Measurement of plasma hydrogen sulfide in vivo and in vitro

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Abstract

The gasotransmitter hydrogen sulfide is known to regulate multiple cellular functions during normal and pathophysiological states. However, a paucity of concise information exists regarding quantitative amounts of hydrogen sulfide involved in physiological and pathological responses. This is primarily due to disagreement among various methods employed to measure free hydrogen sulfide. In this article, we describe a very sensitive method of measuring the presence of H_2S in plasma down to nanomolar levels, using monobromobimane (MBB). The current standard assay using methylene blue provides erroneous results that do not actually measure H_2S. The method presented herein involves derivatization of sulfide with excess MBB in 100 mM Tris–HCl buffer (pH 9.5, 0.1 mM DTPA) for 30 min in 1% oxygen at room temperature. The fluorescent product sulfide-dibimane (SDB) is analyzed by RP-HPLC using an eclipse XDB-C18 (4.6×250 mm) column with gradient elution by 0.1% (v/v) trifluoroacetic acid in acetonitrile. The limit of detection for sulfide-dibimane is 2 nM and the SDB product is very stable over time, allowing batch storage and analysis. In summary, our MBB method is suitable for sensitive quantitative measurement of free hydrogen sulfide in multiple biological samples such as plasma, tissue and cell culture lysates, or media.

Keywords

Antioxidant; Thiol; Bimane; Protocol; Plasma; Free radicals

Hydrogen sulfide (H_2S) is commonly recognized as a colorless, flammable, water-soluble gas with an offensive odor of rotten eggs [1,2] produced from a variety of sources, but also found in mammalian tissues, where it is generated during cysteine metabolism [3]. H_2S is produced predominantly by tissue-specific enzymes [4–6], including cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE), and 3-mercaptosulfurtransferase (MST). Fig. 1 demonstrates the various formation pathways of hydrogen sulfide, modified from Hughes et al. [7]. CBS and CSE are pyroxidal-5′-phosphate-dependent enzymes, which use L-cysteine as their principal substrate. CBS is most abundantly expressed in brain, whereas the activity of CSE is highest in liver, kidney, and blood vessels [8–12]. MST can produce H_2S

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or oxidize its sulfur to sulfide and thiosulfate in the mitochondria and can also transfer sulfur from 3-mercaptopyruvate to thiocysteine in the cytosol [13,14].

Similar to other gaseous mediators (e.g., nitric oxide and carbon monoxide), H_2S plays an important role in cardiovascular, neuronal, and endocrine systems including inflammatory processes [5,15–23]. Hydrogen sulfide has also been reported to participate in the pathogenesis of a variety of disorders, such as Alzheimer disease, Down syndrome, and ulcerative colitis [24,25]. However, specific cellular and molecular mechanisms of hydrogen sulfide are still unclear and the concentration-dependent effect of hydrogen sulfide on these responses remains largely unknown.

Accurate and reliable measurement of biologically free hydrogen sulfide concentrations can provide critical information regarding the amounts associated with various normal or abnormal biochemical processes. However, precise measurement of hydrogen sulfide in multiple biological matrices and in high-throughput fashion is controversial. In the past few years, much attention has been focused on measuring hydrogen sulfide by head-space gas analysis [26], spectrophotometric determination [27–29], and a silver sulfide or polarographic sensor [12,30,31]. Because of oxidation of sulfide and reaction of sulfide with a number of different species (e.g., superoxide radical, hydrogen peroxide, peroxynitrite, etc.) these assays have yielded highly variable results for determination of absolute hydrogen sulfide in biological samples resulting in little consensus for concentrations of hydrogen sulfide associated with normal or pathophysiological processes. In this study, we sought to develop a fluorimetric, reverse-phase (RP)-HPLC analytical method that stabilizes biological free hydrogen sulfide from oxidation and chemical reaction while being able to detect low levels of the molecule. Here we report that biological free hydrogen sulfide is rapidly derivatized with excess monobromobimane in a pH-, oxygen-, and metal-dependent manner resulting in a stable sulfide-dibimane product that can be sensitively measured by RP-HPLC coupled with fluorescence detection. This method has been rigorously evaluated against the commonly used methylene blue method of hydrogen sulfide detection in biological samples from our laboratory for quantitative measurement of hydrogen sulfide under several different conditions. This new detection method allows for reliable, sensitive, high-throughput measurement of biological free hydrogen sulfide that should substantially aid investigators in critically evaluating hydrogen sulfide concentration effects under various biological conditions.

Principles

Monobromobimane reacts quickly with hydrogen sulfide under basic conditions at room temperature to produce sulfide-dibimane. Bimane is a hydrophobic molecule, and sulfide-dibimane is more hydrophobic than most physiological thiols. This characteristic allows sulfide-dibimane to be separated by RP-HPLC with a gradient elution and analyzed by fluorescence detection. The unstable nature of hydrogen sulfide in solution makes measurement and analysis for biological models difficult. Derivatization with monobromobimane allows for a quick and accurate representation of sulfide present in the biological medium assayed.

Materials

1. Monobromobimane (MBB; Sigma–Aldrich, Cat. No. B4380).

2. Sodium sulfide (Alfa Aesar, Cat. No. 65122). *Note.* The commercial source of sulfide is extremely important for the preparation of assays and standard curves. Hydrogen sulfide and sodium sulfide should have a white color; if it is yellow it is not to be used. White sulfide products may contain impurities such as sulfite and thiosulfate formed by oxidation of the sulfide. In these experiments anhydrous sodium sulfide from Alfa Aesar was found to remain pure for several months in a vacuum desiccator [7].

3. *N*-ethylmaleimide (NEM; Sigma–Aldrich, Cat. No. 04259-5 G).

4. (4) 2-Mercaptoethanol (Sigma–Aldrich, Cat. No. M7154).

5. Ethyl acetate (Sigma–Aldrich, Cat. No. 650528).

6. Methanol (MeOH; Sigma–Aldrich, Cat. No. 34860).

7. Alltech Prevail SPE C18 cartridge (Grace, Cat. No. 605430).

8. Microtainer plasma separator tubes (BD Biosciences, Cat. No. 365958).

9. Sulfosalicylic acid (SSA; Sigma–Aldrich, Cat. No. S2130).

10. Acetonitrile (CH_3CN; Sigma–Aldrich, Cat. No. 34851).

11. Trifluoroacetic acid (TFA; Thermo Scientific, Cat. No. 28903).

12. Zinc acetate solution (Sigma–Aldrich, Cat. No. 383058-500 G).

13. *N,N*-dimethyl-*p*-phenylenediamine sulfate (Sigma–Aldrich, Cat. No. 186384-25 G).

14. $FeCl_3$ (Sigma–Aldrich, Cat. No. 157740-5 G).

15. 1.5-ml tube (VWR, Cat. No. 20170–038).

16. PCR tube (Molecular Bioproducts, Cat. No. 34129).

17. Glass tube (National Scientific, Cat. No. 4011).

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. HPLC system: Shimadzu Prominence ultra fast liquid chromatograph equipped with fluorescence detector (HPLC 20A prominence).

5. Hypoxic chamber (Coy Laboratory Products, Inc.; large glove box).

Protocol

Preparation of MBB stock solution and calibration standard

1. Place plastic 15-ml tubes, 15 1.5-ml Eppendorf tubes, 100-μl and 1-ml pipettes, and ~30 ml CH_3CN in a 50-ml tube in hypoxic chamber. (*Note.* An argon chamber can also be used in conjunction with an oxygen meter to monitor chamber oxygen concentration.)

2. Purge the chamber with nitrogen gas to 1% O_2.

3. Deoxygenate the CH_3CN by bubbling with argon for 10 min. (*Note.* Vacuum can also be used to degas the solvents.)

4. Turn off the room light. (*Note.* MBB solution should be kept protected from light; exposure to light may result in photolysis of MBB resulting in formation of fluorescent bimane [32].)

5. Place the MBB vial in the hypoxic chamber.

6. Prepare a 10 mM solution of MBB in CH_3CN (2.71 mg/ml).

7. Add the calculated volume of deoxygenated CH_3CN and vortex the tube to make sure that all the MBB has been dissolved.

8. Take 1 ml of the MBB solution and transfer to the prepared Eppendorf tubes. Take the tubes out of the hypoxic chamber.

9. Place the tubes containing MBB solution at −20 °C.

10. Prepare standard sulfide solutions fresh in the hypoxic chamber, in the concentration range of 1.0 to 200 nM or μM.

Synthesis of sulfide-dibimane

1. Add 4 ml of a 6 mM sodium sulfide solution to a 50-ml tube with 10 ml of 100 mM deoxygenated Tris–HCl buffer (pH 9.5, 0.1 mM diethylenetriaminepentaacetic acid (DTPA)).

2. Slowly add 5 ml of a 10 mM MBB solution, stirring continuously.

3. Incubate for 30 min in 1% O_2 at room temperature.

4. Quench excess MBB by adding 1 ml of 2-mercaptoethanol.

5. Extract the mixture with 10 ml of ethyl acetate and transfer organic layer into a 50-ml tube. (*Note.* In our hands approximately 80% of the initial sulfide-dibimane is recovered in the ethyl acetate layer (data not shown).)

6. Evaporate organic layer under nitrogen stream, then dissolve crude product in 6 ml of water:MeOH mixture (10:90).

7. Purify sulfide-dibimane on an Alltech Prevail SPE cartridge:

a. Condition the cartridge by passing 6 ml of water, followed by 3 ml of water:MeOH mixture (50:50), 3 ml of pure MeOH, and again 6 ml of pure water.

b. Load the crude product onto the cartridge

c. Wash the cartridge with 4×3 ml of water.

d. Wash the cartridge with 2×3 ml of MeOH:water mixture (10:90).

e. Wash the cartridge with 3×3 ml of MeOH:water mixture (50:50). Sulfide-dibimane should elute in this step.

f. Wash the cartridge with 2×3 ml of MeOH:water mixture (20:80), and then wash the cartridge with 2×3 ml of MeOH.

g. After assaying with RP-HPLC, evaporate the solvent in the fractions containing pure sulfide-dibimane (MW 446.54 g/mol). The resulting residue should have a yellow tint.

Extinction coefficient determination for sulfide-dibimane

1. Prepare a 0.25 M solution of sulfide-dibimane using one of the following solvents: water, pH 4.5 HCl solution, methanol, or ethyl acetate (this provides an extinction coefficient for the solvents your sulfide-dibimane (SDB) may be dissolved in while using this protocol).

2. Measure absorbance values from 300 to 800 nm (only a single peak was observed between 300 and 450 nm).

3. From the results in Fig. 2 the extinction coefficients were calculated according to the following equation: C [mol L^{-3}]= $A/(\varepsilon$ [L^3 mol^{-1} cm^{-1}]×L [cm]).

4. The extinction coefficient values for the various solvents are reported in Table 1.

Preparation of calibration standards

1. Prepare a series of dilutions of purified sulfide-dibimane solution to give 5, 10, 50, 100, and 200 nM standards (weigh powder, verify concentrations via extinction coefficient).

2. Transfer 200 μl of these standards into HPLC vials containing 0.2-ml inserts and place in the autoinjector and keep temperature at 4 °C.

3. Inject 10 μl of these standards into RP-HPLC coupled with fluorescence detector.

4. Plot the peak area of sulfide-dibimane versus the amount of sulfide-dibimane injected and fit the curve by linear regression analysis.

Derivatization reaction of hydrogen sulfide with monobromobimane

Hydrogen sulfide reaction conditions with monobromobimane were optimized using 6 μM anhydrous sodium sulfide solution as the hydrogen sulfide donor.

1. Prepare the reaction buffer: 100 mM Tris–HCl buffer (pH 9.5, 0.1 mM DTPA). Degas the solution with nitrogen gas for 30 min, and keep in the hypoxic chamber (1% O_2) for 10 min.

2. Prepare 6 μM sodium sulfide solution: 46.824 mg sodium sulfide is dissolved in 10 ml of degassed water and then diluted accordingly in the hypoxic chamber.

3. Prepare 200 mM sulfosalicylic acid solution: 508.44 mg sulfosalicylic acid is dissolved in 10 ml of water.

4. In a reactor combine the following:

   a. 30 μl of 6 μM sodium sulfide solution,

   b. 70 μl of reaction buffer,

   c. 50 μl of 10 mM MBB.

5. Incubate these vials for 30 min at room temperature in the hypoxic chamber.

6. Add 50 μl of 200 mM sulfosalicylic acid solution to stop the reaction.

7. Quantify the amount of sulfide-dibimane by RP-HPLC with fluorescence detection.

Similarly, to optimize the effects of other experimental conditions, hydrogen sulfide was reacted with monobromobimane under various conditions including 0–1 h reaction time, pH 7.0–9.5 reaction buffer, 1–21% oxygen in the hypoxic chamber, and various reaction tubes (1.5-ml tube, PCR tube, and glass tube).

Stability of sulfide-dibimane

1. Prepare various pH solutions: pH 4.5, pH 8, pH 9.5.

2. Prepare 12 μM sulfide-dibimane solution using the various pH solutions.

3. Treat the mixtures with or without 50 mM NEM.

4. At various time points, withdraw an aliquot (200 μl each) of these solutions and analyze by RP-HPLC. For greater sample reproducibility, keep the time constant.

Quantification of sulfide-dibimane by RP-HPLC with fluorescence detection

1. Prepare two mobile phases: (A) water containing 0.1% (v/v) TFA and (B) 99.9% CH_3CN, 0.1% (v/v) TFA. Both of the mobile solutions should be filtered through 0.2-μm membrane filter.

2. Inject 10 μl of sample into the HPLC system with an Agilent Eclipse XDB-C18 column (5 μm, 80 Å, 4.6×250 mm) equilibrated with 15% CH_3CN in water containing 0.1% (v/v) TFA.

3. Set a flow rate of 0.6 ml/min.

4. Fluorescence detection should be set with 390 nm (excitation) and 475 nm (emission), using a gain of 4×.

5. Separate monobromobimane and sulfide-dibimane using the gradient shown in Table 2.

6. The retention times for the sulfide-dibimane and the monobromobimane peaks are 15.75 and 16.80 min, respectively.

7. Measure the amount of hydrogen sulfide from linear plots of the HPLC peak areas of sulfide-dibimane vs known concentration of sulfide solution.

Effect of NEM on hydrogen sulfide reaction with monobromobimane

1. Prepare 100 mM NEM stock solution.

2. Incubate respective final concentrations of NEM (0, 10, 50, 100, 250, 500, or 1000 μM) with 6 μM sodium sulfide for 5 min.

3. Transfer 30 μl of the NEM/sodium sulfide sample into 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% O_2 in the hypoxic chamber at room temperature.

6. Add 50 μl of 200 mM SSA solution to stop the reaction.

7. Use 5 μl of the reaction solution for RP-HPLC analysis.

Measurement of sulfide by methylene blue [18]

1. Prepare the following solutions.

    a. Zinc acetate solution: 1 g dissolved in 100 ml degassed water.

    b. *N,N*-dimethyl-*p*-phenylenediamine sulfate solution: 7.4094 mg dissolved in 1 ml of 7.2 mM HCl.

    c. FeCl$_3$ solution: 4.866 mg dissolved in 1 ml of 1.2 mM HCl.

2. Mix 75 μl of sample with 250 μl of 1% (w/v) zinc acetate and 425 μl degassed water in a test tube.

3. Add 133 μl of 20 mM *N,N*-dimethyl-*p*-phenylenediamine sulfate in 7.2 mM HCl and 133 μl of 30 mM FeCl$_3$ in 1.2 mM HCl.

4. Incubate for 10 min at room temperature.

5. Add 250 μl of 10% (v/v) trichloroacetic acid to the reaction mixture and pellet by centrifugation at 12,000 rpm for 5 min.

6. Perform UV wavelength scan from 400 to 800 nm.

Detection of hydrogen sulfide in the plasma

1. Place BD microtainer plasma separator tubes (No. 365958) with lithium heparin on ice.

2. Collect blood from mice by retro-orbital bleed using heparinized plastic capillary tubes (do not use glass hematocrit tubes) directly into plasma separator tubes.

3. Centrifuge at 3000 rpm for 2 min at 4 °C.

4. Transfer 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 CH_3CN) to the PCR tube.

5. Incubate the solution in 1% O_2 in the hypoxic chamber 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 precipitate 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. Transfer 100 μl of the supernatant to an HPLC vial equipped with a 200 μl insert, seal the vial.

9. Place in the autosampler with the tray cooled to 4 °C until analysis by RP-HPLC. (*Note.* In our hands a loss of sulfide-dibimane occurs if stored at room temperature; approximately 5% loss after 48 h at room temperature.)

10. The value obtained after analysis by RP-HPLC is multiplied by a factor of 6.6. This factor is included to account for the various dilutions made to the sample before its injection and analysis on RP-HPLC. (A 30-μl sample of plasma is diluted with 70 μl of buffer, 50 μl of MBB, and 50 μl of sulfosalicylic acid, resulting in a 200/30 or 6.6 dilution factor.)

Calculations and results

Measurement techniques for analyzing hydrogen sulfide

Of the identified biological gasses H_2S has the largest dipole moment (H_2S, 0.97; NO, 0.16; CO, 0.13; O_2, 0), suggesting that it is the least likely to diffuse across cellular membranes. Ionization of H_2S may account for its reduced ability to diffuse through the lipid bilayer compared to O_2 or CO. In an aqueous solution, free hydrogen sulfide is a weak acid with two acid dissociation constants. The pK_a values for the first and second dissociation steps of H_2S are 7.04 and 19±2, respectively (Fig. 3A). Depending on the pH of the surrounding environment H_2S may exist as different species. At physiological pH and 37 °C, about 20% of the sulfide is present as H_2S, whereas at pH 7.4 and 25 °C about 40% of the sulfide is present as H_2S. At pH 9.5, free hydrogen sulfide mainly exists as HS^- [7]. In vivo the pH is favorable for sulfide to exist primarily as H_2S and its highly reactive anion, HS^- [33].

The fluorescent reagent MBB has been widely used to measure various thiol-containing species through alkylation [34]. As shown in Fig. 3B, S-alkylation occurs twice with sulfide, forming SDB. MBB readily reacts with HS^- at alkaline pH allowing for free HS^- measurement under its most stable conditions. Because of hydrophilic differences among the thiol-dibimane derivatives, sulfide-dibimane can be separated and assayed via RP-HPLC.

Moreover, the highly fluorescent nature of sulfide-dibimane (λ_{ex} =390 nm, λ_{em} = 475 nm) allows low concentrations of hydrogen sulfide to be measured easily.

HS^- can also be measured at acidic pH, by reacting with *N,N*-dimethyl-*p*-phenylenediamine or with ferric chloride ($FeCl_3$) to form methylene blue (MB) (Fig. 3C), and this is the most common technique currently in use [35]. This approach can be problematic, making it a less than ideal methodology for measuring biologic levels of H_2S. One issue is that the final product is blue in color and is measured at 670 nm [35]; at this wavelength significant interference by other colored substances has been documented [35]. Another issue is that sample acidification liberates acid-labile sulfur pools [36] that can introduce artifact into the measurement, thereby diminishing sensitivity in detecting free HS^-. Because of these issues with the MB method, there is a clear need for a selective and sensitive method to quantify free biological H_2S in a large-batch format.

Method sensitivity

The MBB method involves the reaction of hydrogen sulfide with monobromobimane under alkaline conditions to form SDB, involving a 2:1 stoichiometry of MBB to sulfide. Sulfide-dibimane and monobromobimane can be analyzed by RP-HPLC using an excitation wavelength of 390 nm and emission wavelength of 475 nm (Fig. 4A). The retention time of sulfide-dibimane and monobromobimane is 15.8 and 16.7 min, respectively, providing distinct separation of SDB and excess MBB. Measurement of sulfide-dibimane increased linearly with increasing concentrations of sulfide (Fig. 4B). The minimum detectable level of sulfide was approximately 5 nM. The injection volume used was 10 μl; therefore, the limit of detection (signal-to-noise ratio=3) for sulfide-dibimane by the MBB method was 0.02 pmol. Using the MB method of hydrogen sulfide detection, the detection limit was 2 μM, making the MBB method of detection 3 orders of magnitude more sensitive (Fig. 4C).

Effects of pH, trace metals, and oxygen concentration on sulfide derivatization

Thiol groups are highly reactive and therefore highly susceptible to oxidation by ambient oxygen. It is therefore necessary to determine the effect oxygen has on derivatization of hydrogen sulfide. All reaction solutions were purged with nitrogen for 20 min to ensure deoxygenation. Deoxygenated solutions will slow the oxidation of sulfide; however, it may still be catalyzed by trace impurities of transition metals, such as iron and copper [33,37]. To ensure chelation of trace metals DTPA was also added into the reaction buffer, after the nitrogen-degassing procedure. Here we examined the effects of DTPA on H_2S derivatization. Fig. 5A demonstrates that the highest reaction percentage was achieved after 30 min of incubation, with or without 0.1 mM DTPA, under 1% oxygen. Using 0.1 mM DTPA during the derivatization of sulfide yielded the largest percentage of product formed. Therefore, 0.1 mM DTPA was applied in all the subsequent experiments. Oxygen concentration and pH of reaction buffer affect sulfide derivatization. At 21% oxygen with a pH of 9.5 the maximum reaction percentage was achieved, with approximately 70% reaction (Fig. 5B). At 10% oxygen, a pH of 9.5 was once again the optimal pH for maximum yield after sulfide derivatization, reaching a plateau around 80% (Fig. 5C). At 1% oxygen and a pH of 9.5 the optimal amount of sulfide derived plateaued near 90%, making it the ideal oxygen tension (Fig. 5D).

Effects of reaction vessel and NEM on sulfide derivatization

To estimate potential sulfide in various reaction vessels a 1.5-ml tube, a PCR tube, and a glass tube were used as reactors with 30 μl of 0, 30, and 600 μM sulfide solution. Sulfide derivatization was performed under 1% oxygen. After an incubation of 30 min, 50 μl of 200 mM SSA solution was added into the various reaction vessels and sulfide-dibimane in these samples was assayed by RP-HPLC. Fig. 6A shows that the 1.5-ml tube and the PCR tube contain more sulfide than the glass tube at 30 and 600 μM, making glass a poor choice as a reaction vessel. Fig. 6A also shows the background sulfide levels in the 1.5-ml tube to be higher than in the PCR tube, making the PCR tube the best choice as a reaction vessel. The rest of the experiments were performed using the PCR tube as the standard reaction vessel.

NEM is a thiol-reactive compound commonly used to modify –SH groups and widely used to protect thiols from oxidation. Therefore, the effect NEM exerts on sulfide derivatization was studied. Before sulfide derivatization, 0–1000 μM NEM was added to the sulfide stock solution. Fig. 6B demonstrates that NEM can affect the H_2S/monobromobimane reaction and has a direct impact on the sulfide-dibimane produced in an inversely proportional manner. These data clearly indicate that experiments using NEM to block the biological effects of H_2S may interfere with follow-up measurements of H_2S concentration.

Stability of SDB (derivatized sulfide)

Sulfide-dibimane is the resultant product of the alkylation reaction between sulfide and MBB. The derivatization process occurs under alkaline conditions and is stabilized with an acidic pH. Figs. 7A, B, and C show hydrolysis of the SDB mixture increasing with increasing pH values, with only 5% SDB remaining after a 20-h incubation at pH 9.5. At this alkaline pH the introduction of NEM further destabilizes the SDB product. A pH of 8.0 results in a slightly more stable SDB solution, with the majority of the product remaining after a 20-h incubation. By far the best pH to use for stability of SDB is 4.5. In this acidic environment the SDB formed from the derivatization reaction is stable at 4 °C for up to 2 weeks (Fig. 7D).

Stability of hydrogen sulfide in experimental buffer

Hydrogen sulfide exists in three forms: hydrogen sulfide (H_2S), hydrogen sulfide anion (HS^-), and sulfide anion (S_2^-). To estimate free hydrogen diffusion in plasma, sulfide stability in Tris–HCl buffer (100 mM, pH 9.5) was compared to that of phosphate-buffered saline (PBS; pH 7.4). This specifically addresses the question of whether the Tris–HCl buffer at pH 9.5 is the best derivatization buffer to use for biological specimens. Fig. 8A shows that the most stable conditions observed for H_2S are 1% oxygen in Tris–HCl buffer (100 mM, pH 9.5). This stability of H_2S in solution is maintained for approximately 20 min. Fig. 8B shows that under optimal conditions (1% oxygen and Tris–HCl buffer 100 mM, pH 9.5) H_2S is stabilized in plasma for at least 15 min. Using the Tris–HCl buffer in the presence of either 21 or 1% oxygen results in a significant difference in detection efficiency of H_2S in plasma (Fig. 8C). Statistics were performed using Prism GraphPad software; the test used for Fig. 8C was a Student t test using a 95% confidence interval.

Effect of protein on hydrogen sulfide determination

Hydrogen sulfide serves as a gaseous mediator, so distinguishing bound H_2S from free pools is an important measurement. To determine what role plasma proteins play in free H_2S pools a 20 micromolar sulfide solution was mixed with plasma. Fig. 9A demonstrates that with protein present in the plasma H_2S is quickly scavenged. Fig. 9B shows that, after extraction of proteins (MW >20 kDa) from the plasma, H_2S loss is much slower. Fig. 9C presents the sulfide amounts extracted from these samples, illustrating that time as well as protein concentration in the measured biological sample is of the utmost importance in obtaining an accurate measurement. Statistics were performed using Prism GraphPad software; the test used for Fig. 9C was a two-way ANOVA with a Bonferroni posttest using a 95% confidence interval.

Method sensitivity in an animal model

The MB method results in a sulfide product detectable in the 660–680 nm wavelength range. High concentrations of H_2S (1 mM) in our hands result in a definite peak in this wavelength range (Fig. 10A). However, using a much lower concentration (50 μM) and again scanning in this wavelength range resulted in no detectable peak (Fig. 10B). Fig. 10C shows a comparison of the MBB and MB methods after a 50 μM injection of Na_2S. The MBB method resulted in a sulfide peak at 1 min postinjection that rapidly decayed by 10 min and returned to baseline by 30 min. The MB method shows a small peak at 1 min; however, the MBB method provided much more detailed results.

Method sensitivity in murine mutant models characterized by lower H_2S levels

Both sulfide analysis techniques were applied to measure basal hydrogen sulfide in the plasma of C57Bl/6 J, $CSE^{-/+}$, and $CSE^{-/-}$ mice. CSE is a pyridoxal phosphate-dependent enzyme catalyzing the desulfhydration of L-cystine and a β-disulfide elimination reaction, which results in the production of thiocysteine. Thiocysteine reacts with cysteine to form sulfide. The MBB method of analysis provided clear differentiation between the three genotypes (Fig. 11A). Fig. 11B shows the expected results; the heterozygote knockout of CSE resulted in less sulfide, and the homozygote knockout resulted in the least measured sulfide. Fig. 11C shows the measurement of the same animals using the MB method, resulting in no clear differentiation between sulfide peaks. Fig. 11D shows that the MB method is unable to distinguish sulfide levels between the CSE heterozygote and the wild-type mice. The complete knockout mutant resulted in a significant decrease in sulfide levels compared to wild type. The MBB method is by far the most sensitive and representative of bioavailable H_2S in plasma. Statistics were performed using Prism GraphPad software. The test used for Figs. 11B and D was a one-way ANOVA with a Bonferroni posttest using a 95% confidence interval.

Caveats

Sulfide solutions should be limited in exposure to oxygen, because of the high reactivity of hydrogen sulfide to oxidation. For this purpose degassing all solutions is essential before analysis of samples.

Monobromobimane is a light-sensitive reagent so during derivatization the H_2S/MBB solution should be reacted in the dark.

Hydrogen sulfide readily binds to glass; therefore, high-quality polypropylene plastic tubes should be used for reactions. It is also important to use plastic capillary tubes coated with heparin when collecting the blood from mice.

This article reports a method for the measurement of sulfide concentration by hydrogen sulfide derivatization with MBB. Based on the experimental conditions set forth in this article hydrogen sulfide is accurately measured to low nanomolar levels compared to other widely used methods such as methylene blue.

Wintner et al. have published a paper using monobromobimane to measure sulfide levels in rat blood, yet their results are considerably different from ours [38]. In their study, glass reaction vessels were used for the derivatization of hydrogen sulfide with MBB. In our hands, glass significantly depleted hydrogen sulfide from the solution, resulting in decreased levels measured by MBB. However, our method did not require evaporation steps and consistently produced narrow band peaks of the same widths as Wintner's group in our HPLC analysis. Wintner et al. also purged the reaction vessel of oxygen using nitrogen gas; however, the amount of oxygen was not precisely measured during their procedure, whereas we maintained a 1% oxygen atmosphere throughout the derivatization process. The buffer solution used in the Wintner paper was pH 8.0; however, we determined that a buffer pH of 9.5 yields faster and more efficient derivatization of hydrogen sulfide with MBB. In summary, our methodology has identified specific variables to be cognizant of when analyzing hydrogen sulfide. Through careful comparison of hydrogen sulfide derivatization with MBB versus that of methylene blue, we clearly demonstrate that the methylene blue method does not produce a bona fide hydrogen sulfide peak at low physiological concentrations and that the MBB method is ideally sensitive and stable for biological detection in numerous samples.

Acknowledgments

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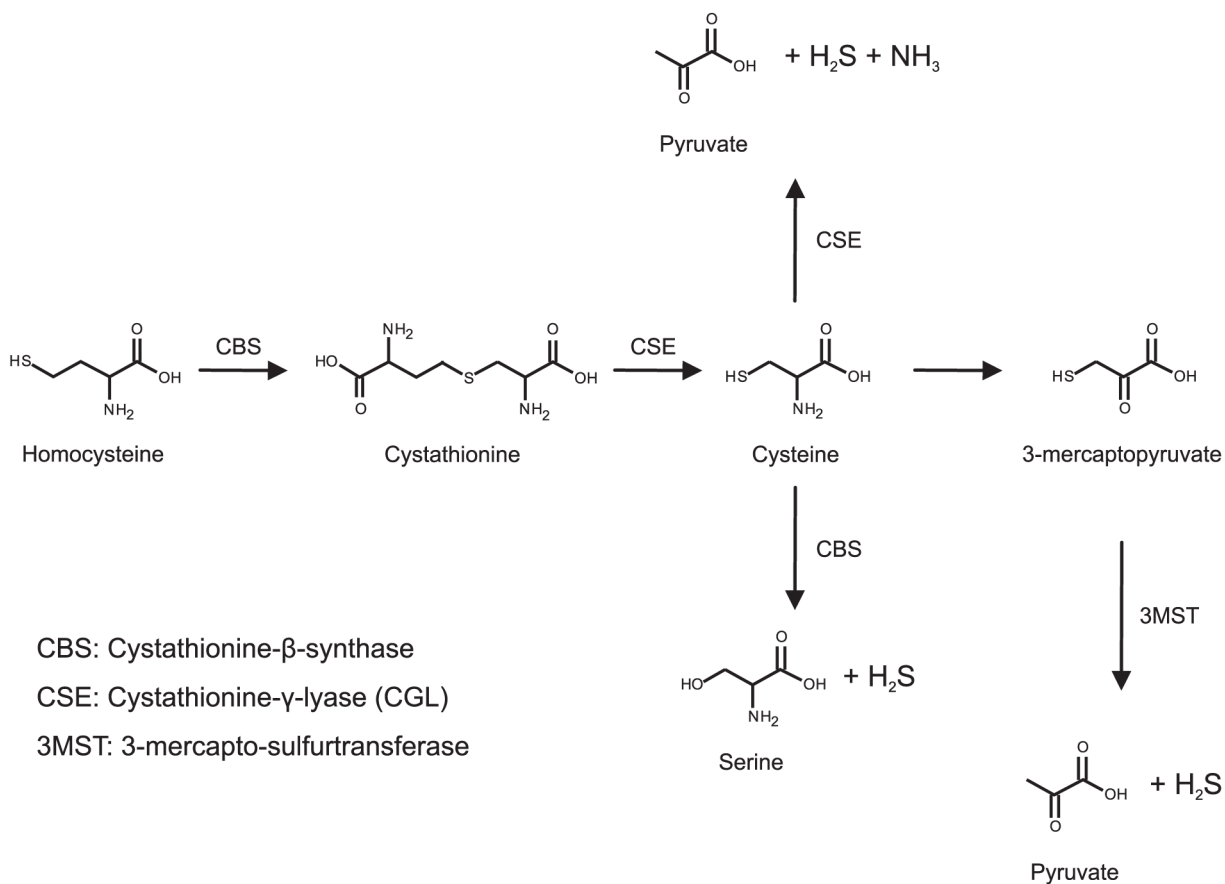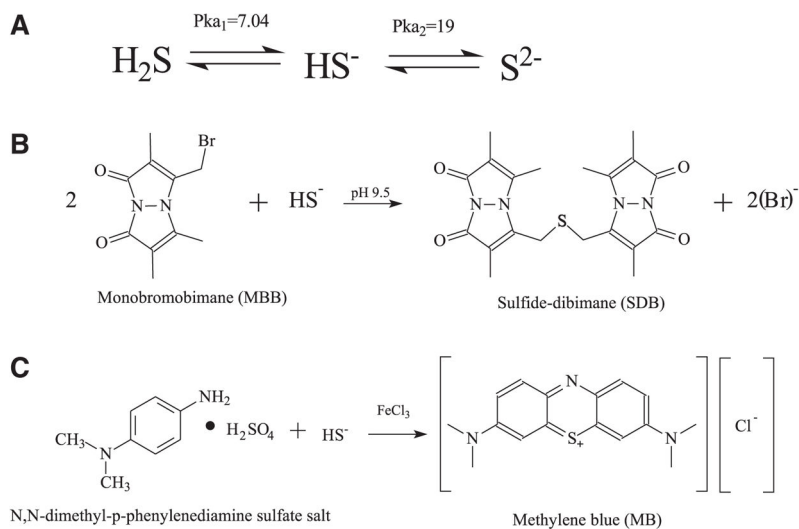
Fig. 1.
Schematic representation of the formation of H$_2$S depicting three divergent pathways.
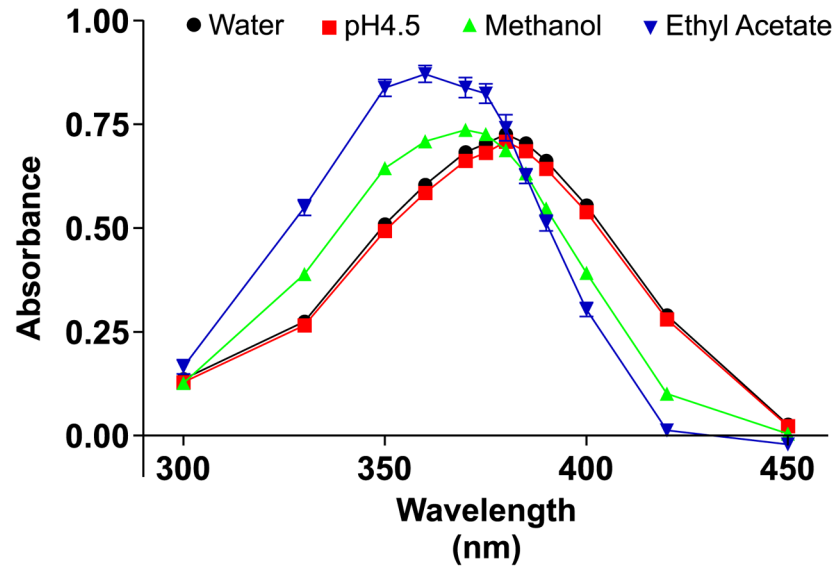
Fig. 2.
Absorbance curves of sulfide-dibimane dissolved in water, HCl (pH 4.5), methanol, or ethyl acetate. Analyzed from 300 to 450 nm.

Fig. 3.
Schematic representations of (A) the dissociation and (B, C) the derivatization of hydrogen sulfide with (B) monobromobimane or (C) *N,N*-dimethyl-*p*-phenylenediamine sulfate, forming sulfide-dibimane or methylene blue, respectively.
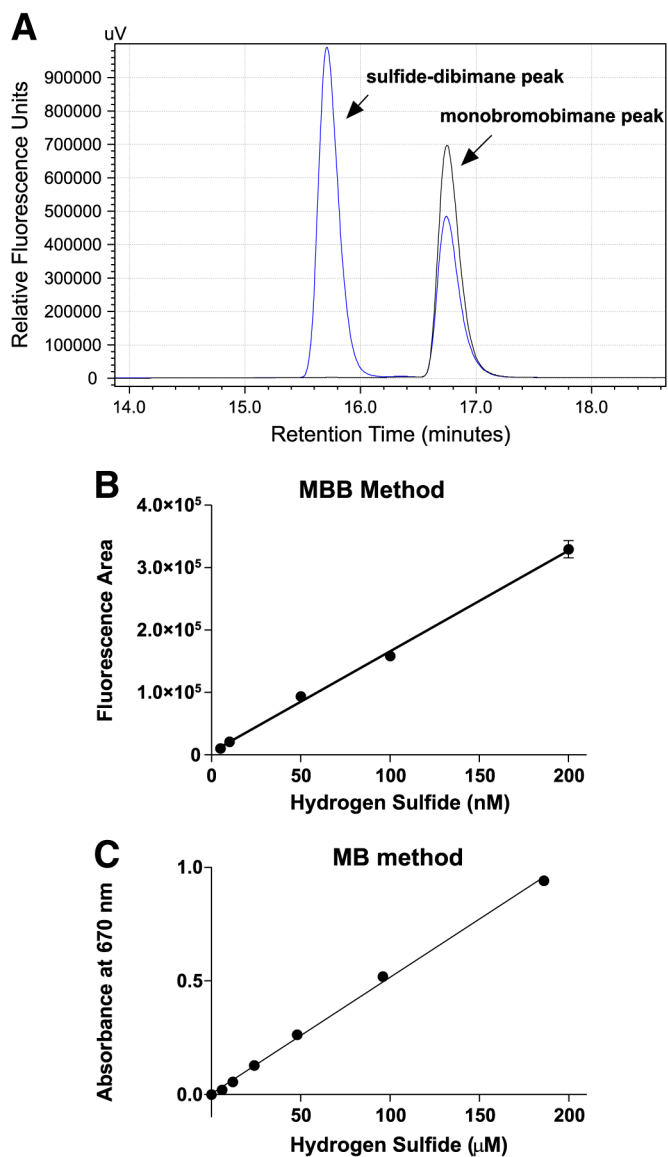
Fig. 4.
Comparison of MBB and MB methods. (A) HPLC spectrum of sulfide-dibimane (SDB) and monobromobimane (MBB). (B) Linear relationship between the fluorescence area of the SDB peak and the concentration of hydrogen sulfide in the MBB method. (C) Linear relationship between the absorbance at 670 nm and the concentration of sulfide in the MB method. *n*=6.
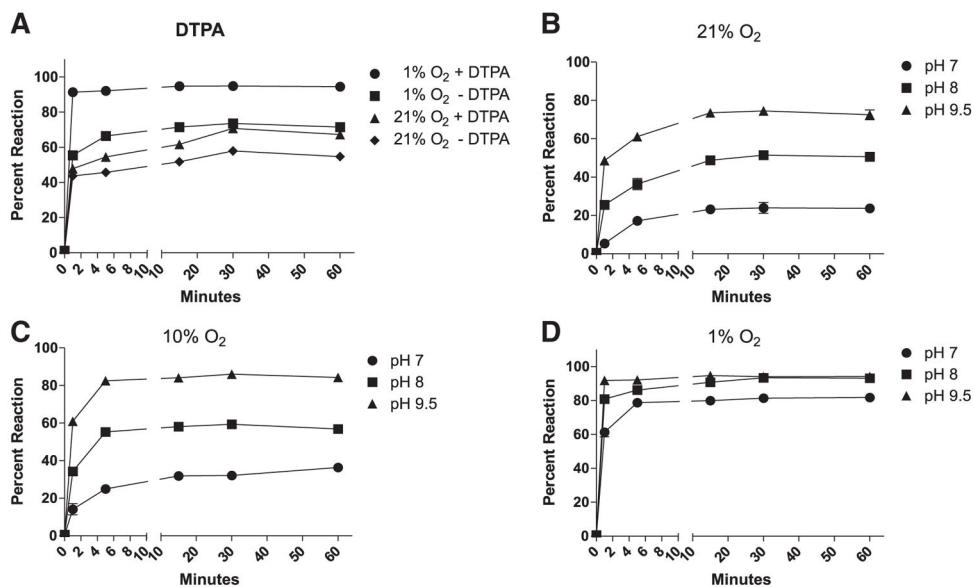
Fig. 5.
Effects of pH, oxygen, and DTPA on the derivatization of hydrogen sulfide with MBB. SDB was analyzed via RP-HPLC with fluorescence detection. (A) The effect DTPA has on the derivatization of hydrogen sulfide using MBB. (B) The derivatization of hydrogen sulfide (6 μM) with MBB and DTPA over time using a pH of 7, 8, or 9.5 and an oxygen concentration of 21%. (C) The derivatization of hydrogen sulfide (6 μM) with MBB and DTPA over time using a pH of 7, 8, or 9.5 and an oxygen concentration of 10%. (D) The derivatization of hydrogen sulfide (6 μM) with MBB and DTPA over time using a pH of 7, 8, or 9.5 and an oxygen concentration of 1%. n=6.
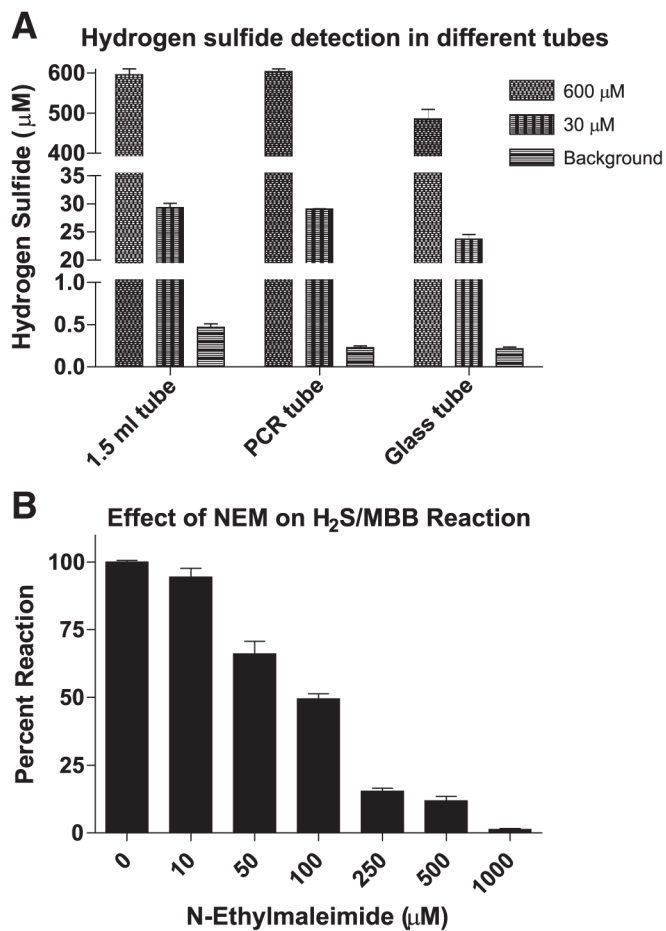
Fig. 6.
Hydrogen sulfide recovery from various reaction vessels and the effect NEM has on the derivatization of H_2S. (A) The amount of hydrogen sulfide measured after adding 30 or 600 μM, using a 1.5-ml tube, a PCR tube, or a glass tube as a reaction vessel. (B) The effect of increasing concentrations of NEM on the derivatization of hydrogen sulfide. $n=6$.
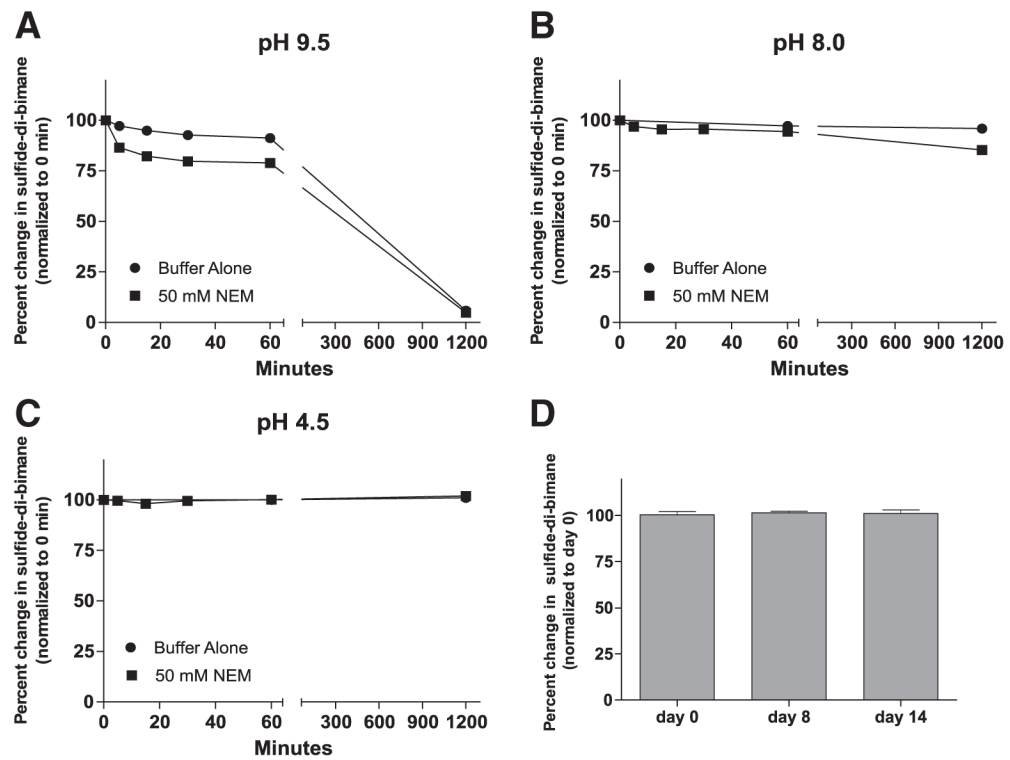
Fig. 7.
Stability of SDB in the presence or absence of NEM at various pH values. (A) The stability of SDB at pH 9.5. (B) The stability of SDB at pH 8.0. (C) The stability of SDB at pH 4.5. (D) At pH 4.5 SDB is stable up to 2 weeks. $n=6$.
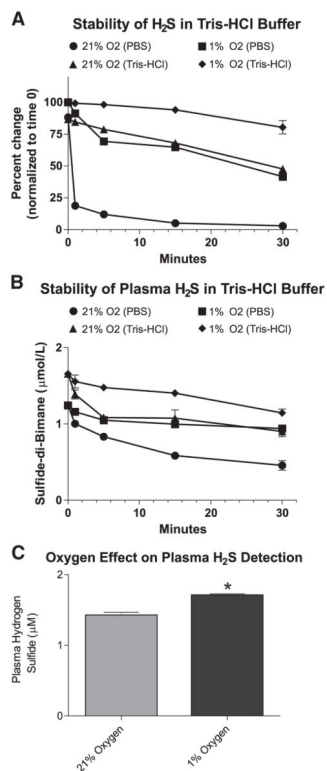
Fig. 8.
Effect of derivatization buffer on hydrogen sulfide level. (A) The effects of two different oxygen amounts (1 and 21%) and two different buffer systems (PBS, pH 7.4, and Tris–HCl, pH 9.4) on the stability of hydrogen sulfide over 30 min. (B) The effects of two different oxygen amounts (1 and 21%) and two different buffer systems (PBS, pH 7.4, and Tris–HCl, pH 9.4) on the stability of hydrogen sulfide in plasma over 30 min. (C) The effects of 1 and 21% oxygen on hydrogen sulfide concentration. *$p<0.05$, $n=6$.
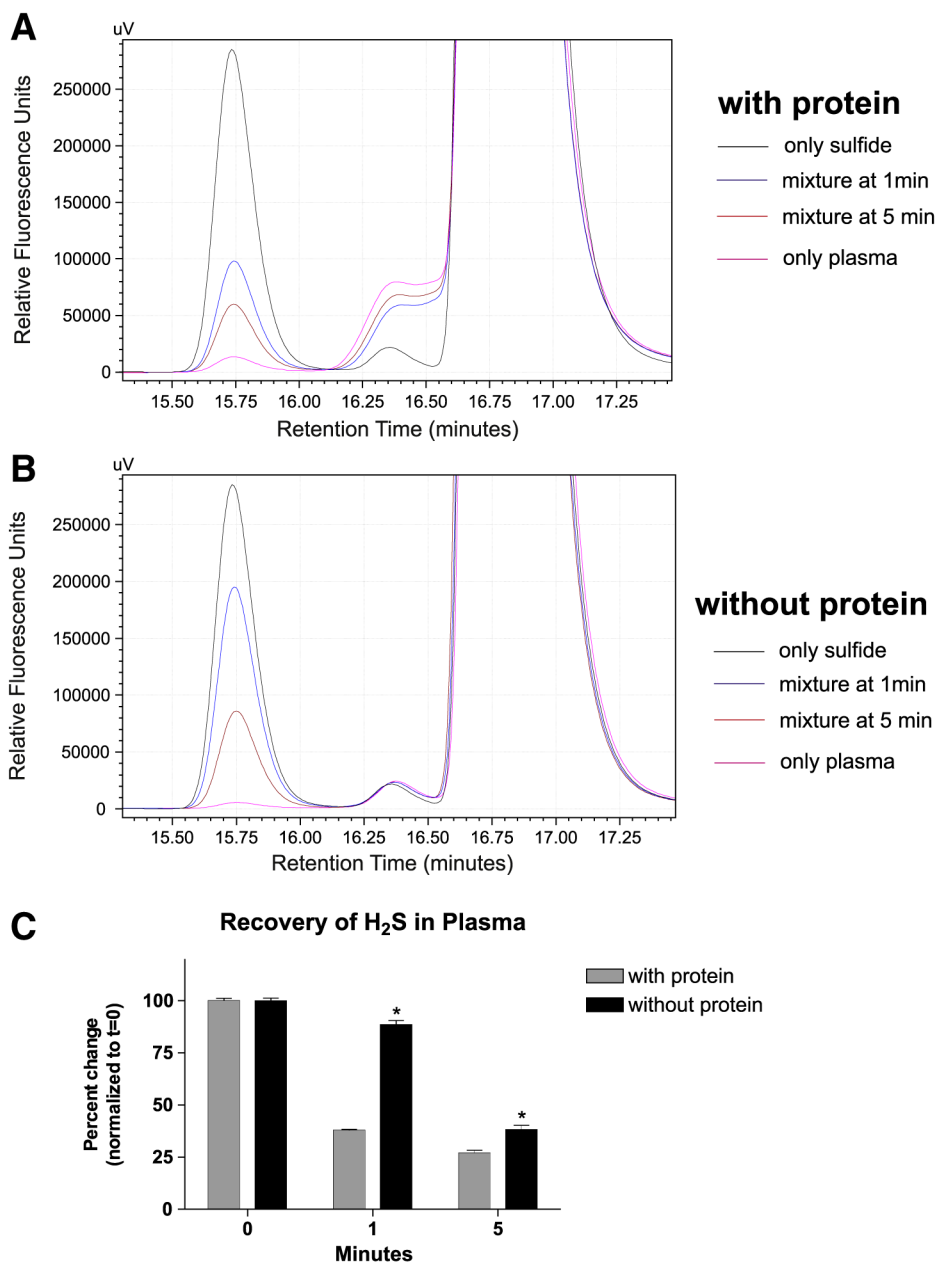
Fig. 9.
Effect of plasma on hydrogen sulfide recovery. (A) The results of HPLC analysis of hydrogen sulfide after mixture with protein-rich plasma at 1 and 5 min. (B) The results of HPLC analysis of hydrogen sulfide after mixture with plasma (no protein) at 1 and 5 min. (C) Side-by-side comparison of mixing with plasma, either containing protein or not, at 1 and 5 min. *$p<0.05$, *n*=6.
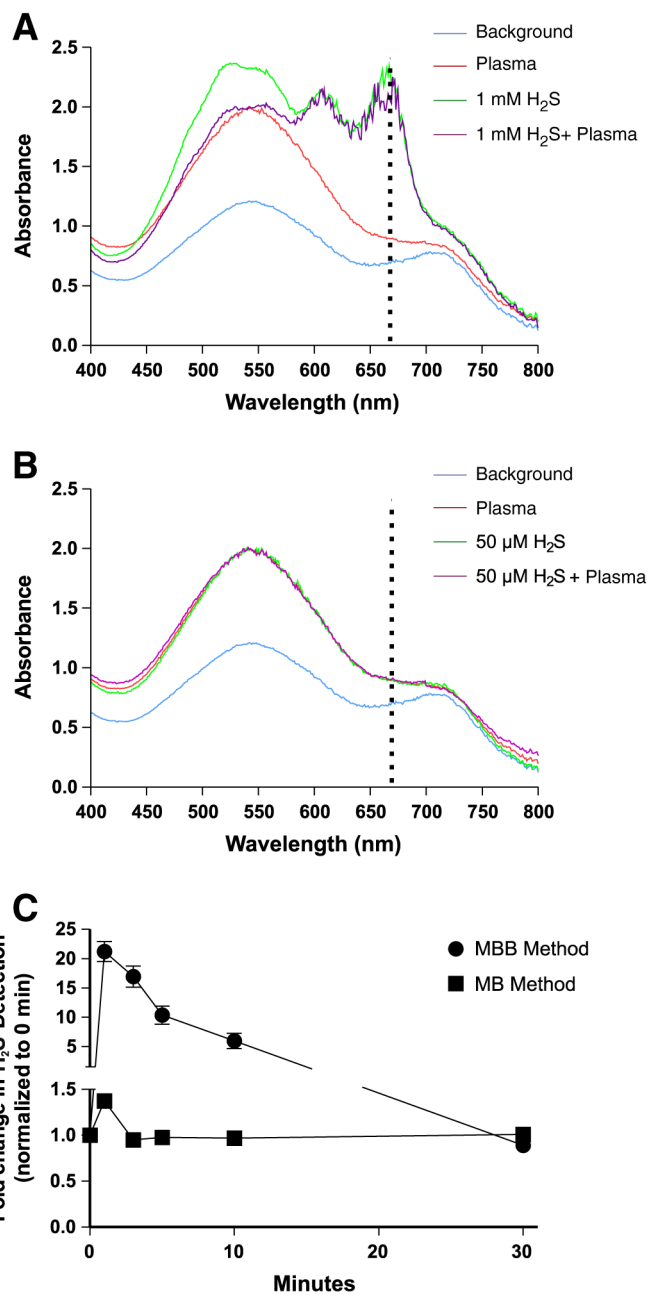
Fig. 10.
Comparison of hydrogen sulfide analysis between MBB and MB methods. (A) The peak of hydrogen sulfide in the range 660–680 nm, using the MBB method. (B) The peak of hydrogen sulfide in the range 660–680 nm, using the MB method. (C) The difference in detection efficiency of the MBB and MB methods after administration of Na_2S to a C57Bl/6 J mouse. $n=3$.
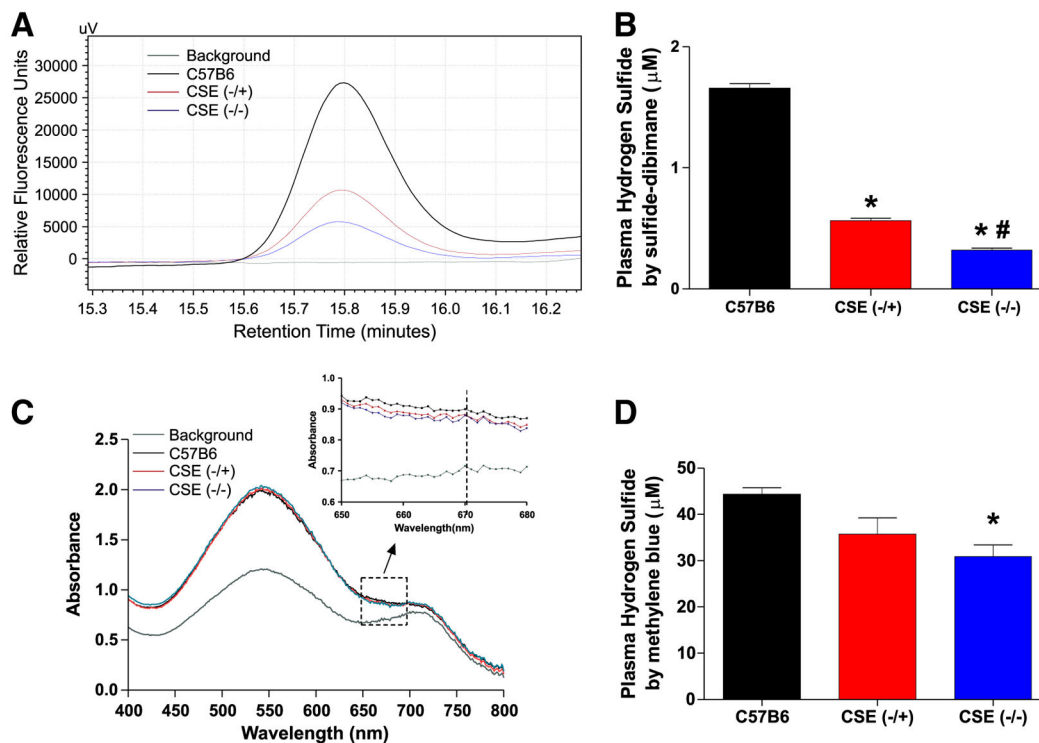
Fig. 11.

Comparison of hydrogen sulfide analysis between the MBB and the MB methods in CSE mutant mice. (A and B) Clear differences in the amounts of sulfide measured using the MBB method in C57Bl/6 J, CSE heterozygote knockout, and CSE homozygote knockout mice are visible. (C and D) Analysis of hydrogen sulfide levels in C57Bl/6 J, CSE heterozygote knockout, and CSE homozygote knockout mice using the MB method showing no clear differences in sulfide levels. *$p<0.05$, compared to control; #$p<0.05$, CSE$^{-/+}$ compared to CSE$^{-/-}$. $n=6$.

Table 1

Extinction coefficient values for the various solvents used.

Solvent	Wavelength λ_{max} (nm)	E (λ_{max}) (L^3 mol^{-1} cm^{-1})
Water	380	4812.156
pH 4.5	380	4694.152
Methanol	370	4883.257
Ethyl acetate	360	5773.096

Table 2

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