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# REVERSED-PHASE ION-PAIR LIQUID CHROMATOGRAPHIC PROCEDURE WITH ELECTROCHEMICAL DETECTION FOR THE ANALYSIS OF URINARY THIOSULPHATE

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SUMMARY

A method using high-performance liquid chromatography with mercury-based electrochemical detection has been developed for the determination of thiosulphate in urine. The chromatographic separation is based upon ion-pair formation between thiosulphate and tetrabutylammonium and reversed-phase chromatography. The method was compared with an earlier reported colorimetric assay and found to be superior with respect to specificity and sensitivity.

## INTRODUCTION

Urine from human subjects contains low concentrations of thiosulphate [1], which is a metabolic conversion product of sulphite [2]. Moreover, very high urinary concentrations of thiosulphate are found in subjects with sulphite oxidase deficiency [3], a rare hereditary disorder. An acquired form of sulphite oxidase deficiency was recently found in a patient on prolonged total parenteral nutrition [4] as demonstrated by a heavy thiosulphaturia.

However, studies on the excretion of thiosulphate under normal and pathological conditions have been hampered by a lack of reliable analytical methods. An early method [5] for the determination of urinary thiosulphate based on the precipitation of the nickel—ethylenediamine complex of thiosulphate apparently gives too high results. A more specific colorimetric method based on cyanolysis of thiosulphate to thiocyanate in the presence of cupric ions [6] was adopted for determinations on human urine as a screening test for sulphite oxidase deficiency [7]. However, this method is not sufficiently sensitive for

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determination of normal or moderately raised urinary thiosulphate concentrations. A more sensitive method for the determination of urinary thiosulphate was developed [8] by combination of ion-exchange techniques and the original cyanolysis method [7], but it is fairly laborious and time-consuming. This challenged us to investigate alternative methods and we now describe a procedure whereby urinary thiosulphate is determined by ion-pair high-performance liquid chromatography (HPLC) followed by electrochemical detection.

## EXPERIMENTAL

### *Materials*

Sep-Pak $C_{18}$ cartridges were obtained from Waters (Milford, MA, U.S.A.). Tetrabutylammonium hydrogen sulphate (TBA) was a product of E. Merck (Darmstadt, G.F.R.) and methanol HPLC grade was from Rathburn Chemicals (Walkerburn, Great Britain). A stock standard solution of thiosulphate, 100 mmol/l, was prepared from a Titrisol ampoule (E. Merck) and working standards, 100 μmol/l or less, were prepared daily with the mobile phase as diluent.

### *Urine collection and preparation*

Urine was collected for 24 h with thymol—isopropanol [9] as a preservative. A 2.5-ml aliquot of urine was passed through a Sep-Pak $C_{18}$ cartridge, and the first 0.5 ml was discarded. After five-fold dilution with the mobile phase used for HPLC, the sample was ready for chromatography.

### *Chromatographic conditions*

We used a Constametric III pump from LDC (Riviera Beach, FL, U.S.A.) and a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 sample injector with a 100-μl sample loop. All separations were performed on a RSiL $C_{18}$ HL column (10 μm, 250 × 4.6 mm) from Alltech (Deerfield, IL, U.S.A.). The mobile phase was prepared from 85 vols. of a solution containing sodium phosphate (2 m*M*), disodium EDTA (0.1 m*M*) and TBA (17.6 m*M*) adjusted to pH 6.0 with NaOH, and 15 vols. of methanol. The mobile phase was suction-filtered through a 0.5-μm cellulose acetate filter, type EH (Millipore, Bedford, MA, U.S.A.) before use and delivered to the column at a flow-rate of 1 ml/min at room temperature. A mercury-based electrochemical detector was constructed according to the description of Rabenstein and Saetre [10] and operated at a potential of 0.0 V of the working electrode vs. saturated calomel electrode. The polarographic current was registered by an LC-2A Amperometric Controller (Bioanalytical Systems, West Lafayette, IN, U.S.A.) connected to a recorder via an electronic filter and amplifier (Model 1021 A, Spectrum, Newark, DE, U.S.A.). Peak heights were measured for quantitative evaluation of thiosulphate. Capacity ratios, $k'$, were calculated from the retention time, $t_R$, and the time for the non-sorbed peak, $t_0$, by $k' = (t_R - t_0)/t_0$. The time for the non-sorbed peak was determined by injecting 100 μl of water and measuring the time from injection to the first deviation of the recording from baseline.

*Comparison method*

The thiosulphate assay reported by Sörbo and Öhman [8] is based on cyanolysis of thiosulphate to thiocyanate in the presence of cupric ions followed by colorimetric determination of thiocyanate as the ferric thiocyanate complex. Preformed thiocyanate and compounds interfering with cyanolysis are removed by chromatography on ion-exchange resins. However, the original method had to be modified as the manufacturer of the resin Lewatit MP 7080 (Bayer, Leverkusen, G.F.R.) suddenly changed the manufacturing process for this resin. Whereas earlier batches of this resin had a high affinity for thiocyanate [11], later batches showed a very low affinity for this ion. We have now found that another weakly basic anion-exchange resin Amberlyst A 21 (Rohm & Haas, Philadelphia, PA, U.S.A.) can replace MP 7080 in the method for thiosulphate assay. Furthermore, we observed that compounds inhibiting the cyanolysis reaction were removed by chromatography on Amberlyst A 21. This permitted the omission of the initial chromatography step on the resin AG 3 necessary in the original method. As Amberlyst A 21 is delivered as fairly coarse particles, it should be ground in a laboratory mill before use.

Our modified method for thiosulphate assay was as follows. Urine was adjusted to pH 4.0 with acetic acid and a 25-ml aliquot taken for analysis. To another 25-ml aliquot of urine was added 0.10 ml sodium thiosulphate (10 m*M*) as an internal standard. Each sample was then applied to a 5 × 0.7 cm column of Amberlyst A 21 ($Cl^-$) which was washed with 10 ml of water. Elution of thiosulphate was then performed with 20 ml of a solution containing ammonia (2.5 *M*) and ammonium sulphate (0.2 *M*) and the column was washed with 10 ml of water. To the combined effluent and washing was added 1.0 ml of KCN (0.5 *M*) followed by 0.5 ml of $CuCl_2$ (1.0 *M*). The reaction mixture was transferred to a 2.5 × 0.7 cm column of Amberlyst A 21 (free base) which was washed with 10 ml of water and 10 ml of nitric acid (0.3 *M*). The washings were discarded and the thiocyanate formed in the cyanolysis reaction was eluted with 5.0 ml of a solution containing ferric nitrate (0.25 *M*), nitric acid (3.25 *M*) and methanol (30%). The absorbance of the eluate was then determined at 470 nm in a 1-cm cuvette. A blank correction was obtained by addition of 50 µl of mercuric nitrate (1 *M*) in nitric acid (4.8 *M*) and determination of the resulting blank absorbance. We have verified on a number of human urine samples that the modified method gives results in close agreement with those of the original procedure.

## RESULTS

*Chromatography of standard solutions*

Due to the ionic nature of thiosulphate, an ion-pairing agent (TBA) must be present in the mobile phase in order to obtain retention during reversed-phase chromatography. The mobile phase also contained phosphate buffer, a small amount of EDTA to prevent on-column oxidation of thiosulphate and methanol as an organic modifier in order to obtain suitable retention times. We verified in this connection that addition of methanol up to 20% final concentration had no noticeable effect on the sensitivity of the detector. In

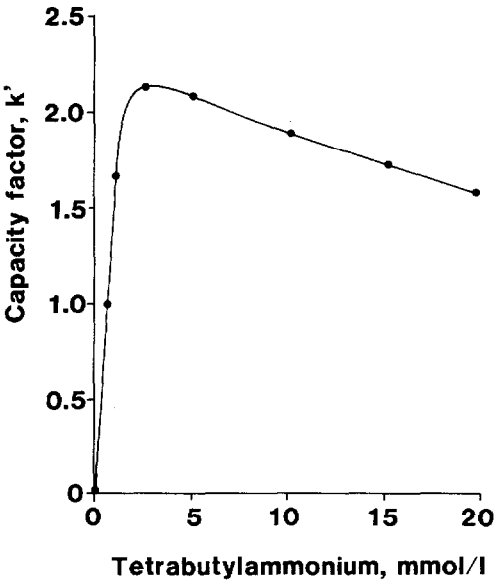


Fig. 1. Effect of TBA on the retention of thiosulphate. Other chromatographic conditions as in the standard procedure.

preliminary experiments we studied the effect of pH between 3 to 7 and found that $k'$ for thiosulphate slightly decreased from pH 3 to pH 7, but that at the lower pH the thiosulphate peak was broad and tailing. Satisfactory peaks were obtained at pH 6.0 which was consequently used in further experiments. The effect of TBA on the retention of thiosulphate was then studied and a $k'$-maximum at about 3 m*M* TBA was observed (Fig. 1). A definite maximum and not a plateau value was obtained with increasing concentration of the ion-pairing agent. This is often obtained in ion-pair chromatography and the underlying mechanism has been discussed, for example by Knox and Hartwick [12].

*Chromatography of urine*

Urine samples were initially analyzed using a TBA concentration of 5 m*M*. The thiosulphate peak was identified from its retention time determined with a standard solution and by the peak enhancement technique. However, the thiosulphate peak obtained at this TBA concentration was tailing (Fig. 2a), suggesting the presence of an unresolved peak. In fact, when the TBA concentration was raised to 15 m*M*, the shape of the thiosulphate peak improved and a minor peak appeared close to the thiosulphate peak (Fig. 2b). As higher TBA concentrations did not improve the results, a concentration of 15 m*M* was used in the final procedure. The standard curve (Fig. 3) was slightly non-linear. We thus found it convenient to calculate the thiosulphate concentrations of unknown samples from the standard curve using non-linear regression calculations with a desk-top computer [13]. Before chromatography, urine samples were passed through Sep-Pak columns to remove non-polar constituents, which otherwise might irreversibly bind to the reversed-phase column. Although not proven, we surmise that the Sep-Pak treatment of urine results in a prolonged

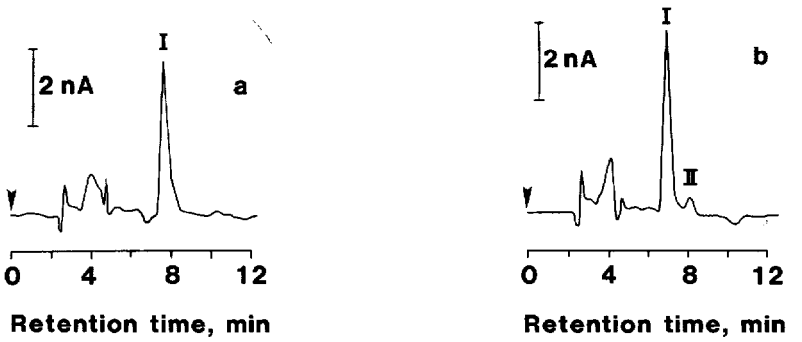


Fig. 2. Representative chromatograms of urine. (a) Mobile phase containing 0.05 *M* TBA. (b) Mobile phase containing 0.15 *M* TBA. Peak I = thiosulphate. Peak II = unknown component, resolved from thiosulphate at higher TBA concentrations.

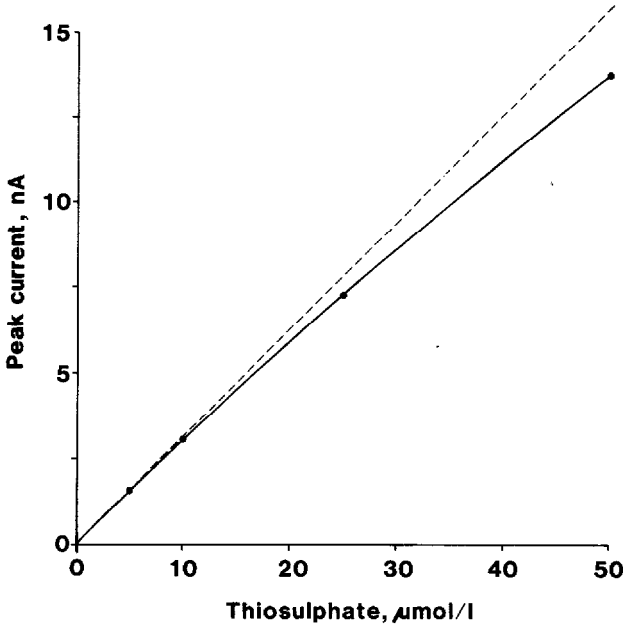


Fig. 3. Standard curve for thiosulphate.

life of the analytical column. As verified by experiments with standard solutions, the recovery of thiosulphate in this step was quantitative.

*Precision and sensitivity*

The intra-assay precision of the method was evaluated by analyzing ten aliquots of a urine sample. The result, 19.9 ± 0.82 µmol/l (mean ± S.D.), corresponds to a coefficient of variation of 4.2%. Similarly, the inter-assay precision was determined by analyzing the same urine on ten different days, giving 21.4 ± 1.1 µmol/l (mean ± S.D.), or a coefficient of variation of 5.3%. The sensitivity of the method was evaluated by ten injections of "simulated urine" [14] devoid of thiosulphate. The blank values thus obtained had an S.D. value corresponding to a thiosulphate concentration of 0.16 µmol/l, and the

detection limit was thus 0.3 μmol/l if defined as twice the S.D. of blank determinations.

*Recovery*

Thiosulphate was added to eleven urine samples of known thiosulphate concentrations (range 3.0—27.6 μmol/l) to increase the latter by 20 μmol/l. Renewed thiosulphate determinations on these samples gave a recovery of thiosulphate corresponding to 88.4 ± 4.2% (mean ± S.D.). When similar experiments were conducted with "simulated urine" a recovery of 98.0 ± 2.2% (mean ± S.D.) was obtained. The somewhat lower recovery obtained with authentic urine is thus probably due to a matrix effect. As the effect was small it was neglected in routine determinations.

*Stability*

When five freshly voided urine samples were analyzed for thiosulphate and then kept for 24 h at room temperature with thymol—isopropanol as a preservative, the repeated determination of thiosulphate gave 95.2 ± 6.0% (mean ± S.D.) of the original value. Furthermore, when aliquots of a normal urine sample were stored at −20°C, no significant change of the initial value was observed after 45 days of storage.

*Method comparison*

When urine samples from healthy subjects were analyzed by the present method and by an earlier described colorimetric method [8], the latter was found to give somewhat higher values (Fig. 4). In the light of the satisfactory recovery of thiosulphate obtained with the HPLC method, the colorimetric method apparently overestimates urinary thiosulphate, especially in the lower normal range.

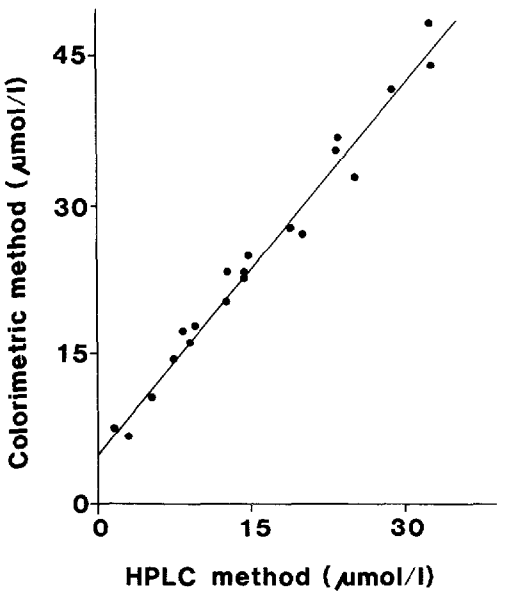


Fig. 4. Correlation between present method and colorimetric method for determination of thiosulphate in urine. Regression line: $Y = 1.23X + 5.23$ ($r$=0.99).

TABLE I

THIOSULPHATE EXCRETION IN TRAUMA PATIENTS

| Diagnosis | Thiosulphate (μmol per 24 h) | |
|---|---|---|
| | Colorimetric method | HPLC |
| Burn + septicemia | 146 | 1.1 |
| Burn + septicemia | 278 | 5.5 |
| Multiple trauma + septicemia | 167 | 2.1 |
| Multiple trauma + meningitis | 112 | 6.9 |

The unspecificity of the colorimetric method was dramatically illustrated by results obtained on certain patient samples where the colorimetric method gave considerably higher values than the HPLC method (Table I). Apparently, these patients excreted an unknown compound which behaved as thiosulphate in the colorimetric method. Furthermore, we observed that this compound in contrast to thiosulphate was not precipitated as a nickel—triethylenediamine complex [5] and was not retained on an organomercurial adsorbent [15]. However, its chemical identity remains to be established. The (few) patients who excreted this compound suffered from thermal or mechanical trauma complicated by a serious bacterial infection (Table I) and were treated with heavy doses of antibiotics. However, the mechanism behind the excretion of the thiosulphate-mimicking compound is unknown.

*Urinary excretion in normal subjects*

The excretion of thiosulphate in twelve healthy males and eight healthy females on a free diet was found to be 27.3 ± 21.5 μmol per 24 h and 18.1 ± 13.1 μmol per 24 h (mean ± S.D.), respectively. There was no significant difference between the sexes and the results were consequently combined giving an overall mean as 23.6 ± 18.7 μmol per 24 h. This is somewhat lower than the previously reported value of 31.7 ± 12.8 μmol per 24 h obtained with the colorimetric method [8].

## DISCUSSION

Thiosulphate is detected in the present HPLC method with a mercury electrode, which is unfortunately not commercially available. Recently, an HPLC method for the determination of thiosulphate was reported [16] which used a commercially available glassy carbon working electrode. However, the method was only applied to the analysis of aqueous solutions of thiosulphate. We found in preliminary experiments that although a glassy carbon detector may be used for thiosulphate analysis of aqueous solutions, it was not applicable to the direct analysis of urine due to interference from a number of electroactive substances.

The present HPLC method is apparently more specific than the earlier colorimetric method. This is supported by the fact that it gives lower values for normal urine than the earlier colorimetric method and the demonstration of a

compound in urine from certain patients which interferes in the colorimetric determination. On the other hand, with the HPLC method we have confirmed (unpublished observations) a previous report [17] that subjects with the metabolic disorder mercaptolactatedisulphiduria excrete normal amounts of thiosulphate. This is of special importance with respect to the suggested biochemical abnormality underlying this condition [17].

The HPLC method has other advantages over the colorimetric method such as better precision and higher sensitivity. Furthermore, a smaller amount of urine is required for the HPLC method, which is an advantage when urine from newborn infants needs to be analysed. The HPLC method is also less time-consuming and consequently a higher number of samples can be processed in one working day.

It should be pointed out that thiosulphate in human urine is sufficiently stable to permit 24 h urine collection at room temperature, as demonstrated by the present investigation. On the other hand, we have confirmed (unpublished results) a previous observation by Gunnison et al. [18] that thiosulphate is fairly unstable in rat urine. This may be explained by a heavy contamination with faecal bacteria which is difficult to avoid during collection of rat urine in a metabolic cage.

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