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Note

Determination of S-sulfocysteine in urine by high-performance liquid chromatography

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S-Sulfocysteine (cysteine-S-sulfonic acid) was first obtained by Clarke [1] as a reaction product from cystine and sulfite and was later identified [2] as an intermediate in the conversion of inorganic sulfate to organic sulfur compounds by moulds. It was furthermore reported that sulfocysteine was present in the urine of rats after injection of cystine [3], and remarkably high concentrations of sulfocysteine have been found in the urine of the blotched Kenya genet [4]. This compound has also been demonstrated in plasma and urine from animals exposed to sulfur dioxide or sulfite in the diet [5, 6], and high concentrations of sulfocysteine are present in the urine of patients suffering from sulfite oxidase deficiency [7].

When present in high concentrations in biological material, sulfocysteine may be determined with a conventional amino acid analyzer [7, 8]. A thin-layer chromatographic screening technique has also been reported for the detection of elevated concentrations of sulfocysteine caused by sulfite oxidase deficiency [9]. Furthermore, a gas chromatographic method for the determination of sulfocysteine after enzymatic hydrolysis of sulfonated proteins has been reported [10]. Unfortunately, neither of these methods is sufficiently sensitive and specific for the determination of the fairly low concentrations of sulfocysteine present in the urine of normal human beings. Sulfocysteine is a thiosulfate ester and we recently developed a liquid chromatographic method for the determination of urinary thiosulfate using a mercury-based electrochemical detector [11]. However, this mode of detection was not applicable to urinary sulfocysteine. Liquid chromatography with fluorometric detection was then considered as an attractive alternative, provided that a fluorescent derivative of sulfocysteine could be prepared. Pre-column derivatization with

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o-phthalaldehyde in the presence of mercaptoethanol [12], succesfully used for the determination of amino acids in physiological fluids, appeared unsuitable to the determination of sulfocysteine, as the latter would react with mercaptoethanol to give cysteine [13], which yields an *o*-phthalaldehyde derivative with low fluorescence [12]. On the other hand, the Dns derivatives of amino acids [14] show very strong fluorescence and the preparation of Dns-sulfocysteine would not be expected to cause problems.

We have now developed a method for the determination of urinary sulfocysteine based on a preliminary separation of sulfocysteine from interfering compounds by ion-exchange chromatography followed by its conversion to the Dns derivative. The latter is then determined by high-performance liquid chromatography with fluorometric detection.

EXPERIMENTAL

Materials

Sulfocysteine synthesized as described previously [15] was chromatographically pure when examined by our liquid chromatographic procedure. On the other hand, we found that a commercially available product (Pierce, Rockford, IL, U.S.A.) contained an unidentified impurity which reacted with Dns chloride. The latter was a product from Fluka (Buchs, Switzerland). Ion-exchange resins AG 50W-X8 (H^+, 100—200 mesh) and AG 3-X4A (Cl^-, 200—400 mesh) were obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.) and used as received.

Procedure

Human urine was collected for 24 h with thymol—isopropanol as a preservative. A 1-ml aliquot was applied to a 3.2 × 1.0 cm column of AG 50W-X8 and the column was washed with 6 ml of water. The combined effluent and washings were neutralized with 1 *M* sodium hydroxide solution and applied to a 3.2 × 1.0 cm column of AG 3-X4A. The column was washed with 8 ml of water and the effluent and washings were discarded. The sulfocysteine was then eluted with 8 ml of 1 *M* sodium chloride solution. A 1.5-ml aliquot of the eluate was then transferred to a 10-ml test tube with a PTFE-lined screw-cap and 0.5 ml of 0.12 *M* lithium carbonate solution, adjusted to pH 9.5 with hydrochloric acid, and 1 ml of a solution of Dns chloride (1.5 g/l) in acetonitrile, were added. After vortex-mixing, the reaction mixture was left in the darkness overnight at room temperature and was then taken to dryness at 60°C using a Vortex Evaporator (Buchler Instruments Inc., Fort Lee, NJ, U.S.A.). The residue was then suspended in 0.5 ml of the mobile phase used for chromatography as described below, by vortex-mixing for two 3-min intervals separated by a 3-min sonification period. After centrifugation, 100 μl of the clear supernatant were taken for chromatography.

Our liquid chromatographic system consisted of a Constametric III pump (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 sample injector with a 100-μl sample loop, an Apex Silica column, 5 μm, 250 × 4.5 mm (Jones Chromatography, Llanbradach, Great Britain) and a Fluoromonitor III fluorometric detector (Laboratory

Data Control) with a strip chart recorder. The detector was operated with a mercury lamp and a 360-nm excitation filter and a 418–700-nm emission filter. The mobile phase was toluene–pyridine–acetic acid–ethanol (8:0.2:2:1) and was delivered to the column at a flow-rate of 1 ml/min at room temperature. Peak heights were measured for quantitative determination of sulfocysteine and a standard curve was prepared from sulfocysteine solutions of known concentrations.

RESULTS AND DISCUSSION

Preliminary experiments showed that sulfocysteine was easily converted to its Dns derivative using a procedure slightly modified from that of Tapuhi et al. [14]. As the fluorescence of Dns-amino acids is considerably lowered in polar solvents [16] we decided to use normal-phase liquid chromatography on a silica column for the separation of Dns-sulfocysteine from other Dns derivatives of urinary compounds. The mobile phase system benzene–pyridine–acetic acid–methanol described by Bayer et al. [17] was taken as a starting point for further development of the method. However, we replaced benzene with the less toxic toluene and obtained a better separation of sulfocysteine with a higher concentration of acetic acid than in the original phase system. Furthermore, we observed that the retention time of sulfocysteine varied from day to day when methanol was a component of the system. When ethanol was substituted for methanol the retention time became more reproducible, presumably due to a decreased esterification rate of ethanol with acetic acid. When liquid chromatography was attempted on urine samples after Dns derivatization, no Dns-sulfocysteine could be demonstrated due to the presence of interfering compounds.

Chromatography on a cation-exchange resin eliminated neutral and basic amino acids [2], but liquid chromatography after Dns derivatization of the pretreated sample still gave unsatisfactory results. A subsequent anion-exchange chromatographic step [18] removed remaining interfering compounds. A typical chromatogram of human urine after this clean-up procedure is shown in Fig. 1.

The identity of the peak attributed to Dns-sulfocysteine was verified by subjecting the material present in this peak to two-dimensional thin-layer chromatography on silica gel G (E. Merck, Darmstadt, G.F.R.) using the solvent system [19] methyl acetate–isopropanol–ammonia (9:7:4) in the first dimension and methyl ethyl ketone–propionic acid–water (15:5:6) in the second. Only one fluorescent component which migrated as authentic Dns-sulfocysteine was found in this peak.

The standard curve was linear at least up to concentrations of sulfocysteine corresponding to 15 μmol/l of urine. The recovery of authentic sulfocysteine added to urine samples of known sulfocysteine concentration to increase the latter by 5 μmol/l was 91 ± 11% (mean ± S.D., n = 5) and the detection limit was estimated at 0.1 μmol/l. The intra-assay precision evaluated from seven replicate analyses of a urine sample was 2.9% (coefficient of variation) and the inter-assay precision obtained from analysis of a urine sample on seven different days was 8.1%. The excretion of sulfocysteine in ten healthy males

and ten healthy females on a free diet was 11.8 ± 5.5 μmol per 24 h and 8.0 ± 2.7 μmol per 24 h (mean ± S.D.), respectively. As there was no significant difference between the sexes the results were combined giving an overall mean of 9.9 ± 4.7 μmol per 24 h (range 3.9—25.1 μmol per 24 h).

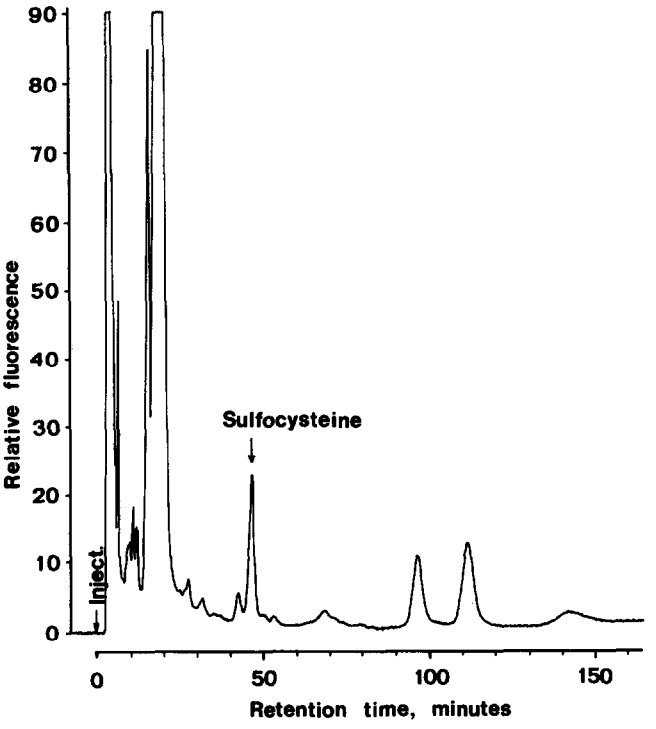

Fig. 1. Chromatogram of human urine.

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