CHROM. 5360

Chromatography of cysteine and glutathione derivatives on a Dowex 1/Sephadex mixed-bed column

As reported previously[1], Dowex 1 X8 anion-exchange resin may be employed to separate many sulfinic and sulfonic acids which co-chromatograph on cation-exchange resins such as those normally employed in automatic amino acid analyzers. However, even on Dowex 1 X8, many important amino acids and peptides co-chromatograph and we had difficulty while trying to measure sulfinic and sulfonic acid derivatives of cysteine and glutathione. Cysteic acid had the same elution time as glutathione disulfide which was always present in our samples. It was thought that addition of Sephadex G-10 to the resin bed would retard the smaller molecules enough to make a separation possible.

Materials and methods

Most of the amino acids used were purchased from Calbiochem or Mann Research Laboratories. Analysis showed that the cysteine sulfinic acid was not a hydrate as indicated on the label. Preparation of some of the compounds has been described previously[1]. Glutathione sulfinic acid was prepared as described by CALAM AND WALEY[2]. It was purified by chromatography on a column of Dowex 1 X8, formate form, using a formic acid gradient[3]. The sulfinic acid was detected by the sodium iodoplatinate test. Glutathione sulfonic acid was made by oxidising glutathione with performic acid and purified as described in the literature[4]. A mixture containing cysteine and glutathione disulfides and trisulfides was prepared by the method of FLETCHER AND ROBSON[5,6] whereby the thiols were reacted with free sulfur dissolved in chloroform and ethanol. After evaporation of the solvent the residue was dissolved in water, filtered, and freeze dried.

The amino acid analyzer was a modified Technicon[1], model NC-1, and the standard 0.6 × 140 cm glass columns were used to contain the resin. The mixed-bed column was prepared by mixing Dowex 1 X8 (minus 400 mesh) anion-exchange resin (40 ml) previously converted to chloroacetate form, and Sephadex G-10 (25 ml). A slurry of the mixture in water was poured into the glass column and packed as rapidly as possible by applying air pressure in order to prevent separation of the Dowex and the Sephadex. An "autograd" gradient elution device was used to produce a suitable gradient. Water containing BRIJ-35 (10 ml/l) and thiodiglycol (5 ml/l), and sodium monochloroacetate solution (1 *M*, pH 5.5) containing BRIJ-35 (10 ml/l) were used in the "autograd". Using seven chambers, with 100 ml in each, the volume (ml) of chloroacetate was increased gradually from chambers 1 to 7 as follows: 0, 5, 20, 40, 60, 80, 100.

Results and discussion

The elution of the sulfinic and sulfonic acids of cysteine and glutathione from Dowex 1 X8 at room temperature is illustrated in Fig. 1. As illustrated, the cysteic

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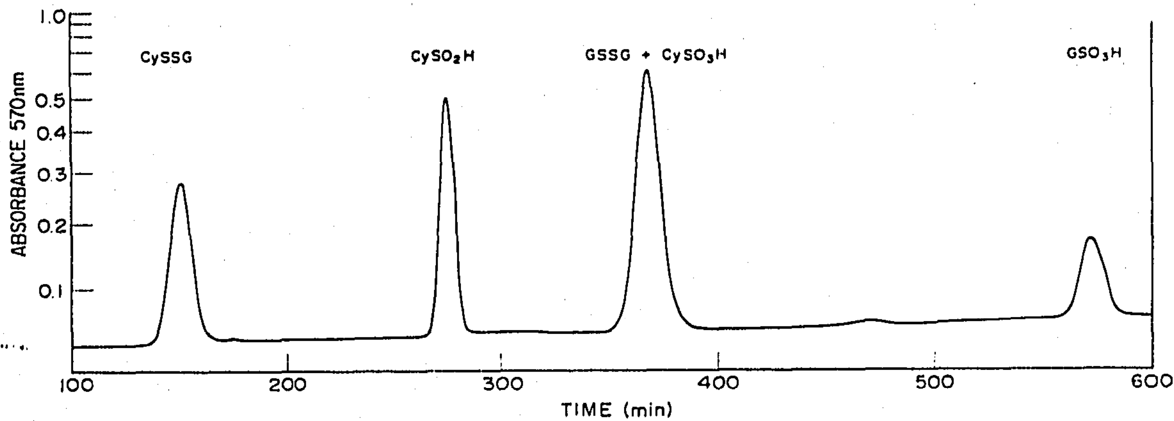

Fig. 1. Chromatography of disulfides and sulfinic and sulfonic acids on a column of Dowex 1 at room temperature and 30 ml/h buffer flow rate.

acid and glutathione disulfide peaks coincided and this was usually the case on Dowex 1 columns. Increasing the column temperature from room temperature to 45° and decreasing the buffer flow rate from 30 to 20 ml/h produced a partial separation but the peaks became broad and the elution times increased to an undesirable extent. Columns with mixed beds of Dowex 1 X8 and Sephadex G-10 were prepared and tested with the same mixture of amino acids. The proportion of Sephadex in the column was increased until a good separation was obtained. Good chromatograms were achieved using a buffer flow rate of 20 ml/h and a column temperature of 45°. Under these conditions the run was still reasonably fast due to the reduction in the amount of anion-exchange resin in the column. Fig. 2 shows the elution pattern obtained with a column bed consisting of 8 parts Dowex 1 X8 and 5 parts Sephadex G-10. The amino acid mixture contained *ca.* 0.25 μequiv. of each amino acid except for glutathione sulfonic acid which was half strength. Several of our standard amino acids were chromatographed on the Dowex 1/Sephadex column and Table I compares the elution times with a Dowex 1 column of the same dimensions. The yields shown in the table are relative to glutamic acid; the actual yields may vary de-

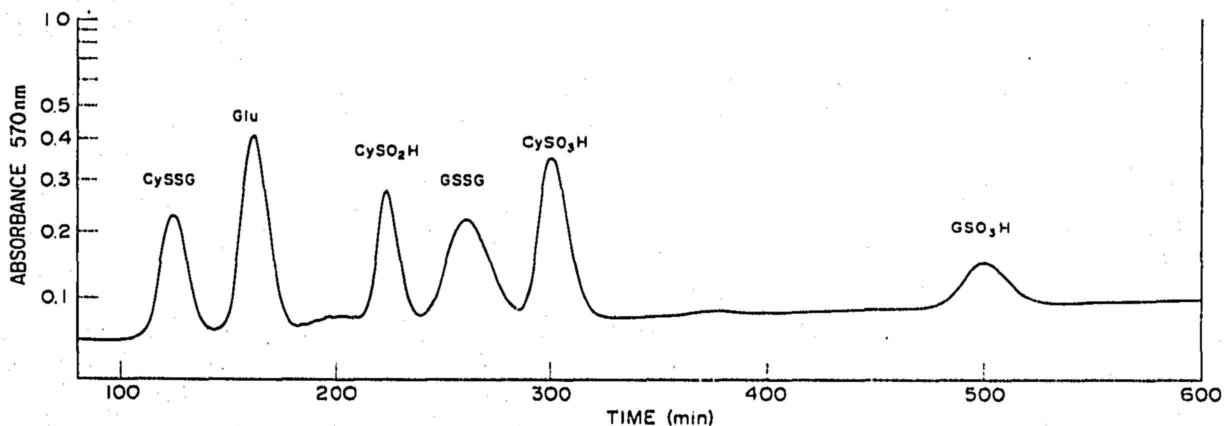

Fig. 2. Chromatography of disulfides and sulfinic and sulfonic acids on a mixed-bed column of Dowex 1 and Sephadex G-10 at 45° and 20 ml/h buffer flow rate.

TABLE I

ELUTION OF ACIDS FROM DOWEX 1/SEPHADEX AT 45° AND 20 ML/H AND FROM DOWEX 1 AT ROOM TEMPERATURE AND 30 ML/H

Compound	*Abbreviation*	*Elution time (min)*		*Yield (hw/0.25 μ equiv.)*
		Dowex/ Sephadex	*Dowex 1*	
Cystine	CySSCy	35	30	7.5
Cysteine-glutathione disulfide	CySSG	125	150	9.5
Glutamic acid	Glu	160	230	13.5
Cysteine sulfinic acid	$CySO_2H$	225	275	10.9
Glutathione disulfide	GSSG	260	365	10
Cysteic acid	$CySO_3H$	300	365	10.5
Glutathione sulfinic acid[a]	GSO_2H	375	480	10
Glutathione sulfonic acid	GSO_3H	500	575	10.0
Alanine thiosulfonic acid	$CySO_2SH$	505	615	—
Alanine thiosulfuric acid	$CySSO_3H$	540	660	8.0

[a] A trace of GSO_2H can be seen in the runs illustrated in Figs. 1 and 2.

pending on the system used, flow rates, etc. The glutathione sulfinic acid was impure but it was assumed to have a color yield similar to the sulfonic acid by analogy with other sulfinic and sulfonic acids. The color yield of glutathione sulfonic acid was checked by hydrolyzing a sample and measuring the yields of glycine, glutamic and cysteic acids.

The mixed-bed column had the good elution properties of the ion-exchange resin used combined with the molecular sieve features of Sephadex G-10. After a period of time the column bed appeared to pack down and the elution times increased but the elution pattern did not change significantly. It may be necessary to repour the column after several months or to fill it with fresh Dowex 1/Sephadex mixture. Similar separations should be possible using anion-exchange Sephadex but these materials are not available at present in a molecular exclusion range corresponding to Sephadex G-10. One anion-exchange Sephadex was tested, QAE-Sephadex A-25,

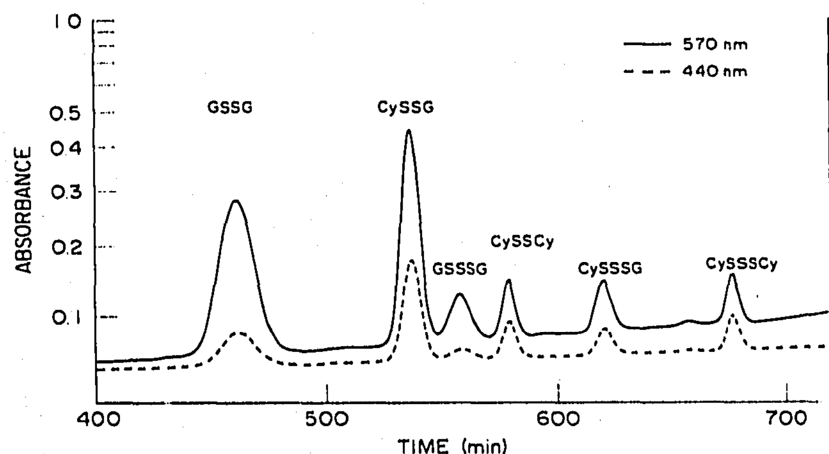

Fig. 3. Chromatography of disulfides and trisulfides of cysteine and glutathione on "Chromobeads" at 60° and 30 ml/h buffer flow rate.

but it was unsuccessful and tended to shrink as the ionic strength of the eluant was increased.

While working on glutathione derivatives it became necessary to determine the elution times of glutathione trisulfide and the mixed trisulfide of cysteine and glutathione. A mixture of these compounds, together with the corresponding disulfides, was chromatographed on "Chromobeads" type A. This column was operated at 60° with the gradient solution described previously[6]. The elution pattern is illustrated in Fig. 3. As before, the ratio of the absorption at 570 nm to the absorption at 440 nm was useful for identifying derivatives of cystine. This mixture of disulfides and trisulfides was also resolved successfully on a shorter column (0.6 × 75 cm) of chromobeads type C2 with a buffer flow rate of 30 ml/h. The order of elution of the amino acids was unchanged but the elution times were reduced to: GSSG, 235; CySSG, 275; GSSSG, 285; CySSCy, 295; CySSSG, 320; CySSSCy, 355. The disulfides were formed in greater yield than the trisulfides whereas the opposite effect was observed when the trisulfides of cysteine and penicillamine were made by the same method.

In conclusion, Sephadex can be added to an ion-exchange resin in order to improve the separation where compounds of significantly different molecular weights are involved. It should be possible to apply this technique to the separation of other substances which differ sufficiently in ionic properties and molecular weights.

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1 J. W. PURDIE, J. P. FARANT AND R. A. GRAVELLE, *J. Chromatogr.*, 23 (1966) 242.
2 D. H. CALAM AND S. G. WALEY, *Biochem. J.*, 85 (1962) 418.
3 C. DAVIES, R. D. HARTLEY AND G. J. LAWSON, *J. Chromatogr.*, 18 (1965) 47.
4 W. O. KERMACK AND N. A. MATHESON, *Biochem. J.*, 65 (1957) 45.
5 J. C. FLETCHER AND A. ROBSON, *Biochem. J.*, 87 (1963) 553.
6 J. W. PURDIE, R. A. GRAVELLE AND D. E. HANAFI, *J. Chromatogr.*, 38 (1968) 346.

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