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H₂S Analysis in Biological Samples Using Gas Chromatography with Sulfur Chemiluminescence Detection

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Abstract

Hydrogen sulfide (H₂S) is a metabolite and signaling molecule in biological tissues that regulates many physiological processes. Reliable and sensitive methods for H₂S analysis are necessary for a better understanding of H₂S biology and for the pharmacological modulation of H₂S levels *in vivo*. In this chapter, we describe the use of gas chromatography coupled to sulfur chemiluminescence detection to measure the rates of H₂S production and degradation by tissue homogenates at physiologically relevant concentrations of substrates. This method allows separation of H₂S from other sulfur compounds and provides sensitivity of detection to ~15 pg (or 0.5 pmol) of H₂S per injected sample.

1. INTRODUCTION

Reliable detection of hydrogen sulfide (H₂S) in biological samples is challenging due to its volatility, redox lability, and most importantly, its low steady-state concentrations (Kabil & Banerjee, 2010). These difficulties combined with the use of methods that are not specific for measurement of free sulfide at physiological pH, have resulted in reports in the literature of H₂S concentrations spanning five orders of magnitude ranging from the tens of nanomolar to hundreds of micromolar (Olson, 2009). This ambiguity in the physiologically relevant concentration of H₂S has, in turn, resulted in the use of widely varying concentrations of sulfide donors to elicit physiological effects. H₂S is a product of sulfur metabolism (Kabil & Banerjee, 2010; Kabil, Vitvitsky, & Banerjee, 2014) and is synthesized and degraded by mammalian tissues at relatively high rates (Jurkowska et al., 2014; Vitvitsky, Kabil, & Banerjee, 2012). The methods used for H₂S detection have been discussed in Olson (2012) and include colorimetric analysis monitoring methylene blue formation, use of a sulfide ion-selective or a polarographic electrode, gas chromatography (GC) with flame photometric or sulfur chemiluminescence detection, ion chromatography, HPLC analysis of the monobromobimane derivative of sulfide with fluorescence detection, and the use of sulfide-sensitive fluorescent dyes. Using the GC-coupled sulfur chemiluminescence detection method for H₂S, recent studies have converged on low nanomolar concentrations of H₂S in biological samples (Furne, Saeed, & Levitt, 2008; Kabil, Vitvitsky, Xie, & Banerjee, 2011; Levitt, Abdel-Rehim, & Furne, 2011; Vitvitsky et

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al., 2012). In this chapter, we describe the application of the GC-based sulfur chemiluminescence method for H₂S detection in biological samples.

2. PRINCIPLE OF THE GC-COUPLED SULFUR CHEMILUMINESCENCE METHOD

Analysis of H₂S levels in biological samples using GC with sulfur chemiluminescence detection relies on the equilibration of H₂S between the liquid sample and gas phases in a hermetically sealed sample chamber. H₂S present in the gas phase is measured following GC separation from other sulfur compounds and detected using a 355 sulfur chemiluminescence detector (Agilent Technologies, Santa Clara, CA). In the detector, the sample undergoes combustion in the furnace generating sulfur monoxide, which reacts with ozone in the reaction cell to form sulfur dioxide emitting light (Fig. 1). The light intensity is measured using a photomultiplier tube and the signal is linearly proportional to the quantity of sulfur in the sample. The GC method allows separation of H₂S from different natural sulfur compounds (Fig. 2) (Levitt, Furne, Springfield, Suarez, & DeMaster, 1999). The sensitivity of the instrument allows detection of 0.5 pg of sulfur and the linear range spans approximately four orders of magnitude based on the vendor's information.

2.1. Limitations of the GC method

The sensitivity of the method is influenced by parameters such as column type, injection volume, injection mode, and gas flow rate, which affect the amount of sample that reaches the detector. Hence, these parameters must be adjusted to optimize sensitivity prior to sample analysis. A second issue that influences the sensitivity of this method is that low H₂S concentrations necessitate injection of relatively large samples volumes. Modern GC capillary columns with a maximal available inner diameter of 0.53 mm are not suitable for injection of more than 1 ml of a gas sample. Packed columns allow injection of up to 10 ml of gas samples and significantly enhance sensitivity of H₂S analysis. Unfortunately, the newest 355 sulfur chemiluminescence detector does not interface with packed columns.

3. PROTOCOL FOR GC-COUPLED SULFUR CHEMILUMINESCENCE DETECTION OF H₂S

3.1. Materials

- NORM-JECT polypropylene syringes (Henke Sass Wolf, Tuttlingen, Germany)
- Plastic three-way stopcocks (Smiths Medical ASD, Dublin, OH)
- Sleeve stopper septa for 5 mm NMR tubes (white, 1.5 × 3.9 i.d. × o.d., Sigma Aldrich, St. Louis, MO)
- Tedlar gas sample bag (Jensen Inert Products, Coral Spring, FL)
- Gas-tight glass syringe with PTFE plunger and stainless steel needle (Agilent Technologies, Santa Clara, CA)
- H₂S calibration mixture, 40 ppm H₂S in N₂ (Cryogenic Gases, Detroit, MI)

- Mixture of natural sulfur gases in N₂ (Cryogenic Gases, Detroit, MI)
- Ultrahigh purity grade helium, H₂, and N₂ cylinders (Cryogenic Gases, Detroit, MI)
- Zero grade air cylinder (Cryogenic Gases, Detroit, MI)
- DB-1 capillary column (30 m × 0.53 mm × 1.0 μm, Agilent Technologies, Santa Clara, CA)

3.2. Calibration standards

For instrument calibration, gas cylinders containing either 40 ppm H₂S in N₂ or a mixture of 10 ppm each of carbonyl sulfide (COS), methanethiol (CH₃SH), ethanethiol (CH₃CH₂SH), and dimethylsulfide (CH₃SCH₃) in N₂ are obtained from Cryogenic Gases (Detroit, MI, USA). For routine instrument calibration, ~1 l of 40 ppm H₂S in N₂ was kept in a Tedlar gas sample bag in which the valve was sealed with sleeve stopper septum. The H₂S concentration in the bag was stable over the course of the day and the bag was refilled daily with fresh calibration standards.

3.3. Sample manipulation

H₂S disappears rapidly if kept in glass containers or if exposed to rubber (e.g., stoppers). Hence, disposable polypropylene syringes were utilized for sample handling. The sample is placed in the barrel of a syringe and the syringe is sealed with a plunger. The syringe tip is fitted with a three-way stopcock, which is used to make the sample anaerobic by flushing the syringe with N₂. During sample incubation, the top of the three-way stopcock is sealed with a small-sleeve stopper septum. Gas aliquots for measurement of H₂S levels are collected from the syringe through the septa and injected into the GC using a gas-tight glass syringe with a PTFE plunger and stainless steel needle. Since H₂S levels are stable in glass syringes for at least 5 min, they are suitable for sample injection. Aliquots of standard H₂S mixture (40 ppm in N₂) are added when needed, through the septa into the syringes filled with N₂ to obtain different dilutions for instrument calibration.

3.4. Chromatography conditions

Gas samples were injected into an Agilent 6890 Series Gas Chromatograph equipped with a DB-1 capillary column. The following chromatographic conditions were found to provide optimal sensitivity and resolution: manual splitless injection, inlet temperature 105 °C, total gas flow rate 29.9 ml min⁻¹, column gas flow rate 2 ml min⁻¹, detector outlet temperature 150 °C. Helium is used as a carrier gas. The oven temperature is maintained at 30 °C during sample injection and analysis, increased to 110 °C at the end of the run at a rate of 20 °C min⁻¹, and maintained at 110 °C for 2 min before returning to the initial conditions. The oven temperature is increased to 110 °C in order to clean the column from possible contamination with organic substances and water present in the injected samples.

H₂S analysis in a single sample takes ~2 min. The total run cycle including the temperature gradient and restoration to initial conditions takes ~20 min. In our hands, the linear range of this method extends over two orders of magnitude (Fig. 3), and the sensitivity of detection is ~15 pg (i.e., 0.5 pmol) of H₂S per injection. We estimate that sensitivity could be enhanced ~5-fold by direct sample injection onto the column and even further, using a packed column.

4. ANALYSIS OF BIOLOGICAL SAMPLES

4.1. Monitoring H₂S production in tissue homogenate

Substrates for H₂S production (cysteine, homocysteine, or both for cystathionine β -synthase and γ -cystathionase and mercaptopyruvate and a reductant such as dithiothreitol or dihydrolipoic acid for mercaptopyruvate sulfurtransferase) are added to tissue homogenates prepared as described (Kabil et al., 2011; Vitvitsky et al., 2012). The sample with a total volume of 0.5 ml is placed in the barrel of a 20-ml polypropylene syringe attached to a three-way stopcock. The syringe is sealed with a plunger and made anaerobic by flushing the headspace with N₂ five times using the three-way stopcock (Fig. 4) and filled with N₂ to a total volume (liquid+gas) of 20 ml. Then, the stopcock is disconnected from the source of N₂ and sealed with a sleeve stopper septum, and the syringe is incubated for 20 min at 37 °C with gentle shaking (75 rpm). Control samples in which the tissue homogenate is replaced by buffer are prepared and incubated in parallel. Aliquots (200 μ l) from the gas phase of the reaction syringes are collected using a gas-tight syringe through the sleeve stopper septum attached to the stopcock, and injected into the GC. For samples containing a high concentration of H₂S, a 20-fold dilution with N₂ is used to prevent column overloading. For this, 0.5 ml of the sample is injected into a syringe filled with 9.5 ml of N₂. Then, 200 μ l of the diluted sample is injected into the GC.

Different dilutions of the H₂S gas calibration mixture (40 ppm in N₂) are prepared using N₂. Aliquots (200 μ l) of the varying H₂S dilutions in N₂ are injected into the GC and the corresponding peak areas are used to generate a standard curve. The concentration of H₂S in the injected sample is calculated from the peak area using the calibration coefficient obtained from the standard curve. The total amount of H₂S in syringes needed to estimate the H₂S production rates in tissues is calculated as described below in Section 4.3. It is important for H₂S concentration estimations and for instrument response linearity that the injection volume of the samples and calibration gas mixture are the same.

Typically, since peaks other than H₂S are not observed in these samples, multiple injections can be made during a single run cycle (Fig. 5). For this, the oven temperature in the GC is maintained at 30 °C for 20 min during which several (up to 10) injections of sample and calibration gas aliquots can be made. Following this, the temperature is increased to complete the run cycle. Usually, the calibration mixture containing 40 ppm H₂S in N₂ is injected with each analytical run to control for variability.

4.2. Monitoring H₂S degradation in tissue homogenates

Tissue homogenate (0.5 ml) is placed in the barrel of a 20-ml polypropylene syringe attached to a three-way stopcock and the syringe is sealed with a plunger. For anaerobic assays, the syringe headspace is flushed five times with N₂ using a three-way stopcock (Fig. 4) and then filled with N₂ to a total volume (liquid+gas) of 10 ml. Then, 10 ml of the H₂S gas mixture (40 ppm in N₂) corresponding to 17.9 nmoles H₂S is added to the syringe using the three-way stopcock to give a final volume of 20 ml. For aerobic assays, the syringe headspace is filled with air to a total volume (liquid+gas) of 10 ml and 10 ml of the H₂S gas mixture (40 ppm in N₂) is added using a three-way stopcock, to give a final volume of 20

ml. The syringes are incubated at room temperature (25 °C) with stirring and 200 µl aliquots are collected from the gas phase over 20 min and as described above, multiple samples and the calibration standard are injected per GC run cycle (Fig. 6). The concentration of H₂S in the samples is determined from the peak area using a calibration coefficient as described in the next section.

4.3. Estimation of H₂S production and degradation rates

To determine the rates of H₂S production and degradation, the total amount of H₂S in the reaction syringe needs to be calculated taking into account its equilibration between the gas and liquid phases and its dissociation at the pH of the reaction mixture. In the samples described above, the amount of H₂S in the gas phase is equal to the H₂S concentration in the sample aliquots multiplied by the total gas phase volume (i.e., 19.5 ml). The amount of dissolved H₂S present in the liquid phase is calculated by multiplying the concentration of H₂S in the gas phase by 1.6, which is the equilibrium ratio between the concentration of H₂S in the gas and liquid phases at 37 °C (Furne et al., 2008) and by the volume of the liquid (0.5 ml). The amount of dissolved sulfide anion (HS⁻) in the liquid phase is calculated using a pK_a of 6.8 for ionization of H₂S in water at 37 °C (Hershey, Plese, & Millero, 1988). The total amount of H₂S in the sample is then the sum of the amounts of H₂S in the gas phase, dissolved H₂S, and HS⁻. The pK_a for H₂S is influenced by the ionic strength (Almgren, Dyrssen, Elgquist, & Johansson, 1976; Hershey et al., 1988; Millero, 1986) and is expected to decrease from 6.8 in water to ~6.6 at physiologically relevant salt concentrations. Under our experimental conditions with a gas:liquid ratio of 39:1, the majority (~80%) of the H₂S in the reaction mixture is present in the gas phase at pH 7.4. Since adjusting the pK_a value from 6.8 to 6.6 changes the total H₂S value by <10%, a pK_a value of 6.8 was employed in our calculations. The rate of H₂S production is then determined by subtracting the sum of H₂S amounts in the gas and liquid phases in the control from the sample and dividing that value by the incubation time and the wet weight of the tissue. Since the H₂S degradation kinetics are measured at 25 °C a value of 2.0 for the equilibrium ratio for H₂S in the gas versus liquid phase (Furne et al., 2008) and a pK_a value of 7.0 (Hershey et al., 1988) are used in the calculations.

The total amount of sulfide in the reaction mixture (S_T) needed to calculate the rate of H₂S production and degradation is the sum of the sulfide amounts in the gas (S_{Gas}) and liquid (S_{Liq}) phases of the mixture (Eq. 1).

$$S_T = S_{Gas} + S_{Liq} \quad (1)$$

From the peak area of the sample injected into the GC, one can calculate the concentration (S_[Gas]) and the amount (S_{Gas}) of H₂S in the gas phase of the reaction mixture using Eqs. (2) and (3).

$$S_{[Gas]} = A/C \quad (2)$$

$$S_{\text{Gas}} = S_{[\text{Gas}]} * V_{\text{Gas}} = A * V_{\text{Gas}} / C \quad (3)$$

Here A is the peak area of H_2S in the sample, C is the calibration coefficient (i.e., the peak area corresponding to 1 M H_2S) obtained from the standard curve and V_{Gas} is the volume of the gas phase in the reaction mixture. The total sulfide amount in the liquid phase of the reaction mixture (S_{Liq}) is the sum of the amount of dissolved undissociated ($S_{\text{H}_2\text{S}}$) and ionized (S_{HS^-}) forms (Eq. 4).

$$S_{\text{Liq}} = S_{\text{H}_2\text{S}} + S_{\text{HS}^-} \quad (4)$$

The amount of dissolved sulfide depends on the concentration in the liquid phase of the undissociated ($S_{\text{H}_2\text{S}}$) and dissociated (S_{HS^-}) forms and on the liquid volume (V_{Liq}) (Eqs. 5 and 6).

$$S_{\text{H}_2\text{S}} = S_{[\text{H}_2\text{S}]} * V_{\text{Liq}} \quad (5)$$

$$S_{\text{HS}^-} = S_{[\text{HS}^-]} * V_{\text{Liq}} \quad (6)$$

The concentration of undissociated H_2S in the liquid phase is determined using the equilibrium ratio (R) between the gas and liquid phases (Eq. 7) and depends on the temperature.

$$S_{[\text{H}_2\text{S}]} = R * S_{[\text{Gas}]} \quad (7)$$

Similarly, the concentration of the dissolved ionized sulfide (S_{HS^-}) is determined using the pK_a value for the dissociation of H_2S to sulfide anion (Eq. 8) taking into account the pH of the liquid phase.

$$S_{[\text{HS}^-]} = S_{[\text{H}_2\text{S}]} * 10^{(\text{pH} - \text{pK}_a)} \quad (8)$$

The total amount of sulfide in the reaction mixture is obtained from the sample peak area (A), the calibration coefficient (C), the volumes of the gas (V_{Gas}) and liquid (V_{Liq}) phases, the pH of the liquid phase, the equilibrium ratio for H_2S between the gas and liquid phase, and the pK_a value of H_2S (Eq. 9), which simplifies to Eq. (10).

$$\begin{aligned}
S_T &= S_{\text{Gas}} + S_{\text{Liq}} = S_{\text{Gas}} + S_{\text{H}_2\text{S}} + S_{\text{HS}^-} \\
&= S_{[\text{Gas}]} * V_{\text{Gas}} + (S_{[\text{H}_2\text{S}]} + S_{[\text{HS}^-]}) * V_{\text{Liq}} \\
&= S_{[\text{Gas}]} * V_{\text{Gas}} + (R * S_{[\text{Gas}]} + R * S_{[\text{Gas}]} * 10^{(\text{pH}-\text{pKa})}) * V_{\text{Liq}} \quad (9) \\
&= S_{[\text{Gas}]} * V_{\text{Gas}} + R * S_{[\text{Gas}]} \left(1 + 10^{(\text{pH}-\text{pKa})} \right) V_{\text{Liq}} \\
&= A * V_{\text{Gas}} / C + (R * A / C) \left(1 + 10^{(\text{pH}-\text{pKa})} \right) * V_{\text{Liq}} \\
&= (A / C) \left(V_{\text{Gas}} + R * V_{\text{Liq}} \left(1 + 10^{(\text{pH}-\text{pKa})} \right) \right)
\end{aligned}$$

$$S_T = (A / C) \left(V_{\text{Gas}} + R * V_{\text{Liq}} \left(1 + 10^{(\text{pH}-\text{pKa})} \right) \right) \quad (10)$$

5. ADDITIONAL TECHNICAL DETAILS

5.1. Column conditioning

The protocol recommended for conditioning a new capillary column includes heating it to 250 °C. However, this procedure is very detrimental for the catalytic element in the sulfur chemiluminescence detector and leads to a significant drop in detector sensitivity. Hence, the column must be disconnected from the detector and the inlet to the detector must be plugged with an external nut and no-hole ferrule before the column is heated to 250 °C. In our experience, heating the capillary column to >200 °C causes a significant loss in the detector sensitivity and requires replacement of the catalytic element in the reaction furnace, i.e., the small ceramic tube, if the sulfur chemiluminescence detector is not disconnected from the column.

5.2. Additional gas purification

The sensitivity of the detector declines rapidly if the helium and hydrogen carrier gases are not purified prior to their use due to the presence of trace contaminants, particularly hydrocarbons. To increase the lifetime of the small ceramic tube, inline cartridges can be used to remove contaminants from the gases. Use of the Big Universal Trap RMSH-2 cartridge for helium and the Big Universal Trap RMSHY-2 cartridge (Agilent Technologies) for hydrogen helps maintain detector sensitivity with regular usage over a period of a year. Zero grade air does not require additional purification.

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Figure 1.

Scheme showing analysis of sulfur compounds using GC coupled to a sulfur chemiluminescence detector (SCD). Sulfur compounds in the sample are separated on a GC column prior to entering the SCD. In the reaction furnace, the samples undergo combustion in an air and hydrogen mixture producing sulfur monoxide. In the reaction cell, sulfur monoxide reacts with ozone to produce sulfur dioxide and light emission. The latter is detected using a photomultiplier tube (PMT).

Figure 2.

Analysis of a mixture of sulfur gases using GC-SCD. The mixture (200 μ l total injection volume) contained 2.7 ppm H₂S, 5 ppm carbonyl sulfide (COS), 5 ppm 3-methanethiol (CH₃SH), 5 ppm ethanethiol (CH₃CH₂SH), and 5 ppm 5-dimethylsulfide (CH₃SCH₃) in N₂.

Figure 3.

Linear dependence of the H_2S peak area on H_2S amount. The symbols and line represent the experimental data and linear fit, respectively. Each experimental point represents the mean $\pm$ SD of 2–4 independent measurements. In most cases, the standard deviation is equal to or less than the symbol size. Samples (200 μ l) containing different amounts of H_2S were prepared by dilution of the stock solution (40 ppm H_2S) with N_2 .

Figure 4. Scheme showing setup for sample preparation. The solid arrows depict the direction of N₂ flow, the movement of the syringe plunger and movement of the three-way stopcock valve during flushing of the syringe with N₂. The dashed arrow indicates the position where the sleeve stopper septum is attached during sample incubation.

Figure 5.

A representative chromatogram of H_2S production by murine liver homogenate at different cysteine concentrations. Peaks 1–3 were obtained after anaerobic incubation for 20 min of the homogenate (pH 7.4, 37 °C) with 0.1, 0.2, and 0.5 mM cysteine, respectively. The arrow indicates the control sample lacking cysteine.

Figure 6.

A representative chromatogram showing the kinetics of aerobic H₂S degradation by murine liver homogenate. The numbers correspond to samples (200 μ l) removed at 1, 2, 3, 5, 8, 10, and 15 min following incubation of liver homogenate with H₂S at 25 °C. The calibration peak (12.2 ng H₂S) is denoted by Std. The inset shows the kinetics of H₂S disappearance. The amount of H₂S at $t = 0$, shows the amount of H₂S added to the reaction mixture.