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Ion-pair high-performance liquid chromatography of cysteine and metabolically related compounds in the form of their S-pyridinium derivatives

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Abstract

A procedure was developed for converting cysteine, glutathione, homocysteine, acetylocysteine, N-(2-mercaptopropionyl)glycine and its metabolite 2-mercaptopropionic acid into their S-pyridinium derivatives for determination by paired-ion reversed-phase high-performance liquid chromatography. The thiol compounds were derivatized with 2-chloro-1-methylpyridinium iodide within a few minutes at room temperature. The thiol group reacted smoothly with the reagent in the pH 8.2 buffer to form an S-pyridinium derivative showing strong UV absorption with a maximum at 314 nm. The reaction mixture was injected directly into a chromatograph without purification and detected spectrophotometrically at 314 nm. The six thiols in the pmol range were separated and determined in a single run on an octadecyl-bonded silica column under isocratic conditions using 0.175 *M* citrate buffer containing 10 mmol/l sodium octanesulphonate (pH 2.8), acetonitrile and methanol (82:6:12, v/v/v) as the mobile phase. Linear calibration graphs were obtained for concentrations of the thiols between 1 and 50 μmol/l. The detection limits ranged from 0.75 pmol for acetylocysteine to 2.1 pmol for 2-mercaptopropionic acid and the relative standard deviations were equal to or better than 9.0% at the 1 μmol/l thiol level and 0.86% at the 50 μmol/l level. Optimum derivatization reaction conditions and HPLC separation conditions were elucidated.

1. Introduction

Cystein and metabolitically related amino acids are substances of great biological importance. L-Cysteine participates in a number of biochemical processes that depend on the particular reactivity of the thiol group. The high nucleophilicity of the thiol function facilitates the role of cysteine as an active site, covalent catalyst, among others in papain and glyceraldehyde-3-phosphate dehydrogenase [1], and allows the cysteine residue of glutathione to accept and detoxify electrophiles during mercapturic acid biosynthesis [2,3] and peroxide reduction [4] or free radical scavenging [5]. Interest in both the physiological and pharmacological roles of SH-containing amino acids has resulted in the development of a number of different methods for their separation and determination.

Most analytical procedures for biological aminothiols involve some form of derivatization followed by separation by a chromatographic method, mostly high-performance liquid chromatography (HPLC), with various detection tech-

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niques. Most of the methods presented up to 1986 have been described in reviews [6,7]. These methods include UV measurement with precolumn derivatization [8,9], fluorimetric detection of monobromobimane (mBrB) derivatives [10–12], *o*-phthalaldehyde (OPA) derivatives [13], derivatives of halosulphonylbenzofurazans (SBD-F and SBD-Cl) [14,15], dansylaziridine [16], N-substituted maleimide [17] and a recycling postcolumn reaction using glutathione reductase and Ellman's reagent [18], detection by the sulphydryl–disulphide exchange reaction in the postcolumn system [19,20] and electrochemical detection [21,22]. More recent publications report data on enlargement of the bimane family of reagents by introducing *p*-sulphobenzoyloxybromobimane [23] as a membrane-impermeable reagent for the derivatization of thiols, likewise a fluorogenic oxazole-based [24] reagent, chiral derivatization with 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate for UV detection [25] and electrochemical detection after derivatization with 3,5-di-*tert*-butyl-1,2-benzoquinone [26] or silver nitrate [27] or without derivatization [28,29].

In spite of the popularity of various precolumn derivatization methods, there have been many reports describing various shortcomings of the procedures, and to date no one method has been shown to overcome all the problems. For example, OPA gives a derivative with cysteine, unlike with the other amino acids containing a primary amino group, with minimal fluorescence [13]. This problem may be overcome by oxidizing cysteine to cysteic acid, which when reacted with OPA–2-mercaptoethanol (MCE) yields a derivative with fluorescent properties similar to those of the other amino acid derivatives [13]. However, the conditions for these two reaction are very different, one being oxidizing and the other reducing, and it is difficult to obtain between-run reproducibility of cysteine derivatization with OPA–MCE following its oxidation; moreover, this procedure makes impossible the determination of cysteine in the presence of cystine without pretreatment of the sample with an SH-blocking reagent [30]. Many of the techniques suffer from incomplete reactions owing to the presence of some solvents or common buffer salts, and highly absorbing hydrolysis products, by-products or the reagent itself, unless an extraction step is incorporated into the procedure prior to the chromatographic analysis [26] or the molar excess of reagent is carefully limited.

In a search for an ideal derivatization reagent or highly automated techniques that are readily available, which could relax the criteria for a practical reagent, we focused our studies on developing a new reagent for the HPLC of aminothiols that minimizes or eliminates the drawbacks mentioned above. Recently we published results on the determination by spectrophotometry of cysteine and related compounds in the form of their S-pyridinium derivatives with the use of 2-chloro-1-methylpyridinium iodide (2-CMPI) as a derivatization reagent [31]. In this paper we report the usefulness of 2-CMPI as a UV derivatization reagent for the separation and determination of biologically important aminothiols by means of ion-pair HPLC.

2. Experimental

2.1. Apparatus

The HPLC system consisted of a Hewlett-Packard Series 1050 isocratic pump, a Hewlett-Packard Series 1050 variable-wavelength detector and a Hewlett-Packard HP 3394A integrator. The samples were injected using a Rheodyne Model 7125 injection valve fitted with a 20-μl loop. The column (250 × 4.0 mm I.D.) was prepacked with 5-μm diameter Spherisorb ODS-2 and operated at a flow-rate of 0.7 ml/min. The analytical column was fitted with a clean-up column (20 × 2.1 mm I.D.) packed with ODS Hypersil (30 μm). UV spectra were recorded on a Carl Zeiss Jena UV–Vis spectrophotometer (1-cm cells).

2.2. Chemicals and reagents

Reduced glutathione (GSH) and L-cysteine (CSH) were obtained from Reanal (Budapest,

Hungary) and DL-homocysteine (HSH), N-acetyl-L-cysteine (ACSH), N-(2-mercaptopropionyl)glycine (MPG) and 2-mercaptopropionic acid (PrSH) from Fluka (Buchs, Switzerland). Ion-pairing reagents (sodium 1-butane-, 1-hexane-, 1-octane- and 1-decanesulphonate) were supplied by Sigma (St. Louis, MO, USA). Other chemicals were purchased from Baker (Deventer, Netherlands).

2-Chloro-1-methylpyridinium iodide (2-CMPI) was prepared as described previously [32]. For aminothiol derivatization prior to HPLC, a 0.01 *M* aqueous solution of 2-CMPI was used.

Standard thiol solutions

Stock standard solutions (0.01 *M*) of the aminothiol compounds were prepared in water or dilute HCl and assayed with HMB [33]. Working standard solutions were prepared daily by dilution with water containing 1 mmol/l of EDTA.

Buffers

Citrate and phosphate buffers of various ionic strength and pH were prepared with purified water. At each pH value, the electrode was calibrated with standard pH solutions. After controlling the pH, the buffers were filtered through a 0.2-μm filter under vacuum.

2.3. Sample derivatization

In a 10-ml calibrated flask were placed an aliquot of sample and 3 ml of 0.1 *M* phosphate derivatization buffer (pH 8.1), then 1 ml of working reagent solution was added. The flask was stoppered, mixed by inversion and put aside for 15 min. The mixture was then diluted to volume with water and a 20-μl aliquot was injected into the liquid chromatographic system. The derivatization procedure was applied to working standard solutions of aminothiols to obtain a calibration graph.

2.4. Assay procedure

An aliquot of the sample solution was subjected to the derivatization procedure and 20 μl of the final analytical solution were injected into the liquid chromatograph in triplicate. The peak areas were measured and the amount of each analyte of the sample was then calculated by interpolation on the calibration graph.

2.5. Chromatography

HPLC separation were carried out under isocratic conditions on a Spherisorb ODS-2 reversed-phase column operated at a flow-rate of 0.7 ml/min. For routine determination of five aminothiols (GSH, CSH, HSH, ACSH and MPG) in a single run, a mobile phase consisting of 0.175 *M* citrate buffer containing 10 mmol/l of sodium octanesulphonate (pH 2.8) and methanol (80:20, v/v) and a detector wavelength of 314 nm were found to be appropriate, allowing an adequate separation of the five S-pyridinium derivatives. With more complicated mixtures, *e.g.*, when 2-mercaptopropionic acid (PrSH, metabolite of MPG) was present, the mobile phase was modified by addition of 6% of acetonitrile at the expense of methanol. Excess reagent elutes last in the form of a small peak (low ε at the recommended analytical wavelength) and does not interfere with the chromatogram (see Fig. 9).

3. Results

3.1. Optimization of aminothiol derivatization

The proposed derivatization reaction, shown in Fig. 1, takes advantage of the high nucleophilicity of thiols; they react rapidly in aqueous solution with 2-chloro-1-methylpyridinium iodide (2-CMPI) to form stable thioethers (S-pyridinium derivatives). These derivatives exhib-

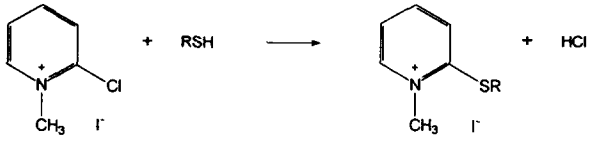

Fig. 1. Reaction of aminothiols (RSH) with 2-chloro-1-methylpyridinium iodide (2-CMPI).

it a well defined maximum at 314 nm in the UV spectrum as a consequence of the bathochromic shift from the reagent maximum (Fig. 2). Of the three functionalities of the amino acids potentially able to undergo nucleophilic attack at the 2-position of the pyridinium ring, in aqueous solution, unlike in anhydrous conditions [34], only the sulphydryl group reacts. This means that no multiple derivatives are formed, as was demonstrated previously [31].

Buffer type and pH

The yields of the aminothiol derivative formation in standard mixtures were studied using 0.1 *M* phosphate derivatization buffers with pH ranging from 7 to 10. Derivative yields for glutathione *versus* time and reaction pH are plotted in Fig. 3a and demonstrate that the recoveries reach a maximum after 5 min in the pH range 8.1–9.0, but at lower pH the reaction is slower. At higher pH (data not shown), the reagent tends to hydrolyse to 1-methyl-2-pyridone (MP) with an absorption maximum at 290 nm. For subsequent assays, 0.1 *M* phosphate derivatization buffer of pH 8.1 was used. Under these conditions, MP would not be expected to

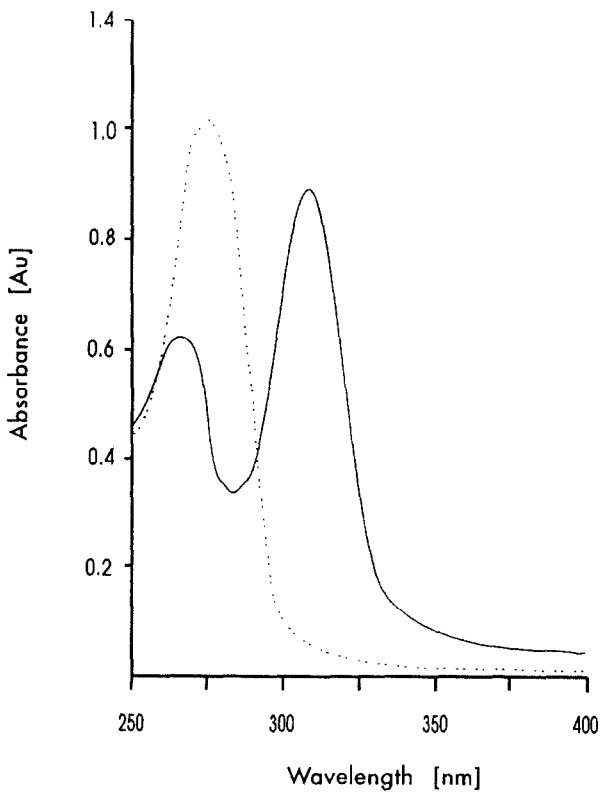

Fig. 2. Absorption spectra of 0.8 μmol of GSH (solid line) and a blank solution (dotted line) treated according to the proposed derivatization procedure. Cells with an optical path length of 1 cm were used.

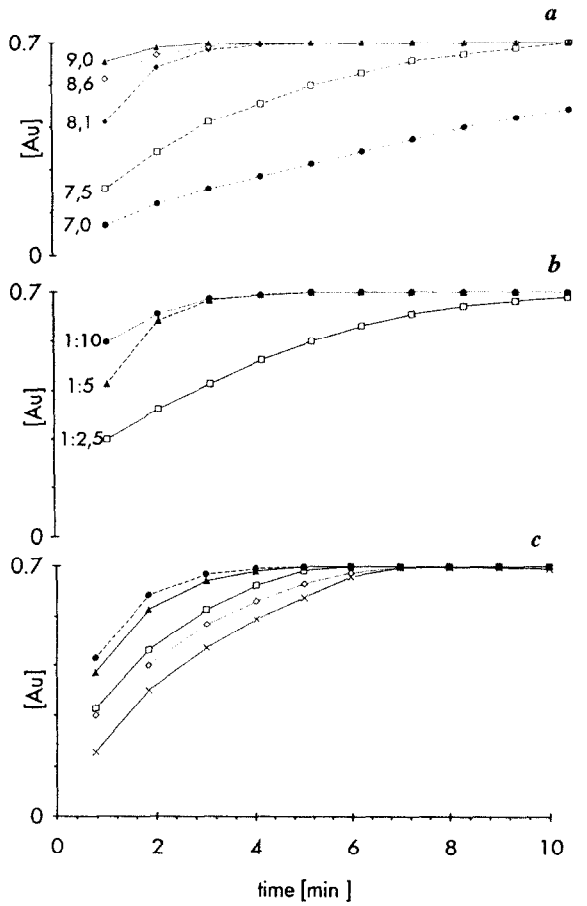

Fig. 3. Kinetics of aminothiol derivative formation in 0.1 *M* phosphate derivatization buffer performed by spectrophotometry; 0.8 μmol of each aminothiol, λ = 314 nm. (a) Effect of derivatization buffer pH and time on aminothiol derivative yields for glutatione. Molar ratio of the reagent to glutathione = 5. (b) Effect of reagent excess and time on aminothiol derivative yields for glutathione in 0.1 *M* phosphate derivatization buffer (pH 8.1). (c) Time course of the reaction of 2-CMPI with several aminothiols (pH 8.1). Fivefold reagent excess with respect to each aminothiol. × = ACSH; □ = CSH; ● = GSH; ◇ = HSH; ▲ = MPG.

interfere in the separation of the target thiols in the calibration range. However, with concentrations of thiols close to the detection limit, when the reagent excess is high, an MP peak appears on the chromatogram (see Fig. 9c).

Effect of the reagent excess and time of the reaction on aminothiol derivative yield

Fig. 3b shows that using a fivefold molar excess of the reagent with respect to glutathione afforded a maximum yield after 3 min, and increasing the excess of reagent beyond this level had no effect on the recoveries. With a 2.5-fold molar excess the derivative was obtained in 60% yield, and in 96% yield after 10 min. The reaction was found to slow with an increase in ionic strength of the reaction environment (0.0–2.0 mol/l range checked, NaCl added; data not shown). As can be seen in Fig. 3c, the rates of 2-CMPI derivative formation decrease in the order GSH > MPG > CSH > HSH > ACSH.

3.2. Derivative stability

Peak areas for the derivatized aminothiols (reaction mixture kept at ambient temperature or in a refrigerator at 4°C) were monitored for at least 10 days and no significant changes were observed. Hence the aminothiol derivatives have sufficient stability to allow for manual or automated chromatographic analysis.

3.3. Optimization of chromatographic conditions for separation of aminothiol derivatives

Highly ionic compounds, such as the present S-pyridinium derivatives of aminothiols could not be satisfactorily separated under standard reversed-phase conditions owing to their very short retention times. They elute close to the solvent front (Fig. 4a), which makes any separation or determination impossible. The only useful alternative was an ion-pair approach. In addition to pH, the effect of the organic modifier, the pairing ion concentration of the eluent and the lipophilicity (length of the alkyl chain) of the pairing ion on retention and resolution were studied.

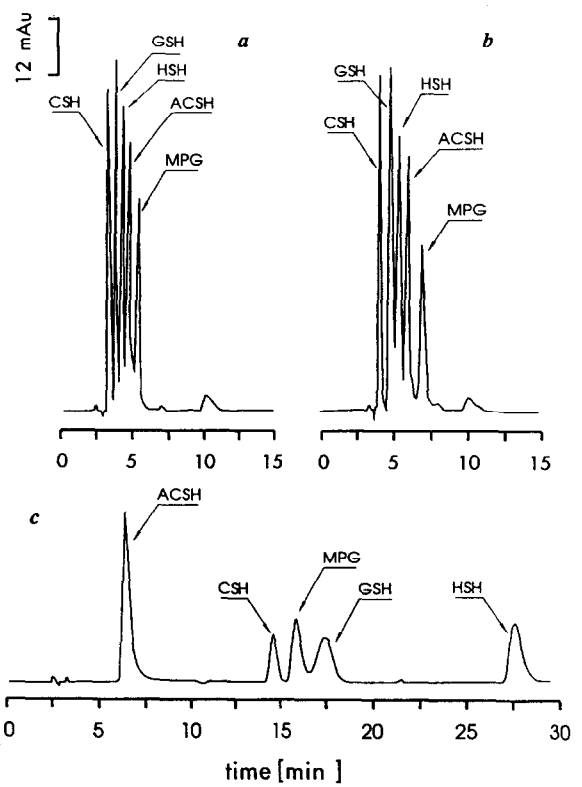

Fig. 4. Chromatograms of the derivatized aminothiol mixture obtained with 0.1 *M* citrate buffer (pH 2.8)–methanol (80:20, v/v) mobile phase containing (a) no ion-pair reagent, (b) 12 mmol/l of B-6 and (c) 10 mmol/l of B10.

Effect of buffer pH and ionic strength

The effect of pH was studied within the range 2.5–7.5, which was imposed by well known fact that outside this range a silica-based stationary phase (ODS-2) could be seriously damaged by aqueous buffer–mobile phase systems. We investigated the effect of the pH of the mobile phase containing 8 mmol/l of octanesulphonate on the capacity factors of all solutes concerned. As can be seen from Fig. 5, the capacity factors of all the solutes increase with decreasing pH in the range 2.5–3.0. In the pH range 3.0–7.5 the capacity factors of HSH and CSH increase and the other three (ACSH, MPG and GSH) elute very close to the void volume, having capacity

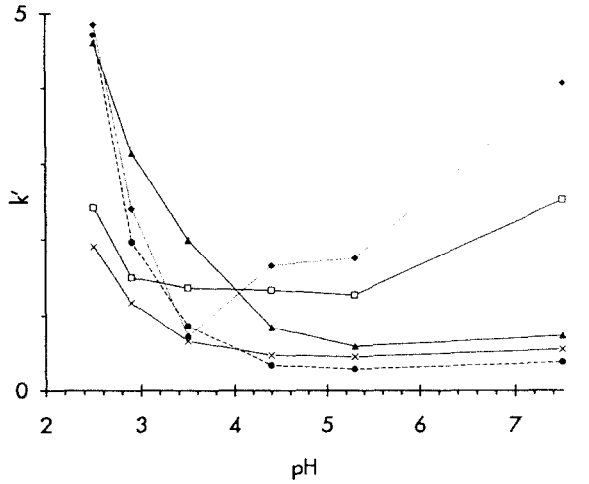

Fig. 5. Variation of the capacity factors of a mixture of aminothiol derivatives as a function of the eluent pH. Mobile phase: 0.1 *M* citrate buffer containing 8 mmol/l of B-8–methanol (80:20, v/v). Symbols as in Fig. 3 except ◆ = HSH.

factors smaller then 1. Therefore, we chose a mobile phase of pH 2.8 for routine HPLC target thiol determination, fulfilling the first criterion for good chromatographic separation, $1 < k' < 10$.

The effect of the ionic strength of the citrate buffer in the concentration range 0.05–2 *M* was also studied. An increase in the ionic strength of the buffer decreased the retention (data not shown), and the concentration of 0.175 *M* chosen constitutes a necessary compromise between maximum detectability and chromatographic resolution.

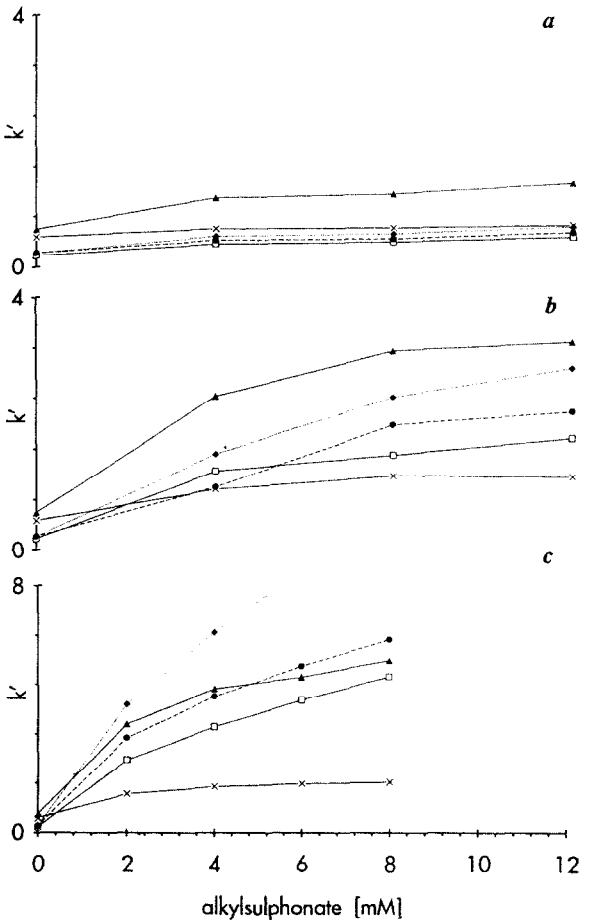

Fig. 6. Capacity factors (k') of aminothiol derivatives as a function of mobile phase sulphonate concentration: (a) hexanesulphonate; (b) octanesulphonate; (c) decanesulphonate. Mobile phase as in Fig. 4. Symbols as in Fig. 5.

Lipophilicity and concentration of ion-pair agent

The use of four alkanesulphonate pairing ions [1-butane- (B-4), 1-hexane- (B-6), 1-octane- (B-8) and 1-decanesulphonate (B-10)] of different lipophilicity was evaluated. The mobile phase concentration of the pairing ions was varied from 1 to 12 m*M*. In Fig. 6 the solute capacity factors are plotted against the mobile phase sulphonate concentration. In the case of sodium hexanesulphonate (B-6) only N-(2-mercaptopropionyl)-glycine (MPG) becomes more retained in the B-6 concentration range 4–12 m*M*, and the capacity factors of the four remaining analytes were virtually unchanged (Figs. 4b and 6a). A similar situation occurred when B-4 as an ion-pair agent was added (data not shown). Sodium octanesulphonate (B-8) enhanced the retention of all solutes and in the concentration range 8–12 m*M* (Figs. 6b and 7a) the capacity factors and resolutions guarantee a good chromatographic separation ($1 < k' < 10$, $R_s > 1.5$). When B-8 was replaced with B-10 a dramatic increase in retention was observed in all instances except ACSH and owing to the high k' values broad peaks were formed, worsening the quantification (Figs. 4c and 6c).

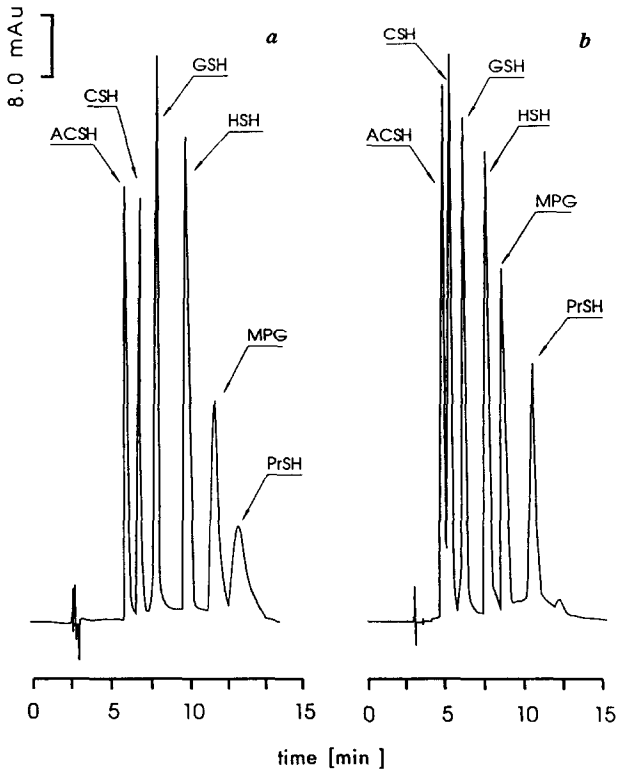

Fig. 7. Chromatograms obtained during the optimatization of the composition of the ternary mobile phase for the separation of six derivatized biological thiols. Mobile phase: (a) methanol–0.175 *M* citrate buffer containing 10 mmol/l of B-8 (20:80, v/v); (b) acetonitrile–0.175 *M* citrate buffer containing 10 mmol/l of B-8 (14:86, v/v).

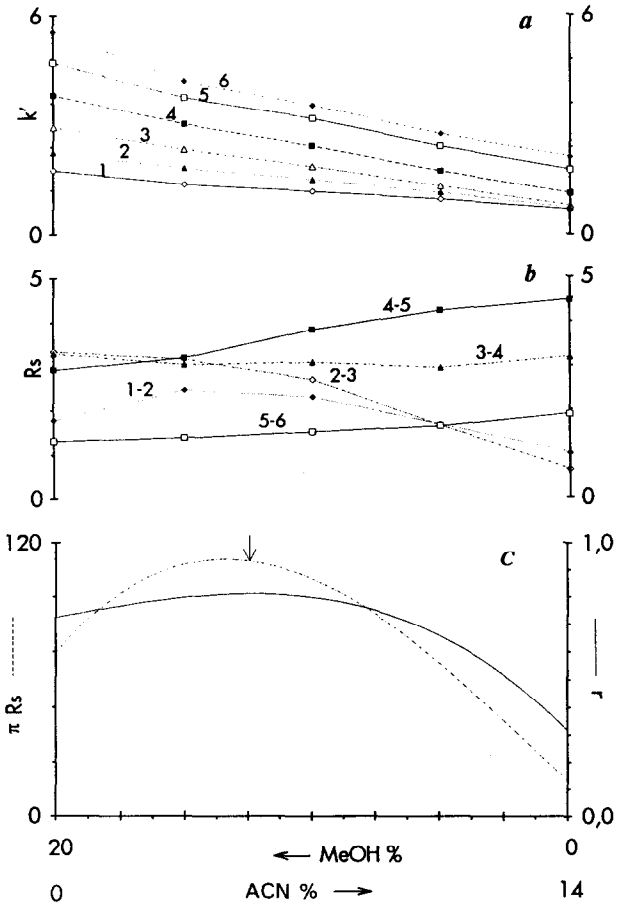

Fig. 8. Final phase selection diagrams for the ternary optimization problem of the separation of six biological thiols: (a) variations of capacity factors; (b) variations of resolutions; (c) response lines of resolution product (πR_s) and normalized resolution product (r). Composition of aqueous component of the mobile phase as in Fig. 7. 1 = ACSH; 2 = CSH; 3 = GSH; 4 = HSH; 5 = MPG; 6 = PrSH.

Effect of organic modifier

Organic modifiers such as acetonitrile, methanol, 2-propanol and tetrahydrofuran were added to the mobile phase in order to check their influence on the separation quality. Addition of 2-propanol and tetrahydrofuran caused tailing and broadening of the peaks of MPG and its metabolite PrSH, so these solvents were not considered further. As expected, an increase in acetonitrile content, at the expense of methanol, maintaining the same solvent strength, produced a linear decrease in all k' values. This is shown in Fig. 8a, where 100% methanol (far left) represents the mobile phase methanol–buffer (20:80, v/v) and 100% acetonitrile (far right) represents acetonitrile–buffer (14:86, v/v). Chromatograms corresponding to these two extreme mobile phase compositions obtained during the optimization procedure are shown in Fig. 7a and b. The best binary separation is achieved with 20% methanol (Fig. 7a), but still a better separation of the tailing peaks of MPG and PrSH would be desirable. The plots of R_s values for several pairs of peaks *versus* ternary solvent compositions are shown in Fig. 8b. These plots show that with an increasing content of acetonitrile in the mobile phase up to 50% of methanol does not cause

much change in resolution, and further increases in acetonitrile concentration lead to poorer resolution of less retained solutes (ACSH, CSH and GSH) and improved resolution of more retained solutes (HSH, MPG and PrSH).

For the prediction of the optimum ternary mobile phase composition, a phase selection diagram (Fig. 8c) was constructed from the chromatograms shown in Fig. 7 and three others, using the criteria r (normalized resolution product) and πR_s (resolution product). The r and

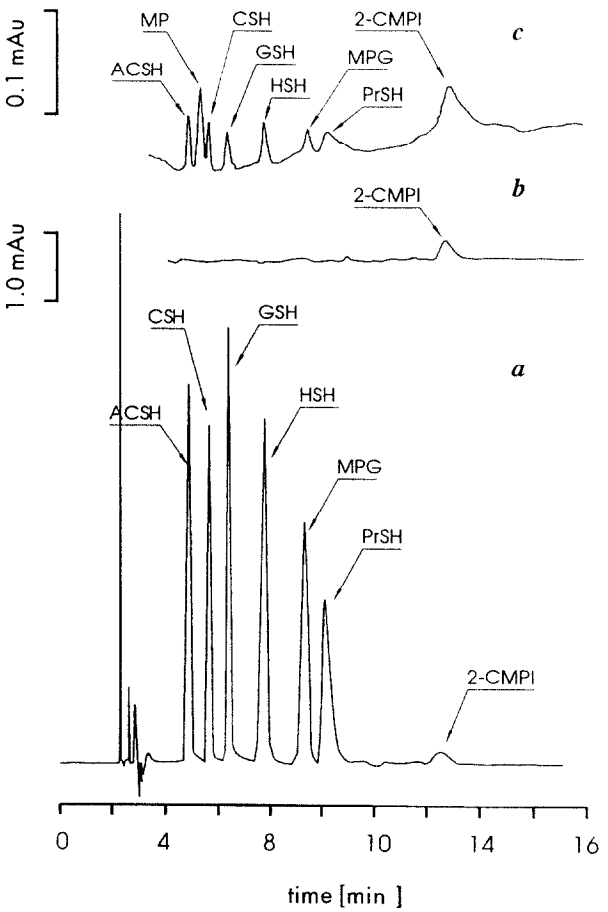

Fig. 9. Optimized chromatography for the derivatized biological thiol standard mixture: (a) 1 nmol for injection (2-CMPI to thiol ratio = 7:1); (b) blank; (c) 4 pmol for injection (2-CMPI to thiol ratio = 1750:1). Mobile phase: acetonitrile–metahnol–0.175 *M* citrate buffer containing 10 mmol/l of octanesulphonate (6:12:82, v/v/v). Detection wavelength: 314 nm.

πR_s values for the given chromatograms were calculated according to the following equations [35]:

$$r = \prod_{i=1}^{n-1} \left(\frac{R_{si,i+1}}{R_s} \right)$$

$$\pi R_s = \exp\left(\sum \ln R_s \right)$$

$$R_s = \frac{k'_2 - k'_1}{k'_1 + k'_2 + 2} \cdot \frac{\sqrt{N}}{2}$$

where n is the number of peaks, k' the capacity factor, N the number of theoretical plates, R_s the resolution and $\bar{R}_s$ the average R_s value.

The point marked with an arrow in Fig. 8c corresponds to the final optimum composition. The chromatogram shown in Fig. 9a was run to verify the optimum composition. This chromatogram is the final result of the procedure, and it does indeed yield a satisfactory distribution of the peaks over the chromatogram.

3.4. Analytical parameters

A linear calibration graph was obtained over the range 1–50 μmol/l with relative standard deviations ≤9.2% at the 1 μmol/l and ≤0.86% at the 50 μmol/l thiol level. The correlation coefficients for response linearity were ≥0.99885. The detection limits ranged from 0.75 pmol for ACSH to 2.1 pmol for PrSH. Detailed data are given in Table 1.

4. Discussion

A six-component mixture of biologically important thiols was taken as an example to demonstrate the usefulness of the method. All the solutes are considered to be of interest and must be separated from each other. The method introduces a new reagent for derivatization of biological thiols for HPLC with superior properties to those of some currently employed reagents, and permits the separation and determination of the six compounds at the pmol level in a single isocratic HPLC run.

Table 1
Analytical parameters

Thiol[a]	Correlation coefficient[b]	Relative standard deviation (%)[c]	Detection limit (pmol)[d]
ACSH	0.99930	2.71; 0.86	0.75
CSH	0.99951	1.28; 0.75	0.94
GSH	0.99997	1.04; 0.79	0.88
HSH	0.99974	1.56; 0.72	0.79
MPG	0.99885	1.15; 0.70	1.5
PrSH	0.99979	9.21; 0.57	2.1

Detection wavelength, 314 nm.
[a] Samples of standard thiol mixtures were in the concentration range 1–50 μmol/l.
[b] $n = 6$.
[c] For bottom and top of the calibration range; $n = 5$.
[d] As calculated from a 4-pmol injection (Fig. 9c) of final analytical solution and based on a signal-to-noise ratio of 3.

The reagent possesses several advantages: high selectivity as it reacts only with thiol group and no multiple derivatives are formed with sulphur amino acids; high reactivity even in the presence of dissolved salts; compatibility with aqueous samples; lack of interference with the chromatogram due to a bathochromic shift of the absorption maximum in the UV spectrum from 275 nm (reagent) to 314 nm (derivative), ensuring a resolution of better than 1.5 for all peaks of interest; low rate of hydrolysis under the recommended conditions; the absorption maximum of the derivatives (analytical wavelength of the detector) falls in the region (314 nm) where common impurities of solvents are UV transparent; and a simple derivatization procedure and high stability of derivatives, allowing automation. Quantitative conversion to S-pyridinium adducts simply requires the addition of the reagent to a buffered sample followed by a few minutes of waiting.

The disadvantage of this method, compared with direct liquid chromatographic approaches with the use of electrochemical detection, is its inability to determine disulphide metabolites, unless they are converted into the corresponding thiols using a reductant such as tri-*n*-butylphosphine and subsequent derivatization of the sulphydryl to form an ultraviolet chromophore in a parallel run.

With regard to sensitivity, owing to the relatively high molar absorptivity of S-pyridinium derivatives ($\varepsilon = 1 \cdot 10^4$ l mol^{-1} cm^{-1}) the method can compete with HPLC methods using fluorescence detection generally considered as one order of magnitude more sensitive than those with UV detection. The detection limits with the recommended method are almost the same as those with bimane (*ca.* 1 pmol) [12] or ammonium-7-fluorobenzo-2-oxa-1,3-diazole-4-sulphonate (0.1–1.4 pmol) [14], lower than those with dansylaziridine (*ca.* 15 pmol) [16] or ethacrynic acid (30 pmol) [9] and higher than those with an oxazole-based reagent (*ca.* 1 fmol) [24].

Such a powerful derivatization reagent for biological thiols as 2-chloro-1-methylpyridinium iodide is likely to find numerous applications in analytical chemistry and biochemistry. In this work the usefulness of the reagent was tested for the separation and determination of compounds in standard solutions, but experiments on the analysis of biological samples are near the completion and will be presented elsewhere.

Acknowledgements

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