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Short communication

Urinary thiosulfate determined by suppressed ion chromatography with conductimetric detection

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Abstract

Thiosulfate is a naturally occurring product of sulfur metabolism. Assays of urinary thiosulfate have been based on the reaction with cyanide to form thiocyanate. However, matrix interferences and background variation in endogenous thiocyanate excretion place serious constraints on this method for determination of physiological amounts of thiosulfate in urine. We describe a column-switching ion chromatographic separation for urinary thiosulfate that allows for sensitive and accurate detection by ion conductimetry. In 20 adult volunteers, we found a lower urinary thiosulfate (8.50 ± 7.39 μmol/24 h, mean ± S.D.) than others have described, although the upward skew of the results (median, 6.90; range, 0.84–32 μmol/24 h) was similar. However, we have not observed any of the interferences and the sensitivity of our technique (< 0.2 μmol/24 h) allows for detection of thiosulfate in all control samples. This sort of methodological improvement will be essential for any study of physiological thiosulfate metabolism.

1. Introduction

Thiosulfate is a normal constituent of human urine. Its synthesis and secretion are poorly understood, but the main source appears to be a reaction between sulfite species and sulfane sulfur, which is catalyzed by the enzyme rhodanese or thiosulfate–sulfurtransferase (EC 2.8.1.1.) [1]. Sörbo [2] developed a method for thiosulfate determination based on copper-catalyzed cyanolysis of the sulfide moiety to form thiocyanate, followed by colorimetry of a ferric thiocyanate complex. This method has been useful in detecting greatly increased thiosulfate concentrations associated with inborn errors of metabolism [3], but suffers from significant interferences in various urine matrices, particularly when excess phosphate and sulfur-containing drug products are present [4,5]. Subsequently, clean-up procedures have been reported that reduce this interference substantially [6], but they are still insufficiently sensitive to determine baseline levels of urinary thiosulfate in all subjects. Electrochemical detection can provide the needed sensitivity and specificity, but the only reported procedure includes several clean-up steps, and requires reversed-phase, ion-pair HPLC and a mercury electrode [7]. Our experience with trace sulfate determination in

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various biological fluids by conductimetry after ion chromatography [8,9] suggested that this method might be a superior alternative. Bak et al. [10] have reported ion chromatographic determination of thiosulfate in microbial cultures with UV absorbance detection, but the high concentrations of UV-absorbing organics would make this unsuitable for urine assays. Moreover, interference from the anions predominating in urine (chloride, sulfate, phosphate) preclude a simple column configuration for detection of the sub-micromolar quantities of thiosulfate expected in some samples. We have therefore developed a column-switching method that obviates these interferences and allows rapid and sensitive determination of thiosulfate excretion levels in normal healthy adults.

2. Experimental

2.1. Instrumentation

All samples were analyzed on a DX-500 Ion Chromatograph (Dionex Instruments (Canada), Mississauga, Ont., Canada). To prevent leaching of metal ions, the system was equipped with Teflon (PTFE) plumbing throughout. The configuration included a GP-40 gradient pump connected alternatively to the 50 × 4 mm I.D. Ionpac AG4A-SC guard column and thence to the 250 × 4 mm I.D. Ionpac AS4A-SC analytical column (nominal particle size 12 μm; both from Dionex Instruments), or to the analytical column only. A WCP-1 auxiliary pump was plumbed so that its eluent stream passed alternatively through the guard column only or was directed to waste. The eluent from the analytical column was directed to the ASRSI anion self-regenerating suppressor column, and thence to an ED40 conductivity detector. An LC-30 oven was thermostatted to 30°C to improve baseline stability. The samples were injected manually and the concentrations calculated from standard curves using electronic integration and regression routines (Dionex PeakNet software).

2.2. Chemicals and reagents

All chemicals used in this study were of reagent or HPLC grade purchased from local suppliers. All distilled, deionized water used had a resistance of >15 MΩ and contained no detectable thiosulfate. Sep-Pak Plus C_{18} cartridges were purchased from Millipore Waters Canada (Mississauga, Ont., Canada).

2.3. Chromatographic conditions

The mobile phase contained sodium carbonate (10 mmol/l) and sodium bicarbonate (10 mmol/l); degassing was achieved and monitored online. The flow-rate was set at 1.0 ml/min for the auxiliary pump and 2.0 ml/min for the primary pump. The column-switches were set for 0.1 and 1.5 min after injection (25-μl volume). With this configuration, the void volume and early eluting anions passing through the guard column between 0.0 and 0.1 min are directed to waste. Anions moderately adsorbed to the guard column, including thiosulfate, pass from the guard column after 0.1 min through to the analytical column and detector. Anions that remain on the guard column longer than 1.5 min are redirected to waste. This "heart-cut" method reduces matrix interference and substantially reduces baseline noise. The electrical conductivity cell was set to give a maximum response at 300 μS and the regeneration current for the suppressor was set at 300 mA.

2.4. Methods for comparison

For comparison, samples were assayed by two versions of the colorimetric cyanolysis method. The first method was based on the report by Shih et al. [3] describing appropriate pH conditions for the reaction and the inclusion of urine blanks to reduce interference from preformed thiocyanate and compounds that react with ferric ion. Our only modification was the addition of a pre-extraction step with a C_{18} solid-phase cartridge, described by Kågedal et al. [7] but performed here as described by Voroteliak et al. [6].

The second method was based exclusively on the report from Voroteliak et al. and uses the cyanolysis and thiocyanate detection reagents in reverse order when blanking.

2.5. Urine samples

One large volume of urine with significant amounts of thiosulfate was divided into multiple aliquots to assess recovery, within- and between-run analytical variation, and to serve as a quality control sample. Random urines from 5 female (median age, 38 yr; range, 27–61 yr) and 15 male (median age, 43 yr; range, 28–63 yr) adult volunteers were assayed. Values were normalized by urinary creatinines determined by automatic instrumentation with an enzymatic method [11]. To assess within-person and between-person variation, urines were collected from 5 males (median age, 52 yr; range, 39–61 yr) and 15 females (median age, 47 yr; range, 25–64 yr) for a 24-h period, followed by a single voided early-morning sample.

2.6. Sample processing

It was noted that a commercially available preservative based on boric acid (b.a. tablets, Oti Specialties, Santa Monica, CA, USA) generated a small but distinct peak ($t_R = 1.41$ min) that did not interfere with thiosulfate. This preservative was used for the 24-h collections only. Samples were analyzed on the day of collection or stored at −20°C and thawed, well mixed and centrifuged at 10 000 *g* for 10 min prior to analysis. A portion of the supernatant was passed through a C_{18} Sep-Pak Plus cartridge. After discarding the first 1.0 ml, 40 μl was used to fill the injection loop. Individual concentrations are the mean of duplicates unless otherwise stated.

3. Results and discussion

With column switching, urinary thiosulfate eluted as a clean, symmetric peak, ($t_R = 3.97$ min), well resolved from other anions of interest (Fig. 1, middle tracing). Also detectable in urine

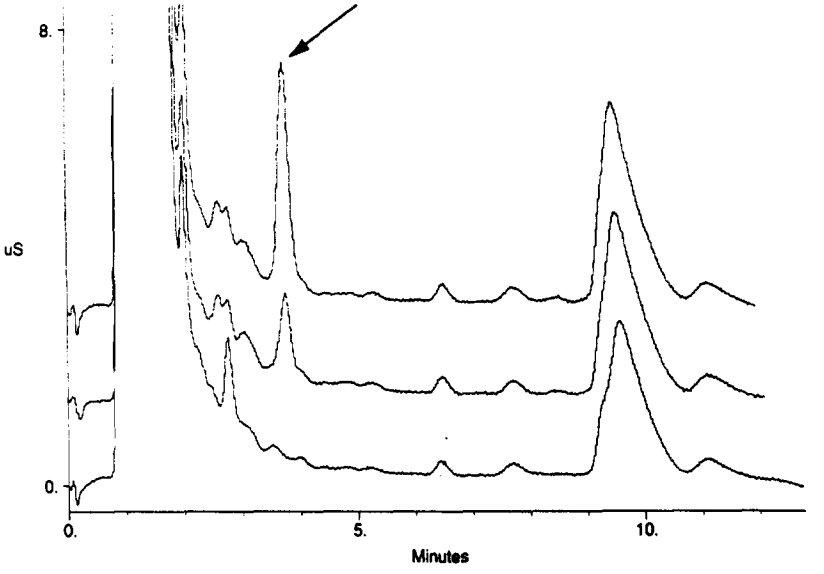

Fig. 1. Thiosulfate ion chromatograms. The middle tracing shows a typical urine chromatogram with thiosulfate eluting at 3.8 min (arrow). The top tracing shows the effect of adding 50 μmol/l to the same sample which had a nominal concentration of 27.2 μmol/l. The bottom tracing shows a sample to which H_2O_2 was added (see text).

were sulfate ($t_R = 1.68$ min), oxalate ($t_R = 2.01$ min) and thiocyanate ($t_R = 7.65$ min). Authentic thiosulfate added to urine co-eluted with the peak of interest. To confirm that the eluting peak in urine samples was entirely thiosulfate, standards and urine samples were mixed with 1% (v/v) hydrogen peroxide. This treatment oxidizes thiosulfate to sulfate, causing the disappearance of thiosulfate matched by a corresponding increase in the sulfate peak. In the urine samples, the conductivity profile reverted to baseline, as expected (see bottom tracing in Fig. 1); in the standard, no thiosulfate was detectable and the expected sulfate peak was observed (results not shown).

For a sample containing 29 μmol/l, we found an intra-assay coefficient of variance of 4.5% ($n = 16$) and an inter-assay coefficient of variance of 11.7% ($n = 16$). The detection limit of this method (background + 3σ) was 0.28 μmol/l. This was used as the concentration for the occasional sample in which some thiosulfate appeared to be present.

Comparative data on thiosulfate recoveries are shown in Table 1. For eight matched urines, the IC-based urinary thiosulfate was modestly lower but not significantly different for that determined by Shih et al. [3]. However, only those urines giving measurable amounts of thiosulfate with the colorimetric method could be compared. There was a statistically significant correlation ($r = 0.81$, $p = 0.013$)) between methods, but regression analysis showed that slope of the line, expected to be close to 1.0, was 0.71 (95% confidence interval: 0.21–1.21) and there were discrepancies of more than 100% in some values. We were not at all able to use the modified method of Voroteliak et al. [6] for baseline physiological thiosulfate determinations because the blanks frequently gave higher absorbance readings than the samples. The investigators themselves have acknowledged this to be a problem (T.H.J. Florin, personal communication), but point out that accuracy improves substantially if thiosulfate concentrations are elevated. Indeed, we noticed that recoveries of added authentic thiosulfate were much more comparable. For 5 and 10 μmol/l additions, respectively, IC-based recoveries were 100.1 ± 2.8% and 95.0 ± 3.8% and not different from 100%. Mean recoveries obtained with both colorimetric methods were similar (Table 1), but the variation with the method of Voroteliak et al. [6] was quite high. In the end, we were unable to convince ourselves that methods based on physico-chemical detections (our ion conductimetry method and the electrochemical method of Kågedal et al. [7]) generate values comparable to those determined by cyanolysis-based colorimetric methods. Therefore, we did not pursue further correlative studies.

Our reference values for urinary thiosulfate excretion are compared with those reported in the literature (Table 2). In 24-h urines, we detected significantly less thiosulfate by conductimetry (8.50 ± 7.39 μmol/24 h) than Kågedal et al. [7] reported (23.6 ± 18.7 μmol/24 h) using an electrochemical method ($p < 0.002$, Student's t-test). Whether this represents a difference in collection methods is not clear; our mean 24-h

Table 1
Comparison of colorimetric and chromatographic methods for thiosulfate determination

	Concentration (mean ± S.D.) (μmol/l)		
	Shih et al. [3]	Voroteliak et al. [6]	Ion chromatography
Urine thiosulfate ($n = 8$)	10.1 ± 6.3	ND	8.3 ± 5.4
	% Recovery		
+5 μmol/l ($n = 4$)	95.5 ± 3.4	102 ± 38	100.1 ± 2.8
+10 μmol/l ($n = 4$)	103.3 ± 1.8	103 ± 28	95.0 ± 3.8

ND = not detectable.

Table 2
Urinary thiosulfate excretion in adult volunteer subjects

	Daily excretion (μmol/24 h)		Excretion index (μmol/mmol creatinine)	
	Mean ± S.D.	Median (range)	Mean ± S.D.	Median (range)
Present study				
24-h collections ($n = 20$)	8.50 ± 7.39	6.90 (0.84–32)	1.12 ± 1.13	0.77 (0.06–4.1)
Matching early AM specimens ($n = 19$)[a]	–	–	0.80 ± 0.70	0.59 (0.02–3.0)
Random specimens ($n = 20$)	–	–	1.17 ± 0.78	0.90 (0.21–3.2)
Other studies				
Kågedal et al. [7] ($n = 20$)	23.6 ± 18.7	–	–	–
Mårtensson [12] ($n = 10$)				
fed	–	–	1.99 ± 0.66	–
fasted	–	–	0.88 ± 0.44	–

[a] One outlier was removed for statistical analyses.

volumes (1400 ± 741 ml/24 h) were not significantly less than expected for a group of middle-aged volunteers. However, we did see the same wide variation amongst our volunteers (biological C.V. = 87%) with a very substantially skewed distribution. We would suggest that 24-h excretion data be reported with medians and ranges where possible.

We also examined the relationship between thiosulfate and creatinine concentrations in our samples. Like Sörbo and Öhman [4], we found a modest but significant correlation between them ($r = 0.37$, $p < 0.02$, $n = 39$). We also found that the thiosulfate/creatinine ratios in our 24-h samples were not significantly different from our series of random urine collections [1.12 ± 1.13 μmol/mmol ($n = 20$) vs 1.17 ± 0.78 μmol/mmol ($n = 20$), Student's $t = 0.47$, $p = 0.64$]. When we compared early-morning (fasted) samples from the same volunteers contributing the 24-h samples, we found that the thiosulfate/creatinine ratios were lower (0.69 ± 0.50 μmol/mmol) but the difference was not significant. However, a strong within-person correlation was observed (Spearman's $r = 0.71$, $p < 0.0001$), suggesting that the data obtained from a morning fasting sample provides between-person information similar to the 24-h sample. As others have noted, we found no significant difference in ratios between men and women (1.05 ± 0.71 vs. 0.93 ± 0.85 μmol/mmol, Student's $t = 0.47$, $p = 0.64$).

Using a colorimetric assay, Mårtensson [12] found that the ratio fell from 1.99 to <1.0 with a 24-h fast. The control value is higher than we found, but the decline with fasting is in keeping with the decreased ratios we observed in fasted early-morning samples. We would presume that their higher control values are due to a methodological difference. This presumption is supported by examination of control data obtained with their electrochemical detection method in another study [13]. In that study, which looked at the influence of bacterial flora on thiosulfate excretion in only six subjects, the mean thiosulfate/creatinine ratio in controls was close to 1.1. In fact, those authors comment that the colorimetric method overestimates thiosulfate excretion and frequently gives inconsistent results.

4. Conclusion

We have assayed thiosulfate by a novel combination of heart-cut ion chromatography with conductimetric detection and found that considerably less of this compound is naturally present than previous studies would indicate. Considering that interferences with chemically based detection methods are reported as difficult to eliminate [5], this is not surprising. It would appear that we have followed the trend of

downward revision for reference values of thiosulfate excretion that has followed each improvement in assay methodology. Our method should allow for better comparisons of clinical data. There is continuing interest in the role of thiosulfate as a marker of sulfite oxidase deficiency as it applies to inborn errors of sulfite oxidase deficiency and, perhaps more commonly, to the postulated relative sulfite oxidase deficiency in individual's sensitivity reactions to sulfites in food and beverages.

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