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Analytical Measurement of Discrete Hydrogen Sulfide Pools in Biological Specimens

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Abstract

Hydrogen sulfide (H_2S) 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 pharmaco-therapeutic manipulation. Amongst the challenges facing the field is the accurate measurement of biologically active H_2S. We have recently reported that the typically used methylene blue method and its associated results are invalid and do not measure bonafide H_2S. The complexity of analytical H_2S measurement reflects the fact that hydrogen sulfide is a volatile gas and exists in the body in different forms, including a free form, an acid labile pool and as bound sulfane sulfur. Here we describe a new protocol to discretely measure specific H_2S pools using the monobromobimane method coupled with RP-HPLC. This new protocol involves selective liberation, trapping and derivatization of H_2S. Acid-labile H_2S is released by incubating the sample in an acidic solution (pH 2.6) of 100 mM phosphate buffer with 0.1 mM DTPA, in an enclosed system to contain volatilized H_2S. Volatilized H_2S 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 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 H_2S measurement performed in an analogous manner 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 H_2S measurement from the acid liberation protocol alone compared to that of TCEP plus acidic conditions. In summary, our new method protocol allows very sensitive and accurate measurement of the three primary biological pools of H_2S including free, acid labile, and bound sulfane sulfur in various biological specimens.

Keywords

monobromobimane; volatile sulfur compounds; HPLC; blood; plasma

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Introduction

Hydrogen sulfide (H_2S) is a ubiquitous gaseous signaling molecule that plays an important role in numerous cellular functions and has become of focus of many research endeavors including pharmaco-therapeutic 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 H_2S gas and the remainder is almost all hydrosulfide anion (HS^-) with a negligible contribution of S^{2-} [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 different volatile sulfur pools. It is increasingly clear that these pools, besides free hydrogen sulfide, are important in regulating the amount of bioavailable sulfur with the most relevance being the acid labile and bound sulfane sulfur pools [10, 11]. Figure 1 illustrates the different volatile sulfide pools with associated chemical species.

Sulfane sulfur refers to divalent sulfur atoms bound only to other sulfur, though at they may bear an ionizable hydrogen at some pH values. These include thiosulfate $S_2O_3^{2-}$, persulfides R-S-SH, thiosulfonates R-S(O)-S-R′, polysulfides R-S_n-R, polythionates $S_nO_6^{2-}$ and elemental sulfur S^0 [10]. Acid labile sulfide, the other major pool, consists of sulfur present in the iron sulfur clusters contained in iron-sulfur proteins (non-heme) 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 storing 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 H_2S 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 [10], sulfide ion -selective electrodes, polarographic sensors[16], gas chromatography [13, 17] and HPLC in conjunction with fluorimetric based methods using monobromobimane to derivatize free H_2S [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

bonafide 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 H_2S along with the volatility and spontaneous oxidation of H_2S may help explain these apparent paradoxes.

Earlier attempts to characterize the different biologic pools of sulfur have utilized MBB in conjunction with 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 due to various problematic issues such as pH, volatilization and oxidation. Here we report a new protocol method that measures all relevant biologic hydrogen sulfide pools namely free hydrogen sulfide, acid labile sulfide and bound sulfane sulfur. These 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 fluorescent 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 of H_3PO_4 and 70 μM of KH_2PO_4, pH 2.6, 0.1 mM DTPA) causing release of free hydrogen sulfide into the headspace of a vacutainer tube from the acid labile pool. After removal of this solution, 100mM Tris-HCL buffer (pH 9.5, 0.1mM DTPA) is added and hydrogen sulfide gas is re-dissolved back into the buffer and the sulfide level is measured by the MBB method. This 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. While dithiothreitol (DTT) has been used by others[10, 11, 21, 23]for this purpose, TCEP is water soluble, non-volatile, 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 prior to 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 de-halogenation of monobromobimane by TCEP with the creation of a fluorescent product has been reported with the potential for interference [25], while 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 has 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 prior to re-trapping the volatilized hydrogen sulfide gas in alkaline solution for subsequent reaction with MBB.

Materials

1. Monobromobimane (MBB, 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 (CH_3CN, Sigma-aldrich, Cat. No. 34851)
6. Trifluoroacetic acid (TFA, Thermo Scientific, Cat. No. 28903)
7. BD Vacutainer (Becton, Dikinson and Company, Cat. No. 366703)
8. 1ml plastic syringe
9. PCR tube (Molecular Bioproducts, Cat. No. 34129)
10. 3.5 inch 25 gauge spinal needle (BD #405180)
11. ½ inch 30 gauge needle (BD# 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. HPLC system: Shimadzu Prominence Ultra Fast Liquid Chromatography (UFLC) equipped with fluorescence detector (HPLC 20A prominence)
5. Hypoxic chamber (Coy Laboratory products Inc.; large glove box)
6. Nutating mixer- a hybrid mixing device 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 minutes at 4°C for murine samples and 1500 RCF for 4 minutes at 4°C for human samples.
4. Derivatization reaction of sulfide with monobromobimane was performed at 1% O_2 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 CH_3CN) 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 sec. 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 RP-HPLC system with an Agilent Eclipse XDB-C18 column (5μm, 80 Å, 4.6 mm×250 mm) equilibrated with 15% CH_3CN in water containing 0.1% (v/v) TFA.
9. Fluorescence detection should be set with 390 nm(excitation) and 475 nm (emission)
10. Monobromobimane and sulfide-dibimane are separated using the gradient of two mobile phases identified in Table 1: (A) water containing 0.1% (v/v) TFA and (B) 99.9% CH_3CN, 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 minutes, 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 retrapping, we determined the amount of hydrogen 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 4ml 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. In one tube solution is removed through the cap with 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) was added into BD vacutainer tube; In the other tube 25 μl of 3M NaOH solution was added for adjust pH to ∼9.5.
5. Incubate these tubes for 30 min on the nutating mixer.
6. Measure sulfide level in a BD vacutainer tube by MBB method.
7. Also 20 μl of these samples were mixed with 200 μl of 100 mM KCN (dissolved into 10 mM NaOH), and then A_{335} was measured [28], further development of a red color was 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 of Na_2S (final concentration) was incubated with the mixture of plasma and 0.1 M Tris-HCl buffer (pH 9.5, v/v 1:9) under hypoxic conditions (1% O_2), the A_{335} was measured by spectrophotometry at 0, 10, 30 and 60 mins [30].

Effect of TCEP on hydrogen sulfide reaction with monobromobimane

1. Prepare 100 mM TCEP stock solution in H_2O.
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 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 different pH solutions.
3. Treat the mixtures with or without 1 mM TCEP.
4. At various time points, aliquots (200 μl each) of these solutions were withdrawn and analyzed by RP-HPLC. For greater sample reproducibility, keep the time constant.

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

1. 50 μl of plasma is added separately into two sets of 4ml BD vacutainer tubes.

2. 450 μl of 100 mM phosphate buffer (pH 2.6, 0.1 mM DTPA) is added to one and to the other 450 μl of 100 mM phosphate buffer (pH 2.6, 0.1 mM DTPA and 1 mM TCEP) is added.
3. Incubate these tubes on the nutator for 30 min.
4. Remove solution through the cap with 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 BD vacutainer tube, using a 30G needle and incubating for 30 min on the nutator.
6. Remove solution and measure sulfide level in a BD vacutainer tube by MBB method.
7. Calculate acid-labile sulfide and sulfane sulfur pools.
    a. Acid labile sulfide= value obtained by acid liberation protocol – the free hydrogen sulfide value.
    b. Bound sulfane sulfur=value obtained by TCEP treatment- the value obtained by acid liberation protocol.

Similarly, to optimize the effects of other experimental conditions, hydrogen sulfide was 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, Louisiana 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 the Louisiana State University (LSU) Health Shreveport, Shreveport, Louisiana via protocol H11-110. Human subjects were males between 23-34 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. 2A the highest recovery percentage was achieved after 30 min of trapping headspace sulfide gas, establishing this timeframe as optimal. Next we sought to demonstrate that percentage recovery of acid volatilized hydrogen sulfide was optimal as demonstrated in Fig. 2B, where the sulfide content of 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. 2C 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+/- 0.0029 μM that is slightly above the reaction solution background level that was 0.00725+/- 0.0018 μM. This does not represent a significant fraction of H2S 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. 3A demonstrates that the measured hydrogen sulfide is decreased when the trapping process occurred 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 this loss of hydrogen sulfide is attributed 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. 3B and 3C.

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 effect of different concentrations of TCEP on sulfide derivatization was studied. Before sulfide derivatization, 50 mM or 1 mM TCEP was added to the sulfide stock solution containing 40μM sodium sulfide. Fig. 4A and 4B demonstrates that TCEP can affect the sulfide-monobromobimane 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.4C shown 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.3D 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 either in solution alone or in a mixture of 5 μM sodium sulfide plus 25 μM DATS.

We next examined the stability of the sulfide-dibimane product in the presence of TCEP. The reaction between hydrogen sulfide and MBB occurs under alkaline conditions and is then terminated and stabilized with 200mM sulfosalicylic acid. Fig.4E shows 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.

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

Free, acid-labile sulfide and bound sulfane sulfur levels were measured in the plasma of wild type C57BL/6J, CSE gene deficient mice and healthy human volunteers. Fig.5A showed that CSE knockout mice, known to be defective in hydrogen sulfide production, show a significant decrease in free and acid-labile sulfide compared to wild type mice, but no significant difference in bound sulfane sulfur. In Fig. 5B, we found that the free hydrogen sulfide pool in plasma from healthy human volunteers to be in the low nanomolar range but that the acid labile pool was in the low micromolar range. The bound sulfane sulfur pool was found to be significantly smaller similar to the murine models above. 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 function.

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- accomplished by immediate collection of samples into vacuum tubes that should be maintained at all times without plasma after volatilization and the transfer of reagents and solutions done via a needle inserted into the rubber stop-cock of the vacuum tubes hence avoid loss of vacuum and gas samples [31].
2. Binding of H_2S to experimental vessels is minimized by using polypropelene reaction vessels.
3. Lithium heparin used as anticoagulant as this has been shown to be less prone to cause hemolysis than EDTA[32].
4. Samples should be transported at 4 degree centigrade 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. 1% oxygen environment is maintained throughout the reaction protocol with acid and reducing agents as well as the MBB reaction process.

Conclusion

While the field of hydrogen sulfide measurement continues to evolve with modifications of various methods, report of different fluorescent probes[33-35] as well as applications of new technologies such as nanotubes and quantum dots[36, 37]. The new method described here opens the door to accurate, quantitative and scalable measurement of discrete pools of hydrogen sulfide from primary volatile sulfide pools. Use of this protocol in both experimental and clinical specimens will further enhance our understanding of hydrogen sulfide bioavailable equivalents during pathophysiological events and provides a critical

analytical detection method for pharmacologic endeavors to manipulate the levels of hydrogen sulfide for therapeutic purposes.

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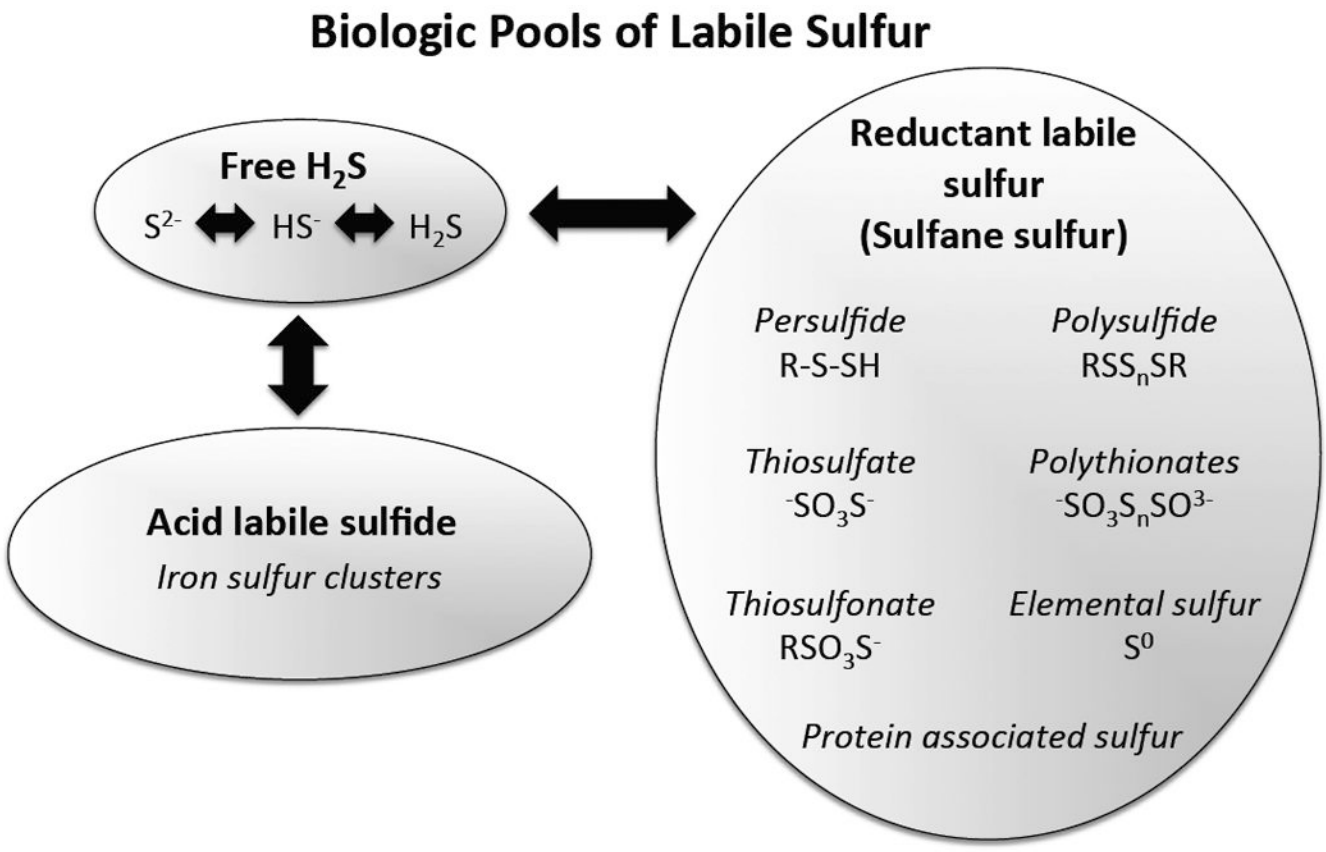

Fig.1.
Biological Pools of Labile Sulfur.

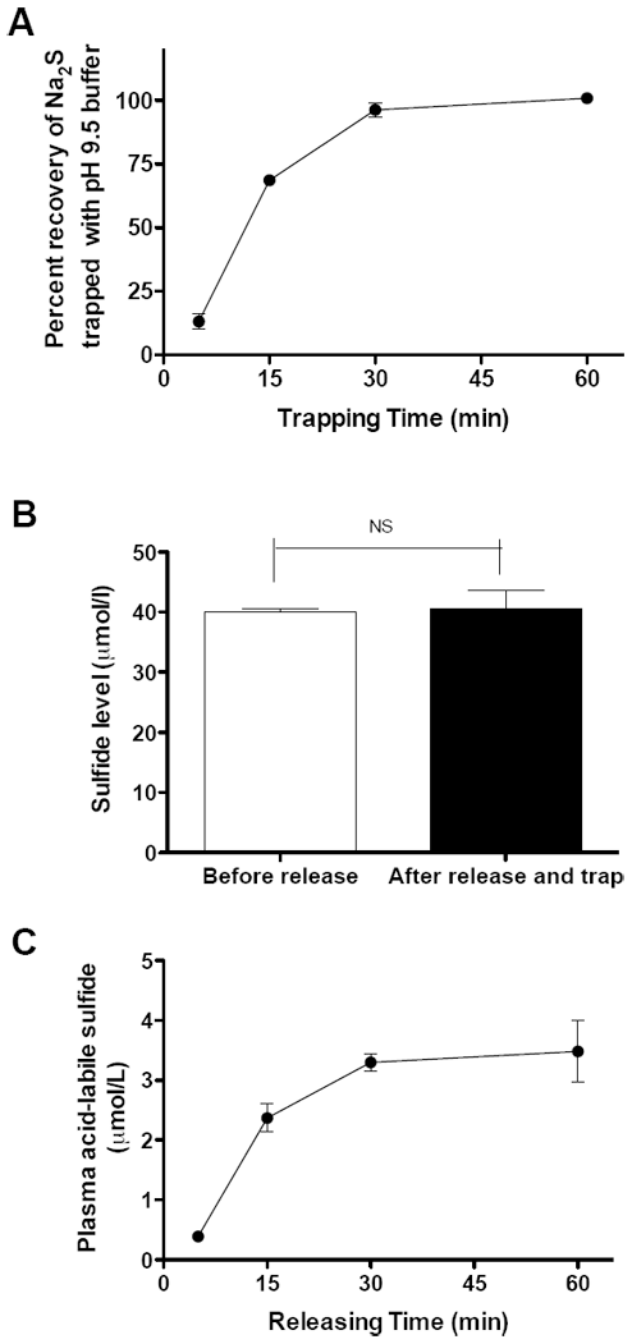

Fig.2.

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 pH 2.6 phosphate buffer for 30 min, hydrogen sulfide gas was re-trapped by 500 μL of 0.1 M Tris-HCl buffer (pH 9.5, 0.1 mM DTPA) with a peak recovery time of 30 minutes. (B) Effect of sodium sulfide release and re-trapping on hydrogen sulfide recovery. The hydrogen sulfide detected from 40 μM of sulfide was compared before release and after releasing and re-trapping. Both releasing and trapping times were 30 minutes. (C)

Effect of plasma on release of sulfide. 50 μL of plasma was incubated with 450 μL of 0.1M pH 2.6 phosphate buffer for different lengths of time and then re-trapped for 30 min demonstrating optimal release at 30 minutes.

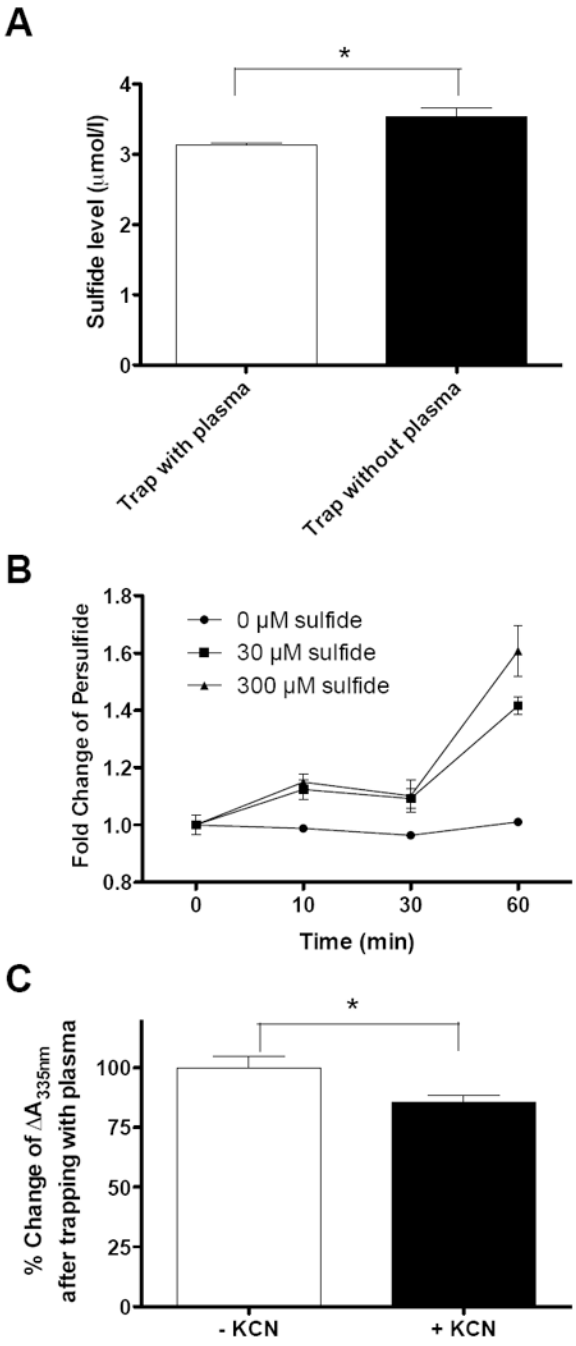

Fig.3.

Effect of plasma proteins on trapping hydrogen sulfide gas. (A) Comparison of sulfide level between samples trapped with plasma remaining in reaction vessel and samples trapped after removal of plasma. After 50 μL of plasma was incubated with 450 μL of 0.1M pH 2.6 phosphate buffer for 30 min, in the first group ~25 μL of 3 M NaOH solution was added directly to plasma to adjust 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 30min. (B) Effect of sulfide on protein persulfide

formation. 0, 30, 300 μM of sodium sulfide was incubated with plasma and 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.

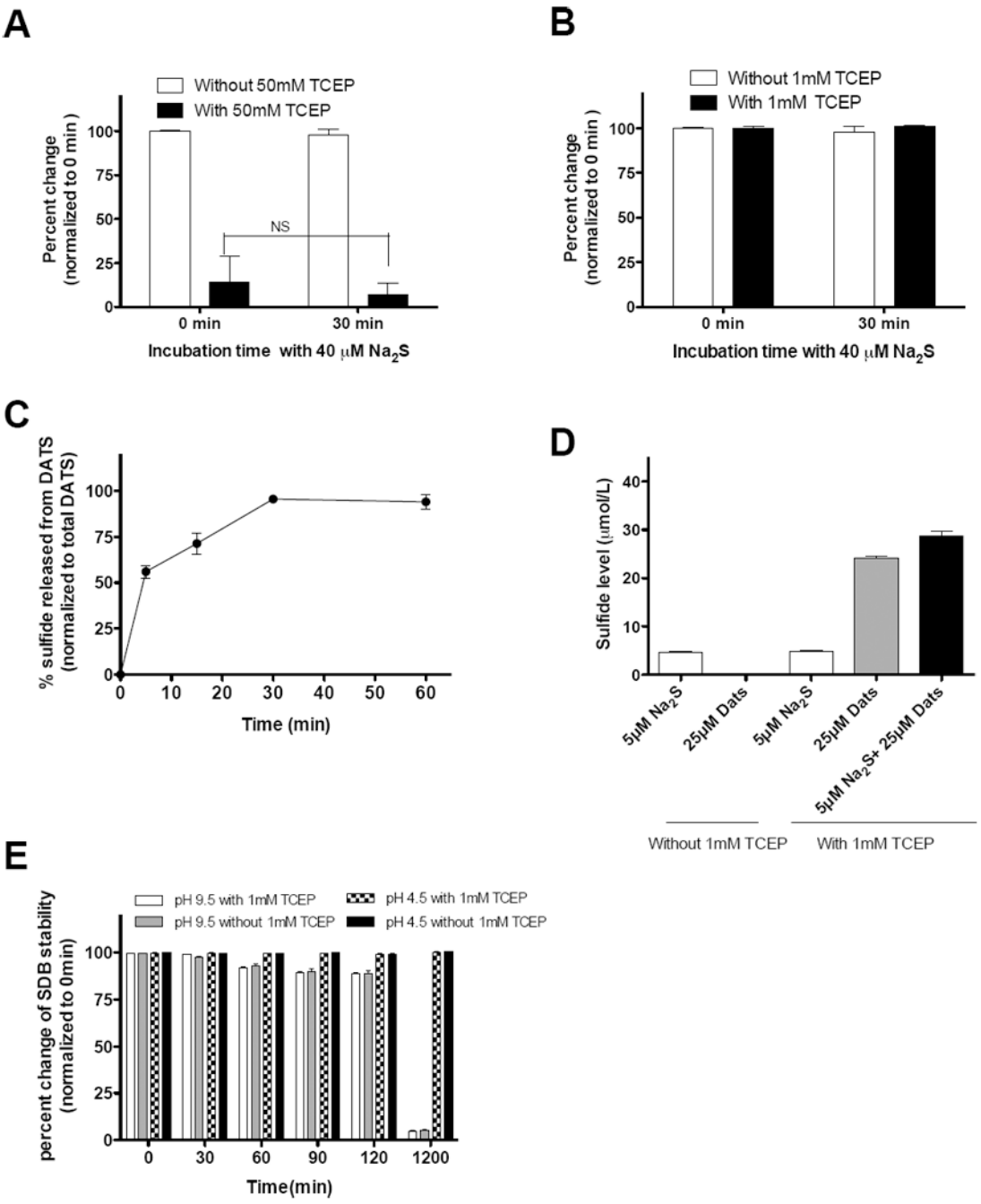

Fig.4.

Effect of TCEP on the derivatization of hydrogen sulfide with MBB. 40 μM sodium sulfide was reacted with MBB solution in the presence of 50mM TCEP (A) or 1 mM TCEP (B). (C) Effect of TCEP on diallyl trisulfide (DATS) reduction. 50 μL of 25 μM DATs was incubated with 450 μL of 0.1M phosphate buffer (pH 2.6, 1mM TCEP) at different time points, and the resulting hydrogen sulfide was trapped with 0.1M Tris-HCl buffer for 30min. (D) Efficiency of the protocol including reducing, releasing and trapping. 5 μM Na2S and 25 μM Dats were used with and without TCEP. (E) Effect of TCEP on stability of SDB across pH range.

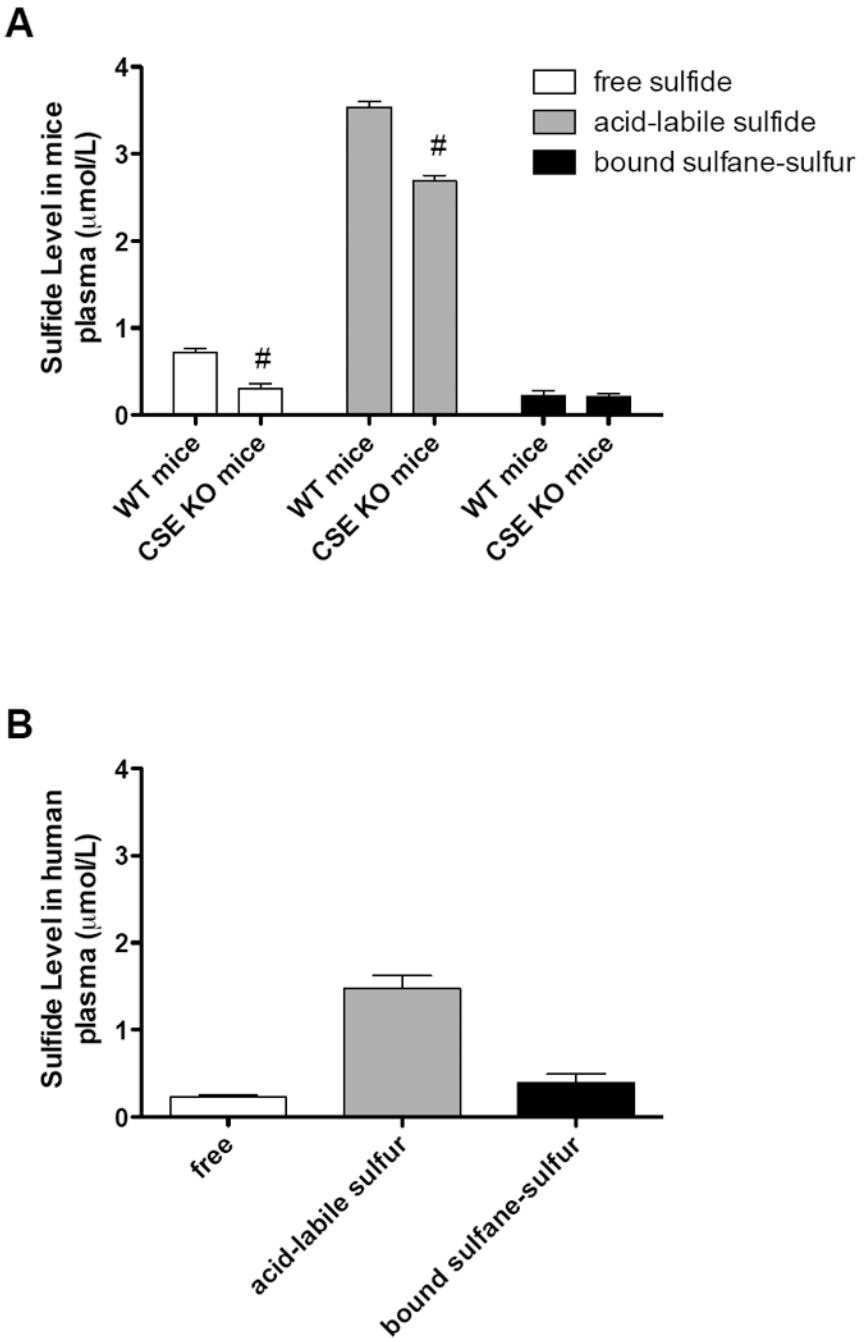

Fig.5.

Levels of free hydrogen sulfide, acid-labile sulfur and bound sulfane-sulfur in murine and human plasma. (A) C57BI/6J mice plasma in comparison with CSE-/- mice. (B) Human plasma from healthy male human controls.

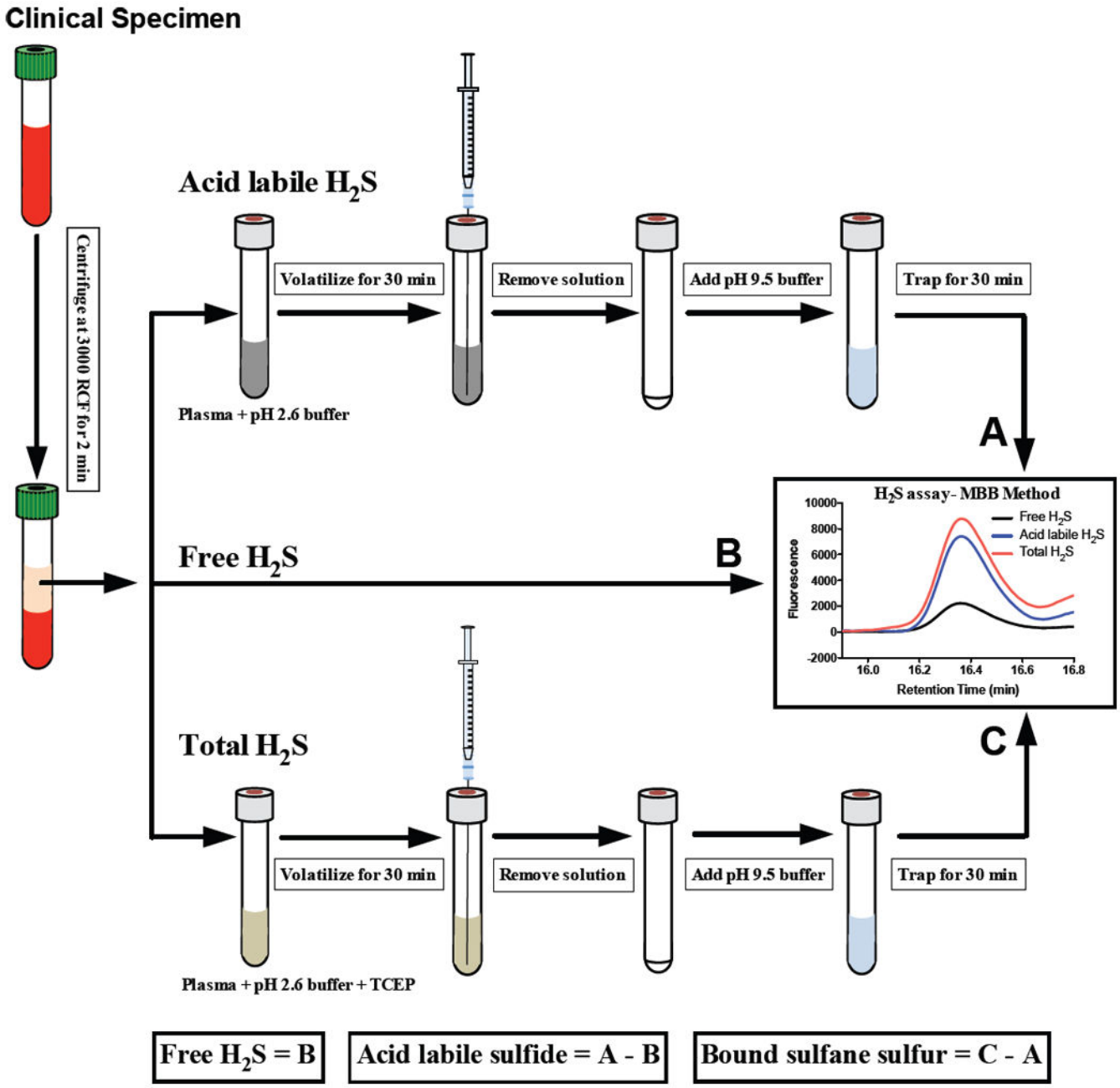

Fig.6.
Method overview illustration.

Table 1

Mobile Phase Gradient Table

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