

## II.1.2 Hydrogen sulfide and its metabolite

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### Introduction

Hydrogen sulfide ( $\text{H}_2\text{S}$ ) is a colorless gas with the smell of putrid eggs; it can exist in both non-ionic and ionic forms in aqueous solution. The ratio of the nonionic form to the total ionized one is influenced by concentration of hydrogen ion in the solution. Under acidic conditions,  $\text{H}_2\text{S}$  does not ionized and evaporated from water; under alkaline conditions it is easily ionized and retained in the solution.

As toxic effects of  $\text{H}_2\text{S}$ , it (at higher than 700 ppm) acts on the central nervous system causing generalized poisoning, and also shows localized inflammatory effects on the wet mucous membranes of the eye and respiratory organs.  $\text{H}_2\text{S}$  poisoning together with oxygen deficiency is most frequent in industries; the former is also occurring at sewers, sewage treatment institutions, petroleum refineries, sodium sulfide factories, and zones of volcanos and spas. The poisoning can also occur by ingesting a pesticide of the lime-sulfur mixture or bath salts including sulfur.

It is necessary to analyze  $\text{H}_2\text{S}$  in blood of a poisoned patient to verify its poisoning. The analytical methods for  $\text{H}_2\text{S}$  can be classified into two groups; methods for detecting nonionic  $\text{H}_2\text{S}$  under acidic conditions and those for detecting an ionized form of  $\text{H}_2\text{S}$  under alkaline conditions. In this chapter, a method of GC with a flame photometric detector (FPD) for analysis of the nonionic  $\text{H}_2\text{S}$  and a method of GC/MS for the ionized form with derivatization are presented.

$\text{H}_2\text{S}$  is easily oxidized to thiosulfate and sulfate in a human body [1–3]. The levels of sulfate in blood and urine of non-poisoned subjects are relatively high, making sulfate difficult to be used as an indicator of  $\text{H}_2\text{S}$  poisoning. However, thiosulfate can be used as the indicator of the poisoning [4–9], because its endogenous levels in human blood and urine are usually low<sup>a</sup>. Therefore, a method for detecting this metabolite is also presented.

### GC analysis of Hydrogen sulfide ( $\text{H}_2\text{S}$ ) in blood

See [10].

### Preparation of the standard stock solution of $\text{H}_2\text{S}$

- i. One gram of sodium sulfide nonahydrate ( $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ , Wako Pure Chemical Industries, Ltd., Osaka, Japan and many other manufacturers) is placed in a volumetric flask (100 mL) and dissolved in purified water<sup>b</sup>, which had been degassed by bubbling with nitrogen, to make 100 mL solution.
- ii. A 25-mL volume of iodine solution [0.1 N (=0.05 M) standard solution available from Wako Pure Chemical Industries, and other manufacturers] is placed in an Erlenmeyer flask, fol-



lowed by addition of 1 mL of concentrated HCl and 10.0 mL of the above  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  solution, and left at room temperature for 10 min.

- iii. The iodine in the above solution is titrated using the titer(f)-known sodium thiosulfate solution [0.1 N=0.1 M, standard solution available from many manufacturers] in the presence of the starch color reactant (1 g of starch is mixed with 10 ml water, which is put in 100 mL hot water with stirring, boiled for 1 min and cooled) using a biuret titrator.
- iv. A volume of the sodium thiosulfate solution (0.1 M) to be required for the above titration is assumed to be (a) mL; separately, at the step ii), 10 ml of distilled water is added in place of 10 ml of the  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  solution as a blank test and the following titration procedure is exactly the same as described above. A volume of the sodium thiosulfate solution (0.1 M) to be required for the titration of the blank test is assumed to be (b) mL.
- v. The volume of  $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$  solution prepared at the first step to be used for making the final standard solution of  $\text{H}_2\text{S}$  is:  $[89.3 / (b-a)f]$  mL. This volume of the solution is placed in a 100-mL volume volumetric flask, followed by dilution with the purified water degassed with nitrogen to make the final 100 mL solution; this standard stock solution contains 152  $\mu\text{g/mL}$  of  $\text{H}_2\text{S}$ .

## GC conditions

GC: an instrument with a flame photometric detector (FPD) and with a filter for sulfur; column: a glass packed column (3 m  $\times$  3 mm i.d.); packing material: diatomite treated with acid and silane (60–80 mesh) and coated with 25% 1,2,3-tris(2-cyanoethoxy)propane (TCEP)<sup>6</sup>; column temperature: 70 °C; injection temperature: 150 °C; carrier gas: nitrogen; its flow rate: 50 mL/min.

## Procedure

- i. One milliliter of whole blood is placed in a 10-mL volume glass centrifuge tube with a ground-in stopper.
- ii. Five milliliters of cold acetone and 0.5 ml of 20% HCl solution are added to the above centrifuge tube and mixed well.
- iii. The tube is centrifuged at 3,000 rpm for 5 min to remove sediment at low temperature; the supernatant fraction is decanted to another glass tube.
- iv. The supernatant fraction is diluted 5–20 fold with acetone. A 1–3  $\mu\text{L}$  aliquot of it is injected into GC.
- v. Using a double-logarithmic graph, a external calibration curve is drawn with  $\text{H}_2\text{S}$  concentration (0.05–2.0  $\mu\text{g/mL}$ ) on the horizontal axis and with peak height (cm) on the vertical axis in advance. The concentration ( $\mu\text{g/mL}$ ) of  $\text{H}_2\text{S}$  in a test sample is calculated using the calibration curve.

## Assessment of the method

When  $\text{H}_2\text{S}$  in a blood specimen is extracted by the headspace method, the  $\text{H}_2\text{S}$  gas in the headspace is decomposed according to heating temperature and time, resulting in variation in data obtained. However,  $\text{H}_2\text{S}$  is relatively stable in the acetone solution acidified with HCl. The  $\text{H}_2\text{S}$



concentration in blood was measured in an H<sub>2</sub>S poisoning case by this method [11]. The detection limit is 0.1 µg/mL; the sensitivity is satisfactory. However, the retention time of H<sub>2</sub>S is as short as 0.7 min; it overlaps peaks of pentane and hexane. The retention time of acetone is 3.8 min.

## GC/MS analysis

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See [8, 12–14].

## Reagents and their preparation

- H<sub>2</sub>S standard stock solution: its preparation is the same as described in the above GC analysis section.
- 5 mM Tetradecyldimethylbenzylammonium chloride (TDMBA, Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan)<sup>d</sup> / borax-saturated aqueous solution: 36.8 mg of TDMBA is dissolved in 20 mL of purified water, which has been degassed with nitrogen and saturated with sodium tetraborate.
- 20 mM Pentafluorobenzyl bromide (PFBBBr, GL Sciences, Tokyo, Japan and other manufacturers) solution: 104 mg of PFBBBr is dissolved in 20 mL toluene.
- 10 µM 1,3,5-Tribromobenzene (TBB, Wako Pure Chemical Industries and others) solution (internal standard, IS): 31.5 mg TBB is dissolved in 100 mL ethyl acetate; the solution is diluted 100-fold with ethyl acetate.

## GC/MS conditions

See [8].

Column: HP-5 (30 m × 0.32 mm i.d., film thickness 0.25 µm, Agilent Technologies, Palo Alto, CA, USA); column temperature: 100° C (2 min) → 10° C/min → 220° C (5 min); injection temperature: 220° C; ion source temperature: 210° C; carrier gas: He; its flow rate: 2 mL/min; injection mode: splitless; ionization mode: EI; electron energy: 70 eV; ionization current: 300 µA.

## Procedure

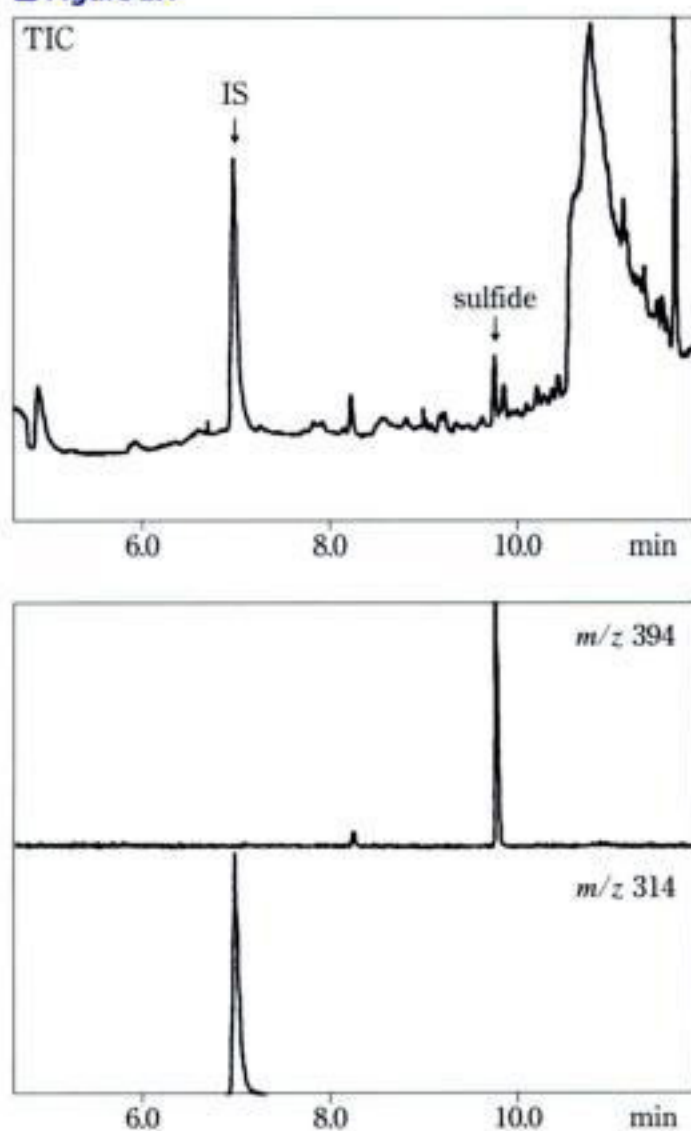
- A 0.8-mL volume of 5 mM TDMBA aqueous solution, 0.5 mL of 20 mM PFBBBr toluene solution and 2.0 mL of 10 µM TBB ethyl acetate solution are placed in a 10-mL volume glass centrifuge tube with a ground-in stopper.
- A 0.2-mL volume of blood is added to the above mixture and vortex-mixed for 1 min.
- A 0.1-g aliquot of solid potassium dihydrogenphosphate is added to the mixture <sup>e</sup> and vortex-mixed for 10 s.
- The tube is centrifuged at 2,500 rpm for 5 min; the supernatant fraction is transferred to a small vial with a screw cap to serve as test solution.

- v. A 1- $\mu$ L aliquot of the solution is injected into GC/MS.
- vi. A calibration curve is constructed with sulfide concentration ( $\mu\text{g/mL}$ ) on the horizontal axis and with the area ratio of the peak at  $m/z$  394 (the derivative of sulfide) to that at  $m/z$  314 (IS) on the vertical axis. The concentration of sulfide ( $\mu\text{g/mL}$ ) in a specimen is calculated with this curve.

## Assessment of the method

► Figure 2.1 shows a total ion chromatogram (TIC) and mass chromatograms for the sulfide derivative (retention time 9.8 min) and IS (7.0 min) [8]. In the present GC/MS analysis for the derivative of sulfide<sup>f</sup> using PFBBBr as a derivatization reagent, it is not necessary to extract sulfide from blood beforehand; the method is highly sensitive, allows the final identification of the compound and thus is useful to verify its poisoning. Since  $\text{H}_2\text{S}$  is produced in putrefied blood and also by decomposition of cysteine [15, 16], it is necessary to construct a calibration curve by adding sulfide to blood obtained from healthy subjects<sup>g</sup>. The detection limit is 0.2  $\mu\text{g/mL}$  in

■ Figure 2.1



TIC and mass chromatograms of a derivative of sulfide obtained from blood of a victim who died of hydrogen sulfide poisoning.  $m/z$  394: the derivative of sulfide;  $m/z$  314: IS.



the scan mode and 0.02 µg/mL in the SIM mode. Using the present GC/MS method, the changes in sulfide concentration in blood during storage in a refrigerator or a freezer were reported [14, 15]; sulfide poisoning cases were also reported [7–9, 17–19].

## Toxic concentrations

In the survived cases, blood should be sampled from patients as soon as possible after exposure to H<sub>2</sub>S gas, because H<sub>2</sub>S is rapidly metabolized in a human body. In the experience of the author et al., sulfide could not be detected from blood specimens sampled from six survived patients 4–15 h after exposure [7, 9].

► *Table 2.1* summarizes H<sub>2</sub>S concentrations in blood of fatal poisoning cases. Ikebuchi et al. [11] detected 0.31 µg/mL of H<sub>2</sub>S from blood obtained at autopsy from a victim, who had died of poisoning by H<sub>2</sub>S gas evaporated from polluted water at an industrial waste disposal facility. Kimura et al. [17] autopsied 3 of 4 victims, who had died of poisoning by H<sub>2</sub>S developed from dark slime accumulated in a seawater-introducing pipe at a flatfish farm, and detected 0.08–0.5 µg/mL of sulfide from their blood obtained. The author et al. also experienced cases, in which one subject had died by exposure to H<sub>2</sub>S gas developed from slime in an underground waste water tank of a hospital [7], in which one subject had died of H<sub>2</sub>S added for conversion of glutathione copper into glutathione at a glutathione-refinery factory [9], and in which one subject had died of poisoning by volcano gas flowing backward into an oil-separating tank at a geothermal power plant [8]; the blood concentrations of sulfide detected from these victims were 0.13–0.45 µg/mL. In addition, the author et al. [15] made animal experiments, in which rats were exposed to 550–650 ppm of H<sub>2</sub>S gas; the mean blood concentration of H<sub>2</sub>S in the rats (n=5) killed by H<sub>2</sub>S poisoning was 0.38 µg/mL.

The fatal blood concentrations of sulfide were also measured for humans and rats after oral ingestion of sulfide or polysulfide<sup>h</sup>; as shown in ► *Table 2.2*, the concentrations of sulfide after oral ingestion were more than 20 times higher than those after exposure to H<sub>2</sub>S gas [18, 19].

■ **Table 2.1**

**Blood concentrations of hydrogen sulfide (H<sub>2</sub>S) in fatal poisoning cases after exposure to its vapor**

No.	Place of incident	Concentration (µg/mL)	Ref.
1	Industrial waste disposal facility	0.31	[11]
2	Flatfish farm	0.08–0.50 (3 victims)	[17]
3	Underground waste water tank of a hospital	0.22	[7]
4	Glutathione-refinery factory	0.13	[9]
5	Geothermal power plant	0.45	[8]
Rat experiments (exposed to 550–650 ppm H <sub>2</sub> S)		0.38	[15]



**■ Table 2.2**  
**Blood concentrations of sulfide in fatal poisoning cases after oral ingestion of sulfide or polysulfide**

No.	Poison ingested	Concentration (μg/mL)	Ref.
1	Sulfide	30.4	[19]
2	Polysulfide	32.0	[18]
3	Polysulfide	131	[18]
Rat experiments			
	Sulfide	10.2	[19]
Rat experiments			
	Polysulfide	16.6	[18]

**GC/MS analysis of thiosulfate (a metabolite of hydrogen sulfide) in blood and urine**

See [5, 8].

**Reagents and their preparation**

- Standard solution of sodium thiosulfate: its 0.1 M solution is commercially available (Wako Pure Chemical Industries and other manufacturers), or it can be easily prepared in a laboratory.
- 200 mM Ascorbic acid solution: 352 mg of ascorbic acid is dissolved in purified water to prepare 10 mL solution.
- 5% NaCl solution: 500 mg NaCl is dissolved in purified water to prepare 10 mL solution.
- 20 mM Pentafluorobenzyl bromide (PFBBr) solution: 104 mg of PFBBr is dissolved in acetone to prepare 20 mL solution.
- 25 mM Iodine solution: 317 mg of iodine is dissolved in ethyl acetate to prepare 100 mL solution.
- 40 μM 1,3,5-Tribromobenzene (TBB) solution (IS): 31.5 mg of TBB is dissolved in 100 mL ethyl acetate; 4 ml of the solution is diluted 25-fold with ethyl acetate to prepare 100 mL solution.

**GC/MS conditions**

Column: HP-5 (30 m × 0.32 mm i.d., film thickness 0.25 μm, Agilent Technologies); column temperature: 100° C (2 min)→ 10° C/min→ 220° C (5 min); injection temperature: 220° C; ion source temperature: 210° C; carrier gas: He; its flow rate: 2 mL/min; injection mode: splitless; ionization mode: EI; electron energy: 70 eV; ionization current: 300 μA.



## Procedure

- i. A 0.05-mL volume of 200 mM ascorbic acid, 0.05 mL of 5% NaCl aqueous solution and 0.5 mL of 20 mM PFBBBr acetone solution are placed in a 10-mL volume glass centrifuge tube with a ground-in stopper.
- ii. A 0.2-mL volume of blood or urine<sup>i</sup> is added to the above mixture and vortex-mixed for 1 min.
- iii. A 2.0 mL volume of 25 mM iodine ethyl acetate solution and 0.5 mL of 40  $\mu$ M TBB ethyl acetate solution are also added to the mixture and vortex-mixed for 30 s.
- iv. The tube is centrifuged at 2,500 rpm for 5 min; and left at room temperature for 1 h. Then, the supernatant fraction is transferred to a small vial with a screw cap to serve as test solution.
- v. A 1- $\mu$ L aliquot of the solution is injected into GC/MS.
- vi. A calibration curve is drawn with thiosulfate concentration ( $\mu$ mol/mL) on the horizontal axis and with the area ratio of the peak at  $m/z$  426 (the derivative of thiosulfate) to that at  $m/z$  314 (IS) on the vertical axis. The concentration of thiosulfate ( $\mu$ mol/mL) in a test specimen is calculated with this curve.

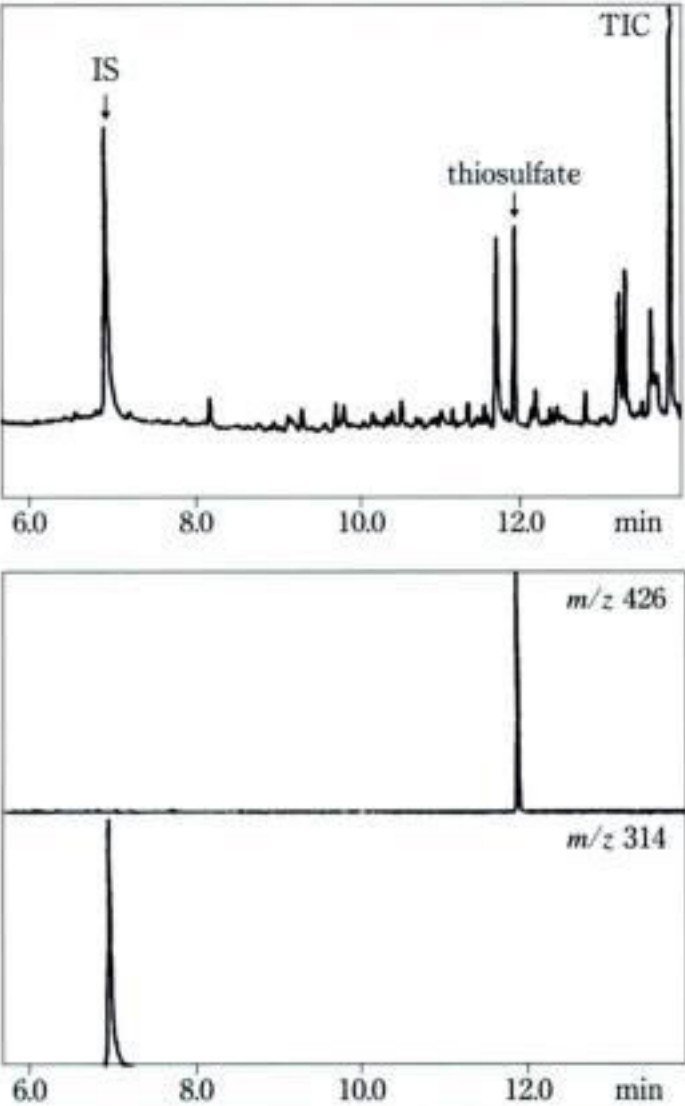
## Assessment of the method

➤ Figure 2.2 shows a TIC and mass chromatograms for the thiosulfate derivative<sup>i</sup> (retention time 11.9 min) and IS (7.0 min) [8]. This method does not require any special pretreatment, and sensitive identification and quantitation can be achieved like in the case of GC/MS assays of sulfide described before. The detection limit was 0.02  $\mu$ mol/mL in the scan mode, and 0.002  $\mu$ mol/mL in the SIM mode. Using the present GC/MS method, the changes in thiosulfate concentration in blood and urine during storage in a refrigerator were reported [14]; H<sub>2</sub>S poisoning cases were also reported [7–9].

## Toxic concentrations

As shown in ➤ Table 2.3, the author et al. [7] could not detect thiosulfate from blood of four survived patients after exposure to H<sub>2</sub>S gas at a recycled paper manufacturing factory; the blood specimens had been sampled 6–15 h after the exposure. However, 0.12–0.43  $\mu$ mol/mL of thiosulfate could be detected from urine in 3 of the 4 patients. In a case in which 2 subjects were exposed to H<sub>2</sub>S gas during working in a close position to an instrument for excluding acidic gas at an ammonia- manufacturing factory, thiosulfate could not be detected from blood of both patients sampled 4–5 h after the exposure, but 0.18 and 0.50  $\mu$ mol/mL thiosulfate could be detected from their urine [9]. In the survived cases of animal experiments in which rabbits were exposed to 100–200 ppm H<sub>2</sub>S gas, 0.061  $\mu$ mol/mL of thiosulfate could be detected from blood sampled just after the exposure, followed by a trace amount of the metabolite 2 h after the exposure; while in urine of rabbits, about 1  $\mu$ mol/mL of thiosulfate could be detected 1–2 h after the exposure, followed by 0.51  $\mu$ mol/mL 4 h after the exposure and further decrease according to time, but a small but higher peak of thiosulfate than the control peak could be detected even after 24 h [6]. These data show that the measure-

■ **Figure 2.2**



TIC and mass chromatograms of a derivative of thiosulfate obtained from blood of a victim who died of hydrogen sulfide poisoning. *m/z* 426: the derivative of thiosulfate; *m/z* 314: IS.

■ **Table 2.3**

Concentrations of thiosulfate in urine of survivors after exposure to H<sub>2</sub>S

No.	Place of incident (interval between exposure and sampling)	Concentration (μmol/mL)	Ref.
1	Recycled paper manufacturing factory (6–15 h)	0.12–0.43 (3 victims)	[7]
2	Ammonia-manufacturing factory (4–5 h)	0.18, 0.50 (2 victims)	[9]
Rabbit experiments		0.51 (5 animals)	[6]
(exposed to 100–200 ppm H <sub>2</sub> S for 60 min)			
(exposure-to-sampling interval: 4 h)			



■ Table 2.4

Concentrations of thiosulfate in blood after death by H<sub>2</sub>S poisoning

No.	Place of incident	Concentration (μmol/mL)	Ref.
1	Underground waste water tank of a hospital	0.025	[7]
2	Glutathione-refinery factory	0.058	[9]
3	Geothermal power plant	0.143	[8]
	Rabbit experiments (exposed to 500–1,000 ppm H <sub>2</sub> S)	0.080	[6]

ments of thiosulfate in urine are more effective than those in blood especially in survived cases.

➤ Table 2.4 shows the thiosulfate contents in blood of fatal victims exposed to H<sub>2</sub>S gas. The three cases are the same as those shown in ➤ Table 2.1 [7–9]. Their blood concentrations of thiosulfate were 0.025, 0.058 and 0.143 μmol/mL, respectively. As animal experiments, rabbits were exposed to 500–1,000 ppm H<sub>2</sub>S gas until death. The mean blood concentration of thiosulfate in the poisoned rabbits was 0.080 μmol/mL [6]. However, thiosulfate could not be detected from rabbit urine, probably because of their sudden death due to exposure to H<sub>2</sub>S. It can be thus concluded that the measurements of thiosulfate in blood are more effective than those in urine for such sudden death cases.

## Notes

- Kawanishi et al. [20] analyzed thiosulfate in urine and plasma of 5 healthy subjects; thiosulfate concentrations in urine and plasma were 31.2 μmol/24 h (0.0288 μmol/mL) and 0.00268 μmol/mL, respectively. The author et al. [5] also detected 0.007 μmol/mL (mean value) of thiosulfate from urine of 12 healthy subjects; while the level in blood was below the detection limit (0.003 μmol/mL).
- Since H<sub>2</sub>S can be decomposed by oxygen dissolved in water, the purified water degassed with nitrogen gas is used. The purified water after boiling, followed by cooling to room temperature, can be also used.
- A similar packing material can be purchased from GL Sciences, Tokyo, Japan.
- The reagent is a quaternary ammonium compound to be used as a phase-transfer-catalyst. Another group reported a polymer-bound tributylmethylphosphonium chloride for such a type of catalysis [13].
- Under alkaline conditions, sulfur-containing compounds, such as cysteine and glutathione, in blood decompose to produce sulfide. To suppress these reactions, the pH of the mixture is made acidic.
- The derivatization reaction of sulfide is:  

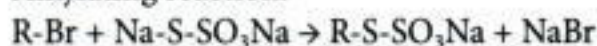
$$2\text{R-Br} + \text{Na}_2\text{S} \rightarrow \text{R-S-R} + 2\text{NaBr}$$
R = pentafluorobenzyl
- McAnalley et al. [21] analyzed blood sulfide for 100 subjects without any exposure to H<sub>2</sub>S; the results were not greater than 0.05 μg/mL. The author et al. [15] found that the blood sulfide levels were markedly influenced by postmortem intervals and by temperatures of specimens



for storage. When blood specimens are sampled within 24 h after death and stored at not higher than 20° C, the postmortem production of H<sub>2</sub>S can be suppressed; the sulfide concentration in blank blood was not greater than 0.01 µg/mL. When the specimens are stored in a refrigerator or in a freezer, the postmortem production of H<sub>2</sub>S due to putrefaction could be suppressed even for the blood specimens sampled from a cadaver with a postmortem interval of more than 24 h.

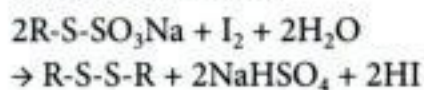
- h) When polysulfide is ingested orally, the unchanged compound can be detected from blood [18].
- i) Blood is the suitable specimen for fatal poisoning cases; while urine is suitable for survived cases after poisoning.
- j) The derivatization reaction for thiosulfate is shown as follows. It consists of two-step reactions; the first one is alkylating reaction and the second one oxidation reaction.

Alkylating reaction:



R = pentafluorobenzyl

Oxidation reaction:



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