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Cyanide is a toxic chemical that may be introduced to living organisms as a result of both legal and illicit uses of cyanide. Exposure to cyanide can be verified by analyzing cyanide or one of its break-down products from biological samples. This verification is important for medical, law-enforcement, forensic, research, and veterinary purposes. This review will identify common problems associated with the analysis of cyanide and its metabolites, discuss current bioanalytical techniques used for verification of cyanide exposure, and briefly address the metabolism and toxicokinetics of cyanide and its break-down products in biological systems.

Introduction

Cyanide is toxic in humans, animals, and fish and exposure can occur in various ways. Many substances are potential sources of cyanide exposure including edible and non-edible plants, industrial operations, fires, and cigarette smoke. Although the primary natural source of cyanide poisoning is from plants (*1-7*), other natural sources include volcanoes, bacteria, and fungi (*1, 8-13*). Man-made sources include malfunctioning catalytic converters, residential and commercial fires involving the burning of plastics, cigarette smoke, as well as illicit uses of cyanide (*14*). Additionally, hundreds of thousands of tons of cyanide are manufactured annually in the U.S. alone for industrial uses, including chemical syntheses, electroplating, plastics processing, paint manufacturing, gold and silver extraction, tanning, and metallurgy.

Along with many legal industrial uses of cyanide, multiple illegal uses of cyanide exist. Recently, terrorist acts involving cyanide have been the most publicized illicit uses of cyanide. In 1982, cyanide was placed in bottles of Tylenol in the Chicago area, killing seven people (*1*). In 1995 in Tokyo, an acid and a cyanide salt were found in several subway restrooms in the weeks following the release of nerve agents (*2*). Another illegal use of cyanide is the capture of fish for subsequent sale in the live fish trade (*15*). Cyanide is used at sub-lethal doses to temporarily stun fish, making them easier to catch (*16*). The practice of using cyanide in this manner has been found in a number of countries, and has an adverse affect on coral reefs (*15*). The cyanide is toxic to algae that are necessary for coral to survive and also produces many adverse secondary effects, such as killing smaller fish species. With this and other destructive practices used during capture of cyanide stunned fish, irreversible damage to coral reefs has and continues to occur (*17*).

As industrial and illicit applications of cyanide increase, the need for rapid, sensitive, and specific analytical methods to assess cyanide exposure will be amplified. The goals for this review are to briefly discuss current bioanalytical techniques used for verification

of cyanide exposure and to identify common problems associated with the analysis of cyanide and its metabolites in biological samples. This review does not include methods to test for cyanide in environmental or industrial matrices (e.g., surface waters, minerals, process streams). A number of related reviews have been published (*8, 15, 18-22*).

the gills or intestine (*15*). Little is known about the metabolism of cyanide in fish, and more research is necessary to fully understand the similarities and differences of mammals and fish, but considering that cyanide's toxic effects are similar in both mammals and fish, a number of metabolic pathways may also be similar. As the majority of research on cyanide metabolism has been done on

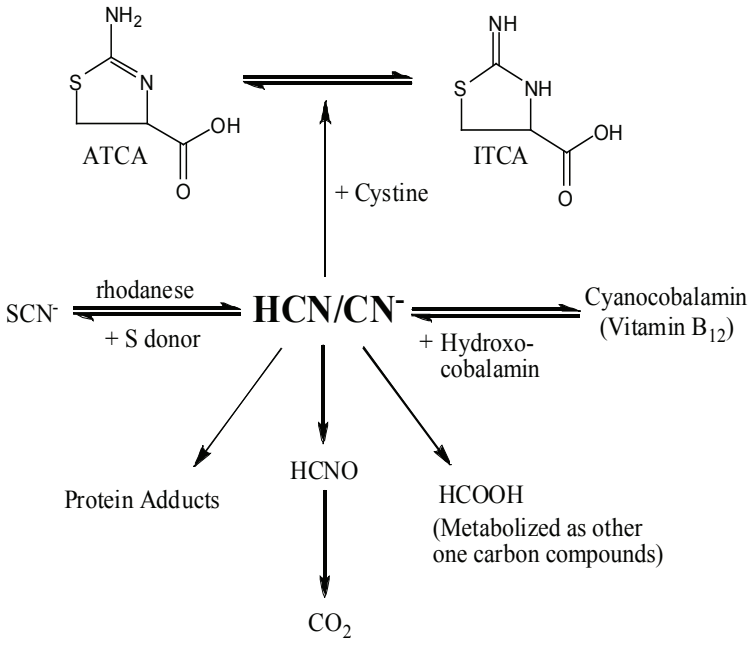


Figure 1. Human metabolism of cyanide.

Cyanide metabolism and toxicokinetics

Although there are other chemical forms of cyanide, it is hydrogen cyanide (HCN) that is the primary toxic agent, regardless of its origin. The toxic effects of cyanide can be traced to interference of aerobic metabolism (*18*). This occurs when cyanide blocks terminal electron transfer by binding to cytochrome oxidase for both mammals and fish (*18, 23*). For mammals, cyanide ion (CN^-) is acquired through ingestion while hydrogen cyanide is acquired through inhalation or absorption through the mucous membranes or skin. For fish, cyanide is absorbed through humans, the following text summarizes cyanide metabolism in humans.

Once absorbed, cyanide is quickly transferred to the blood and metabolized through a number of processes, as shown in Figure 1. The major pathway for cyanide metabolism is conversion of cyanide to thiocyanate (SCN^-) in the presence of a sulfur donor (e.g., thiosulfate) (*24*). This reaction is catalyzed by the enzyme rhodanese (*18, 25, 26*). About 80% of the initial cyanide dose is converted to thiocyanate, which is subsequently excreted in the urine. Other minor metabolic

pathways are the conversion of cyanide to 2-amino-2-thiazoline-4-carboxylic acid (ATCA; the tautomeric form of ATCA is 2-iminothiazolidine-4-carboxylic acid – ITCA; Figure 1) in the presence of cystine (*18, 26-28*), and the reversible reaction of cyanide with hydroxocobalamin to form cyanocobalamin. (Note: Throughout the text, the ATCA/ITCA tautomeric pair will be referred to as ATCA.) The production of ATCA may predominate when sulfur donors become depleted or in tissues where rhodanese is sparse. Other minor pathways for metabolism include the creation of one-carbon metabolites and protein adducts (i.e., reaction of a chemical species with a protein to form a chemical bond that modifies the parent protein) (*18, 29*). Each metabolite in Figure 1 could potentially be used as a marker for cyanide exposure.

The toxicokinetics and metabolism of an analyte must be considered when an analytical technique is used to determine exposure to the analyte. A main consideration for determining a target for confirmation of exposure is the half-life of the biomarker of interest. Table 1 lists the half-lives of cyanide and thiocyanate for acute exposures of a number of mammalian species. If the half-life of a toxic agent is short, as with cyanide ($t_{1/2}$ = 0.34-1.28 hours), it can be difficult to determine exposure by direct analysis of the toxic agent if significant amounts of time have elapsed. Thiocyanate offers a longer half-life ($t_{1/2}$ = 4.95-5.8 hours). The half-lives of ATCA and cyanide-protein adducts are unknown, although for protein-adducts, a half-life similar to that of the parent protein could be expected (e.g., 20-25 days for human serum albumin) assuming the adduct is stable (*30, 31*). It should be noted that chronic exposure to cyanide increases the apparent half-life of cyanide and thiocyanate (*32*). This is most likely due to the depletion of sulfur donors over the course of the chronic exposure with subsequent reduction of cyanide transformation by this metabolic pathway. The activity of rhodanese could also be reduced over the course of the chronic exposure, leading to longer half-lives of cyanide.

As seen by the half-lives of cyanide among a number of mammals in Table 1, the toxicokinetics of cyanide is somewhat species dependent. Therefore, although generalities concerning the toxicokinetics and metabolism of cyanide can be made, half-life values cannot be used directly from other species. To more fully understand the relationship of cyanide metabolism and toxicokinetics of sparsely studied species (e.g., most fish species), more research should be undertaken to determine relationships between organisms of interest.

Table 1. Reported half-lives of cyanide and thiocyanate from acute exposures of cyanide.

Cyanide/ Metabolite	Species	$t_{1/2}$ (hr)	Reference
Cyanide	Human	0.34-1.00	(*34*)
	Rat	0.64	(*35*)
	Pig	0.54	(*35*)
	Goat	1.28	(*35*)
Thiocyanate	Rat	5.80	(*35*)
	Pig	4.95	(*35*)
	Goat	13.9	(*35*)

Special considerations for the analysis of cyanide and its metabolites

Considering the typical half-life of cyanide for acute exposure (Table 1), it may be difficult to determine cyanide concentrations in blood or tissue for long periods of time following exposure. When analyzing cyanide from blood (the preferred method for determination of cyanide exposure in large species), analysis must occur soon after exposure since the concentration of cyanide in the blood decays within minutes to hours. A number of issues limit the direct analysis of cyanide from blood, including rapid cyanide detoxification processes, the difficulty of establishing steady state cyanide levels with time, and the volatility and nucleophilic properties of cyanide. Although limited, direct analysis of cyanide from blood is still useful in that it may be the only biomarker capable of indicating exposure to cyanide within the initial minutes following exposure (*33-35*).

Although traces of cyanide in urine (*36, 37*), saliva (*25, 38*), and expired air (*39-42*) have been found, direct analysis of cyanide from these matrices is even more limited. It is difficult to assess decay of cyanide concentrations in tissues, because levels of rhodanese are highly variable between organs (*18*). Considering the highly nucleophilic nature of cyanide ion, free cyanide concentrations are likely to be very low in tissues, although this does not preclude some reservoir of bound cyanide within the tissues. Any cyanide analysis technique would have to consider conversion of bound cyanide to free cyanide prior to analysis.

As an alternative to direct analysis of cyanide, thiocyanate and ATCA may be determined in urine, saliva, tissue, and blood. Cyanide-protein adducts have also been found in human blood proteins. These markers may offer an advantage in half-life (Table 1 for thiocyanate), and correlation of thiocyanate and ATCA concentrations to cyanide exposure have been examined (*43-49*). Each of these markers has advantages and disadvantages when considering the detection of cyanide exposure.

The advantages to measuring thiocyanate are that appreciable concentrations may be found shortly following exposure and it has a longer half-life than cyanide (*35*). However, thiocyanate is naturally found in biological fluids, and while this is a condition of all cyanide metabolites, thiocyanate levels are normally quite high and can be inconsistent (*25, 43, 50-55*). Large variation in background thiocyanate concentrations makes it difficult to determine low-level cyanide exposure. Also, Ballantyne (*56*) found that concentrations of thiocyanate in blood varied inconsistently during storage at a number of different temperatures and that analytical recovery of thiocyanate from whole blood was difficult. Large and variable concentration may indicate that thiocyanate is involved in a number of biological processes in addition to cyanide metabolism. Indeed, significant use of thiocyanate by biological processes other than cyanide metabolism has been established (*24, 57, 58*).

ATCA may also be used as an alternative for determination of cyanide exposure. An advantage to using ATCA is that it is stable in biological samples for months at freezing and ambient temperatures (45, 48). It also has been found that ATCA is not metabolized further (18, 47, 59) and therefore, may be a lasting signature of cyanide exposure. However, relatively few techniques have been described to analyze ATCA from biological matrices (45, 48, 60, 61) and relatively few studies have been performed to evaluate the relationship between ATCA and cyanide exposure (45, 48). With more knowledge of ATCAs behavior with relation to cyanide exposure, ATCA's stability and applicability

to sensitive analytical techniques may prove beneficial.

Recently, cyanide-protein adducts have been discovered (*29*). If these proteins are stable, they could serve as long-lived markers of cyanide exposure. Disadvantages of this analysis include costly instrumentation, limited research pertaining to the behavior of these adducts, and the difficulty and length of sample preparation. Even considering these disadvantages, cyanide adducts are extremely promising for providing a long-lived biomarker of cyanide exposure, especially if a less complex and less costly method of analysis can be developed.

Multiple factors must be considered when choosing which analyte to target for verification of cyanide exposure. Analysis of cyanide or any one of its metabolites has advantages and disadvantages. Table 2 compares cyanide and its metabolites in terms of some factors important for verification of cyanide exposure.

Species considerations for verification of cyanide exposure

For humans, determination of cyanide exposure has been suggested or attempted from blood (*20, 25, 37, 43, 45, 62-97*), urine (*36, 37, 45, 48, 89, 95, 98*), saliva (*25, 38, 51, 93, 99*), expired air (*39, 42*), and tissue (post-mortem) (*74, 96, 100*) samples. Blood may be the most versatile biological sample used to determine exposure to cyanide, because the analysis of cyanide, thiocyanate, ATCA, and cyanide-protein adducts can be performed on blood samples. Saliva, urine, or tissue may be more appropriate depending on the analytical method and other factors, including ease of obtaining the sample. If determination of cyanide exposure is to be done from non-human species, obviously saliva and urine samples will be difficult to obtain. Therefore, blood and tissues are the most likely samples to be analyzed to determine cyanide exposure. The choice of blood or tissue is determined by a number of factors, including the size of the species. For example, most fish species would not have sufficient quantities of blood to analyze, therefore making the analysis of

Table 2. Comparison of cyanide and its metabolites for verification of cyanide exposure.

Cyanide/ Metabolite[a]	Half-lives	Toxicokinetic Data	Storage Stability	Biological Sample[b]	Species[c]
CN	Minutes-hours	Some in a few species	Low	Blood, Urine, Saliva, Tissue, Expired Air, Rumen	Human, Fish, Cow, Mouse, Rat, Guinea Pig, Goat, Horse
SCN	Hours	Limited	Medium	Blood, Urine, Saliva, Tissue, Milk, Gastric Fluid, Cerebrospinal Fluid	Human, Fish, Rat, Mouse, Pig, Goat, Horse
ATCA	Unknown	None	High	Blood, Urine, Feces, Saliva, Tissue	Human, Fish, Rat
CN-protein adducts	Presumably days-months	None	Unknown	Blood	Human

[a] CN – cyanide, SCN – thiocyanate, ATCA – 2-amino-2-thiazoline-4-carboxylic acid.
[b] Cyanide or the metabolite has been analyzed from this matrix with an analytical method reported in at least one research article or in the authors' laboratories.
[c] Cyanide or the metabolite has been analyzed from this species with an analytical method reported in at least one research article or in the authors' laboratories.

tissue samples indispensable. Although tissue may be the only choice for certain species, most published methods for determination of cyanide exposure are for analysis of biological fluids. While some methods developed for biological fluids may fail for tissue analysis, a number of these bioanalytical methods can be slightly modified for analysis of tissues. Another consideration is that rates of cyanide metabolism may be inconsistent between organs because of variable rhodanese and sulfur donor concentrations (*18*). Therefore, careful selection of tissue, depending on the analyte to be determined, can be extremely important. Also, analytes must be extracted from the tissue for most analytical methods. Therefore, an extra extraction and solid sample processing may be necessary for tissue analysis.

The detection of cyanide and its metabolites in biological samples

The determination of cyanide, thiocyanate, ATCA, and cyanide-protein adducts in biological fluids and tissues, is useful for forensic, clinical, research, law enforcement, and veterinary purposes. Methods of analysis include spectrophotometric or fluorescence methods (*37, 41, 43, 44, 51, 60, 61, 67, 76, 77, 82, 87, 89, 99-129*), electrochemical methods (*90, 130-139*), gas chromatography (*33, 38, 45, 62, 63, 66, 70, 78, 79, 81, 83, 86, 94, 124, 140-158*), and liquid chromatography techniques (*37, 46, 48, 69, 80, 84, 88, 89, 93, 95, 97, 98, 133, 159-171*). Choosing from the many available types of methods and biomarkers of cyanide exposure, complicated by numerous discrepancies in the literature between these methods makes selection of an analytical method for a specific purpose nontrivial. Factors that will influence the initial choice of which biomarker and analytical technique to use are cellular absorption and detoxification kinetics, sampling and analysis time, sample storage time and conditions, sample matrix, interferences, sensitivity, available instrumentation and equipment, expertise, and cost. Table 3 describes some differences between the groups of methods listed above in terms of these considerations.

Sample preparation and storage

Careful sample preparation of biological samples containing cyanide or its metabolites is a key element to producing accurate results. (Note: It is always necessary to consider the volatility of HCN when working with samples that may have significant concentrations of cyanide and the dangers that it may pose to laboratory personnel.) A major problem in the analysis of cyanide and thiocyanate is their interconversion, which occurs during sample preparation and storage and leads to inaccurate results. The amount of cyanide within the sample can be altered during storage by up to 66% in 14 days, depending on the storage temperature (*20, 33, 77, 172-175*). A number of researchers have attempted to address this problem. In preparation of samples for gas chromatographic (GC) analysis, Seto et al. (*79*) demonstrated that artificial formation of HCN from thiocyanate in blood occurred, and later showed that ascorbic acid prevents artifactual cyanide formation at temperatures below 63°C (*175, 176*). Sano et al. (*88*) found that, under the conditions studied, pretreatment of blood samples with water and methanol was successful in preventing artifactual formation of cyanide from thiocyanate. There are a number of methods to help prevent artificial formation of cyanide during storage, and if samples containing cyanide are to be stored before analysis, these methods should be considered (see *Suggested procedures for delayed analysis of biological samples* section). The stability of ATCA in biological samples under a number of storage conditions has been established (*45, 48*).

Earlier methods of cyanide analysis involved extensive sample preparation in which the sample was acidified (typically with sulfuric or phosphoric acid), and HCN was transferred to alkaline solution by distillation or microdiffusion. This served to concentrate the cyanide and to separate it from potential interferences (*33, 43, 70, 77, 82, 105-107, 125, 144, 151*). Buffered hydroxocobalamin (*67, 177*) or methemoglobin (*100*) solutions have also been used to capture liberated HCN. This pre-treatment method can be used prior to most analytical techniques for the determination of cyanide. For example, this procedure has been used prior to ion-selective electrode (ISE) analysis (*15*). For GC analysis of HCN, the procedure of liberating HCN by acidification (without capture in solution) is extensively used prior to headspace analysis (*33, 70, 94, 144*). It should be noted that when using methods that liberate HCN, rubber septa or stoppers can react with gaseous HCN. Therefore, polytetrafluoroethylene septa should be used (*64, 151*).

Individual pretreatment steps (i.e., derivatization) are generally necessary for detection of cyanide by spectrophotometry or fluorescence. For example, Lundquist and Sörbo (*123*) used a modification of the König reaction (Figure 2; discussed in more depth in the *Spectrophotometric, luminescence, and atomic absorption methods* section) to spectrophotometrically determination blood cyanide concentrations by high-performance liquid chromatography (HPLC) and 2,3-napthalenedialdehyde (NDA) and taurine have been effectively used with HPLC-fluorescence or as a stand-alone fluorescence method to produce highly sensitive methods for the determination of cyanide (*36, 37, 69, 84, 88, 159*). Other derivatization schemes will be discussed with individual analytical methods below.

Thiocyanate sample preparation is normally limited to derivatization that is intended to increase a specific detector's response to the ion. While initial sample preparation is not common, ion exchange columns could be used to separate thiocyanate from biological sample components. Thiocyanate is weakly spectrophotometrically active, but it is normally derivatized with a strong absorber or fluorophore prior to analysis because of its weak absorbance. For example, 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one has been used effectively for the fluorometric determination of SCN by HPLC (*163*). Others have also used variations of the König reaction to produce stronger spectrophotometric signals (*95, 118, 123*).

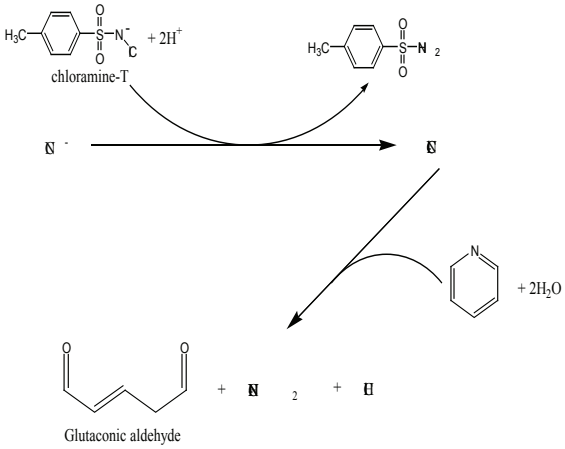
Multiple methods have been proposed for the simultaneous analysis of cyanide and thiocyanate. Methods for simultaneous analysis are generally limited to chromatographic methods and preparation can involve derivatization. For example, Kage et al. (*83*) used pentafluorobenzyl bromide as the derivatizing agent for simultaneous GC-mass spectrometric (MS) analysis of cyanide and thiocyanate, and Funazo et al. (*146*) quantitatively methylated cyanide and thiocyanate for GC analysis with a nitrogen-phosphorous detector (NPD). Multiple authors have used other derivatization techniques for GC analysis of cyanide or thiocyanate in biological samples (*145, 150, 153, 154*).

ATCA has been mainly prepared for analysis using cation exchange solid-phase extraction columns and individual pretreatment steps, depending on the analytical technique. Lundquist et al. (*48*) and Logue et al. (*45*) both used cation exchange solid phase extraction columns to separate ATCA from a number of components in biological samples. Lundquist et al. (*48*) further purified disulfides from samples

by reduction and subsequent separation with another column. Both Bradham et al. (*60*) and Lundquist et al. (*48*) heated ATCA in strong basic solution to open the ring structure of ATCA and produce a thiol group. Bradham et al. (*60*) then used hydroxymercuribenzoate, and subsequently diphenylthiocarbazone, to produce a colored product that was analyzed spectrophotometrically. Lundquist et al. (*48*) derivatized ATCA (after opening the ring) with a coumarin derivative and analyzed by HPLC. After using a cation exchange column (discussed above), Logue et al. (*45*) prepared ATCA for GC-MS analysis by derivatizing with a silylating agent.

Sample preparation for cyanide-protein adducts involved isolation of the protein of interest with subsequent enzymatic digestion of the adducted protein. Fasco et al. (29) used this technique to analyze protein fragments after digestion with trypsin. Although this method was time-consuming and required highly powerful instrumentation, the authors were able to detect protein-cyanide adducts from the plasma fraction of human blood.

Figure 2. König reaction for the spectrophotometric analysis of cyanide.



Spectrophotometric, luminescence, and atomic absorption methods

Early spectrophotometric methods of cyanide analysis from biological fluids were often based on the König synthesis (Figure 2) (39, 101-107, 178). König dye synthesis involves oxidation of cyanide using chloramine-T (82, 109, 118, 125), hypochlorite (87, 110, 123) or bromine water (41, 101, 102, 119) to form a cyanogen halide (see Figure 2). The cyanogen halide is then reacted with an aromatic amine (normally pyridine) to produce a glutaconic aldehyde product that is measured in the visible region. These methods have adequate sensitivity, but they lack specificity due to interferences from other chemical species commonly present during the analysis of cyanide, especially thiocyanate and thiosulfate (179). Also, they often require lengthy microdiffusion preparations and the products are unstable. Modifications have been developed that yield more stable reagents and increased precision for this type of reaction (41, 44, 87, 115-117, 119, 125).

Spectrophotometric analysis for thiocyanate is often a variation of the König reaction described above. Hypochlorite and thiocyanate react to form the cyanogen chloride, then either pyridine-malononitrile (76) or barbituric acid-pyridine reagent (95, 118, 123) can be used as coupling agents. Cyanogen chloride can also be reacted with isonicotinic acid to produce a glutaconic aldehyde. Condensation of this aldehyde with two molecules of 1,3-dimethylbarbituric acid produces a dye which can be analyzed spectrophotometrically (89). Other early methods (108) involved oxidation of the thiocyanate to hydrogen cyanide, with aeration into alkali solution, permitting the determination of cyanide as described above for the König reaction. The modified König reaction was also applied to the simultaneous analysis of cyanide and thiocyanate (101,

102). Nagashima (109) used the differences in the rates of the reaction of cyanide and thiocyanate with chloramine-T and variations in pH dependence for the simultaneous spectrophotometric determination of cyanide and thiocyanate.

ATCA has also been analyzed spectrophotometrically. Bradham et al. (60) analyzed ATCA from urine as described above. Limitations for this method include interference from a number of species (including cyanide ions) and it is time intensive.

Fluorescence (*37, 110-114*) methods have also been applied to the determination of cyanide in biological fluids. As with the spectrophotometric methods, fluorescence methods also require extraction techniques to isolate cyanide and eliminate interferences from blood. A number of sensitive fluorometric assays to determine cyanide, free of thiosulfate interference (a problem with a number of spectrophotometric methods) have been developed with greater sensitivity than spectrophotometric methods (*110, 111, 114*). One specific fluorometric method of analysis of cyanide is reaction of cyanide with NDA and taurine to create a highly fluorescent molecule that can be used for the fluorometric determination of cyanide (*37, 159*). Cyanide from whole blood has also been determined by flow injection chemiluminescence (*180*). Acidification and distillation (as mentioned above) was used to separate cyanide from interfering whole blood components and a microchip based reactor was used to mix reagents to produce chemiluminescence.

Although non-chromatographic fluorescence methods for the determination of thiocyanate and ATCA have not been published, the possibility of derivatization with fluorescent derivatizing agents exists. For instance, 3-bromomethyl-7-methoxy-1,4-benzoxazin-2-one and coumarin dyes have been used effectively for the HPLC-fluorometric determination of thiocyanate (*163*) and ATCA (*48*), respectively. Cyanide-protein adducts have not been analyzed by fluorescent methods.

Atomic absorption (AA) methods are indirect methods of cyanide or thiocyanate analysis. A metal is added to a sample and a metal complex is formed with the analyte. This complex either precipitates or is extracted into an organic solvent and subsequently analyzed by AA. For example, cyanide has been analyzed by complexing an iron(II)-phenanthroline, and extracting the complex in chloroform for subsequent AA analysis (*181*). Chattaraj and Das (*127*) used this technique with flame AA by forming a complex with copper to determine thiocyanate from biological fluids. This technique has also not been used to determine ATCA or cyanide-protein adducts.

Electrochemical, ion-selective electrode, and biosensor methods

Many electrochemical methods for the detection of cyanide and thiocyanate exist, but few of these methods have been applied to their analysis in biological samples (*15, 90, 131, 134, 135, 182*). Benefits of using electrochemical methods are high sensitivity (for some methods) and quick analysis time. However, they can be subject to multiple interferences from many organic and inorganic ions, including sulfide, ClO_4^-, NO_2^-, N_3^-, and I^- (*130, 183*). Electrochemical methods can also be hampered by narrow working concentration ranges and may require large sample sizes (*132, 137*). Westley and Westley (*130*) used a silver rotating disk electrode, and a dropping mercury electrode for the voltammetric determination of cyanide and thiocyanate in biological samples, including plasma, tissue, and whole blood. Electrochemical detection

can also be used for analysis of cyanide and thiocyanate with ion chromatography (*133*).

Polymeric membrane-based ion selective electrodes (ISEs) have been developed to address some of the issues limiting electrochemical analysis of cyanide and thiocyanate (*21, 132, 136, 183-188*). ISEs can exhibit rapid response, high sensitivity, wide linear range, low cost, and they are usually simple to operate. The polymeric membrane in ISEs contains an ion carrier that interacts selectively with the analyte. To selectively analyze cyanide or thiocyanate, the ion carrier must strongly interact with the analyte anion and weakly interact with other anions. This interaction is often enhanced by the use of a metal-ligand interaction. Although many ISEs exist, they are somewhat more limited for anions compared with cations (*183*).

ISEs have been developed for cyanide analysis, but few have been used for analysis of cyanide from biological samples. This is due to their interaction with multiple ion interferences. If these interferences can be removed, then ISE methods can be used for the determination of cyanide from biological samples. In fact, the standard method for analysis of cyanide from fish tissues is based on ISEs (*184*). For this method, tissue samples are prepared by homogenizing, acidifying, and distilling internal organs of fish species (similar to those mentioned above in the *Sample preparation and storage* section). Cyanide present in the homogenized tissue is converted to HCN and captured in an alkaline solution following distillation. A cyanide ISE is then used to analyze for cyanide based on its interaction with silver. Another ISE test using gold disc electrodes coated with a sulfonated tetrafluoroethylene copolymer was recently developed by Lindsay and O'Hare for the analysis of cyanide in blood without sample pretreatment (*185*).

Thiocyanate, due to its lipophilicity, is highly suited for electrostatic interaction techniques, and ISEs have been used successfully for the selective determination of thiocyanate (*21*). ISEs have been described that exhibit good agreement with values determined by ion exchange chromatography (*136*) or spectrophotometric methods (*132, 183, 186, 187*), and were used to analyze thiocyanate from serum (*136*), urine (*132, 183, 186-188*), and saliva (*132, 183, 186, 188*). These include an ISE based on crystal violet thiocyanate or methylene blue thiocyanate in nitrobenzene (*136*), and a highly selective thiocyanate polymeric membrane sensor that contained a nickel(II)-azamacrocycle complex coated on a graphite electrode (*132*). The Ni(II)-electrode showed a great enhancement in selectivity coefficients and detection limits from previously reported electrodes and was successfully used for the analysis of thiocyanate in urine, saliva and milk. Other electrodes which demonstrated good selectivity for thiocyanate in biological samples were a poly(vinyl chloride) (PVC) membrane electrode based on a nickel-hexaazacyclotetradecane derivative (*186*), a PVC membrane electrode with an unsymmetrical nickel(II) macrocyclic complex as an ion carrier (*183*), and a graphite electrode based on iron phthalocyanine membranes with sodium tetraphenylborate as a lipophilic anionic additive (*187*).

Many biosensors (small detection devices normally based on biological activity toward an analyte) exist for the determination of cyanide, including microbial cyanide sensors, sensors based on the enzyme inhibition of cyanide, and sensors based on cyanide degrading enzymes. Most biosensors have advantages of being portable, low cost, easy to use, and can have high selectivity. Limitations of biosensors include degradation of biological components that make up these sensors,

inconsistent electrochemical signals, and difficulty producing sufficient quantities and activities of enzymes or microbes on which these sensors depend. Most of the biosensors developed for cyanide analysis have not been applied to the analysis of biological samples, although a method for the determination of cyanide in fish was recently described (*15*). Organs of the fish were homogenized with NaOH and a fungal enzyme extract was used to produce formate from metal-cyanide complexes. Then formate dehydrogenase and nicotinamide adenine dinucleotide (NAD+) were added to convert the formate into CO_2, which reduced NAD+ to NADH. NADH was monitored spectrophotometrically and used to determine the amount of cyanide in the sample.

Currently, biosensors have not been applied to thiocyanate detection from biological samples. Also, electrochemical and biosensor methods have not been applied to analyze for ATCA and cyanide-protein adducts from biological samples.

Liquid Chromatography

The complex nature of biological matrices, small concentrations of cyanide and its metabolites, and the high number of species that interfere with spectrophotometric, luminescent, and electrochemical methods have necessitated analysis of cyanide and its metabolites by more powerful methods. Liquid chromatographic techniques can determine trace amounts of an analyte and can efficiently separate analytes from interfering components in the matrix. Chromatographic techniques, both liquid and gas, also have the ability to simultaneously analyze for cyanide and thiocyanate. For these reasons, they have gained popularity in analysis of cyanide and cyanide metabolites in biological samples.

Three types of liquid chromatography have been used to analyze cyanide: reverse-phase high-performance liquid chromatography (RP-HPLC) (*36, 46, 48, 80, 84, 88, 93, 159, 160, 162-164*), ion chromatography (IC) (*69, 95, 97, 98, 133, 138, 164-169, 189-191*), and capillary electrophoresis (*139*). RP-HPLC methods are common, but generally require pretreatment steps for each anion or multiple post-column reagents. Ion chromatography is often used for thiocyanate analysis, and although these methods are categorized separately, ion chromatography is often a modified RP-HPLC method (i.e., a column modifier is added to the mobile phase to create an ionic stationary phase). Some common detectors used in the liquid chromatographic analysis of cyanide or its metabolites include spectrophotometric (*46, 89, 95, 160, 166*), fluorescence (*37, 84, 88, 93, 159, 163, 164*), electrochemical (*162, 165, 168, 191*), or mass spectrometric (*29, 80*) detection.

Several groups have adapted the spectrophotometric detection of cyanide and thiocyanate in blood and urine based on the König reaction to RP-HPLC (*89, 95, 161, 166*). This reaction has also been used for HPLC with fluorometric detection. Toida et al. (*84*) analyzed cyanide in blood at picomole levels with HPLC and fluorometric detection by using a variant of the König reaction, replacing pyridine with pyridine-barbituric acid. Fluorescence detection was also used for the determination of cyanide in human erythrocytes and whole blood using RP-HPLC with pre-column derivatization with NDA and taurine (*88*). A number of other fluorometric HPLC methods have been developed for the analysis of cyanide and its metabolites from biological fluids (*69, 88, 93, 159, 163*). Thiocyanate has also been analyzed using RP-HPLC with fluorometric detection. Tanabe et al. (*93*) used HPLC with fluorometric detection to determine

thiocyanate in saliva and serum based on the formation of fluorescent cerium (III) from cerium (IV) by a redox reaction. RP-HPLC has also been used for the simultaneous detection of cyanide and thiocyanate. Using pentafluorobenzylbromide as derivatizing agent, Liu and Yun (*46*) simultaneously determined cyanide and thiocyanate in blood and milk. Mass spectrometric detection was used by Tracqui and Tamura (*80*) after microdiffusion sample preparation followed by derivatization with NDA and taurine for HPLC-MS analysis of cyanide in blood.

ATCA and cyanide-protein adducts have also been analyzed with RP-HPLC (*29, 48*). Lundquist (*48*) used HPLC with fluorometric detection for the determination of ATCA in urine. Although this method was time consuming, it was able to detect ATCA in the urine of smoking individuals. RP-HPLC with mass spectrometric detection has been used to determine cyanide and blood-protein adducts. Fasco et al. (*29*) used RP-HPLC with tandem mass spectrometric detection to analyze cyanide-adducted proteins from human plasma. The method involved isolation and enzymatic digestion of cyanide-adducted human serum albumin.

A number of authors have used ion exchange (or ion interaction) chromatography to analyze biological fluids for thiocyanate because of the ease of thiocyanate analysis. Chinaka et al. (*69*) used NDA derivatization of cyanide with an ion exchange column, allowing the simultaneous determination of cyanide and thiocyanate. Lundquist et al. (*95*) used an ion exchange column with visible spectrophotometric detection for the determination of thiocyanate in serum and urine. Connolly et al. (*98*) used ion interaction LC with UV detection to analyze thiocyanate in urine. Other authors have coated normal reverse-phase HPLC columns or used ion-pairing agents to analyze thiocyanate. Examples of this type of analysis include, a RP-HPLC column coated with cetyldimethylamine (*97, 169*), a zwitterionic micellar-coated stationary phase (*189*), and bovine serum albumin as the stationary phase with tartaric acid as the eluent (*190*). Brown et al. (*164*) used a cetylpyridinium coated reverse-phase column to create an ion-exchange column for the analysis of thiocyanate from rainbow trout plasma in a pharmacokinetic study of thiocyanate exposure. Cookeas and Efstathiou (*191*) successfully analyzed thiocyanate in saliva with flow injection and ISE detection using a cobalt-phthalocyanine modified carbon paste electrode. ISEs have also been used to detect thiocyanate in urine following ion chromatography (*133*).

Capillary electrophoresis (CE) has been used successfully for the analysis of thiocyanate in biological fluids (*139*). Glatz et al. (*139*) analyzed blood, urine, and saliva samples for thiocyanate with CE and spectrophotometric detection no sample preparation aside from dilution. CE methods for cyanide, ATCA, and cyanide-protein adducts have not been suggested.

Gas Chromatography

One of the most common methods for analysis of cyanide, thiocyanate, and more recently ATCA is gas chromatography. Common detectors used for analysis of cyanide or its metabolites are the electron capture detector (ECD) (*66, 70, 81, 140, 141, 151, 154, 156, 192*), nitrogen-phosphorus detector (NPD) (*33, 62, 63, 78, 79, 86, 124, 144-147, 150, 152, 155*), and mass spectrometric (MS) detector (*45, 83, 94, 148, 153, 158*). Although most of these methods are used for detection of cyanide in blood some groups have applied gas chromatography techniques for other biological matrices and for the detection of thiocyanate and ATCA.

For the detection of cyanide from biological matrices, no specific derivatization is necessary as HCN is volatile. Therefore, the most common pre-analysis step in GC analysis of cyanide is sampling of cyanide from the sample head space. Either equilibrium or dynamic headspace methods can be used to prepare a sample for GC analysis (*63, 66, 70, 79, 81, 94, 124, 141, 143, 149, 152, 158*). Another pre-analysis step for cyanide analysis is cryogenic oven trapping, which has been used to trap liberated HCN into headspace with high resolution and sensitivity (*86, 147*). Because thiocyanate and ATCA are non-volatile, they require chemical modification (normally derivatization) to allow analysis by GC. Chemical modification is also necessary for analysis of cyanide by ECD (because of its poor response) or for simultaneous analysis of cyanide and thiocyanate. Therefore, a number of pretreatment steps have been developed to facilitate the analysis of cyanide, thiocyanate, and ATCA by GC.

In GC analysis, the NPD permits the sensitive and specific detection of nitrogen-containing compounds, and has been used for the detection of cyanide and thiocyanate in plasma, urine and saliva (*79, 83, 145, 146, 150*). However, this detector may be unstable at times, and is less sensitive than some other types of GC detectors. This has led to the creation of analytical methods that take advantage of more stable and sensitive detectors such as the ECD and MS. When using ECD detectors in GC analysis of HCN, derivatization is required before analysis. Derivatization of cyanide has been performed by alkylation of cyanide (*83*) or the conversion of HCN into cyanogen chloride by choramine-T oxidation (Figure 2) (*70, 151, 156*). Kage and coworkers (*83*) used GC-ECD to simultaneously analyze cyanide and thiocyanate using an extractive alkylation technique.

One of the most sensitive methods for analysis of cyanide, thiocyanate, and ATCA is GC-MS (*83, 94*). With MS detection, stable isotope standards (e.g. ^{13}CN for ^{12}CN) can be used to correct for matrix effects common to cyanide and cyanide metabolites. This can eliminate the need for standard addition techniques and matrix matching. Dumas et al. (*94*) used stable isotope standards with GC-MS and head space analysis to analyze cyanide concentrations. Kage and co-workers also used GC-MS to analyze both cyanide and thiocyanate simultaneously (*83*).

ATCA has also been analyzed by GC-MS. Logue et al. (*45, 157*) analyzed ATCA in plasma and urine using GC-MS by first converting the non-volatile metabolite into a volatile form using trimethylsilyl-trifluoroacetamide. Cyanide-protein adducts have not been analyzed by GC-MS.

Suggested procedures for delayed analysis of biological samples

For cyanide analysis, a sample should be collected quickly after exposure and cyanide analysis should be performed as soon as possible because of the rapid detoxification of cyanide from blood samples (discussed above). However, if analysis of cyanide cannot be performed quickly and storage of biological samples is necessary, the following should be considered:

1) *Volatility and nucleophilicity of cyanide.* As described above, HCN is volatile and cyanide ion is nucleophilic. Tightly sealed vials, low temperatures, high pH, and the addition of preserving agents are common procedures that have been used to prevent evaporative loss of cyanide. Storing samples at low temperatures is extremely important to reduce evaporative loss, and slow biochemical reactions. However, there

are many discrepancies in the literature when evaluating the stability of cyanide in biological fluids under various conditions (43, 87, 131, 172, 179). Generally, nucleophilic losses are reduced by adding sequestering agents (e.g., hydroxocobalamin) or chemicals that produce sequestering agents (e.g. sodium nitrite to produce methemoglobin) (87, 123, 193). One method found to improve cyanide stability is the addition of silver ions to biological samples (87).

2) *Cyanide concentration varies in biological components.* Cyanide in blood primarily resides in erythrocytes (red blood cells) (*64, 82, 87, 174, 194*) by binding to methemoglobin, forming cyanomethemoglobin. Cyanide may also be present in plasma, especially if cyanide concentrations exceed erythrocyte concentrations (*82, 87*). To ensure accurate cyanide concentrations when analyzing blood, collection containers that contain anticoagulants (e.g., heparin) should be used to prevent clotting. Analysis of cyanide from tissues requires knowledge of the behavior of cyanide in specific organs since the enzyme that catalyzes conversion of cyanide to thiocyanate has highly variable concentrations depending on the organ. Therefore, in specific organs, there will be little to no cyanide because of extremely fast metabolism to thiocyanate.

3) *Potential for cyanide formation during storage.* Artifactual formation of cyanide may also occur in biological samples depending on storage conditions (*77, 172-174*). It has been suggested that oxyhemoglobin (*175*), thiocyanate oxidase (*173, 174*), and white blood cells (*77*) may oxidize thiocyanate to cyanide and these reactions are dependent on the temperature and pH of the sample. Microorganisms may also be responsible for cyanide production and low temperature storage will help to eliminate their growth (*173*).

These considerations are common to all the analytical methods for analysis of cyanide from biological samples and certainly contribute to discrepancies in similar studies in the literature. For post-mortem analysis of cyanide, production and transformation of cyanide must be considered when interpreting results along with other considerations discussed above (*74, 195-198*).

Some of the same issues must also be considered for thiocyanate, as a number of problems with storage of samples to be analyzed for thiocyanate have been found (*56*). This may be due to interconversion of thiocyanate and cyanide over time and the removal and production of thiocyanate by biological processes other than cyanide detoxification (*24, 58*). It has been suggested that ATCA is not involved in other biological processes and it has been found to be stable during storage (*18, 45, 47, 48, 59*). While ATCA may not have the storage issues of cyanide and thiocyanate, there is little information on its toxicokinetics, which currently limits its use as a biomarker for cyanide exposure. Also, cyanide-protein adducts have recently been discovered, and therefore little information about the toxicokinetics and stability of these adducts is known.

Conclusions

The analytical determination of cyanide and its metabolites is not an easy task due to chemical properties, biological activities, and limited research. Numerous methods have been developed and each has its own advantages and disadvantages. However, they

have all provided insight into the verification of cyanide exposure from analysis of biological samples. Table 3 provides a comparison of analytical techniques for analysis of cyanide or its metabolites based on sensitivity, specificity, sample size, capacity, expertise necessary to perform the method, and cost. Some other key pieces of information should also be considered prior to choosing a method to perform: 1) Was preservation of cyanide and its metabolites during storage addressed?; 2) Were typical interferences for the biological matrix of interest removed?; 3) Were analysis procedures that could result in the loss of cyanide or its metabolites used (i.e., heating or acidification)?

For the analysis of cyanide, the largest inconsistency in the literature is analysis with different preservation techniques. Regardless of the analytical method, a preserving technique needs to be considered so that accurate concentrations of cyanide can be found (*75, 87, 112, 123, 128, 197-200*). If cyanide is analyzed, biological samples should be collected and analyzed as soon as possible for confirmation or refuting cyanide exposure. One also needs to consider that all biological samples will contain endogenous levels of cyanide (and its metabolites). Therefore, baseline levels of the analyte measured should be known prior to concluding an exposure occurred. Problems with direct analysis of cyanide, including short half-lives, artifactual formation of cyanide, and interconversion of cyanide and thiocyanate, contribute to difficulties in the analysis of cyanide for all analytical methods designed to determine cyanide in biological samples.

While cyanide is most often analyzed to determine cyanide exposure, one should consider using analytical techniques that analyze for cyanide metabolites as well as those that analyze for cyanide directly. Although cyanide metabolites may offer longer half-lives, they also have a number of drawbacks. For thiocyanate, the main drawback is large and variable background concentrations in biological samples. Other disadvantages include are interconversion of cyanide and thiocyanate, and the use of thiocyanate by other biological processes not directly related to cyanide metabolism. For ATCA and cyanide-protein adducts, the main drawback is the limited amount of research available on toxicokinetics and relationships of these metabolites to cyanide exposure. Care should be taken when choosing an analytical method to consider not just the parameters of the analytical method but also the toxicokinetics of cyanide and its metabolites.

Table 3. Analytical Methods to determine cyanide and its metabolites in biological fluids.[a]

Technique[b]	Sub-category	Analyte[c]	Matrices	Sensitivity[d]	Specificity[d]	Sample Size[d]	Capacity[d]	Expertise[d]	Cost[d]	Refs.
UV-Vis	None	CN, SCN, ATCA	Blood, Urine, Saliva	Low	Low	0.5-1 mL	Medium	Low	Low	(*41, 44, 60, 61, 76, 77, 82, 87, 89, 101-105, 108, 109, 115-119, 123, 178, 179*)
Lumin-escence	Fluorescence	CN	Blood	Medium	Medium	0.5-1 mL	Medium	Medium	Low-Medium	(*77, 110-114, 124*)
	Chemi-luminescence	CN	Blood	Medium-High	Medium	3 µL	Medium	Medium	Low	(*180*)
Electro-chemistry	None	CN, SCN	Blood, Saliva, Tissue	Medium-High	Medium-High	1-5 mL	Medium-High	Low	Very Low -Low	(*15, 21, 90, 130-137, 182, 183, 185-187, 201, 202*)
AA	Indirect AA	SCN	Blood, Saliva	Medium-High	Medium	1 mL	Low	Medium	Medium-High	(*127*)
Biosensor	None	CN, SCN	Blood, Urine, Saliva	Medium-High	Medium-High	1-5 mL	Medium-High	Low	Very Low -Low	(*15*)
LC	RP-HPLC UV	CN, SCN	Blood, Urine	Medium	High	10-100 µL	High	Medium-High	Medium	(*89, 95, 160, 166*)
	RP-HPLC fluorescence	CN, SCN, ATCA	Blood, Urine, Saliva	High	High	10-100 µL	High	High	Medium	(*37, 84, 88, 93, 159, 163, 164*)
	RP-HPLC electrochemical	SCN	Urine	Medium-High	High	10-100 µL	High	Medium-High	Medium	(*162, 165, 168*)
	RP-HPLC mass spectrometric	CN	Blood	Very High	Extremely High	10-100 µL	High	Very High	High	(*80*)
	RP-HPLC MS-MS	CN-protein adduct	Blood	Extremely High	Extremely High	10-100 µL	Low	Extremely High	Extremely High	(*29*)
	IC	CN, SCN	Blood, Urine, Saliva	Medium	High	1-100 µL	High	High	Medium	(*97, 167, 169*)
	CE	SCN	Blood	High	High	1-10 nL	High	High	Medium-High	(*138, 167*)
GC	GC-NPD	CN, SCN	Blood, Urine, Saliva	High	Very High	1-10 µL	Medium	High	High	(*33, 62, 63, 78, 79, 86, 124, 144-147, 150, 152, 155*)
	GC-ECD	CN, SCN	Blood, Urine, Saliva	Very High	Very High	1-10 µL	High	High	High	(*66, 70, 81, 140, 141, 151, 154, 156, 192*)
	GC-MS	CN, SCN, ATCA	Blood, Urine, Saliva, Tissue	Very High	Extremely High	1-10 µL	High	High	Very High	(*45, 83, 94, 148, 153, 158*)

[a] This table is meant to give a general overview of analytical techniques to analyze cyanide and its metabolites along with a general idea about parameters specific to each analysis technique. Parameters of specific methods within a particular analysis technique may be outside of those listed.

[b] UV-Vis – ultraviolet visible spectrophotometry, AA – atomic absorption, LC – liquid chromatography, RP – reverse phase, MS-MS – tandem mass spectrometric detection, IC – ion chromatography, CE – capillary electrophoresis, GC – gas chromatography, NPD – nitrogen phosphorous detector, ECD – electron capture detector.

[c] CN – cyanide, SCN – thiocyanate, ATCA – 2-amino-2-thiazoline-4-carboxylic acid.

[d] These parameters are related to the general instrumental technique used and not each individual method of analysis.

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