ELSEVIER

Journal of Chromatography B, 676 (1996) 29–34

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Separation of urinary thiols as tributyltinmercaptides and determination using capillary isotachophoresis

Mieczysław Wroński

Department of Chemical Technology and Environmental Protection, University of Łódź, Pomorska 18, 91-416 Łódź, Poland

Received 16 May 1995; revised 7 September 1995; accepted 19 September 1995

Abstract

The essential steps in the assay included electrolytic reduction of disulphides, neutralization, extraction of thiols with 0.1 *M* tributyltin hydroxide in octane, stripping of the extract with 2% acetic acid, fixing the washed-out amino thiols to a cation exchanger, elution with 2 *M* hydrochloric acid, oxidation with bromine and evaporation. The remaining octane extract was decomposed by dodecanethiol, the mercapto acids were washed out, oxidized with bromine and evaporated. Both residues were dissolved in water and analysed using capillary isotachophoresis at pH 3.0. Cysteamine was extracted from reduced urine at ca. pH 10, decomposed by dodecanethiol and re-extracted to boric acid followed by determination as a cation. The presence of the following thiols in urine has been confirmed: mercaptoacetic acid, 3-mercaptolactic acid, 2-mercaptopropionic acid, acetylcysteine, mercaptoethanol, cysteine, homocysteine and an un-identified amino thiol. Cysteamine and 3-mercaptopropionic acid could not be detected. Captopril, homocysteine and cysteine were determined quantitatively.

Keywords: Thiols; Tributyltinmercaptides; Mercaptoacetic acid; 3-Mercaptolactic acid; 2-Mercaptopropionic acid; Acetylcysteine; Mercaptoethanol; Cysteine; Homocysteine; Penicillamine; Cysteamine; Captopril

1. Introduction

Many different techniques have been suggested for the determination of thiols in biological materials, including the use of either fluorogenic and UV derivatization [1–3] or electrochemical detection [4,5]. Sörbo and Hannestad [6] developed a gas chromatographic method for the determination of 3-mercaptolactic acid, mercaptoacetic acid and acetylcysteine in urine.

Capillary isotachophoresis and capillary zone electrophoresis have been used relatively rarely for the analysis of thiols, in spite of their many advantages. Holloway [7] and Ling and Baeyens [8] suggested the use of fluorogenic bimane derivatives. Mizobuchi et al. [9] developed a method for the determination of cystine, homocystine and cystathionine in urine using separation on a cation exchanger. Pure mercapto acids, their disulphides and corresponding sulphonic acids can be easily determined by isotachophoresis [10,11]. However, biological materials contain many ionic species which usually entirely overlap with the small amounts of mercapto acids or sulphonic acids. For that reason successful and reliable isotachophoretic analysis must be preceded by a separation and fractionation of thiols to separate them as much as possible from foreign ions. Extraction with tributyltin (TBT) hydroxide to form TBT mercaptides, which are soluble in organic solvents, has been recommended for the separation

0378-4347/96/$15.00
SSDI 0378-4347(95)00404-1

of thiols from aqueous solutions [12–15]. TBT hydroxide dissolved in an organic solvent behaves as a liquid anion exchanger with a great affinity for the mercapto group. Thus thiols can be separated from a large excess of sulphate, chloride and many other ions. Saturation of the aqueous solution with ammonium sulphate increases the extraction efficiency of thiols.

The TBT mercaptides can either be decomposed by hydrochloric acid or replaced by another thiol such as dodecanethiol that is present in excess. However, the liberated thiols are still contaminated with anions co-extracted with TBT. These foreign ions can be removed from the extract by washing with diluted acetic acid and then fixing the washed-out amino thiols to a cation exchanger. The mercapto acids remain in the extract and can be obtained in an almost pure state. The fixed amino thiols are separated from foreign ions by washing, and eluted with hydrochloric acid. These amino thiols can be analysed either directly, e.g. cysteamine, or as sulphonic acid. The great advantage of this procedure is the almost complete removal of interferences. The pure background in isotachophoresis assures the high sensitivity and separativity of the measurements.

2. Experimental

The preparation of reagents was described in other papers [12–16].

2.1. Reduction of disulphides

The apparatus for electrolytic reduction [17] consisted of a cooling water jacket, a lead cathode round a ceramic tube containing a lead anode in 5% (v/v) 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 was carried out at room temperature at a current density of 0.01 A/cm^2 of cathode surface for 45 min. The anolyte should be renewed for every reduction because of migration of chloride.

2.2. Titrimetric determination of cysteine and mercapto acids [18]

To a 5-ml volume of reduced urine, 0.1 ml of 0.1 *M* EDTA and discoloured with *o*-hydroxymercuribenzoate (HMB) dithiofluorescein [16] were added and titrated with triethanolamine just to the appearance of a blue colour. The solution was titrated with 0.2 m*M* HMB until the colour disappeared. The consumption of HMB, V_1 ml, corresponded to the total thiol content. Another 5 ml of reduced urine were processed in the same way but before titration with HMB, 0.2 ml of 1 *M* formaldehyde was added. The consumption of HMB, V_2 ml, corresponded this time to mercapto acids and neutral thiols and the difference between the two values corresponded to the sum of cysteine, homocysteine and cysteinyl-glycine but it could be roughly assumed to correspond only to cysteine. Thus the concentration of cysteine (c) in nmol/ml is given by

$$c = 0.04(V_1 - V_2) \quad (1)$$

2.3. Extraction

To a 25-ml volume of reduced urine 0.2 ml of 0.1 *M* EDTA and ca. 150 mg of ascorbic acid were added and the solution was titrated with triethanolamine in the presence of phenol red indicator to the first change of colour. The extraction was performed by adding 15 g of ammonium sulphate and shaking for 2 min with 6 ml of 0.1 *M* TBT hydroxide in octane. The required pH for the aqueous phase was ca. 7.0. The aqueous phase was removed and the remainder was washed three times with 2 ml of water and 5 ml of clear extract, taken through a small ball of cotton, and then transferred into another separating funnel.

Glutathione was not extracted by TBT in octane. The mercaptoacids were extracted with an efficiency of about 90%, whereas the homocysteine efficiency was about 70%. A second extraction would have increased the last value to above 90%, but as the reproducibility of the results was, in general, satisfactory only one extraction was retained. The extraction of amino

thiols not containing carboxylic groups must be performed from alkaline solution.

2.4. Separation of amino thiols (cysteine and homocysteine)

A 5-ml sample of the extract was shaken three times with 2 ml of 2% (v/v) acetic acid and once with 2 ml of water. All washings were put in turn on a small column of 0.8 cm I.D. containing 0.8 cm^3 of cation exchanger Dowex WX4 in the H^+ form. The column was washed with 5 ml of water, the fixed amino thiols were eluted with 8.0 ml of 2 *M* hydrochloric acid and the eluate was collected in a 10-ml vial, oxidized with a drop of 1 *M* bromine in carbon tetrachloride and evaporated at 85°C at normal pressure on a thermostated plate with holes. The residue was dissolved in 0.25 ml of water and 10 μl was used for anion isotachophoretic analysis. Cysteine and homocysteine peptides were hydrolysed in the course of evaporation. The action of bromine on homocysteine resulted in the formation of two compounds moving at different rates, one being homocysteic acid and the other one probably a cyclic sultam shown in Fig. 1. In the course of evaporation with 2 *M* hydrochloric acid the last compound was converted to homocysteic acid.

2.5. Separation of mercapto acids and neutral thiols

The mercaptides in the remaining extract were decomposed by adding 0.7 ml of 1 *M* dodecanethiol in octane (purified by washing with 0.2 *M* potassium hydroxide) and the free thiols were re-extracted into water by shaking three times for 1 min with 2 ml of water. This extract was washed with 2 ml of octane (in order to remove the traces of dodecanethiol), oxidized with bromine and evaporated at 75°C at normal pressure. The residue was dissolved in 0.25 ml of water and 10 μl was used for isotachophoretic analysis. To avoid serious losses of acetylcysteine and mercaptolactic acid, the extract in procedure 4 should be washed only once with 2 ml of 2% acetic acid and twice with 2 ml of water.

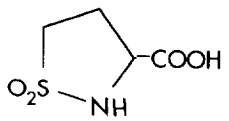

Fig. 1. Cyclic sultam formed by oxidation of homocysteine with bromine.

The sulphonic acids derived from acetylcysteine and 2-mercaptopropionic acid cannot be resolved at pH 3.0. When, however, the sample is evaporated with 5 ml of 2 *M* hydrochloric acid after isotachophoretic analysis, the sulphonic acid of acetylcysteine will be hydrolysed leaving the other constituent unchanged. Acetylcysteine content can then be calculated by difference.

2.6. Determination of penicillamine in the presence of homocysteine

To a 25-ml volume of reduced urine, ca. 150 mg of ascorbic acid were added and the sample was titrated with 2 *M* potassium hydroxide containing 1 m*M* EDTA to a colour change of added phenolphthalein. After the addition of 0.2 ml of acrylonitrile, the sample was left aside for 3 min at room temperature and the pH was then adjusted to ca. 6.5 by titration with 1 *M* sulphuric acid in the presence of phenol red. A 15-g amount of ammonium sulphate was added to the solution, followed by extraction and separation as described in procedures 3 and 4.

Homocysteic acid and dimethylcysteic acid could not be resolved by the isotachophoretic method and only the sum of both compounds could be determined. There was, however, a great difference between rate constants of parent thiols in reaction with acrylonitrile [15]; e.g. at pH 9.0 the rate constants (l mol^{-1} min^{-1}) are: cysteine 23, homocysteine 18.6, penicillamine 0.35. It was thus possible to eliminate homocysteine leaving penicillamine almost unchanged as is demonstrated in Fig. 3. The treatment with acrylonitrile also eliminated all remaining mercapto acids and neutral thiols leaving phosphoric

acid and pyrophosphoric acid unchanged, and this can be used for their identification.

2.7. Separation of amino thiols from alkaline solution

A volume of 25 ml of reduced urine containing 150 mg of ascorbic acid was titrated with 2 *M* potassium hydroxide plus 1 mM EDTA to a full change of the colour of phenolphthalein. The solution was extracted with 5 ml of 0.1 *M* TBT hydroxide in octane by shaking for 3 min. If the red colour disappeared some more potassium hydroxide was added. After phase separation the lower phase was removed and 1 ml of *n*-butanol was added to the upper layer which was composed of foam and emulsion. This resulted in good separation after shaking. The organic phase was washed twice with 2 ml of water, the 5 ml of clear extract was taken through a cotton ball and placed in a vial, 0.7 ml of 1 *M* dodecanethiol in octane was added and the amino thiols were re-extracted to 0.5 ml of 5% boric acid by shaking for 3 min. A 10-μl sample was then used for isotachophoretic analysis.

2.8. Isotachophoretic analysis

The measurements were carried out using an isotachophoretic analyser ZK_2 from Labeco, Spišská Nová Ves (Slovak Republic) with preseparation and analytical columns: lengths 90 and 160 mm, inner diameters 0.8 and 0.3 mm, and the following buffers: anions, leading: 114 mg of beta-alanine in 100 ml of 0.01 *M* hydrochloric acid plus 0.2% PEG 200 as an additive; terminating: 0.01 *M* glucuronic acid. Cations, leading: 0.02 *M* acetic acid plus 0.01 *M* sodium hydroxide, 0.2% PEG 200; terminating: 0.01 *M* tetrabutylammonium bromide. The mobilities (u), expressed in 10^{-5} cm^2 s^{-1} V^{-1} units, were calculated [19,20] using as standards trichloroacetic acid for anions and tetrabutylammonium for cations by means of the following equations:

$$\text{anions: } u = 79(1 + 1.18\,h)^{-1},$$

$$\text{cations: } u = 51.9(1 + 1.70\,h)^{-1} \qquad (2)$$

where h=relative step height with regard to the standard. The relative step height of trichloroacetate with regard to glucuronic acid was 0.440 at pH 3. The driving current in the preseparation column was 250 μA, and in the analytical column it was 50 μA. During the determination of homocysteic acid, the excess of cysteic acid was cut off and only the remains of it were brought into the analytical column and registered using a conductivity detector.

3. Results and discussion

The urinary mercapto acids and neutral thiols are shown in Fig. 2, the mercaptoamino acids in Fig. 3 and the amino thiols in Fig. 4. The following components may be assumed as constant constituents of the urine: mercaptoacetic acid, 2-mercaptopropionic acid, 3-mercaptolactic acid, acetylcysteine, mercaptoethanol, cysteine and homocysteine. Apart from thiols, traces of extracted phosphoric acid and pyrophosphoric acid can be identified.

The best approach for identification of thiols and of the sulphonic acids derived from them is the calculation of mobility at a given pH. The method used for this calculation in this paper can be explained by the following example. In Fig. 2 step height of 1-(3-sulpho-2-methyl-1-oxopropyl)-proline is 121 mm, and total step height is 196 mm. Relative step height for glucuronic acid is 121/196=0.617, and it is 0.617/0.44=1.40 for trichloroacetate. The mobility is given by 79/(1+1.4×1.18)=29.8. In [11] the reported mobility is 30.0.

Hydrolysis of urine before reduction for 48 h at 90°C in 1 *M* hydrochloric acid did not bring, apart from hydrolysis of acetylcysteine, any remarkable changes. As can be seen in Fig. 3, the steps for homocysteic acid and for dimethylcysteic acid are well shaped and almost identical. The treatment with acrylonitrile eliminates homocysteine but not penicillamine. It follows that homocysteine and penicillamine can be determined in the presence of each other.

Fig. 4 demonstrates the steps of amino thiols

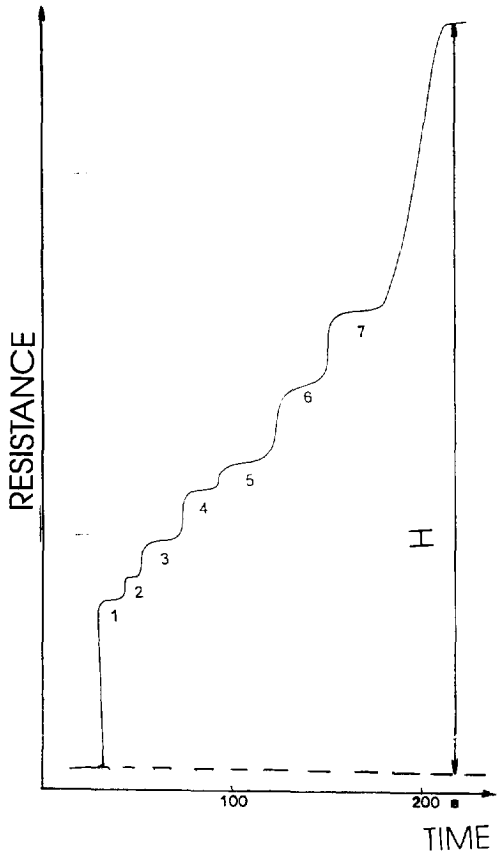

Fig. 2. Isotachopherogram of sulphonic acids derived from thiols separated from urine. 1=pyrophosphoric acid, u=48.7; 2=sulphoacetic acid, u=45.9; 3=sulpholactic acid, u=43.0; 4=hydroxyethanesulphonic acid, u=39.4; 5=mixed zone containing acetylcysteic acid and 2-sulphopropionic acid, u=37.4; 6=phosphoric acid, u=33.3; 7=1-(3-sulpho-2-methyl-1-oxopropyl)-proline from added captopril, u=29.8.

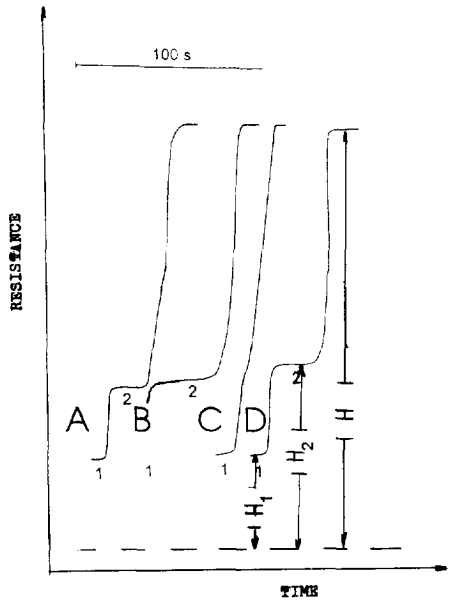

Fig. 3. Isotachopherograms of sulphonic acids derived from cysteine (the greater part is cut off), homocysteine and penicillamine. H=total step height=184 mm; H_1=step height of cysteic acid=100 mm, u=32.1; H_2=step height of homocysteic acid and dimethylcysteic acid=123 mm, u=28.3. A=cysteic acid and homocysteic acid; B=homocysteic acid after adding homocysteine to a concentration of 10 nmol/ml in excess to urine; C=reduced urine treated before extraction with acrylonitrile; D=urine containing 11 nmol/ml of penicillamine treated with acrylonitrile.

separated from alkaline solution. In natural samples the presence of cysteamine could not be detected but the response for added cysteamine is evident. It is interesting to note the occurrence of an unknown amino thiol, different from thiamine. Table 1 shows the results of examination of quantitative analysis of three thiols by described procedures. Increasing concentrations of thiols were added to normal urine. The endogenous homocysteine content of urine samples was subtracted from the homocysteine enriched samples.

The results obtained by examination of seven different samples of the same origin but taken at different times are the following: range of cysteine content, 144–320 nmol/ml; range of homocysteine content, 5–14 nmol/ml; the ratio of homocysteine to cysteine content, 0.036; S.D., 0.005. Hence, in order to avoid any influence of the dilution of urine on the results, it is suggested that the results should be expressed as the ratio to cysteine content.

Acknowledgments

The author thanks Miroslaw Makowski for technical assistance.

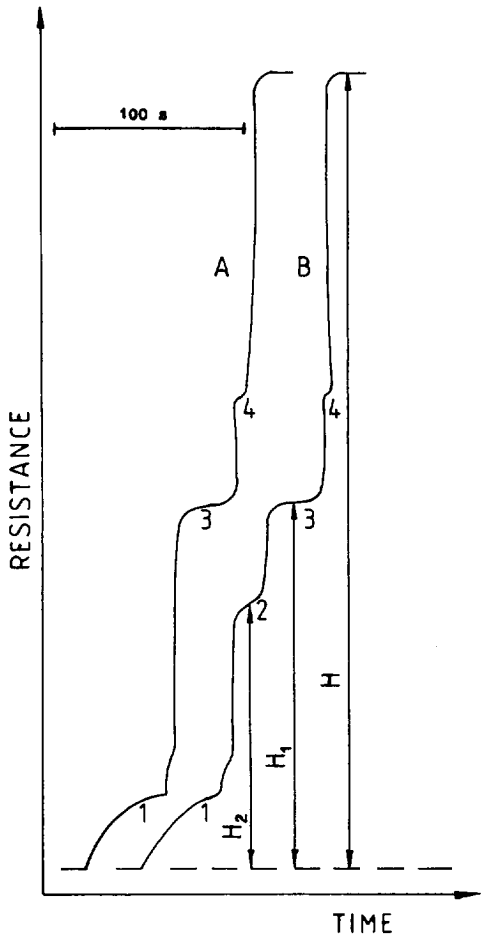

Fig. 4. Isotachophoregrams of mercapto amines separated from alkaline urine. Step heights: H=208 mm, H_1=95 mm, H_2=69 mm. 1 and 4=contaminations; 2=cysteamine, u=33.2; 3=un-identified amino thiol, u=29.2. A=normal urine, B=urine with added cysteamine.

Table 1
Assessment of the results of determination.

No.	Thiol	Range (nmol/ml)	a	b	R.S.D.	C.V. (%)
1	Captopril	13–50	0.61	4.27	1.6	4.2
2	Cysteamine	8–100	1.40	0.67	1.4	5.6
3	Homocysteine	3–20	−1.20	2.18	1.9	8.9

Linear calibration function: $t=a+bc$; t=step length, s; c=concentration, nmol/ml; driving current=50 μA; R.S.D.=residual standard deviation (measure of the scatter of the information values about the calibration line); C.V.=coefficient of variation; n=7.

References

[1] D.W. Jacobsen, V.J. Gatautis and R. Green, Anal. Biochem., 178 (1989) 208.
[2] M.A. Mansoor, A.M. Svardal and P.M. Ueland, Anal. Biochem., 200 (1992) 218.
[3] S. Sypniewski and E. Bald, J. Chromatogr. A, 676 (1994) 321–330.
[4] R.F. Bergstrom, D.R. Kay and J.G. Wagner, J. Chromatogr., 222 (1981) 445.
[5] S.B. Thomson and D.J. Tucker, J. Chromatogr., 382 (1968) 247.
[6] B. Sörbo and U. Hannestad, Clin. Chim. Acta, 95 (1979) 189.
[7] C.J. Holloway, J. Chromatogr., 390 (1987) 101.
[8] B.L. Ling and W.R.G. Baeyens, Anal. Chim. Acta, 255 (1991) 283.
[9] N. Mizobuchi, T. Ageta, K. Sasaki and H. Kodama, J. Chromatogr., 382 1986) 321.
[10] M. Wroński, J. Chromatogr., 657 (1993) 165.
[11] M. Wroński, J. Chromatogr. A, 672 (1994) 273.
[12] M. Wroński, J. Chromatogr., 248 (1982) 363.
[13] M. Wroński, J. Chromatogr., 307 (1984) 416.
[14] M. Wroński, Chem. Anal. (Warsaw), 34 (1989) 213.
[15] M. Wroński, Chem. Anal. (Warsaw), 37 (1992) 231.
[16] M. Wroński, Talanta, 24 (1977) 347.
[17] M. Wroński, Talanta, 26 (1979) 976.
[18] M. Wroński, Biochem. J., 104 (1967) 978.
[19] F.M. Everaerts, J.L. Beckers and Th.P.E.M. Verheggen, Isotachophoresis – Theory, Instrumentation and Application, Elsevier, Amsterdam, 1976.
[20] P. Boček, M. Deml, P. Gebauer and V. Dolnik, Analytical Isotachophoresis, VCH, Weinheim, 1988.