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# Determination of dimethyl trisulfide in rabbit blood using stir bar sorptive extraction gas chromatography-mass spectrometry

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ABSTRACT

Cyanide poisoning by accidental or intentional exposure poses a severe health risk. The current Food and Drug Administration approved antidotes for cyanide poisoning can be effective, but each suffers from specific major limitations concerning large effective dosage, delayed onset of action, or dependence on enzymes generally confined to specific organs. Dimethyl trisulfide (DMTS), a sulfur donor that detoxifies cyanide by converting it into thiocyanate (a relatively nontoxic cyanide metabolite), is a promising next generation cyanide antidote. Although a validated analytical method to analyze DMTS from any matrix is not currently available, one will be vital for the approval of DMTS as a therapeutic agent against cyanide poisoning. Hence, a stir bar sorptive extraction (SBSE) gas chromatography – mass spectrometry (GC–MS) method was developed and validated for the analysis of DMTS from rabbit whole blood. Following acid denaturation of blood, DMTS was extracted into a polydimethylsiloxane-coated stir bar. The DMTS was then thermally desorbed from the stir bar and analyzed by GC–MS. The limit of detection of DMTS using this method was 0.06 μM with dynamic range from 0.5–100 μM. For quality control standards, the precision, as measured by percent relative standard deviation, was below 10%, and the accuracy was within 15% of the nominal concentration. The method described here will allow further investigations of DMTS as a promising antidote for cyanide poisoning.



## 1. Introduction

Cyanide ($LD_{50,\ human}$ = 1.5 mg/kg for an oral exposure; $LC_{50,\ human}$ = 524 ppm for a 10 min inhalation exposure to HCN) is a rapidly acting, highly toxic compound that inhibits cytochrome c oxidase and subsequently causes cellular hypoxia, which may eventually result in death [1–6]. It can be introduced into the body by a number of different ways, such as consumption of cyanogenic plants and fruits [7–9] (e.g., cassava roots, yam, sorghum, bitter almonds), inhalation of hydrogen cyanide gas from fire [10] (i.e., burning of acrylonitrile, polyurethane, wool, silk, rubber produces HCN) and cigarette smoke, occupational exposures (e.g., the 2015 warehouse explosion in Tianjin, China [11,12]) and from use of cyanide as a suicide, homicide, or chemical warfare agent [13–15] (e.g., in World War I, II, Tokyo subway attack, etc. [1]). The availability of cyanide, due to its versatile use in industrial processes (e.g., electroplating and plastic processing) and its rapidly acting nature, makes it an important threat to mankind [1,16]. Currently, there are three major classes of cyanide therapeutics that are approved by the U.S. Food and Drug Administration (FDA): methemoglobin generators, direct sequestering agents, and sulfur donors [3,17–19].

Sodium nitrite, primarily classified as a methemoglobin generator, is generally agreed to function by indirect sequestration of cyanide [20]. Nitrite oxidizes ferrous (2+) iron to ferric (3+) iron in hemoglobin to form methemoglobin, which strongly binds cyanide to form cyanomethemoglobin. Methemoglobin serves as a temporary binding site for cyanide ion, and thus transiently decreases free cyanide in the bloodstream. Another recently proposed alternative mechanism of action is the conversion of nitrite to nitric oxide, which can then displace cyanide bound to cytochrome c oxidase [21,22]. After displacement, cyanide is subsequently converted to less harmful compounds through normal metabolism or neutralized via a combination therapy (e.g., thiosulfate) [22]. With either

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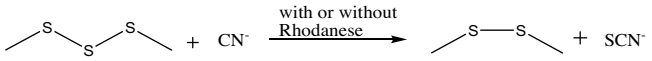


**Fig. 1.** Schematic representation of the reaction of DMTS and cyanide to form dimethyl disulfide (DMDS) and thiocyanate.

detoxification mechanism, the major limitation of sodium nitrite is the production of methemoglobin. Excessive methemoglobin production (>30%) is a health risk, especially in children, leading to headaches, cyanosis, fatigue, coma, and even death [17]. Additionally, conversion of hemoglobin to methemoglobin lowers the oxygen carrying capacity of the blood, which can exacerbate carbon monoxide-induced reduction in oxygen carrying capacity in smoke-inhalation victims [3,20].

While sodium nitrite indirectly binds cyanide, hydroxocobalamin acts by directly sequestering cyanide [3,23,24]. The high affinity of cyanide towards the cobalt atom in hydroxocobalamin allows the formation of cyanocobalamin, which is easily excreted from the body in the urine. Although administration of hydroxocobalamin produces only mild side effects, it requires a high dose for optimum therapeutic effect (5 g administered over 15 min) [24]. Therefore, hydroxocobalamin typically needs to be administered intravenously by trained personnel over a long period of time, which severely limits its applicability during mass casualty events [3,17].

Sodium thiosulfate is the only currently approved sulfur donor, the third class of cyanide antidote, for treatment of cyanide poisoning. It donates a sulfur to cyanide, converting it to minimally toxic and renally excreted thiocyanate [25,26]. Although sodium thiosulfate has few adverse effects, its antidotal activity is mainly limited by its short biological half-life, small volume of distribution, and its dependence on rhodanese to aid sulfur transfer [3]. Rhodanese is a sulfur transferase enzyme primarily located in mitochondria of the liver and kidneys, with low concentrations in the brain, an organ most susceptible to cyanide-induced histotoxic anoxia. The limited lipophilicity of thiosulfate as a result of its anionic charge, also adversely impacts its ability to penetrate the cell and reach the mitochondrial rhodanese [3].

Considering the serious limitations of the current antidotes, several investigations have been in progress to develop the next generation of cyanide therapeutics [19,25–29]. One promising approach is the development of a sulfur-donating compound that works effectively with or without rhodanese [25,26]. Based on this approach, numerous synthetic and naturally occurring sulfur donors have been evaluated for in-vitro and in-vivo efficacy [3], with dimethyl trisulfide (DMTS) suggested as the most promising next generation sulfur donor for treatment of cyanide poisoning. The reaction by which DMTS detoxifies cyanide into thiocyanate is shown in Fig. 1. The rhodanese sulfur transfer mechanism is well-discussed in the literature [30]. However, the mechanism of direct DMTS sulfur transfer is not well understood, but is known to occur [31]. Moreover, the high lipophilicity of DMTS permits its effective penetration of the cell membrane and the blood brain barrier, allowing better in-vivo antidotal efficacy than thiosulfate [31]. Recent in vitro studies demonstrate that, compared to sodium thiosulfate, DMTS is 43 times more effective at detoxifying cyanide in the presence of rhodanese [31]. Whereas, in absence of rhodanese, the difference in efficacy is even higher, with DMTS producing 79 times greater efficacy than thiosulfate [31]. These results are consistent with in vivo studies, where the therapeutic antidotal ratio of DMTS was more than triple of what was observed for thiosulfate at the same dose. The in vivo and in vitro efficacy data confirm that DMTS is a superior cyanide countermeasure compared to the present sulfur donor therapy of thiosulfate.

Despite the potential advantages of DMTS, the lack of a validated analytical method for its analysis may limit vital studies for therapeutic translation of DMTS. The only relevant report in regards to analysis from a biological matrix was published by Shirasu and coworkers, where DMTS was identified as a source of sulfurous malodor in fungating cancer wounds [32]. However, the concentrations of DMTS were not well quantified, and the method was not validated. Besides this single study, DMTS has mainly been identified as a naturally occurring compound contributing to pungent odors in vegetables such as garlic, soy, cabbage, broccoli, and cauliflower [32–36]. In addition, it has also been detected or quantified from fermented and aged food (cheese) and drinks (milk, beer [37], sake, and wine), where it most likely comes from oxidation of methanethiol, a bacterial degradation product of methionine [32,38–42]. The analysis of DMTS is typically accomplished using headspace analysis or solid-phase microextraction with GC–MS. While these analytical techniques provide direction for the analysis of DMTS from blood, a validated analytical method is not currently available (from any matrix), but is critical for further development of this promising antidote. Therefore, the objective of the current study was to develop a validated method for the analysis of DMTS from whole blood. To accomplish this objective, a stir bar sorptive extraction (SBSE) GC–MS analysis technique for analysis of DMTS from rabbit whole blood samples was developed.

## 2. Experimental

### 2.1. Reagents and standards

All reagents were at least reagent grade unless otherwise noted. Methanol (LC–MS grade) and nitric acid were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Reverse-osmosis water was purified to 18.2 MΩ-cm using a polishing unit from Lab Pro, Labconco (Kansas City, KS, USA). Dimethyl trisulfide (DMTS) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Gerstel Twister® stirbars (film thickness 0.5 mm, length 10 mm) were purchased from Gerstel, Inc. (Linthicum, MD, USA). Isotopically labeled internal standard (IS), dimethyl-d6-trisulfide (DMTS-d6), was acquired from US Biological Life Sciences (Salem, MA, USA). DMTS stock solution was prepared fresh for each experiment. The internal standard solution was prepared from a 10 mM stock solution in methanol stored at −30 °C.

### 2.2. Biological fluids

For method development and validation, rabbit whole blood (EDTA anti-coagulated) was purchased from Pelfreeze Biological (Rogers, AR, USA) and stored at −80 °C until used. Rabbit whole blood was used to develop the method presented here because we planned to utilize a rabbit model developed by our collaborators to prove the applicability of the analytical method for the analysis of blood DMTS concentrations. However, at the time we were finalizing the method validation, efficacy studies of DMTS were transitioned to a mouse model. Male Charles River (North Carolina, USA) CD-1 mouse (18–20 grams) blood from DMTS efficacy studies was gathered at Sam Houston State University. DMTS was intramuscularly administered at 200 mg/kg. The mice were anesthetized (after 10 min) and placed on isoflurane. Blood was collected intravenously using a heparinized Pasteur pipette and transferred to heparinized 1.5 mL centrifuge tubes. An aliquot of blood (~150 μL) was then frozen and shipped on dry ice to South Dakota State University. Upon receipt, samples were stored at −80 °C until ready for analysis. Due to the limited volume of mouse blood, each sample (100 μL) was transferred to a clean vial and diluted in DI water to 500 μL before extraction and analysis.

All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals [43] by an Association