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Analytical measurement of discrete hydrogen sulfide pools in biological specimens

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ABSTRACT

Hydrogen sulfide (H₂S) is a ubiquitous gaseous signaling molecule that plays a vital role in numerous cellular functions and has become the focus of many research endeavors, including pharmacotherapeutic manipulation. Among the challenges facing the field is the accurate measurement of biologically active H₂S. We have recently reported that the typically used methylene blue method and its associated results are invalid and do not measure bona fide H₂S. The complexity of analytical H₂S measurement reflects the fact that hydrogen sulfide is a volatile gas and exists in the body in various forms, including a free form, an acid-labile pool, and bound as sulfane sulfur. Here we describe a new protocol to discretely measure specific H₂S pools using the monobromobimane method coupled with RP-HPLC. This new protocol involves selective liberation, trapping, and derivatization of H₂S. Acid-labile H₂S is released by incubating the sample in an acidic solution (pH 2.6) of 100 mM phosphate buffer with 0.1 mM diethylenetriaminepentaacetic acid (DTPA), in an enclosed system to contain volatilized H₂S. Volatilized H₂S is then trapped in 100 mM Tris-HCl (pH 9.5, 0.1 mM DTPA) and then reacted with excess monobromobimane. In a separate aliquot, the contribution of the bound sulfane sulfur pool was measured by incubating the sample with 1 mM TCEP (tris(2-carboxyethyl)phosphine hydrochloride), a reducing agent, to reduce disulfide bonds, in 100 mM phosphate buffer (pH 2.6, 0.1 mM DTPA), and H₂S measurement was performed in a manner analogous to the one described above. The acid-labile pool was determined by subtracting the free hydrogen sulfide value from the value obtained by the acid-liberation protocol. The bound sulfane sulfur pool was determined by subtracting the H₂S measurement from the acid-liberation protocol alone compared to that of TCEP plus acidic conditions. In summary, our new method allows very sensitive and accurate measurement of the three primary biological pools of H₂S, including free, acid-labile, and bound sulfane sulfur, in various biological specimens.

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Introduction

Hydrogen sulfide (H₂S) is a ubiquitous gaseous signaling molecule that plays an important role in numerous cellular functions and has become the focus of many research endeavors, including pharmacotherapeutic manipulation [1–5]. Hydrogen sulfide is produced predominately from cysteine, by two pyridoxal-5'-phosphate-dependent enzymes, cystathionine-β-synthase and cystathionine-γ-lyase, as well as by 3-mercaptosulfurtransferase. Hydrogen sulfide can diffuse across cellular membranes without the need for a specialized transporter [4,6]. At pH 7.4 and temperature of 37 °C, 18.5% of free hydrogen sulfide exists as H₂S gas and the remainder is

almost all hydrosulfide anion (HS⁻) with a negligible contribution of S²⁻ [7,8]. It is long been known that the sulfur exists in the body in several forms, ranging from a fully reduced divalent state as sulfide to a fully oxidized hexavalent state as sulfate [1,9,10]. Measurement of biologic sulfur has focused on measuring sulfide, i.e., in the reduced divalent state, in part because of difficulties in accurately measuring other states. Additionally, sulfur equivalents in the reduced divalent state are very reactive within biological matrices, resulting in sulfide equivalents being present in various volatile sulfur pools. It is increasingly clear that these pools, in addition to free hydrogen sulfide, are important in regulating the amount of bioavailable sulfur, with the most relevant being the acid-labile and bound sulfane sulfur pools [10,11]. Fig. 1 illustrates the various volatile sulfide pools with associated chemical species.

Sulfane sulfur refers to divalent sulfur atoms bound only to other sulfur, although they may bear an ionizable hydrogen at some pH values. These include thiosulfate S₂O₃²⁻, persulfides

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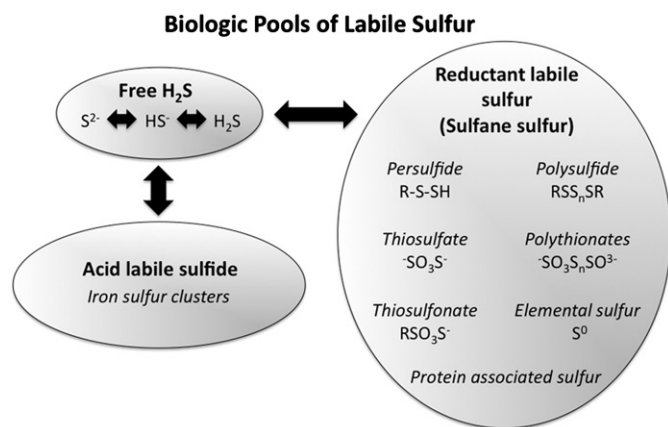


Fig. 1. Biological pools of labile sulfur.

R-S-SH, thiosulfonates R-S(O)-S-R', polysulfides R-S_n-R, polythionates S_nO₆²⁻, and elemental sulfur S⁰ [10]. Acid-labile sulfide, the other major pool, consists of sulfur present in the iron-sulfur clusters contained in iron-sulfur proteins (nonheme), which are ubiquitous in living organisms and include a variety of proteins and enzymes including rubredoxins, ferredoxins, aconitase, and succinate dehydrogenase [10,12]. Some have claimed that the process of acid liberation may also release hydrogen sulfide from persulfides, which have traditionally been classified as sulfane sulfur [13]. This pool of sulfur has been postulated to be a "reversible sulfide sink" and may be an important storage pool, which regulates the amount of bioavailable free hydrogen sulfide that is available [14], though others have suggested that bound forms may be more important in storage and release of exogenously administered sulfide [11].

The Achilles heel of the sulfide field has been the lack of precise methodology for the accurate and reproducible measurement of hydrogen sulfide both in vivo and in vitro. A variety of methods to measure free H₂S have been employed, with divergent results [10,13,15]. These include a spectrophotometric derivatization method resulting in methylene blue formation, variations of this using high-performance liquid chromatography (HPLC) [10], sulfide ion-selective electrodes, polarographic sensors [16], gas chromatography [13,17], and HPLC in conjunction with fluorimetric-based methods using monobromobimane (MBB) to derivatize free H₂S [14,18]. The levels so determined range from nanomolar to hundreds of micromolar concentrations [10,15]. This is due to the fact that the previously favored methylene blue method of hydrogen sulfide detection results in interference from bound sulfide pools coupled with the fact that this method is subject to chemical artifacts and unable to measure bona fide hydrogen sulfide at analytical or physiological concentrations [18]. Compelling arguments against micromolar concentrations reported by older studies [19] are further reiterated by the effect of local nanomolar concentrations affecting biologic function [20]. Indeed, the existence of storage pools of sulfide that can release H₂S along with the volatility and spontaneous oxidation of H₂S may help explain these apparent paradoxes.

Earlier attempts to characterize the various biologic pools of sulfur have utilized MBB in conjunction with dithiothreitol (DTT) as a reducing agent [10,21] or have focused on the free hydrogen sulfide and acid-labile pools alone [11,13]. These study results were limited because of various problematic issues such as pH, volatilization, and oxidation. Here we report a new protocol that measures all relevant biologic hydrogen sulfide pools, namely free hydrogen sulfide, acid-labile sulfide, and bound sulfane sulfur. The results highlight the ability to analytically and

comprehensively measure hydrogen sulfide bioavailability in biologic specimens.

Principles

The fluorescent reagent MBB has been widely used to measure various thiol-containing species through alkylation [22]. S-alkylation occurs twice with sulfide under alkaline conditions, forming sulfide dibimane. Our lab has previously published an analytical method of measuring free plasma hydrogen sulfide in vivo and in vitro by derivatization of sulfide with an excess of monobromobimane under alkaline, 1% oxygen, trace-metal-free conditions with RP-HPLC separation and fluorescence detection of the sulfide dibimane product [18].

The release of hydrogen sulfide from the acid-labile pool requires a pH less than 5.4 [11]. Thus the determination of acid-labile sulfide involves acidification of the sample, performed by adding 450 μl of 100 mM phosphate buffer (30 μM H₃PO₄ and 70 μM KH₂PO₄, pH 2.6, 0.1 mM diethylenetriaminepentaacetic acid (DTPA)), causing release of free hydrogen sulfide into the headspace of a Vacutainer tube from the acid-labile pool. After removal of this solution, 100 mM Tris-HCl buffer (pH 9.5, 0.1 mM DTPA) is added and hydrogen sulfide gas is redissolved back into the buffer and the sulfide level is measured by the MBB method. The result reflects both free hydrogen sulfide and hydrogen sulfide released from the acid-labile pool.

The sulfane sulfur component is determined by treatment with tris(2-carboxyethyl)phosphine hydrochloride (TCEP), which cleaves disulfide bonds to liberate the sulfane sulfur atom. Although DTT has been used by others [10,11,21,23] for this purpose, TCEP is water soluble, is nonvolatile, reduces disulfide bonds more rapidly, and has been shown to be very stable across a wide range of pH (2.0–9.5), unlike DTT [24]. TCEP also does not have a thiol moiety and has the additional advantage of not requiring removal before reaction with MBB, in contrast to DTT, which contains a thiol moiety and has been reported to have small amounts of sulfide contaminants [21].

However, reductive dehalogenation of monobromobimane by TCEP with the creation of a fluorescent product has been reported, with the potential for interference [25]; although this product can be extracted by methylene chloride, it is not necessary as HPLC analysis of the reaction mixture separates this product from sulfide dibimane. We found that the product of TCEP and MBB has a retention time of 11.2 min (data not shown), whereas the product of sulfide and MBB, i.e., sulfide dibimane, has a retention time of 16.5 min, and MBB alone has a retention time of 17.6 min. To overcome TCEP consumption of MBB as well as any inhibitory effects on the dye as have been reported with iodoacetamide and maleimide dyes [26], we used a ratio of greater than 10:1 MBB to TCEP.

Persulfide formation normally occurs at alkaline pH at room temperature, and persulfide can be measured by absorbance at 335 nm using a spectrophotometer [27]. Free hydrogen sulfide released into the headspace can react with plasma proteins to form persulfide as reported below using spectrophotometry. Thus, removal of plasma from the reaction vessel after volatilization of hydrogen sulfide into the headspace is necessary before retrapping the volatilized hydrogen sulfide gas in alkaline solution for subsequent reaction with MBB.

Materials

- 1) Monobromobimane (Sigma-Aldrich, Cat. No. B4380)
- 2) Sodium sulfide (Alfa Aesar, Cat. No. 65122); this product has been demonstrated to have superior purity compared to other sources [8]

- 3) Microtainer plasma separator tubes (BD Biosciences, Cat. No. 365958)
- 4) Sulfosalicylic acid (SSA; Sigma–Aldrich, Cat. No. S2130)
- 5) Acetonitrile (CH₃CN; Sigma–Aldrich, Cat. No. 34851)
- 6) Trifluoroacetic acid (TFA; Thermo Scientific, Cat. No. 28903)
- 7) BD Vacutainer (Becton–Dickinson, Cat. No. 366703)
- 8) 1-ml plastic syringe
- 9) PCR tube (Molecular Bioproducts, Cat. No. 34129)
- 10) 3.5-in. 25-gauge spinal needle (BD Cat. No. 405180)
- 11) 0.5-in. 30-gauge needle (BD Cat. No. 305106)

Instrumentation

- 1) Pan mass balance (0.1 mg sensitivity; Mettler; AG104)
- 2) Vortex mixer (Thermolyne; Maxi Mix II)
- 3) SmartSpect Plus spectrophotometer (Bio–Rad)
- 4) Shimadzu Prominence ultrafast liquid chromatography HPLC system equipped with fluorescence detector (HPLC 20A Prominence)
- 5) Hypoxic chamber (Coy Laboratory Products; large glove box)
- 6) Nutating mixer — a mixing device hybrid of an orbital shaker and a rocker that allows thorough mixing of contents without plasma or tissue lysate protein foaming (VWR; Model S0500)

Protocol

Sample preparation for detecting free hydrogen sulfide [18]

- 1) Place BD Microtainer plasma separator tubes with lithium heparin for murine samples and BD Vacutainer with lithium heparin for human samples on ice.
- 2) Collect blood using plastic syringes or plastic capillary tubes (do not use glass) directly into plasma separator tubes.
- 3) Centrifuge at 3000 rcf for 2 min at 4 °C for murine samples and 1500 rcf for 4 min at 4 °C for human samples.
- 4) Start the derivatization reaction of sulfide with monobromobimane at 1% O₂ in a hypoxic chamber by transferring 30 µl of plasma, 70 µl of Tris–HCl (100 mM, pH 9.5, 0.1 mM DTPA), and 50 µl of MBB solution (10 mM, in CH₃CN) into the PCR tube.
- 5) Incubate the mixture at room temperature for 30 min.
- 6) Stop the reaction by adding 50 µl of 200 mM ice-cold sulfosalicylic acid solution (to stop the reaction and precipitating protein) and vortex for 10 s. Place tubes on ice for 10 min.
- 7) Centrifuge the tubes at 12,000 rpm at 4 °C for 10 min.
- 8) Inject 10 µl of the supernatant into the RP–HPLC system with an Agilent Eclipse XDB–C18 column (5 µm, 80 Å, 4.6 × 250 mm) equilibrated with 15% CH₃CN in water containing 0.1% (v/v) TFA.
- 9) Set fluorescence detection at 390 nm (excitation) and 475 nm (emission).
- 10) Separate monobromobimane and sulfide dibimane using the gradient of two mobile phases identified in Table 1, (A) water containing 0.1% (v/v) TFA and (B) 99.9% CH₃CN, 0.1% (v/v) TFA, at a flow rate of 0.6 ml/min.
- 11) The retention times for the sulfide dibimane and the monobromobimane peaks are 16.5 and 17.6 min, respectively.
- 12) Measure the amount of hydrogen sulfide from linear plots of the HPLC peak areas of sulfide dibimane versus known concentration of sulfide solution.

Effect of plasma protein on trapping volatilized hydrogen sulfide

To test the interaction of plasma proteins with the released hydrogen sulfide in the headspace of the Vacutainer during alkaline re trapping, we determined the amount of hydrogen

Table 1
Mobile-phase gradients.

Time (min)	(%) phase A	(%) phase B
0	85	15
5	65	35
16	45	55
23	30	70
24	10	90
26	10	90
28	85	15

sulfide detected when the plasma was retained in the Vacutainer as opposed to removal and measured the formation of persulfides in the plasma.

- 1) 50 µl of plasma is added separately into two sets of 4-ml BD Vacutainer tubes.
- 2) 450 µl of 100 mM phosphate buffer (pH 2.6, 0.1 mM DTPA) is added to these tubes.
- 3) Incubate these tubes on the nutating mixer for 30 min.
- 4) From one tube the solution is removed through the cap with a 25-gauge spinal needle and 1-ml plastic syringe, and then 500 µl of 100 mM Tris–HCl buffer (pH 9.5, 0.1 mM DTPA) is added into the now empty BD Vacutainer tube. To the other tube 25 µl of 3 M NaOH solution is added to adjust the pH to ~9.5.
- 5) Incubate these tubes for 30 min on the nutating mixer.
- 6) Measure sulfide level in the BD Vacutainer tubes using the MBB method.
- 7) Also, 20 µl of these samples can be mixed with 200 µl of 100 mM KCN (dissolved into 10 mM NaOH), and then A₃₃₅ is measured [28]; further development of a red color is facilitated by adding 200 µl of ferric nitrate solution (dissolved 2 g of Fe(NO₃) · 9H₂O in 20 ml of 65% nitric acid) [29].

Similarly, 0, 30, or 300 µM Na₂S (final concentration) is incubated with the mixture of plasma and 0.1 M Tris–HCl buffer (pH 9.5, v/v, 1/9) under hypoxic conditions (1% O₂). The A₃₃₅ is measured by spectrophotometry at 0, 10, 30, and 60 min [30].

Effect of TCEP on hydrogen sulfide reaction with monobromobimane

- 1) Prepare 100 mM TCEP stock solution in H₂O.
- 2) Incubate respective final concentrations of TCEP (1 and 50 mM) with 20 µM sodium sulfide for 10 min.
- 3) Transfer 30 µl of the TCEP/sodium sulfide sample into a PCR tube with 70 µl of reaction buffer (100 mM Tris–HCl, 0.1 mM DTPA, pH 9.5).
- 4) Add 50 µl of 10 mM MBB solution.
- 5) Incubate for 30 min at 1% O₂ in the hypoxic chamber at room temperature.
- 6) Add 50 µl of 200 mM SSA solution to stop the reaction.
- 7) Use 10 µl of the reaction solution for RP–HPLC analysis.

Stability of sulfide dibimane

- 1) Prepare different pH solutions: pH 4.5, pH 9.5.
- 2) Prepare 12 µM sulfide dibimane solution using the different pH solutions.
- 3) Treat the mixtures with or without 1 mM TCEP.
- 4) At various time points, withdraw aliquots (200 µl each) of these solutions and analyze them by RP–HPLC. For greater sample reproducibility, keep the time constant.

Sample preparation for detecting acid-labile sulfide and bound sulfane sulfur

- 1) Add 50 μl of plasma separately into two sets of 4-ml BD Vacutainer tubes.
- 2) Add 450 μl of 100 mM phosphate buffer (pH 2.6, 0.1 mM DTPA) to one tube and to the other add 450 μl of 100 mM phosphate buffer (pH 2.6, 0.1 mM DTPA, and 1 mM TCEP).
- 3) Incubate these tubes on the nutator for 30 min.
- 4) Remove the solution through the cap with a 25-gauge spinal needle or other similar long needle and 1-ml plastic syringe.
- 5) Trap the sulfide gas by adding 500 μl of 100 mM Tris-HCl buffer (pH 9.5, 0.1 mM DTPA) into the BD Vacutainer tube,

using a 30-gauge needle, and incubating for 30 min on the nutator.

- 6) Remove the solution and measure the sulfide level in the BD Vacutainer tube by the MBB method.
- 7) Calculate the acid-labile sulfide and sulfane sulfur pools:
 - a. Acid-labile sulfide is the value obtained using the acid-liberation protocol minus the free hydrogen sulfide value.

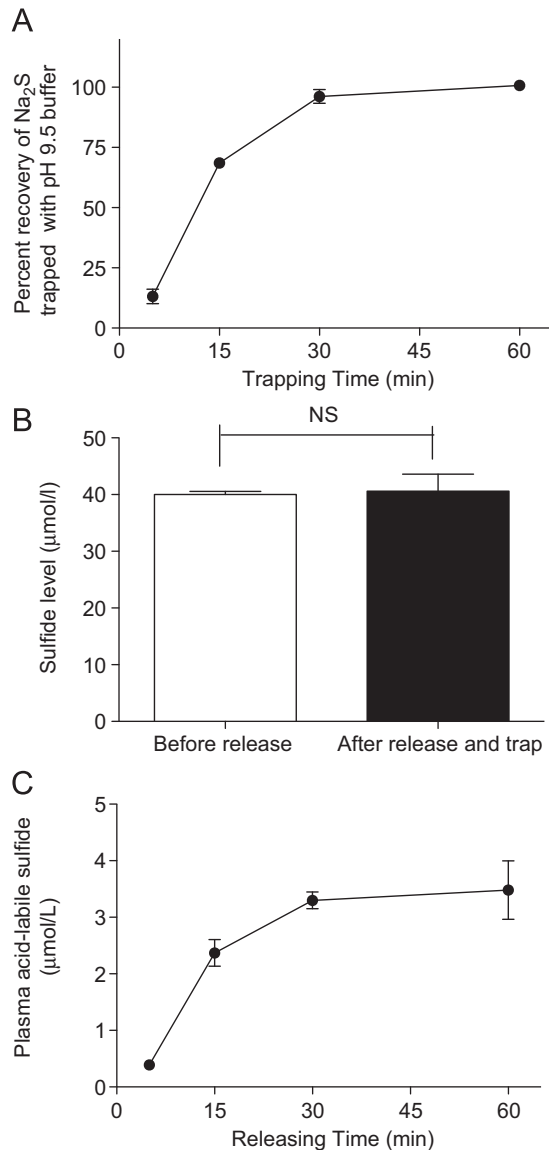


Fig. 2. Optimization of the measurement techniques for the sulfide pools. (A) Effect of trapping time on hydrogen sulfide recovery. After 50 μl of 40 μM sodium sulfide was incubated with 450 μl of 0.1 M phosphate buffer, pH 2.6, for 30 min, hydrogen sulfide gas was retrapped by 500 μl of 0.1 M Tris-HCl buffer (pH 9.5, 0.1 mM DTPA) with a peak recovery time of 30 min. (B) Effect of sodium sulfide release and retrapping on hydrogen sulfide recovery. The hydrogen sulfide detected from 40 μM sulfide was compared before release and after releasing and retrapping. Both releasing and trapping times were 30 min. (C) Effect of plasma on release of sulfide. 50 μl of plasma was incubated with 450 μl of 0.1 M phosphate buffer, pH 2.6, for various lengths of time and then retrapped for 30 min, demonstrating optimal release at 30 min.

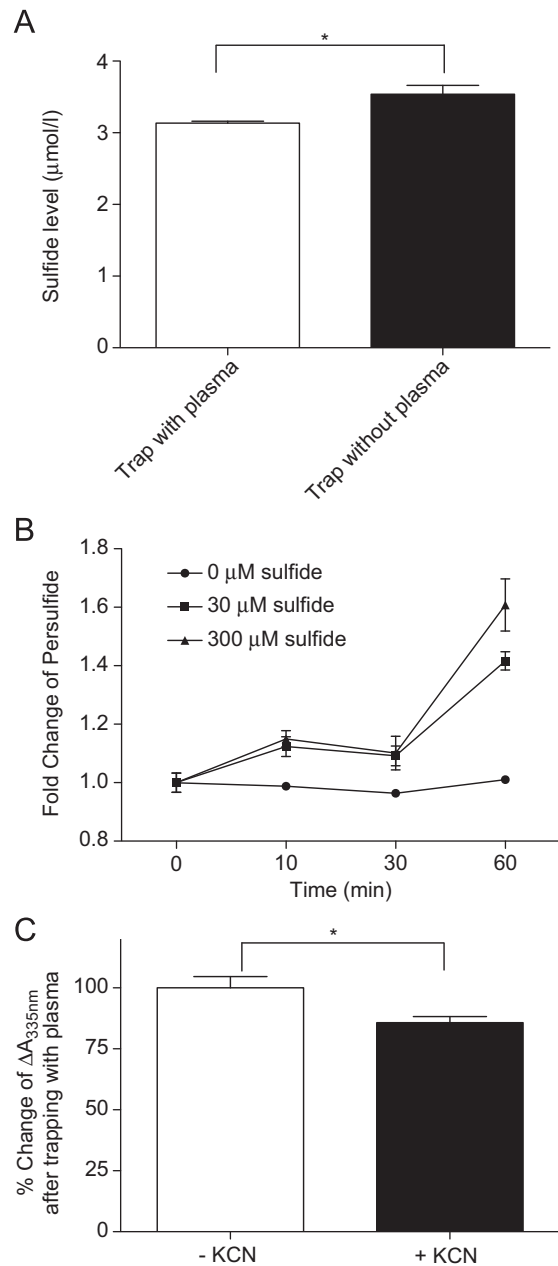


Fig. 3. Effect of plasma proteins on trapping hydrogen sulfide gas. (A) Comparison of sulfide level between samples trapped with plasma remaining in the reaction vessel and samples trapped after removal of plasma. After 50 μl of plasma was incubated with 450 μl of 0.1 M phosphate buffer, pH 2.6, for 30 min, in the first group ~ 25 μl of 3 M NaOH solution was added directly to the plasma to adjust the pH to 9.5 and then trapped for 30 min. In the other group, the plasma was removed from the reaction vessel and the released hydrogen sulfide was trapped by 500 μl of 0.1 M Tris-HCl buffer for 30 min. (B) Effect of sulfide on protein persulfide formation. 0, 30, and 300 μM sodium sulfide was incubated with plasma and the resulting generation of persulfide was measured at various time points. (C) Effect of sulfide on protein persulfide formation. Samples trapped with plasma had KCN added and then absorbance at 335 nm was measured.

- b. Bound sulfane sulfur is the value obtained from TCEP treatment minus the value obtained using the acid-liberation protocol.

Similarly, to optimize the effects of other experimental conditions, hydrogen sulfide can be released and trapped under various conditions including 0–1 h releasing time, 0–1 h reducing time, and 0–1 h trapping time in the hypoxic chamber.

Animal and human approvals

The use of animals for this study was approved by the Institutional Animal Care and Use Committee of the Louisiana State University (LSU) Health–Shreveport (Shreveport, LA, USA) via Protocol P-12-011. All animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals*. The enrollment of male

healthy human subjects was approved by the Institutional Review Board of LSU Health–Shreveport via Protocol H11-110. Human subjects were males between 23 and 34 years of age.

Calculations and results

Measurement techniques for analyzing acid-labile sulfide and bound sulfane sulfur

To establish the efficiency of the acid-liberation technique and alkaline Tris–HCl buffer trapping of the headspace sulfide gas, we sought to determine the optimal trapping time with a known molar solution of sodium sulfide. As demonstrated in Fig. 2(A), the highest recovery percentage was achieved after 30 min of trapping headspace sulfide gas, establishing this time frame as optimal. Next

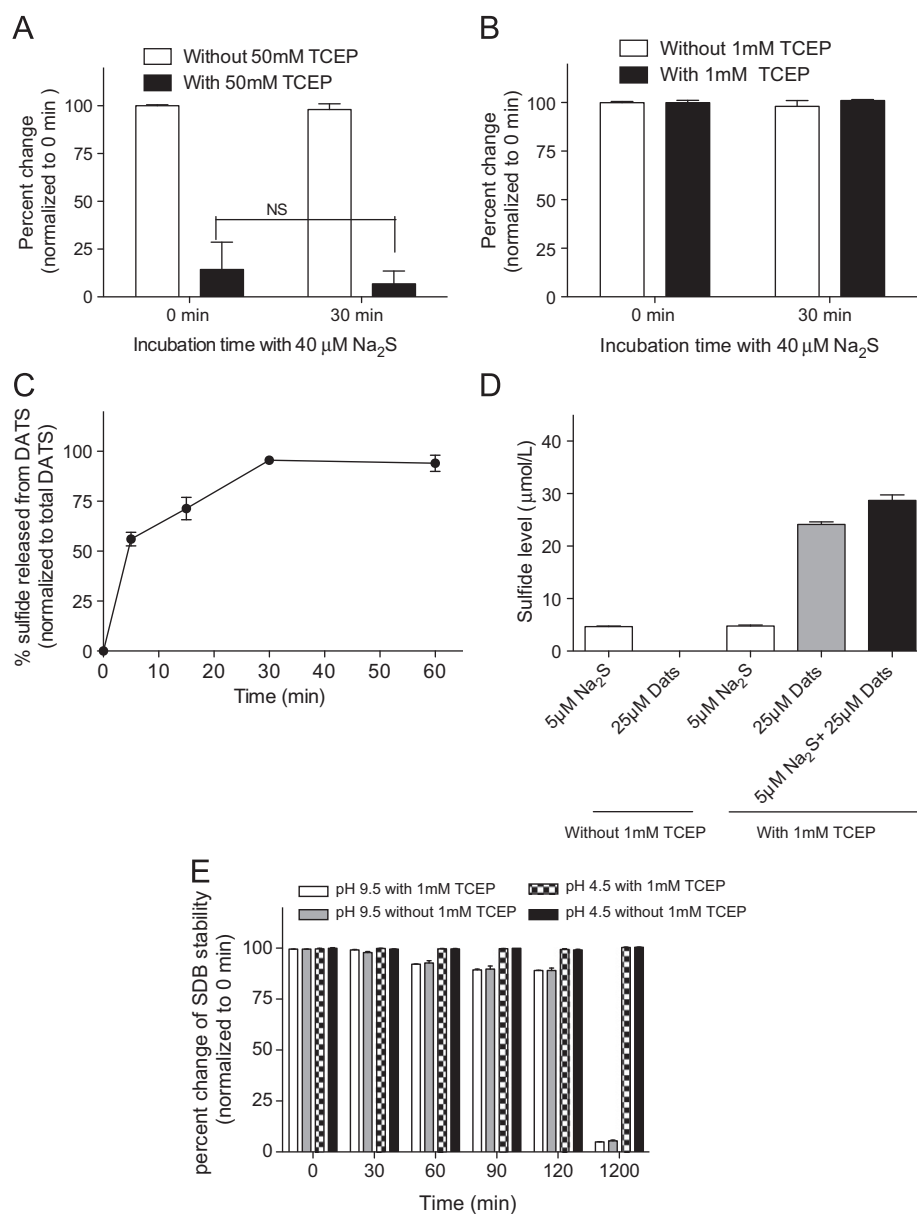


Fig. 4. Effect of TCEP on the derivatization of hydrogen sulfide with MBB. 40 μM sodium sulfide was reacted with MBB solution in the presence of (A) 50 mM TCEP or (B) 1 mM TCEP. (C) Effect of TCEP on diallyl trisulfide (DATS) reduction. 50 μl of 25 μM DATS was incubated with 450 μl of 0.1 M phosphate buffer (pH 2.6, 1 mM TCEP) at various time points, and the resulting hydrogen sulfide was trapped with 0.1 M Tris–HCl buffer for 30 min. (D) Efficiency of the protocol including reducing, releasing, and trapping. 5 μM Na_2S and 25 μM DATS were used with and without TCEP. (E) Effect of TCEP on stability of SDB across pH range.

we sought to demonstrate that percentage recovery of acid volatilized hydrogen sulfide was optimal, as demonstrated in Fig. 2(B), in which the sulfide content of a known molar solution of sodium sulfide was measured directly and after acid release and alkaline re-trapping. Next, we sought to identify the optimal time for release of hydrogen sulfide after acidification of plasma, as shown in Fig. 2(C). Plasma acid-labile sulfide is maximally released after incubation with 100 mM phosphate buffer (pH 2.6) for 30 min. Therefore, 30 min of releasing time and 30 min of trapping time were applied in all the subsequent experiments. Furthermore, the residual hydrogen sulfide in the plasma after volatilization by acidification was measured and found to be $0.0125 \pm 0.0029 \mu\text{M}$, which is slightly above the reaction solution background level, $0.00725 \pm 0.0018 \mu\text{M}$. This does not represent a significant fraction of H_2S detected in the headspace after acid volatilization.

Effect of plasma proteins on trapped hydrogen sulfide

To determine the potential reaction between plasma proteins and volatilized hydrogen sulfide, headspace hydrogen sulfide gas was trapped in 100 mM Tris-HCl buffer (pH 9.5, 0.1 mM DTPA) without and with removal of plasma. Fig. 3(A) demonstrates that the measured hydrogen sulfide is decreased when the trapping process occurs with the plasma remaining in the reaction vessel as opposed to removal and replacement with the 100 mM Tris-HCl (pH 9.5) buffer. We confirmed that this loss of hydrogen sulfide is attributable to the formation of protein persulfides by measurement of persulfide formation using the cyanolysis method compared to known molar concentrations of sodium sulfide reacted with plasma (Fig. 3(B) and (C)).

Effect of TCEP on sulfane sulfur reduction and MBB derivatization reaction of hydrogen sulfide

TCEP is a strong reducing agent and can react with MBB as mentioned above. Therefore, the effects of various concentrations of TCEP on sulfide derivatization were studied. Before sulfide derivatization, 50 or 1 mM TCEP was added to the sulfide stock solution containing $40 \mu\text{M}$ sodium sulfide. Fig. 4(A) and (B) demonstrate that TCEP can affect the sulfide-monomobimane reaction in the presence of 50 mM TCEP, but there is no significant effect in the presence of 1 mM TCEP. We postulate that at higher TCEP concentrations, the TCEP reaction with MBB limits the amount of MBB available to react with sulfide, hence the importance of maintaining a ratio of 1:10 TCEP to MBB.

Diallyl trisulfide (DATS) is an organic polysulfide compound found in garlic that acts as a sulfide donor. DATS was used to verify the efficiency of TCEP reduction of the disulfide bonds. Fig. 4(C) shows that 95% of DATS is reduced to free sulfide after incubation with 100 mM phosphate buffer (pH 2.6, 0.1 mM DTPA and 1 mM TCEP) for 30 min. Fig. 4(D) demonstrates that DATS is stable in the absence of TCEP but with TCEP there is complete reduction of and an expected release of hydrogen sulfide with 100 mM phosphate buffer (pH 2.6, 0.1 mM DTPA and 1 mM TCEP) for 30 min. This was demonstrated both in solution alone and in a mixture of $5 \mu\text{M}$ sodium sulfide plus $25 \mu\text{M}$ DATS.

We next examined the stability of the sulfide-dibimane product (SDB) in the presence of TCEP. The reaction between hydrogen sulfide and MBB occurs under alkaline conditions and is then terminated and stabilized with 200 mM sulfosalicylic acid. Fig. 4(E) shows that hydrolysis of the SDB is increased at pH 9.5 in the presence of 1 or 0 mM TCEP, with only 5% SDB remaining after a 20-h incubation. In the acid buffer, however, SDB is stable at 4°C in the presence of 1 or 0 mM TCEP.

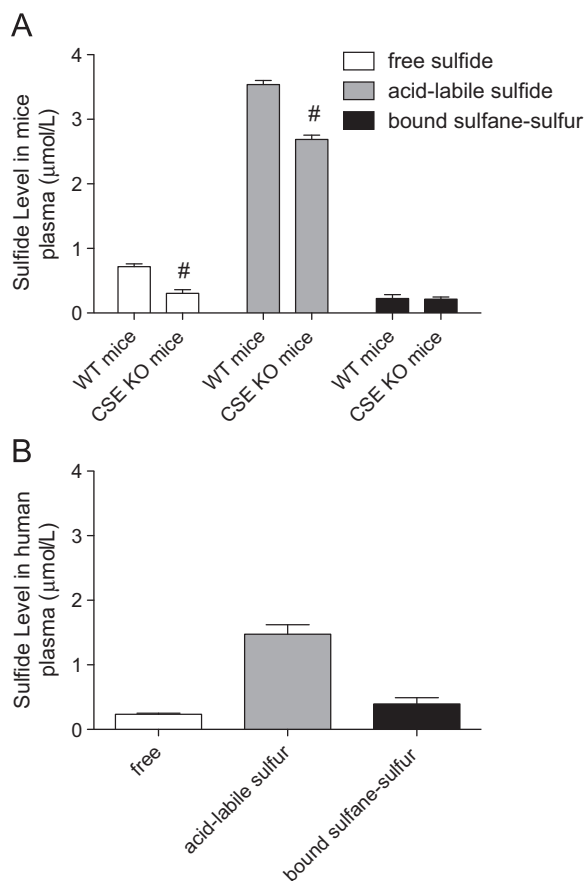


Fig. 5. Levels of free hydrogen sulfide, acid-labile sulfur, and bound sulfane sulfur in murine and human plasma. (A) C57BL/6J mouse plasma in comparison with that of CSE^{-/-} mice. (B) Human plasma from healthy male human controls.

Comparison of free sulfide, acid-labile sulfur, and bound sulfane sulfur in murine and human plasma samples

Free sulfide, acid-labile sulfur, and bound sulfane sulfur levels were measured in the plasma of wild-type C57BL/6J, CSE gene-deficient mice and healthy human volunteers. Fig. 5(A) shows that CSE knockout mice, known to be defective in hydrogen sulfide production, show a significant decrease in free and acid-labile sulfur compared to wild-type mice, but no significant difference in bound sulfane sulfur. In Fig. 5(B), we show that the free hydrogen sulfide pool in plasma from healthy human volunteers is in the low-nanomolar range but that the acid-labile sulfur pool is in the low-micromolar range. The bound sulfane sulfur pool was significantly smaller, similar to the murine models. The low-micromolar levels of the acid-labile pool in plasma from both mice and humans, in contrast to the high-nanomolar levels of free hydrogen sulfide, support the theory of a reversible sulfide sink into and from which hydrogen sulfide can be stored or released to effect biologic functions.

Caveats

Important general experimental considerations with respect to hydrogen sulfide measurement [13,18] remain relevant and should be carefully monitored:

- 1) Volatilization of hydrogen sulfide from the samples is accomplished by immediate collection of samples into vacuum tubes that should be maintained at all times without plasma after

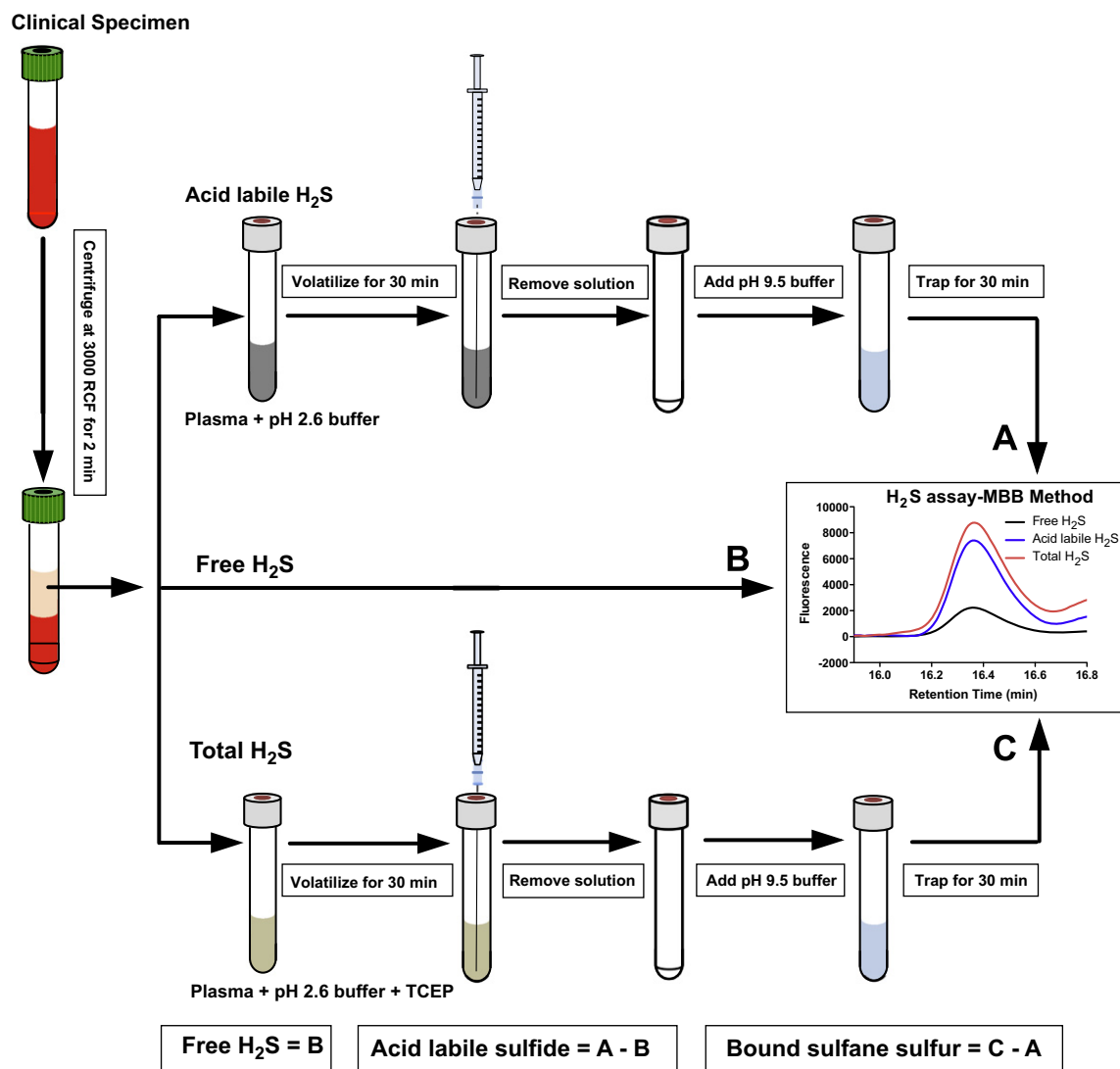


Fig. 6. Method overview illustration.

- volatilization and the transfer of reagents and solutions is done via a needle inserted into the rubber stop-cock of the vacuum tubes, hence avoiding loss of vacuum and gas samples [31].
- 2) Binding of H₂S to experimental vessels is minimized by using polypropylene reaction vessels.
 - 3) Lithium heparin is used as anticoagulant as it has been shown to be less prone to causing hemolysis than EDTA [32].
 - 4) Samples should be transported at 4 °C to minimize enzymatic production or degradation of hydrogen sulfide.
 - 5) MBB is a light-sensitive reagent, so derivatization should be performed in the dark.
 - 6) A 1% oxygen environment is maintained throughout the reaction protocol with acid and reducing agents as well as the MBB reaction process.

Conclusion

The field of hydrogen sulfide measurement continues to evolve, with modifications of various methods, reports of various fluorescent probes [33–35], and applications of new technologies such as nanotubes and quantum dots [36,37]. The new method

described here (Fig. 6) opens the door to accurate, quantitative, and scalable measurement of discrete pools of hydrogen sulfide from primary volatile sulfide pools. Use of this protocol on both experimental and clinical specimens will further enhance our understanding of hydrogen sulfide bioavailable equivalents during pathophysiological events and provide a critical analytical detection method for pharmacologic endeavors to manipulate the levels of hydrogen sulfide for therapeutic purposes.

Acknowledgments

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