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Analysis of potential cyanide antidote, dimethyl trisulfide, in whole blood by dynamic headspace gas chromatography–mass spectroscopy

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ABSTRACT

Cyanide is a rapidly acting and highly toxic chemical. It inhibits cytochrome c oxidase in the mitochondrial electron transport chain, resulting in cellular hypoxia, cytotoxic anoxia and potentially death. In order to overcome challenges associated with current cyanide antidotes, dimethyl trisulfide (DMTS), which converts cyanide to less toxic thiocyanate *in vivo*, has gained much attention recently as a promising next-generation cyanide antidote. While there are three analysis methods available for DMTS, they each have significant disadvantages. Hence, in this study, a dynamic headspace (DHS) gas chromatography–mass spectroscopy method was developed for the analysis of DMTS from rabbit whole blood. The method is extremely simple, involving only acidification of a blood sample, addition of an internal standard (DMTS-d_6) and DHS-GC–MS analysis. The method produced a limit of detection of 0.04 μM for DMTS with dynamic range from 0.2 to 50 μM. Inter- and intraassay accuracy (100 ± 15% and 100 ± 9%, respectively), and precision (<10% and <9% relative standard deviation, respectively) were good. The validated method performed well during pharmacokinetic analysis of DMTS from the blood of rats treated with DMTS, producing excellent pharmacokinetic parameters for the treatment of cyanide exposure. The method produced significant advantages over current methods for analysis of DMTS and should be considered as a "gold standard" method for further development of DMTS as a potential next-generation cyanide countermeasure.

1. Introduction

Hydrogen cyanide (HCN) is an extremely poisonous chemical with a human LD_{50} of only 150–173 ppm via inhalation for a 30-min exposure. Because of its toxicity, HCN has been used as a chemical warfare agent, but it is more commonly found in smoke from cigarettes or fires and in food items [1–3]. Despite its serious health and safety related concerns, both forms of cyanide (HCN and CN^-, inclusively represented as CN) are widely used in the chemical industry to produce polymers, to fabricate synthetic fibers, for electrolysis, to extract minerals, for electroplating, and for pest control [2,4,5]. Because of its industrial uses, the approximate global industrial need for CN is 1.1 million tons per year [4]. CN exposure can occur through inhalation, ingestion and absorption through the skin. Once CN is absorbed, it quickly distributes throughout the body and moves through cells to strongly bind with cytochrome c oxidase. CN blocks the ability of oxygen to bind to cytochrome c oxidase, thereby terminating oxidative phosphorylation. As a result, glycolysis becomes the preferred method of energy production, increasing the lactic acid concentration in cells, resulting in metabolic acidosis. As a result, the toxic outcomes of CN poisoning include cellular hypoxia, cytotoxic anoxia, cardiac arrest, and death [1,3,4,6].

Three antidotes have commonly been used to treat CN poisoning: sodium nitrite (methemoglobin/nitric oxide generator), hydroxocobalamin (direct binding agent), and sodium thiosulfate (sulfur donor). They are commercially available as Cyanokit® (hydroxocobalamin) and Nithiodote® (the combination of sodium thiosulfate and sodium nitrite), and both are administered intravenously (IV). Hydroxocobalamin has a strong affinity towards CN, binding it to form cyanocobalamin (Vitamin B_{12}), which is water soluble and readily excreted from the body [7]. Although hydroxocobalamin has a rapid onset of action and is generally considered safe, it has some limitations. It requires on-site reconstitution, is

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expensive, and has a large recommended dose (5 g administered by IV infusion over 15 min). It also must be administered at a rate of approximately 15 mL/min by trained personnel, which limits its acceptability in mass-exposure situations. Moreover, a second dose of 5 g is sometimes required over a prolonged period (15 min to 2 h) depending upon the severity of poisoning [7–9].

Sodium nitrite, which is often used in combination with sodium thiosulfate, antagonizes CN activity by generating methemoglobin and by forming nitric oxide (NO). It oxidizes Fe^{2+} to Fe^{3+} in hemoglobin to form methemoglobin. Methemoglobin has a strong affinity to CN and serves as a temporary binding site. Methemoglobin then transiently removes free CN as cyanomethemoglobin [3,7]. Although methemoglobin generation is classically considered nitrite's protection mechanism, it can also undergo biotransformation to form NO, which can directly displace CN from cytochrome c oxidase, and reverse its toxicity [10]. Sodium nitrite is commercially available for IV administration (300 mg/10 mL vial at a rate of 2.5–5 mL/min) in combination with sodium thiosulfate (Nithiodote®), with a second dose (150 mg at a rate of 2.5–5 mL/min) potentially required, depending upon the severity of poisoning [9]. Unlike hemoglobin, methemoglobin cannot carry oxygen and its excess production (>30%) leads to headaches, cyanosis, fatigue, coma, and death in severe cases. Moreover, it causes hypotension and should not be used for smoke-inhalation patients with high carboxyhemoglobin (<10%) [3], which also reduces the oxygen-carrying capacity of blood. Therefore, supplemental oxygen and methylene blue may also be administered to convert methemoglobin back to hemoglobin, following nitrite administration [3,11,12].

Sodium thiosulfate converts CN to less toxic thiocyanate in presence of rhodanese (a mitochondrial thiosulfate sulfurtransferase enzyme). The thiocyanate is subsequently excreted in urine [8,13]. Although sodium thiosulfate is safer than sodium nitrite, it has a delayed onset of action. Therefore, it is administered with sodium nitrite as Nithiodote® to overcome this limitation [3]. It is administered intravenously (12.5 g/50 mL vial) and requires a large dose (12.5 g administered by IV infusion over 10 min) immediately following the administration of sodium nitrite, with a second dose of 6.25 g potentially required [9]. Like hydroxocobalamin and sodium nitrite, trained medical personnel are necessary for administration, which limits the use of sodium thiosulfate in mass casualty CN-poisoning events.

The limitations of currently available antidotes have necessitated the search for next-generation CN antidotes. Recently, dimethyl trisulfide (DMTS) has shown impressive efficacy against CN toxicity and has other advantages over current treatments [1,14,15]. DMTS, which is found in some natural and processed foods (e.g., garlic and soy sauce), converts CN to thiocyanate and is 43 times more effective than thiosulfate in presence of rhodanese [7,16–20]. In the absence of rhodanese, DMTS shows even higher relative efficacy (79 times) [7]. Animal studies have shown that DMTS is up to 3 times more effective at treating CN poisoning than sodium thiosulfate, as measured by antidotal potency ratio (APR) [21]. Because it is less dependent on rhodanese and has higher cell membrane permeability than thiosulfate, DMTS promises to be more effective as a sulfur donor. It can also be administered intramuscularly (IM), which allows feasible use in a mass-casualty scenario [22].

Three methods of analysis of DMTS from biological samples have been published, including stir bar sorptive extraction (SBSE) GC–MS [7], headspace solid-phase microextraction (HS-SPME) GC–MS, and HPLC-UV techniques [23]. Although these methods are effective, each approach has significant disadvantages. The SBSE method features simple acidification of blood, extraction of DMTS via an SBSE stir bar, and analysis of the stir bar via thermal desorption GC–MS. While it is highly sensitive (LOD = 0.06 μM), this method does have the disadvantages of requiring sorptive stir bars to extract DMTS from the blood matrix, making it a relatively costly technique (even though the stir bars can be reused multiple times) and the sample preparation is relatively lengthy. The HPLC-UV method involves defibrinating blood, addition of DMDS (used as IS), precipitation of blood proteins by centrifugation (14,000 × *g* at 4 °C for 5 min), and analysis of supernatant by HPLC using C8 column and a UV detector at 215 nm. While this technique has the advantage of more straightforward and affordable sample preparation, it is 193 times less sensitive than the SBSE technique, with a reported LOD of approximately 11.56 μM (reported as 1.46 μg/mL). Also, DMTS is converted to DMDS during its reaction with CN, so it should not be used as an IS in DMTS-treated and CN-exposed individuals. For increased sensitivity, the same authors used an SPME technique which featured homogenized brain samples (in aqueous polysorbate 80 or HPLC grade ethanol), extraction of DMTS via direct immersion SPME with a polydimethylsiloxane (PDMS) sorbent, and analysis via GC–MS. While the reported LOD (213 ng/g of brain, which is 1.49 μM) is much lower than the HPLC method, it is still about 25 times less-sensitive than the SBSE technique. Additionally, similar to SBSE, SPME requires relatively expensive microextraction components. To overcome the limitations of current DMTS analysis methods, there is a need to develop a simple, sensitive, and less costly technique to analyze DMTS from whole blood.

Therefore, the objective of this study was to develop and validate a simple and effective DHS-GC–MS method for the detection of DMTS in rabbit whole blood which combines the advantages of each of previous published methods. The availability of such a method would accelerate further development of DMTS as a possible next-generation CN antidote.

2. Experimental

2.1. Materials

All reagents and solvents were of reagent grade unless mentioned otherwise. Methanol (LC–MS grade) and sulfuric acid (certified ACS plus) were purchased from Fisher Scientific (Fair Lawn, NJ, USA). Reverse-osmosis water used for this study was passed through a polishing unit (Lab Pro, Labconco, Kansas City, KS, USA) and had a resistivity of 18.2 MΩ−cm (referred to as deionized (DI) water). DMTS and internal standard (IS) dimethyl-d_6 trisulfide (DMTS-d_6) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and US Biological Life Sciences (Salem, MA, USA), respectively. Freshly-prepared DMTS stock solution in methanol (200 mM) was used for each experiment and the standard DMTS container was kept in the dark at ambient temperature. As-received IS solution was diluted in methanol to produce a stock solution of 100 mM, which was further diluted to 1 mM in DI water, and stored in −30 °C freezer (Isotemp plus freezer, Fisher Scientific, NJ, USA). The thermal desorption tube, filled with Tenax® TA sorptive material, was obtained from Gerstel Inc. (Linthicum, MD, USA). The sorbent was conditioned using a TC 2 tube conditioner and Aux-controller 163 (Gerstel Inc., MD, USA) for 8 h at 315 °C under 68 psi of ultra-high purity (UHP) 5.0 grade nitrogen gas (A-OX Welding Supply Co., Sioux Falls, SD, USA) before use for the first time. One TD tube was used for the entirety of the current study.

2.2. Biological samples

Rabbit whole blood (Non-sterile with EDTA), obtained from Pel-Freez Biologicals (Rogers, AR, USA), was used for method development and validation, and was kept in a −80 °C freezer (TSU series, Thermo scientific, NJ, USA) until used. Following validation, the effectiveness of the DMTS method was evaluated by ana-

lyzing blood from a DMTS pharmacokinetic study performed at United States Army Medical Research Institute of Chemical Defense (USAMRICD, Aberdeen Proving Ground, MD, USA). Male Sprague Dawley® SAS-400 rats (Charles River Laboratories, Kingston, NY, USA), implanted with jugular vein catheters, were used for this study. Rats, weighing between 250–350 g, were IM injected with a proprietary formulation of DMTS to the right caudal thigh muscle. Multiple blood draws were performed from each catheterized rat. A maximum of 7 blood draws for each rat were randomly distributed among the cohorts at times: 0 (blank control), 2, 5, 10, 15, 30, 60, 90, 120, 180, 240, 300 and 360 min post-DMTS IM injection. Note: The total maximum blood volume drawn from each rat did not exceed 700 μL. Negative control samples (blank controls) were drawn from rats prior to DMTS injection. For each sample, 50 μL of blood was collected into a syringe from the jugular vein catheter, and the blood samples were prepared as below (in the "Preparation of DMTS standards and samples" section). The prepared samples were then flash frozen and shipped to South Dakota State University (Brookings, SD, USA) on dry ice for analysis. Samples were kept at −80 °C until analyzed. All animals were handled and housed in accordance with the Guide for the Care and Use of Laboratory Animals published by the United States National Institutes of Health (NIH Publication No. 85-23, Revised 1996). The experimental protocol was approved by Institutional Animal Care and Use Committee at USAMRICD.

2.3. Preparation of DMTS standards and samples

The stock solution of DMTS (200 mM in methanol) was diluted to 4, 10, 15, 20, 40, 100, 150, 200, 400, 700, and 1000 μM with DI water to produce working standard solutions. Whenever needed, 40 μM aqueous IS was prepared from a 1 mM stock solution in methanol and was either added directly to acidified blood (see below) or spiked (100 μL) into DMTS working standard solutions (1:1 mixture of DMTS:IS).

Two techniques were used to prepare blood for DHS analysis: "large volume" and "small volume" sample preparation techniques. For preparation of samples where a large volume of blood is available, 450 μL of whole blood (i.e., rabbit blood for this study) was transferred to a 4-mL HPLC vial with 500 μL of 0.4% sulfuric acid. The mixture was then vortexed for 10 s at 252 × g to ensure proper coagulation and denaturation of proteins. Note that acid denaturation prior to the addition of DMTS and/or IS is a crucial step in DMTS sample preparation to mitigate the quick and irreproducible degradation of DMTS in non-denatured blood. Finally, 50 μL of a DMTS:IS mixture (for standards) or an IS solution (for samples) was added to the acidified blood (950 μL). The sample was then vortexed for 10 s at 252 × g, and an aliquot of the sample solution (100 μL) was placed in a 20-mL HS vial with a polytetrafluoroethylene-lined septum for analysis.

For the preparation of blood samples from the animal studies where only a small volume of blood was available, 0.4% sulfuric acid (950 μL) was added to a 2-mL centrifuge tube along with 50 μL of whole blood (e.g., rat blood in this study) with mixing. IS (25 μL of 6 μM) was then added. The sample was mixed and flash frozen. For preparation of standards for the small volume protocol, acidified blood (25 μL) was spiked with 50 μL of a mixture of DMTS:IS, and then vortexed for 10 s to ensure proper mixing. An aliquot of this preparation (900 μL) was placed in a 20-mL HS vial for analysis.

In both approaches, the concentrations of DMTS in the acidified blood calibration standards were 0.2, 0.5, 0.75, 1, 2, 5, 7.5, 10, 20, 35, and 50 μM, whereas, the IS concentration was fixed at 2 μM (final concentration). When analyzing the whole blood of DMTS-treated animals, acidification and addition of the IS was performed immediately following the collection of blood to ensure minimal degradation of DMTS.

2.4. DHS-GC–MS analysis of DMTS

Analysis of prepared samples was performed using DHS via a Gerstel MPS sampler (Gerstel Inc., Linthicum, MD, USA) coupled with an Agilent GC–MS (Agilent Technologies, Wilmington, DE, USA) consisting of a 6890N series gas chromatograph, and a 5975 series mass selective (MS) detector. The HS vial was initially incubated at 40 °C for 1 min, the septum was punctured using dual needles and nitrogen was delivered through the headspace of the vial to carry DMTS to the adsorptive material (Tenax® TA) where it was collected. The trapping and incubation temperatures were set at 28 and 40 °C, respectively. The transfer heater temperature was set at 75 °C. After trapping for 10 min, the TDU tube was inserted into the TDU and was heated from 30 °C to 280 °C at a rate of 12 °C/s. The analyte was desorbed at 275 °C and transferred to a cooled injection system (CIS) PTV-type inlet, where it was trapped at −100 °C on a CIS liner. The CIS liner was then heated to 275 °C at a rate of 12 °C/s to transfer the analyte to the GC column before returning to its initial temperature.

A DB5-MS bonded-phase column (30 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA) was used with helium as the carrier gas at a flow rate of 1.0 mL/min and a column head pressure of 10.0 psi. The initial GC oven temperature was 30 °C and was held for 1 min, then elevated at a rate of 120 °C/min up to 250 °C, where it was held constant for 2 min, before returning to its initial temperature. The retention times for both DMTS and IS were about 3.5 min. The GC was interfaced with an MS detector with electron ionization (EI). DMTS and IS were identified and quantified using selective-ion-monitoring (SIM) mode at *m/z* of 126 ($[CH_3\text{-}S\text{-}S\text{-}S\text{-}CH_3]^+$, quantification) and 111 ($[CH_3\text{-}S\text{-}S\text{-}S]^+$, identification), and 132 ($[CD_3\text{-}S\text{-}S\text{-}S\text{-}CD_3]^+$, quantification) and 114 ($[CD_3\text{-}S\text{-}S\text{-}S]^+$, identification), respectively.

2.5. Calibration, quantification and limit of detection

The proposed method was validated following the Food and Drug Administration (FDA) bioanalytical method validation guidelines [24–26]. Stock solutions of both DMTS (200 mM) and IS (100 mM) were prepared in methanol, which were further diluted with DI water to prepare the calibration standards (0.2, 0.5, 1, 2, 5, 10, 20, and 50 μM) and quality-control (QC) standards (0.75, 7.5, and 35 μM) for DMTS in denatured rabbit blood. Each calibration standard was prepared in triplicate, where the concentration of IS was 2 μM. To construct a calibration curve, the average peak area signal ratios of DMTS to IS were plotted as a function of calibrator concentrations. Peak areas of both DMTS and IS were calculated by manual integration from baseline to baseline in Agilent ChemStation software (Santa Clara, CA, USA). To evaluate the calibration behavior of DMTS, a total of six preliminary calibration curves were constructed. Both linear ($y = mx + c$) and log-log ($\log y = m \log x + \log a$) regressions were used, with goodness-of-fit (GOF) quantified by percent residual accuracy (PRA) [27]. PRA was used, in conjunction with the coefficient of determination (R^2), to more effectively quantify the GOF. R^2 is predominantly based on the GOF of the highest concentration 2 or 3 calibrators and doesn't accurately quantify GOF throughout the calibration range. The disadvantages of R^2 are exacerbated when using a geometric series of concentrations for calibration standards and when calibration curves span 2–3 orders of magnitude, as in this study. Therefore, for a more accurate representation of the goodness-of-fit, PRA was reported in the manuscript, along with R^2. A "good" fit of the calibration model throughout the calibration range will produce PRA values of 90–100%. Preliminary studies and the validation experiment

confirmed that a log-log curve fit best described the calibration behavior of DMTS, as also reported by Manandhar et al. [7].

The upper and lower limits of quantification (ULOQ and LLOQ) were investigated using two inclusion criteria: (1) calibrator precision of <10% relative standard deviation (RSD), and (2) calibrator accuracy of ±15% of the nominal concentration compared to the concentration back-calculated from the calibration curve. In parallel with the calibration standards, three QCs (0.75, 7.5 and 35 μM as low, medium, and high, respectively) were also prepared in the same way (not included in the calibration curve) and were analyzed in quintuplicate each day for 3 days in order to calculate the intraassay (within same day) and interassay (over 3 days, within 5 calendar days) accuracy and precision. The LOD, which was defined as the lowest DMTS concentration to reproducibly produce a signal-to-noise ratio (S/N) of 3, was calculated by analyzing blood samples spiked with DMTS concentrations below the LLOQ. Noise was calculated as peak-to-peak noise in blank solutions over the retention time of DMTS.

2.6. Selectivity and stability

Selectivity of the method was evaluated by confirming the absence of significant signals above the baseline in the blank over the retention time of DMTS, as determined by comparing blank rabbit blood and DMTS-spiked rabbit blood samples by the procedure described earlier (see section "Calibration, quantification and limit of detection"). Resolution of the DMTS peak was also calculated as a measure of selectivity by standard methods [28].

Short- and long-term storage stability of DMTS was evaluated in triplicate using denatured rabbit blood spiked with HQC and LQC concentrations at different storage conditions over multiple time periods. For short-term stability, both QCs were evaluated in the autosampler and under multiple freeze–thaw (FT) cycles. The autosampler stability was evaluated by placing triplicates of low (0.75 μM) and high (35 μM) QCs on the autosampler of the GC at ambient temperature and analyzing them at approximately 0, 2, 5, 10, and 24 h following preparation to ensure the integrity of DMTS during the course of analysis. Because of the evaporative loss of DMTS in blood, IS was also incorporated to track the change of analyte signal with respect to time, but it was not considered when evaluating the autosampler stability, as it would correct for the loss of DMTS in blood during the analysis. For FT analysis, four sets of LQCs and HQCs were prepared in triplicates without IS. Three sets of QCs were stored in a −80 °C freezer, while one set was analyzed on the same day after adding IS. After 24 h, all QC samples were thawed at room temperature (RT) by running tap water (about 25 °C) over the base of the sample vials. To one set of thawed QCs, IS was added, the samples were centrifuged at 252 × *g* for 10 s and then analyzed. The remaining two sets of QCs were placed back in the −80 °C freezer for 24 h. This freeze-thaw cycle was repeated two more times to evaluate three FT cycles.

Bench-top stability and long-term stability studies for DMTS at −20 and 4 °C have been reported by Manandhar et al. [7] and were not evaluated in this study. Manandhar et al. [7] recommended to immediately add acid to a blood sample, add IS, snap freeze, and keep samples at −80 °C in order to store blood samples for DMTS analysis, but only performed a 5-day stability study to confirm this recommended procedure. Therefore, we extended this experiment to 90 days to evaluate how long DMTS can be stored under these conditions before inaccurate results are found. For this study, triplicates of LQC and HQC concentrations of DMTS in blood were prepared for storage and analyzed after 0, 1, 2, 5, 10, 20, 30, 45, 60 and 90 days at −80 °C. Both QCs were prepared in bulk (30 mL) in 50-mL Falcon® conical tubes to minimize sample-to-sample variation in the original QC concentrations. IS was spiked before storage, and from these bulk QCs, 10 sets of LQCs and HQCs each were prepared in triplicate by simply transferring 1 mL of sample to a 4-mL HPLC vial for each replicate. One set of IS-treated QCs was analyzed on the same day (i.e., Day "0"), and the rest were snap-frozen using a dry ice–acetone bath before storing them in a -80 °C freezer. After 24 h, another set of QCs (in triplicate) was thawed and analyzed (Day 1). The same protocol was followed for the rest of the samples. For all the stability experiments, stability of DMTS was calculated as a percentage of the initial ("Day 0") signal. DMTS was considered stable if the signal from the stored sample was within ±10% of the initial signal.

2.7. Matrix effect

Matrix effects for the analysis of DMTS in blood were evaluated by comparing an aqueous calibration curve with a calibration curve in blood. The assessment of matrix effects reveals the direct or indirect alteration of response due to the presence of interfering substances in the blood sample. The slopes (m) of the log-log plots for the calibration curves in blood and aqueous solution were used to evaluate and quantify (i.e., m_{blood}/m_{aq}) the matrix effect. The value of (m_{blood}/m_{aq}) equal to 1 indicates no matrix effect, whereas less than 1 and greater than 1 represent suppression and enhancement effects, respectively. The effectiveness of IS to compensate the matrix effect was also evaluated by comparing the ratio of m_{blood}/m_{aq} of non-corrected curves with the IS-corrected curves.

3. Results and discussion

3.1. GC–MS analysis of DMTS

The analytical method presented here features simple and quick sample preparation for the analysis of DMTS from whole blood. The sample preparation involves only 3 steps: 1) treatment of whole blood with acid (0.4% H_2SO_4), 2) addition of IS, and 3) mixing. An aliquot of the resulting mixture is transferred to a HS vial and analyzed via DHS-GC–MS. During DHS, the TDU tube (filled with Tenax® TA sorbent) dynamically adsorbs chemicals within the headspace vapor, including DMTS. This process allows exhaustive extraction of the DMTS from the blood. The DMTS is then thermally desorbed into the GC–MS by heating the TDU tube. The selected ion chromatograms of DMTS-spiked blood (0.5 μM, at *m/z* = 111 and 126), IS-spiked blood (2 μM, at *m/z* = 114 and 132) and non-spiked blood (*m/z* = 126 and 132) are plotted in Fig. 1. As shown in Fig. 1, the method produced good selectivity, with DMTS and IS eluting at approximately 3.50 min and the blank containing no coeluting peaks at this time. The closest consistent peaks of DMTS-spiked blood eluted at about 3.40 (*m/z* = 126) and 3.55 (*m/z* = 111) min, producing resolutions (R_s) of 2.38 (quantification ion) and 1.22 (identification ion), respectively. The peak shapes for both DMTS and IS were sharp and symmetrical with peak asymmetry factors of <1.3 and <1.2, respectively.

Table 1 shows the analytical description of each previously published method for DMTS analysis from biological matrices as compared to the current method. The main advantages of the current method are: 1) rapid and simple sample preparation, 2) sensitivity, 3) small required sample volume, and 4) minimal consumable use. First, the overall sample preparation time is only 10 min, with chromatographic analysis lasting approximately 12 min. Therefore, using the current method, roughly 72 parallel samples could be processed and analyzed in a 24-h period. Quick blood sample preparation is vital for DMTS to minimize its unwanted loss, and the reported sample preparation time (i.e., 10 min) is the shortest time necessary for any published method. Second, the current method is extremely sensitive, allowing detection and quantification as low as 0.04 and 0.2 μM, respectively,

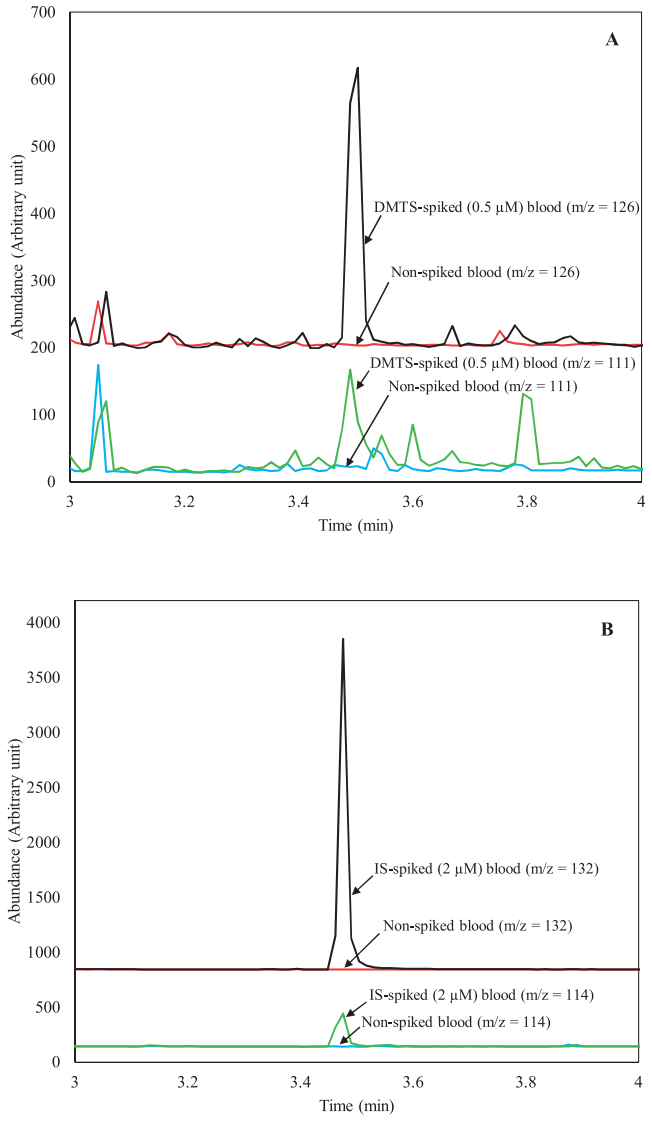

Fig. 1. SIM mode GC–MS chromatograms of A) DMTS-spiked blood (0.5 µM) and non-spiked blood at *m/z* of 126 (quantification ion, *upper trace*) and *m/z* of 111 (qualification ion, *lower trace*), and B) IS-spiked blood (2 µM) and non-spiked blood at *m/z* of 132 (quantification ion, *upper trace*) and at *m/z* of 114 (qualification ion, *lower trace*).

for the large volume approach, and limits of detection and quantification of 0.06 and 0.2 µM, respectively, for the small volume technique. This is the lowest LLOQ for the current methods by at least 2.5 times. Third, the required volume of blood for the method presented here is only 50 µL (as described in 'small' volume technique), which is the smallest blood volume required for any of the current DMTS analysis methods (Table 1). Fourth, the current approach necessitates only one TD tube (Tenax® TA) for the extraction of DMTS during the analysis of multiple samples, which makes the extraction process more consistent and less demanding than that of the other methods in Table 1. The need for multiple stir bars and SPME fibers makes the SBSE and SPME methods more costly since the Tenax® tube and a single stir bar or SPME fiber are approximately the same cost. Moreover, compared with the stir bars and SPME fibers, the Tenax® does not directly contact the biological samples, allowing greater longevity. Therefore, the Tenax® tube can be reused many more times than the stir bars or SPME fibers before it degrades (e.g., a single Tenax® sorbent tube was used for every sample in the current study with no observed loss in performance). Besides these main advantages, the DHS technique requires no organic solvent, whereas the HPLC technique requires extraction with acetonitrile, and mobile phase constitutes of water and acetonitrile in the ratio of 35:65 v/v.

3.2. Dynamic range, limit of detection, and sensitivity

Initially, the linearity of the method was evaluated in the range of 0.2–200 µM in blood. Multiple calibration curves were constructed to analyze the calibration behavior of DMTS by plotting the concentration of DMTS versus the signal ratio (i.e., peak area of DMTS at *m/z* 126 divided by the corresponding peak area of IS at *m/z* 132) as linear and log-log regressions over multiple calibration ranges. We confirmed that the calibration behavior of DMTS was best described by a log-log relationship. Manandhar et al. [7] also showed similar behavior of DMTS, which was explained by increased formation of the 126 (*m/z*) fragment at higher concentrations of DMTS in the ionization chamber (i.e., enhancement of MS ionization process at higher DMTS concentrations), independent of the sample preparation process. The largest two calibration standards (100 and 200 µM) did not meet the accuracy and/or precision inclusion criteria, resulting in a quantification range of 0.2 µM (LLOQ) to 50 µM (ULOQ). This linear range spanned over two orders of magnitude, as is desired for analysis of biological samples [29,30]. Moreover, the LLOQ (0.2 µM) was much lower than the LLOQs for the HS-SPME-GC–MS and HPLC-UV methods reported by Kiss et al. [23], and is 2.5 times lower than the SBSE-GC–MS method of Manandhar et al. [7] (see Table 1).

Table 2 shows the calibration curve equations of three separate calibration curves prepared over a 3-day period with their corresponding R^2 and PRA values. All three calibration curves were found to be highly stable in terms of slope, R^2, PRA, accuracy and precision.

The LOD of DMTS for the current method was 0.04 µM (large volume technique), which is 1.5 times lower than that of the Manandhar et al. [7] method, and significantly better than the reported LODs of Kiss et al. methods (1.49 and 11.56 µM [23]). This high

Table 1
Comparison of some important features of published and the current method for the analysis of DMTS from biological matrices.

Study	Analytical technique	Biological sample	Sample volume required (µL)	Sample preparation time (min)	LOD (µM)	LLOQ (µM)	ULOQ (µM)
Kiss et al. [23]	HS-SPME-GC-MS	Brain	588	30[a]	1.49	4.51[b]	38.0[b]
	HPLC-UV	Blood	495	25[a]	11.6	35.2[c]	396[c]
Manandhar et al. [7]	SBSE-GC-MS	Blood	450	70	0.06	0.5	100
Current method	DHS-GC-MS	Blood	450[d]	10	0.04[d]	0.2	50
			50[e]		0.06[e]		

[a] Total estimated sample preparation time.
[b] Reported as 645–5435 ng_{DMTS}/g_{brain}.
[c] Reported as 4.45–50 µg/mL.
[d] Large volume technique.
[e] Small volume technique.

Table 2
Curve equations and related values.

Day	Equation	m	PRA	R^2	Accuracy (%)	Precision (%RSD)
Day-1	log y = 1.07 log x – 0.76	1.07	90.0	0.9962	100 ± 14.9	<9.3
Day-2	log y = 1.13 log x – 0.80	1.13	95.8	0.9989	100 ± 11.8	<7.5
Day-3	log y = 1.15 log x – 0.83	1.15	90.0	0.9965	100 ± 14.6	<9.3

Table 3
Intra- and inter-assay accuracy and precision for analysis of DMTS in spiked rabbit blood.

Conc (μM)	Intra-assay						Inter-assay	
	Accuracy (%)[a]			Precision (%RSD)[a]			Accuracy (%)[b]	Precision (%RSD)[b]
	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3		
0.75	100 ± 7.8	100 ± 11.1	100 ± 2.9	5.9	3.7	6.1	100 ± 5.4	8.3
7.5	100 ± 1.6	100 ± 14.1	100 ± 13.5	3.8	2.9	2.7	100 ± 8.7	8.6
35	100 ± 5.4	100 ± 5.8	100 ± 1.7	6.5	6.0	9.0	100 ± 3.2	7.9

[a] QC method validation (n = 5).
[b] Average of three different days of QC method validation (n = 15).

sensitivity is very important to accurately quantify DMTS in blood samples at typical doses of DMTS [31].

3.3. Accuracy and precision

Although DMTS is very unstable and volatile in nature, the accuracy and precision, reported in Table 3, were excellent relative to other methods of DMTS analysis from blood. To perform the study, three QC standards (0.75, 7.5 and 35 μM) were analyzed in quintuplicate on three different days within six calendar days. FDA method validation guidelines were followed to evaluate the results [25,32,33] and the obtained intraassay accuracy (100 ± 8–15%) and precision (<10% RSD), and inter-assay accuracy (100 ± 9%) and precision (<9% RSD), were remarkable.

3.4. Matrix effect and storage stability

To evaluate the matrix effect, two types of calibration curves were constructed (non-IS corrected and IS-corrected curves) in both blood and in aqueous samples. The ratio of slopes (m_{blood}/m_{aq}) was 0.90 (Table 4), indicating only a minor matrix effect for the analysis of DMTS in blood. Moreover, the IS-corrected slope ratio (m_{blood}/m_{aq}) was 0.98, revealing the importance of IS for correcting for any loss of analyte signal during DMTS analysis process.

The short-term stability of DMTS in blood was evaluated in the autosampler over 24 h at ambient temperature and under multiple FT cycles. Results obtained from the autosampler stability showed that both QC concentrations of DMTS were unstable during the investigated time period. Without IS correction, the loss of DMTS signals from both QCs were approximately 50% of time zero after 10 h, which was expected, and likely caused mainly by the rapid enzymatic degradation of DMTS in blood. After 24 h, signal loss was exacerbated, with only about 16% of DMTS signal from both QCs recovered. Although the raw DMTS signals were unstable, the IS-corrected signals showed the necessary stability (≥90%) over the entire 24 h. Therefore, we recommend leaving prepared samples on the autosampler for periods of time not longer than 10 h, but if necessary, the IS correction can provide accurate quantification of DMTS over 24 h of storage on the autosampler. The FT stability test showed that DMTS is lost with each FT cycle, with almost 40% of DMTS signal loss after the first FT cycle. Similar outcomes were also reported by Manandhar et al. [7], reasoning that this is due to complex enzymatic activity. So, thawing and re-freezing blood samples should be avoided, but if it is necessary, then IS should be added during sample preparation to correct the loss of DMTS signal during thawing process.

Table 4
Slopes obtained from non-IS corrected and IS-corrected calibration curves of DMTS.

Calibration curve	m_{blood}	m_{aq}	m_{blood}/m_{aq}	Remark
Non-IS corrected	0.98	1.09	0.90	Minor matrix effect
IS-corrected	1.13	1.15	0.98	Corrected matrix effect

For long-term stability investigations, low and high QCs of DMTS-spiked blood with IS were stored at −80 °C, and the samples were analyzed periodically for 90 days. The purpose of this study was to evaluate the effectiveness of the storage protocol recommended by Manandhar et al. [7], where acid is used to denature blood proteins, IS is added to correct for loss of DMTS signal, and blood is flash frozen and stored at −80 °C. Our results showed that the DMTS signal loss (without IS correction) from both QCs was about 40% over 60 days, which was in agreement with the findings from the FT stability. Over the next 30-day period the non-IS-corrected signal loss was drastic (i.e., almost 90% loss compared with Day "0"). However, IS-corrected DMTS signals were consistent (100 ± 10% of Day 0), again showing the value of the IS to provide accurate DMTS concentrations during storage.

Considering the storage stability results in aggregate, similar to Manandhar et al. [7], we recommend immediate acid denaturation of biological samples, addition of DMTS-d_6 IS, snap freezing of the mixture and storage at −80 °C. These frozen samples are stable for 60 days with IS correction. Multiple thawing and refreezing of samples should be avoided, and prepared samples should be stored on the autosampler ≤10 h.

3.5. Pharmacokinetic study of DMTS-treated animals

The validated DHS-GC–MS method was applied to the analysis of blood samples from a pharmacokinetic study of DMTS in treated rats. Fig. 2 shows the chromatograms of treated and untreated rat blood. DMTS was detected at 3.65 min, whereas no peak was found at the retention time of DMTS in the blood of untreated rats. This slight shift in retention time is due to the installation of a new column before the analysis of pharmacokinetic study samples.

Fig. 3 shows the plasma concentration-time curve of DMTS pharmacokinetics in rats. In terms of the DHS-GC–MS analysis of DMTS, the relatively low DMTS concentrations found in the blood of IM-treated rats mean that both the HPLC-UV and SPME-GC–MS techniques (Table 1) would not allow quantification of DMTS-spiked blood concentrations, even at C_{max}, without extensive modification. Also, while it is a possibility that the SBSE-GC–MS

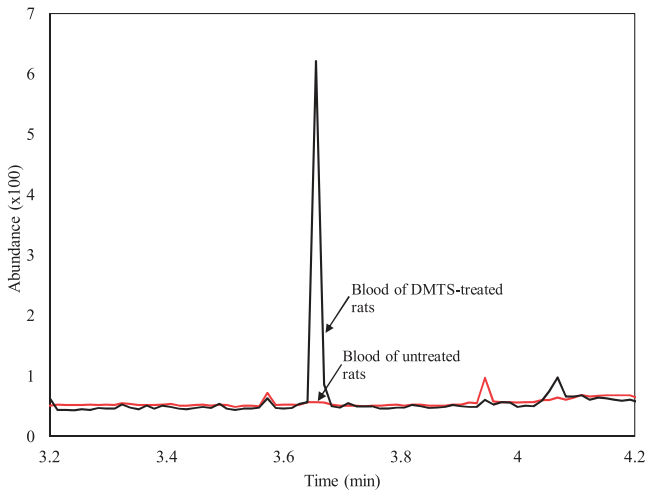

Fig. 2. Representative GC–MS chromatograms of the blood of DMTS-treated and untreated rats (*m/z* 126).

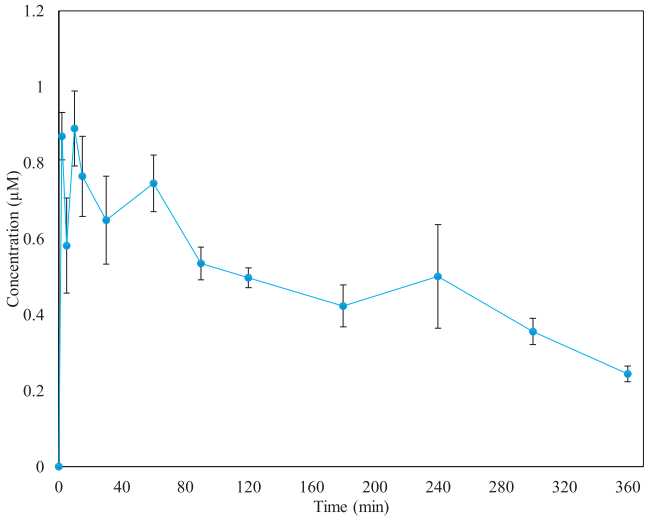

Fig. 3. Blood concentration-time behavior of DMTS following IM administration of DMTS solution to rats. Each value is expressed as mean ± SEM (n = 5).

method may allow quantification of DMTS, the method would need to be modified to allow analysis of lower volumes of blood.

The pharmacokinetic results, although preliminary, reveal that DMTS is absorbed and distributed very quickly with a C_{max} (the maximum concentration of a drug achieved in the plasma following dose administration) and t_{max} (the time at which C_{max} is attained) of 0.89 ± 0.09 μM and 10 min, respectively. Moreover, the blood DMTS concentration at the earliest time point (2 min) (0.87 ± 0.06 μM) was not significantly different from maximum concentration. This quick distribution of DMTS from IM administration is promising, in that it should allow fast treatment of CN throughout the body by untrained personnel. Additionally, DMTS showed a long elimination half-life ($t_{1/2}$ = 630 min or 10.5 h), with an estimated elimination rate constant (k_E) of 0.0011 min^{-1}. This behavior allows early and continuous protection for long periods of time after DMTS treatment. Overall, this preliminary pharmacokinetic data showed impressive behavior of DMTS for the treatment of CN poisoning, most notably quick onset of action and slow elimination.

4. Conclusions

A simple, relatively affordable, and highly sensitive DHS-GC–MS method for the rapid analysis of DMTS in blood was developed. When considered in aggregate, this validated method is superior to other published approaches in terms of its ability to detect and quantify low concentrations of DMTS (0.04 and 0.2 μM, respectively), its simple and rapid sample preparation scheme (<10 min), and the low required blood volume (50 μL). Relative to other methods of DMTS analysis from blood, the method presented here produced consistently good recovery, high sensitivity, excellent accuracy, outstanding precision, and minimal matrix effect. Moreover, the recommended storage procedure allows analysis of blood samples for DMTS up to 60 days following collection. Because of its advantages, this method should be considered the current "gold-standard" method for the analysis of DMTS blood concentrations from drug development studies of DMTS.

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