High Performance Liquid Chromatographic Determination of Bound Sulfide and Sulfite and Thiosulfate at Their Low Levels in Human Serum by Pre-column Fluorescence Derivatization with Monobromobimane

Tadayasu Togawa,[a] Masahiro Ogawa,[a] Masashi Nawata,[a] Yuki Ogasawara,[b] Koji Kawanabe,[a] and Shinzo Tanabe*,[b]

Departments of Analytical Chemistry[a] and Hygienic Chemistry,[b] Meiji College of Pharmacy, 1–22–1, Yato-cho, Tanashi-shi, Tokyo 188, Japan.

Received March 17, 1992

A sensitive and specific high performance liquid chromatographic method for the determination of sulfide, sulfite, and thiosulfate was established. Inorganic sulfur anions were converted into fluorescent derivatives with monobromobimane. The derivatives were separated on a coupled column chromatography with a reversed-phase octadecyl silica column connected with a weakly basic anion exchanger column by isocratic elution with acetic acid solution (pH 3)–acetonitrile (13:3, v/v) containing 25 mM $NaClO_4$. The method was applied to the determination of bound sulfide and sulfite and thiosulfate in normal human serum. Thiosulfate could be determined directly by use of an ultrafiltered sample. For the determination of bound sulfide and sulfite, the pretreatment step with continuous flow gas dialysis was effective for the sample after releasing sulfide and sulfite by reduction with dithiothreitol. The limits of quantification by the present method were 0.05 μM for thiosulfate, 0.5 μM for bound sulfide, and 0.2 μM for bound sulfite.

Keywords bound sulfide; bound sulfite; thiosulfate; coupled column chromatography; pre-column fluorescence derivatization; monobromobimane; dithiothreitol reduction; flow gas dialysis; human serum

Introduction

Inorganic sulfur anions, sulfide, sulfite, and thiosulfate are produced from sulfur-containing amino acids as intermediary metabolites in mammalian systems by a complex pathway which has not been fully characterized.[1)] Koj *et al.* proposed a thiosulfate cycle in which the sulfur derived from sulfur-containing amino acids is incorporated into sulfate after it is converted into sulfide, sulfite, and thiosulfate in rat liver.[2)]

Thus, several metabolic pathways generating sulfide, sulfite, and thiosulfate *in vitro* are known, but the biological roles of these sulfur anions *in vivo* are not clearly understood; their best functions *in vitro* have been considered to be the sulfur substrate in cyanide detoxication[3)] and the physiological source of the sulfur in the iron–sulfur cluster enzymes.[4,5)] This is because no reliable analytical method for endogenous levels of sulfide, sulfite, and thiosulfate is available.

Recently, a few methods for the determination of each, sulfide, sulfite and thiosulfate, in endogenous levels have been developed. Sulfide levels in the brain tissues of human, rat, and cattle have been measured by employing gas dialysis pretreatment and ion chromatography with electrochemical detection (ECD),[6)] and by reversed-phase ion-pair high performance liquid chromatography (HPLC) with pre-column derivatization based on the formation of methylene blue.[7)] Thiosulfate in normal human urine and plasma has been measured by reversed-phase HPLC-ECD.[8,9)] On the other hand, it is known that sulfite is a very reactive ion *in vivo* and is mainly present in a state of *S*-sulfonates by reaction with endogenous disulfide compounds.[10,11)] Togawa *et al.*[12)] have developed the determination method of total *S*-sulfo groups in rabbit plasma by reversed-phase HPLC with pre-column derivatization, after the conversion of an *S*-sulfo group into sulfite by reduction with dithiothreitol (DTT). Although each method has its advantages, application of these techniques to determine individual analytes in the sample is inconvenient, troublesome, and time-consuming, especially when the object compounds are minor components. Recently, Fahey *et al.*[13–15)] reported methods for the determination of biological thiols at the picomole level by means of HPLC with pre-column fluorescence derivatization using monobromobimane (MBB) synthesized by Kosower *et al.*[16)] as a fluorescent labeling reagent for the thiol group. In the reactions, sulfide, sulfite, and thiosulfate also react with MBB to form fluorescent derivatives. Shea and Howell[17)] have modified Fahey's method and applied the method to the determination of exogenous thiosulfate in human plasma and urine at a detection limit as low as 3.2 μM, with gradient elution using a paired ion solvent system. The main disadvantage of their method, however, was that the thiosulfate peak could not be separated completely from one of several peaks arising either from impurities or from fluorescent MBB hydrolysis products, and consequently, the detection limit of the assay was not as good as that obtained for the thiols.

In this paper, we report a method for the determination of sulfide, sulfite, and thiosulfate at concentrations below 1 μM by means of a coupled column HPLC using a reversed-phase octadecyl silica (ODS) and a weakly basic anion exchanger with an isocratic elution, with pre-column fluorescence derivatization using MBB. The application of the method for the determination of endogenous levels of thiosulfate and bound sulfide and sulfite in normal human serum is described.

Experimental

Reagents MBB was synthesized and purified by the method of Kosower *et al.*[16)] Sodium sulfide nonahydrate was obtained from Wako Pure Chemical Co., Osaka (Japan). Sodium salts of sulfite and thiosulfate pentahydrate were purchased from Kanto Chemical Co., Tokyo (Japan). All other chemicals were of analytical grade. Water was distilled and degassed after being passed through an ion exchange column. Stock solutions of sulfide and sulfite (10 mM each) were prepared in 0.1 M of a sodium hydroxide solution containing 5 mM EDTA·2Na and 10 mM glycerol; the concentrations of both ions were calibrated by titration methods.[18)] A 10 mM thiosulfate stock solution was prepared with distilled water. The standard solutions of sulfide, sulfite, and thiosulfate were prepared daily by appropriate dilutions of each stock solution with 0.05 M of a borate buffer (pH 8.0) containing 5 mM EDTA·2Na. DTT solution (20 mM) was prepared in 0.05 M borate buffer (pH 9) containing 5 mM

EDTA·2Na.

Apparatus and HPLC Conditions The HPLC consists of a Hitachi L-6000 pump (Tokyo, Japan), a Rheodyne syringe loading injector (20 μl, U.S.A.), a Shimadzu RF-535 fluorescence detector (12 μl flow-cell, Kyoto, Japan) set at 396 nm (excitation wavelength) and 476 nm (emission wavelength), and an Shodex cc-11 integrator (Showa Denko K. K., Japan). A Nucleosil 100-5 C_{18} (50 × 4 mm i.d., Macherey–Nagel, Germany) was installed between the injection valve and a Nucleosil 5N$(CH_3)_2$ (a dimethylamino-bonded silica resin, 250 × 4 mm i.d.). Acetic acid solution (pH 3)–acetonitrile (13 : 3, v/v) containing 25 mM $NaClO_4$ as the mobile phase was delivered at a flow rate of 1.0 ml/min. Uncorrected fluorescence spectra were measured with a Hitachi F-4010 spectrofluorometer in 10 × 10 mm quartz cells.

Flow Gas Dialysis System and Construction of Gas Dialysis Cell A schematic diagram of the flow gas dialysis system is shown in Fig. 1. The system consists of two pumps, a DM2U pump (pump-1) (Sanuki Kogyo Co., Tokyo, Japan) having two pump heads to provide a 20% phosphoric acid solution, and a Hitachi L-6000 pump (pump-2) which delivers an absorbing solution of 0.1 M NaOH containing 10 mM glycerol and 5 mM EDTA·2Na, a six-port injection valve equipped with a 500 μl loop (Shimamura Instrument Co., Tokyo, Japan), and a gas dialysis cell (homemade). The streams of both phosphoric acid solutions from pump-1 and the absorbing solution from pump-2 were maintained accurately at 0.5 ml/min. A homemade gas dialysis cell consists of a membrane separator with a polytetrafluoroethylene (PTFE) membrane (Toyo Roshi, Ltd., Japan, pore size 0.5 μm) between two parallel Teflon sheets with grooves (groove dimensions: 160 mm length, 5 mm width, and 0.2 mm depth) supported by acryl plates. Both bores (inlet and outlet) of an absorbing solution were 0.5 mm i.d., and those of inlet and outlet of the phosphoric acid solution were 0.5 and 1.0 mm i.d., respectively. The sample (500 μl) was injected into a 20% phosphoric acid stream. The sample stream was mixed with the same phosphoric acid solution, converting sulfide and sulfite to hydrogen sulfide and sulfur dioxide, which diffused through the PTFE membrane to a stream of absorbing solution. After 75 s of the sample injection, the absorbing solution was collected accurately in a vial during the period of 20 s, corresponding to the volume of 0.17 ml.

Fluorescence Derivatization of Sulfide, Sulfite, and Thiosulfate with MBB To a 100 μl sample solution, 100 μl of 5 mM MBB in acetonitrile was added, and the mixture was left for 60 min at room temperature in the dark. The reaction was stopped by the addition of 100 μl of 0.05 M KCl–HCl buffer (pH 2), and 20 μl of the reaction mixture was submitted to HPLC.

Determination of Bound Sulfide and Sulfite in Human Serum A 500 μl portion of serum was placed in a sample tube, and 500 μl of 20 mM DTT was added. The mixture was incubated at 37 °C for 10 min. Then, 500 μl of the reaction mixture was treated by the flow gas dialysis system. A 50 μl portion of the absorbing solution collected was adjusted to pH 8.0 with 50 μl of a solution containing 0.1 M of a borate buffer (pH 8.0) and 1 M HCl (9 : 1), and the entire solution was applied to a fluorescence derivatization reaction with MBB. The reagent blank is similarly constituted except that distilled water was added, instead of the sample. The amounts of bound sulfide in serum was determined by subtracting the sulfide concentration in the reagent blank.

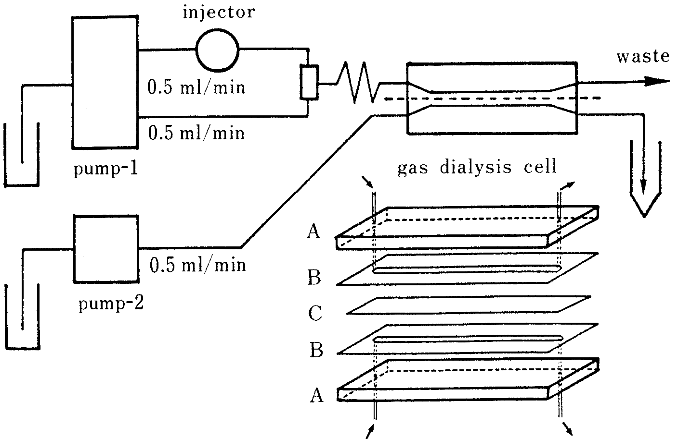

Fig. 1. Schematic Diagram of Flow Gas Dialysis System

Pump-1, 20% H_3PO_4; pump-2, 0.1 M NaOH containing 10 mM glycerol and 5 mM EDTA·2Na. Injection volume, 500 μl. Sampling time, 75—95 s. Gas dialysis cell: A, acryl-plate; B, teflon sheet; C, PTFE membrane; cell volume, 160 μl.

Determination of Thiosulfate in Human Serum A 250 μl portion of serum was mixed with 250 μl of 0.05 M of a borate buffer (pH 8.0), and the mixture was ultrafiltered with an Amicon MPS-1 (Amicon, U.S.A.) by centrifugation at 1000 *g* for 15 min. A 100 μl portion of the filtrate was applied to the fluorescence derivatization reaction.

Results

HPLC Separation of MBB Derivatives of Sulfide, Sulfite, and Thiosulfate The optimum HPLC conditions for the separation of the MBB derivatives of sulfide, sulfite, and thiosulfate with fluorometric detection were investigated with a coupled column system with isocratic elution. The fluorescence excitation and emission spectra of the reaction mixture for all these derivatives were almost identical in maxima (at 396 and 476 nm, respectively) to the manual spectrofluorometric data of the reaction solutions prepared by the method of Shea and Howell.[17] In our preliminary experiments, the mobile phase that consisted of acetic acid solution (pH 3)–acetonitrile containing $NaClO_4$ was chosen to study the retention behavior of the MBB derivatives of these sulfur anions. The retention time variation obtained with different proportions of acetic acid solution (pH 3) and acetonitrile and at various concentrations of $NaClO_4$ in acetic acid solution (pH 3)–acetonitrile (13 : 3) are shown in Fig. 2. The proportion of acetonitrile and the concentration of $NaClO_4$ had a marked influence on the separation of the sulfide-MBB derivative (Fig. 2A) and on both the sulfite and thiosulfate derivatives (Fig. 2B), respectively.

Figure 3 shows a typical chromatogram of a standard mixture of sulfide, sulfite, and thiosulfate obtained by coupled column HPLC with the optimized mobile phase. The MBB derivatives of sulfide, sulfite, and thiosulfate were well separated from the peaks due to either impurities or products of MBB hydrolysis at retention times of 13, 16 and 19 min, rspectively.

Optimization of Pre-labelling In order to optimize the pre-column derivatization conditions, concentration of MBB and reaction time were both examined in a 0.05 M borate buffer (pH 8.0) at room temperature, using the established HPLC system. Variation of the concentrations of the MBB reagent showed that the maximum peak area for each fluorescent MBB derivative was obtained at a

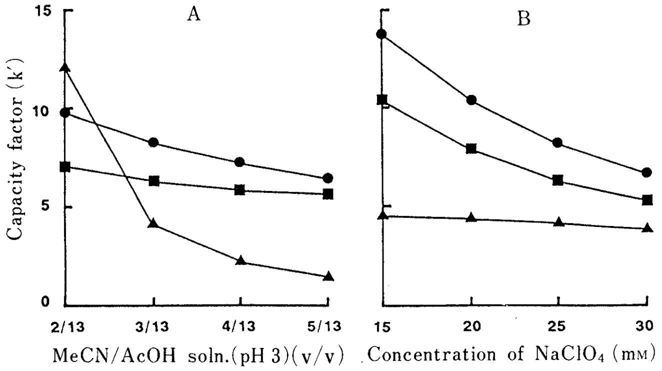

Fig. 2. Effect of the Volume Ratio of Acetonitrile and Acetic Acid (A) and the Concentration of $NaClO_4$ (B) on the Separation of Sulfide (▲), Sulfite (■), and Thiosulfate (●)

HPLC conditions are given in the Experimental section.

Table I. Linear Range, Precision, and Detection Limits

Anion	Linear range (μM)	Slope	Intercept	N	r	C.V. (%)[a]		Detection limit (μM, $S/N=3$)
						0.5 μM	5.0 μM	
Sulfide	0.1—10.0	0.278	0.077	6	0.999	2.21	1.30	0.04
Sulfite	0.05—10.0	0.886	0.016	6	0.999	1.25	0.85	0.02
Thiosulfate	0.05—10.0	0.761	0.047	6	0.999	1.02	0.68	0.02

a) $n=5$.

Table II. Analytical Results and Recoveries of Thiosulfate in Human Serum

Sample No.	Thiosulfate (μM)	Recovery (%)	
		1.0 μM[a]	5.0 μM[a]
1	0.690	97.3	97.9
2	0.579	104.6	97.4
3	0.660	93.2	93.5
4	0.563	101.8	96.2
5	0.537	107.1	95.1
Mean ± S.D.	0.606 ± 0.066	100.8 ± 5.59	96.0 ± 1.78

a) Amount added.

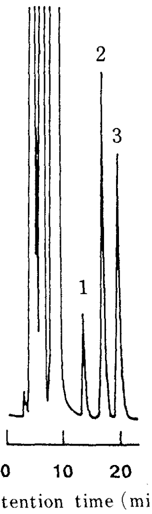

Fig. 3. Chromatogram of Standards

1, sulfide (0.5 μM); 2, sulfite (0.5 μM); 3, thiosulfate (0.5 μM). HPLC conditions are given in the Experimental section.

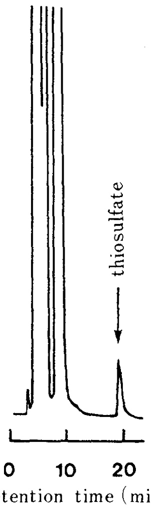

Fig. 4. Chromatogram of Human Serum Treated with Ultrafiltration

HPLC conditions are given in the Experimental section.

concentration of 5 mM. By using the above reagent concentration, the peak areas of both MBB derivatives of sulfide and thiosulfate reached a maximum rapidly, but the sulfite derivative required about 40 min. Therefore, the reaction time of 60 min was chosen. The MBB derivatives of sulfide, sulfite, and thiosulfate were stable in the weakly acidic solution treated with a 0.05 M of a KCl–HCl buffer (pH 2) for at least 24 h in the dark at room temperature. Table I shows the data of linear range, precision, coefficients of correlation, and detection limits for all three fluorescent MBB derivatives. Each calibration graph obtained for 20 μl injections passed through the origin.

Determination of Thiosulfate in Human Serum The present method was applicable to the determination of thiosulfate in human serum. Human serum was deproteinized by ultrafiltration using an Amicon MPS-1 micropartion system. Figure 4 shows the chromatogram for an MBB derivatized sample of ultrafiltered human serum. Only the thiosulfate peak was completely separated from the other peaks. Table II shows the analytical results of endogenous thiosulfate in five normal human sera, and also the analytical recoveries of thiosulfate from the spiked samples at two different levels. The mean recoveries of thiosulfate in the concentrations of 1 and 5 μM was 100.8 and 96.0%, with coefficients of variation (C.V.) of 5.5 and 1.7%, respectively.

Determination of Bound Sulfide and Sulfite in Human Serum Free sulfide and sulfite were not detected from the ultrafiltered serum sample as shown in Fig. 4, and also could not be recovered from the spiked samples because of their strong binding to a biological matrix. In this study, bound sulfide and sulfite were released from serum by DTT reduction. Figure 5A shows the chromatogram of human serum spiked with known amounts of sulfide and sulfite. Many thiol peaks due to the reduction of various disulfide compounds co-eluted with the sulfide and sulfite peaks. For removal of these interferences, a continuous flow gas dialysis system, as shown in Fig. 1, was employed as a pre-treatment of the samples treated with DTT. Figures 5B and 5C show the chromatograms of blank and serm sample obtained by a combination of the DTT reduction and the gas dialysis pre-treatment. Bound sulfide and sulfite were separated completely without any interference from biological thiols.

The calibration curves for the sulfide and sulfite standards with gas dialysis pre-treatment were linear in the

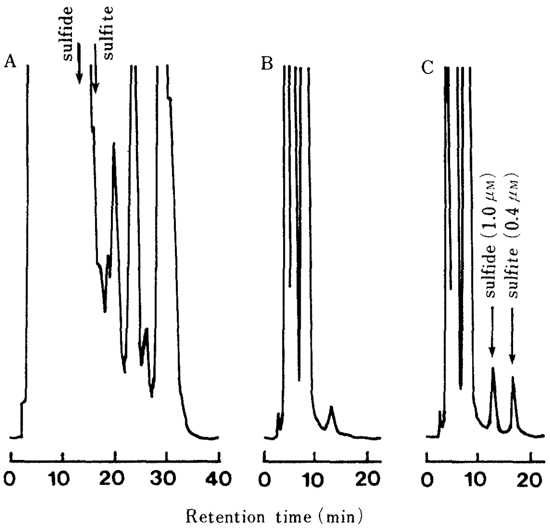

Fig. 5. Chromatograms of Human Serum with (B and C) and without (A) Gas Dialysis Treatment

A, human serum spiked with sulfide and sulfite at 1 μM each; B, DTT; C, human serum. HPLC conditions are given in the Experimental section.

Table III. Recovery Tests of Sulfide and Sulfite from Human Serum

Amount added (μM)	Recovery (%)[a] Mean ± S.D. Sulfide	Recovery (%)[a] Mean ± S.D. Sulfite
1.0	103.2 ± 22.7	101.1 ± 11.3
5.0	99.5 ± 2.8	99.5 ± 9.2

[a] $n = 5$.

Table IV. Determination of Bound Sulfide and Sulfite in Human Serum

Sample No.	Sulfide (μM)	Sulfite (μM)
1	2.31	ND[a]
2	1.02	0.41
3	1.26	0.80
4	1.21	0.21
5	0.71	0.47
Mean ± S.D.	1.30 ± 0.60	0.47 ± 0.25

[a] Not detected.

range of 0.5—20 and 0.2—20 μM, respectively. The relative standard deviations for 10 replicate procedures for the standard samples containing 2 μM each were found to be 1.4 for sulfide and 2.9% for sulfite, respectively. The recoveries of sulfide and sulfite added to human serum at the concentrations of 1 and 5 μM were showed in Table III. Table IV shows the concentrations of bound sulfide and sulfite in normal human serum.

Discussion

This study has provided a sensitive and selective method that permits the determination of bound sulfide and sulfite and thiosulfate at their low levels in normal human serum.

First we attempted to separate the MBB derivatives of sulfide, sulfite, and thiosulfate by reversed-phase ODS HPLC in the absence and the presence of tetrabutylammonium hydroxide (TBA) in a mobile phase of acetonitrile–water (3:7, v/v). In the solvent systems, both MBB derivatives of sulfite and thiosulfate having a negative charge in the molecules were retained by the column when TBA was present in the mobile phase, but complete separation from the peaks attributed to fluorescent MBB hydrolysis products could not be achieved in the same manner as in the result of Shea's study.[17] On the other hand, the sulfide derivative was separated completely from the extraneous peaks. Full separation of the sulfite and thiosulfate derivatives from the fluorescent interfering peaks was obtained by a weakly basic anion exchange HPLC on a dimethylammonium-type column with a mobile phase of acetic acid solution (pH 3) containing 5% (v/v) acetonitrile and 10 mM $NaClO_4$ in the system, the sulfide-MBB derivative eluted at void volume together with the extraneous peaks. From these results, the reversed-phase ODS column was directly connected to the dimethylamino silica column for the separation of these derivatives. In the established HPLC conditions, the fluorescent MBB derivatives of sulfide, sulfite, and thiosulfate were separated simultaneously without any interference (Fig. 3). It must be emphasized here that commercial MBB should be used for experiments after purification by column chromatography[16] since the MBB is contaminated with small amounts of dibromobimane (DBB); otherwise, one of the fluorescent degradation products derived from DBB gives a peak with the same retention time as the sulfide-MBB derivative.

We were able to separate and measure endogenous thiosulfate in serum easily by the use of an ultrafiltered sample. The thiosulfate peak in the serum sample was identified from its retention time as determined from the standard solution. Influence from biological thiols was also checked by the experiment in which thiols are blocked by the reaction with *N*-ethylmaleimide (NEM) prior to derivatization with MBB; however, no significant decrease in the thiosulfate peak was observed and no other peaks attributable to biological thiols appeared in the chromatogram of the serum sample (Fig. 4). It is generally known that thiols in serum are rapidly autoxidized to form disulfides and thioesters, unless the sample is deproteinized immediately under an acidic condition.[19] The excellent recovery shown in Table II suggests that thiosulfate in serum is present in the free form. The thiosulfate values obtained in the present method ranged from 0.54—0.69 μM for normal human serum. The values are somewhat lower than previously reported values (2.41—3.04 μM), which were obtained by HPLC with a dual electrochemical detector (DECD) achieved by recording the differences in the response of the two electrodes.[9] The reason for such a difference has not been clarified here, but it seems likely that the selectivity of the HPLC-DECD method is affected by the difference in potential between the two electrodes using different mode detection. At any rate, the proposed method seems to be superior to HPLC-DECD[9] in both sensitivity and selectivity.

Sulfide, as well as sulfite, may also be present in a bound state to serum because there was no successful recovery of sulfide from the spiked serum sample. The existence state of sulfide *in vivo* has not yet been clarified, although protein-associated sulfur was found in certain rat tissues.[20] Bound sulfide and sulfite could be liberated easily from the matrix by the reduction with DTT as reported by Togawa *et al.*,[12] but disulfide compounds also underwent the reduction simultaneously to form the corresponding thiols, followed by derivatization with MBB, as shown in Fig. 5A. An aeration[21] or a diffusion method[22] is frequently applied to clean up sulfide and sulfite from the complex matrix. When the generation of H_2S and SO_2 gases was carried out in Conway microdiffusion cells according to the procedure of Ishizaka,[22] no good recovery was obtained for sulfite concentrations of less than 5 μM (below 80%), although the recovery of sulfide was good at the lower concentration of 2 μM. We modified the Goodwin's continuous flow gas dialysis pretreatment system[6] in the following way and were able to assay bound sulfide and sulfite simultaneously in human serum with the DTT reduction. We utilized a homemade flow dialysis cell. The sample treated with DTT was reacted in a 20% phosphoric acid carrier stream to release the gases (Fig. 1); for concentrations greater than 20% phosphoric acid there was no change in response when a given sulfide or sulfite standard was injected. The absorbing solution used was 0.1 M NaOH in which 10 mM glycerol and 5 mM EDTA·2Na were added to prevent the autoxidation of sulfide and sulfite (Fig. 1).

The effect of gas dialysis pre-treatment is quite evident from Fig. 5C as no significant interfering peaks were observed. Thiosulfate decomposes gradually in acid solution with an evolution of acidic gaseous sulfur dioxide. Under our gas dialysis condition, however, the production of acidic sulfur dioxide from thiosulfate was below 0.3% and thiosulfate showed no interference at concentrations up to at least 10 μM in the determination of sulfite. Although the recovery across the dialysis membrane in this system was 55% for sulfide and 45% for sulfite, respectively, the continuous flow gas dialysis has proved to be a rapid pretreatment method (approximately 100 samples per hour) for determining low levels of bound sulfide and sulfite in biological samples. The membrane used can also withstand at least 100 injections. In this work, DTT was used for the liberation of bound sulfide and sulfite from human serum; however, the commerical DTT used was always contaminated with small amounts of sulfide (0.002% or less, Fig. 5B). The amount of bound sulfide in serum should be determined by subtracting the concentration of sulfide in the DTT reagent; therefore, the lower precision for the recovery test of sulfide was given when a small amount (1 μM level) was added to the serum (Table III). In five healthy subjects, the mean concentrations of bound sulfide and sulfite in serum were 1.30 and 0.47 μM, respectively, as given in Table IV. It is noteworthy that sulfide and sulfite which are liberated with the DTT reduction exist as a normal constituent of human serum, even though these levels are very low. This finding is presumably the first report on the simultaneous determination of bound sulfide and sulfite in human serum. The binding feature of sulfide and sulfite into a biological matrix is under investigation. The present method will be suitable for quantitative characterization of the physiological pool of sulfur available for biological systems.

References

1) A. J. L. Cooper, *Ann. Rev. Biochem.*, **52**, 187 (1983).
2) A. Koj, J. Frendo, and Z. Janik, *Biochem. J.*, **103**, 791 (1967).
3) J. Westley, "Enzymatic Basis of Detoxication," Vol. II, ed. by W. B. Jakoby, Academic Press, Inc., New York, 1980, p. 245.
4) F. Bonomi, S. Pagani, P. Cerbetti, and C. Cannella, *Eur. J. Biochem.*, **72**, 17 (1977).
5) S. Pagani, M. Eldridge, and R. R. Eady, *Biochem. J.*, **244**, 485 (1987).
6) L. R. Goodwin, D. Francom, F. P. Dieken, J. D. Taylor, M. W. Warenycia, R. J. Reiffenstein, and G. Dowling, *J. Anal. Toxicol.*, **13**, 105 (1989).
7) J. C. Savage and D. H. Gould, *J. Chromatogr.*, **526**, 540 (1990).
8) B. Kåegedal, M. Källberg, J. Mårtensson, and B. Sörbo, *J. Chromatogr.*, **274**, 95 (1983).
9) T. Kawanishi, T. Togawa, A. Ishigami, S. Tanabe, and T. Imanari, *Bunseki Kagaku*, **33**, E295 (1984).
10) C. McArdle, *Biochem. J.*, **102**, 211 (1967).
11) G. S. Rao and G. Gorin, *J. Org. Chem.*, **24**, 749 (1959).
12) T. Togawa, Y. Akiyama, K. Kaneko, A. Nakamura, and T. Imanari, *Anal. Sci.*, **2**, 477 (1986).
13) R. C. Fahey, G. L. Newton, R. Dorian, and E. M. Kosower, *Anal. Biochem.*, **107**, 1 (1980).
14) R. C. Fahey, G. L. Newton, R. Dorian, and E. M. Kosower, *Anal. Biochem.*, **111**, 357 (1981).
15) G. L. Newton, R. Dorian, and R. C. Fahey, *Anal. Biochem.*, **114**, 383 (1981).
16) E. M. Kosower and B. Pazhenchevsky, *J. Am. Chem. Soc.*, **102**, 4983 (1980).
17) M. Shea and S. Howell, *Anal. Biochem.*, **140**, 589 (1984).
18) W. P. Kilroy, *Talanta*, **30**, 419 (1983).
19) R. F. Bergstrom, D. R. Kay, and J. G. Wagner, *Life Sci.*, **27**, 189 (1980).
20) U. Hannestad, S. Margheri, and B. Sörbo, *Anal. Biochem.*, **178**, 394 (1989).
21) K. Fugita, M. Ikuzawa, T. Izumi, T. Hamano, Y. Mitsuhashi, Y. Matsuki, T. Adachi, H. Nonogi, T. Fuke, H. Suzuki, M. Yoyoda, Y. Ito, and M. Iwaida, *Z. Lebensm. Unters. Forsch.*, **168**, 206 (1979).
22) O. Ishizaka, "Experimental Methods of Microdiffusion Analysis," Nankodo Co. Ltd., Tokyo, 1969.