Elevated Levels of Serum Sulfite in Patients with Chronic Renal Failure

HIROSHI KAJIYAMA,* YOSHIHISA NOJIMA,* HIDEKI MITSUHASHI,* KAZUE UEKI,* SHIGEO TAMURA,* TETSUO SEKIHARA,† RYOUJI WAKAMATSU,‡ SHINTARO YANO,§ and TAKUJI NARUSE*

*Third Department of Internal Medicine, Gunma University School of Medicine, Maebashi, † Shirane Clinic, Numata, ‡ Nishikatagai Clinic, Maebashi; and § Wakaba Hospital, Maebashi, Gunma, Japan.

Abstract. Sulfite, a well known air pollutant, is toxic for humans, especially those with sulfite hypersensitivity. Sulfite is also generated endogenously, during normal metabolism of sulfur-containing amino acids. Mammalian tissues contain the enzyme sulfite oxidase, which detoxifies both endogenous and exogenous sulfite by oxidation to sulfate. Deficiency of sulfite oxidase in humans is fatal, demonstrating its physiologic importance. Nevertheless, information about serum and tissue levels of sulfite in normal and pathologic conditions is limited. Using a sensitive HPLC assay, it is shown here that sera from patients with chronic renal failure (CRF) contain significantly higher amounts of sulfite than those from healthy subjects. Mean ± SD of serum sulfite in healthy subjects (n = 20) was 1.55 ± 0.54 μM, whereas those in patients under maintenance hemodialysis (HD patients; n = 44) and CRF patients before introducing dialysis therapy (pre-HD patients; n = 33) were 3.23 ± 1.02 μM (P < 0.01) and 3.80 ± 3.32 μM (P < 0.01), respectively. Among pre-HD patients, serum sulfite was positively correlated with serum creatinine (r = 0.714, P < 0.0001), and negatively with serum albumin (r = −0.407, P = 0.0188), hematocrit (r = −0.524, P = 0.0017), and total cholesterol (r = −0.375, P = 0.0318). There was no significant association between sulfite and patient age, gender, or leukocyte counts. Multiple regression analysis revealed serum creatinine as the sole independent predictor of serum sulfite levels. Each HD treatment was associated with approximately 27% reduction in serum sulfite levels, suggesting the presence of a dialyzable form in serum. Thus, these results indicate that reduced glomerular filtration is a factor that determines serum sulfite levels. Chronic elevation in serum sulfite levels might contribute to tissue or organ dysfunction in patients with CRF.

Sulfur dioxide is an air pollutant released into the atmosphere during the combustion of fossil fuel (1,2). Sulfur dioxide can be converted to sulfite upon contact with fluids lining the air passages. Sulfite and its related compounds, such as metabisulfite, are also widely used in food preservation as antimicrobial agents and antioxidants (3). In addition, endogenous sulfite is generated during the normal metabolic processing of sulfur-containing amino acids or drugs (4,5). The toxic effects of sulfite on mammals have been studied extensively (6–11). It can cause allergic reactions in humans; most commonly, bronchoconstriction in asthmatics (6). Mammalian tissues contain sulfite oxidase, which catalyzes the oxidative detoxification of sulfite (12). Children with hereditary deficiency in this enzyme develop mental retardation, neurologic symptoms such as spastic quadriplegia, and early death (4,13,14). Thus, sulfite levels in the body must normally be tightly regulated.

We and others have recently demonstrated that human or rabbit neutrophils produce sulfite spontaneously or in response to stimulation with the bacterial endotoxin lipopolysaccharide (15–17). We also found that in vivo administration of lipopolysaccharide into rats induced a significant increase in serum sulfite concentration (15). These results strongly suggest that sulfite is not only an exogenous toxic substance and an endogenous metabolite, but also acts as a mediator of neutrophil function with antimicrobial and proinflammatory activities. However, the effects of sulfite on cellular functions are unclear. Moreover, little is known about the role of sulfite in various pathophysiologic conditions.

In the present study, we determined sulfite concentrations in sera from patients with chronic renal failure (CRF) using reversed-phase HPLC, a sensitive assay for serum sulfite recently developed by Ji et al. (18). Compared with healthy subjects, CRF patients had significantly higher levels of serum sulfite. A positive correlation between serum sulfite and creatinine among predialysis CRF patients demonstrates that reduced renal function is a contributing factor for elevated serum sulfite. Hemodialysis (HD) treatment was associated with a temporary reduction of serum sulfite, suggesting that at least a portion of serum sulfite is in a dialyzable form.

Materials and Methods

Patients

This study was performed on healthy volunteers (n = 20), patients with CRF not receiving dialysis (pre-HD patients; n = 33), and
patients undergoing maintenance HD (HD patients; n = 44). HD was performed three times a week (12 to 15 h per week) using bicarbonate dialysate. Half of our patients (n = 22) were dialyzed with cellulose tricarboxylic membranes, and the others with polysulfone membranes (n = 6), vitamin E-modified membranes (n = 10), or miscellaneous (n = 6). Median duration ± SD of HD treatment was 43.5 ± 42.4 mo (0.5 to 254 mo). Patient profiles are summarized in Table 1. As shown, the age and gender of the three groups were comparable, except for the mean age of HD patients, which was higher than those of control and pre-HD subjects (P < 0.05). Patients showing evidence of intercurrent infection and those taking antibiotics were excluded from the study. All patients gave informed consent. To assess dietary protein intake, the protein catabolic rate (PCR) was calculated for HD patients according to the method described previously (19).

Sample Preparation

Blood samples were drawn from the antecubital veins of healthy subjects and CRF patients and from the arterial side of the arteriovenous fistula of HD patients before and after HD. All patients were fasted overnight before blood collection. After separation, serum was immediately subjected to sample preparation as described previously (15,18). In brief, serum samples (100 μL) were mixed with 70 μL of 0.212 M sodium borohydride in 0.05 M Tris-HCl (pH 8.5) and incubated at room temperature for 30 min. This procedure is critical for the reductive release of protein-bound sulfite, and sodium borohydride was freshly prepared in each experiment. Preliminary experiments revealed that sulfitc release was dependent on the incubation period with borohydride and reached a plateau level in 30 min. The samples were then mixed with 10 μL of 70 mM monobromobimane in acetonitrile. After incubation for 10 min at 42°C, 50 μL of 1.5 M perchloric acid solution was added to the mixture followed by vortex mixing. The protein precipitates were removed by centrifugation at 12,400 × g for 10 min at room temperature. The supernatant was immediately neutralized by adding 10 μL of 2 M Tris, gently mixed, and centrifuged again at 12,400 × g for 10 min. Ten microliters of the neutralized supernatant was injected onto HPLC column (Hitachi 655-A11 system), as described below. All procedures of sample preparation were completed within 2 h after serum separation.

Determination of Serum Sulfite Concentration using Reversed-Phase HPLC with Fluorescence Detection

Samples were resolved on 4 × 250 mm C8 reversed-phase column (5-μm packing; GL Science, Tokyo, Japan). The column was equilibrated with methanol:acetic acid:water (5.0:0.25:94.75, by volume, pH 3.4) and developed with a gradient of methanol in acetic acid:water (0.25:94.5, by volume) at a flow rate of 0.8 ml/min as follows: 0 to 5 min, 30 ml/L; 5 to 13 min, 30 to 350 ml/L; 13 to 23 min, 350 to 620 ml/L; 23 to 24 min, 620 to 1000 ml/L; 24 to 29 min, 1000 ml/L; 29 to 30 min, 1000 to 30 ml/L; and 30 to 34 min, 30 ml/L. Sulfite-bimane was detected by excitation at 390 nm and emission at 472 nm with use of a cutoff filter and eluted at 45 ml/L of methanol. Standard solutions of sulfite were prepared fresh for each assay by dissolving sodium sulfite in Hanks’ balanced salt solution, and a calibration curve was obtained by measuring relative fluorescence intensity of sulfite-bimane as described previously (15). Linearity was obtained over a concentration range of sulfite from 0.12 to 125 μM. Each serum sample was assayed in duplicate. The intra- and interassay coefficients of variation were 2.7 and 10.5%, respectively.

Table 1. Clinical characteristics of the subjects

<table>
<thead>
<tr>
<th>Variable</th>
<th>Healthy Subjects</th>
<th>Pre-HD Patients</th>
<th>HD Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
<td>33</td>
<td>44</td>
</tr>
<tr>
<td>Male/female</td>
<td>12/8</td>
<td>20/13</td>
<td>25/19</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>55.9 ± 20.3</td>
<td>54.9 ± 15.0</td>
<td>65.3 ± 12.4b</td>
</tr>
<tr>
<td>Disease</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>7</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>CGN</td>
<td>11</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>PCK</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Other or unknown</td>
<td>13</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

* Results are given as mean ± SD. HD, hemodialysis; DM, diabetes mellitus; CGN, chronic glomerulonephritis; PCK, polycystic kidney.

b P < 0.05.

Statistical Analyses

Statistical analyses were performed using commercially available personal computer software, StatView 4.5. Data are presented as mean ± SD. Differences between mean values in study groups were evaluated by the t test. Correlation between two variables was examined by simple regression analysis. Independent associations between one dependent and two or more independent variables were assessed by multiple regression analysis. The difference was considered significant at P < 0.05.

Results

Ji et al. recently established a sensitive method for measuring sulfite concentration in biologic fluids (18). Using this method, they showed that normal human serum contained sulfite at a concentration of 4.87 ± 2.49 μM (mean ± SD). We also examined serum sulfite levels in the healthy Japanese population. The mean ± SD of serum sulfite in 20 Japanese subjects was 1.55 ± 0.54 μM, which is considerably lower than that determined by Ji. There was no significant correlation between serum sulfite levels and subjects’ age (r = 0.04, P = 0.867).

We next measured sulfite in sera from 77 patients with CRF. Forty-four patients were undergoing maintenance HD therapy (HD patients). The remaining 33 did not yet require dialysis (pre-HD patients) and had serum creatinine levels ranging from 1.5 to 13.3 mg/dl (mean 5.47 ± 3.55). Compared with healthy control subjects (Figure 1), both HD and pre-HD patients had significantly higher levels of serum sulfite (P < 0.01). The means (±SD) in HD and pre-HD patients were 3.23 ± 1.02 and 3.80 ± 3.32 μM, respectively. There was no significant difference in serum sulfite concentration between pre-HD and HD patients.

As shown in Figure 1, the level of serum sulfite was widely distributed in pre-HD patients, ranging from 0.70 to 13.5 μM. Therefore, we attempted to determine which factors contribute serum sulfite levels among pre-HD patients. Table 2 gives a simple correlation between serum sulfite and other variables. Serum sulfite levels correlated positively with serum creatinine concentration (Figure 2A) (P < 0.0001), and inversely with serum albumin (P = 0.0188), total cholesterol (P = 0.0318), and hematocrit (Figure 2B) (P = 0.0017). Sulfite showed no significant association with age, gender, serum total protein...
levels, or leukocyte counts. Multiple regression analysis was also performed to evaluate independent factors affecting serum sulfite levels (Table 3). In this analysis, only the serum creatinine levels contributed significantly to the variability of sulfite levels. The mean ± SD of PCR in 31 HD patients was 0.89 ± 0.16 g/kg per d. There was no significant correlation between PCR and serum sulfite levels among these patients (r = 0.099, P = 0.5945).

We finally examined serum sulfite levels before and after HD treatment in 10 HD patients. Each HD for these patients was performed using the cellulose triacetate membrane. As shown in Figure 3, HD treatment led to the significant reduction (P < 0.01) in serum sulfite levels by an average of 27% (95% confidence interval, 17.7 to 33.9%). This suggests that serum sulfite is dialyzable to some extent.

**Discussion**

In the current study, we measured serum concentration of sulfite in healthy Japanese subjects and patients with CRF according to the method developed by Ji et al. (18). When compared with healthy subjects, patients with CRF had a significantly higher serum sulfite concentration. Abnormally high levels in serum sulfite have been demonstrated in patients with congenital deficiency in sulfite oxidase and in patients with hypersensitivity to sulfite (4,20). Our present study indicates that the elevation of serum sulfite levels is not restricted to these uncommon pathologic conditions.

In our hands, sera from healthy control subjects contained sulfite at a concentration of 0.1 to 2.2 μM. Although levels in each individual were within the normal reference range (0 to approximately 9.85 μM) determined by Ji et al. (18), the mean value in our study was approximately 30% of that reported by Ji et al. Togawa et al. (21) reported a far lower value (0.47 ± 0.25 μM) as the mean concentration of serum sulfite in healthy Japanese subjects, although their assay system was completely

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**Table 2. Simple correlation between serum sulfite and other variables in pre-HD patients**

<table>
<thead>
<tr>
<th>Variable</th>
<th>r  Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.138</td>
<td>0.4453</td>
</tr>
<tr>
<td>Gender (Female = 0, Male = 1)</td>
<td>−0.128</td>
<td>0.4761</td>
</tr>
<tr>
<td>Creatinine</td>
<td>0.714</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Leukocyte counts</td>
<td>0.061</td>
<td>0.7373</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>−0.524</td>
<td>0.0017</td>
</tr>
<tr>
<td>Total protein</td>
<td>−0.137</td>
<td>0.4461</td>
</tr>
<tr>
<td>Albumin</td>
<td>−0.407</td>
<td>0.0188</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>−0.375</td>
<td>0.0318</td>
</tr>
</tbody>
</table>

**Table 3. Multiple regression analysis of factors affecting serum sulfite levels in pre-HD patients**

<table>
<thead>
<tr>
<th>Independent variables</th>
<th>β Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>hematocrit</td>
<td>−0.035</td>
<td>0.8618</td>
</tr>
<tr>
<td>creatinine</td>
<td>0.636</td>
<td>0.0016</td>
</tr>
<tr>
<td>albumin</td>
<td>−0.43</td>
<td>0.7972</td>
</tr>
<tr>
<td>total cholesterol</td>
<td>−0.081</td>
<td>0.5962</td>
</tr>
<tr>
<td>r^2</td>
<td>0.520</td>
<td>(P = 0.0003)</td>
</tr>
</tbody>
</table>

* r^2, multiple coefficient of determination.
different from ours. Possible explanations for the disparity in normal reference ranges between our study and that of Ji include methodologic, ethnic, dietary, and other environmental differences. Although dietary intake is certainly a source of serum sulfite (18), it is unlikely that Japanese food and beverages contain less sulfite as a preservative. Moreover, among HD patients, PCR was not significantly correlated with serum sulfite levels ($r = 0.099, P = 0.5945$), suggesting that dietary protein intake contributes little, if any, to the variation in serum sulfite levels among these patients. One possible and likely cause could lie in the effectiveness of the borohydride treatment to reductively release serum protein-bound sulfite. Because borohydride is relatively unstable at pH 8.5, incomplete release of sulfite could account for the difference. At present, however, it is unclear which of these factors is most important.

Among pre-HD patients, the level of serum sulfite was well correlated with that of serum creatinine. Thus, the reduced glomerular filtration may be a cause of elevated serum sulfite concentration. Indeed, sulfate, an oxidative product of sulfite, accumulates in the serum and contributes to acedia in patients with end-stage renal failure (22). We also reported that sulfite production by activated neutrophils was dependent on the sulfate concentration in culture medium (15). Hence, the higher serum concentration of sulfate in CRF patients may shift the equilibrium between sulfite and sulfate toward sulfate. It is also possible that enzyme activity of sulfite oxidase is impaired in CRF patients. In addition, metabolism of sulfur-containing amino acids such as methionine, cysteine, and homocysteine has been reported to be aberrant in CRF patients (23,24). Because sulfite is an intermediary metabolite in the normal processing of these amino acids (4,5), this may be an alternative mechanism by which serum sulfite is elevated in CRF patients.

In our current protocol, sulfite was measured as total serum sulfite which contains both free and protein-bound forms (18). Because sulfite easily reacts with disulfide bonds of proteins and small molecules such as cysteine, the half-life of free sulfite in healthy individuals is thought to be short. At present, however, it remains unknown how much free sulfite is contained in sera in healthy subjects and CRF patients. There was approximately a 27% reduction in serum sulfite levels after HD treatment. This suggests that at least a part of serum sulfite exists as a dialyzable form. Although neutrophil activation commonly occurs during HD, from our current available data the rate of sulfite production during dialysis is unknown, because we have not measured sulfite contained in the dialysate.

Although sulfite is widely used as preservative and antioxidant in food, beverages, and pharmaceuticals, excessive sulfite is highly toxic for human cells and tissues. An extreme example is a congenital disease of sulfite oxidase deficiency (4,13,14). Individuals suffering from this genetic disorder develop severe neurologic abnormalities, dislocated ocular lenses, mental retardation, attenuated growth of the brain, and early death (4,13,14). It is not clear whether the brain damage occurs as a result of toxic levels of sulfite, the absence of sulfate, or a combination of both. A recent study by Reist et al. (8) showed that sulfite exerts toxic effects on cultured neuronal cells directly or in combination with peroxynitrite. Another target organ of sulfite is the lung. It has been well established that exposure to sulfite can cause bronchial asthma and other chronic lung diseases (6). Its damaging effects to the lung have been proposed to involve the generation of sulfite radicals such as $\text{SO}_3^{-}, \text{SO}_4^{2-},$ and $\text{SO}_5^{2-},$ as well as inactivation of $\alpha$-antiproteinase (7,9). Sulfite was also demonstrated to directly activate neutrophils, leading to enhanced migration and generation of oxygen radicals (25–27). Thus, the sulfite concentration must be tightly regulated to maintain homeostasis in humans. It remains unknown whether a rise (up to 10-fold increase above normal range) in serum sulfite plays a role in organ and tissue dysfunction in CRF patients. Oral sulfite loading in healthy subjects results in a transient increase in serum sulfite concentration to 38 to 112 $\mu$M in 30 min that returned to basal levels within 3 h without any adverse reactions (18). However, the possibility cannot be ruled out that chronic and sustained elevation of serum sulfite in CRF patients may be more harmful than acute and transient rise in healthy individuals. More information regarding the effects of sulfite on cellular function and precise determination of sulfite levels in tissues and organs will be necessary to address this issue.

Acknowledgment

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References


![Figure 3. Changes in serum sulfite levels after HD treatment in 10 patients receiving maintenance HD. Statistical analysis was performed by paired t test.](Image)


