SULFUR-CONTAINING AMINO ACIDS: CHROMATOGRAPHY ON CATION AND ANION EXCHANGE RESINS WITH AN AUTOMATIC ANALYZER*

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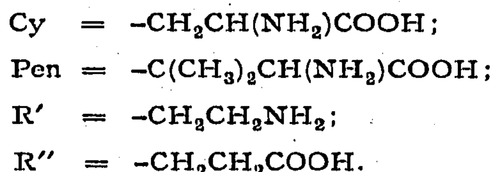
(Received December 21st, 1965)

INTRODUCTION

In a recent publication, DE MARCO *et al.*[1] described a method for examining a number of sulfur-containing amino acids with emphasis on sulfinic, sulfonic, thiosulfonic and thiosulfuric acids. However, some of the compounds could not be separated by their method, which involved chromatography on a column of cation-exchange resin, and had to be determined indirectly. We have been using columns of anion-exchange resin to separate and estimate compounds of this type in conjunction with an amino acid analyzer[2]. This paper reports the results obtained together with the chromatographic behaviour of a number of mixed disulfides and related compounds on cation-exchange resin. The following abbreviations are used in the formulation of compounds:


Cy = $-CH_2CH(NH_2)COOH$;
Pen = $-C(CH_3)_2CH(NH_2)COOH$;
R′ = $-CH_2CH_2NH_2$;
R″ = $-CH_2CH_2COOH$.

The usual abbreviations, GSSG and GSH, are used for oxidised and reduced glutathione, respectively.

EXPERIMENTAL AND RESULTS

Materials

Cystine, cysteine sulfinic acid ($CySO_2H$), cysteic acid, cysteine, homocystine, homocysteic acid, penicillamine, penicillaminic acid ($PenSO_3H$), glutathione (ox.), glutathione (red.) and taurine were commercial products obtained from CalBiochem or Mann Research Laboratories.

The following compounds were prepared by methods described in the literature: $CySSO_3Na$[3], $(CySO_2S)_2Ba$[4], $CyCH_2SSO_3Na$[3], $(CySSSO_3)_2Ba$[5], and CySSSCy[6].

Homocysteine sulfinic acid ($CyCH_2SO_2H$) was prepared by decomposing homocystine-S-monoxide with dilute ammonium hydroxide solution and isolating the product by chromatography on Dowex 1 × 8 ion-exchange resin with formic acid[7]. Penicillamine sulfinic acid ($PenSO_2H$) was produced by irradiating an aqueous

* DCBRL Report No. 491.

solution of PenSH with γ-rays and separating the products by chromatography on Dowex 1 resin. The compound was not characterized but it was eluted in the expected position and gave a positive test with iodoplatinic acid. Penicillamine (ox.) (PenSSPen) was prepared by bubbling oxygen through an aqueous solution of PenSH till a test for thiols was negative (3 days) then crystallizing the product. Mixed disulfides were prepared using the following reaction[8,9]: $RSOSR + 2\,R'SH \rightarrow 2\,RSSR' + H_2O$. The intermediates required, cystine-S-monoxide and homocystine-S-monoxide were prepared as described by SAVIGE *et al.*[6].

The trisulfide CySSSCy was prepared from the monoxide by reaction with H_2S[6].

The mixed disulfide, CySSG, was kindly supplied by W. F. FORBES.

Cation-exchange chromatography

The amino acid analyzer (Technicon Chromatography Corp.) had 15 mm tubular flow cells and a 0.6 × 140 cm column. The resin supplied was "Chromobeads" Type A (Cation-exchange). The instrument was used as recommended by the manufacturer except that the glass reaction coil was replaced by a Teflon capillary tube (18 gauge, 100 ft.) which was heated in a refluxing water bath[10]. Introduction of nitrogen into the stream of reagents was then unnecessary and all of the sample from the ion-exchange column reacted with ninhydrin and passed through the flow cells.

Stock solutions of disulfides in 1% HCl (2.5 mE per litre) were stored at 5° and diluted 5 times before use. (E is the equivalent weight with respect to ninhydrin positive groups). Solutions of sulfhydryl compounds such as CySH were prepared with thiodiglycol present as an antioxidant. Table I gives the elution times and yields of some disulfides etc.

TABLE I

ELUTION OF DISULFIDES ETC. FROM CHROMOBEADS CATION-EXCHANGE RESIN

Column temperature = 60° throughout

Compound	*Time (min)*	*Yield (hw, 0.25 μE)*	*Absorption ratio 440/570 mμ*	*Remarks*
Norleucine	660	13.8	0.2	Reference standard
L-CySH	265	2.5 (440 mμ)	2.3	
D-PenSH	240	—	0.3	Oxidised to PenSSPen
GSH	142	4.3	0.25	
L,L-CySSCy	510	7.0	0.6	
DL,DL-$CyCH_2SSCH_2Cy$	720	12.6	0.3	
DL,DL-PenSSPen	590, 600	—	0.2	Twin peaks
D,D-PenSSPen	590	15.0	0.2	Single peak
GSSG	300	9.7	0.2	Broad peak, 9 cm
L,D-CySSPen	540	8.1	0.35	
L-CySSR'	900	—	0.6	Impure
L-CySSR"	460	9.7	0.5	Broad peak, 7 cm
DL,D-$CyCH_2SSPen$	690	7.0	0.25	Trace of PenSSPen
DL,L-$CyCH_2SSCy$	660	8.8	0.5	
DL-$CyCH_2SSG$	610	—	0.25	Impure
L-CySSG	410	9.5	0.35	
L,L-CySSSCy	640	5.1	0.8	

Anion-exchange chromatography

Dowex 1-x8, minus 400 mesh (BioRad Laboratories) anion-exchange resin has been used successfully as described previously[2]. The resin was converted to the chloracetate form with sodium monochloroacetate solution, rinsed and packed in a glass column 0.6 × 140 cm identical to that used for the "Chromobeads". For standards, operation of the column was similar to the procedure used for the "Chromobeads" column. The compounds being examined were put on in aqueous solution and the column was eluted with a linear gradient of water to 1 *M* sodium monochloroacetate solution (600 ml). The gradient mixture was prepared from water containing 10 ml BRIJ-35 and 5 ml thiodiglycol per litre and neutral (pH 6) sodium monochloroacetate solution containing 10 ml BRIJ-35 per litre. Thiodiglycol was necessary to prevent oxidation of sulfinic acids. The column was operated at room temperature throughout with a flow rate of 30 ml per h. Elution times of some of the compounds examined are reported in Table II.

TABLE II

ELUTION OF ACIDS FROM DOWEX 1 ANION-EXCHANGE RESIN AT ROOM TEMPERATURE

Compound	*Time (min)*	*Yield (hw, 0.25 µE)*	*Remarks*
Glutamic	200	15.3	Reference standard
$CySO_2H$	240	12.6	
$CySO_3H$	320	12.0	
$CySO_2SH$	540	—	Impure, broad, 8 cm
$CySSO_3H$	580	8.6	Broad peak, 8 cm
$CySSSO_3H$	880	—	Very broad peak, 15 cm
$CyCH_2SO_2H$	225	—	Impure
$CyCH_2SO_3H$	320	12.2	
$CyCH_2SSO_3H$	585	—	Impure
$PenSO_2H$	245	—	Impure
$PenSO_3H$	300	9.2	
$R'SO_3H$	80	11.2	
$R'SO_2H$	50	12.0	
GSSG	320	9.7	Broad peak, 8 cm
CySSR″	250	9.6	
Aspartic	210	12.6	

Fig. 1 illustrates the separation obtained with cystine and a number of related acids (0.25 µE of each). Glutamic acid was added as a reference standard. A shallower gradient with a total volume of 700 ml was used for the chromatograms illustrated as this improved the separation in the region of the sulfinic acids.

Fig. 2 shows the results obtained with a number of acids related to homocysteine and penicillamine.

Application of Dowex 1 column

During a study of the γ-radiolysis of cystine in dilute aqueous solution (3×10^{-4} *M*) at low doses[11], it was necessary to separate and estimate the yield of a number of acidic products. This was achieved using the column of Dowex 1 described above as follows. The reference acid (glutamic), and thiodiglycol (5 drops) were added to 25 ml of the solution after irradiation and the mixture was pumped through the

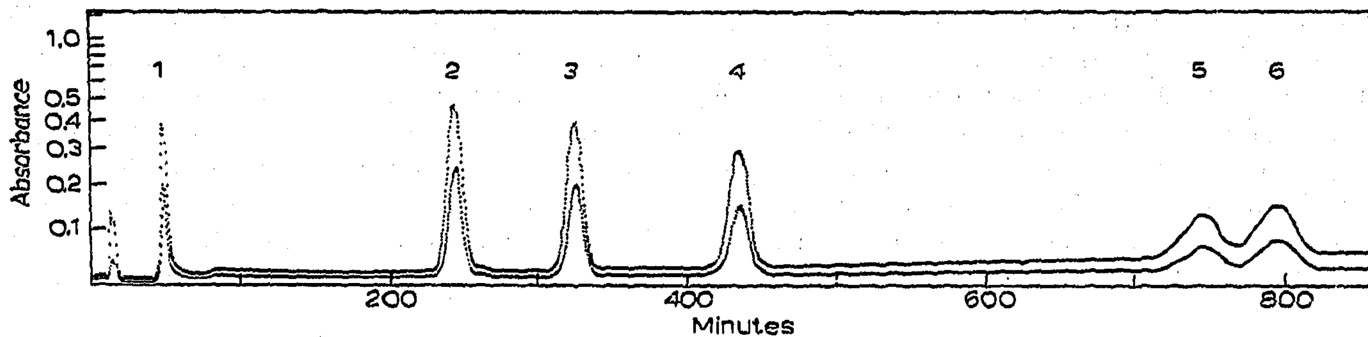


Fig. 1. Chromatography on Dowex 1: 1 = CySSCy; 2 = glutamic acid; 3 = $CySO_2H$; 4 = $CySO_3H$; 5 = $CySO_2SH$; 6 = $CySSO_3H$. Chart speed: 3 in. per h.

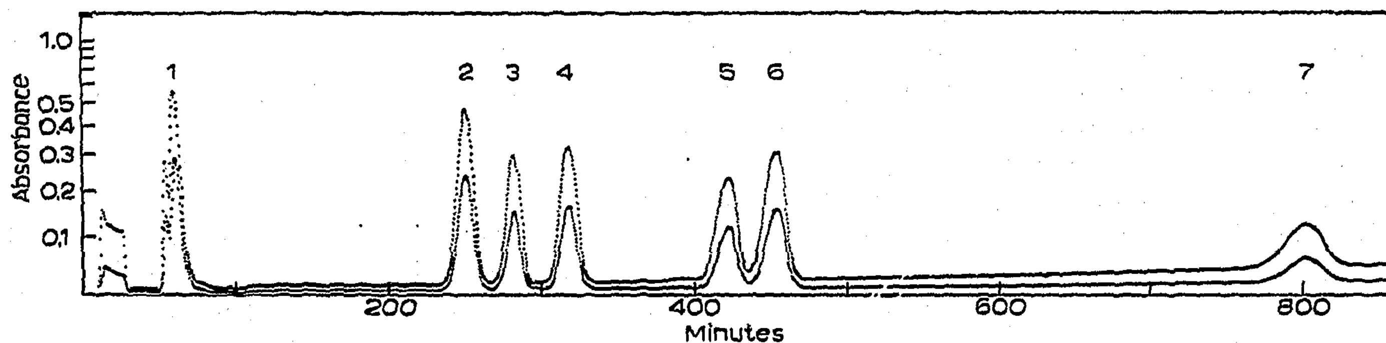


Fig. 2. Chromatography on Dowex 1: 1 = Homocystine; 2 = glutamic acid; 3 = aspartic acid; 4 = $CyCH_2SO_2H$; 5 = $PenSO_3H$; 6 = $CyCH_2SO_3H$; 7 = $CyCH_2SSO_3H$.

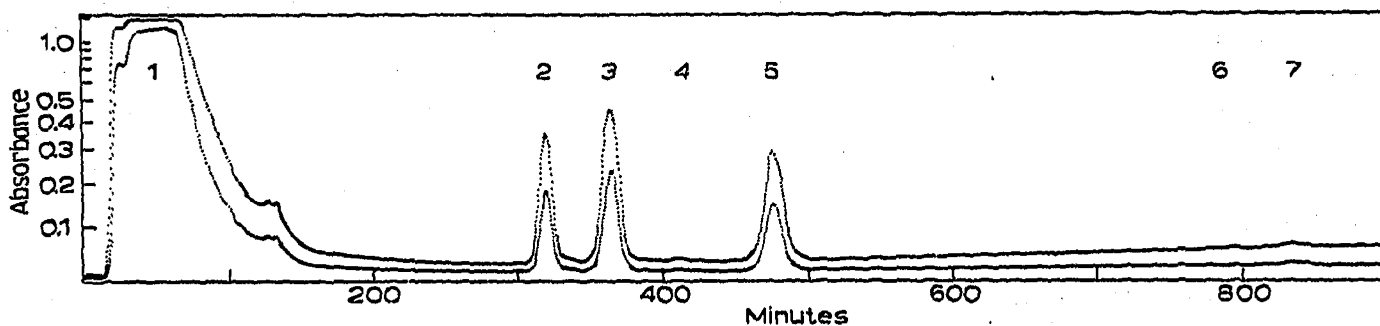


Fig. 3. Irradiated cystine solution (10,000 rads): 1 = Cystine and neutral products; 2 = glutamic acid; 3 = $CySO_2H$; 4 = unidentified product; 5 = $CySO_3H$; 6 = $CySO_2SH$; 7 = $CySSO_3H$.

Dowex 1 column without concentration. The resin retained the acidic compounds and cystine passed through the column. After rinsing with a further 25 ml of water containing BRIJ-35 and thiodiglycol, the column was eluted with aqueous sodium monochloroacetate solution as described above. A typical separation is illustrated in Fig. 3.

In practice the two columns, Dowex 1 and "Chromobeads", were usually operated simultaneously. Two colorimeters (570 mμ and 440 mμ) were used in conjunction with the "Chromobeads" column and the other (570 mμ) was used for the Dowex 1 column[2]. The results were recorded with the standard three point recorder.

DISCUSSION

Chromatography of disulfides and neutral amino acids was straightforward and standard procedures were sufficient. Mixed disulfides were eluted at positions intermediate between those of the corresponding symmetrical disulfides. The ratio of the absorptions at 440 and 570 mμ were useful for identifying derivatives of cystine which had high 440/570 absorption ratios. Cysteine had a particularly high value[12] and the trisulfide CySSSCy was also distinctive in this way (Table I).

Cation-exchange resins were only partially successful for separating mixtures of acids. The method of DE MARCO *et al.*[1], while successful for mixtures of cysteic acid, cysteine sulfinic acid, taurine and hypotaurine, did not resolve mixtures containing cysteic acid, alanine thiosulfonic acid, and alanine thiosulfuric acid since these compounds were not retained by the resin. Anion-exchange resins, on the other hand, retain these strong acids and they can be eluted by buffer solutions or acids approximately in order of their pK_a values[7,13]. MOORE and co-workers[14,15] used a column of Dowex 1 resin to determine cysteic acid produced by oxidation and hydrolysis of proteins. Recently the same method has been used to determine penicillaminic acid[16]. In order to separate a complex mixture of products, however, gradient elution is necessary. Neutral sodium monochloroacetate solution was the most successful of the eluants investigated in the present work. Sodium formate was an equally good eluant but it appeared to interfere with the ninhydrin reaction. Acetate buffer, pH 5.5 eluted the acids from the column but glutamic acid and cysteine sulfinic acid gave peaks with extended shoulders. A similar effect was observed with sodium monochloroacetate solution when the pH was about 4. Most of the sulfinic acids examined were eluted in approximately the same time and this is also true of the sulfonic acids. A separation of at least 15 min is desirable if the yields are to be calculated in the usual way. Improved separation could probably be achieved with a more complex gradient and higher operating temperature but the labile nature of the compounds makes the latter undesirable. The method described has been used successfully in studying the γ-radiolysis of cystine[11].

SUMMARY

Some thiols and mixed disulfides related to cysteine were chromatographed on a cation-exchange resin and the results are reported.

Separation of cysteine sulfinic acid, cysteic acid, alanine thiosulfonic acid, alanine thiosulfuric acid and other compounds of this type on Dowex 1 anion-exchange resin is described.

REFERENCES

1 C. De Marco, R. Mosti and D. Cavallini, *J. Chromatog.*, 18 (1965) 493.
2 J. W. Purdie, *Technicon Symposium, Technicon, Chauncey, New York, September, 1965*, Paper No. 21.
3 B. Sorbo, *Acta Chem. Scand.*, 12 (1958) 1990.
4 C. De Marco, M. Coletta, B. Mondovi and D. Cavallini, *Ital. J. Biochem.*, 9 (1960) 3.
5 T. W. Szczepkowski, *Roczniki Chem.*, 35 (1961) 563.
6 W. E. Savige, J. Eager, J. A. Maclaren and C. M. Roxburgh, *Tetrahedron Letters*, (1964) 3289.
7 C. Davies, R. D. Hartley and G. J. Lawson, *J. Chromatog.*, 18 (1965) 47.
8 La V. D. Small, J. H. Bailey and C. J. Cavallito, *J. Am. Chem. Soc.*, 69 (1947) 1710.
9 A. Schöberl and H. Gräfje, *Ann.*, 617 (1958) 71.
10 P. B. Hamilton, *Anal. Chem.*, 35 (1963) 2055.
11 J. W. Purdie, in preparation.
12 A. Wainer, personal communication.
13 G. J. Lawson and J. W. Purdie, *Mikrochim. Acta*, 3 (1961) 415.
14 E. Schram, S. Moore and E. J. Bigwood, *Biochem. J.*, 57 (1954) 33.
15 S. Moore, *J. Biol. Chem.*, 238 (1963) 235.
16 K. Borner, *Z. Physiol. Chem.*, 341 (1965) 264.