Determination of Total Serum Sulfite by HPLC with Fluorescence Detection

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An estimated 500 000 individuals in the US, mostly steroid-dependent asthmatics, suffer severe adverse reactions to sulfites in foods, beverages, and pharmaceutical products. In an attempt to understand the pathogenesis of sulfite hypersensitivity, we have developed an assay for the determination of total serum sulfite by utilizing: (a) reductive release of serum protein-bound sulfite; (b) derivatization of free sulfite with monobromobimane; (c) separation of sulfite–bimane from thiol–bimanes by reversed-phase HPLC; and (d) quantitation of sulfite–bimane by fluorescence detection. The detection limit of this assay was 0.44 μmol/L serum sulfite. The intra- and interassay CVs for total serum sulfite at 5.4 μmol/L were 8.1% and 22.0%, respectively. The standard addition method was used to determine total serum sulfite in normal subjects. More than 70 samples were prepared in 2–3 h, followed by automated overnight analysis. The mean concentrations (±SD) of total serum sulfite in female (n = 41) and male (n = 35) donors were 4.63 ± 2.33 and 5.16 ± 2.68 μmol/L, respectively (not statistically significant: P = 0.368). The combined mean concentration of total sulfite in both sexes was 4.87 ± 2.49 μmol/L. There was no correlation between total serum sulfite and total serum cysteine, cysteinylglycine, homocysteine, subject age, serum cobalamin, or serum folic acid. The reference range (mean ± 2 SD) for total serum sulfite in normal subjects is 0–9.85 μmol/L.

Indexing Terms: sulfite–bimane/monobromobimane/chromatography, reversed-phase/sulfite hypersensitivity

Sulfur dioxide and other sulfur compounds naturally cycle through the biosphere, but as a result of increased industrial activities over the past several decades, unnatural entry of sulfur into the biosphere will soon equal or even surpass natural sources (1). The tragic consequences of the London “killer fogs” earlier this century were due in part to the presence of oxidized forms of sulfur produced during the burning of coal and other fossil fuels (2). Sulfur dioxide and sulfite salts, known generically as “sulfiting agents,” have been widely used by the food, beverage, and pharmaceutical industries as antioxidants, antibrowning agents, and antimicrobials (3, 4). Until recently, sulfiting agents were given generally-recognized-as-safe status, which allowed their use in foods and beverages without disclosure. It was reasoned that ingested sulfite, a normal sulfur metabolite of cysteine catabolism (the average adult produces ≥1 g of sulfite per day), would be rapidly oxidized to sulfate by the mitochondrial enzyme sulfite oxidase and excreted in the urine without untoward effects. Indeed, this is what happens in normal healthy subjects. However, some individuals are hypersensitive and suffer life-threatening reactions after ingesting sulfites. Reports of several fatalities associated with exposure to food or pharmaceutical sulfites during the past 20 years have forced the Food and Drug Administration to severely restrict the use of these agents (5, 6).

Although the true incidence is unknown, an estimated 3–5% of the 10 million asthmatics in the US are hypersensitive to sulfiting agents (7, 8). Because of its unknown pathogenesis, specific skin or serological tests of sulfite hypersensitivity are not available (9). Currently, the only effective diagnosis of sulfite hypersensitivity is by single- and double-blinded provocative sulfite challenge tests (9, 10). A sulfite challenge test has significant risk and may provoke bronchospasm or other anaphylactic reactions. Although there are colorimetric methods for determining sulfites in foods and beverages (11, 12), these methods lack the sensitivity necessary for determining sulfite in human serum. Recently Togawa et al. (13) reported a sensitive method for determining thiosulfate, sulfate, and sulfide in human serum by using a combination of flow gas dialysis and HPLC with fluorescence detection. In the present work, a rapid, specific, and sensitive assay for determining total serum sulfite in >70 samples per day has been developed by using HPLC with fluorescence detection (14). A reference range in healthy individuals is also reported.

Materials and Methods

Materials. The following reagents were purchased from commercial sources: sodium borohydride (Aldrich Chemical Co., Milwaukee, WI); sodium hydroxide, sodium carbonate, potassium dichromate (J. T. Baker, Phillipsburg, NJ); sodium sulfite, hydrochloric acid, sodium thiosulfate, acetic acid, perchloric acid, methanol (Fisher Scientific, Fair Lawn, NJ); EDTA, disodium salt (Eastman Kodak, Rochester, NY); iodine (E. Merck, Darmstadt, Germany); monobromobimane (mBrB) (Calbiochem–Behring Diagnostics, La Jolla, CA, and Molecular Probes, Eugene, OR); potassium iodide, cysteine, glutathione, cysteinylglycine, Trizma
base (Tris), and L-homocysteine thiolactone (Sigma Chemical Co., St. Louis, MO). L-Homocysteine was prepared from L-homocysteine thiolactone by the method of Hatch et al. (15). Briefly, 10 mL of 15 mmol/L L-homocysteine thiolactone in 30 mmol/L KOH was hydrolyzed under argon at 45 °C for 12 min. After neutralization with 0.15 mL of 1.0 mol/L HCl, homocysteine was stored anaerobically in a Thunberg tube at −20°C for up to 1 week. All reagents were analytical-reagent or ACS-grade unless indicated and all solvents used were HPLC-grade.

Assessment of sodium sulfite purity. Sodium sulfite (250.0 mg) was dissolved in 50 mL of standardized 50 mmol/L iodine in a glass-stoppered flask. After 10 min, 1.0 mL of 1.0 mol/L HCl was added to the solution and excess iodine was titrated with standardized 50 mmol/L sodium thiosulfate with 10 g/L starch solution as an indicator. The iodine and sodium thiosulfate solutions were standardized by iodometry. Sulfite calibrators were prepared fresh for each assay by dissolving 6.3 mg of sodium sulfite in 10.0 mL of 0.10 mmol/L Na₂EDTA, pH 5.5.

Preparation of sulfite–bimane and thiol–bimane calibrators. Stock solutions of cysteine, homocysteine, cysteinylglycine, or sulfite (5.00 mmol/L in water) were diluted 1:10 with 1.0 mmol/L Na₂EDTA (pH 6.0). A stock solution of 20.0 mmol/L mBrB in acetonitrile was diluted 1:10 with the same buffer. Thiol–bimanes and sulfite–bimane were prepared by adding 0.20 mL of 0.5 mmol/L sulfite (or thiol) to 0.10 mL of 2.00 mmol/L mBrB and 0.70 mL of sodium bicarbonate buffer (14.3 mmol/L ammonium bicarbonate containing 14.3 mmol/L Na₂EDTA, pH 8.0). After the solutions were incubated at 42°C for 10 min, 0.1 mL of 100 mL/L acetic acid was added. The thiol–bimane and sulfite–bimane calibrators were stored at 4°C in foil-wrapped tubes for up to 6 months.

Blood collection and human donors. Blood was collected by venipuncture from apparently healthy male (n = 34) and female (n = 41) donors (≥12 h postprandially). The blood was drawn into 10-mL red-top evacuated tubes, left at room temperature for 1 h, and centrifuged at 2100g at 2°C for 15 min. Sera were stored at −70°C until analysis. The procedures used on human subjects in this study were in accordance with ethical standards and were approved by the Institutional Review Board of the Cleveland Clinic Foundation.

Equipment. The HPLC system consisted of two Spectroflow 400 pumps and a Spectroflow 980 programmable fluorescence detector with gradient controller (Applied Biosystems, Foster City, CA). Samples were injected by an autosampler (SP 8875; Spectra-Physics, San Jose, CA). Data were recorded and integrated with a Chromjet integrator (Spectra-Physics). Samples were resolved on a 4.6 × 250 mm C₈ reversed-phase column (no. 235332, 5-μm packing; Beckman Instruments, Fullerton, CA) and equipped with a 15 × 3.2 mm Brownlee RP-8 NewGuard column (no. 0711-0090, 7-μm packing; Applied Biosystems).

Sample preparation and HPLC analysis. Human serum (100 μL) was treated with 70 μL of 0.212 mol/L sodium borohydride in 0.05 mol/L Tris (pH 8.5) and 10 μL of 70.0 mmol/L mBrB in acetonitrile. After incubating for 7 min at 42°C, 50 μL of 1.5 mol/L perchloric acid was added with vortex-mixing. The protein precipitate was removed by centrifugation at 12 400g for 10 min (23°C). The supernate was neutralized in situ with 20 μL of 2.0 mol/L Tris, gently mixed, and centrifuged again at 12 400g for 8 min. The neutralized supernate (100 μL) was transferred to an autosampler vial, and 20 μL was injected onto the HPLC column. The column was equilibrated with methanol:acetic acid:water (5.00:0.25:94.75, by vol, pH 3.4) and developed with a gradient of methanol as follows: 0–5 min, 30 mL/L; 5–9 min, 30–130 mL/L; 9–11 min, 130–150 mL/L; 11–13 min, 150–1000 mL/L; 13–15 min, 1000 mL/L; 15–17 min, 1000–30 mL/L; and 17–20 min, 30 mL/L at a flow rate of 1.5 mL/min. Sulfite–bimane and thiol–bimane were detected by excitation at 390 nm and emission at >418 nm with use of a cutoff filter.

Sulfite–bimane calibration curve and standard addition method. Standardized solutions of sulfite in 0.1 mmol/L Na₂EDTA (pH 5.5) were prepared fresh for each assay and added (10 μL) to 100-μL aliquots of serum, giving a range of added sulfite from 0 to 90 μmol/L. Unknown serum samples (100 μL) received 10 μL of 0.10 mmol/L sodium sulfite. The calibration curve and unknown samples were analyzed as described above. A calibration curve was run with each batch of samples analyzed.

Sulfite loading test. A healthy male and a healthy female volunteer were given sodium metabisulfite (20 mg/kg body weight) dissolved in 6 oz. of vegetable juice (V-8) by mouth. Blood was drawn immediately before and after ingestion of the sulfite cocktail at intervals of 15 min for the first hour, 30 min for the second hour, and 60 min for the third to sixth hour. Serum samples were processed for total sulfite as described above.

Interference study. Hemolyzed blood samples were analyzed to determine the effect of hemoglobin on the sulfite assay. Five tubes of blood were drawn from a single volunteer at one drawing. Red blood cells were disrupted by adding glass beads to four of the sample tubes and vortex-mixing for different times. Hemolytic sera were obtained by centrifuging the above samples at 2100g at 2°C for 15 min. Serum hemoglobin was measured by the cyanmethemoglobin method (16). Twenty samples with four levels of hemolysis were analyzed.

Statistical analysis. The difference between male and female total serum sulfite concentrations was evaluated with Student’s t-test. The Shapiro–Wilk test for normality was applied to the total serum sulfite distribution data. The relation between total serum sulfite concentration and total serum cysteine, cysteinylglycine, and homocysteine was analyzed with Pearson’s correlation. Because of skewness of the data, nonparametric Spearman’s correlation analysis was
used to evaluate correlations between total serum sulfitae and age, serum cobalamin, and serum folate.

Other methods. Serum protein was measured by the bicinchoninic acid method (17). For the determination of ultraviolet-visible absorbance spectra, relative fluorescence quantum yields, and fluorescence excitation and emission maxima, the sulfitae-bimane and thiol-bimane calibrators were purified by reversed-phase HPLC with ultraviolet detection as described previously (18). Sulfitae-bimane and thiol-bimane calibrators were >98% pure as judged by reanalysis with HPLC with ultraviolet detection and HPLC with fluorescence detection. Fluorescence excitation and emission spectra were obtained by using an Aminco-Bowman spectrofluorometer (Model J4-8960A; SLM Instruments, Urbana, IL). Absorbance spectra were obtained by using an ultraviolet-visible recording spectrophotometer equipped with a printer/plotter (Uvikon 860; Kontron, Everret, MA). Sera were analyzed for cobalamin and folic acid with a kit assay (no. 262226; Becton Dickinson, Orangeburg, NY). The reference range in this laboratory for serum cobalamin is 125–517 pmol/L and for serum folic acid is >5.2 nmol/L. Total serum cysteine, cysteinylglycine, and homocysteine were determined by the method of Jacobsen et al. (18).

Results

Assay Performance

Determination of total serum sulfitae. Previous work from this laboratory demonstrated that precolumn derivatization of low-molecular-mass thiols present in serum (cysteine, cysteinylglycine, homocysteine, and glutathione) with mBrB followed by HPLC with fluorescence detection could be used to quantify the thiols (19). To identify sulfitae in serum, we synthesized and purified sulfitae-bimane by reversed-phase HPLC and characterized its absorbance and fluorescence properties. The absorbance spectrum of HPLC-purified sulfitae-bimane exhibited maxima at 231 nm (relative absorbance = 1.00), 259 nm (0.73), and 392 nm (0.32), as shown in Fig. 1A. The uncorrected excitation and emission fluorescence spectra of sulfitae-bimane (Fig. 1B) exhibited maxima at 392 and 479 nm, respectively. The absorbance, fluorescence, and HPLC properties of cysteine-S-bimane, cysteinyl-(S-bimane)glycine, homocysteine-S-bimane, and glutathione-S-bimane have been described previously (18, 20, 21). Sulfitae- and thiol-bimane calibrators were used to identify the mBrB derivatives of sulfitae and other low-molecular-mass thiols in human serum as shown in Fig. 2A and B. As shown in Fig. 2B, the retention times for cysteine-S-bimane, sulfitae-bimane, homocysteine-S-bimane, and glutathione-S-bimane calibrators were 6.85, 7.76, 9.67, and 10.78 min, respectively (the cysteinyl-(S-bimane)glycine calibrator was not present in this run). In human serum the retention times for the bimane adducts of cysteine, cysteinylglycine, sulfitae, homocysteine, and glutathione were 6.70, 7.39, 7.82, 9.67, and 10.70 min, respectively (Fig. 2A). Reaction blanks in which water replaced serum had no identifiable peaks in the sulfitae-bimane area (Fig. 2C).
Optimization of assay conditions. The concentration of each reagent used in the assay was optimized to achieve the greatest possible sensitivity. The optimal concentrations of sodium borohydride and mBrB during reduction–derivatization were 82.4 and 3.89 mmol/L, respectively. Higher concentrations of mBrB cause increased baseline noise and interference with the cysteine–bimane peak. Although the formation of sulfite–bimane adduct appeared to be complete within 5 min of incubation at 42°C, the incubation period was extended to 7 min for routine assay. Perchloric acid concentration (0.33 mol/L) was adjusted to give maximum protein precipitation (>99%) without significant volume dilution. Perchloric acid also destroyed excess borohydride in the reaction. The final pH of the perchloric acid supernate was adjusted to 3.0 with 2.0 mol/L Tris to stabilize the sulfite–bimane adduct and to extend HPLC column life. Direct addition of Tris to the assay tube followed by gentle mixing and centrifugation did not result in significant dislocation or resolubilization of the protein pellet. At pH 3.0 the sulfite–bimane product derived from serum was stable for at least 3 days when stored at room temperature in the dark. Serum samples in this study were thawed, assayed, and analyzed by HPLC with fluorescence detection within 24 h.

Linearity and precision. Serum samples from a normal donor pool were supplemented with standardized solutions of sulfite to give final concentrations of added sulfite ranging from 0 to 90 μmol/L. The samples were assayed by the routine procedure described in Materials and Methods. After correcting for endogenous sulfite content, the relative fluorescence intensity due to added sulfite was plotted as shown in Fig. 3. The response to added sulfite was linear over the concentration range shown. Intraassay CVs were 8.1%, 8.6%, and 7.8% for mean sulfite concentrations of 5.71, 13.6, and 55.0 μmol/L, respectively (Table 1). Biweekly analyses of the serum quality controls for a 3-month period gave interassay CVs of 22.0%, 12.6%, and 14.2% for mean concentrations of 5.01, 11.2, and 48.1 μmol/L, respectively.

Analytical recovery and sensitivity. We determined recovery by adding sulfite to single-donor serum samples (endogenous sulfite = 3.08 ± 0.19 μmol/L) to give final concentrations of 1.43, 5.73, 15.3, 22.9, and 45.8 μmol/L. The recoveries of added sulfite were 115%, 108%, 97.6%, 97.3%, and 99.9%, respectively. The detection limit was calculated according to Pizzoferrato et al. (22) and Fuentes-Arderiu (23) by using 0.1 mol/L potassium phosphate (pH 7.4) as a blank. Under these conditions, the detection limit for sodium sulfite was 0.44 μmol/L.

Interference. The effect of hemolysis on the determination of total serum sulfite was investigated. Sulfite concentrations did not appear to be affected by hemolysis (Table 2). Mean total serum sulfite concentrations in samples with increasing concentrations of hemoglobin did not consistently differ from the mean total serum sulfite concentration in a nonhemolyzed sample. Thus, sulfite can be determined in serum samples that have been obtained from hemolyzed blood.

Table 1. Precision of sulfite assay.

<table>
<thead>
<tr>
<th></th>
<th>Sulfite, μmol/L</th>
<th>CV, %</th>
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<tr>
<td>Intraassay</td>
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<tr>
<td>20</td>
<td>5.71</td>
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</tr>
<tr>
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<td>8.6</td>
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<tr>
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<td></td>
</tr>
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<td>12.6</td>
</tr>
<tr>
<td>21</td>
<td>48.1</td>
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Table 2. Effect of hemolysis on total serum sulfite.

<table>
<thead>
<tr>
<th>Degree of hemolysis</th>
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<th>Test no.</th>
<th>Mean sulfite, μmol/L</th>
<th>CV, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.079</td>
<td>4</td>
<td>4.18</td>
<td>3.4</td>
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<tr>
<td>Slight</td>
<td>1.18</td>
<td>4</td>
<td>4.50</td>
<td>2.6</td>
</tr>
<tr>
<td>Moderate</td>
<td>1.67</td>
<td>4</td>
<td>3.84</td>
<td>2.4</td>
</tr>
<tr>
<td>Severe</td>
<td>2.67</td>
<td>4</td>
<td>5.10</td>
<td>3.6</td>
</tr>
<tr>
<td>Most severe</td>
<td>4.80</td>
<td>4</td>
<td>4.65</td>
<td>2.1</td>
</tr>
</tbody>
</table>

* Within every four samples at each hemolysis level. Four means of sulfite with different severity of hemolysis were compared with the mean of sulfite in a nonhemolytic sample by Wilcoxon rank test; results were significant (P < 0.05) in all four cases.

* Comparison by Wilcoxon rank test shows P = 0.05.
Concentration of total serum sulfite was 4.87 ± 2.49 μmol/L. The reference range (mean ± 2 SD) for total serum sulfite in this population was 0–9.85 μmol/L. As shown in Fig. 4, total serum sulfite concentrations appeared to be normally distributed, but were slightly skewed toward higher values. Total serum cysteine, cysteinylglycine, and homocysteine have been previously reported for these subjects (18). No correlations were found between total serum sulfite and total serum cysteine (Pearson correlation $r = -0.0291; P = 0.814$), total serum cysteinylglycine ($r = -0.0604; P = 0.625$), or total serum homocysteine ($r = 0.0437; P = 0.724$). In addition, no correlations were found between total serum sulfite and the subjects’ ages (Spearman correlation $r = 0.1059; P = 0.387$), serum cobalamin ($r = 0.0414; P = 0.737$), or serum folic acid ($r = -0.0997; P = 0.419$).

Oral sulfite-loading studies. Sodium metabisulfite (dose = 20 mg/kg body weight) was dissolved in vegetable juice and given orally to two individuals. There was a rapid rise in total serum sulfite that reached a maximum concentration (112 and 38 μmol/L in subjects 1 and 2, respectively) in ~30 min (see Fig. 5). This was followed by a rapid fall in sulfite concentration in subject 1 and a moderately rapid fall in subject 2. Total serum sulfite concentrations in both individuals approached basal concentrations in ~3 h. However, sulfite concentrations were still slightly increased at the end of 6 h in both subjects.

Discussion

A rapid and sensitive assay for total serum sulfite has been developed, based on: (a) release of protein-bound sulfite by sodium borohydride reduction to liberate sulfite from protein-S-sulfonates; (b) derivatization of free sulfite with mBrB to form sulfite–bimane; (c) separation of sulfite–bimane from low-molecular-mass serum thiol–bimanes by reversed-phase HPLC; and (d) detection and quantitation of sulfite–bimane by fluorometry. We have previously described assays for low-molecular-mass thiols in serum and plasma involving mBrB (18, 19). The present assay for total serum sulfite, however, has been extensively modified to provide greater sensitivity and quicker sample throughput. For example, the borohydride/HCl reducing system described previously for serum thiols (18) could not be used here because acidification of the sample would result in the formation of sulfur dioxide and analyte loss. Chromatography conditions have been modified to avoid interference with serum thiol–bimanes.

Because sulfite reacts with protein disulfide bonds to form protein-S-sulfonates, it is necessary to include a reduction step in the assay to measure “total” serum sulfite. Borohydride has been used previously as a reducing agent to liberate homocysteine from protein in assays for total serum homocysteine and other thiols (19, 24–26). The use of mBrB as a thiol-specific derivatization reagent and its use in HPLC with fluorescent detection is well established (21, 27). A sulfite–bimane adduct, prepared as a calibrator, has been previously reported by Fahey and Newton (21). Total sulfite refers to all forms of sulfite in serum or plasma, including protein-bound sulfonates and low-molecular-mass S-sulfonates such as cysteine-S-sulfonate and free sulfite. Evidence for the in vivo formation of plasma protein-S-sulfonates has been provided by Gregory and Gunnison (28), who demonstrated that sulfite, when infused intravenously into rabbits, could later be isolated as albumin-S-sulfonate and fibronectin-S-sulfonate. The endogenous forms of sulfite in normal human serum remain to be identified. The existence of free sulfite exits in normal human serum has not been previously demonstrated. However, oral sulfite loading studies are in progress to address these questions. Because sulfite reacts with disulfide bonds of proteins and small molecules such as cysteine, the half-life of free sulfite in normal individuals is probably short. Mansoor et al. have shown transient increases in free reduced homocysteine in plasma after oral loading.
with either methionine (29) or homocysteine itself (30). However, free reduced homocysteine decreased relatively rapidly to a low concentration because of its interaction with serum proteins.

Sulfite has been difficult to determine in normal human serum because it is present at relatively low concentrations and is a chemically reactive species. Gunnison and Palmer (31, 32) used a colorimetric method based on a slight modification of the West and Gaeke method (33) to determine blood sulfite in experimental animals. This method detected free sulfite in plasma but did not determine protein-S-sulfonate. We attempted to measure human serum sulfite with this method and were unable to detect free sulfite. To measure protein-S-sulfonate, Gunnison and Benton used cyanide to release sulfite from protein-S-sulfonates (34, 35). Free sulfite was then determined after dialysis. The cyanide/dialysis method is time-consuming, labor-intensive, and impractical for clinical diagnostic assays. Although the methods described by Grant (36) and West and Gaeke (33) are suitable for determining relatively high aqueous concentrations of sulfite, they lack sufficient sensitivity for human serum.

In experimental animals the concentrations of protein-bound sulfite in nonfasting rats (n = 11) and rhesus monkeys (n = 5) were 8.0 ± 4.0 and 3.0 μmol/L, respectively (32). Acosta et al. (37), using the method of Grant (36) to determine plasma sulfite concentration, reported a very high concentration of sulfite in a sulfite-sensitive individual. In their report, the sulfite concentration in plasma before sodium metabisulfite loading was -90 mg/L (1.13 mmol/L). After metabisulfite loading with a capsule containing 200 mg of sodium metabisulfite, plasma sulfite peaked at -2.13 mmol/L 6 min after ingestion and fell to baseline within 60 min. Although sulfite metabolism may be impaired in patients with sulfite hypersensitivity (38), the basal and postload plasma sulfite values in this single patient are 100 to 500 times higher than the sulfite values reported in this study for normal individuals. Togawa et al. (13) reported that the mean concentration of total serum sulfite in five normal subjects was 0.47 ± 0.25 μmol/L by an assay based on dithiothreitol reduction of serum, flow gas dialysis to isolate sulfur dioxide, conversion back to sulfite in sodium hydroxide, mBrB derivatization, and HPLC analysis with fluorescence detection. The method gave high sensitivity and good resolution with tandem C_{18} reversed-phase and ion-exchange columns. The mean serum sulfite concentration reported by Togawa et al. is ~10% of the mean serum sulfite concentration found in this study. Although ethnic and dietary backgrounds of the study subjects could explain at least some of the difference, the disparity is more likely related to methodologies. For example, dithiothreitol was used as a reducing agent in the Togawa study and sodium borohydride in this study.

In the present study the mean concentrations of total serum sulfite in female and male donors were not statistically significant. The combined mean concentra-

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902 CLINICAL CHEMISTRY, Vol. 41, No. 6, 1995
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