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Note

Determination of sulfide in brain tissue and rumen fluid by ion-interaction reversed-phase high-performance liquid chromatography

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Hydrogen sulfide is present in many environments, either as a naturally occurring gas or as a by-product of industry [1]. Since sulfide is known to be a toxicant, the detection and quantitation of this substance in biological samples is often necessary.

Many methods for the determination of sulfide have been developed. Precipitation of sulfide from aqueous solutions by zinc acetate is well known [2]. Barium and cadmium sulfate turbidimetry has also been used to a limited extent for sulfide quantitation [3]. Aliphatic sulfide [4] and aqueous inorganic sulfide [5] concentrations have been determined by iodine titrations or colorimetric methods. These methods, however, lack sensitivity and do not lend themselves easily to the analysis of biological samples.

Gas chromatography (GC) with flame photometric detection has also been employed for the determination of hydrogen sulfide [6–8]. This method is much more sensitive than the wet methods mentioned above; however, aside from the expense of the detector, the method suffers from interferences which lead to spurious and irreproducible results [9]. Although methods have been devised for purifying the samples prior to GC analysis [10], these added steps are time-consuming and are not amenable to biological samples. An extractive alkylation method has been reported [11], and although this method circumvents the problems of detection and interferences, it suffers the drawback of being cumbersome and expensive in terms of materials and time.

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The recent method of Goodwin et al. [12], which employs ion chromatography following gas dialysis of biological samples, offers the advantage of preconcentration of analyte; however, the method lacks simplicity and requires specialized apparatus.

Reversed-phase high-performance liquid chromatography (HPLC) with fluorescence detection has also been employed recently for sulfide determination. The *o*-phthaldialdehyde method of Mopper and Delmas [13] offers low sensitivity, but the isoindole derivatives are unstable. Vetter et al. [14] have modified the procedure of Fahey and Newton [15] in order to separate the bimane derivatives of sulfur-containing compounds in biological materials. Although this method is an excellent approach to the separation and determination of compounds containing sulfur, it lacks specificity and is therefore not required in all instances.

A sulfide-specific method which has enjoyed widespread use is that of methylene blue. Two equivalents of *p*-N,N-dimethylphenylenediamine, together with an oxidizing agent, react quickly with hydrogen sulfide to produce the methylene blue. This compound is reportedly stable for several days when stored in the dark, but slowly decomposes in sunlight [16]. Methylene blue absorbs light at 670 nm in aqueous acidic solution and has been determined predominantly by spectrophotometric detection. Sensitivity has been in the low micromole range [17]. This method has been used to analyze air and gases [18], water samples [19], paper pulp [20] and plant materials [18]. A disadvantage of spectrophotometric determination by the methylene blue method is that there are several interfering substances which impede analysis.

Recently, reversed-phase HPLC has been used to separate derivatized sulfide (methylene blue) from these impurities [21], although applications have not been reported. In that the methylene blue method is simple and quick, and since reversed-phase HPLC is common to many laboratories, this approach offers many advantages.

During the course of our investigation of neurologic disease in cattle, we were confronted with the need to evaluate sulfide concentrations during the development of the disease. This study describes the application of pre-column derivatization of sulfide with reversed-phase HPLC separation and spectrophotometric detection of methylene blue to determine sulfide in bovine brain tissue and rumen fluid.

EXPERIMENTAL

Instrumentation and reagents

The liquid chromatograph consisted of a Beckman Model 110 A isocratic pump, Valco Model C 10 W ten-port injector equipped with a 100-μl sample loop, Hamilton PRP-1 column (Reno, NV, U.S.A.; 250 mm $\times$ 4.6 mm I.D., 10 μm particle size) with a PRP guard column (Pierce, Rockford, IL, U.S.A.),

Isco Model UA-5 detector (Lincoln, NE, U.S.A.) equipped with a 660-nm filter set and a Hewlett-Packard Model 3390 A integrator (Palo Alto, CA, U.S.A.). The flow-rate was kept constant at 1.5 ml/min.

The N,N-dimethyl-*p*-phenylenediamine (Sigma, St. Louis, MO, U.S.A.) stock solution was prepared by dissolving 12.0 g in 80 ml of 9.0 *M* aqueous sulfuric acid. The working solution was made fresh daily by diluting 1.0 ml stock solution to a final volume of 40 ml with 9.0 *M* sulfuric acid. The oxidizing solution consisted of 50 g ferric chloride dissolved in 100 ml distilled water.

Sulfide standards were prepared fresh for each linear regression determination by serial dilution of a 10 m*M* solution of sodium sulfide in degassed (helium bubbling for 2 min) Millipore Milli-Q water.

The mobile phase consisted of 35% HPLC-grade acetonitrile (J.T. Baker, Phillipsburg, NJ, U.S.A.), 0.5% reagent-grade acetic acid and 64.5% Milli-Q water. The final concentration of *p*-toluenesulfonic acid (Eastman Kodak, Rochester, NY, U.S.A.) was 5.0 m*M*. All mobile phases were filtered through a 0.45-μm membrane and degassed under vacuum in an ultrasonic bath prior to use.

Procedure

Rumen fluid. Rumen fluid was collected via stomach tube from cattle adapted to a roughage diet. A 200-μl volume of 5% zinc acetate was added to 10.0 ml of each rumen fluid sample prior to storage. To 500 μl of this rumen fluid were added 1.00 ml of water, 500 μl of amine solution and 20 μl of oxidizing solution. The vessel was immediately closed and shaken briefly. After a 20-min reaction period, the mixture was centrifuged for 1 min at 4000 *g*. A portion of the centrifugate was filtered through a 0.45-μm membrane filter for immediate injection.

The samples for determining the coefficient of variation were prepared as follows. A 50-ml volume of rumen fluid from a normal steer was centrifuged for 1 min at 1500 *g*. To a 17.0-ml aliquot of the centrifugate were added 1.0 ml of 1% aqueous zinc acetate and 2.00 ml of 50 μ*M* sulfide standard. To a second 17.0-ml aliquot of the centrifugate were added 1.0 ml of 1% aqueous zinc acetate and 2.00 ml of water. The spiked and unspiked samples were divided into 2.5-ml aliquots and frozen at a $-20\,^{\circ}$C until use. Each day, a set of serial dilution standards, from 1.00 m*M* to 500 n*M*, were derivatized and a calibration equation was derived. The spiked and unspiked samples were derivatized and injected three times each to determine the recovery and coefficient of variation. The injection-to-injection variability was calculated by injecting the same derivatized sample ten times.

Brain tissue. A 400-mg cerebrocortical gray matter was homogenized in 2.00 ml of 20 m*M* aqueous zinc acetate, 1.0% Brij-35 (non-ionic detergent, Pierce). To this homogenate were added 2.0 ml of 100 m*M* borate buffer, pH 12. For analysis, 500 μl of amine solution were added to 1.00 ml of homogenate and 500 μl water. A 20-μl volume of oxidizing solution was introduced and the

vessel was immediately closed, shaken and left undisturbed for 30 min. The solution was centrifuged at 4000 *g* for 1 min and a portion of the centrifugate was passed through a 0.45-μm syringe filter for analysis.

For coefficient of variation determination, one 20.0-ml homogenate, 100 mg gray matter per 1 ml, was divided into two 9.0-ml aliquots. To one aliquot was added 1.0 ml of 10 μM sulfide standard, and to the other was added 1.0 ml of water. Both the spiked and unspiked homogenates were divided into 2.5-ml portions and frozen at $-20\,^{\circ}C$ until use. Each day, two samples of the spiked and unspiked homogenate were derivatized and compared to a standard linear regression equation for that day.

RESULTS AND DISCUSSION

Haddad and Heckenberg [21] found that a 1-min reaction period was sufficient for sulfide standards. This is not true for biological samples, however. Much of the sulfide present is bound, and the acid liberation is slower than the formation of methylene blue from free sulfide. The optimum reaction time for brain tissue was found to be around 30 min. The liberation of sulfide in rumen fluid is somewhat quicker: a 20-min reaction period is sufficient.

The coefficient of variation (calculated by dividing the standard deviation by the mean) was excellent for both rumen fluid and brain tissue: 1.8 and 1.6%, respectively. The recovery, calculated by dividing the concentration actually found by the expected spike concentration, was 100% for rumen fluid and 86% for brain tissue. The tissue may be lower than desired due to the high pH of the buffer used to store the samples. Studies are under way to test this hypothesis. Injection-to-injection variability was found to be 0.8% for rumen fluid and 0.1% for brain tissue. Table I lists the results. Figs. 1 and 2 are typical chromatograms of derivatized normal bovine brain tissue and rumen fluid.

Although an octadecyl column was used for previous studies [21], it was reasoned that due to the acidic nature of the injected sample, the PRP-1 col-

TABLE I

REPRODUCIBILITY OF THE METHYLENE BLUE HPLC METHOD

Parameter	Rumen fluid	*n*	Brain tissue	*n*
Coefficient of variation	1.8%	10	1.6%	8
Recovery	$100 \pm 1\%$	10	$86 \pm 3\%$	8
Injection-to-injection repeatability	0.8%	10	0.1%	7
Normal sulfide level		6		6
Mean	56.2 μM		166 nmol/g	
Standard deviation	18.6 μM		31.0 nmol/g	
Range	21.8–70.4 μM		104–191 nmol/g	

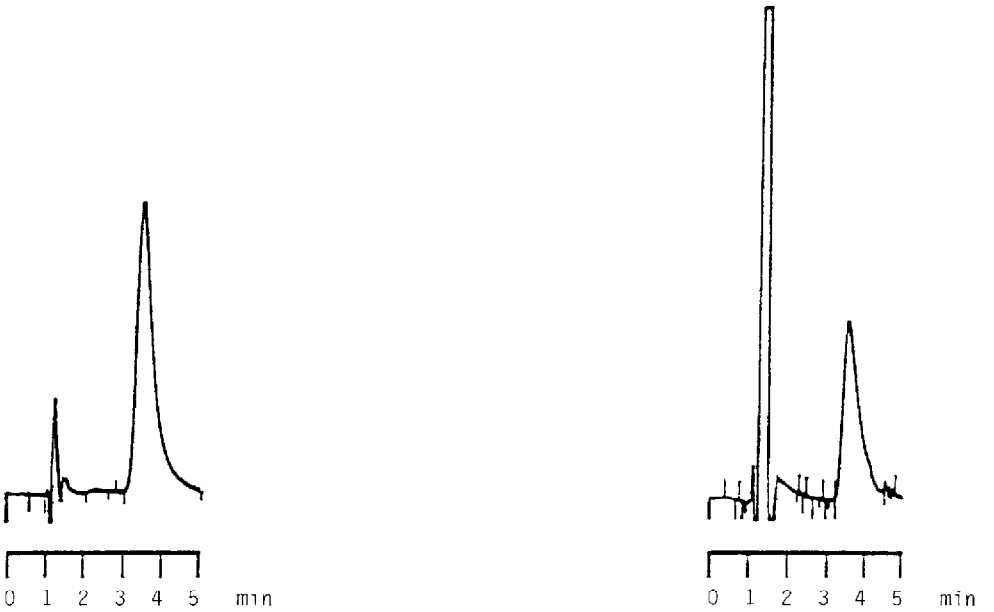

Fig. 1. Chromatogram of derivatized normal bovine rumen fluid. Mobile phase, 35% acetonitrile, 0 5% acetic acid, 64.5% water, 5.0 m*M* *p*-toluenesulfonic acid; retention time of methylene blue, 3.93 min with a flow-rate 1.5 ml/min, detector attenuation range, 0.5; integrator attenuation, 7

Fig. 2. Chromatogram of derivatized normal bovine cerebrocortical gray matter. Mobile phase and flow-rate same as in Fig. 1, detector attenuation range, 0 1; integrator attenuation range, 6.

TABLE II

AVAILABILITY OF AMINO ACID SULFUR FOR THE METHYLENE BLUE REACTION

Derivatization and chromatographic conditions are the same as for coefficient of variation and recovery studies.

Sample	Resulting methylene blue concentration (μM)	Percentage of total sulfur
1 0 m*M* Glutathione	3.79	0.4
1.0 m*M* Methionine	0.00	0.0
1 0 m*M* Cysteine	7.13	0.7
1 0 m*M* Cystine	2.13	0 2

umn would be more durable. The styrene–divinylbenzene stationary phase will withstand conditions from pH 1 to 13, while it is suggested that the octadecylsilane columns can be used between pH 2.5 and 7.5 [22]. Futhermore, the best chromatographic performance was observed with a PRP-1 column and toluenesulfonic acid as the ion interaction reagent. The 150 mm$\times$4.1 mm I.D., 5

μm particle size PRP-1 column was also found to produce excellent separation and better peak shape than the longer 10 μm particle size column.

Several sulfur-containing compounds were tested for methylene blue production. Solutions of 1.0 m*M* oxidized glutathione, cystine, cysteine and methionine were derivatized in the same manner as sulfide standards. None of these compounds yielded methylene blue concentrations above 1% of the total sulfhydryl moiety present; methionine gave no methylene blue peak; however, other reaction compounds in the mixture absorbed light at 660 nm. Table II displays the data.

CONCLUSION

This method appears to have excellent potential for determining sulfide concentrations in many biological samples. Advantages include the simplicity, sensitivity and reproducibility of the technique.

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