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Measurement of H_2S in vivo and in vitro by the monobromobimane method

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Abstract

The gasotransmitter hydrogen sulfide (H_2S) is known as an important regulator in several physiological and pathological responses. Among the challenges facing the field is the accurate and reliable measurement of hydrogen sulfide bioavailability. We have reported an approach to discretely measure sulfide and sulfide pools using the monobromobimane (MBB) method coupled with RP-HPLC. The method involves the derivatization of sulfide with excess MBB under precise reaction conditions at room temperature to form sulfide-dibimane. The resultant fluorescent sulfide-dibimane (SDB) is analyzed by RP-HPLC using fluorescence detection with the limit of detection for SDB (2 nM). Care must be taken to avoid conditions that may confound H_2S measurement with this method. Overall, RP-HPLC with fluorescence detection of SDB is a useful and powerful tool to measure biological sulfide levels.

1. Introduction

1.1. Properties of hydrogen sulfide

Hydrogen sulfide (H_2S) is a colorless gas with the odor of rotten eggs, and can be oxidized to form sulfur dioxide, sulfates, sulfite and elemental sulfur. Based on its lipophilic property, hydrogen sulfide easily penetrates the lipid bilayer of cell membranes (Wang, 2012), however, it is less membrane permeable than nitric oxide (NO) and carbon monoxide (CO). The difference in membrane permeability between NO, CO and hydrogen sulfide is also reflected by their dipole moments, which have values of 0.16, 0.13 and 0.97, respectively. Hydrogen sulfide is slightly soluble in water and acts as a weak acid with an acid dissociation constant (pK_{a1}) of 7.04 and pK_{a2} of 19 at 37°C (Hughes et al., 2009). It can dissociate into H^+ and hydrosulfide anion (HS^-), which in turn may dissociate to H^+ and sulfide anion (S^{2-}) in the following reaction:

$$\mathrm{H_2S} \leftrightarrow \mathrm{H}^+ + HS^- \leftrightarrow 2\mathrm{H}^+ + \mathrm{S}^{2-}$$

At physiological pH and 37°C, ~20% of sulfide is present as H_2S, whereas at physiological pH and 25°C, ~40% of sulfide is present as H_2S conversely at pH 9.5, hydrogen sulfide mainly exists as HS^- (Hughes et al., 2009; Shen et al., 2011). In vivo, pH favors sulfide existence primarily as H_2S and its highly reactive anion, HS^-.

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1.2. Hydrogen sulfide pools

Hydrogen sulfide is produced from a variety of sources, including chemical reactions (e.g. hydrogen gas and elemental sulfur, ferrous sulfide and HCl, aluminum sulfide and water), sulfate-reducing bacteria, and in mammalian tissues. During hydrogen sulfide production in mammalian tissues, there are three tissue-specific enzymes involved, viz.: cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptosulfurtransferase (MST) (Moore et al., 2003).

Acid-labile sulfide and bound sulfane sulfur are two main forms of hydrogen sulfide stored in mammalian cells. They can release hydrogen sulfide under acidic and on reducing conditions, respectively (Shen et al., 2012). Examples of bound sulfane sulfur include thiosulfate, persulfide, thiosulfonate, polysulfides, polythionates and elemental sulfur. Overall, these different biochemical forms are important for regulating the amount of bioavailable hydrogen sulfide (Ishigami et al., 2009; Wintner et al., 2010).

1.3. Physiological and pathophysiological roles of hydrogen sulfide

Hydrogen sulfide is best known for its toxicity. Indeed, H_2S at high concentrations irreversibly inhibits the respiratory chain by binding to the ferric heme a3 center and the CuB center of cytochrome c oxidase. Similarly, H_2S reacts with oxygenated ferrous hemoglobin and myoglobin and converts them to sulfhemoglobin or sulfmyoglobin, which are unable to carry O_2. However, mounting evidence implicates H_2S as an endogenous signaling molecule that plays important roles in physiological and pathological processes (Kolluru et al., 2013a; Kolluru et al., 2013b; Wang, 2011).

To date, a large spectrum of proteins has been shown to be targeted by H_2S. H_2S post-translationally modifies the regulatory sulfonylurea receptor (SUR) of the ATP-sensitive potassium (K_{ATP}) channel in vascular smooth muscle cells, resulting in an increased potassium flow, resultant hyperpolarization and vasodilation (Tang et al., 2005). The wide distribution of K_{ATP} and its isoforms makes H_2S important for the regulation of heart contractility and rate (cardiomyocytes), sensation (neurons), insulin secretion (β-islet cells) and mitochondrial functions (mitoK_{ATP}). Other ion channels are also shown to be the target of H_2S, including intermediate and small conductance potassium channels (IK_{Ca}/SK_{Ca}), L-type calcium channels and the transient receptor potential cation channel A1 ($TRPA_1$) (Avanzato et al., 2014; Mustafa et al., 2011; Streng et al., 2008; Tang et al., 2013).

Moreover, recent evidence shows vascular endothelial growth factor (VEGF) receptor 2 is modified by H_2S to facilitate its activation after ligand binding (Tao et al., 2013). Meanwhile, smooth muscle cell (SMC) proliferation and survival have been shown to be inhibited by H_2S involving ERK activation, which indicates critical regulation of vascular remodeling by H_2S (Baskar et al., 2008). H_2S also targets and inhibits PTP1B and regulates endoplasmic reticulum (ER) stress (Krishnan et al., 2011). Additionally, persulfidation of p65 by H_2S promotes its nucleus translocation and increases transcription of anti-apoptotic proteins (Sen et al., 2012). Last but not least, H_2S can serve as an anti-oxidant that counter balances with reactive oxygen species, including superoxide, hypochlorous acid, peroxynitrite and lipid peroxidation. Interestingly, H_2S is also thought to interact with the

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other gasotransmitters NO and CO on the levels of enzymatic synthesis, oxidative stress and small adducts, such as HNO, HSNO and GSNO (Nagy et al., 2014).

1.4. Measurement of hydrogen sulfide bioavailability

Accurate and reliable measurement of biological hydrogen sulfide can provide critical information associated with various pathophysiological functions. However, significant uncertainty exists regarding levels of H_2S associated with health, disease, and therapeutics. While there are several reasons for the current uncertainties it is now clear that a wide range of values for hydrogen sulfide have been reported (Levitt et al., 2011; Nagy et al., 2014; Shen et al., 2011; Shen et al., 2012; Zheng et al., 2012).

The methylene blue method is the most commonly reported method used in the literature to measure hydrogen sulfide in biological samples (Zhu et al., 2007). The method is based on spectrophotometry of methylene blue dye after the reaction of sulfide and N, N-dimethyl-p-phenylenediamine. However, this method can be highly problematic, making it inappropriate for measuring biological levels of hydrogen sulfide (Shen et al., 2011). Key problems include: 1) interference of other colored substances, 2) methylene blue dimer and trimer formation, 3) strong acid chemical pretreatment and 4) low sensitivity.

Other analytical methods have been reported but are limited for various reasons. Gas chromatography is sensitive enough to measure physiological sulfide levels, but it potentially liberates loosely-bound sulfide because of irreversible sulfide binding or shifts in phase transition equilibria (Levitt et al., 2011; Ubuka et al., 2001). Sulfide-specific ion-selective electrodes have also been in use to detect H_2S levels in biological samples, with a detection range of 1–10 μM but are prone to fouling and limited sensitivity detection. Lastly, fluorescent probes for intracellular measurement of hydrogen sulfide have greatly evolved in the last couple of years. Yet, a major challenge exists with the regard to interference by other thiol species (Nagy et al., 2014).

The purpose of this chapter is to provide detailed techniques to perform the MBB derivatized method for detecting hydrogen sulfide in various biological matrices. The main methodology used is RP-HPLC with fluorescence detection or in combination with mass spectrometry. Additionally, different sample treatment workflows allow for the separation and quantification of free sulfide, acid-labile sulfide and bound sulfane sulfur (Shen et al., 2012). With a 2.0 nM limit of detection, this method is sensitive and reliable enough for use with most biological samples.

2. Experimental Methods

2.1. Derivatization reaction of H_2S with monobromobimane

The fluorescent reagent monobromobimane (MBB) has been widely used to measure various thiols by alkylation (Newton et al., 1981). As shown in Fig 1A, S-alkylation occurs twice with sulfide, forming sulfide-dibimane. According to our previous report (Shen et al., 2011), free sulfide samples are prepared after sulfide derivatization with MBB.

Procedure—The following steps are performed in a hypoxic chamber (1% O_2) at room temperature.

1) 30μl of sample was added to a PCR tube containing 70μl of 100mM Tris-HCl buffer (pH 9.5, 0.1mM DTPA), followed by addition of 50μl of 10 mM MBB (dissolved in deoxygenated acetonitrile).

2) The reaction was stopped by adding 50μl of 200 mM 5-sulfosalicylic acid after a 30-minute incubation.

3) All derivatized samples are removed from the hypoxic chamber and stored at 4°C until analyzed by RP-HPLC.

Comment and limitations: 1) The reaction buffer should be deoxygenated by using nitrogen gas and kept in a hypoxic chamber (1% O_2, room temperature). 2) Samples should be protected from light. 3) The samples should be kept at 4°C after derivatization of sulfide. 4) For biological samples, the sample lysis buffer cannot include N-Ethylmaleimide (NEM), 2-mercaptoethanol, etc because they can interface with the H_2S/MBB reaction and directly impact SDB formation in an inversely proportional manner.

2.2. H_2S detection in biological samples: effects of sample preparation

Hydrogen sulfide is easily lost from biological samples through volatilization and oxidation, especially at micromolar or lower levels of sulfide. However, using RP-HPLC, we are able to detect changes in sulfide levels in mice after sulfide treatment (Bir et al., 2012; Shen et al., 2011). For the measurement of sulfide in biological samples, it is very important that sample preparation be carefully and consistently performed for accurate measurement.

Currently, there are a number of research groups using pH 8.0 buffer for derivative reaction of sulfide with MBB. Figure 2 demonstrates that the remaining sulfide after the derivative reaction of sulfide with MBB under different pH conditions. During the first sulfide derivative reaction, 98.6% and 84.6% of sulfide were converted to SDB at pH 9.5 and pH 8.0, respectively. After a second sulfide derivative reaction, 1.4% and 11.4% of sulfide were converted to SDB at pH 9.5 and pH 8.0, respectively. These data demonstrate that hydrogen sulfide cannot be completely converted to SDB under reaction conditions at pH 8.0 unlike pH 9.5.

The deoxygenated Tris-HCl buffer (100 mM, pH 9.5, 0.1 mM DTPA) is not only the best derivatization buffer to use for MBB/sulfide reaction, but also serves as an ideal stabilization buffer for sulfide samples in that plasma sulfide levels can be stabilized at room temperature for at least 15 minutes, and in liquid nitrogen for up to four weeks in this solution (Peter et al., 2013; Shen et al., 2011).

Photolysis of MBB will result in formation of fluorescent bimane (Kosower and Kosower, 1987), thus it is important to use dark amber vials and dim room lighting. In addition, sulfide derivatization is affected by the pH of the reaction buffer, oxygen concentration, trace metals and the reaction vessel. As our previous optimization of sulfide derivatization (Shen et al., 2011) shows the highest reaction percentage was achieved in PCR tubes with reaction

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buffer (pH 9.5, 0.1 mM DTPA) in a 1% O_2 hypoxic chamber. NEM is a thiol-reactive compound, which was widely used to protect thiols from oxidation. However, we have shown that the use of NEM to block free thiols may interfere with follow-up measurements of hydrogen sulfide by MBB (Shen et al., 2011). In the laboratory, Ethylenediaminetetraacetic acid (EDTA) is widely used for scavenging metal ions, which is the same role as DTPA. However, we have shown that red cells may lyse in EDTA tubes, resulting in increased levels of sulfide (Peter et al., 2013).

2.3. RP-HPLC with fluorescence detection

In our laboratory, sulfide-dibimane is detected using Shimadzu Prominence HPLC (LC-20), which consists of a binary, high-pressure gradient solvent delivery pump (model LC 20AB), an autosampler equipped with a cooling sample device (model SIL-20AC HT), a fluorescence detector (model RF10-AXL), and data processing software (LCsolution Version 1.23).

Procedure

>1) 10 μl of sample is injected into the RP-HPLC with an XDB-C18 column (4.6 × 250 mm, 80 Å), a guard column (4.6 × 12.5 mm, 80 Å) using the fluorescence detector setting of (ex/em, 390 nm/475 nm).

2) The mobile phase for elution of SDB was composed of water (A, 0.1% trifluoroacetic acid (TFA), v/v) and 99.9% pure acetonitrile (B, 0.1% TFA, v/v), which is applied in the following gradient elution at 0.6 ml/min flow rate (Table 1).

3) With this method, the retention time of SDB and MBB are 16.4 and 17.8 min, respectively (Fig 1B).

Preparation of SDB Standard—The calibration standard of sulfide-dibimane is prepared following our previous report (Shen et al., 2011). Briefly, 4 ml of 6 mM sodium sulfide was added to a 50 ml tube with 10 ml of 100 mM deoxygenated Tris-HCl buffer (pH 9.5, 0.1 mM DTPA) followed by addition of 5 ml of a 10 mM MBB solution. After 30 min of incubation in a 1% O_2 hypoxic chamber at room temperature, 1 ml of 2-mercaptoethanol was added to quench excess MBB. Sulfide-dibimane was extracted by 10 ml of ethyl acetate, evaporated by nitrogen stream, and then purified by an Alltech Prevail SPE cartridge. SDB should be eluted using a 50% methanol solution (v/v). After evaporation of the solvent in the fractions containing pure SDB, dry powder of purified SDB can be weighed and then configured to standard solution at different concentrations. The concentration of purified SDB is verified by spectrophotometry, using the extinction coefficient values of 4883.257 and 4694.125 dm3 mol^{-1} cm^{-1} at 370 nm (methanol solution) and 380 nm (HCl solution), respectively, to calculate an average SDB concentration.

Comment and limitations: 1) Though the fluorescent nature of SDB allows low concentrations of hydrogen sulfide to be easily measured, the prepared samples should be kept in the dark and 4°C in the HPLC autosampler. 2) Prepared samples should be centrifuged before being injected into the HPLC. 3) Increasing the acetonitrile level in the

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mobile phase will decrease the elution time of SDB, and conversely decreasing the acetonitrile level will lengthen the retention time of SDB. It should also be noted that other thiol derivatives (e.g. cysteine, glutathione, homocysteine and thiosulfate) can be simultaneously detected with this fluorescent system. 4) All phase solutions should be filtered or be HPLC grade solvents. 5) The column should be equilibrated and never allowed to dry. 6) Sample injection cannot be overloaded into the column. 7) Though sulfide solution is unstable, a known concentration of sulfide solution can also be used as a calibration standard.

2.4. H_2S and sulfide pool detection in biological samples

Hydrogen sulfide may exist in three biological forums: free hydrogen sulfide, acid-labile sulfide and bound sulfane sulfur (Ishigami et al., 2009; Johnson et al., 2005; Shen et al., 2012; Ubuka, 2002). At pH 7.4 and 37°C, 18.5% of free hydrogen sulfide exists as H_2S gas and the remainder is almost all hydrosulfide anion (HS^-) with a negligible contribution of sulfide anion (S^{2-}) (Hughes et al., 2009). Acid-labile sulfide consists of sulfur present in the iron-sulfur clusters contained in iron-sulfur proteins (Johnson et al., 2005). Bound sulfane sulfur includes thiosulfate, persulfide, thiosulfonates, polysulfides, polythionates, and elemental sulfur (Ubuka, 2002). A previous limitation of the sulfide field has been the lack of precise methodology for accurate and reproducible measurement of hydrogen sulfide and sulfide pools in biological samples. We developed an approach to measure them (Shen et al., 2012) which involves selective liberation, trapping and derivatization of hydrogen sulfide, which is illustrated in Figure 3.

Procedure—The following steps are performed in a hypoxic chamber (1% O_2) at room temperature.

1) 50 µl of sample is transferred into two vacutainer tubes, which are labeled A and B.

2) For measurement of acid-labile sulfide, 450 µl of 100 mM phosphate buffer (pH 2.6, 0.1 mM DTPA) is added into tube A. For measurement of bound sulfane sulfur, 450 µl of 100 mM phosphate buffer (pH 2.6, 0.1 mM DTPA and 1 mM TCEP) is added into tube B.

3) All vacutainer tubes are put onto a rocker and rocked for 30 min.

4) All of the solutions in the vacutainer tubes are removed by a 1 ml syringe with a spinal needle so that the solution may be removed without inverting the tube.

5) Volatilized hydrogen sulfide is then trapped by adding 500 µl of 100 mM Tris-HCl buffer (pH 9.5, 0.1 mM DTPA).

6) All vacutainer tubes are then put onto the rocker and rocked for 30 min.

7) Derivatization reactions of sulfide with MBB are performed following the procedure in section 2.1.

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8) After HPLC assay, the acid-labile sulfide level is calculated using the A value minus the free sulfide value. The bound sulfane sulfur level is calculated using the B value minus the A value.

Comment and limitations: 1) TCEP solution should be freshly made. 2) Volatilized hydrogen sulfide should be immediately trapped.

2.5. Confirmation of HPLC & sulfide-dibimane by mass spectrometer

Hydrogen sulfide and sulfide pools were detected using monobromobimane in biological samples using RP-HPLC with a fluorescence detector (Shen et al., 2011; Shen et al., 2012; Wintner et al., 2010). A LCQ-Deca ion trap mass spectrometer was used to identify the SDB peak measured by RP-HPLC (Shen et al., 2014). Additionally, non-radioactive isotopic ^{34}S can be used to generate sodium sulfide to detect changes in sulfide distribution in biological samples (Shen et al., 2014). The mass spectra of SDB and ^{34}S-labeled SDB produced by ESI (+) MS are shown in Fig.4A and 4B, respectively, with the major abundant ions identified. The m/z of 415.17 and 417.16 correspond to the molecular ion of SDB and ^{34}S-labeled SDB, respectively. For SDB and ^{34}S-labeled SDB, a similar MS2 fragment is produced from the 415.17→192.94 or 417.16→192.91 transitions. ^{34}S-labeled SDB can also be used as an internal standard to sensitively and accurately measure biological sulfide levels by selected reaction monitoring (SRM).

Procedure

1) 3 μl of the 1.0 mg/ml IS stock solution (^{34}S-labeled SDB), 30 μl of SDB samples, and 267 μl of 50% acetonitrile were added into the empty tube, which was then vortexed for 30 s and injected into the mass spectrometer.

2) Electrospray data were acquired using a Finnigan LCQ Deca mass spectrometer. The following conditions were used for MS: the ionization mode was positive electrospray ionization (ESI); sheath gas is 10 units; sweep gas is 5 units; maximum inject time is 400 ms; isolation width is 1.0; capillary temperature is 250 °C; spray voltage is 5.0 kV; the ion gauge pressure was 2.41×10^{-5} Torr; a selective reaction monitoring (SRM) mode was used, in which ions of SDB and ^{34}S-labeled SDB were selected at m/z 415.1 and 417.1 as the precursor ion, and m/z 192.9 and 192.9 as product ions, respectively; with an analysis time of 20 min. Full scan mass spectra were acquired from 150 to 500 amu at unit mass resolution. Collision energy was 35 V, isolation width was 1.0, and the activation Q was 0.25. The automatic gain control (AGC) was activated and the value was set to 5×10^{6}.

3) All acquired data were analyzed with Xcalibur software (Thermo Fisher Scientific, Inc.).

Comment and limitations: 1) Proper quantitative MS methods are developed employing SRM along with defined internal standards to account for sample and run fluctuations.

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3. Summary

The monobromobimane method for measuring H_2S by RP-HPLC with fluorescence detection is a useful and sensitive quantitative method to measure sulfide levels in biological samples. This method is also advantageous in that workflow modification can be used to measure all biochemical forms of sulfide, including free sulfide, acid-labile sulfide and bound sulfane sulfur from the same sample. While the MBB method has many advantages, important facts must be kept in mind, including:

1) Monobromobimane is a light-sensitive reagent so the derivatization of the sulfide and MBB solution should be performed in the dark.

2) All solutions must be deoxygenated before preparation of sulfide samples.

3) Hydrogen sulfide readily binds to glass, therefore, high-quality polypropylene plastic tubes should be used for preparation of samples.

4) The chemical derivatization reaction of H_2S with MBB is heavily influenced by pH, time, oxygen tension and volatilization, which all affect the amount of sulfide measured.

5) In the acidic environment the SDB formed from sulfide derivatization is stable at 4°C.

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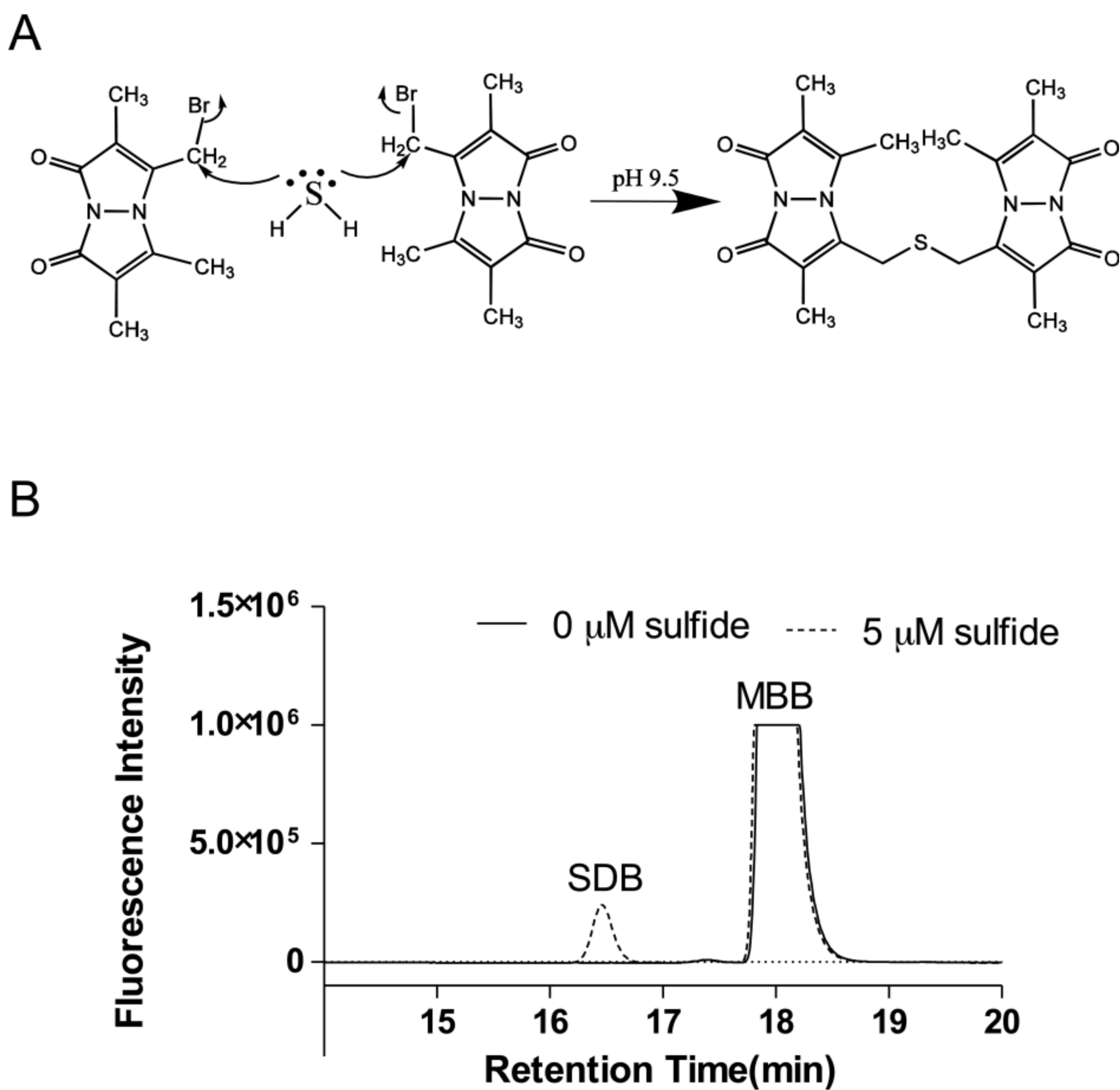

Figure 1.

Measurement of hydrogen sulfide using RP-HPLC with fluorescent detector. (A) Schematic representation of the derivatization of hydrogen sulfide with monobromobimane, forming sulfide-dibimane via S-alkylation. (B) HPLC spectrum of sulfide-dibimane and monobromobimane.

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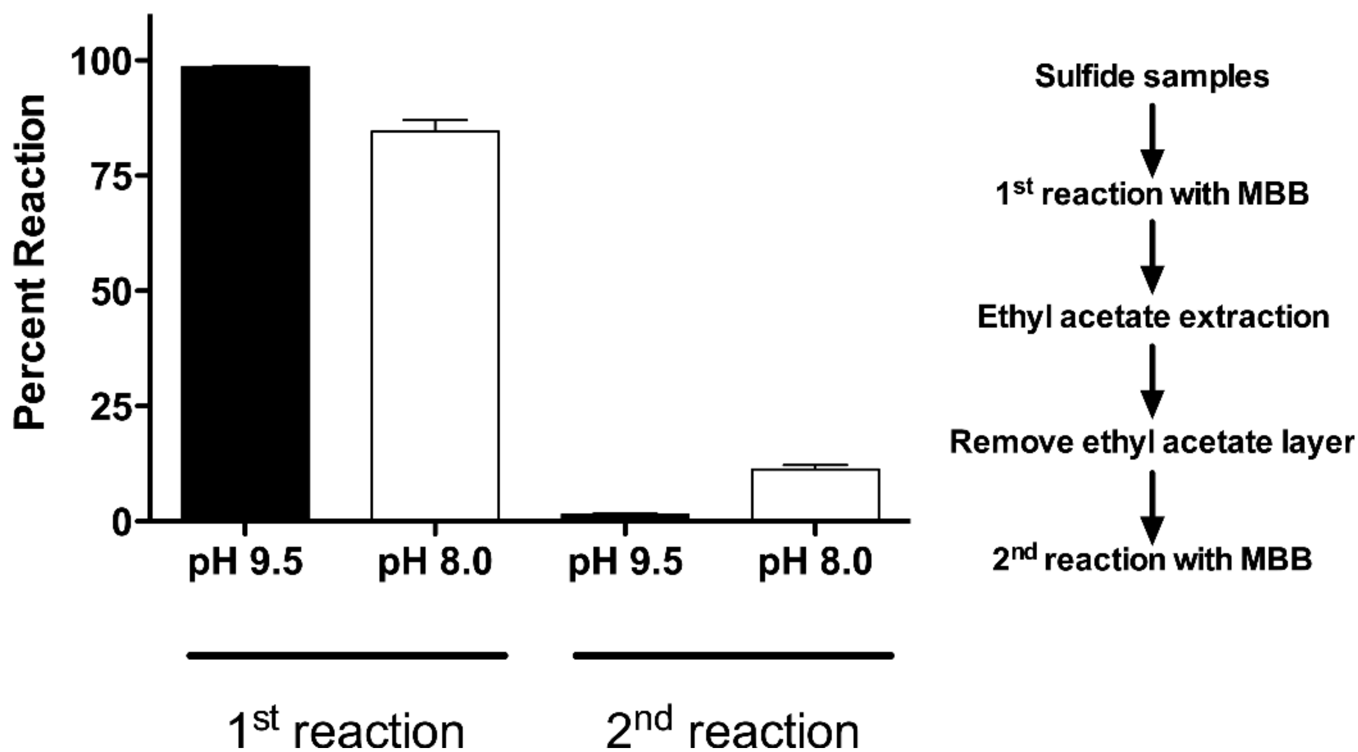

Figure 2.

Comparison of remaining sulfide after the derivatization of hydrogen sulfide with MBB under different pH. 10 μM sulfide solutions were reacted with MBB for 30 min at pH 9.5 and pH 8.0, respectively. After ethyl acetate extraction, the remaining sulfide was reacted with MBB again at pH 9.5 and pH 8.0, respectively.

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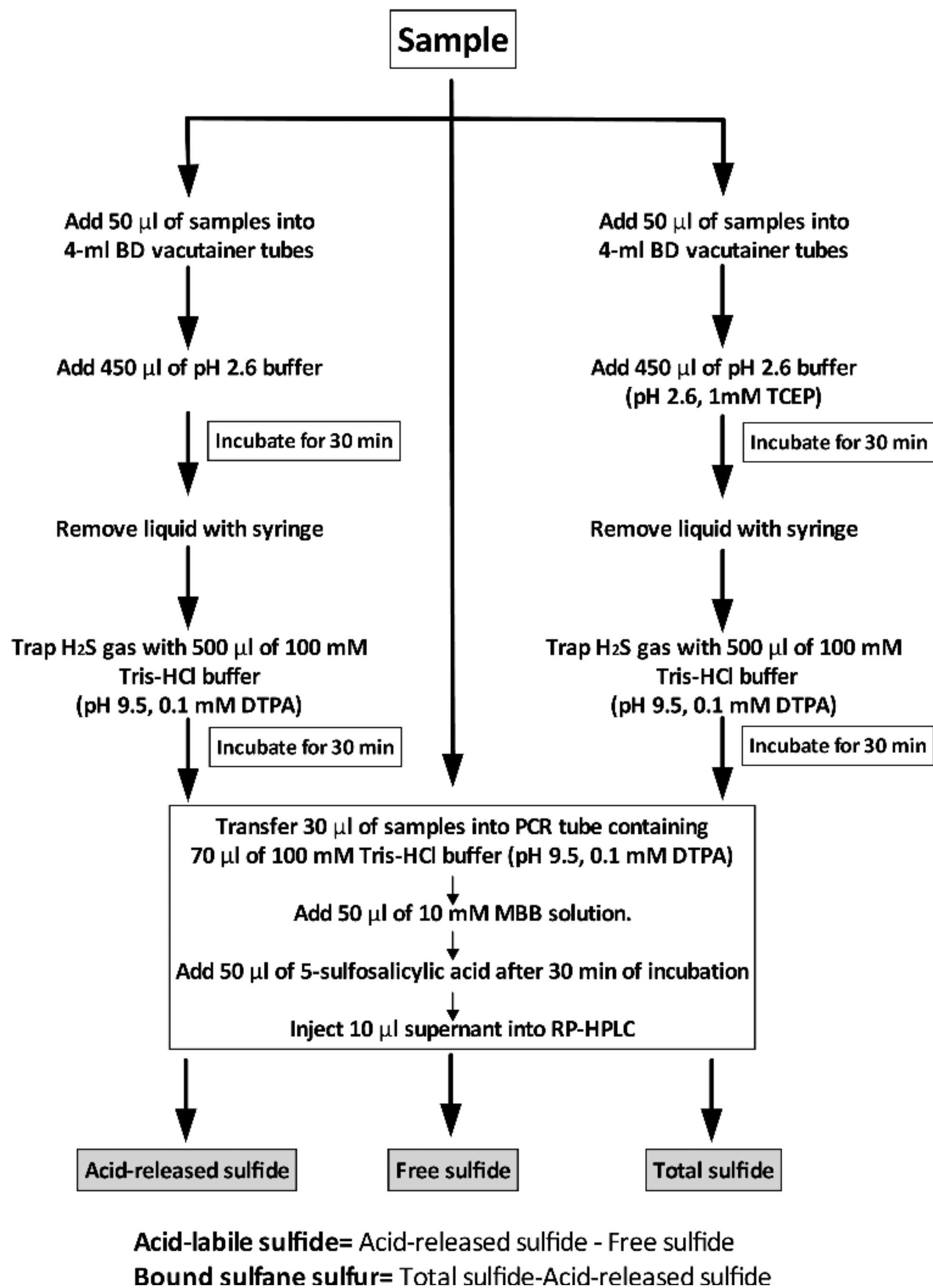

Figure 3.

Schematic illustration of the workflow for sulfide measurement.

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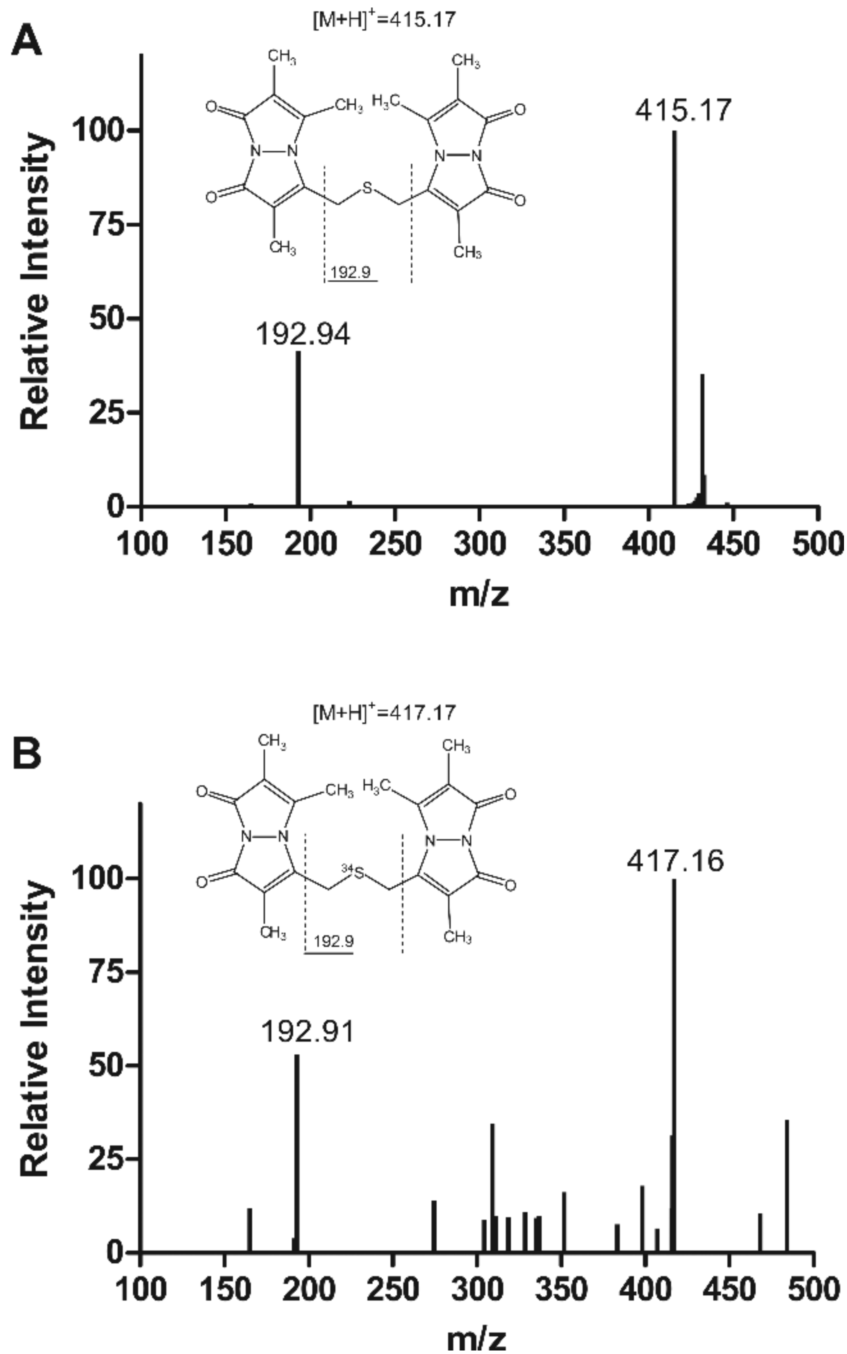

Figure 4.

ESI (+) product ion mass spectra of SDB (A) and ^{34}S-labled SDB (B) with identification of the abundant ions. Molecular ion of SDB and ^{34}S-labled SDB $[M+H]^+$ correspond to 415.17 and 417.16, respectively. Insets, structure of SDB (A) and ^{34}S- labled SDB (B) with abundant fragments indicated.

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Table 1

Mobile phase gradient table

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