that can be present as either HCN or CN^-, yet primarily exists as an HCN at neutral pH values.[18, 31] While cyanide (both HCN and CN^- are represented inclusively as CN herein) is commonly known as a poison, humans are likely exposed to low concentrations of CN in their daily lives as a result of diet and passive or active inhalation (e.g., fire, cigarette smoke, and exhaust contain HCN).[10, 31, 41] Additionally, industrial use of CN also increases the risk of exposure. The use of CN in gold and silver mining, electroplating, industrial wastewater, chemical manufacturing, and pesticide production necessitates the worldwide production of nearly 140,000 tons per year.[31, 137, 138]

CN is readily absorbed through the gastrointestinal tract, skin, and the mucous membrane of the respiratory tract. The primary mechanism of CN toxicity involves combining with trivalent iron of cytochrome oxidase, a vital respiratory chain enzyme, to disrupt cellular respiration. Specifically, CN binds to the heme A group of cytochrome c oxidase to block terminal electron transfer and inhibit oxidative metabolism, altering critical cellular ion homeostasis and potentially resulting in rapid onset of histotoxic anoxia, coma with seizures, apnea, cardiac arrest, and death.[74, 101, 139, 140]

Living organisms can metabolize low concentrations of CN into less toxic compounds which are excreted with physiological fluids. For example, CN is metabolized in-vivo to multiple compounds, including 2-amino-2-thiazoline-4-carboxylic acid (ATCA), 2-amino-thiazoline-4-oxoaminoethanoic acid (ATOEA), α-ketoglutarate cyanohydrin (α-KgCN), and thiocyanate (SCN^-).[31, 72, 84] SCN^- is the major metabolic product of CN (i.e., accounting for 80% of CN metabolism) and is formed from the reaction of CN with a sulfur donor (e.g., thiosulfate), catalyzed by rhodanese. SCN^- is approximately 200 times less toxic than

CN and has a longer half-life, making it a longer-lived marker for cyanide exposure than CN itself.[120, 141-147] Although SCN^- has some advantages over CN as a marker of exposure, SCN^- also comes with major drawbacks. One major disadvantage of SCN^- is that it is present in common food sources and is a product of other biological processes besides CN metabolism. This means that elevated levels of SCN^- may not necessarily indicate cyanide exposure and, therefore, may not correlate to cyanide toxicity. Also, differences in diet produce large variabilities in biofluid concentrations of SCN^- for individuals. Therefore, it is challenging to ascertain whether "elevated" SCN^- concentrations result from CN exposure or another source.[84, 148] Overall, while SCN^- can be a useful in the context of other clinical and exposure data, including blood CN concentrations and clinical symptoms of CN exposure, it may not be very effective as an early biomarker of CN exposure. Therefore, the simultaneous action of CN and SCN^- is crucial to help determine the occurrence of CN exposure.[51, 149]

The importance of CN toxicity to humans has encouraged the development of a multitude of detection techniques for CN, SCN^-, and other biomarkers of CN exposure [18, 150]. Common to all methods of CN analysis, CN's volatility and reactivity make it a difficult analyte to reliably detect and quantify. Endogenous concentrations of SCN^- are also notably high and variable in biofluid analyses. Thus, the correlation between SCN^- levels and CN exposure is complicated by this variability. Despite the fact that there are numerous techniques for analyzing CN and SCN^- independently, only a few methods have been established for their simultaneous analysis in biological fluids.[151-154] These methods are summarized in Table 1.[4, 89, 98, 99, 111, 120, 155-157]

Table 2.1. Comparison of the important characteristics and features of methods simultaneous analysis of CN and SCN^- from biological fluids.

Biofluid(s)	Analytical Technique	Sample volume	Time of analysis (h)	LOD (μM)		Study
				CN	SCN^-	
Human saliva	GC-MS	3 mL	0.9	1.0	5.0	Paul and Smith[132]
Human urine	HPLC-UV	100 mL	1.0	0.2	0.2	Imanari et al.[158]
Human RBC/plasma	HPLC-FLD	200-400 μL/100 μL	7.0	0.02	0.02	Toida et al.[159]
Human blood	IC-UV-FLD	100 μL	1.5	0.004	0.09	Chinaka et al.[102]
Swine plasma	GC-MS	100 μL	1.8	1.0	0.05	Bhandari et al.[98]
Swine plasma	HPLC-FLD	500 μL	0.48*	0.01	0.005	Luo et al.[96]
Swine plasma	HPLC-MS/MS	200 μL	0.50	0.01	0.05	Bhandari et al.[18]
Human ante- and postmortem blood	HPLC-MS/MS	100 μL	0.20	0.219 and 0.352	0.605 and 0.509	Current study

* The CBM synthesis required 6.5 h.

Each of the techniques listed in Table 1 have drawbacks, including matrix interference, high LODs, labor costs, complexity, and time-consumption. Imanari, Toida, and co-workers.[158, 159] developed two similar high-performance liquid chromatography (HPLC) methods for the simultaneous analysis of CN and SCN^- in urine, red blood cells (RBCs), and plasma. While the method for urine analysis only required 1 h to complete, a much longer sample preparation time (i.e., > 4 hr) was needed for use of this method for RBC/plasma samples. Simultaneous measurement of CN and SCN^- in blood was reported by Chinaka et al.[102] In this method, CN was derivatized for ion chromatography separation with fluorometric detection, and unreacted SCN^- was detected spectrophotometrically. While the method produced a very low LOD for CN, other anions commonly found in blood interfered with SCN^- analysis. Paul and Smith [132] developed a gas chromatography-mass spectrometry ((GC-MS) approach for simultaneous analysis of CN and SCN^- from human saliva after the treatment of both anions with pentafluorobenzyl bromide (PFB-Br). The method had several drawbacks, including high variability, low derivatization efficiency, limited applicability, and relatively high LODs. Bhandari et al. [98] extended the Paul and Smith method to swine plasma. The approach produced exceptionally high levels of accuracy, precision, and low LODs. Unfortunately, the analysis required a considerable amount of time (i.e., nearly two hours) and produced high levels of carryover. Therefore, a different approach featuring HPLC-MS/MS was developed by the same group for simultaneous analysis of plasma CN and SCN^-.[18] In this method, CN was chemically modified with naphthalene-2,3-dicroboxaldahyde (NDA) and taurine (Fig. 1A), and SCN^- was derivatized with MBB (Fig. 1B). While the method had advantages of rapid analysis time, low LODs, and high selectivity, the main drawback of this method was its applicability for the

analysis of CN and SCN^- in plasma only. Another method was more recently developed by Luo et al.[96] utilizing a fluorescent label for determination of CN and SCN^- in swine plasma.. The method had the advantage of rapid analysis time, but the major drawback of the method, as with the Bhandari et al. method, is that it was developed for plasma when CN is primarily associated with erythrocytes. Another drawback of this method is that a large amount of plasma (500 µL) is required.

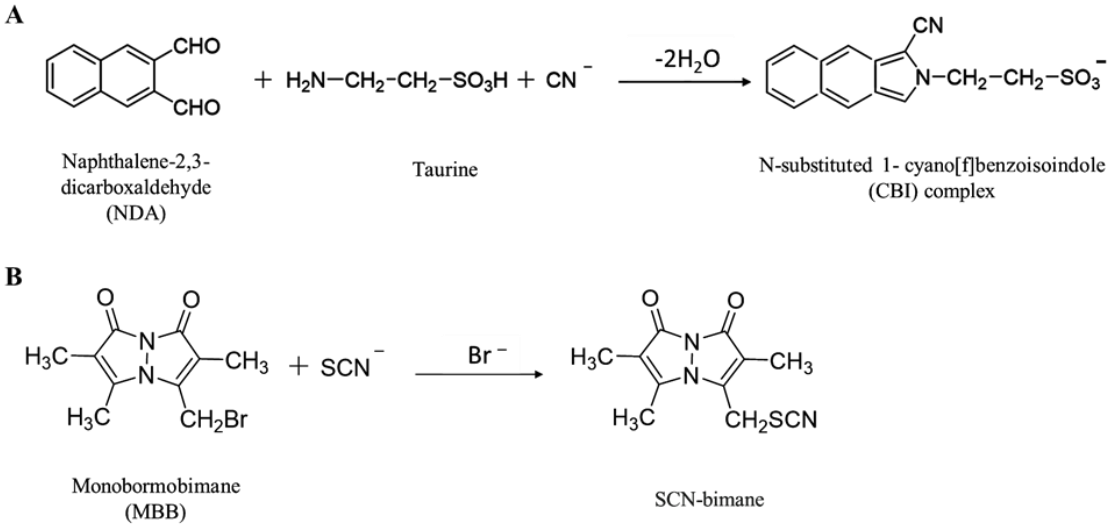

Figure 2.1. Chemical modification of CN and SCN^- for LC-MS/MS analysis. A) NDA and taurine in the presence of cyanide form a highly fluorescent isoindole (i.e., N-substituted 1-cyano [f] benzoisoindole; CBI); B) SCN^- and monobromobimane react to form SCN-bimane.

The relatively rapid onset of toxic effects from CN poisoning, the fact that CN exposure symptoms mimic other medical conditions, and the limitations of currently available methods for simultaneous detection of CN and SCN^- necessitates a rapid, sensitive, accurate, and reliable approach that can concurrently quantify CN and SCN^- in antemortem (i.e., before death) and postmortem human whole blood. The goal of this work was to develop a rapid and robust HPLC-MS/MS method for the simultaneous determination of CN and

SCN^- in ante- and postmortem blood as a complementary method to those already established, with the advantage of analysis of whole blood as a more appropriate method for accurate and simultaneous determination of CN and SCN^-. The analysis of a whole blood retains information on SCN^- while providing more comprehensive information on CN exposure which is not accessible when analyzing serum or plasma. Additionally, the approach negates the necessity to separate serum or plasma from other blood components, making it valuable in clinical settings and diagnostic applications and saving time and effort.

The method of analysis was designed for the purpose of evaluation of acute human cyanide poisoning via simultaneous analysis of CN and SCN^-. Therefore, spiked human blood was used for method development and validation in this study. Due to the ethical constraints of exposure of humans to CN, swine were used to evaluate the effectiveness of the method in determining acute cyanide poisoning. While the blood of human smokers and non-smokers may have been used since significant differences in CN and SCN^- concentrations have been found based on CN exposure through cigarette consumption, these differences are small and highly variable. Additionally, only limited control of individual diets of humans can be achieved, which would contribute to high variability in background SCN^- concentrations.

2. Experimental

2.1. Materials

2.1.1. Reagents and Standards

All solvents used were HPLC grade. All reagents used were analytical standard grade. Potassium cyanide, sodium hydroxide (NaOH), sulfuric acid (H_2SO_4), potassium dihydrogen phosphate (KH_2PO_4), and dibasic potassium phosphate (K_2HPO_4) were purchased

from Fisher Scientific (Hanover Park, IL). NDA was obtained from TCI America (Portland, OR). 2-aminoethane sulfonic acid (taurine) and sodium metaborate tetrahydrate ($NaBO_2 \cdot 4H_2O$) were purchased from Alfa Aesar (Ward Hill, MA). Potassium thiocyanate was purchased from Acros Organics (Morris Plains, NJ, USA). MBB was purchased from Fluka Analytical through Sigma-Aldrich (St. Louis, MO, USA). Ellman's reagent (5,5′-dithiobis 2-nitrobenzoic acid) was obtained from Thermo Scientific (Hanover Park, IL, USA). Isotopically labeled internal standards (IS), $NaS^{13}C^{15}N$ and $Na^{13}C^{15}N$, were acquired from Isotech (Miamisburg, OH, USA). Ammonium formate was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pyrrolidine was purchased from Acros Organics (Morris Plains, NJ, USA). Purified water was obtained from a water PRO PS polisher (Labconco, Kansas City, KS, USA) at a resistivity of 18.2 MΩ-cm.

Caution: CN is poisonous to humans and animals; therefore, CN solids and solutions must be handled with care. CN is released as HCN from solutions with pH values near or below the pKa of HCN (pKa = 9.3 under standard conditions, while it is 9.2 at 37 °C), thus, all aqueous standards containing CN were prepared in 10 mM NaOH to ensure CN remained as non-volatile CN. Additionally, all CN solutions were handled in a well-ventilated hood as an extra precaution.

Single KCN and KSCN stock solutions (10 mM each) were prepared in 10 mM NaOH and diluted into a 1 mM mixture of KCN and KSCN with 10 mM of aqueous NaOH. The obtained mixture was diluted to the desired working concentrations with 10 mM aqueous NaOH for all experiments. Phosphate borate buffer (0.01 M; pH 8.0) and NaOH (0.01 M) were prepared in deionized water and transferred into a plastic container for benchtop storage. H_2SO_4 (2 M) was prepared in deionized water and ethanol and stored at room

temperature. An NDA stock solution (0.002 M) was prepared in 0.01 M phosphate/borate buffer (pH 8.0) and 40% methanol and stored in an amber vial at room temperature for up to six months. (Note: it was important for the NDA to be completely dissolved in the methanol before adding the buffer.) A taurine solution (0.1 M) was prepared in 0.01 M phosphate/borate buffer (pH 8.0) and stored at room temperature for up to 3 months. An Ellman's reagent solution (0.01 M) was prepared in ethanol, pyrrolidine solution (0.01 M) was prepared in deionized water, and an MBB solution (0.04 M) was prepared in 0.01 M phosphate/borate buffer (pH 8.0). These solutions were stored at 4 °C in the dark up to three months, two weeks, and four days, respectively.

2.1.2. Biological Fluids

For development of the method of analysis of human (postmortem) blood, whole blood was obtained from two cadaveric donors: one a Caucasian female and one American Indian female (ages: 86 and 73, respectively). The blood was anticoagulated with ethylenediaminetetraacetic acid (EDTA) and tested negative for Hepatitis B virus (HBV), Hepatitis C virus (HCV), and Human Immunodeficiency virus (HIV). The donors had no known use of tobacco and no known history of alcohol abuse. The blood was collected by Discovery Life Sciences (Huntsville, Alabama, United States) and shipped on dry ice to the Department of Chemistry and Biochemistry at South Dakota State University. Upon receipt, all blood samples were stored at -80 °C until thawed for method development and validation.

Method development of the analysis technique for whole (antemortem) blood utilized human whole blood drawn from a healthy individual caucasian male (age: 69) with EDTA as an anticoagulant by Innovative Research (Novi, Michigan, United States) in an FDA-licensed collection facility. The blood was tested via FDA-approved methods (Viral

Testing) and found negative for Human Immunodeficiency Virus RNA (HIV-1 RNA), antibodies to Human Immunodeficiency Virus (Anti-HIV 1/2), antibodies to Hepatitis C Virus (HCV), Hepatitis C Virus RNA (HCV RNA), Hepatitis B Virus (HBV DNA), Hepatitis B Surface Antigen (HbsAg), and Syphilis. The blood was shipped on dry ice to South Dakota State University. Upon receipt, blood samples were stored at -80 °C until analysis.

To evaluate the effectiveness of the method for determining CN and SCN^- in the ante- and postmortem blood of a cyanide exposed animal, whole blood samples from five citrate anti-coagulated pigs (about 50 kg each) were obtained through the Department of Emergency Medicine at the University of Colorado (Anschutz Medical Campus, Aurora, CO). The pigs were exposed to intravenous cyanide at a rate of 0.17-0.2 mg/kg/min until 6 minutes post apnea. Blood samples were drawn prior to CN exposure, at 1 minute following CN exposure for antemortem blood, and 1 hour following CN exposure for postmortem blood for all pigs. Whole blood samples were collected in clean centrifuge tubes with EDTA anticoagulant, flash frozen, and shipped on dry ice to South Dakota State University. Upon receipt, all blood samples were stored at -80 °C until analyzed. All animals were cared for in compliance with the "Principles of Laboratory Animal Care'' formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Institutes of Health (National Academic Press, 1996) [160]. The CN exposure study was approved by UCD's Institutional Animal Care and Use Committee (IACUC).

2.2. Methods

2.2.1. Sample Preparation

Antemortem and postmortem blood samples were prepared for HPLC-MS/MS analysis via sample preparation scheme outlined by the flow chart in Figure 2. First, the blood sample (100 µL of blank, standard, or sample) was spiked with 10 µL of a $Na^{13}C^{15}N$ and $NaS^{13}C^{15}N$ mixed IS solution (500 µM each). The blood was vortexed and divided into two portions where 25 µL was used for CN analysis and 75 µL was used for SCN^- sample preparation as detailed below.

2.2.2. Preparation of Cyanide for Analysis

CN was prepared for analysis based on active microdiffusion by modifying a previously developed method reported by Bortey-Sam, et al.[85] Briefly, NDA (2 mM), taurine (100 mM), and pyrrolidine (10 mM; 200 µL each) were each directly added to the reagent chamber of a two-chamber sample preparation cartridge which allows active air flow from the sample chamber to the reagent chamber (see Bortey-Sam et al.[85] for details). Ante- or postmortem blood (25 µL) was placed in a sample chamber and diluted with 80 µL of deionized water. H_2SO_4 (2 M, 200 µL) was added to the sample chamber of the sample preparation cartridge to convert CN in the blood to HCN(g). The significant binding of CN to proteins such as hemoglobin can slow the release of HCN, therefore, the purpose of acidifying the blood sample was to ensure the complete conversion of CN to HCN. The sample and reagent chambers were immediately capped. Carrier gas (i.e., room air at 200 mL/min) was pumped through the sample chamber into the capture chamber to transfer HCN(g) to the capture solution. In the capture chamber, the NDA and taurine reacted with CN to form a CN-NDA-taurine complex based on the reaction shown in Figure 1A.

2.2.3. Preparation of Thiocyanate for Analysis

Ante- or postmortem blood (75 µL) was added to a 2-mL microcentrifuge vial. Acetonitrile (1000 µL) was added to the sample to precipitate proteins and the vial was vortexed for 1 min and then centrifuged for 10 min (-5 °C) at 13,200 rpm (16,200×g; Thermo Scientific Legend Micro 21R Centrifuge, Waltham, MA, USA). An aliquot (750 µL) of the supernatant was then transferred to a 4-mL glass screw top vial and dried under N_2 (g) for approximately 10 min at room temperature (RT) (Reacti-vap III, Pierce, Rockford, IL, USA). After drying, the sample was reconstituted with 75 µL of 10 mM aqueous ammonium formate. An aliquot (50 µL) of Ellman's reagent (10 mM) was added for quenching excess free sulfhydryl groups (thiols) moieties[18] in solution and vortex-mixed (1 min). An aliquot (50 µL) of MBB (4 mM) was then added to produce the SCN-bimane complex shown in Figure 1B.

2.2.4. The Preparation of CBI and SCN-bimane Complex

An aliquot (550 µL) of the CN capture chamber solution was transferred into a 4-mL glass screw top vial along with 175 µL of the prepared solution containing the SCN-bimane complex and mixed. The mixture was filtered with a 0.22 µm polytetrafluoroethylene (PTFE) into a150-µL glass insert for subsequent HPLC-MS/MS analysis.

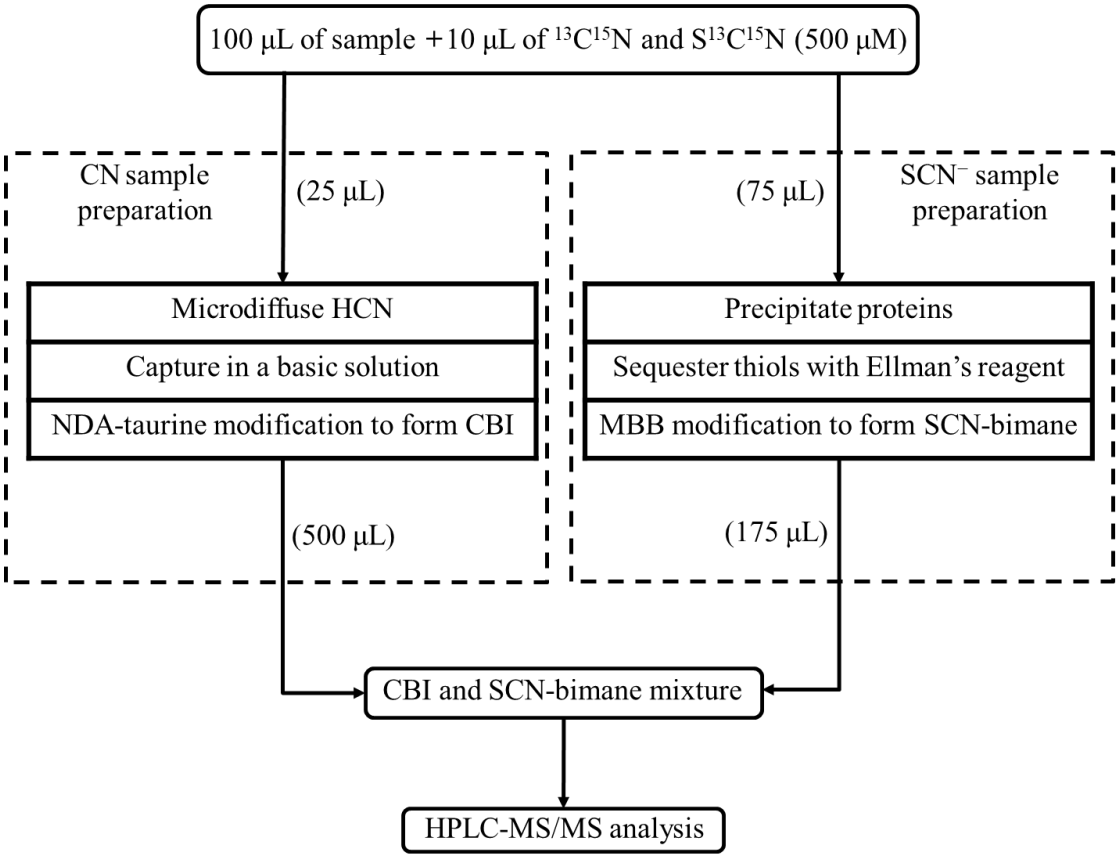

Figure 2.2. Flowchart representation of CN and SCN^- sample preparation of ante- and postmortem human blood for LC-MS/MS analysis. NDA: 2,3-naphthalene dialdehyde, taurine: 2-aminoethane sulfonic acid, MBB: Monobromobimane, and CBI: N-substituted 1-cyano [f] benzoisoindole complex.

2.2.5. HPLC-MS/MS Analysis

Prepared samples were simultaneously analyzed for CBI and SCN-bimane using a Shimadzu HPLC (LC20AD, Shimadzu Corp., Kyotu, Japan) coupled with an AB Sciex Q-trap 5500 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA). Separation was achieved by reversed phase (RP) chromatography using a ZORBAX RRHT Eclipse Plus C_{18} column (100×3.0 mm, 1.8 µm, 95 Å) protected by a ZORBAX RRHD Eclipse Plus C_{18} UHPLC guard column (3.0 mm, 1.8 µm, 95Å) (both acquired from Agilent, California, USA). Each chromatographic analysis was carried out using 10 mM

ammonium formate in water (Mobile phase A) and 10 mM ammonium formate in methanol (Mobile phase B) as mobile phases. An aliquot (10 µL, injection volume) of the prepared sample was separated with gradient elution at a flow rate of 0.3 mL/min and 40 °C as follows: the column was initially equilibrated with 50% B, linearly increased to 100% B over 3 minutes, maintained at 100% B for 1 minute, and then decreased linearly back to 50% B over 1 minute, where the mobile phase composition was held constant for 2 minutes to re-equilibrate the column between samples. A computer workstation running Analyst™ software 1.4.1. (Farmingham, MA, USA) was used for data acquisition and peak integration.

Detection of CBI and SCN-bimane was achieved by an AB Sciex Q-trap 5500 MS/MS with multiple reaction monitoring (MRM) was used for the analysis of the analytes using electrospray ionization (ESI) operated in negative ion mode. MRM parameters for CN and $^{13}C^{15}N$ quantification and identification ions were optimized for maximum sensitivity and set as follows: nitrogen (20 psi) was used as the curtain gas. The ion source was operated at -4500 V, a temperature of 500 °C, and a pressure of 10 psi and 0 psi for nebulizer (GS1) and drying (GS2) gases, respectively. For CN, the m/z ratio of 298.6 corresponds to the molecular ion of the CBI complex. The transitions 298.6 → 190.7 and 298.6 → 80.9 were adopted as the quantification and identification transitions, respectively. The corresponding transitions 300.6→192.7 and 300.6→80.9 were selected for isotopically labeled cyanide serving as the IS signals. For SCN^-, the m/z ratio of 248.0 corresponds to the molecular ion of the SCN-bimane complex. The transitions 248.0 → 111.0 was selected as the quantification transitions and 248.0 → 124.1 was used as the identification transition, using the corresponding transitions for isotopically labeled cyanide as IS signals, 250.0 →111.0

and 250.0 →126.1. The collision cell was operated with a medium collision gas pressure and subjected to an entrance potential (EP) of -10 V, a declustering potential (DB) of -70.0 V, a collision energy (CE) of -25.0 V, a collision cell exit potential (CXP) of -19.0 V, and a 100 ms dwell time (DT). MRM parameters for SCN^- and $S^{13}C^{15}N$ quantification and identification ions were set as follows: curtain gas set at 20 psi, ion source temperature of 500 °C, GS1 at 10 psi and GS2 at 0 psi, ionization voltage at -4500 V. The collision cell was operated with a medium collision gas pressure with an EP of -10 V, a DB of -185.0 V, a CE of -19.0 V, a CXP of -19.0 V, and a 100 ms DT.

2.2.6. Calibration, Quantification, and LOD

The calibration and quality control (QC) standards were prepared from an aqueous CN and SCN^- stock solution mixture (10 mM). All the calibration standards for CN (5, 10, 20, 50, 100, 200, and 500 μM) and SCN^- (10, 20, 50, 100, and 200 μM) were prepared in ante- and postmortem human whole blood. The peak area signal ratios (i.e., the peak area of the analyte transition divided by the peak area of the IS transition) were plotted as a function of calibrator concentration. Both non-weighted and weighted ($1/x$ and $1/x^2$) linear calibration curves were fit using least squares and a $1/x^2$ weighted linear fit was found to best describe the calibration data for CN and SCN^-.[98]

To delimit the range of calibration, the upper limit of quantification (ULOQ) and the lower limit of quantification (LLOQ) were defined by investigation of calibrators which satisfied the following inclusion criteria: (1) a percent relative standard deviation of <10% (as a measure of precision) and (2) a percent deviation within ±20% back-calculated from the nominal concentration of each calibration standard (as a measure of accuracy). All calibrators and other standard analysis were performed in triplicate for all validation

experiments except when otherwise denoted. The goodness-of-fit (GoF) for the calibration curve was calculated via percent residual accuracy (PRA or %RA). According to Manandhar et al.,[161] PRA equally weighs the accuracy of all calibrators into a single numerical value. The coefficient of determination (R^2) was also calculated as a conventional GoF measurement. However, it is important to note that R^2 primarily reflects the GoF for the highest calibrators (2-3) and, therefore, may not provide a reliable indication of the overall GoF throughout the entire calibration range. A PRA of 100% indicates a "perfect" fit while ≥ 90% is indicative of a "good" fit for a calibration model throughout the calibration range.

Four QC standard concentrations were prepared in ante- and postmortem blood for CN (15, 60, and 250 μM as low, medium, and high concentrations QCs, respectively) and SCN^- (15, 60, and 150 μM as low, medium, and high, respectively) and were analyzed in quintuplicate (N =5) each day for 3 days (within 9 calendar days). These QC standards were analyzed in parallel with the calibration standards. Intraassay precision and accuracy of the method were assessed by analyzing replicates of the QC standards from each day's analysis. Interassay precision and accuracy of the method were calculated by comparing the QC standards from three separate days. The intraassay and interassay investigations were performed within seven calendar days.

Commonly, biological samples contain endogenous concentrations of cyanide and its metabolites, including thiocyanate.[18, 31, 98, 162] Thus, it is difficult to establish realistic LODs due to these endogenous background concentrations of CN and SCN^-. For this study, the LODs signals were estimated by analyzing multiple non-spiked ante- and postmortem human whole blood samples and averaging the signal one minute directly prior and one

minute directly following the analyte peak. The signal LOD was then calculated as the sum of the blanks mean and three times of their standard deviation SD (i.e., $LOD_{signal} = \bar{x} + 3 \cdot s_{blank}$). The concentration-based LOD was calculated by converting the calculated LOD signal to its corresponding concentration via the calibration equation based on peak height of calibrators.

2.2.7. Selectivity and Stability

The ability to differentiate and quantify CBI and SCN-bimane in the presence of other blood components (assay selectivity) was determined by comparing blank antemortem and postmortem blood (each in triplicate) with spiked antemortem and postmortem blood (15, 60, and 250 μM, triplicate) by the procedure described earlier. The peaks for SCN-bimane and CBI were observed around 2.25 and 3.94 min, respectively. Endogenous concentrations of CN and SCN^- make definitive evaluation of assay selectivity difficult, similar to LOD. Therefore, selectivity was evaluated by comparing retention times and MRM ions (both quantitation and identification ions) of CBI and SCN-bimane in aqueous solution and ante- and postmortem blood and evaluating if the chemical components in the whole blood interfered with the signals of CBI and SCN-bimane.

Many previous studies have evaluated and reported on the long-term and freeze-thaw stability of CN and SCN^-. Therefore, this was not investigated in the current study. For more in-depth studies of the stabilities of CN and SCN^-, reader may consult previous studies.[18, 31, 160, 163-167] We did evaluate the stability of CN and SCN^- for method-specific storage conditions. The short-term storage stability (i.e., bench-top stability) of CN and SCN^- was evaluated by spiking blood with high and low QC concentrations of CN and SCN^-, testing one set of QCs immediately, and leaving other sets for 0, 2, 4, 8, 12, and 24 hr on the bench-

top before preparation for analysis. It should be noted that IS were added to the QCs directly prior to sample preparation to correct for variations due to sample preparation and instrumental errors but to not correct for instability of the analytes. The autosampler stability of CBI and SCN-bimane was evaluated for prepared CN and SCN^- QC standards (high, medium, and low for CN and high and low for SCN^-) after placing the prepared QC standards in the LC autosampler at 15 °C and analyzing at approximately 0, 2, 4, 8, 12, and 24 hr. CN and SCN^- were considered stable if the calculated concentrations were within ±10% of the initial signal.

2.2.8. Matrix Effect and Recovery

Matrix effects were investigated by comparing calibration curves prepared in aqueous solution, human antemortem, and postmortem human blood. The slopes of the ante- and postmortem curves were compared with the aqueous calibration curve slopes to determine the matrix effects.

Recoveries of CN and SCN^- were determined as a percentage by comparing peak areas obtained from the spiked antemortem and postmortem blood samples at low, medium, and high QC concentrations which was processed through the sample preparation procedure, compared with the equivalent concentration of QCs spiked in an aqueous solution (i.e., QCs were prepared at concentrations equivalent to these that would be produced assuming no loss of analyte during sample preparation). Note: The purpose of the recovery experiment was to evaluate the incomplete recovery of CN and SCN^- during sample preparation, therefore, IS was not used in the recovery calculation. Incorporation of IS in the recovery calculation would correct for the loss of CN and SCN^-.

3. Results and Discussion

3.1. LC-MS/MS Analysis of Cyanide and Thiocyanate

While the HPLC-MS/MS method described by Bhandari et al.[18] was used as starting point for the current method, it was exclusively modified to allow analysis of CN and SCN^- concentrations in ante- and postmortem whole blood samples. A summary of the sample preparation scheme is shown in Figure 2. In the present approach, CN was rapidly prepared via microdiffusion and chemical modification with NDA and taurine to produce a CBI complex (Fig. 1A) following the method described in Section 2.2.2. The analysis of SCN^- was conducted via reacting MBB with SCN^- (Fig. 1B) following the method described in Section 2.2.3. While Ellman's reagent does not react with SCN^-, it reacts with free thiols in amino acids, soluble proteins, peptides, and other molecules.[18] Conversely, MBB reacts with both SCN^- and free thiols. Therefore, the use of Ellman's reagent was critical to react with free thiols prior to addition of MBB, since without it, they heavily interfered with the analysis. Following addition of Ellman's reagent, MBB could selectively target SCN^- to form SCN-bimane for analysis. After the final reaction products were produced, the prepared sample portions were mixed, and HPLC-MS/MS was used to determine the concentrations of CN and SCN^- in antemortem and postmortem blood. Representative HPLC-MS-MS chromatograms of the quantification and identification transitions from the analysis of CN and SCN^- spiked human ante- and postmortem blood (i.e., analyzed as CBI and SCN-bimane) are shown in Figure 3. The chromatographic analysis of CBI, SCN-bimane, and their corresponding IS reaction products produced sharp and predominantly symmetrical peaks. The peak asymmetry factors (A_s) for CBI were <1.03 in postmortem blood and <1.04 in antemortem blood. Peak asymmetries for SCN-bimane were <1.07 in postmortem blood and <1.05 in antemortem blood. Tailing factors (T_f) for CN, SCN^-, $^{13}C^{15}N$ and

$S^{13}C^{15}N^-$ were <2.0 for ante- and postmortem blood. The number of theoretical plates (N) was approximately 3.5×10^5 for CN (as CBI) and 3.3×10^4 for SCN^- (as SCN-bimane), indicating excellent column efficiency. Both CBI and SCN-bimane were not retained in the column and they eluted promptly within a short time frame, with no interfering peaks.

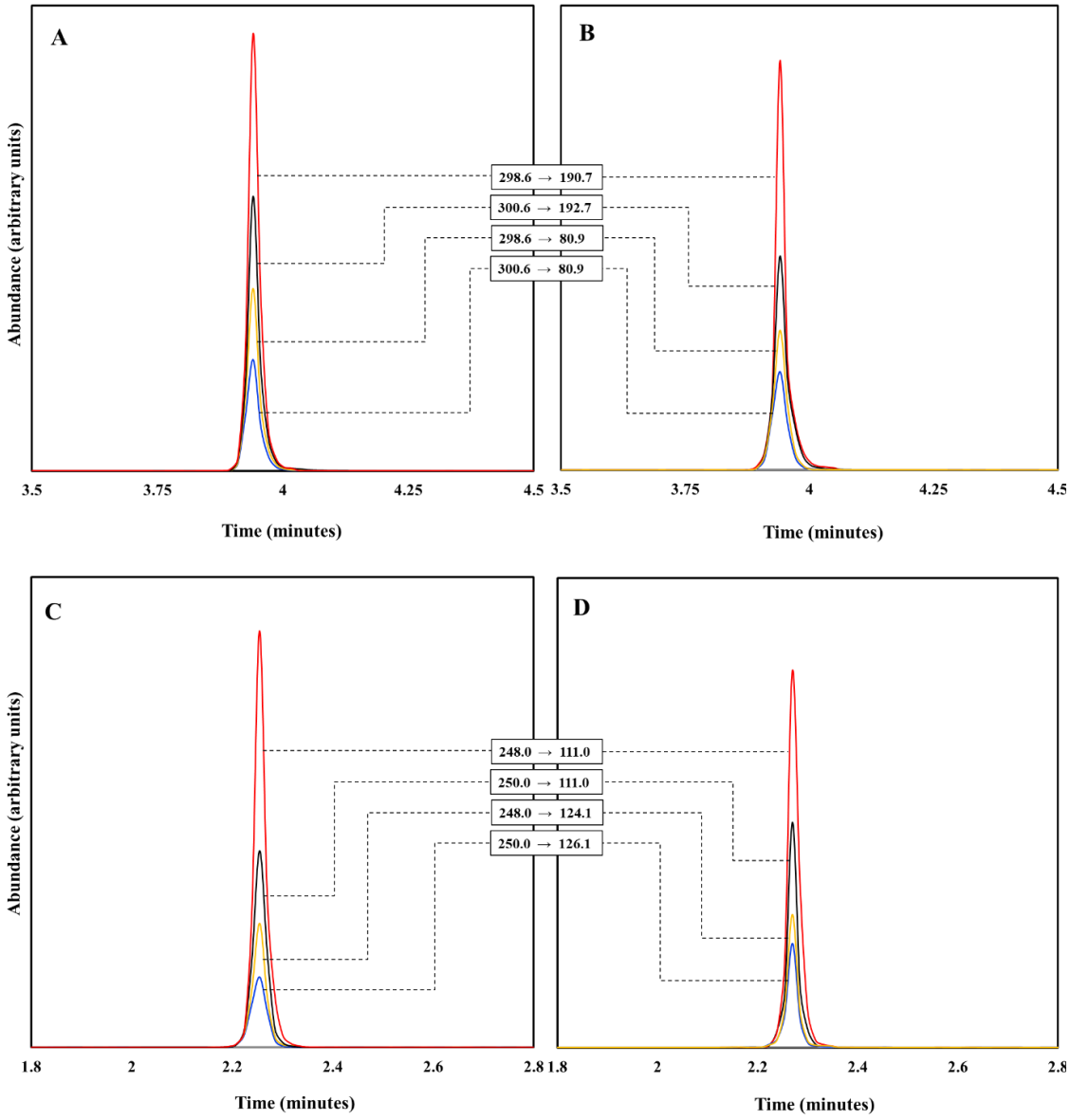

Figure 2.3. HPLC-MS/MS chromatograms of 10 µM CN and SCN^- spiked into ante- and postmortem blood and analyzed via the current method. Chromatograms of CBI and CBI-internal standard from postmortem (A) and antemortem (B) blood are plotted. Chromatograms of SCN-bimane and SCN-bimane internal standard from postmortem (C) and antemortem (D) blood are also shown. Both target analyte and internal standard transitions are

plotted. The MRM transitions for CBI are 298.6→190.7 and 298.6→80.9 and for CBI-internal standard are 300.6→192.7 and 300.6→80.9. The MRM transitions for SCN-bimane are 248.0→111.0 and 248.0→124.1 and for SCN-bimane internal standard are 250.0→111.0 and 250.0→126.1. CN as CBI and SCN^- as SCN-bimane eluted from the column at approximately 3.93 and 2.28 min, respectively, in the ante- and postmortem blood.

The overall sample preparation time for CN was less than 1.5 min for microdiffusion, and for SCN^- the overall preparation time was around 23 min. The chromatographic analysis run time was approximately 6.5 min (including equilibrium for the following sample) for a total analysis time of approximately 40 min for a single sample. For processing multiple samples in parallel, an estimated 220 samples can be prepared and analyzed within a 24-hr timeframe.

3.2. Calibration and quantification

In human antemortem blood, calibration standards of CN at 0.5, 1, and 2 μM were below the LLOQ, leading to a linear dynamic range of 5 to 500 μM. Meanwhile, calibration standards of SCN^- 0.5, 1, 2, 5, and 500 μM were outside of the linear range, resulting to a linear dynamic range of 10 to 200 μM. In human postmortem blood, CN and SCN^- calibration standards at 0.5, 1, 2, 5, and 10 μM were below the LLOQ for CN, while calibration standards at 0.5, 1, 2, and 500 μM were outside the quantification range for SCN^-, resulting in linear dynamic ranges from 10 to 500 and 5 and 200 μM for CN and SCN^-, respectively. Note that endogenous levels of CN and SCN^- affect the LLOQs of the method, such that the LLOQs reported are artificially elevated. The linear ranges for both CN and SCN^- were acceptable considering the typical concentrations of CN and SCN^- found in blood. The calibration curve equations of three separate calibration curves for both CN and SCN^- in ante- and postmortem blood (Table 2) were generally stable. Specifically, the %RSDs of

the $m_{antemortem\ blood}$ and $m_{postmortem\ blood}$ were 4.6% and 5.8%, respectively for CN. For SCN^-, the %RSDs of the $m_{antemortem\ blood}$ and $m_{postmortem\ blood}$ were 10% and 5%, respectively. PRA values for antemortem blood were ≥ 90% and ≥ 91% for CN and SCN^-, respectively. For postmortem blood, PRA values were ≥ 90% and ≥ 94% for CN and SCN^-, respectively. The PRA values for all the calibration curves (≥ 90%) indicated a good fit of all thelinear calibration model for the entire dynamic range.[161]

Table 2.2. Comparison of the stability of curve equations and related values for CN and SCN^- analysis from spiked human ante- and postmortem blood over 3 days.

Sample	Analyte	Day	Equation	R^2	PRA (%)
Antemortem blood	CN	1	y =0.0148x + 0.0573	0.968	91.86
		2	y = 0.0181x + 0.0388	0.951	90.07
		3	y = 0.0166x + 0.0311	0.965	91.80
	SCN^-	1	y = 0.0173x + 0.0413	0.993	96.65
		2	y = 0.0178x + 0.0319	0.988	94.29
		3	y = 0.0186x + 0.0212	0.991	94.83
Postmortem blood	CN	1	y = 0.0132x - 0.0318	0.977	90.04
		2	y = 0.0119x - 0.0066	0.992	91.05
		3	y = 0.0129x - 0.0082	0.956	93.85
	SCN^-	1	y = 0.0166x + 0.1231	0.999	96.92
		2	y = 0.0199x + 0.2148	0.969	95.22
		3	y = 0.0202x + 0.1098	0.926	91.31

3.3. LOD, Accuracy, and Precision

Using a standard noise-based method [168], we estimated the LODs for antemortem human whole blood samples were 219 nM and 605 nM for CN and SCN^-, respectively. The postmortem blood samples had slightly higher LODs of 352 nM and 509 nM for CN and SCN^-, respectively. These values represent the minimum concentration of CN and SCN^- that this method can reliably detect with a high degree of confidence without the interference of endogenous concentrations of CN and SCN^-.

The accuracy and precision of the method were evaluated through the quintuplicate analysis of low, medium, and high concentration QCs on three different days over a period of nine calendar days. The obtained results were evaluated following the Food and Drug Administration (FDA) standards for method validation. The method was accurate and precise across all conditions tested. The accuracy and precision reported in Table 3 are the aggregates of all QCs standard analysis for ante- and postmortem human whole blood, respectively. The obtained intra- and interassay accuracies for CN and SCN^- in antemortem blood were remarkable considering the complexity of the matrix. The intra- and interassay accuracies for CN and SCN^- in postmortem human blood were slightly worse than antemortem blood, but still met the gools of the analysis. In addition, the method demonstrated good intra- and interassay precision, with relative standard deviations of all quality control samples being less than 15% for CN and SCN^- in both ante- and postmortem human whole blood. The accuracy and precision of the method indicates that the method is reproducible and accurate over multiple runs, which is an important characteristic of any reliable laboratory method.

Table 2.3. Method intra- and inter-assay accuracy and precision of cyanide and thiocyanate analysis from spiked ante- and postmortem whole blood.

Sample	Analyte	QC Concentration (μM)	Intraassay						Interassay	
			Accuracy (%) [a]			Precision (%RSD) [a]			Accuracy (%) [b]	Precision (%RSD) [b]
			Day 1	Day 2	Day 3	Day 1	Day 2	Day 3		
Antemortem blood	CN	15	98.6	93.1	87.5	13.6	12.1	9.6	93.1	<13.0
		60	87.8	99.9	102.4	7.9	6.9	6.7	96.7	<9.7
		150	94.3	103.1	100.3	5.2	6.7	9.3	99.2	<8.2
	SCN^-	15	115.3	107.9	105.2	2.6	12.3	11.7	109.5	<10.4
		60	100.9	95.8	111.7	8.9	10.9	8.0	102.8	<11.3
		150	88.9	91.3	108.3	4.8	10.1	7.4	96.1	<11.9
Postmortem blood	CN	15	102.9	105.8	91.0	11.7	11.5	12.7	99.9	<13.6
		60	89.9	109.9	111.3	6.4	9.9	11.1	103.7	<13.5
		150	90.7	112.2	103.1	5.7	9.6	14.8	102.0	<13.9
	SCN^-	15	89.9	103.5	87.0	12.3	13.5	11.9	93.5	<14.9
		60	108.6	107.7	112.8	4.4	2.5	8.2	109.7	<6.0
		150	113.7	113.8	111.7	5.3	6.6	3.6	113.1	<5.4

[a]QC method validation (n = 5).

[b]Average of three different days of QC method validation (n = 15).

3.4. Stability

The inherent volatility and reactivity of CN imparts limited stability under ordinary storage conditions. The tendency of CN to exist as HCN in biological fluids leads to its rapid depletion from biological specimens. In addition, CN may be produced or utilized through single-carbon metabolism, and microbial metabolism has been shown to alter CN levels. Another factor reducing CN stability is its nucleophilicity, allowing CN to participate in biological reactions, including protein binding.

SCN^- is a byproduct of cyanide detoxification in biological systems, which demonstrates more stability under physiological conditions. This stability of SCN^- is supported by its linear structure and strong bonds. While is relatively inert compared to CN and it is not volatile, the stability of SCN^- can still be affected by a variety of factors, including pH, temperature, and the specific chemical or biological environment it inhabits.

For the bench-top stability experiment, CN in ante- and postmortem blood was stable for up to 4 h in spiked non-denatured blood where the measured concentrations of CN were within 10% of the initial concentration for all QCs. Similarly, CBI was also stable for up to 4 h on the autosampler for all the QCs tested. SCN^- was stable on the bench-top in both ante- and postmortem blood for up to 8 h in all the QCs tested. Likewise, SCN-bimane was stable for up to 8 h on the autosampler for all the QCs tested. Although CN and SCN^- were considered unstable by 4 and 8 hrs, respectively, the $^{13}C^{15}N$ and $S^{13}C^{15}N^-$ accurately corrected signal degradation for CN and SCN^- on both the benchtop and autosampler over the 24-h time period tested.

The findings from investigations of the short-term stability of CN and SCN^- indicate that samples should not be stored under ambient conditions for more than 4 hrs prior to

sample preparation. Additionally, the CBI and SCN-bimane are only stable on the autosampler for 4 and 8 hrs, respectively. While CN, SCN^-, CBI, and SCN-bimane showed limited stability, the IS corrected for this instability over the entire 24-hr period tested.

3.5. Matrix Effect and Recovery

The matrix effect was assessed by constructing calibration curves of CN and SCN^- in DI water, antemortem and postmortem blood, and evaluating the slopes of the ante- and postmortem blood calibration curves (non-corrected) compared to aqueous samples (i.e., m_{blood}/m_{aq}). The ratios of slopes for CN were 1.22 and 1.23 in ante- and postmortem blood, respectively. For SCN^-, the matrix effect was 1.40 in antemortem blood and 1.37 in postmortem blood. The increase in slope is likely due to the unique physicochemical properties of the blood matrix compared to DI water, which can affect the behavior of the endogenous CN and SCN^- concentrations, ultimately enhancing the observed response compared to pure DI water. The dissociation of CN from the cyanmethemoglobin complex (i.e., CN forms a cyanmethemoglobin complex upon binding with methemoglobin) may explain the observed signal enhancement for CN [169, 170]. Cyanmethemoglobin is a stable hemoglobin derivative that can dissociate slowly into free CN and methemoglobin [171, 172]. This dissociation may result in a noticeable increase in free CN concentration, thereby increasing the amount of CN captured during microdiffusion and resulting in the enhanced signal observed. For SCN^-, the ionic strength and pH differences between purified water and blood may favor the nucleophilic substitution reaction in the prepared blood matrix. Additionally, the complex composition of blood, compared to deionized water, introduces variations in viscosity, density, and other physical properties, which potentially influence the behavior of CN and SCN^- ions. To evaluate if the use of the IS minimized the effect of the matrix

on the calibration curves, the ratios of $^{13}C^{15}N$ and $S^{13}C^{15}N^-$ corrected calibration curve slopes were evaluated. As expected, the $^{13}C^{15}N$ and $S^{13}C^{15}N^-$ IS corrected for these matrix effects, producing slope ratios of near 1: 0.96 and 1.02 for CN and 0.90 and 0.93 for SCN^- in ante- and postmortem blood, respectively.

The recoveries of CN in antemortem blood for low, medium, and high QCs were 63%, 68%, and 39%, respectively, with those for postmortem blood of 72%, 64%, and 73%. The low recovery may be explained by the loss of CN during microdiffusion and/or the nucleophilicity of CN ion. The SCN^- recoveries in antemortem blood for low, medium, and high QCs were 143%, 133%, and 80%, respectively. The recoveries of SCN^- in postmortem blood were 695%, 203%, and 75%, respectively. The notably high recovery percentages observed for the low and medium QCs concentrations of SCN^- in the ante- and postmortem, can primarily be attributed to endogenous CN and SCN^-. Specifically, for the low and medium QCs, the exogenously introduced CN and SCN^- concentrations are likely not substantially greater than the endogenous level, which results in artificially high recovery values (i.e., the endogenous CN and SCN^- disproportionately affect the low and medium QCs). Conversely, for the high QC, where larger quantities of CN and SCN^- are introduced, the endogenous concentrations are comparatively small, resulting in a recovery percentage that is within the expected range. The IS readily corrected for inconsistent recoveries, producing "corrected recoveries" of 97%-105% and 96%-100% for CN and 98%-102% and 91%-97% for SCN^- in ante- and postmortem blood, respectively.

3.6. Application of the Method

The validated LC-MS/MS method presented here was used to analyze CN and SCN^- from ante- and postmortem whole blood of CN-exposed swine. Representative LC-MS/MS

chromatograms for the analysis of CN and SCN^- from antemortem and postmortem blood of the CN-exposed (upper red trace) and non-exposed (lower black trace) pigs are shown in Figure 4. The CN (i.e., as CBI) eluted at 3.93 min and SCN^- (i.e., as SCN-bimane) eluted at 2.28 min as sharp peaks with slight tailing. As expected, signals above baseline noise were observed from endogenous CN and SCN^- in nonexposed pigs but signals produced from cyanide-exposed pigs were obviously larger for CN and somewhat increased for SCN^-.

To evaluate the ability of the method to verify CN exposure, the blood of multiple pigs was analyzed prior to and following CN exposure. Figure 5 compares the average CN and SCN^- concentrations for nonexposed and cyanide-exposed pigs in ante- and postmortem blood. As seen in Figure 5, the blood CN concentrations of CN and SCN^- are elevated following CN exposure. To evaluate the statistical significance of the observed differences (i.e., nonexposed and cyanide-exposed swine samples), a t-test was employed. The computed p-values in antemortem blood were < 0.0001 for both CN and SCN^-. For postmortem blood, the resultant p-values for CN and SCN^- were < 0.0001 and < 0.004, respectively. While both CN and SCN^- were able to verify CN exposure, the background concentrations of SCN^- were higher than the CN background concentrations. Specifically, the concentration of CN increased in the antemortem and postmortem blood of cyanide-exposed pigs by 9.4x times and 4.5x times the concentration of CN for nonexposed animals, respectively. Conversely, while the absolute concentration of SCN^- is higher than CN, the increase in SCN^- from the nonexposed animals is smaller. Specifically, the signal for SCN^- increased in both the antemortem and postmortem blood by <3x the concentration for nonexposed animals. Moreover, the background concentrations of SCN^- were more variable, with

antemortem and postmortem. Relative standard deviations of 10.3% and 12.4%, respectively, as compared to 4.7%, for both ante- and postmortem blood CN concentrations. Although the diet of the swine was controlled, the variation of SCN^- is likely caused by differences in daily dieting consumption as and variability in metabolism of SCN^- and CN-containing foods for individual pigs. For both swine and humans, SCN^- is both ingested directly and produced from detoxification of CN as an essential defense mechanism that has evolved to mitigate the harmful effects of cyanide. Therefore, differences in dietary habits and metabolism between individuals can lead to variability in SCN^- concentrations. While use of SCN^- alone may not be well-suited for verification of CN exposure, using CN and SCN^- as complementary biomarkers of exposure may mitigate the risk of false positives and/or inconclusive results.

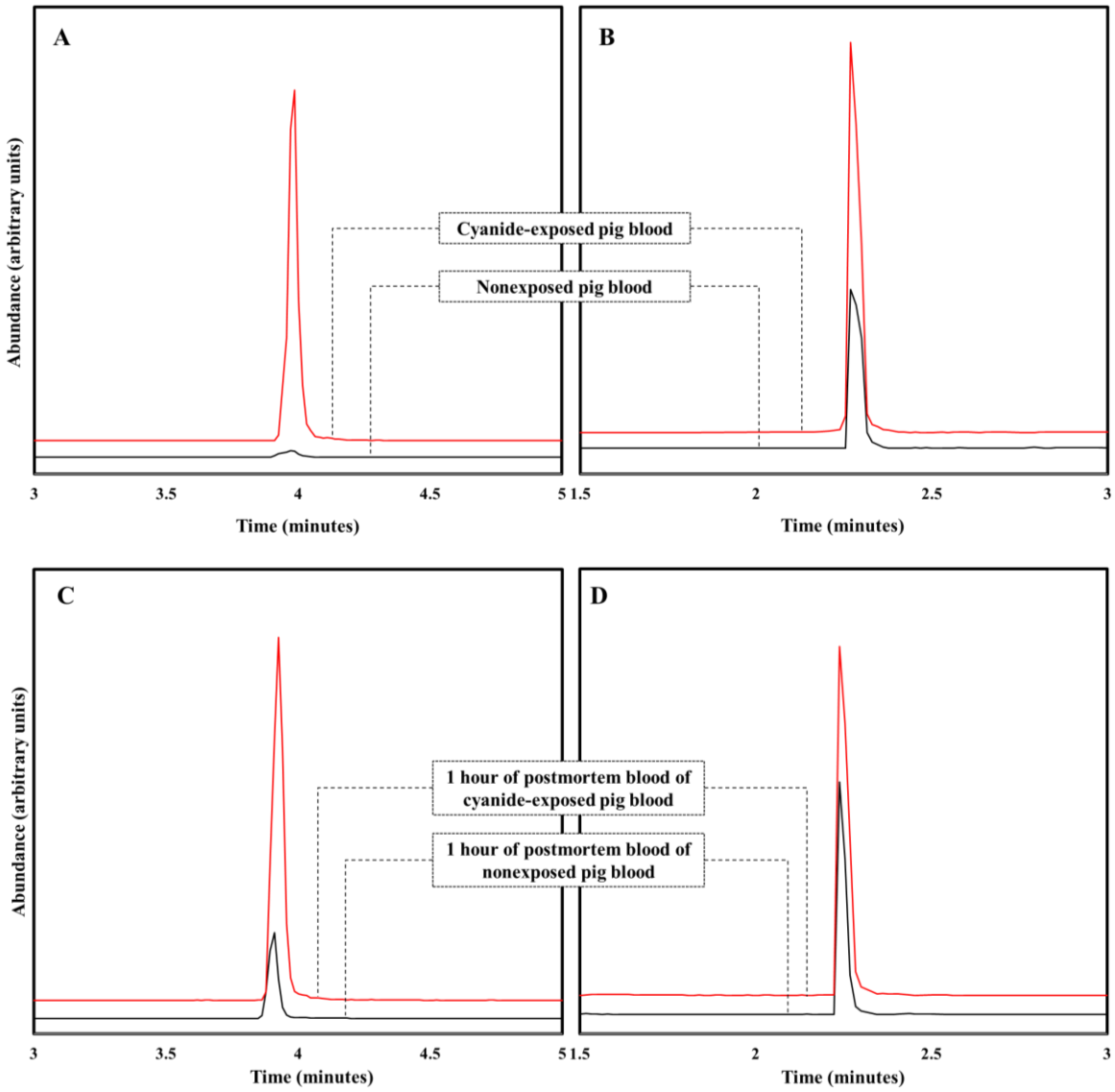

Figure 2.4. Representative chromatograms of CBI (from CN) and SCN-bimane (from SCN^-) in antemortem (A and B) and postmortem (C and D) pig blood. Panel A: shows CN analysis in antemortem blood, panel B: SCN^- analysis in antemortem blood, panel C: CN analysis in postmortem blood, and panel D: shows SCN^- analysis in postmortem blood. The potassium cyanide-exposed pig (1.7-2.0 mg/kg/min; upper trace) and blood obtained pre-exposure (lower trace) are shown. The chromatograms represent the signal response of the MRM transition 298.6→190.7 for CBI and 248.0→111.0 m/z for SCN-bimane.

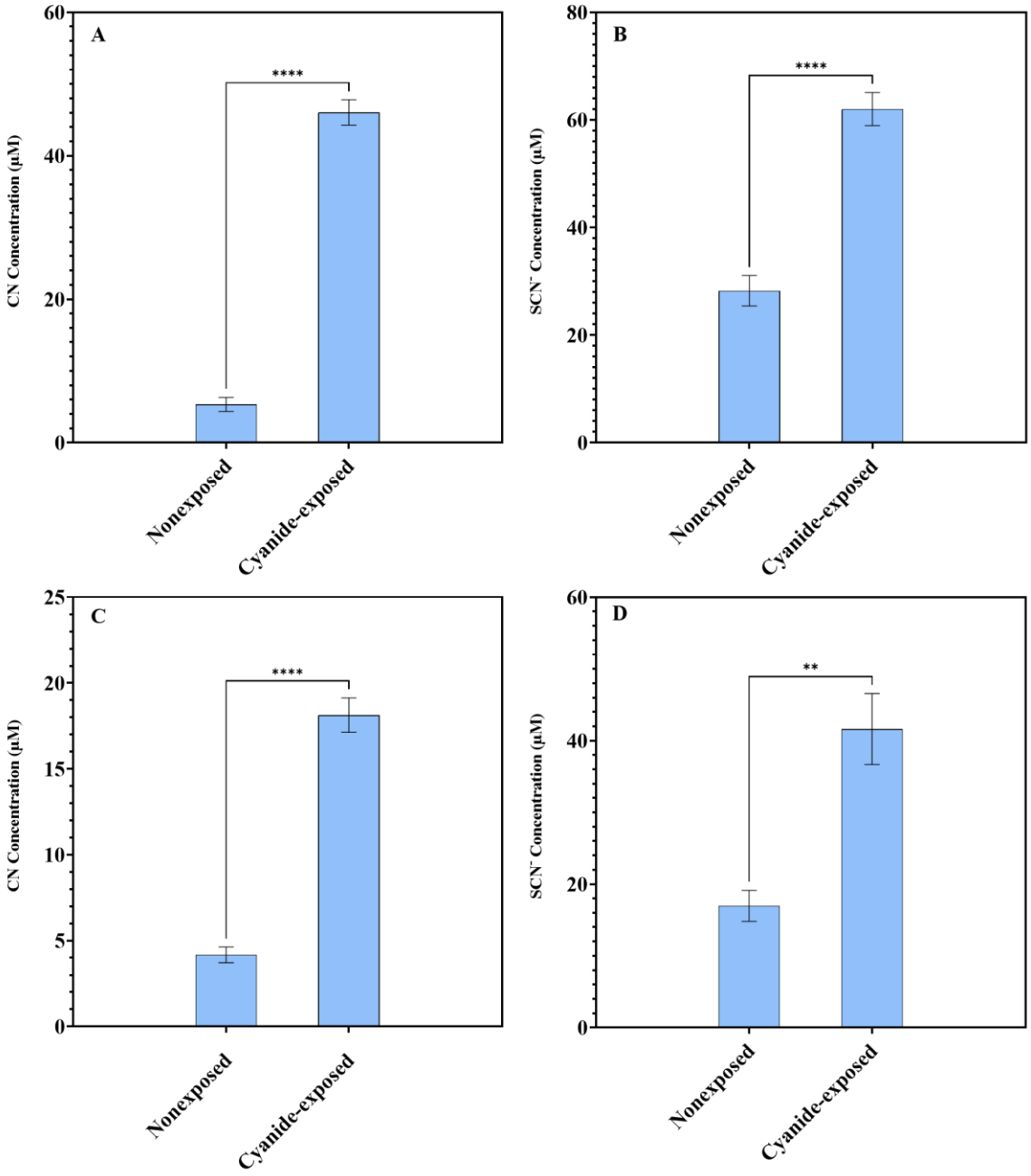

Figure 2.5. Average CN and SCN^- concentrations from pigs prior to and following CN exposure. A and B represent the mean concentrations of CN (A) and SCN^- (B) in the blood of 5 pigs prior to CN exposure (labeled as nonexposed) and antemortem blood (taken at 1 min following CN exposure). C and D represent the mean concentrations of CN (C) and SCN^- (D) in the blood of 4 pigs prior to CN exposure (i.e., labeled as nonexposed) and postmortem blood (i.e., taken at 1 hr following euthanasia of CN-exposed pigs). Note: error bars denote the standard error of the mean. The four asterisks (****) represent a value < 0.0001 and the two asterisks (**) represent a value < 0.01.

4. Conclusions

A simple, sensitive, selective, and cost-effective HPLC-MS/MS method for the rapid analysis of CN and SCN^- was developed and validated in ante- and postmortem whole blood. The method features simple sample preparation, has the ability to simultaneously detect CN and SCN^- at low levels, and proved useful for their detection from the ante- and postmortem whole blood of cyanide-exposed pigs. To the best of our knowledge, this is the first HPLC-MS/MS method for the rapid simultaneous analysis of CN and SCN^- in human ante- and postmortem whole blood. The availability of this method now allows simultaneous quantification of CN and SCN^- blood concentrations in human whole blood, which will inform toxicokinetic studies, forensic investigations, and enhanced outcomes for victims of cyanide poisoning.

5. Acknowledgments

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6. Declarations

The authors declare that they have no conflict of interest. Antemortem human blood collected from donors in FDA-licensed collection centers located in the United States. All antemortem human blood donors were consented prior to participation and were screened and approved by Innovative Research Inc. (46430 Peary Court Novi, MI 48377). Postmortem human blood samples were obtained and processed through Discovery Life Sciences Inc. (800 Hudson Way, Suite 1700 Huntsville, AL 35806). Post-mortem sites are not required to obtain IRB approval as there is no increased risk to the subject. HHS defines a human subject as a "living individual". However, consent from either by the subject prior to passing or next of kin post-mortem was obtained.

All animals were cared for in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Institutes of Health (National Academic Press, 1996). The University of Colorado's Institutional Animal Care and Use Committee (IACUC) approved the CN exposure study (ethics committee: Carter Severance, Genna Nault, Tara Hendry-Hofer, Nathan Wetmore). It complied with the regulations and guidelines of the Animal Welfare Act and the American Association for Accreditation of Laboratory Animal Care.

3. CHAPTER 3. CAN THE CYANIDE METABOLITE, 2-AMINOTHIAZOLINE-4-CARBOXYLIC ACID, BE USED FOR FORENSIC VERIFICATION OF CYANIDE POISONING?

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Abstract

Forensic verification of cyanide (CN) poisoning by direct CN analysis in postmortem blood is challenging due to instability of CN in biological samples. CN metabolites, thiocyanate (SCN^-) and 2-aminothiazoline-4-carboxylic acid (ATCA), have been proposed as more stable biomarkers, yet it is unclear if either is appropriate for this purpose. Therefore, this study evaluated the behavior of CN, SCN^-, and ATCA in postmortem swine (N=8) stored at 4 °C and postmortem blood stored at 25 °C (room temperature, RT) and 37 °C (typical human body temperature, HBT). Following CN poisoning, the concentration of each CN biomarker increased well above the baseline. In postmortem, swine CN concentrations declined rapidly ($t_{1/2}$ = 34.3 h) versus SCN^- ($t_{1/2}$ = 359 h, 15 days) and ATCA ($t_{1/2}$ = 544 h, 23 days). CN instability accelerated as the storage temperature increased: RT ($t_{1/2}$ = 10.7 h) and HBT ($t_{1/2}$ = 6.6 h) reducing CN to below baseline level within 50, 74, and 120 h for postmortem swine at 4 °C, and post-mortem blood at RT and HBT, respectively. SCN^- and ATCA were much more stable than CN at all storage conditions. In postmortem swine, the $t_{1/2}$ of SCN^- and ATCA were 15 and 23 days, respectively. For RT and HBT, SCN^- produced $t_{1/2}$s between 18-19 days and ATCA produced $t_{1/2}$s between 24-27 days. While SCN^- and ATCA each produced $t_{1/2}$s > 18 days, endogenous levels of SCN^- were much larger and more variable than ATCA. Overall, ATCA exhibited the most desirable forensic marker of CN poisoning (e.g., the longest half-life, and largest increase above baseline levels for the CN biomarkers tested, and most stable background concentrations).

1. Introduction

Cyanide (HCN or CN^-, inclusively represented herein as CN) is a potent lethal chemical agent due to its toxic characteristic profile (i.e., CN inhibits cellular respiration and blocks the electron transport chain of cytochrome c oxidase).[18, 31, 61, 88, 98, 173] Because of its toxicity, ease of use, and ready availability (e.g., approximately 750,000 tons of CN is produced annually for extensive industrial applications in the U.S.),[174, 175] CN has been commonly used as a poison. In cases of CN poisoning, the determination of the cause of death has long been recognized as a significant and continuing problem in the field of forensic toxicology.[2, 3, 31, 33, 57, 91, 173, 176-185]

CN poisoning can occur through various routes of exposure, such as inhalation, ingestion, or skin contact.[22, 31, 51, 57, 155, 174, 175] The clinical symptoms of CN poisoning (e.g., chest pain, confusion, dizziness, eye pain, difficulty breathing, headache, abdominal cramping, nausea, rapid or slow heart rate, rapid or slow breathing, restlessness, shortness of breath, vomiting, weakness, and wheezing) are generally nonspecific, and often mimic other common illnesses such as flu or food poisoning.[18, 22, 31, 57, 98, 155, 174, 175] Therefore, CN poisoning can be easily confused with other medical conditions or causes of toxicity or death.[9, 14, 16, 22, 91] More specific indicators of CN poisoning are a "bitter almond" odor from the victim and pink lividity (i.e., a pinkish or rosy skin discoloration during postmortem examination). These indicators may help investigators identify CN-related fatalities but are difficult to identify, may rapidly disappear, and may be difficult to detect for certain individuals.[31, 51, 57, 155, 186] For example, pink livity can arise from alternative conditions such as carbon monoxide poisoning. Additionally, the olfactory smell of bitter almonds is contingent upon highly variable circumstances, including the quantity of CN present and its metabolic

breakdown. It is also estimated that only 60% of people can recognize the “biter almond” smell of CN.[14, 81, 185, 187-190] Because of the nature of CN poisoning, it is often not immediately apparent to investigators that CN may have been utilized. Consequently, the collection and/or analysis of blood samples, which can serve as crucial evidence, is typically delayed until there is a growing suspicion of cyanide involvement.[100, 191] The duration of this delay can vary significantly, ranging from a few days to several weeks before samples are finally secured for analysis.[79, 100, 186] Additionally, even if collection of blood is done immediately, it may not be analyzed for days, and in the interim, may be stored under non-ideal conditions.

While it is possible to directly analyze CN from biological samples in an attempt to verify CN poisoning, it is difficult to use CN as a biomarker in all but the earliest times following exposure.[31, 51, 52] This is because CN is highly unstable in biological matrices due to its chemical characteristics, including reactivity, volatility, and active metabolism of CN by normal biological processes. CN readily participates in certain reactions due to its nucleophilic nature. CN can bind tightly to iron and other metals and reacts readily with disulfides and thiol moieties in biological molecules.[31, 51, 177] The volatility of CN is another factor contributing to its loss from biological samples. CN exists predominantly as HCN under biological conditions (pK_a = 9.3 under standard conditions, and 9.2 at 37 °C). If blood samples are not properly handled and stored, HCN can quickly off gas from the sample.[31, 51, 88, 177, 192] Aside from its chemical reactivity and volatility, CN is metabolized efficiently by the human body (i.e., the half-life ($t_{1/2}$) of CN in humans ≈ 20-60 min). This rapid metabolism and chemical reactivity often return CN to background levels quickly.[31, 51, 99] If there is either an extended postmortem period before collection of samples or

inadequate/prolonged storage before analysis, CN concentrations will likely decrease considerably, potentially back to baseline.[31, 51, 140] Another problem inherent in CN determination is the presence of small (< 10 μM), but ubiquitous, endogenous concentrations in biological samples. These CN concentrations are present based on diet and natural metabolic processes.[18, 31] Therefore, to link CN poisoning to toxicity or death, blood concentrations of CN must be significantly elevated above background levels. Overall, the chemical and biological characteristics of CN make its direct use as an indicator of CN poisoning challenging unless blood samples are gathered, properly stored, and analyzed soon after exposure, which is not possible in most cases.[31, 100]

An alternative to analyzing CN directly is to analyze its metabolites, thiocyanate (SCN^-) and 2-aminothiazoline-4-carboxylic acid (ATCA), as biomarkers of CN poisoning (Figure 1).[31, 52, 79] SCN^-, the major metabolic product of CN, accounts for approximately 80% of CN metabolism.[85, 96, 132] It is formed through a rhodanese-catalyzed reaction of CN with a sulfur donor, such as thiosulfate. Compared to CN, SCN^- is approximately 200 times less toxic and exhibits a longer half-life ($t_{1/2} \approx$ 96-192 hr), making it a more persistent biomarker for CN exposure.[31, 88, 98] However, while SCN^- offers the advantages of abundance and stability, there are significant limitations for its use as a biomarker for CN exposure mainly due to its large and highly variable background concentrations.[18, 31, 51, 88] Notably, SCN^- is naturally present in common food sources and can arise from biological processes unrelated to CN metabolism.[31, 52, 61] Consequently, elevated levels of SCN^- may not result from CN poisoning and do not necessarily correlate with CN toxicity, especially under uncontrolled conditions.[18, 31, 79, 81, 98, 120] Therefore, distinguishing between elevated SCN^- concentrations due to CN exposure versus other natural sources is challenging. While

SCN^- can be valuable in conjunction with blood CN concentrations and clinical symptoms, it has not been proven effective as a useful biomarker of CN exposure.

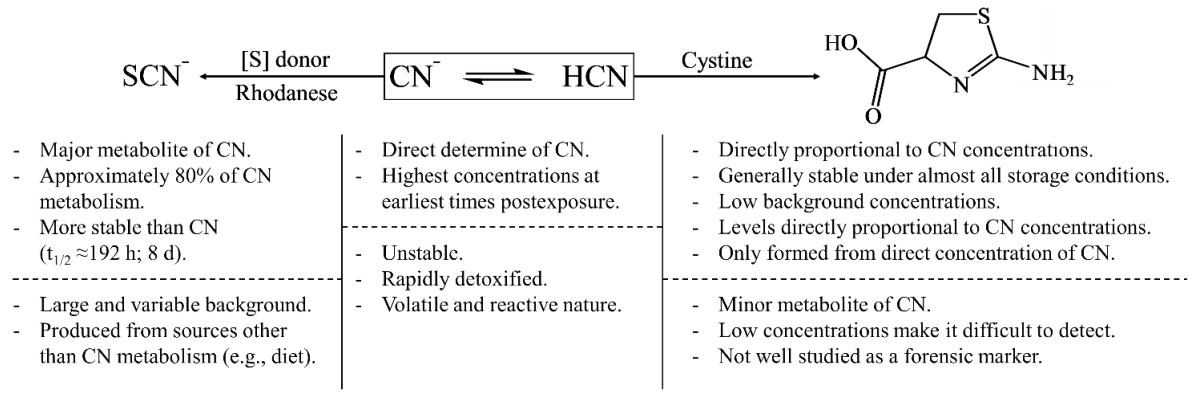

Figure 3.1. Characteristics of the biomarkers of CN exposure for forensic analysis. Dashed lines separate characteristics which are advantages (above the dashed line) and disadvantages for use of the biomarker for forensic purposes.

The in-vivo conversion of cyanide to ATCA (Figure 1), via direct reaction of CN with cystine, was initially reported by Wood and Cooley.[193] ATCA is a marker of CN exposure which is generally stable under almost all storage conditions and has low background concentrations in biological fluids.[31, 81] ATCA accounts for only a small amount of CN metabolism, but the concentration of ATCA is directly proportional to CN concentrations.[31, 79, 81] Moreover, besides the cyanide-mediated pathway, no other endogenous pathways for ATCA production within the human body have been identified.[51, 79, 81, 86] Extended studies of ATCA stability in biological samples have shown remarkable preservation of ATCA concentrations for prolonged periods at all temperatures tested.[31, 51, 86] Consequently, ATCA has garnered significant attention as a promising biomarker for forensic determination of CN poisoning.

While evidence is mounting that ATCA may be a highly stable and reliable marker of CN exposure, the stability of ATCA in biological samples has only been evaluated under

controlled storage conditions, which may not be similar to postmortem decay. Therefore, the objective of this study was to determine if ATCA is valuable as a marker to verify CN poisoning under typical situations where blood samples are not immediately obtained and/or analysis is delayed. Additionally, the behavior of CN and SCN^- was also evaluated. By examining these biomarkers in parallel, our study aimed to directly assess their interrelationships, offering valuable insights into their dynamics and their advantages and disadvantages as indicators of CN poisoning under the identical conditions.

2. Experimental

2.1. Materials

2.1.1. Reagents

All reagents used in this study were of analytical standard grade. The solvents, HPLC-grade methanol (MeOH), phosphoric acid (H_3PO_4, 85%), ammonium hydroxide (NH_4OH, 29% by weight), LC-MS grade and HPLC-grade acetonitrile (ACN), formic acid (CH_2O_2, 98%), and ammonium acetate ($C_2H_3O_2NH_4$) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Potassium cyanide, sodium hydroxide (NaOH), sulfuric acid (H_2SO_4), potassium dihydrogen phosphate (KH_2PO_4), and dibasic potassium phosphate (K_2HPO_4) were purchased from Fisher Scientific (Hanover Park, IL). Potassium thiocyanate was purchased from Acros Organics (Morris Plains, NJ, USA). 2,3-naphthalene dialdehyde (NDA) was obtained from TCI America (Portland, OR). 2-aminoethane sulfonic acid (taurine) and sodium metaborate tetrahydrate ($NaBO_2 \cdot 4H_2O$) were purchased from Alfa Aesar (Ward Hill, MA). Monobromobimane (MBB) was purchased from Fluka Analytical through Sigma-Aldrich (St. Louis, MO, USA). Ellman's reagent (5,5′-dithiobis-2-nitrobenzoic acid) was obtained from Thermo Scientific (Hanover Park, IL, USA). Isotopically labeled internal standards, $NaS^{13}C^{15}N$ and $Na^{13}C^{15}N$, were acquired from Isotech (Miamisburg,

OH, USA). The ATCA internal standard ($C_3{}^{13}CH_6{}^{15}N_2O_2S$; ATCA-$^{13}C,^{15}N_2$) was obtained from Toronto Research Chemical, Inc. (North York, Canada). The mixed-mode cation-exchange sorbent (Oasis-MCX®) was purchased from Waters Corporation (Milford, MA, United States). Ammonium formate (NH_4HCO_2) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Pyrrolidine was purchased from Acros Organics (Morris Plains, NJ, USA). Purified water with a resistivity of 18.2 MΩ-cm was obtained using a water PRO PS polisher from Labconco (Kansas City, KS, USA).

2.1.2. Standard Solutions

Phosphate borate buffer (0.01 M; pH 8.0) and NaOH (0.01 M) were prepared in deionized water and placed into a plastic bottle and stored on the benchtop. A solution of H_2SO_4 (2 M) was prepared using deionized water and ethanol and was kept at room temperature for storage. A stock solution of NDA (0.002 M) was prepared in a phosphate/borate buffer (0.01 M; pH 8.0) along with 40% methanol. NDA was dissolved completely in methanol before adding the buffer to ensure proper dissolution, transferred to an amber vial, and stored at room temperature, where it was stored for up to six months. A taurine solution (0.1 M) was prepared in phosphate/borate buffer (0.01 M; pH 8.0) and stored for up to 3 months at room temperature. An Ellman's reagent solution (0.01 M) was prepared in ethanol, pyrrolidine solution (0.01 M) was prepared in deionized water, and an MBB solution (0.04 M) was prepared phosphate/borate buffer (in 0.01 M; pH 8.0). These solutions were stored at 4 °C in the dark up to three months, two weeks, and four days, respectively. NH_4OH (5%) was prepared from NH_4OH (29%) and diluted with MeOH. ATCA-$^{13}C,^{15}N_2$ was prepared and diluted in methanol to produce a 50 µM standard. Ammonium acetate (0.005 and 0.03 M) was prepared in deionized water. These solutions were stored at 4 °C.

KCN and KSCN stock solutions (10 mM each) were prepared in 10 mM NaOH and diluted into a 1 mM mixture of KCN and KSCN with 10 mM of aqueous NaOH. The obtained mixture was diluted to the desired working concentrations with 10 mM aqueous NaOH for all standards. The calibration standards for CN and SCN^- were prepared at concentrations of 10-500 µM and 5-200 µM, respectively. The calibration standards for ATCA were prepared and at concentrations of 30-300 µM. Prior to use, the working standard solutions for CN, SCN^-, and ATCA were prepared by diluting the suitable stock solutions with swine postmortem blood to achieve the desired concentrations.

Caution: it is crucial to be aware that CN poses a significant risk to both humans and animals due to its highly toxic nature. Therefore, handling CN solids and solutions requires utmost care and caution. When solutions fall below a pH of about 10 (i.e., pKa of CN is 9.2), CN is released as HCN(g). To prevent this potentially hazardous situation, all aqueous standards containing CN were prepared in a 10 mM NaOH solution, ensuring the non-volatile state of CN. Furthermore, all CN solutions were exclusively handled within a well-ventilated hood.

2.2. Methods

2.2.1. CN Exposures

Note: The animals used for this study were control animals used in a separate research study for the evaluation of a potential next-generation cyanide antidote. Our study utilized blood samples from these animals to add value to the drug-development study under ethical principles of maximizing the utility of existing samples and minimizing the need for additional animal use.

Blood samples from eight pigs (Species: *Sus scrofa*; Breed: Yorkshire cross; Weight: 45-55 kg) were obtained at the Department of Emergency Medicine at the University of Colorado (Anschutz Medical Campus, Aurora, CO). Before CN exposure, blood samples were drawn to establish a baseline for a "zero" time point. Pigs were then exposed to intravenous CN at 0.2 mg/kg/min and after some times were euthanized with sodium pentobarbital. Two pigs (Pigs 2 and 8) met euthanasia criteria at 40- and 38-min following CN exposure, respectively. All other animals were euthanized after a 90-min observation period. Heparin (10,000 units) was administered in an attempt to limit coagulation of the animal's blood postmortem. Following euthanasia, animals were then transferred to a walk-in cold room where they were stored at 4°C to obtain blood samples. Blood samples were collected from a central venous catheter placed in the external jugular as follows: the catheter was flushed with 5 mL normal saline to clear the catheter, 10 mL of blood was collected and discarded to minimize the potential for contamination of the sample, then a 3 mL blood sample was collected for analysis. Following blood collection, the jugular catheter was flushed with 10 mL of normal saline followed by 3 mL of heparin to prevent clotting within the catheter. Blood samples were collected into heparinized blood collection tubes at 1, 2, 4, 24, 48, 72, 120, and 168 h after death where possible. Despite heparinization, clotting blood blocked most catheters before the end of the experiment. Table 1 shows the blood samples collected for each pig and the N-value at each time point. Following collection, blood samples were flash-frozen in liquid nitrogen, stored in a -80 °C freezer, and then shipped on dry ice to South Dakota State University. Upon receipt, all blood samples were stored at -80 °C until analyzed.

All animals were cared for in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Institutes of Health (National Academic Press, 1996). The University of Colorado's Institutional Animal Care and Use Committee (IACUC) approved the CN exposure study which complied with the regulations and guidelines of the Animal Welfare Act and the American Association for Accreditation of Laboratory Animal Care.

Table 3.1. Sampling time points following euthanasia where postmortem blood was collected for the analysis of CN, SCN^-, and ATCA in a group of eight pigs. "Background" indicates a blood drawn from the pig prior to CN exposure for the purpose of analyzing the endogenous levels of CN, SCN^-, and ATCA. A check mark (✓) indicates the successful collection of a blood sample at that specific time point for the corresponding pig.

Pigs	"Background"	Blood draw time following euthanasia (h)							
(45-55 kg)	Blood Draw	1	2	4	24	48	72	120	168
1	✓	✓	✓	✓	✓				
2	✓	✓	✓	✓					
3	✓	✓	✓	✓	✓	✓	✓	✓	✓
4	✓	✓	✓	✓					
5	✓	✓	✓	✓	✓	✓	✓		
6	✓	✓	✓	✓					
7	✓	✓	✓	✓	✓	✓	✓		
8	✓	✓	✓	✓					
N-Value	8	8	8	8	4	3	3	1	1

2.2.2. Preparation of CN and SCN^- for Analysis

Postmortem blood samples were prepared for HPLC-MS/MS analysis of CN and SCN^- following the established method developed by Alluhayb et al.[194] where detailed

instructions and additional information can be found. In brief, the blood samples were thawed at room temperature, vortexed for 1 minute, and centrifuged at 3,600 g (Sorvall™ Legend™ X1 Centrifuge with TX-400 Rotor Adapter, Thermo Scientific™) prior to analysis. Postmortem blood (100 µL of control or sample) was obtained and subsequently spiked with a $Na^{13}C^{15}N$ and $NaS^{13}C^{15}N$ mixed internal standard solution (500 µM each, 10 µL). After thorough vortexing, the blood sample was divided into two portions for CN (25 µL) and SCN^- (75 µL) sample preparation.

CN was prepared for analysis using active microdiffusion. Reagent solutions (200 µL each) containing NDA (2 mM), taurine (100 mM), and pyrrolidine (10 mM) were added to the reagent chamber of a two-chamber sample preparation cartridge. Postmortem blood (25 µL) was added to the sample chamber and diluted with 50 µL of water. Aqueous H_2SO_4 (200 µL) was added to the sample chamber. The sample and reagent chambers were sealed, and air was passed through the sample chamber into the capture chamber. This facilitated the transfer of HCN(g) to the capture solution. Within the capture chamber, the CN reacted with NDA and taurine to form a CN-NDA-taurine complex. For SCN^-, postmortem blood (75 µL) was treated with ACN (1000 µL) to precipitate proteins. After vortexing for 1 min and centrifugation for 10 min (-5 °C) at 16,700 g (Sorvall™ Legend™ Micro 21R Microcentrifuge with 24 x 1.5/2.0mL Rotor with ClickSeal™ Biocontainment Lid, Thermo Scientific™), the supernatant (750 µL) was transferred and dried. The dried sample was reconstituted with 10 mM aqueous ammonium formate (75 µL). Ellman's reagent (10 mM, 50 µL) was added to react with free thiols, followed by vortex-mixing. Subsequently, MBB (4 mM, 50 µL) was added to form the SCN-bimane complex.

The prepared CN and SCN^- solutions, 550 µL of the CN capture chamber solution and 175 µL of the prepared SCN^- solution, were combined for subsequent HPLC-MS/MS analysis in a 4-mL glass screw top vial. The mixture was then filtered through a 0.22 µm polytetrafluoroethylene (PTFE) filter into a 150-µL glass insert for follow-on HPLC-MS/MS analysis.

2.2.3. Preparation of ATCA for Analysis

Postmortem blood samples were prepared for HPLC-MS/MS analysis of ATCA following the established method of Giebułtowicz et al.[86] with slight modifications. Briefly, the blood samples were thawed, vortexed, and centrifuged at 3,600 g prior to analysis. Postmortem blood (100 µL of control or sample) was placed in an Eppendorf test tube and mixed with DI water (100 µL) for 1 min by hand, followed by 3 min of vortexing. Cold ACN (750 µL) was added, vortexed for 2 minutes, and placed in a freezer for 20 min at -20 °C. Then, the sample was centrifuged for 5 min at 16,700 g. Subsequently, the supernatant (800 µL), H_3PO_4 (85%, 20 µL), ATCA-^{13}C,$^{15}N_2$ (100 µM, 50 µL), and ACN (130 µL) were combined in a separate Eppendorf test tube containing solid MCX sorbent (10 mg). The mixture was then placed on a laboratory shaker for 5 minutes. The sorbent was washed separately with ACN (1 mL) and ammonium acetate (500 µL of 0.03 M), with vortexing for 1 min and centrifugation for 5 min at 16,700 g after each wash. Subsequently, NH_4OH (5%, 500 µL) was added and vortexed for 30 min to elute the ATCA from the MCX sorbent. The supernatant (450 µL) was transferred to a glass test tube and evaporated to dryness using a nitrogen stream at 40 °C. The sample was then reconstituted with ACN (350 µL) and ammonium acetate (0.005M, 15 µL) and transferred to an HPLC-MS/MS vial for analysis.

2.2.4. HPLC-MS/MS Conditions for Analysis of CN, SCN^-, and ATCA

Prepared samples were analyzed using a Shimadzu HPLC (LC20AD, Shimadzu Corp., Kyotu, Japan) coupled with an AB Sciex Q-trap 5500 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) to detect CBI and SCN-bimane using negative ionization, and ATCA using positive ionization. Data acquisition and peak integration for analytes was carried out using Analyst™ software 1.4.1. (Framingham, MA, USA).

For CBI and SCN-bimane, HPLC was conducted with a ZORBAX RRHT Eclipse Plus C_{18} column (100×3.0 mm, 1.8 µm, 95 Å) protected by a ZORBAX RRHD Eclipse Plus C_{18} UHPLC guard column (3.0 mm, 1.8 µm, 95 Å) (both from Agilent Technologies, California, USA). For the separation of CBI and SCN-bimane, a gradient of 10 mM ammonium formate in water (Mobile phase A) and 10 mM ammonium formate in methanol (Mobile phase B) was used. The details of the method, including MS parameters, are given in Alluhayb et al.[194]

For ATCA, an Atlantis Premier BEH Z-HILIC Column (50 mm x 2.1 mm, 5 µm) (Waters Corporation, Milford, MA). The mobile phases consisted of HPLC-grade water with 0.1% formic acid as eluent A and acetonitrile with 0.1% formic acid as eluent B at a flow rate of 0.5 mL/min and 40 °C. Detailed MS parameters are given in Giebułtowicz et al.[86]

2.2.5. Stability Kinetics

Kinetic parameters were assessed using the methodologies established and detailed by the World Health Organization and Shargel et al.[195, 196] The concentration-time curves were utilized to calculate the elimination half-life ($t_{1/2}$) and the elimination constants (K_e)

through interpolation. To determine the ratio $C_{max}/C_{baseline}$, the maximum blood concentration was divided by the endogenous (baseline) concentration.

2.2.6. Stability of CN, SCN^-, and ATCA in Sampled Postmortem Blood

To evaluate the stability of CN, SCN^-, and ATCA in postmortem blood under potentially important conditions for forensic analysis, we stored aliquots of the 2-h postmortem blood sample at room temperature (RT) and typical human temperature (HBT) and measured CN, SCN^-, and ATCA immediately following, and 1, 2, 4, 8, 12, 24, 48, 72, 120, 168, and 240 h (10 days) of storage. The stability of the biomarkers of CN was calculated as a percentage of the initial concentration of the respective analyte.

3. Results and discussion

3.1. Stability of CN, SCN^-, and ATCA in Postmortem CN-exposed Pigs

Pigs were exposed to a potentially lethal concentration of CN and euthanized when they met approved criteria or after 90 min. Immediately following euthanasia, they were stored in a walk-in cold room at 4 °C and blood was drawn at multiple times (1-168 h) following euthanasia. The blood was analyzed for CN, SCN^-, and ATCA to evaluate the stability of these markers of CN poisoning over 7 days. Figure 2 (solid lines), S1A, and S2A show the stability of CN, SCN^-, and ATCA in blood collected from postmortem CN-exposed pigs.

The initial concentrations of CN, SCN^-, and ATCA (i.e., prior to CN exposure) from antemortem blood (i.e., prior to death) were 2.5, 8.1, and 0.74 μM, respectively. Measurable concentrations of each of these analytes were expected since it is well known that biological samples contain endogenous concentrations of CN and its metabolites.

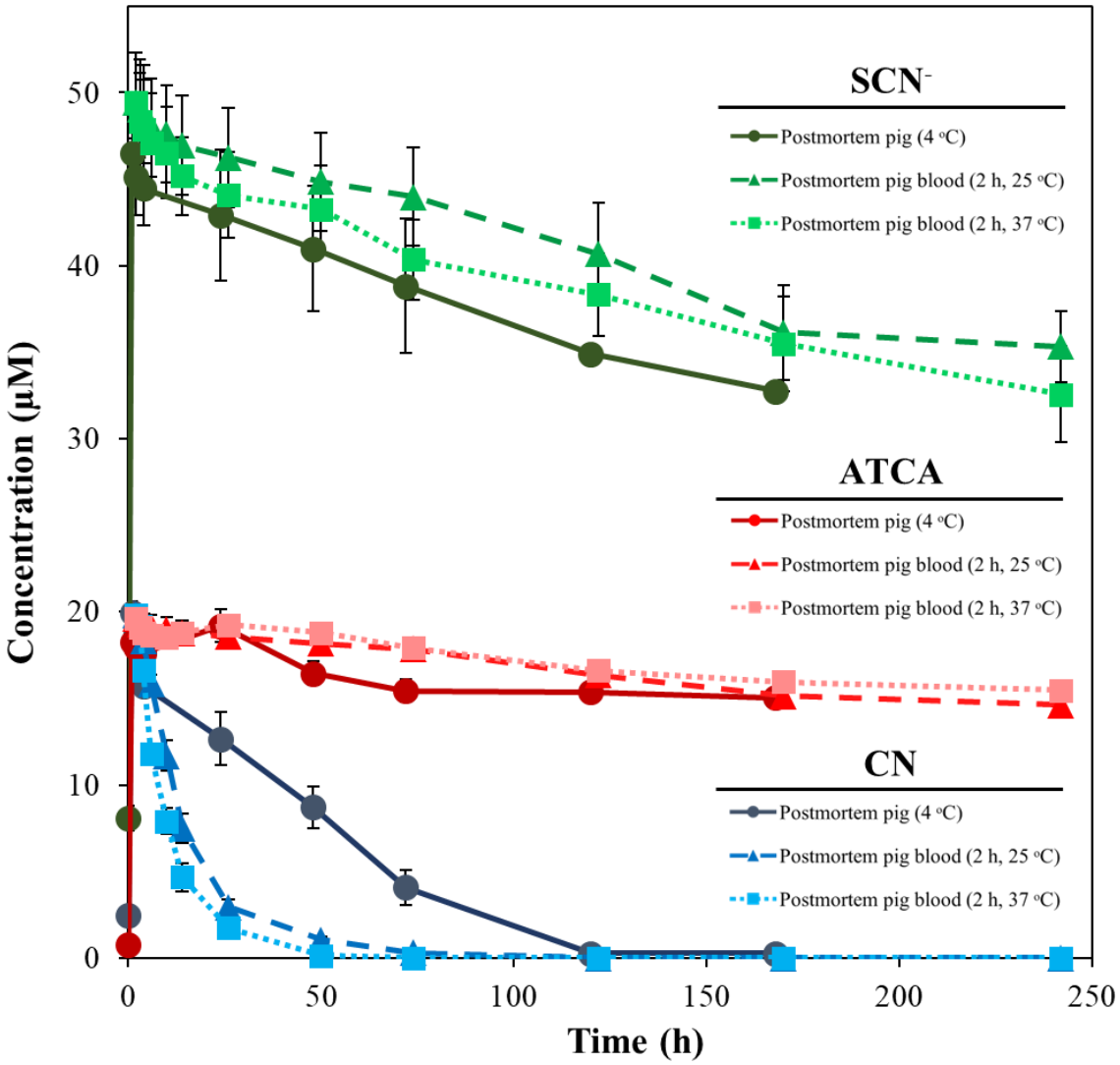

Figure 3.2. The stability of CN, SCN^-, and ATCA in postmortem CN-exposed swine. The circles connected via solid lines represent the stability of these compounds in postmortem pigs. Blood stored at room temperature (25 ºC) is indicated by triangles and dash lines, while that stored at a typical body temperature (37 ºC) is shown by squares connected by dotted lines. Error bars represent standard error of mean (SEM) (N = 8).

Following CN exposure, the initial postmortem blood sample, obtained 1 h postmortem, showed dramatic escalations of blood concentrations of CN and its metabolites compared to background. Table 2 lists the ratios of the initial concentration of each CN biomarker (i.e., 1 h postmortem blood) compared to its respective background concentration

(C_{1h}:$C_{background}$) (i.e., concentrations from antemortem blood collected from the same animal prior to CN exposure). The C_{1h}:$C_{background}$ ratio decreases from ATCA > CN > SCN^-. Therefore, ATCA is the most elevated marker of CN exposure compared to its background concentration even though SCN^- has the highest absolute blood concentration at this time. A higher C_{1h}:$C_{background}$ ratio is advantageous for use as a marker of CN exposure since it is easier to differentiate elevated levels from background concentrations. The C_{1h}:$C_{background}$ ratio for SCN^- is approximately 5 times lower than for ATCA because of the relatively high background concentrations of SCN^-. The high background concentrations of SCN^- were expected based mainly on dietary exposure to SCN^-. While the increase for all markers of CN exposure at 1 h could be interpreted as evidence that each of these markers could be used to confirm acute exposure to lethal CN concentrations, it is important to note that the conditions in this study were ideal for preserving forensic information. These conditions include animals housed under controlled conditions (including a controlled diet), known background concentrations of each marker for each animal, immediate storage of animals at 4 °C following euthanasia, and deliberate handling of blood samples with immediate freezing and subsequent storage at –80 °C until analysis. In a more realistic situation, a victim's diet is not controlled, the background concentrations of these biomarkers are not available, and it is unlikely that blood samples are immediately gathered and properly stored. It should be noted that CN drops below its background concentrations at 120 h. This is likely because generation of CN from diet or normal metabolism has ceased, and redistribution occurs postmortem.

Following the immediate sharp rise in the concentration of CN it relatively rapidly decreases to baseline levels by 120 min, whereas ATCA and SCN^- exhibit a steady, slow,

decrease in the postmortem swine over the course of the experiment, maintaining well above the initial blood concentration. As shown in Figure 2, S1A, and S2A, CN decreased markedly faster than SCN^- and ATCA, producing the lowest $t_{1/2}$ = 34.3 h of the markers of CN exposure (Table 2 and Figure S2A). The rapid decrease in CN concentrations is consistent with multiple other studies which also observe a rapid decrease in CN concentrations in blood under a variety of storage conditions.[197-202] The inherent instability of CN is problematic for its use as a forensic biomarker of CN poisoning.

Table 3.2. Stability/ kinetic parameters for CN, SCN^-, and ATCA in postmortem blood of CN-exposed pigs (stored at 4 °C) and in postmortem swine blood stored at 25 °C and 37 °C.

Environment	Analyte	$t_{1/2}$ (h)	$t_{1/2}$ (day)	C_{1h} / $C_{background}$
Pig (stored at 4 °C)	CN	34.3	1.4 d	11
	SCN^-	359	15 d	5.2
	ATCA	544	23 d	26
2 h postmortem pig blood (stored at 25 °C)	CN	10.7	0.5 d	NA
	SCN^-	432	18 d	
	ATCA	573	24 d	
2 h postmortem pig blood (stored at 37 °C)	CN	6.63	0.3 d	NA
	SCN^-	450	19 d	
	ATCA	653	27 d	

The alternative markers of CN exposure analyzed in this study, SCN^- and ATCA, showed more stability versus CN in postmortem pigs, but the half-life of ATCA ($t_{1/2}$ = 544.3 h = 23 d) was nearly double that of SCN^- ($t_{1/2}$ = 326.4 h = 15 d) (Table 2 and Figure S2A). The increased stability of SCN^- and ATCA is advantageous for forensic purposes it

allows a longer window-of-opportunity to verify if the cause of death is CN poisoning. Therefore, because ATCA is the most stable marker of CN poisoning, it is more advantageous for forensic purposes, especially if ideal conditions cannot be achieved.

3.2. Stability of CN, SCN^-, and ATCA in Postmortem Blood at Elevated Temperatures

Under more typical circumstances of CN poisoning, deceased victims would not be immediately transitioned to cold storage (4 °C). Additionally, the time between a poisoning event and cooled storage may be lengthy and/or cooled storage may not even occur. Therefore, the postmortem stability of CN, SCN^-, and ATCA in victims of CN poisoning at RT and HBT is of interest. Figure 2 shows the stability of CN, SCN^-, and ATCA in 2 h postmortem blood samples of CN-exposure pigs stored at RT (dashed lines) and HBT (dotted lines) over 10 days (240 h). CN exhibited pronounced instability at these temperatures, measured as a rapid decrease in CN concentrations with near complete elimination by 74 h, as illustrated in Figures 2, S1B, S1C, and S2C. The stability of CN in postmortem blood at RT and HBT is much lower than in postmortem swine stored at 4 °C. The $t_{1/2}$s of CN at RT and HBT (Table 2 and Figure S2B and C) show the rapid degradation of CN in postmortem blood at elevated temperatures. Compared to in postmortem swine at 4 °C, postmortem blood produces a 3x and 5x decrease in $t_{1/2}$ for RT and HBT, respectively.

The stability of SCN^- and ATCA in postmortem blood (at RT and HBT) was much higher than CN (Figure 2, S1B, and S1C). To quantify this relationship, the $t_{1/2}$s for each biomarker and storage condition pair are reported in Table 2. The $t_{1/2}$s for SCN^- and ATCA are approximately 40x and 53x higher than CN at RT, respectively, and 68x and 99x higher than CN at HBT for SCN^- and ATCA, respectively. In a real-world scenario, where ideal

conditions are rare, this study indicates that ATCA and SCN^- offer more persistent markers of CN poisoning, with ATCA being about 1.5 x more stable than SCN^-.

3.3. ATCA is a Reliable Postmortem Biomarker of CN Poisoning

The relatively low stability of CN in postmortem CN-exposed pigs and in postmortem blood at RT and HBT presents a major problem for direct analysis of CN as a marker of CN poisoning. Specifically, CN concentrations rapidly declined, particularly at RT and HBT. The rapid degradation of CN can lead to challenges in forensic analyses, particularly when trying to ascertain whether an individual was exposed to lethal concentrations of CN. The inherent instability of CN not only affects its potential as a consistent marker but also casts a shadow on the reliability of forensic analysis of CN when CN poisoning is suspected as a cause of death. Thus, if direct analysis of CN is to be used for this purpose, immediate sample collection, proper storage, and timely analysis is imperative to ensure trustworthy forensic information.

The degradation behavior of SCN^- and ATCA was comparable under the conditions tested, but ATCA was about 1.5 x more stable. Although SCN^- can be considered to have acceptable stability, the major disadvantage of SCN^- for forensic analysis is its high and variable endogenous concentrations (i.e., SCN^- produced the highest background concentration). Elevated concentrations of SCN^- relative to CN and ATCA are based on efficient metabolism of CN and SCN^- consumed via diet. The dependence of background concentrations of SCN^- on diet is disadvantageous since it is more difficult to determine if elevated SCN^- concentrations are produced from CN exposure. Further, while the absolute concentrations of SCN^- following CN exposure were the largest in this study (Figure 2), the increase in SCN^- relative to its baseline concentration was small compared to CN and ATCA

(Table 2). Factors such as age, health, genetics, and gut microbiota also influence the conversion of CN to SCN^-, leading to increased variability among individuals. Therefore, SCN^- suffers from large and variable background concentrations and limited increase relative to its respective background concentration. Therefore, while SCN^- can be a valuable marker for assessing CN exposure in the context of other evidence, elevated concentrations are likely not conclusive evidence of CN poisoning under most conditions.

ATCA demonstrated the slowest elimination dynamics among the three analytes, maintaining concentrations nearest the initial postmortem swine blood draw (1 h) throughout the study (10 days) while it was the most stable marker, it still decreased over time under all study conditions. This decrease is likely be due to redistribution of ATCA following death or changes in the postmortem blood matrix. Regardless of the time elapsed and inherent postmortem blood transformation over time, ATCA levels remain remarkably consistent. This distinguishes it from CN and SCN^-, which can show more significant degradation. Moreover, the large increase in ATCA above background levels is advantageous for forensic purposes, providing a longer runway to confidently determine elevated levels of ATCA. Based on its properties of detectability, stability, and relative increase directly related to CN exposure, ATCA can be used as a forensic marker to verify CN exposure.

While this study answered an important question in the field of forensic analysis related to CN poisoning, there are other questions important to definitely answer: 1) Is ATCA the most advantageous marker when blood is obtained from the heart (i.e., the heart is a common location to draw blood postmortem), and 2) Is ATCA the most appropriate marker when death from CN poising is delayed (i.e., when active metabolism of ATCA occurs over a significant amount of time). There have been studies that shed light on question 2,

such as Bhandari et al,[88] which showed ATCA tracked CN concentrations, producing a $t_{1/2}$ of only 40.7 and 13.9 min in rabbits and swine, respectively. ATCA concentrations decline faster when metabolism is active because it is excreted through normal metabolic pathways. Overall, active metabolism directly impacts the persistence of ATCA. Therefore, confirmation of ATCA's behavior during active metabolism must be considered. For question 3, our study did not address the effect of biological decay on the behavior of CN, SCN^-, or ATCA. For example, after death, CN can continue to be produced due to various biological processes.[192, 199, 203-207] This postmortem production of CN could lead to formation of ATCA in the presence of cysteine. If this process is common, ATCA measured in postmortem blood may not necessarily originate from CN poisoning. Future research is needed to address these questions to solidify the situations where the use of ATCA as a forensic biomarker for cyanide exposure in forensic investigations is appropriate.

4. Conclusions and Future Work

The findings of this study highlight the advantages of ATCA as a biomarker of CN poisoning based on its relative stability in postmortem pigs at 4 °C and in postmortem blood at RT and HBT. ATCA demonstrated the slowest elimination and produced a high C_{1h}:$C_{background}$ ratio. The slow and predictable decline of ATCA over extended intervals provides a long window-of-opportunity to detect ATCA above background levels during postmortem analysis of blood from suspected CN poisoning victims, particularly in real-world scenarios, where ideal conditions are rare. Given its pronounced postmortem stability, ATCA emerged as the best candidate for use as a biomarker of CN poisoning.

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6. Declarations

The authors declare that they have no conflict of interest. All animals were cared for in compliance with the "Principles of Laboratory Animal Care" formulated by the National Society for Medical Research and the "Guide for the Care and Use of Laboratory Animals" prepared by the National Institutes of Health (National Academic Press, 1996). The University of Colorado's Institutional Animal Care and Use Committee (IACUC) approved the CN exposure study (ethics committee: Carter Severance, Genna Nault, Tara Hendry-Hofer, Nathan Wetmore). It complied with the regulations and guidelines of the Animal Welfare Act and the American Association for Accreditation of Laboratory Animal Care.

4. CHAPTER 4. BROADER IMPACTS, CONCLUSIONS, AND FUTURE WORK

1. Broader Impacts

The threat of CWAs, particularly CN, has been used for centuries to kill or incapacitate enemies. With increased public awareness and significant concerns in modern society, there is a need for simple, quick, selective, and efficient analytical methods for detecting CN poisoning. The development of an analytical method to detect CN and its metabolite SCN^- concurrently in whole blood was crucial for confirming acute CN exposure. This method will aid clinicians in verifying CN poisoning in patients. The validated method can inform regulatory limits and policies regarding allowable workplace or environmental CN exposures, enhancing safety standards and reducing health impacts. The technique could also be adapted to assess cyanide contamination in environmental, food, or drinking water samples, providing broader public health benefits.

ATCA mimics CN behavior and appears to be a promising candidate for early forensic verification of CN poisoning. The findings should aid in determining CN and its metabolites from the biofluids of CN poisoning victims. The knowledge that ATCA persists in blood long after death aids retrospective analysis to identify past lethal CN exposures in stored samples. The findings (i.e., postmortem stability of ATCA) support the expanded use of ATCA as a reliable marker of CN exposure when ideal sample collection, storage, and analysis is not feasible.

2. Conclusions

An HPLC-MS/MS method was developed to analyze CN and SCN^- in human ante- and postmortem whole blood. The method is simple, sensitive, selective, and cost-effective, allowing the concurrent detection of CN and SCN^- at low levels. It is the first HPLC-

MS/MS method for concurrent CN and SCN^- analysis in human ante- and postmortem whole blood. This method will be beneficial to be used for forensic investigations and improve outcomes for CN poisoning victims.

Forensic analysis of CN, SCN^-, and ATCA in CN exposed swine postmortem highlights the benefits of ATCA as a biomarker for CN poisoning due to its stability in postmortem swine and postmortem blood samples at elevated temperatures. ATCA produced the slowest elimination rate and a high C_{1h}: $C_{background}$ ratio, providing an extended window for detecting CN poisoning concentrations above background levels during postmortem analysis, providing a wide range of detection opportunities in forensic scenarios where ideal conditions are rare.

3. Future work

The HPLC-MS/MS method developed in this study allows for the sensitive, rapid, and reliable quantification of cyanide and its metabolite thiocyanate in whole blood samples. The method was validated in human blood, but further research is needed to validate it across different human sample biofluids and storage conditions. Furthermore, the method can also be expanded for tissue CN detection, environmental samples, and therapeutic drug monitoring. Long-term studies are needed to assess its utility for chronic CN exposure assessments in occupational settings.

The CN biomarker, ATCA, has shown promise in postmortem stability and persistence, but further research on ATCA kinetics from cardiac blood sampling and active metabolism is needed. Understanding the interactions between metabolism and ATCA production will enhance its utility for exposure confirmation.

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APPENDIX AND SUPPORTING MATERIAL

Can Cyanide Metabolite 2-Aminothiazoline-4-Carboxylic Acid Be Used to Verify Cyanide Poisoning in Postmortem Blood?

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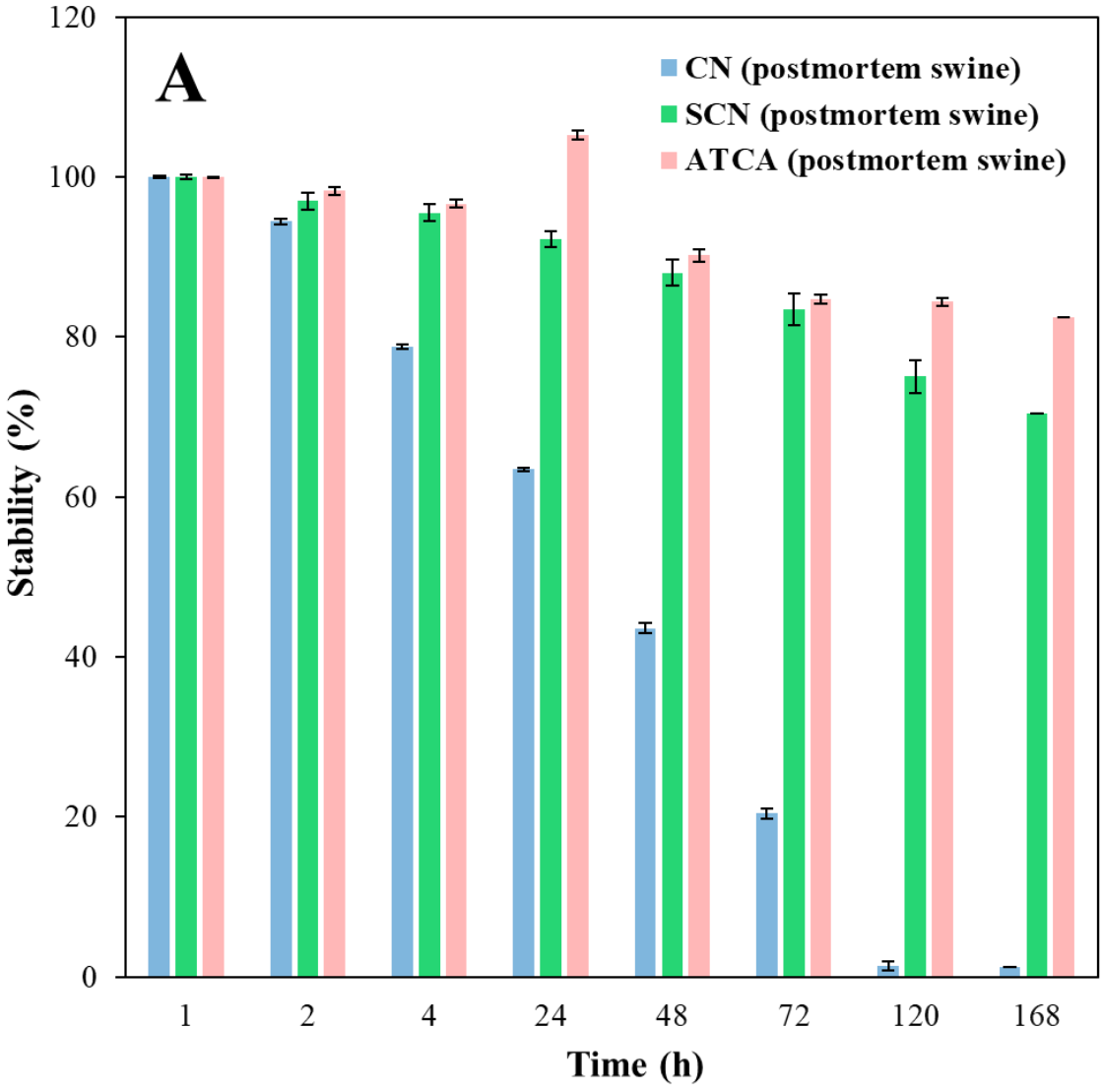
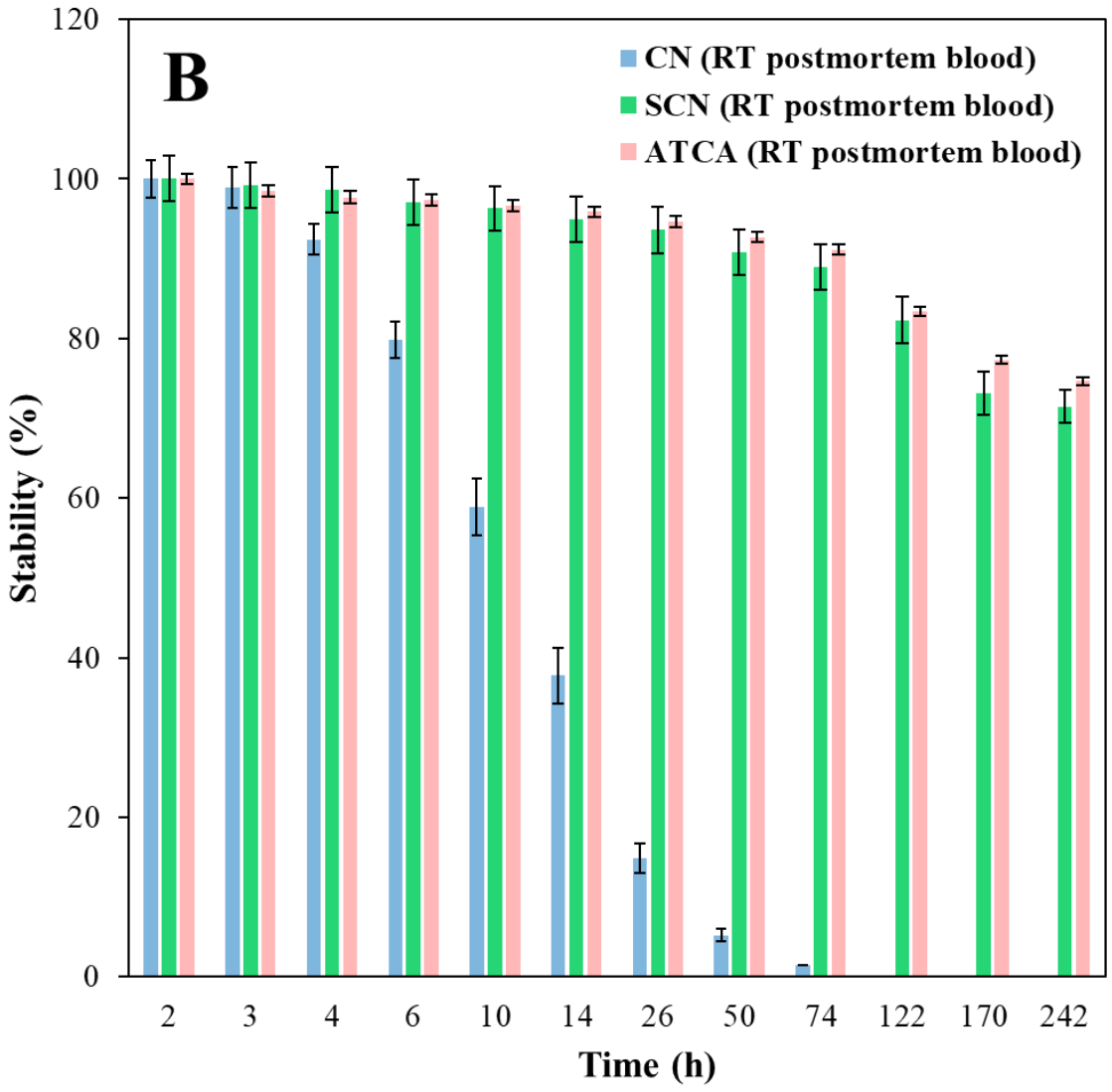
Supplementary Figure S1A demonstrates the superior stability of ATCA compared to CN and SCN^- in postmortem swine (stored at 4 °C) following CN poisoning. While CN concentrations rapidly decreased to near zero by 120 h, and SCN^- declined to 70% and ATCA reduced to 82% of their initial concentrations by 168 h. These data highlight the relative stability of CN, SCN^-, and ATCA in postmortem swine over extended storage times. The marked stability of ATCA supports its use as a reliable biomarker for CN poisoning confirmation.




B
CN (RT postmortem blood)
SCN (RT postmortem blood)
ATCA (RT postmortem blood)
Stability (%)
120
100
80
60
40
20
0
2
3
4
6
10
14
26
50
74
122
170
242
Time (h)

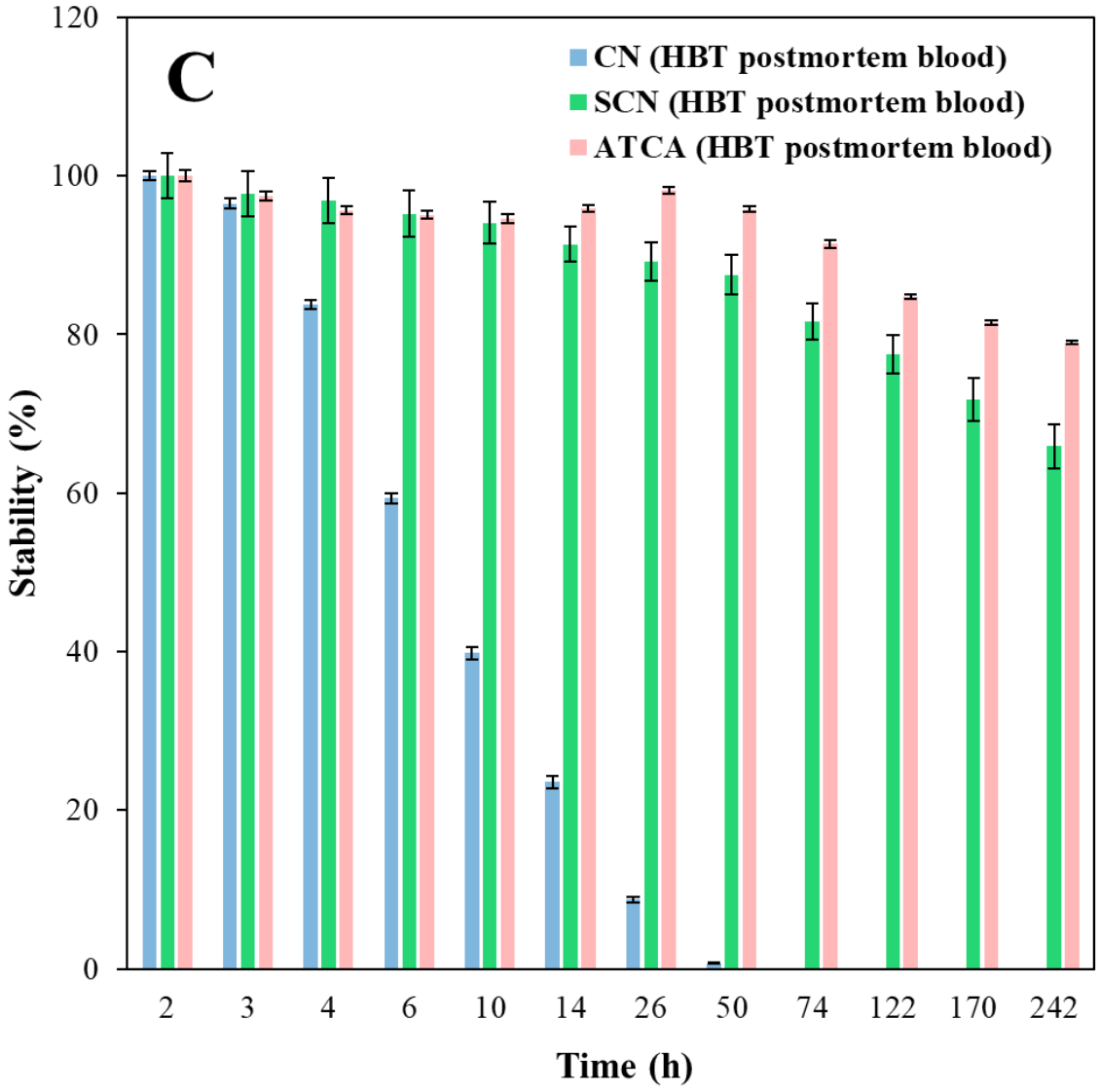
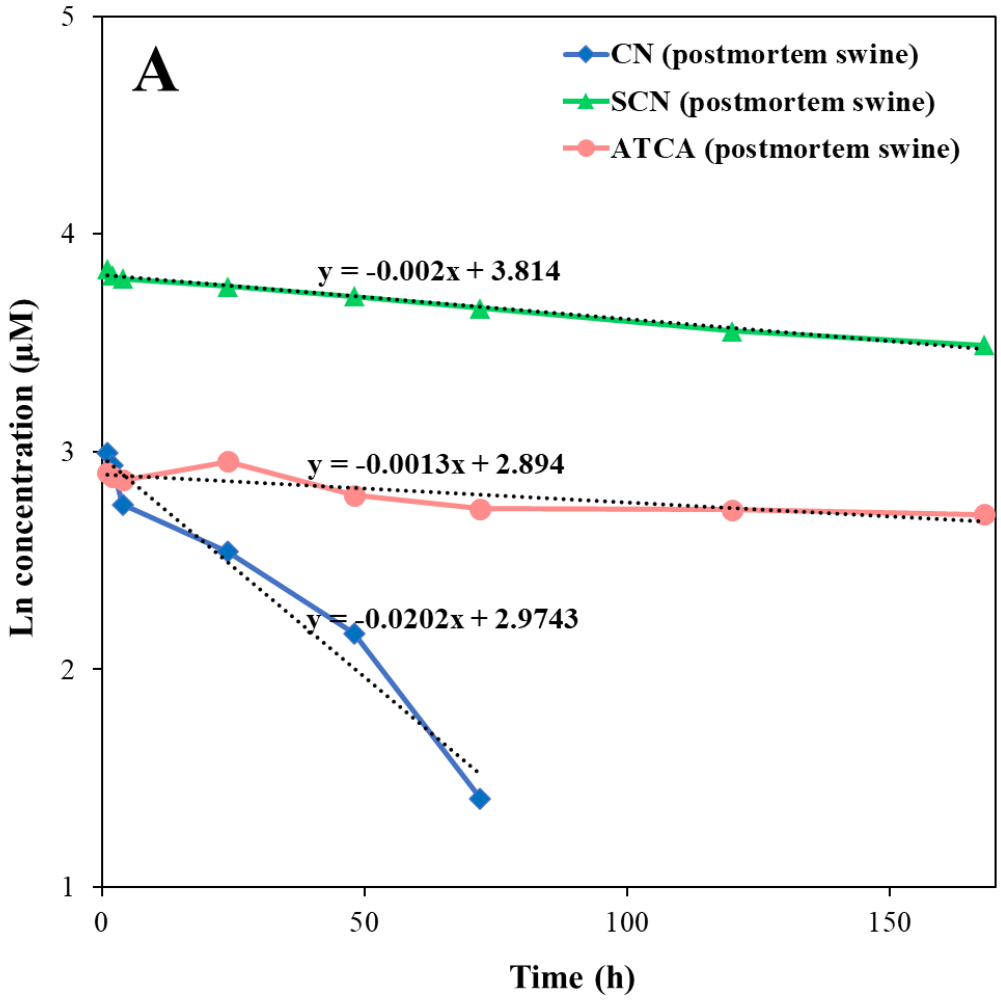


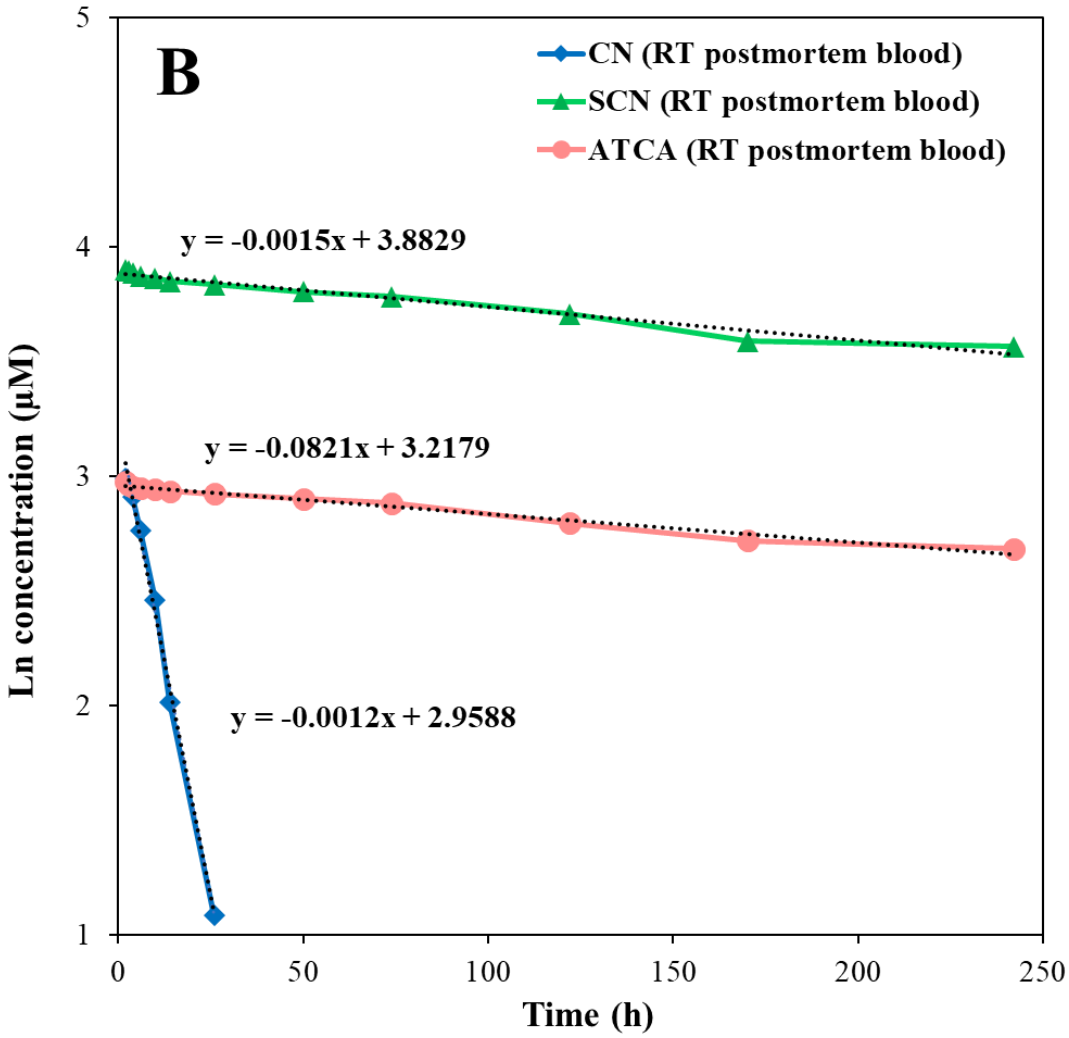
Figure S1. The stability of CN, SCN^-, and ATCA in A) postmortem CN-exposed swine; B) room temperature; C) and typical body temperature. Error bars are plotted as standard error of mean (SEM) (N = 8)

Supplementary Figures S1B and S1C show the stability of CN, SCN^-, and ATCA over time in postmortem blood stored at RT and HBT, respectively. The rapid degradation of CN is clearly illustrated, with concentrations decreasing to near zero by 74 h and 50 h at RT and HBT, respectively. SCN^- and ATCA decline more slowly, however the stability of ATCA is markedly better than SCN^- at HBT. At RT, ATCA and SCN^- maintain

concentrations at 74 h. After 74 h (at HBT), ATCA declined to 79% of its initial concentration but SCN^- dropped to 66%. Together these figures demonstrate the stability of SCN^- and ATCA under elevated storage conditions compared to the inherent instability of CN.

Figure S2A shows the calculation of $t_{1/2}$ for CN, SCN^-, and ATCA in postmortem swine blood stored at 4°C. It is clear that CN has the shortest $t_{1/2}$ of 34.3 h. Though SCN^- is more stable than CN, ATCA exhibits the longest the $t_{1/2}$ of 23 days nearly double that of SCN^- 15 days. These $t_{1/2}$ values highlight the instability of CN versus the persistence of ATCA as a forensic biomarker.




B
CN (RT postmortem blood)
SCN (RT postmortem blood)
ATCA (RT postmortem blood)
y = -0.0015x + 3.8829
y = -0.0821x + 3.2179
y = -0.0012x + 2.9588
Ln concentration (µM)
Time (h)
5
4
3
2
1
0
50
100
150
200
250

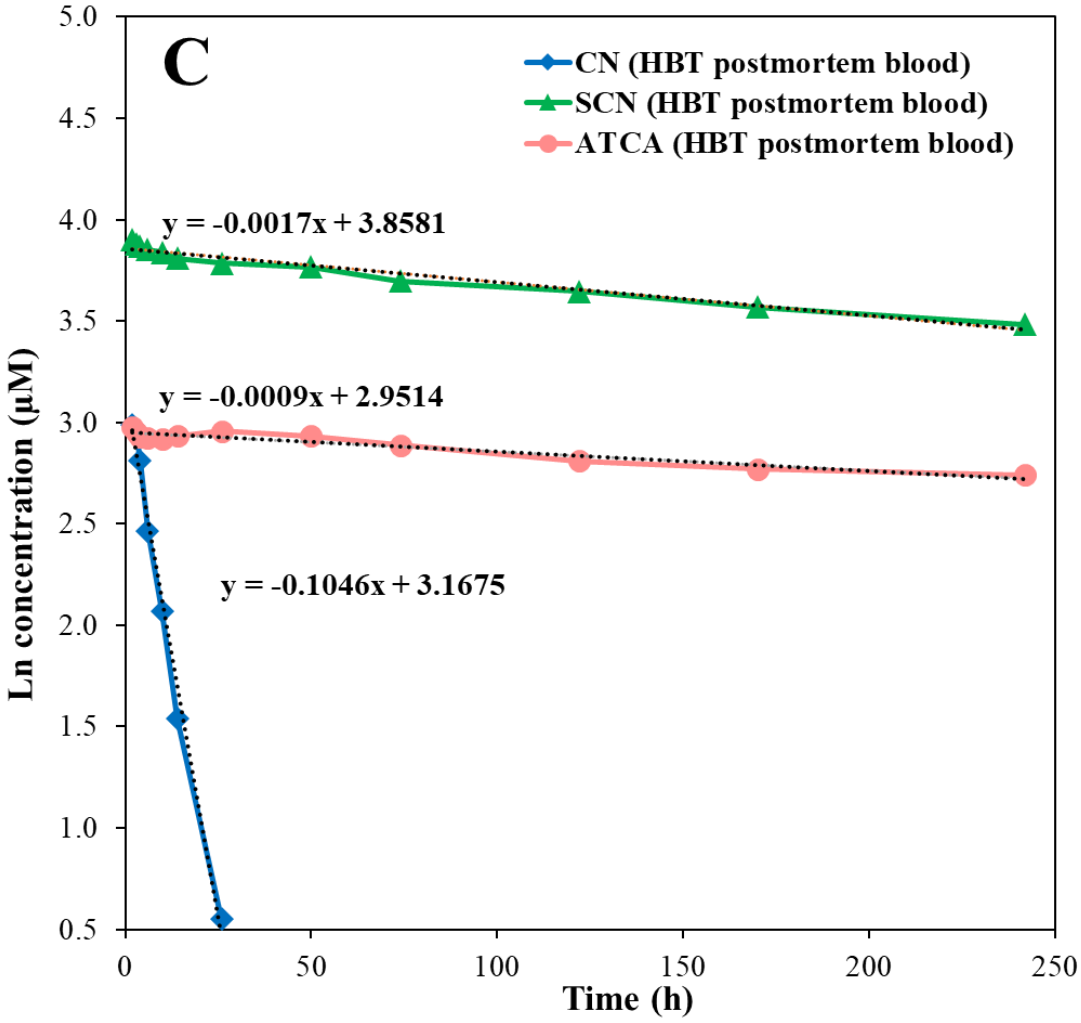


Figure S2. The $t_{1/2}$s for CN, SCN^-, and ATCA in A) postmortem CN-exposed swine; B) room temperature; C) and typical body temperature.

The rapid degradation of CN is further demonstrated in Supplementary Fig S1B and C, which show the $t_{1/2}$s calculation for CN stored in postmortem blood at RT and HBT. As shown in Table 2 and Figures S2B and C, the $t_{1/2}$ of CN drops to 10.7 h and 6.6 h at RT and HBT, respectively. Meanwhile, SCN^- and ATCA remain much more stable, with $t_{1/2}$ values of 18 and 24 days (RT) and 19 and 27 days (HBT) (i.e., SCN^- is ≈40-68x and ATCA is ≈53-99x more stable than CN for these temperature conditions). This quantitative

comparison of $t_{1/2}$ values under different storage conditions highlights the advantages of ATCA as the most stable forensic marker of CN poisoning in postmortem swine.