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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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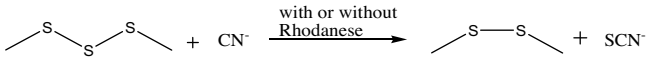

Fig. 1. Schematic representation of the reaction of DMTS and cyanide to form dimethyl disulfide (DMDS) and thiocyanate.

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

for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International accredited institution. The Institutional Animal Care and Use Committee (IACUC) at Sam Houston State University approved the experiment.

2.3. Sample preparation

Blood (450 μL) was added to a 20 mL glass scintillation vial. An aliquot of aqueous nitric acid (1 mL, 90 mM) was added to the blood to lyse the red blood cells and denature the blood proteins. Where appropriate, aqueous DMTS standard (25 μL) was spiked into the denatured blood to achieve the desired final DMTS concentration. Whenever needed, internal standard (25 μL) was spiked into the prepared blood to produce a final concentration of 5 μM DMTS-d6. A PDMS-coated stir bar was then added to the mixture, the sample was capped, and stir bar sorptive extraction was performed for 1 h at 700 rpm. Following extraction, the stir bar was removed from the solution using a 2-inch Teflon-coated magnet. The stir bar was gently dried by dabbing against a delicate task wipe and then transferred into a Thermal Desorption Unit (TDU) tube. The TDU tube was then placed into an auto-sampler for follow-on thermal desorption and GC–MS analysis. *(Note: Because of the rapid enzymatic degradation of DMTS in blood, for all validation experiments, blood was acid denatured before adding DMTS or IS in order to maintain the reported concentration and minimize error occurring from the rapid decomposition. The reported concentration of all DMTS standards, including QCs and calibrators, excludes the acid dilution volume.)*

2.4. GC–MS analysis of DMTS

After SBSE, stir bars were analyzed for DMTS and DMTS-d6 using an Agilent Technologies 7890A gas chromatograph with a 5975C inert XL electron ionization/chemical ionization mass selective detector with a Gerstel MPS 2XL series autosampler. To initiate analysis of the stir bar, the TDU tube with stir bar was transferred to the TDU injector and heated to transfer DMTS to the cooled injection system (CIS). The initial TDU injector temperature was maintained at 60 °C, and increased linearly to 250 °C at a rate of 720 °C/min. The final TDU injector temperature of 250 °C was maintained for 1 min. Desorbed analytes were transferred to the CIS programmable temperature vaporization (PTV) type inlet in splitless desorption mode with a TDU transfer temperature of 200 °C. To transfer the analyte from the CIS to the GC column, the initial CIS temperature (30 °C) was linearly increased to 200 °C at a rate of 12 °C/s before returning to its initial temperature. Lower CIS temperatures, 10 and 0 °C, were evaluated. However, improvement of the chromatography was not observed, likely due to the relatively high boiling point of DMTS (170 °C).

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 to separate components of the sample with nitrogen as the carrier gas at a flow rate of 1 mL/min and a column head pressure of 5.565 psi. The GC oven temperature was initially held at 30 °C for 1 min, then elevated at a rate of 120 °C/min to 250 °C, where it was held constant for 1 min, before returning to its initial temperature. The elapsed time from adding the TDU tube to TDU until the end of the analysis was ~9 min, which included transfer of the analyte from the PDMS stir bar, through the TDU and CIS, and into the column. The actual chromatographic acquisition time was 3.83 min with DMTS and DMTS-d6 eluting at 2.9 min. The GC was interfaced with a mass selective detector using electron ionization with an electron energy of 70 eV. The MS source and quadrupole temperatures were 250 °C and 150 °C, respectively. Selective ion monitoring (SIM) was used to monitor the quantification and identification ions of DMTS (m/z of 126 and 111, respectively) and DMTS-d6 (m/z of 132 and 114, respectively).

2.5. Calibration, quantification, and limit of detection

Validation of this method was performed by generally following Food and Drug Administration guidelines [44–46]. For calibration and quality-control (QC) standards, a combined DMTS and IS aqueous solution was initially prepared to limit loss of DMTS (i.e., loss from evaporation and degradation). Aqueous DMTS standard (100 μL) was transferred via pipette to a 2 mL glass vial, and a cap with a septum was used to immediately seal the vial. To the closed vial, IS (100 μL of 200 μM) was injected using a 1 mL (0.33 × 12.7 mm) syringe, and subsequently mixed to produce a combined standard of DMTS and IS. This standard solution was refrigerated at 4 °C until it was used for the preparation of calibration or QC standards. *(Note: Mixing of DMTS and IS in a closed vial using a syringe was a crucial step to prevent the rapid and uneven evaporative loss of DMTS and IS when opening and closing vials. Additionally, it was important to introduce IS and DMTS to the blood simultaneously to account for the rapid enzymatic degradation of DMTS.)* Calibration and QC standards were prepared by spiking the combined standard (25 μL) into 1475 μL of denatured blood and extracting as previously described in the sample preparation section. Each calibration standard (0.5, 1, 2, 5, 10, 20, 50, and 100 μM) was prepared in triplicate. To obtain a calibration curve, the average peak-area signal ratios of DMTS to IS were plotted as a function of concentration. Peak areas were calculated by manual integration from baseline to baseline in ChemStation software (Agilent Technologies, Santa Clara, CA, USA). Five preliminary calibration curves were constructed to evaluate the calibration behavior of DMTS. Ultimately, a total of ten calibration curves constructed over the course of preliminary studies and validation experiments confirmed that a non-linear power curve fit ($y = ax^b$) best described the calibration behavior of DMTS.

The limit of detection (LOD) was determined by analyzing multiple concentrations of DMTS in blood below the LLOQ. The LOD was defined as the lowest DMTS concentration that reproducibly produced a signal-to-noise ratio of 3. Noise was measured by averaging the peak-to-peak noise in blank solutions over the retention time of the analyte. The lower limit of quantification (LLOQ) and upper limit of quantification (ULOQ) were defined as the lowest and highest concentrations that satisfied the inclusion criteria of <15% relative standard deviation (as a measure of precision), and a percent deviation within ±15% back-calculated from the nominal concentration (as a measure of accuracy) for all calibration standards within the dynamic range. QC standards were prepared and analyzed in quintuplicate at three concentrations not included in the calibration curve: 1.5 μM (low QC), 7.5 μM (medium QC), and 35 μM (high QC). QCs were prepared fresh daily in order to calculate the intra-assay (within same day) and inter-assay (over three separate days, within six calendar days) accuracy and precision.

2.6. Selectivity and stability

The ability to differentiate and quantify DMTS in the presence of other blood components (assay selectivity) was determined by comparing blank blood with DMTS spiked blood. The absence of signals above the baseline in the blank over the elution time of DMTS was indicative of high selectivity.

Short- and long-term stability of DMTS was assessed by analyzing triplicates of low and high QCs in blood at different storage conditions over multiple time periods. For short-term stability, prepared QCs were evaluated in the autosampler, on the bench-top, and under multiple freeze-thaw (FT) cycles. The autosampler stability was evaluated by storing PDMS stir bars after SBSE extraction

on the autosampler (at ambient temperature), and analyzing them at approximately 0, 1, 5, 10, and 24 h. Internal standard was not used for the autosampler stability experiment, as it would correct for the loss of DMTS during the storage time tested. The bench-top stability was evaluated for two different conditions: non-denatured blood and denatured blood. The low and high QCs for both conditions were allowed to stand at room temperature for 0, 1, 2, 4, 8, 12, and 24 h prior to analysis.

For freeze-thaw stability of DMTS, four sets of QCs (low and high) were prepared. One set of QCs was extracted and analyzed on the same day while the other three sets were stored in a freezer at −80 °C. After 24 h, all three sets of QCs were thawed by running ambient tap water over the base of the sample vial. A single set of thawed QCs was then extracted and analyzed, while the remaining two sets were again stored at −80 °C for 24 h. The procedure was repeated two more times to evaluate three freeze-thaw cycles.

Evaluation of long-term stability in blood was conducted at three storage conditions (−80, −20, and 4 °C). The low and high QCs were analyzed in triplicate on the day they were prepared, and after 1, 2, 5, 10, and 30 days. A simple experiment was also performed to test if loss of DMTS would be minimized by snap freezing of blood samples before storage. Triplicates of high QCs in blood were prepared and snap-frozen using a dry ice–acetone bath before storing them at −80 °C. The recovery of these samples was compared to non-snap frozen samples also stored at −80 °C. For bench-top, freeze-thaw, and long-term stability experiments, internal standard was spiked into the blood samples after completion of the storage period and prior to SBSE extraction to correct for sample preparation and instrument variability. We also evaluated the long-term stability of DMTS in PDMS-coated stir bars at −80 °C. For this experiment, all stir bars were extracted at the same time from a single denatured solution (50 mL blood, 100 mL 90 mM HNO_3) of appropriate QC concentration (low and high). For each QC solution, fifteen PDMS stir bars (triplicates for 5 storage days) were anchored on a 3-inch cylindrical magnetic stir bar, which was used to stir the denatured blood solution for 1.5 h to perform SBSE. After extraction, the PDMS stir bars were transferred into 2 mL glass vials and capped. The stir bars were analyzed on the day they were prepared, and after 1, 5, 10, and 40 days. Since IS could not be used for this experiment, triplicate of a 5 μM aqueous DMTS solution was prepared, extracted using SBSE, and analyzed on each day the QCs were evaluated for stability in order to correct for instrument variability over different days. For all stability experiments, signal stability was calculated as a percentage of the initial "time-zero" signal. DMTS was considered stable if the stored sample signal stability percentage was within 10% of time zero.

Finally, to verify if the IS corrects for the loss of DMTS during storage and sample preparation, a 5-day stability test was performed. The QCs (low and high) were prepared by spiking the combined standard of DMTS and IS, and were stored at −80 °C for 1, 2, and 5 days. When ready to analyze, the QCs were thawed, denatured with acid, and extracted using SBSE. The DMTS/IS ratio for each day was calculated and compared to Day 0.

2.7. Recovery and matrix effect

Recovery of DMTS was evaluated by analyzing triplicates of low, medium, and high aqueous QCs, and comparing them with the equivalent QCs in blood. Recovery was calculated as a percentage by dividing the analyte signal in blood by the aqueous signal. Recovery calculated in this manner will be influenced by blood matrix effects, and therefore, may not reflect a true estimate of recovery. A true recovery from blood cannot be measured discretely, but may be estimated once matrix effects are assessed. Matrix effects were assessed by comparing the aqueous calibration curve with the calibration curve of DMTS in blood. The deviation of b in the power-fit equation ($y = ax^b$) gave a measure of the magnitude of the matrix effects.

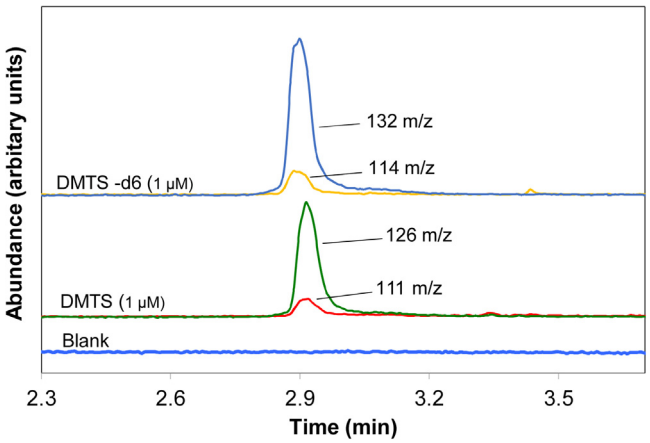

Fig. 2. Total ion GC–MS chromatograms of non-spiked blood (lower trace, listed as "Blank") and selected ion chromatograms of 1 μM DMTS (middle traces) and 1 μM DMTS-d6 (upper traces). DMTS quantification and identification ions (*m*/*z* 126 and 111, respectively) and DMTS-d6 quantification and identification ions (*m*/*z* 132 and 114, respectively) are separately plotted.

3. Results and discussion

3.1. GC–MS analysis of DMTS from rabbit blood

The method presented here includes an easy one-pot sample preparation scheme for extraction of DMTS from whole blood. The whole blood is simply treated with acid to lyse RBCs and denature proteins, and then a PDMS stir bar is directly added to the resulting solution. After an hour of SBSE, the stir bar is then analyzed via GC–MS by thermally desorbing the DMTS in the TDU.

SBSE was chosen for sample preparation of DMTS because it has a relatively high phase ratio, SBSE is a single-step solventless process, and it is typically highly reproducible [47]. Specifically, the analysis of DMTS using SBSE takes advantage of the relatively high octanol-water distribution co-efficient of DMTS (log K_{ow} = 1.87). Assuming equilibrium is reached and the K_{ow} of DMTS is a good estimate of the K_{PDMS}, approximately 54% of the DMTS should reside in the PDMS layer of the stir bar (film thickness 0.5 mm, length 10 mm), when the sample volume is 1.5 mL (i.e., the final sample volume used in the current method). The overall sample preparation time is 1 h 10 min, with thermal desorption and chromatographic analysis lasting approximately 15 min (including equilibration time for the following sample). Using this method, roughly 90 individual samples could be processed and analyzed in a 24 h time period.

The total ion chromatogram (TIC) of blank blood and selected ion chromatograms (*m*/*z* = 111, 114, 126 and 132) of spiked (1 μM DMTS and 1 μM IS) blood are plotted in Fig. 1. The peaks for DMTS and IS eluted at approximately 2.9 min. The method showed excellent selectivity with no interfering or co-eluting peaks in the blank. Quantification ions of DMTS (*m*/*z* of 126) and IS (*m*/*z* of 132), and identification ions of DMTS (*m*/*z* of 111) and IS (*m*/*z* of 114) are plotted in Fig. 2.

3.2. Dynamic range, limit of detection, and sensitivity

Calibration curves for DMTS were constructed in the range of 0.2–200 μM in blood. Upon analysis of multiple calibration curves using linear (non-weighted and weighted), power, and quadratic fits, we determined that the calibration behavior of DMTS followed a power curve ($y = ax^b$). Using a power fit, 0.2 and 200 μM calibra-

Table 1
Curve equations and R^2 values for separate calibration curves prepared over a three-day period.

Days	Equation	R^2
Day 1	$y = 0.1184\ x^{1.18}$	0.9984
Day 2	$y = 0.1321\ x^{1.21}$	0.9992
Day 3	$y = 0.1232\ x^{1.19}$	0.9984

tors did not meet the accuracy and/or precision inclusion criteria, and were excluded. Therefore, the dynamic range for the method was from 0.5 μM (LLOQ) to 100 μM (ULOQ), with a correlation coefficient (R^2) > 0.998 (Table 1). Table 1 shows that the b-term of the calibration equation ($y = ax^b$) remained consistent over the three calibration curves, producing a relative standard deviation of ≤2%, confirming the uniform calibration behavior of DMTS.

The dynamic range of the method spanned over two orders of magnitude, which is typically good for analysis from biological samples [48,49], and should be useful for therapeutic studies where a large range of concentrations is administered. The method achieved an excellent LOD, 60 nM in blood, as compared to other similar methods for therapeutics [19,49,50]. The low limit of detection is most likely attributed to the efficient pre-concentration of DMTS in the PDMS layer of the stir bar.

The non-linear behavior of DMTS was verified by over than 10 calibration curves produced with the method presented here from both blood and aqueous samples, and from direction injection of DMTS calibration standards. Non-linear calibration behavior when directly injecting DMTS calibration standards, suggests that the non-linearity did not stem from the extraction process or from the matrix components. This behavior is most likely caused by either higher concentrations of DMTS enhancing the MS ionization process (i.e., more DMTS molecular ions are produced as the concentration of DMTS in the ionization chamber increases) or adsorptive losses at low DMTS concentrations (i.e., a small amount of high affinity sites binding DMTS, with greater amounts of DMTS overwhelming these high affinity sites). Evaluation of the peak shape and calibration behavior of the low and high concentration calibrators indicated no evidence of adsorptive losses. Therefore, it is most likely that the ionization of the DMTS molecular ion, the ion used for quantification, is enhanced at higher concentrations. This may occur if DMTS molecular ions assist in ionization of more DMTS molecules through energy transfer, similar to chemical ionization.

3.3. Accuracy and precision

The accuracy and precision of the method, as determined by quintuplicate analysis of low (1.5 μM), medium (7.5 μM), and high (35 μM) QCs, on three different days (within a 6-day period), are reported in Table 2. Considering that DMTS is vulnerable to rapid enzymatic degradation and evaporative loss, the accuracy and precision of the method were remarkable. The intra-assay accuracy (100 ± 7–14%) and precision (<2–10% RSD), and inter-assay accuracy (100±11%) and precision (<6% RSD) of this method were excellent and well within the FDA method validation guidelines [44–46,48].

3.4. Matrix effects and recovery

The assessment of matrix effects provides a measure of ion suppression or enhancement leading to loss or gain of analyte signal. It is typically calculated by comparing the slope of the calibration curve in blood with that of the aqueous calibration curve. For a non-linear power trend, the power (b) in $y = ax^b$ translates to the slope in a linear fit. Hence, matrix effects were evaluated by calculating the ratio of power coefficient of the calibration curve in blood to that in aqueous. This ratio, determined as 0.96, indicated that matrix effects were essentially negligible for the analysis of DMTS in blood. The minimal matrix effect can be attributed to the fact that only hydrophobic analytes are efficiently extracted into the PDMS-coated stir bar. This minimizes the interference from other blood components during DMTS analysis. Note that the power coefficients of IS-corrected aqueous and blood calibration curves, were even closer to each other ($Power_{blood}/Power_{aq} = 0.998$), which gives another indication of effectiveness of the IS in correcting for any loss of DMTS signal during the analysis process.

Assay recovery of DMTS for low, medium, and high QCs was 66, 63, and 59% respectively. Incomplete recovery can be partly attributed to the rapid enzymatic degradation of DMTS in blood. Although the acid concentration and volume were optimized for the best recovery, the acid-denaturation process may not suffice in completely halting all enzymatic activity. Additionally, lower recovery can also be explained by the hydrophobic nature of DMTS. Since some components of blood provide a more hydrophobic environment than water, these components may interfere with effective partitioning of DMTS into the PDMS layer of the stir bar. Nevertheless, the recovery for all low, medium, and high QCs was very consistent, and sufficient to achieve detection at concentrations as low as 60 nM. It is unlikely that lower recovery is caused from evaporative loss, since both the aqueous and blood samples undergo same sample preparation steps. Therefore, loss of DMTS from evaporation should be comparable.

3.5. DMTS storage stability

The bench-top stability of DMTS was evaluated for low and high QCs in both non-denatured and denatured blood for 0, 1, 2, 4, 8, 12 and 24 h. At 1 h, DMTS was undetectable in the non-denatured blood, whereas 65% of the signal was recovered from the denatured blood. This observation confirms that enzyme metabolism and/or protein binding comprise a key loss mechanism for DMTS. Although DMTS was detectable in the denatured blood, it is still considered unstable (<90% of the original signal was recovered) at 1 h.

Auto-sampler stability, following stir bar extraction of DMTS, was tested for 0, 1, 5, 10, and 24 h, during which, DMTS was stable for the 24 h time period tested (i.e., the DMTS signals at different time periods randomly deviated above and below the time zero

Table 2
The accuracy and precision for the analysis of DMTS in spiked blood by SBSE-GCMS.

Concentration (μM)	Intra-assay						Inter-assay	
	Accuracy (%)[a]			Precision (%RSD)[a]			Accuracy (%)[b]	Precision (%RSD)[b]
	Day1	Day2	Day3	Day1	Day2	Day3		
1.5	100 ± 12.5	100 ± 6.7	100 ± 13.5	9.4	6.3	2.1	100 ± 10.8	5.9
7.5	100 ± 0.1	100 ± 0.2	100 ± 8.3	0.9	0.6	1.1	100 ± 2.8	4.9
35	100 ± 6.5	100 ± 7.6	100 ± 6.5	0.7	1.2	1.3	100 ± 6.9	0.6

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

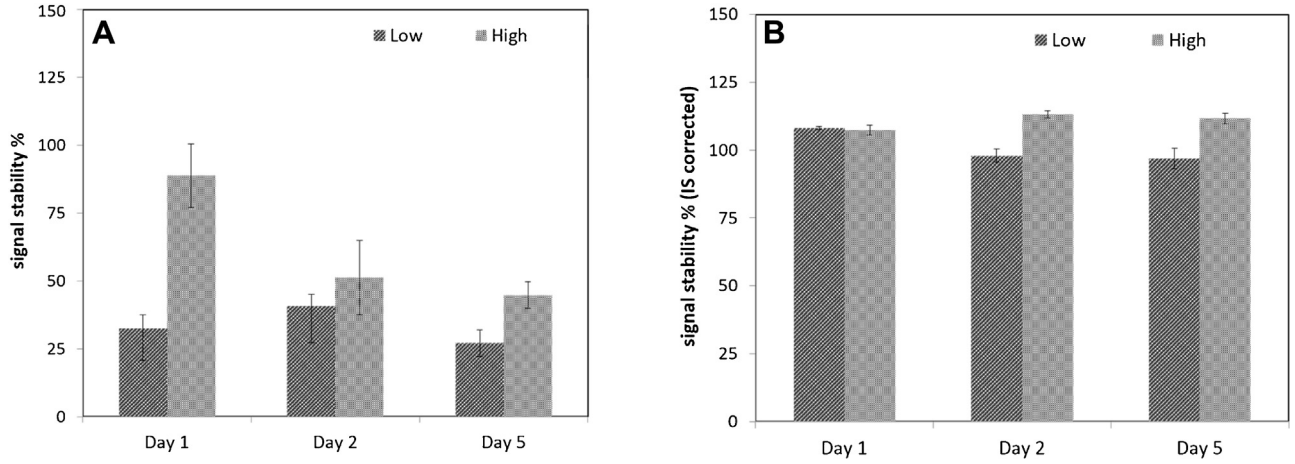

Fig. 3. Evaluation of the ability of the IS to correct for signal loss during storage at −80 °C. (A) DMTS signal stability plotted without IS correction. (B) IS corrected DMTS signal stability. The uncorrected DMTS signal clearly decreases from Day 0 (due to loss during storage and freeze-thaw process) and has high variability (due to variation in instrument sensitivity and stir bars). The IS corrected stability remains consistent throughout the time tested, with the IS correcting the DMTS signal for significant loss mechanisms.

signal; the variability was likely caused by differences between stir bars and slight changes in instrument sensitivity over time).

Long-term stability was evaluated for low and high QCs by storing them at −80, −20, and 4 °C over a 30-day period. DMTS was rapidly removed from blood when stored at −20 and 4 °C, with less than 10% signal recovered after one day relative to the initial time. However, at −80 °C, although some of the DMTS signal was lost (20–50%), the concentration stayed consistent over the 30 day period with no trend of further loss. The rapid loss at higher temperatures (−20 and 4 °C) likely results from the enzymatic activity of blood proteins. At −80 °C, the rapid initial loss may have resulted from DMTS degradation during the delay times between spiking DMTS and complete freezing, and/or during thawing prior to acid addition. In order to reduce the initial delay time of complete freezing, blood samples were snap frozen using dry ice and acetone bath before transferring to a −80 °C freezer. Results from this experiment showed similar recovery for the snap frozen (79 ± 9.6%) and non-snap frozen (72 ± 12.8%) samples. This suggested that the significant loss mainly takes place during the thawing process, and that snap freezing prior to storage does not provide a definite advantage.

Long-term stability of DMTS in the stir bar was evaluated at −80 °C for 0, 1, 5, 10, and 40 days to determine if the samples could be prepared and the stir bars analyzed at a later date. The signal ratio of DMTS to positive control was determined to compare the stability of DMTS over different days. The high QCs were stable in the stir bar for 5 days (i.e. signal within 100 ± 10%), whereas, the low QCs were considered unstable on Day 5. On Day 40, both QCs (high and low) were considered unstable with signal loss of 30–40%.

The freeze-thaw stability test showed that DMTS is lost with each freeze-thaw cycle, which was in agreement with our findings from long-term stability of DMTS in blood at −80 °C. During the freeze-thaw cycle, DMTS is rapidly lost from the blood likely due to the enzymatic activity. Hence, freezing and thawing should be avoided, but if freezing the blood is necessary, then acid should be added immediately after thawing in order to help preserve DMTS.

The 5-day stability study using QCs (low and high) showed that when IS was spiked before storage, the DMTS to IS ratio remained consistent throughout the storage time, verifying that the IS effectively corrected for any loss of DMTS during storage. Fig. 3 shows the ability of the IS to correct for signal loss during storage. Fig. 3A illustrates DMTS stability without IS correction, where DMTS signal is seen to significantly deviate compared to the Day 0 signal. Fig. 3B shows the DMTS to IS signal ratio for all days. When comparing these two graphs, it is obvious that the IS corrects for the large signal loss due to instability of the DMTS.

Overall, our results from the stability studies suggest that blood samples should be denatured with acid, spiked with IS, extracted and analyzed immediately whenever possible. In any circumstance where immediate analysis is not possible, the blood samples should be spiked with IS, frozen as soon as possible, and stored at −80 °C for future analysis. Once the frozen samples are thawed, they should be prepared and extracted immediately. Once extracted into the PDMS layer, DMTS is stable for at least 24 h on the auto-sampler at ambient temperature. While the DMTS is stable in the stir bar at −80 °C for 24 h, it is not recommended to store the stir bar under these conditions unless it is absolutely necessary.

3.6. Analysis of DMTS exposed animals

The validated SBSE GC–MS method was applied to the analysis of DMTS from blood samples of treated mice. The GC–MS chromatograms of treated and untreated mouse blood are shown in Fig. 4. DMTS was detected as a prominent peak at 2.9 min from DMTS- treated mouse blood, whereas no peak was present in the untreated blood. This further verified the selectivity of the method and confirmed its applicability to authentic samples from treated animals.

4. Conclusion

A simple and sensitive analytical method for the determination of DMTS in blood was developed using SBSE-GCMS. The method presented is the first validated method for DMTS analysis from any matrix. The described method is simple, with easy one-pot sample preparation and extraction. The method yielded excellent accuracy and precision, consistent recovery, minimal matrix effects, an excellent detection limit, and a large dynamic range that spanned over 2 orders of magnitude. The ability to store internal standard spiked samples was also demonstrated. The creation of this method is significant, since there are no analytical methods currently available for analysis of DMTS from blood and the first validated method in any matrix. The availability of this method will allow further drug development investigations of DMTS as a promising cyanide antidote.

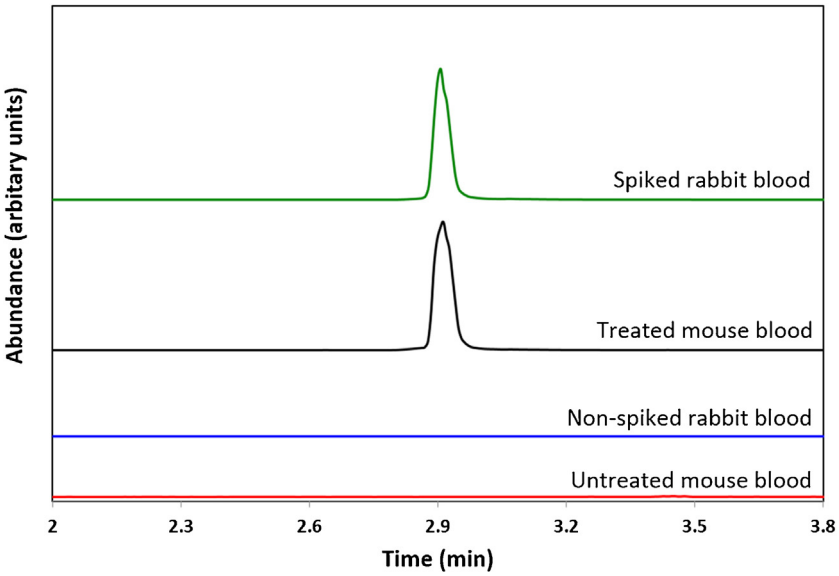

Fig. 4. GC–MS chromatograms (SIM, *m*/*z* 126) for DMTS treated (200 mg/kg, intramuscular administration) and untreated mice blood, and DMTS spiked and non-spiked rabbit blood.

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