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Short communication

Measurement of fecal sulfide using gas chromatography and a sulfur chemiluminescence detector

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Abstract

We describe a simple technique to measure sulfide in fecal homogenates (or any other liquid milieu), which involves acidification followed by the G.C. measurement of H_2S in a gas space equilibrated with a small quantity of homogenate. An internal standard of $Zn^{35}S$ added to the homogenate permits correction for incomplete recovery of H_2S in the gas space. The use of a sulfur chemiluminescence detector, which specifically and sensitively responds to sulfur-containing compounds, greatly facilitates this measurement. Published by Elsevier Science B.V.

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1. Introduction

Sulfide, a product of bacterial metabolism, is found in a variety of biological materials including lake water, sludge, and feces. In addition to its unpleasant odor, H_2S is highly toxic as evidenced by an LD_{50} for rodents that is of the same order as cyanide [1].

The proposal by Roediger et al. [2,3] that sulfide toxicity may play a role in the pathogenesis of ulcerative colitis has stimulated interest in fecal sulfide measurements. The technique commonly used for this purpose was first described by Cline [4] for measurement of sulfide in lake water. In this technique, sulfide released by acidification of the sample binds with two *N*,*N*-dimethyl-*p*-phenylenediamine monomers to produce a phenoxazine, which is then converted to methylene blue. Sulfide concentration is determined by the spectrophotometric intensity of the blue color developed in the reaction.

Our extensive experience with the methylene blue technique indicated that this methodology has shortcomings when applied to fecal material [5]. Homogenates of feces have a background turbidity and color that is difficult to correct for with an appropriate blank. Sufficient dilution to render this turbidity and color negligible may result in unmeasureable sulfide concentrations. In addition, fecal components variably interfere with the reaction producing methylene blue as evidenced by incomplete recovery of sulfide added to feces. Lastly, compounds other than sulfide may contribute to the blue color in the assay. Thus, the accuracy of this labor intensive technique is questionable, particularly at low fecal sulfide concentrations.

In contrast to the situation with liquids, the H_2S concentration of a gas sample can be rapidly and accurately determined via gas chromatography

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(G.C.). The present report describes a simple technique to assess fecal sulfide in which sulfide is converted to H_2S via acidification, and the H_2S of an equilibrated gas space is then quantitated using a G.C. technique.

2. Methods

2.1. Fecal specimens

Freshly passed fecal specimens were weighed and then homogenized in a 2% zinc acetate solution (1 g feces to 5 ml of a zinc acetate). Zinc avidly binds sulfide as insoluble zinc sulfide and hence prevents loss of sulfide via volatilization and/or oxidation.

2.2. Conversion of sulfide to H_2S

All studies were carried out in polypropylene syringes since, in previous studies, we found that H_2S reacted with glass and most plastic and rubber surfaces [6]. The plunger was removed from a 20-ml syringe attached to a stopcock, and a 0.5-ml volume of vigorously stirred fecal homogenate was instilled in to the syringe using a displacement pipette. (This means of adding homogenate to the syringe was used because attempts to instill the homogenate though the stopcock via a 1-ml syringe and needle often resulted in plugging of the needle.) The plunger of the syringe was reinserted and a 0.05 volume of 2% zinc acetate containing about 0.01 μci of ^{35}S (specific activity of about 0.02 μci/nmol) was injected into the 20 ml syringe using a 0.10-ml syringe and a #23 needle inserted through the open stopcock. The labeled sulfide served as an internal standard for measuring the efficiency of H_2S release into the gas space from the homogenate. The plunger was set such that the syringe contained 19 ml of air. The syringe contents were mixed by rotation of the syringe. A 0.5-ml volume of 12 N HCl (contained in a 1-ml syringe) was rapidly instilled into the syringe via a needle inserted though the open stopcock. The needle was immediately withdrawn, the stopcock was sealed, and stirring was obtained via rotation of the syringe. During these mixing maneuvers, care was taken that the solution did not run into the tip of the syringe where it connected with the stopcock, which would make it difficult to aspirate gas (without liquid) through the stopcock. After a 30-min equilibration period at room temperature, a 0.3-ml aliquot of the gas space was analyzed for H_2S, and a 5-ml aliquot was used for analysis for $H_2^{35}S$.

2.3. Measurement of H_2S

The concentration of H_2S was determined by G.C. as follows. A 0.3-ml aliquot of the gas space was injected directly onto a G.C. column using a gas tight syringe. The gas chromatograph (Model 5890, Hewlett-Packard Company, Palo Alto, CA) was equipped with a Teflon column (8′×1/8″, packed with Chromosil 330, Supelco, Bellefonte, PA; maintained at 80° with a N_2 flow-rate of 20 ml/min) and a sulfur chemiluminescence detector (Model 355, Sievers Instruments, Inc., Boulder, CO) that specifically detects sulfur-containing gases. Hydrogen sulfide is the major sulfur gas released during the acidification of the homogenate and elutes in the above G.C. system with a retention time of about 30 s (see Fig. 1). The concentration of H_2S was determined by comparison of the peak area of the unknown with the areas of authentic standards of known concentration.

2.4. Measurement of $H_2^{35}S$

A volume of 5-ml of gas was transferred from the syringe containing the homogenate to a second 20 ml syringe fitted with a stopcock. A 0.3-ml volume of 0.2 N benzethonium hydroxide in methanol was then added to this syringe. (Preliminary studies showed that H_2S was avidly taken up by benzethonium hydroxide.) A volume of 10 ml of Ultima Gold was added to the syringe, the solutions mixed, and the total volume of the mixture was then transferred to a scintillation vial. Radioactivity was then determined via scintillation counting (Packard Instrument Company, Downers Grove, IL). The total radioactivity initially added to the homogenates was determined via the addition of 0.05 ml of the internal standard to a scintillation vial containing 0.3 ml of Hyamine and 10 ml of Ultima Gold.

2.5. Synthesis of ^{35}S-sulfide

Since ^{35}S-sulfide is not commercially available on a routine basis, we synthesized this compound as

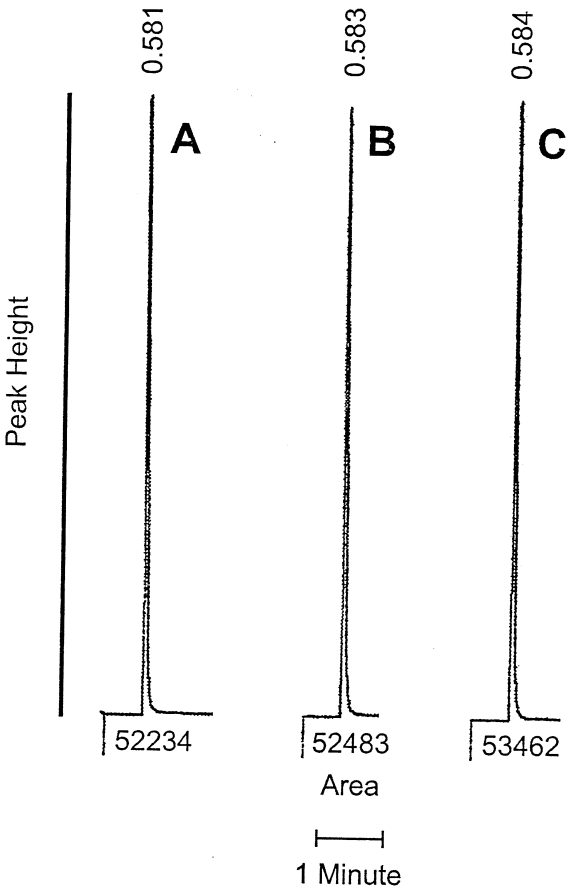

Fig. 1. Gas chromatographic tracings of H_2S concentrations in the gas space following acidification of three aliquots of a fecal homogenate. The numbers above the peaks indicate the retention times (minutes), and the numbers below the peaks indicate the peak area. The measurement is highly reproducible and no interfering peaks are registered by the sulfur chemiluminescence detector.

follows. Freshly passed rat feces were homogenized in a blender (Waring, Eberbach Corporation, Ann Arbor, MI) using one part feces to four parts phosphate-buffered saline, pH 7.0. A total of 50 μCi ^{35}S-cysteine was added to 5 ml of fecal homogenate, and the mixture was incubated in a sealed 50 ml polypropylene syringe along with 30 ml of nitrogen. The gas space was removed at 24 h and H_2 ^{35}S isolated via first passing the 40 ml gas space through a column (glass, 43×5 mm; packed with MTO-Tenax-TA (80/100 mesh, Supelco, Bellefonte, PA)) maintained in dry-ice. Nitrogen (20 ml) was then perfused through the column at 25°C, and the eluant was collected in a second syringe. Analysis by G.C. showed that H_2S was the only sulfur gas present in the column eluate, which was immediately mixed with 10 ml of 2% zinc acetate. Sulfide bound by zinc in this solution is stable for several months.

2.6. Calculation of sulfide concentration of homogenate

The volume of H_2S released into the gas space with acidification was determined from measurement of the concentration of H_2S in the gas space and the 19-ml volume of the space. (The H_2S contributed by the internal standard usually was negligible.) This volume was then corrected for the fraction of the ^{35}S-sulfide internal standard recovered in the gas space. Sulfide concentration of feces was calculated as follows:

Sulfide/0.5 ml of homogenate=[H_2S] in gas space×19 ml/fractional recovery ^{35}S of internal standard in gas space.

Since the homogenate represented a 5-fold dilution of feces, the fecal concentration of sulfide was five times that of the homogenate, and was expressed as μmol/g feces.

2.7. Recovery studies

Recovery studies were carried out with three fecal homogenates. Known quantities of zinc sulfide were added to aliquots of homogenates, and the sulfide concentrations of the unspiked and spiked homogenates were then determined as described above. The observed sulfide content of the unspiked homogenate was subtracted from that of the spiked sample, and this value was then compared with the quantity of sulfide added to the homogenate to calculate recovery.

3. Results

Fig. 1 shows the gas chromatographic tracings of H_2S in the gas space after acidification of three aliquots of a fecal homogenate. It is apparent that this measurement is highly reproducible.

A plot of the H_2S concentration in the gas space following acidification of serial dilutions of a 2.1

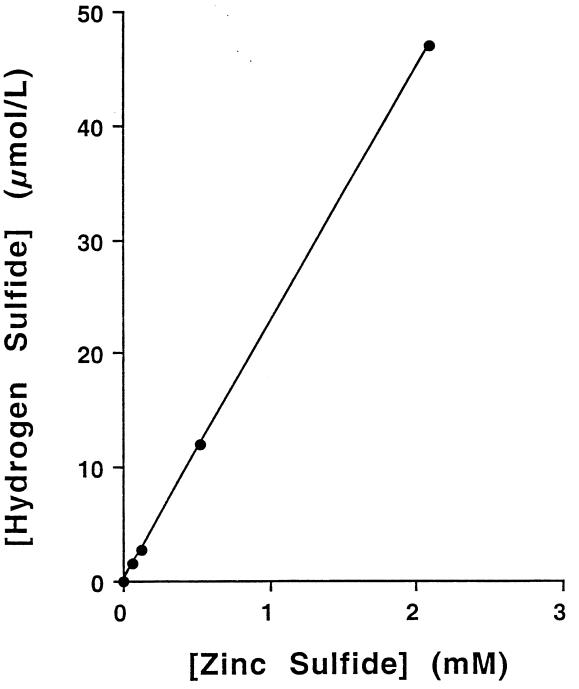

Fig. 2. Observed sulfide concentrations in the gas space following acidification of serial dilutions of a 2.1-m*M* zinc sulfide solution. Each value represents the mean of duplicate measurements.

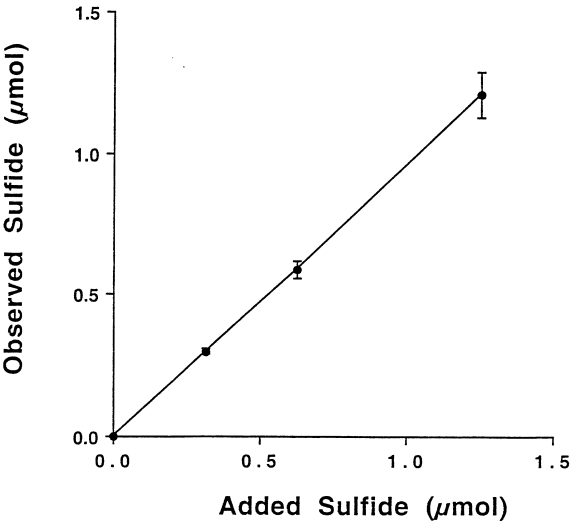

Fig. 3. Recovery of sulfide added to fecal homogenates. The observed increase in sulfide in the fecal sample (sulfide in spiked sample minus that of unspiked sample) is plotted against the quantity of sulfide added to the sample. Data represent the mean±SD of triplicate determinations carried out with three fecal homogenates. Recoveries averaged 96, 94, and 97% for 0.31, 0.62, and 1.25 μmol of sulfide, respectively.

m*M* zinc sulfide standard solution is shown in Fig. 2. The H_2S measured in the gas space was linearly related to sulfide concentration over a 30-fold range of concentrations.

Acidification of ten fecal homogenates resulted in the release into the gas phase of an average of 80±1% of the ^{35}S added to the homogenate to serve as an internal standard. As would be expected, all of this volatile radioactivity eluted from Tenax with H_2S.

The reproducibility of the assay was calculated from triplicate determinations carried out on 19 fecal specimens. The coefficient of variation (SD/mean) of these measurements averaged 0.036±0.034.

The recovery of known quantities of sulfide added to three fecal homogenates is shown in Fig. 3. Recovery averaged 96, 94 and 97%, respectively, for 0.31, 0.62 and 1.25 μmol of added sulfide.

The mean±1 SD of the concentrations of sulfide present in fecal specimens obtained from 10 healthy human subjects was 1.3±0.46 μmol/g wet weight.

4. Discussion

The underlying concept of the assay described in this report-acidification of the sample to convert sulfide to H_2S followed by analysis of the gas space for H_2S — is not original. However, application of this methodology to feces has been limited by the high water:gas solubility ratio of H_2S (approximately 8:1), lack of knowledge concerning the possible influence of non-aqueous fecal components on this distribution ratio, and the possibility that H_2S may be oxidized during the analytical procedure. In an assay in which roughly similar volumes of homogenate and gas are equilibrated, a very large (and possibly, unpredictable) fraction of the H_2S would remain in the aqueous phase, creating the potential for sizable errors if total H_2S were predicted from gas space measurements.

We utilized two modifications of standard methodology to insure that analysis of the gas space provided an accurate assessment of the total sulfide content of homogenate plus gas space. The first ''innovation'' we employed was the use of a G.C.

equipped with a sulfur chemiluminescence detector for the determination of H_2S. This detector very sensitively and specifically responds to sulfur-containing compounds. This specificity makes it possible to inject unmodified gas space onto the chromatograph and measure the rapidly eluting H_2S (elution time of 30 s) with no interference from simultaneously eluting peaks (see Fig. 1). The sensitivity of the detector makes it possible to accurately measure H_2S using a relatively high gas: feces ratio (0.1 g feces to 19 ml gas). As a result, the vast majority of the released H_2S is in the gas rather than the liquid phase. Another advantage of this detector is the broad range over which it linearly responds to H_2S (see Fig. 2). This wide range obviates the need to assay multiple dilutions of homogenates to insure that the H_2S concentration falls within the range of accurate detection.

The second innovative aspect of this assay was the use of zinc ^{35}S-sulfide as an internal standard to measure the efficiency with which sulfide was recoverable as H_2S in the gas space equilibrated with the acidified homogenate. The percentage recovery of the internal standard as $H_2^{35}S$ in the gas space was relatively constant, averaging about $80\pm1\%$ for ten fecal specimens. Thus, it was necessary to increase the observed H_2S content of the gas space by about 20% to yield the true total sulfide content of the homogenate. Utilizing this methodology, recovery studies showed that the observed increase in sulfide content of homogenates closely correlated with the quantity of sulfide added to the homogenate (see Fig. 3).

The use of the internal standard represents the most complicated aspect of what otherwise is an extremely simple assay. If additional studies demonstrate that the recovery of sulfide in the gas phase is always about 80%, the use of the internal standard could be eliminated and a standard correction applied to the observed results. The fractional recovery of sulfide in the gas phase (80%) is that predicted from the relative solubilities of H_2S in acidified water and gas. Thus, if the water: gas ratio is held constant, it seems likely that the use of the internal standard can be eliminated, and a constant 20% correction for incomplete recovery can be employed.

The technique described in this report has several advantages over the commonly used methylene blue method for the measurement of sulfide in feces. The G.C. technique is simple, rapid and requires the use of only one reagent (HCl) and the ^{35}S-sulfide internal standard. Most important, the accuracy of the G.C. analysis is independent of any assumptions concerning chemical reactions that might be influenced by the non-sulfide components of the fecal homogenate. In contrast, fecal components that cause turbidity, interference with color development, and non-specific color development create theoretical as well as practical problems in the methylene blue assay for sulfide. Although the present report deals solely with fecal samples, the G.C. technique should be applicable to measurement of sulfide in any liquid milieu. The high sensitivity of this technique makes it particularly useful for determinations in solutions containing very low sulfide concentrations.

The sulfide concentration of fecal samples obtained from ten healthy subjects averaged 1.38 ± 0.46 μmol/g when measured by our G.C. technique. A wide range of fecal sulfide concentrations for normal human feces have been reported using the methylene blue assay. Reported values (in units of μmol/g) include the following: 0.05 ± 0.01 and 0.21 ± 0.03 in feces that produced and did not produce methane, respectively [7], 0.18 ± 0.08 [8], 0.027 ± 0.007 [9], 0.66 ± 0.06 [10], and 1.33 ± 0.21 [11], and 1.6 ± 0.85 [5]. We conclude from the present study that the true concentration of fecal sulfide probably is at the high end of the range of previously reported values obtained with the methylene blue technique.

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