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Note

Electrophoresis of thiols in cellulose gels

III. Group analysis of urinary thiols

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Urinary thiols and their disulphides comprise a rather long list of compounds that have been identified either as of natural origin including pathological excretion (e.g. cysteine, acetylcysteine, cysteinylglycine, mercaptoacetic acid, lipoic acid, cysteamine, methanthiol, mercaptolactic acid, homocysteine, glutathione), or as resulting from administered medicines (e.g. thiomalic acid, penicillamine, captopril [1, 2]. The analytical methods described in the literature are as a rule limited to the estimation of a single thiol, and no efforts have been encountered to demonstrate a general urinary thiol balance. An attempt to do so is given in this paper.

As previously suggested [3] the thiols may be separated into five groups on the basis of extraction of the free thiols and of their tributyltin (TBT) mercaptides. The thiols of groups 0 and 1 can be extracted with hexane, the thiols of group 2 with butanol; the free thiols of group 3 can not be extracted from aqueous solution. The TBT mercaptides of group 0 in hexane are not decomposed by alkalies whereas those of the group 1 are. The TBT mercaptides of group 2 are soluble in hexane and those of group 3 in butanol. The TBT mercaptides of group 4 are insoluble in organic solvents. Of course there is not a sharp distinction between the groups and the determined group composition depends on the procedure used.

In the present study the urinary thiols are separated into three groups by a procedure involving extraction of TBT mercaptides with butanol followed by stripping with alkalies. Group 4 thiols remain in aqueous solution, group 0 in butanol, and thus the sum of groups 1, 2 and 3 can be calculated by difference.

It has also been suggested to take account of the selective blocking of certain

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aminothiols by formaldehyde [4, 5]. Thiols such as cysteine, cysteamine, homocysteine, penicillamine and cysteine peptides with a free amino group of cysteine are referred to in this paper as active aminothiols. They can be determined as the difference between the total thiol content and the thiol content after adding formaldehyde using titration with *o*-hydroxymercuribenzoic acid (HMB) in the presence of dithiofluorescein as indicator [6].

To complete the thiol balance further separation is suggested on the basis of electrophoresis. In neutral media mercaptoacids can be separated from a mixture of neutral thiols and aminothiols and the mixture can be analysed using selective blocking with formaldehyde.

The thiols extracted as TBT mercaptides have to be recovered for electrophoresis as concentrated solutions, as far as possible free from electrolytes. In this study the thiols of groups 0, 1, 2, and 3 are extracted together as their TBT mercaptides with butanol and the extract is diluted with hexane. Stripping with acetic acid solution provides the samples for electrophoresis in acid solution, and stripping with a solution of barium hydroxide in glycerol followed by neutralization with sulphuric acid provides samples in neutral medium. To prevent oxidation a higher thiol (e.g. hexadecanethiol) has been added.

The thiols can be titrated with HMB at pH 8—11 using dithiofluorescein as indicator or in 0.05—0.1 *M* potassium (or sodium) hydroxide using dithizone as indicator. The titration can be carried out in a test tube even with a $2.5 \cdot 10^{-5}$ *M* solution of HMB. To avoid errors when dealing with urinary thiols the following points should be observed: (1) As the thiols of group 4 react slowly with the dithiofluorescein—HMB complex, the result of direct titration is too low. The HMB must be added in excess. This is not necessary when thiols of group 4 are absent. (2) In the presence of TBT only dithizone can be used as indicator. (3) In sodium hydroxide solution the urinary thiols are rapidly oxidized with air. The titration should be repeated and completed in as short a time as possible. The pure thiols are not so sensitive to oxidation.

EXPERIMENTAL

Reduction

The apparatus for reduction, described in another paper [7], is composed of a cooling water jacket, a lead cathode with each side of 125 cm^2 surface area, a ceramic tube and a lead anode in 2 *M* sulphuric acid. The urine was acidified with 1 ml of 98% sulphuric acid per 100 ml. To prevent foaming a drop of a silicone suppressor was added. The reduction of 100 ml of urine was carried out at room temperature for 2 h with a current of 0.5 A.

Analysis of the reduced urine

Procedure for total thiols. To 2 ml of the sample add 1 ml of 1 *M* triethanolamine and 1 ml of a solution of dithiofluorescein (it should consume about 1 ml of HMB solution per ml), and titrate with 10^{-4} *M* HMB until the blue colour disappears. Repeat the titration adding first the volume of HMB found previously with 20% excess, then add dithiofluorescein until the sample becomes blue and titrate until it becomes colourless again. The HMB should be

added in excess followed by an excess of dithiofluorescein. The result is the difference between the total volume of HMB and the consumption of HMB by the added dithiofluorescein.

Procedure for active aminothiols. Take 4 ml of the sample and titrate as above after adding 0.2 ml of 1 *M* formaldehyde. The concentration of active aminothiols is the difference between the amount of total thiols and the thiols found in this titration.

Extraction

Place 100 ml of reduced urine in a 250-ml separating funnel and dissolve in it 5 g of triethanolamine and 5 g of anhydrous sodium sulphite; the resulting pH should be between 7.5 and 8. Add 25 ml of a 0.06 *M* solution of TBT hydroxide in butanol and mix vigorously for 10 min by a stream of nitrogen. Separate the extraction layer and centrifuge to remove droplets of water and sediments. Add 1 g of sucrose to the extraction layer and store at 4°C with protection from light. Measure the volumes of the extraction and raffinated layers.

Determination of thiols of group 4

Extract 5 ml of the raffinated layer with 2.5 ml of 0.06 *M* TBT hydroxide in butanol. Add to 2 ml of the new raffinated layer 1 ml of 2 *M* potassium hydroxide and titrate with 10^{-4} *M* HMB using dithizone as indicator. Repeat the titration adding first the volume of HMB as found above, then add potassium hydroxide and indicator and finish the titration. The thiols found are assumed to belong to group 4.

Determination of thiols of group 0

Add to 2 ml of the extraction layer 2 ml of hexane and strip two times with 4 ml of 0.5 *M* potassium hydroxide. Take 2 ml of organic layer, add 3 ml of propanol and 0.2 ml of 2 *M* potassium hydroxide and titrate with 10^{-4} *M* HMB using dithizone as indicator. The result calculated for the total volume of the extraction layer corresponds to the group 0 thiols in 100 ml of urine.

Preparation of samples for electrophoresis

For acid medium. Add to 5 ml of extraction layer 4 ml of 0.1 *M* hexadecanethiol in hexane and 0.4 ml of solution prepared by mixing 1 ml of acetic acid with 10 g of glycerol. Shake vigorously for 5 min, remove the upper layer and wash the glycerol layer with 2 ml of hexane.

For neutral medium. Place in a polyethylene test tube 5 ml of the extraction layer, 4 ml of 0.1 *M* hexadecanethiol in hexane and 0.4 ml of a solution prepared by dissolving while heating 2 g of barium hydroxide octahydrate in 10 g of glycerol. Shake vigorously for 5 min, remove the upper layer and wash the glycerol layer with 2 ml of hexane. Neutralize the sample with 2.5 *M* sulphuric acid in 50% (w/w) glycerol in the presence of phenol red.

Determination of mercaptoacids, aminothiols and neutral thiols in neutralized glycerol layer

Carry out electrophoresis in a cellulose gel rod in neutral solution as described previously [3] using a 0.4-ml sample. When the mercaptoacids have

penetrated the gel, wash out the remaining thiols (aminothiols and neutral thiols) with water, dilute to 10 ml, extract with 1 ml of hexane to remove hexadecanethiol, if any, and analyse for total thiols and aminothiols as described above. Continue the electrophoresis with the gel rod in inverted position and control the emerging mercaptoacids by titration with $2.5 \cdot 10^{-5}$ *M* HMB using dithizone as indicator. From the results obtained calculate the percentage composition of thiols.

RESULTS AND DISCUSSION

The distribution of thiols in three samples of urine are given in Table I. Electropherograms obtained show at pH 1.8 one peak of cysteine, and at pH 7.2 two peaks — one corresponding to mercaptoacetic acid and the other to acetylcysteine.

TABLE I

DISTRIBUTION OF THIOLS IN REDUCED URINE (COLUMNS 2—6) AND IN THE SAMPLE PREPARED FOR ELECTROPHORESIS IN NEUTRAL MEDIUM (COLUMNS 7—9)

Sample	Total thiols (μmol/l)	Active aminothiols (μmol/l)	Group composition (%)			Thiol distribution (%)		
			0	1—3	4	Mercapto-acids	Amino-thiols	Neutral thiols
1	475	366	3	65	32	14	79	7
2	185	130	9	81	10	6	62	32
3	245	174	8	77	15	7	71	22

The active aminothiols form about 75% of the total thiols. The cysteine content can be roughly calculated from the percentage composition of thiols. As calculated from previously given results [8], when 25 ml of 0.06 *M* TBT in butanol are used for extraction from 100 ml of neutral solution, only 84% of cysteine will be extracted. Taking into account that cysteine belongs to group 3, its content in sample 1 will be given as $0.79 \times 0.65 \times 475/0.84 = 290$ μmol/l, and the values for samples 2 and 3 amount to 111 and 160 μmol/l, respectively, i.e. 61%, 60% and 65% of total thiols, or 79%, 85% and 92% of active aminothiols.

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