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Note

Problems associated with the high-performance liquid chromatography of thiols

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The linking of electrochemical detection to high-performance liquid chromatography (HPLC)[1] has allowed, for the first time, the rapid chromatographic determination of reduced thiols. This new approach has coincided with the increased need to measure a number of thiol-containing drugs[2–4] in physiological fluids. Historically thiols have proved extremely difficult to chromatograph giving poor peak shapes and oxidizing to various disulphides during analysis. Recent work has employed both reversed-phase[3–6] and ion-exchange HPLC packings[1,2,7,8] for the separation of thiols. In this laboratory we have developed HPLC methodologies for the determination of cysteine and the thiol-containing drugs, D-penicillamine, thiomalate and captopril. This note outlines some of the problems we have encountered with the chromatography of thiols.

MATERIALS AND METHODS

The HPLC system consisted of a Model 300/01 pump (ACS, Luton, U.K.) and either a Model 4A (Bioanalytical Systems, West Lafayette, IN, U.S.A.) or LCA 14 (EDT, London, U.K.) electrochemical detector; both were equipped with gold working electrodes (Bioanalytical Systems). Stainless-steel columns, 10 × 0.46 cm I.D., were packed in-house with 3-μm ODS-Hypersil or 5-μm Spherisorb packings according to the manufacturer's instructions (Shandon, Runcorn, U.K.). 5-μm strong cation-exchange bonded silica was obtained from HSCP (Bourne End, U.K.). HPLC grade solvents were obtained from Rathburn Chemicals (Walkerburn, U.K.) and other chemicals were of analytical grade. Thiomalic acid, D-penicillamine, L-cysteine and the other thiols were obtained from Sigma. Standard solutions were prepared in 0.1 *M* phosphoric acid and injected using a Rheodyne injector with 20-μl loop. Full details of our methods have been previously published[3,4].

RESULTS AND DISCUSSION

Chromatography of thiols

Using reversed-phase columns we have found that most thiols give poor peak

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TABLE I

EFFECT OF pH ON COLUMN EFFICIENCY FOR THIOMALATE

A 10 × 0.46 cm ODS-Hypersil column eluted at 1 ml/min with 0.1 *M* phosphate buffer containing 3% methanol and 1 *M* Na_2EDTA.

pH	*k'*	*Plates/m*
1	1.35	40,390
2	1.39	36,730
3	0.82	22,160
4	0.36	8660
7.4	0.05	No peak

shapes when chromatographed with buffers of neutral pH. At worst multiple peaks have been obtained. Along with other workers[5] we have observed that reversed-phase chromatography at acid pH gives more acceptable chromatography. For example with thiomalate reducing the pH from 4 to 1 increased the apparent column efficiency by 450% (Table I). The cation-exchange column was, of necessity, operated at pH 2–3 and such effects due to the suppression of ionization were not readily apparent.

Even with low pH eluents both columns exhibited marked tailing of the peaks which was not apparent for test mixtures of non-thiols. It was considered likely that the thiols were chelating metals in the system, including those of the column components, since inclusion of disodium EDTA in the buffers markedly improved the peak shape. Using a pH-3 buffered eluate the inclusion of 1 m*M* disodium EDTA raised the apparent efficiency from 32,900 plates/m to 49,200 plates/m for the separation of thiomalate on an ODS column. Similarly, for the separation of D-penicillamine on the cation-exchange column the efficiency was increased from 5570 plates/m to 10,700 plates/m. Cation-exchange columns were invariably less efficient than the reversed-phase columns of the same dimensions. Concentrations of EDTA above 2 m*M* with acid buffers produced a fine precipitate of sequestric acid in the system and causes a rapid pressure build-up due to blocked sinters. Such blockages could be removed by pumping a neutral buffer until the pressure dropped to normal.

It was anticipated that the use of ion-pair agents would improve the chromatography of the acidic thiols. However, for thiomalate, the use of tetrabutylammonium ions led to a rapid loss of sensitivity at the gold working electrode although retention times were altered. Other workers have successfully used ion pairs for the separation of amino-thiols *e.g.* D-penicillamine, when using glassy-carbon electrodes[9,10].

With careful choice of column, pH and buffer thiomalate, cysteine and D-penicillamine can be satisfactorily resolved on ODS columns. Using a gold working electrode they can be determined at low concentrations (100 n*M*). This combination allows their ready determination in plasma and urine on a routine basis[3,4].

Column binding

After many routine determinations of thiomalate we observed that some samples, not believed to contain thiomalate, had peaks eluting at its retention time. Such artifacts had not been encounted in plasma samples during the course of assay de-

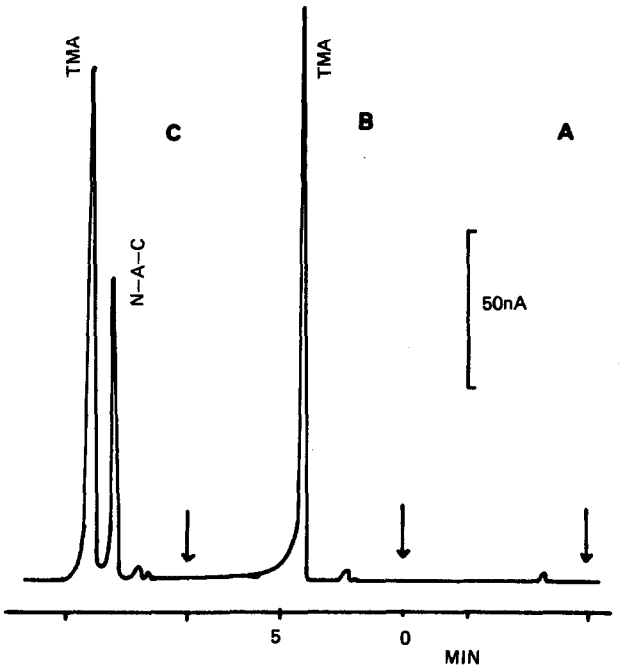

Fig. 1. Displacement of thiomalate from a used ODS column. (A) Blank injection (20 μl) of 0.1 *M* phosphoric acid, (B) injection of 500 pmol of thiomalate, (C) injection of 500 pmol N-acetylcysteine. For chromatographic details see Table I.

velopment. Injection of blank solutions of either eluent or 0.1 *M* phosphoric acid gave no peaks other than a small injection front (Fig. 1A). Injection of standard solutions of thiomalate continued to be reproducible and gave a single peak (Fig. 1B).

During a study on N-acetylcysteine it was observed that more than one peak occurred when standard materials were separated on a ODS column. Further investigation revealed that only one peak could be attributed to N-acetylcysteine. The other peaks were thiomalate and D-penicillamine, which had been previously run on the same Spherisorb-ODS column. To investigate this further, an ODS-Hypersil column which had previously been employed for many hundreds of thiomalate assays was injected with 500-pmol aliquots of a series of freshly-prepared thiol solutions. The results are shown in Table II and a representative chromatogram is shown in Fig. 1C. All the thiols tested were capable of releasing thiomalate from the column. This ability differed with each thiol; D-penicillamine being apparently the most efficient and thioglucose the least. However during the experiments, which were performed in the order given in the table, the amount of thiomalate remaining on the column would have fallen. This may explain why D-penicillamine appeared more effective than dithiothreitol, an efficient disulphide reducing agent. Following overnight cleaning of the column with methanol–water (50:50) the amount of thiomalate released by N-acetylcysteine fell to 35 pmol.

This effect was not observed for captopril, possibly because it requires a buffer

TABLE II

RELEASE OF THIOMALATE FROM A USED COLUMN BY OTHER THIOLS

The column had been used regularly for the determination of plasma thiomalate according to ref. 4.

*Thiol injected**	*Thiomalate released*	
	Peak Height (mm)	*Amount (pmol)*
D-penicillamine	200	2000
L-cysteine	67	670
N-acetylcysteine	127	1270
Thioglucose	38	380
Cysteamine	118	1180
N-acetyl-D-penicillamine	111	1110
2-Mercaptopropylglycine	97	970
Dithiothreitol	160	1600

* 500 pmol of each thiol was injected.

containing 40% methanol for elution from an ODS column. Neither D-penicillamine nor cysteine could be displayed from a used cation exchange column by N-acetylcysteine.

Carry-over is a well known chromatographic problem and is usually caused by contamination with traces of the previous sample from areas such as the injection loop. Carry-over from the column as determined by blank injections is usually slight. In the example reported here a different mechanism appears to be involved. Thiols are strongly absorbed onto some column packings under certain conditions. A number of mechanisms are possible. The displacement of bound thiols by other thiols is typical of many thiol–disulphide interactions. Disulphide bonds onto proteins accumulated on the column from previous samples was unlikely since D-penicillamine bound to ODS materials but not cation-exchange even though the same type of samples were being analysed. Binding to the metal parts of the systems is similarly discounted although the improvements caused by EDTA indicate that some interactions do occur. The binding appears to be directly to ODS packing materials when thiols are chromatographed in buffers of high polarity. Since captopril did not bind in 40% methanol and methanol successfully cleaned the columns it appears, that even a small reduction in polarity will reduce the binding.

CONCLUSIONS

Clearly those chromatographing thiols should be aware of the problems outlined above since most physiological fluids contain large amounts of endogeneous thiols, which will readily displace thiols of interest bound to the column. One indication of possible column binding is the fact that some columns need to be saturated with standard materials before they give reproducible peaks in response to standard injections. To minimise displacement effects, the column should be cleaned with multiple injections of another thiol and then flushed with methanol preferably overnight. The use of assays in which the thiol moiety is chemically derivatised might avoid such problems entirely.

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