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SULFUR-CONTAINING AMINO ACIDS

II. CHROMATOGRAPHY OF DISULFIDES AND TRISULFIDES WITH AN AUTOMATIC ANALYZER*

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SUMMARY

Synthetic mixed disulfides and trisulfides related to cystine were chromatographed on a column of cation-exchange resin. The elution times and colour yields with ninhydrin are reported.

The elution pattern of the disulfides is demonstrated by co-chromatography with several of the common amino acids. Chromatography of trisulfides and unsymmetrical trisulfides is also illustrated.

INTRODUCTION

The biochemistry of sulfur-containing amino acids has been reviewed recently by MAW[1]. Some mixed disulfides occur naturally, *e.g.* cysteine-homocysteine disulfide, and others result from treatment of illnesses, *e.g.* cysteine-penicillamine disulfide. Disulfides involving glutathione also occur naturally and a new mixed disulfide, β-mercaptolactate-cysteine disulfide, has been isolated by AMPOLA *et al.*[2]. Several papers have been published recently on the chromatography of sulfur-containing amino acids. An automatic method for analysis of lanthionine and lysinoalanine has been described by ROBSON *et al.*[3] (It is worth noting that the method also detects cysteine.) Automation of several methods for analysis of sulfur-containing compounds has been described by BARBER[4]. Methods are described for thiols, sulfides, disulfides, etc., and detection of cysteine-homocysteine disulfide is illustrated.

In the course of studying the radiation chemistry of disulfides, we have prepared and examined many sulfur-containing amino acids. In an earlier publication, chromatography of a number of disulfides and related sulfinic and sulfonic acids was reported[5]. Since that time, some new compounds have been synthesised and most of the mixed disulfides have been further purified. This paper reports the chromatographic properties of these compounds using a commercial amino acid analyzer and

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TABLE I

CHROMATOGRAPHY OF THIOLS, DISULFIDES AND TRISULFIDES ON A COLUMN OF 'CHROMOBEADS'
Flow rate 35 ml/h, temperature 60°.

Compound	*Abbreviation*	*Time (min)*	*Yield (hw, 0.25 μE)*	*Ratio (440/570)*
L-Cysteine	CySH	249	3.05	2.25
Glutathione (ox.)	GSSG	275	9.78	0.22
DL + *meso*-Lanthionine	CySCy	296, 319*	2.68 (2.39)	—
D-Penicillamine	PenSH	331	2.20	0.26
L-Cysteine-glutathione disulfide	CySSG	394	10.32	0.33
L-Cystine	CySSCy	473	7.29	0.55
L-Cysteine-D-penicillamine disulfide	CySSPen	504	7.84	0.33
D-Penicillamine disulfide	PenSSPen	545	7.42	0.19
DL + *allo*-Cystathionine	CySHo	548, 554*	impure	—
L-Isoleucine	Ileu	566	13.62	0.19
L-Cysteine trisulfide	CySSSCy	595	5.47	0.66
L-Cysteine-D-penicillamine trisulfide	CySSSPen	616	impure	0.48
L-Cysteine-L-homocysteine disulfide	CySSHo	618	10.23	0.42
L-Homocysteine-D-penicillamine disulfide	HoSSPen	640	9.31	0.27
D-Penicillamine trisulfide	PenSSSPen	655	6.62	0.31
L-Cysteine-L-homocysteine trisulfide	CySSSHo	675	impure	0.52
L-Homocystine	HoSSHo	723	12.77	0.31
D-Penicillamine tetrasulfide	PenSSSSPen	735	impure	0.25
Cysteamine-glutathione disulfide	CyaSSG	737	impure	0.24
L-Homocysteine trisulfide	HoSSSHo	796	11.36	0.35
L-Cysteine-cysteamine disulfide	CySSCya	976	4.65	0.56

* Twin peaks.

the relationship of the disulfides to some of the common amino acids is illustrated. The abbreviations used for the sulfur compounds are explained in Table I.

MATERIALS AND METHODS

Commercially available thiols, sulfides and disulfides were purchased from Calbiochem or Mann Research Labs.

Unsymmetrical disulfides were synthesised using the method of SCHÖBERL AND GRÄFJE[6], *i.e.*

$$2\ R'SH + R''\overset{O}{\overset{\|}{S}}SR'' \rightarrow 2\ R'SSR'' + H_2O$$

This reaction is rapid, without byproducts and gives good yields. A similar method, starting from cystine-S,S-dioxide, was used by ERIKSSON AND ERIKSSON to prepare cysteine-glutathione disulfide[7]. The monoxides of cystine, homocystine and cystamine, which were required for the various mixed disulfides, were prepared by oxidation of the disulfide with peracetic or performic acid as described by SAVIGE *et al.*[8]. In the case of cystamine-S-monoxide, the method described by KLAYMAN AND MILNE[9] was also used and is probably superior. The preparation of cysteine-penicillamine disulfide is described in detail elsewhere[10]. After recrystallization, the mixed disulfides were obtained analytically pure with the exception of cysteamine-glutathione disulfide.

The trisulfides of cysteine, homocysteine and cysteamine were obtained by bubbling H_2S through an aqueous solution of the S-monoxide. After crystallization from aqueous ethanol, pure compounds were obtained. Penicillamine trisulfide (PenSSSPen) was prepared by the method of FLETCHER AND ROBSON[11]; addition of sulfur to PenSH. The product was purified by chromatography on Dowex 50W cation-exchange resin with pyridine–acetic acid buffers[12] and recrystallized to give pure PenSSSPen[10]. In the course of separating the trisulfide, some tetrasulfide (PenSSSSPen) was isolated and its composition confirmed by sulfur analysis. The same method, addition of sulfur to thiols, was utilized to produce mixed trisulfides. Thus a mixture of CySSSCy, CySSSPen, and PenSSSPen was obtained from a mixture of cysteine and penicillamine. Similarly, a mixture containing CySSSHo was prepared. The latter compound was also obtained by bubbling H_2S through an aqueous solution of cystine-S-monoxide and homocystine-S-monoxide.

The amino acid analyzer was a Technicon, Model NC-1, with a column of "chromobeads" Type A cation-exchange resin (0.6 × 150 cm). It was operated as recommended by the manufacturer except for the minor modifications described previously[5]. The column temperature was maintained at 60° throughout the runs as this was satisfactory for the compounds being studied. The "Autograd" gradient elution device was used with 75 ml of buffer in each chamber as follows: 1 to 4, pH 2.875; 5, pH 3.80; 6, pH 3.80 + pH 5.0 (1:2); 7 to 9, pH 5.0. This differs only slightly from the recommended autograd composition and does not affect the order of elution of the compounds described. A buffer flow rate of 35 ml per hour was used.

RESULTS AND DISCUSSION

The results obtained with a number of thiols, sulfides, disulfides and trisulfides are contained in Table I. The yields given are typical yields obtained with the purified amino acids but some variation was observed between different runs, different columns, and different batches of reagent. The yields given are height × width at half height for 0.25 μE where E is the equivalent weight with respect to ninhydrin positive groups. For exact calculations, standards were chromatographed with the same reagents etc., as the compounds being studied. Table I also gives the ratio of the peak heights at 440 and 570 mμ. This ratio was useful in identifying derivatives of cysteine, most of which had higher than average values. It was found that the mixed disulfides were reasonably stable in water (pH 4 to pH 6) and no disulfide exchange

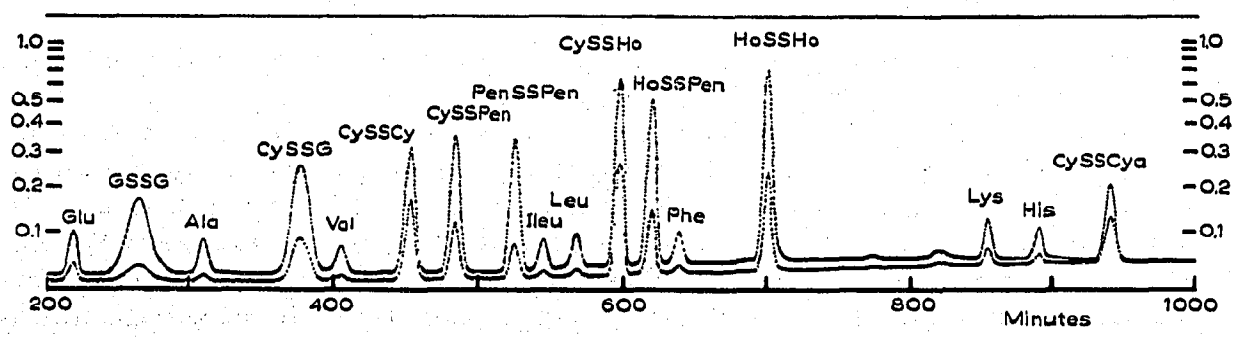

Fig. 1. Chromatography of disulfides and mixed disulfides on a column of cation-exchange resin at 60°. Buffer flow rate, 35 ml/h.

was observed with solutions after two weeks at 5°. Above pH 7 conversion to a mixture occurs and at very low pH acid catalyzed disulfide exchange may take place.

The mixed disulfides were co-chromatographed with the corresponding symmetrical disulfides and several of the natural amino acids. This provided a useful illustration of the elution pattern and the chart obtained is shown in Fig. 1. 0.25 μE of each disulfide was used while the quantity of the other amino acids was kept much smaller (0.025 μE). Chromatography of the trisulfides is shown in Fig. 2 which shows the trisulfides together with the corresponding disulfides and thiols. As with the mixed disulfides, the order of elution of these compounds was reproducible and almost independent of flow rate. The trisulfide of cysteamine was also tested but it was not eluted from the column.

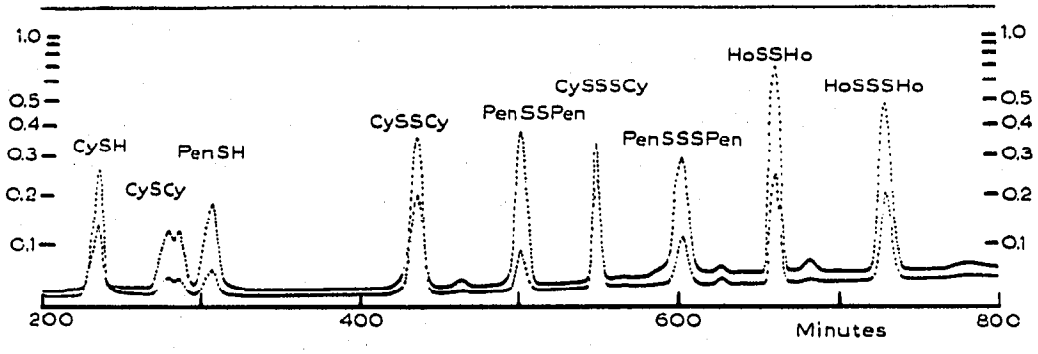

Fig. 2. Chromatography of thiols, sulfides, disulfides and trisulfides on a column of cation-exchange resin at 60°. Buffer flow rate, 35 ml/h.

The result obtained with a mixture containing a mixed trisulfide, CySSSPen, prepared as described above, is illustrated in Fig. 3. The mixed trisulfide was not isolated for characterization but it is likely that the peak occurring between CySSSCy and PenSSSPen is CySSSPen. The mixed trisulfide of cysteine and homocysteine was obtained by two different methods and it behaved in the same manner. In Fig. 3, a compound tentatively identified as the tetrasulfide, PenSSSSPen, co-chromatographed with ammonia. At a pump rate of 30 ml/h it separates from ammonia. The

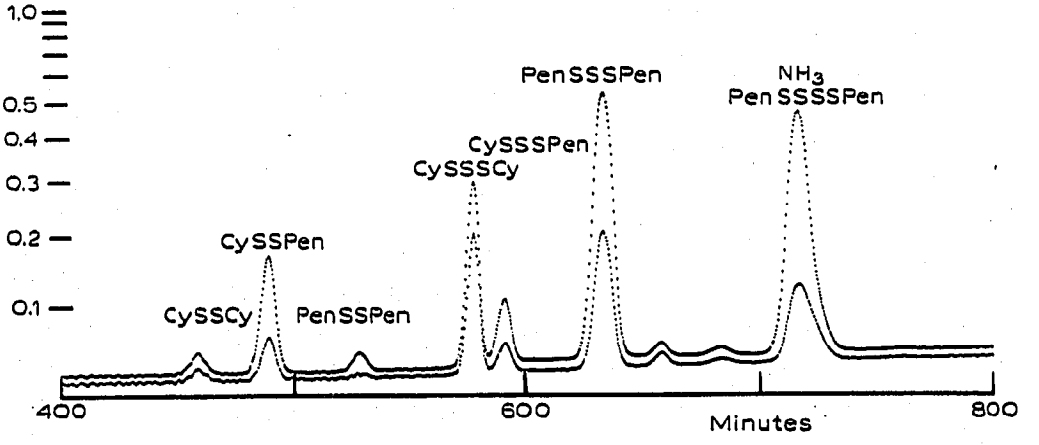

Fig. 3. Chromatography of a mixture containing disulfides, trisulfides and tetrasulfides of penicillamine and cysteine at 60° and 35 ml/h.

two minor peaks between PenSSSPen and PenSSSSPen may be due to CySSSSCy and CySSSSPen. FLETCHER AND ROBSON[11] also obtained a tetrasulfide when they prepared CySSSCy by the sulfur method.

Thus it can be seen that mixed disulfides chromatograph between the corresponding symmetrical disulfides and mixed trisulfides run between the symmetrical trisulfides as would be expected. However the unsymmetrical compounds do not always lie exactly midway between the symmetrical compounds so a synthetic mixture or a specific sulfur reaction may be necessary for satisfactory identification when they are expected.

In general terms, the elution times increase in proportion to the size of the molecule in a series; sulfide, disulfide, trisulfide and tetrasulfide. The amino acid functional groups probably change very little between members in the series. In homocystine for example there are two methylene groups between the sulfur atoms and the functional groups and still the increase in elution time between the disulfide and the trisulfide is similar to the increase in elution time between cystine and cysteine trisulfide. Hence the increase in elution times must be due to adsorption chromatography or to an increasing ability to reach two ionic sites on the resin at the same time. In the absence of more information about these compounds, a more detailed analysis is not possible.

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