Journal of Chromatography, 354 (1986) 155–167
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 266

SOLID-PHASE DERIVATIZATION OF THIOLS

APPLICATION TO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received July 26th, 1985; revised manuscript received October 14th, 1985)

SUMMARY

The use of functionalized silica materials packed in a short column has been investigated for the determination of thiol compounds in solution and in chromatographic eluates. The influences of the silica treatment, grafted disulphide, pH and solvent effects are described. These silica-based postcolumn reagents exhibit high capacities, result in low peak broadening and are compatible with organic solvents. They permit the detection of 0.05 nmol of thiol.

INTRODUCTION

Thiol compounds are of great importance in biochemical processes and in chemical systems. Thus, substantial efforts have been made to design highly specific reagents for their determination[1–12]. Some methods involve the release of highly coloured[5–9] or fluorescent products[10–12]. However, in many cases, the thiols must be separated before derivatization. High-performance liquid chromatography (HPLC) with precolumn[13–17] or postcolumn derivatization[18–20] has been reported as an useful means for the analysis of thiol mixtures; the former technique is often time-consuming and the latter can introduce peak broadening which can affect the resolution, peak shape and height and minimum detection limits.

Within the past few years, novel approaches for performing pre- or postcolumn derivatization without excessive dilution have been described[21,22].

In the case of thiols and of disulphides, thiol reagents immobilized on soft Sepharose gels have been introduced by Studebaker[23,24]. In order further to minimize peak broadening in this technique, the thiol polymers can be replaced by thiol-functionalized silica materials[25,26]; this may allow operations at high flow-rates, and the use of microparticulate silica may improve the derivatization kinetics. We have studied two solid-phase reagents (SPRs) obtained from 10-μm diameter silica particles. After activation with a disulfide R'S–SR', they allow the determination of thiol compounds in solutions or in chromatographic eluates with high sensitivity.

The elution of a small amount of a thiol RSH on a disulphide silica column

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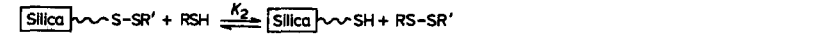
may proceed by two reactions

(1)



(2)

When R' is aromatic, the $R'S^-$ anion is stabilized by resonance in a thione form, and reaction 1 becomes predominant; moreover, the thiol–disulphide interchange can readily be monitored by electronic absorption spectroscopy[5,27]. Thus, we have used aromatic disulphides.

In order to improve the sensitivity of the method, we have investigated the influence of different parameters such as eluent pH, solvent and flow-rate. The nature of the silica surface and that of the radical R' are of great importance; free silanol groups on the silica, may result in additional phenomena such as retention of the released thiol or thione and tailing effects. These drawbacks can be avoided by grafting diol functions onto the silica surface[28]. Thus, by studying two thiol silica supports whose silanol groups are masked or not, and by using two disulphides, 2,2'-dipyridyl disulphide (2 PDS) and 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), whose corresponding thiols have very different solubilities in water, we have been able to prepare silica-based materials and use them as postcolumn solid-phase reagents.

EXPERIMENTAL

Materials

LiChrospher Si 100, 2-thiopyridone, 2-mercaptoethanol and cysteamine hydrochloride were obtained from Merck. (3-Mercaptopropyl)trimethoxysilane, 2 PDS, DTNB and dithioerythritol (DTE) were obtained from Aldrich. L-Cysteine hydrochloride, acetonitrile, sodium dihydrogenphosphate, disodium hydrogenphosphate, toluene and EDTA were purchased from Prolabo. Glycidoxypropyltrimethoxysilane was obtained from Polysciences.

Preparation of mercaptopropyl-bonded silica (support I)

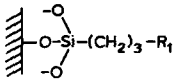
A suspension of LiChrospher Si 100 (1 g) in toluene (9.5 ml) was placed in an ultrasonic bath at 80°C. After addition of 0.5 ml of freshly distilled mercaptopropyltrimethoxysilane, the mixture was kept for 1 h under these conditions. After several washings with toluene and ethanol, the thiol silica was dried at 40°C and used immediately. The thiol content was determined with a 2 PDS solution[29], and the sulphur content by elemental analysis.

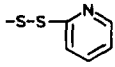
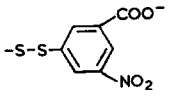
Preparation of mercaptopropyl- and diol-bonded silica (support II)

The introduction of diol functions onto silica was achieved by treating silica with glycidoxypropyltrimethoxysilane in an aqueous medium as described previously[30]. Then, 1 g of diol silica (9% carbon) was suspended in 8 ml of toluene, and placed in an ultrasonic bath at 80°C. After the addition of 2 ml of mercaptopropyltrimethoxysilane, the mixture was kept for 2 h under these conditions. Washing, drying and determination of the thiol and sulphur contents were performed as for product I (Table I).

TABLE I

STRUCTURE AND CHARACTERISTICS OF THE SILICA-BASED SUPPORTS



Support No.	R_1	*S content* (mmol/g)*	*SSR' content** (mmol/g)*
I	–SH	0.36–0.5	–
II	–O–CH_2–CH(OH)–CH_2(OH) and –SH	0.03–0.05	–
III		–	0.35–0.5
IV	–O–CH_2–CH(OH)–CH_2(OH) and 	–	0.025–0.05
V		–	0.35–0.5

* Determined by elemental analysis.
** Determined with a 2 PDS solution.

Instrumentation

For all experiments, the eluent was delivered by an Altex pump (Model 110); thiol samples were injected within an U6K injector (Waters). The released thione was monitored by a 450 (Waters) variable-wavelength detector, and recorded on an Omniscribe recorder (Houston Instrument). The thiol silica was slurry packed in a 5 cm × 4 mm I.D. stainless-steel column[31], and then activated by pumping a $5 \cdot 10^{-3}$ *M* disulphide solution in 0.1 *M* phosphate buffer–ethanol (1:1), at 0.2 ml/min, for 16 h. Three kinds of supports were investigated (see Table I): mercaptopropyl-bonded silica activated by 2 PDS (support III), mercaptopropyl- and diol-bonded silica activated by 2 PDS (support IV) and mercaptopropyl-bonded silica activated by DTNB (support V).

Standard procedure

Thiol solutions were prepared in buffers containing 1 m*M* EDTA, to prevent oxidation. 50-μl Aliquots were injected on the disulphide column. The response was evaluated by measuring the height, H, and/or the surface area, S_1. of the released thione peak at 350 nm for supports III and IV, at 412 nm for support V.

The amount, ρ (%), of the thiol–disulphide interchange was calculated by comparing S_1 with the surface area, S_0, of the peak obtained by injection of the theoretical amount of thione onto a 5-cm length of an inert column (LiChrosorb Diol, Merck):

$$\rho = S_1/S_0 \tag{3}$$

RESULTS AND DISCUSSION

Detection of thiol compounds on a 2 PDS-activated mercaptopropyl silica (support III)

Calibration curve. The injection of a thiol such as DTE on support III yielded a peak at 350 nm corresponding to the released thione (see reaction 1 and Fig. 1a). When increasing concentrations of DTE were injected, the calibration curve derived from the peak surface areas was linear from $5 \cdot 10^{-4}$ to 10^{-5} *M* (lowest detectable concentration), contrary to that obtained from the peak heights which showed a noticeable curvature. This discrepancy is caused by peak asymmetry which is dependent on the injected thiol concentration: when concentrated thiol solutions are injected on the solid-phase reagent (SPR) the thiol–disulphide interchange is more rapid and the released thione is eluted with a sharper peak than with diluted solutions.

Influence of acetonitrile. Different thiols were detected on this support (see

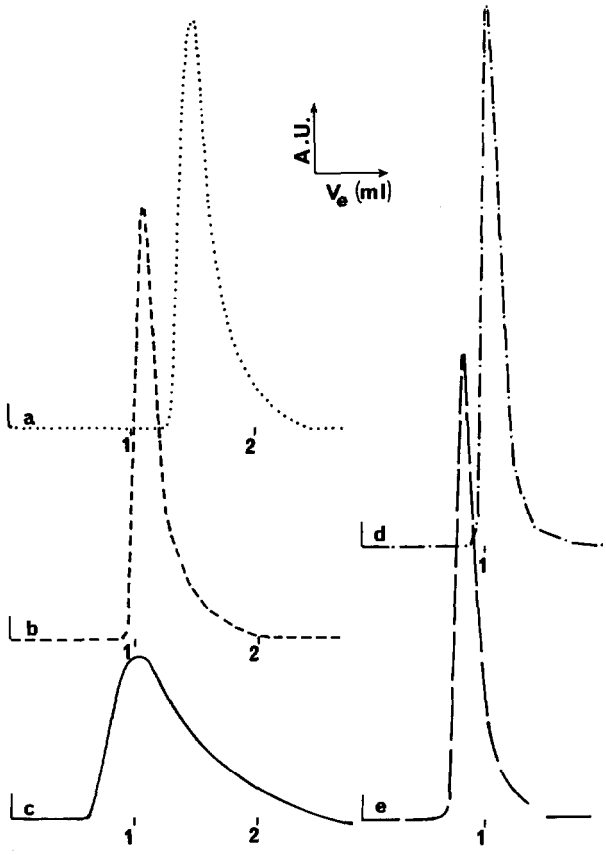


Fig. 1. Response of various solid-phase reactors to 10^{-4} *M* DTE injections. Experimental conditions: columns, 5 × 0.4 cm I.D.; flow-rate, 0.5 ml/min. (a) Support III; 0.15 *M* phosphate buffer, pH 6.0; λ = 350 nm at 0.2 a.u.f.s. (b) Support III; 0.15 *M* phosphate buffer, pH 6.0–acetonitrile (80:20); λ = 350 nm at 0.2 a.u.f.s. (c) 2 PDS-activated thiopropyl Sepharose; 0.15 *M* phosphate buffer, pH 8.0, 10^{-3} *M* EDTA; λ = 350 nm at 0.1 a.u.f.s.(d) Support IV; 0.15 *M* phosphate buffer, pH 8.0; λ = 350 nm at 0.2 a.u.f.s. (e) Support V; 0.15 *M* phosphate buffer, pH 7.95; λ = 412 nm at 0.4 a.u.f.s.

TABLE II

DETECTION OF THIOL COMPOUNDS ON SUPPORTS III–V

Conditions: thiol concentration, 10^{-4} *M*; flow-rate 0.5 ml/min; injection volume, 50 µl; eluent, 0.15 *M* phosphate buffer, 10^{-3} *M* EDTA. V_e = Elution volume. S_1 = Detector response (0.2 a.u.f.s.; chart speed 2.5 cm/min).

Injected thiol	*Support*	*pH*	*Acetonitrile (%)*	V_e *(ml)*	S_1	$\rho = \frac{S_1}{S_0} \cdot 100$
DTE	III	6.0	0	1.46	262	130
			10	1.05	203.5	101
			20	1.09	173.3	86
	IV	6.0	0	1.09	82.6	41
		7.0	0	1.04	193.5	96
		8.0	0	1.1	197.6	98
	V	7.0	0	0.91	270	81
		7.9	0	0.84	324	97
Cysteine	III	6.0	0	1.45	162	79
			10	1.0	159.2	79
			20	1.0	163.2	81
	IV	6.0	0	1.05	90.1	45
		7.0	0	1.0	153.2	76
		8.0	0	1.03	181.4	90
	V	7.0	0	0.9	236	70
		7.9	0	0.83	290	86
Cysteamine	III	6.0	0	1.49	117	58
			10	1.01	102.8	51
			20	1.1	112.9	56
	IV	6.0	0	1.09	65.3	32
		7.0	0	1.05	110.9	55
		8.0	0	1.1	121.0	60
	V	7.0	0	0.91	194	58
		7.9	0	0.8	212	63

Table II). The elution volumes of the released thiones are constant, V_e from 1.45 to 1.49 ml. However, when injected on a 5-cm long LiChrosorb Diol reference column (Merck), 2-thiopyridone is eluted at a lower volume, V_e = 1 ml, and as a sharper peak (see Fig. 2a). This difference results from the reaction kinetics and/or from an affinity of the injected or of the released compounds for the support.

By adding to the eluent a component such as acetonitrile, the retention volume of the released thione decreases, V_e = 1 ml, and tailing effects are reduced (see Fig. 1b). Under these conditions, interactions between the thione and this support are minimized. Moreover, in the presence of acetonitrile the amount of thiol–disulphide interchange, ρ, becomes less than 100% for DTE, but is unaffected for cysteine and cysteamine (see Table II).

One can conclude that, in purely aqueous media, DTE has an affinity toward the modified silica. Due to the presence of two SH groups in the DTE molecule, several reactions with the SPR can be proposed. In the absence of acetonitrile, after reaction 4, DTE is strongly retained by the SPR, and there is a possibility of reaction 5:

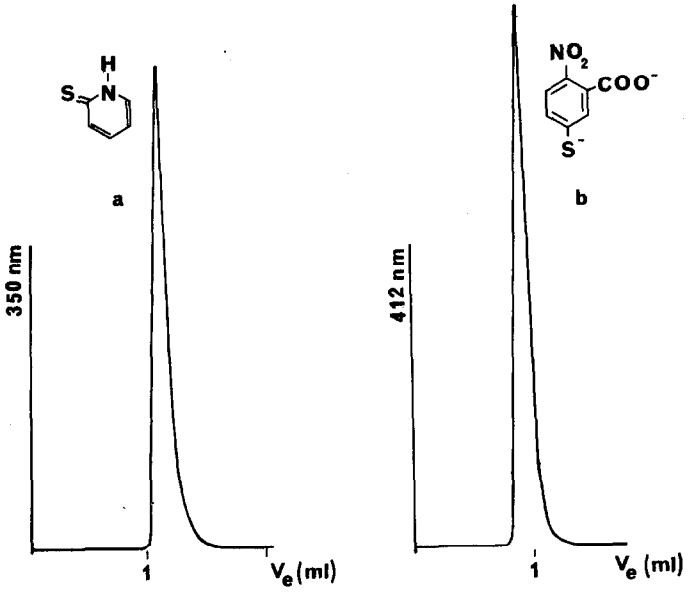
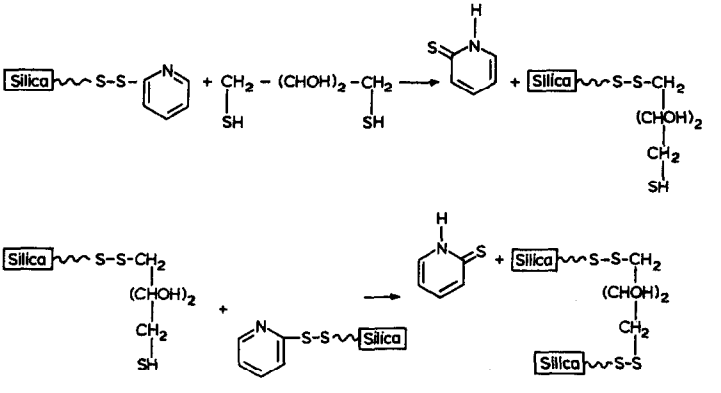


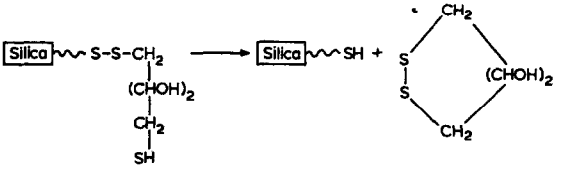
Fig. 2. Injection of 2-thiopyridone (a) and of 5-thio-2-nitrobenzoate (b) anion into a reference column (5 × 0.4 cm I.D.; LiChrosorb Diol, Merck; particle diameter, d_p = 10 μm). Flow-rate: 0.5 ml/min. Eluent: phosphate buffer, pH 8.0.



(4)

(5)

Two moles of thione are released per DTE molecule, which can explain ρ values higher than 100%. In the presence of acetonitrile, after reaction 4, the usual cyclization of the oxidized DTE[32] can take place (reaction 6)



(6)

and leads to the release of one thione per DTE molecule, lowering the ρ value, as observed experimentally.

Influence of flow-rate and pH. The influence of pH has been investigated in order to improve the sensitivity of the detection. The reaction yield is practically constant for pH values between 4.5 and 6.5 and decreases at higher pH. Similarly, by lowering the flow-rate from 1 to 0.2 ml/min, which involves a greater residence time of the thiol compound in the column, there is only a slight increase in the thiol–disulphide interchange.

Thus, under these conditions (0.01 a.u.f.s., maximum sensitivity of the UV detector used), this support permits a detection limit of $5 \cdot 10^{-10}$ mol of thiol.

Detection of thiol compounds on a 2 PDS-activated, mercaptopropyl and diol silica (support IV)

In order to avoid the addition of acetonitrile to the eluent, we have studied support IV whose silanol groups are masked. When thiol solutions are injected on a column filled with this material, the released thione peak is eluted at low retention volumes and presents no tailing effects (see Fig. 1d), demonstrating that adsorption phenomena are minimized with this support.

Influence of flow-rate on calibration curve. With a flow-rate of 1 ml/min, the response of support IV to cysteine injections is linear from $5 \cdot 10^{-4}$ to $5 \cdot 10^{-6}$ *M* (minimum detectable concentration), and the amount, ρ, of the thiol–disulphide interchange is practically constant, 35–40%; the correlation coefficient for 15 injections was found to be $\sigma = 0.999$. At lower flow-rates, ρ decreases when the thiol concentration becomes smaller than 10^{-4} *M* (see Fig. 3). This can be explained by the onset of the reverse reaction of the thiol–disulphide interchange (reaction 1) which prevents a higher extent of reaction under these conditions.

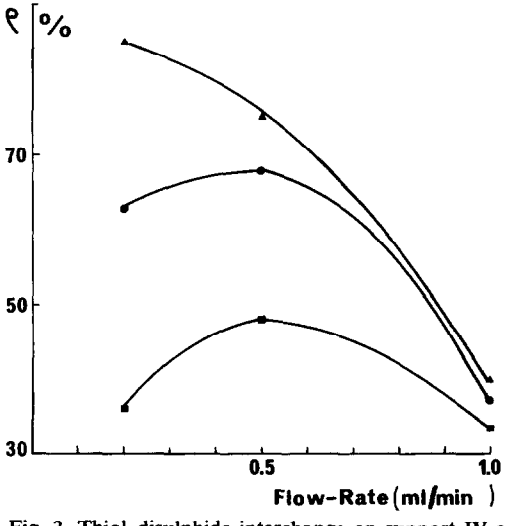
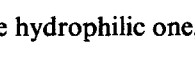


Fig. 3. Thiol–disulphide interchange on support IV as a function of the flow-rate at different cysteine concentrations: ▲, $5 \cdot 10^{-4}$, $2 \cdot 10^{-4}$ and 10^{-4}; ●, $5 \cdot 10^{-5}$; ■, 10^{-5} *M*. Experimental conditions: injection volume, 50 µl; eluent, 0.15 *M* phosphate buffer, 10^{-3} *M* EDTA, pH 7.0.

Influence of pH and of acetonitrile. The detector response at pH 6.0 is lower than with support III, but at higher pH values (up to 8) the released thione peaks are sharper and the amount of the diol–disulphide interchange increases (see Table II). Owing to the presence of the thiol function, the addition of acetonitrile (10–20%) to the eluent has little influence on the retention volumes and on ρ, but may be useful for the detection of poorly water-soluble thiol compounds. Under optimum conditions (pH 8.0, flow-rate = 1 ml/min), $2.5 \cdot 10^{-11}$ mol of thiol can be detected on this support.

For comparison, another thiol silica, whose silanol groups are masked by diol functions, was prepared in the same way as support IV, from LiChrosorb Diol (Merck); this support has a high thiol content ($1.2 \cdot 10^{-3}$ SH groups per g) and has been tested after activation by 2 PDS. The study of the released thione peaks revealed strong retention, even in the presence of acetonitrile in the eluent. This affinity may be due to the presence of a high concentration of $-(CH_2)_3-SSPy$ groups (Py = pyridyl) on the solid phase. Due to this defect, this support was not studied further.

Detection of thiol compounds on a DTNB-activated mercaptopropyl silica (support V)

By replacing the –SPy moiety by a more hydrophilic one,  , support V

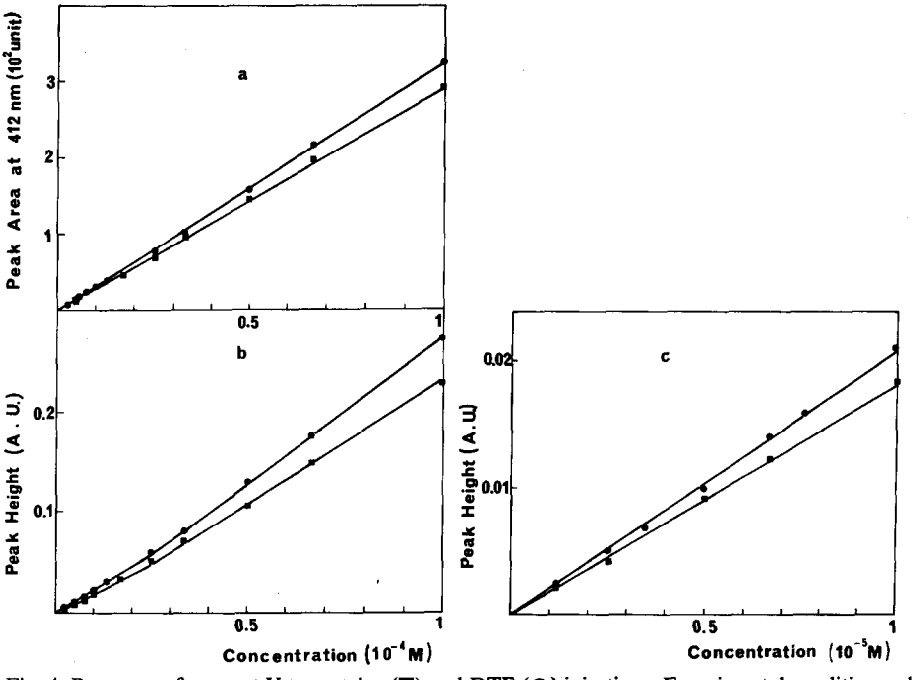


Fig. 4. Response of support V to cysteine (■) and DTE (●) injections. Experimental conditions: eluent, 0.15 *M* phosphate buffer, pH 7.95, 10^{-3} *M* EDTA; flow-rate, 0.5 ml/min; injection volume, 50 μl. (a) Peak area at 412 nm. (b) Peak height at 412 nm (absorbance units). (c) Peak height at 412 nm for thiol concentrations less than 10^{-5} *M* ($7 \cdot 10^2$ units corresponds to 10^4 mm²).

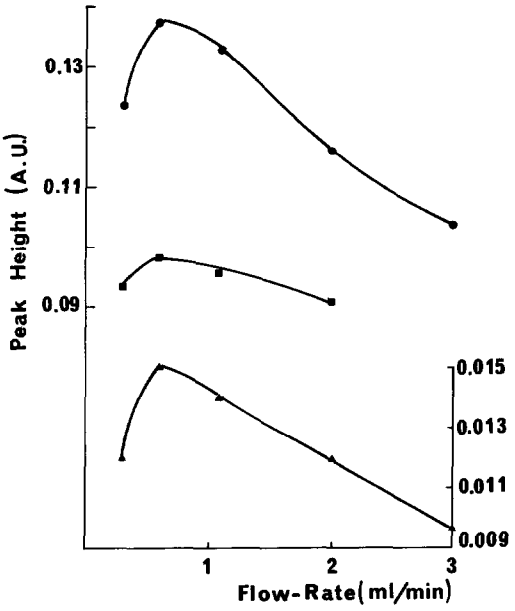


Fig. 5. Response of support V as a function of the flow-rate to different cysteine concentrations: ●, $7 \cdot 10^{-5}$; ■, $4 \cdot 10^{-5}$; ▲, $8 \cdot 10^{-6}$ *M*. Experimental conditions: injection volume, 50 µl; eluent, 0.15 *M* phosphate buffer, pH 7.95, 10^{-3} *M* EDTA.

has been obtained as described under Experimental. When thiol compounds are injected, the released thione, which has a 2-charge at pH values higher than 6.0 (thiol $pK_a = 4.53$[6]), is eluted as a sharp peak (see Fig. 1e) at smaller retention volumes than on other supports (see Table II).

Calibration curve. As shown in Fig. 4a, the response of this SPR to DTE and cysteine is linear from $2 \cdot 10^{-4}$ to 10^{-6} *M* (lowest detectable concentration), when the peak surface area, S_1, is plotted against thiol concentration ($\sigma = 0.999$ for 21 cysteine injections; $\sigma = 0.999$ for 27 DTE injections). The calibration curve obtained with peak heights (Fig. 4b) exhibits a slight change in slope, but can be used for thiol concentrations between $2 \cdot 10^{-5}$ and 10^{-6} *M* (see Fig. 4c).

Influence of pH, flow-rate and acetonitrile. The amount of the thiol–disulphide interchange increases when the pH rises from 6.0 to 8.0 (see Table II). However, at pH higher than 7.5, a spontaneous thione release sometimes took place as revealed by a strong increase in the baseline absorbance.

We have also investigated the influence of the flow-rate. From Fig. 5, it is seen that for any thiol concentration there is an optimum flow-rate between 0.5 and 1 ml/min; at lower flow-rates, a contribution of the reverse reaction (reaction 1) can be invoked.

Between 0.02 and 0.3 *M*, the ionic strength of the eluent has a very small influence on the detector response. It enables detection of thiol compounds whose elution from chromatographic columns requires concentration gradients. For example, thiol separations on a silica-based anion exchanger, prepared in our laboratory[33], are shown in Fig. 6.

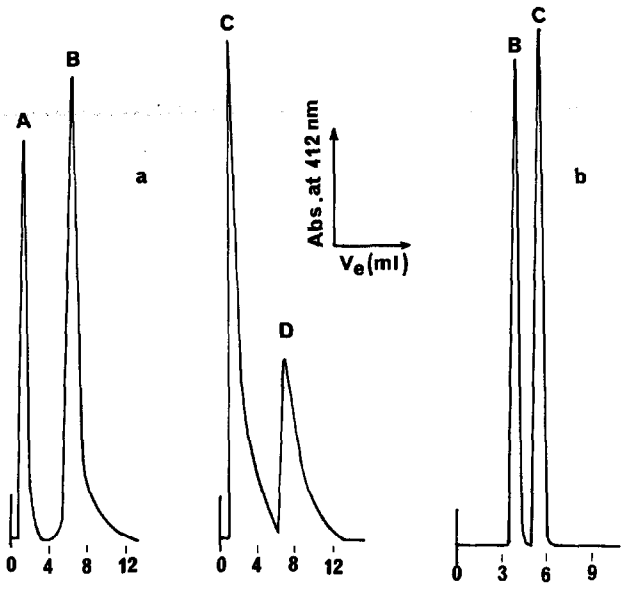


Fig. 6. Detection with support V of thiol mixtures after separation on an analytical column. Experimental conditions: (a) analytical column, anion-exchange silica, 7.5 × 0.4 cm I.D.; eluent, linear gradient from 5 to 95% B in 5 min, A = 0.02 *M* Tris–acetate, pH 8.0, B = 0.02 *M* Tris-acetate, 0.3 *M* sodium chloride, pH 7.7; flow-rate, 0.8 ml/min. (b) Analytical column, C_{18} μBondapak (25 × 0.4 cm I.D.); eluent, acetonitrile–0.15 *M* Tris-HCl, pH 7.9 (20:80); flow-rate, 1 ml/min. Peaks: A = DTE; B = reduced glutathione; C = 2-mercaptoethanol; D = cysteine.

Fig. 7 shows the influence of the addition of organic solvents such as acetonitrile or methanol to the eluent. An organic modifier in amounts as high as 50% (v/v) can be used, without drastic decrease of the detector response. This enables the detection of poorly water-soluble thiol compounds and of thiol mixtures separated on reversed-phase columns. Fig. 6b shows an example of a separation of thiol compounds on a C_{18} μBondapak column, detected after derivatization with support V.

Thus, under optimum conditions (pH 7.9, flow-rate = 0.5 ml/min), $5 \cdot 10^{-11}$ mol of thiol can be detected on support V with the detector presently used.

Peak broadening

As shown in Fig. 1, the silica-based materials result in less peak broadening than thiol Sepharose material tested under the same conditions (Fig. 1c).

In order to evaluate the peak broadening due to the chemical interchange reaction, we have compared the released thione peaks eluted from these SPRs (length 5 cm, d_p = 10 μm) and those obtained by injecting the same amount of the corresponding thione on a 5 cm long LiChrosorb Diol reference column (d_p = 10 μm). From the results summarized in the Table III, it appears that solid-phase reactions induce an increase in the thione peak width of 30% in the best cases (supports IV and V) and of 60% in the worst. This peak broadening could probably be reduced by using smaller silica particles.

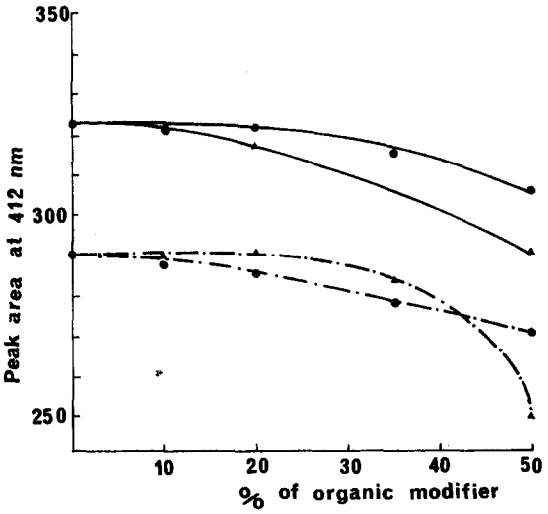


Fig. 7. Response of support V to DTE (——) and cysteine (— · — ·) injections as a function of the organic modifier (●, methanol; ▲, acetonitrile). Experimental conditions: thiol concentration, 10^{-4} *M*; flow-rate, 0.5 ml/min; eluent, 0.15 *M* Tris–HCl, pH 8.0 and an organic modifier; injection volume, 50 µl.

Column lifetime

About 200 10^{-4} *M* thiol solutions have been injected on support IV, corresponding to 1/20 of the SSR′ content of the solid-phase reactor, without noticeable decrease in the response. With supports III and V of greater SSR′ content, the number of injections would be ten-fold higher. We have not yet achieved this number. In spite of this, we have examined the possibility of regenerating support IV: the column was washed with a mercaptoethanol solution (10^{-2} *M*; pH 8.0) until the thione was completely released, and then reactivated by a disulphide. After these operations, the performance of the SPR was found to be reduced: for example, ρ which was close to 95% before regeneration, dropped to 60% afterwards.

It seems that the major limitation in the durability of these reactors is the slow dissolution of the silica in aqueous media, which leads to the occurrence of a small void in the column after use for about 10 weeks.

TABLE III

PEAK WIDTH AT HALF HEIGHT, δ, AS A FUNCTION OF THE NATURE OF THE SUPPORT

Conditions: column, 5 cm × 4 mm I.D.; d_p = 10 µm; thiol concentration, 10^{-4} *M*; flow-rate, 0.5 ml/min.

Support	*Injected compound*	δ *(ml)*
LiChrosorb Diol*	2-Thiopyridone	0.125 ± 0.006
	5-Thio-2-nitrobenzoate	0.138 ± 0.006
Support III**	DTE	0.200 ± 0.006
Support IV*	DTE	0.163 ± 0.006
Support V*	DTE	0.187 ± 0.006

* 0.15 *M* phosphate buffer, pH 8.0.
** 0.15 *M* phosphate buffer, pH 6.0–acetonitrile (80:20).

CONCLUSIONS

Solid-phase reagents for thiol detection based on silica materials can easily be obtained; columns (5 cm × 4 mm I.D.) containing 10–20 μmol SSR (support IV) and 140–200 μmol SSR (supports III and V) have been prepared. These allow numerous detections of thiol compounds without changing or regenerating the support.

Moreover, by using such silica-based SPRs, peak broadening is considerably reduced (see Fig. 1), compared to analogous soft gels. Compatibility with organic solvents such as acetonitrile or methanol is good, which allows detection of poorly water-soluble thiol compounds, and thiol separations on reversed-phase columns.

When using supports IV and V, no interaction of the released thione with the silica support was observed. They are operated at pH values from 7.0 to 8.0. Peaks obtained with the former are somewhat sharper, but the SSR′ content of the columns is lower and linearity of the response is not as good as on support V. Support III gives satisfactory results at low pH values (4.5–6.5), but the addition of 20% acetonitrile to the eluent is recommended.

Despite the slight contribution of the chemical interchange reaction to peak broadening, these solid-phase reactors make it possible to determine thiol compounds with high accuracy, since the minimum detectable amount has been evaluated as $5 \cdot 10^{-11}$ mol with the UV detector presently used.

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