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Application of micro-scale liquid chromatography with fluorescence detection to the determination of thiols

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ABSTRACT

Different thiol compounds of biological and pharmacological interest were separated on a packed reversed-phase fused-silica capillary column and determined with fluorescence detection. Conventional inexpensive liquid chromatographic equipment could be adapted for such purposes, providing a highly efficient analytical system. The different compounds were derivatized with the fluorogenic reagents SBD-F and ABD-F and chromatographed both isocratically and in the gradient mode in order to separate a series of thiols with widely varying polarities. Subsequently the derivatives were measured at $\lambda_{ex.}$ = 380 nm and $\lambda_{em.}$ = 510 nm. Application of the system to biological and pharmacological samples is suggested.

INTRODUCTION

Since the late 1970s, when the groups of Novotny[1] and Ishii[2] introduced capillary columns in liquid chromatography (LC), the development of this technique has shown considerable progress. Numerous laboratories are increasingly interested in its potential, as evidenced by the frequent publication of books[3–8], reviews[9,10] and papers on the developments and/or applications of this technique in different fields. The success of capillary columns is undoubtedly due to their distinct advantages, including the low consumption of mobile phase and stationary phase, better permeability and efficiency, higher mass sensitivity and easy coupling with other

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separation and detection techniques[11,12]. However, micro-LC instrumentation and technology have still to develop their full potential. Their growth has been slow, the problem most often being the LC detection system[13].

In this work, a conventional LC pumping device and fluorescence detector were adapted to micro-LC analysis. This was possible by employing a split-flow technique for delivering the eluent and by using a laboratory-made square quartz cell for performing the micro-LC fluorescence measurements. As a result, a highly sensitive and efficient separation system was obtained, which was applied to the LC–fluorescence determination of several thiol compounds of biological and pharmacological importance. These compounds were first derivatized with the thiol-specific fluorogenic reagents SBD-F and ABD-F[14], developed by Imai's group[15–17], and for which several applications in high-performance LC[18–28] and in thin-layer chromatography[29–36] have been reported during the last 5 years.

EXPERIMENTAL

Chemicals

The thiol-specific fluorogenic reagents ammonium 7-fluoro-2,1,3-benzoxadiazole (SBD-F) and 4-(aminosulphonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) were obtained from Wako (Neuss, F.R.G.). The thiol compounds were purchased from Janssen Chimica (Beerse Belgium), Merck (Darmstadt, F.R.G.), UCB (Leuven, Belgium) and Aldrich (Beerse, Belgium). Captopril was obtained from Squibb (Brussels, Belgium). All compounds were chemically pure and were used without further purification. Acetonitrile and water for the mobile phase were of HPLC grade (Alltech, Deerfield, IL, U.S.A.).

Disodium EDTA (Merck) was added to all thiol and reagent solutions, at a concentration of 2.0 m*M*, to prevent metal-catalysed thiol oxidation.

All other chemicals and solvents were obtained from Merck and UCB.

Sample preparation: derivatization reaction[15–17]

Equal volumes of SBD-F (1.0 m*M* in 0.1 *M* aqueous sodium borate buffer, pH 9.5, containing 2.0 m*M* disodium EDTA) and of thiol solutions were vortex mixed and heated in a water-bath at 60°C for 60 min, followed by cooling in ice–water. The thiol solutions were freshly prepared in the buffer solution mentioned above. A 60–200-nl aliquot of the derivatized samples was subjected to micro-LC.

A reagent blank solution (no thiol present) was similarly prepared and analysed.

The procedure for derivatization of thiols with ABD-F was performed in the same way as with SBD-F, but with heating at 50°C for 5 min at pH 8.0.

Apparatus and chromatography

The isocratic chromatographic set-up consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model 510 HPLC pump equipped with a split-flow system, a 60–200-nl internal volume injector (CI4W; Valco, Houston, TX, U.S.A.) and a 250 × 0.32 mm I.D. fused-silica capillary filled with 5-μm Nucleosil C_{18} or RoSiL C_{18} (Bio-Rad RSL, Eke, Belgium). The split-flow system was obtained by using a conventional HPLC column (5-μm RoSiL C_{18}, 150 × 4.6 mm I.D.) as a solvent by-pass, placing a T-splitter (Valco) between the pump and the conventional–micro-LC connection system. This

arrangement allowed a constant flow-rate of 1–5 μl min^{-1} to be achieved in the micro-column.

Detection of the derivatized samples was performed with a Shimadzu Model RF-535 fluorescence detector (Pleuger, Wijnegem, Belgium) in which a laboratory-made 312-nl square quartz detection cell (0.25 × 0.25 × 5.0 mm) was included. Excitation was at 380 nm and emission at 510 nm. Recording of the signals was performed with a Model 2020-0000 recorder (Linear, Instruments, Reno, NV, U.S.A.), integrating with a Chromatopac C-R3A (Shimadzu, Kyoto, Japan). Chromatography was carried out isocratically at ambient temperature using 0.15 *M* H_3PO_4–CH_3CN (90:10, v/v) as the mobile phase.

The gradient reversed-phase chromatography set-up was similar to that described above, but using a RoSiL C_{18} column (Bio-Rad RSL) and a Varian Aerograph (Walnut Creek, CA, U.S.A.) Model 5560 gradient pump at a flow-rate of 1.5 ml min^{-1} providing a 2.6 μl min^{-1} flow in the micro-LC column. The T-split system was then connected to a conventional 250 × 4.6 mm I.D. 5-μm RoSiL C_{18} HPLC column.

The flow-rate in the micro-LC column was frequently checked by connecting an empty 10-μl syringe to the column end and timing the advance of the liquid meniscus.

RESULTS AND DISCUSSION

Optimization of the chromatographic system

Chromatography was first applied to the separation of cysteine–, homocysteine–, glutathione– and acetylcysteine–SBD derivatives. Following the method suggested by Toyo'oka and Imai[18], isocratic elution using 0.1 *M* sodium phosphate (pH 6.0)–methanol (95:5, v/v) as the mobile phase was tried out. However, the high concentration of buffer solution caused salt precipitation and the eluent composition was therefore modified. Improved selectivity and efficiency of separation were obtained by replacing methanol with acetonitrile and by using a lower concentration of phosphate buffer solution. Finally, by replacing the salt with the corresponding acid, a considerable gain in resolution was obtained. The optimum mobile phase was 0.15 *M* H_3PO_4–CH_3CN (90:10, v/v).

Fig. 1A shows the analysis of four common thiols labelled with SBD-F, achieving their separation in 15 min and requiring only 60–200 nl of sample. The optimum flow-rate was 5 μl min^{-1}. Sample volumes of up to 1 μl may be injected although there appears some peak tailing. The detection cell used provided improved sensitivity and less background noise than when applying "on-column" detection, a technique in which part of the fused-silica column not containing packing material is used as the detection cell[37]. This is the result of using a square instead of a round cell.

The chromatographic conditions mentioned above could also be applied to the separation of the ABD derivatives of the four thiols although when the same mobile phase is used considerably longer retention times are then obtained, *viz.* 7.2, 12.6, 15.0 and 47.6 min for cysteine, homocysteine, glutathione and acetylcysteine, respectively (Fig. 1B). Blank reagent solutions did not provide any peaks in the chromatogram.

Determination of thiols

SBD-and ABD-labelled cysteine, homocysteine, glutathione and acetylcysteine were analysed on a reversed-phase packed fused-silica column and measured by

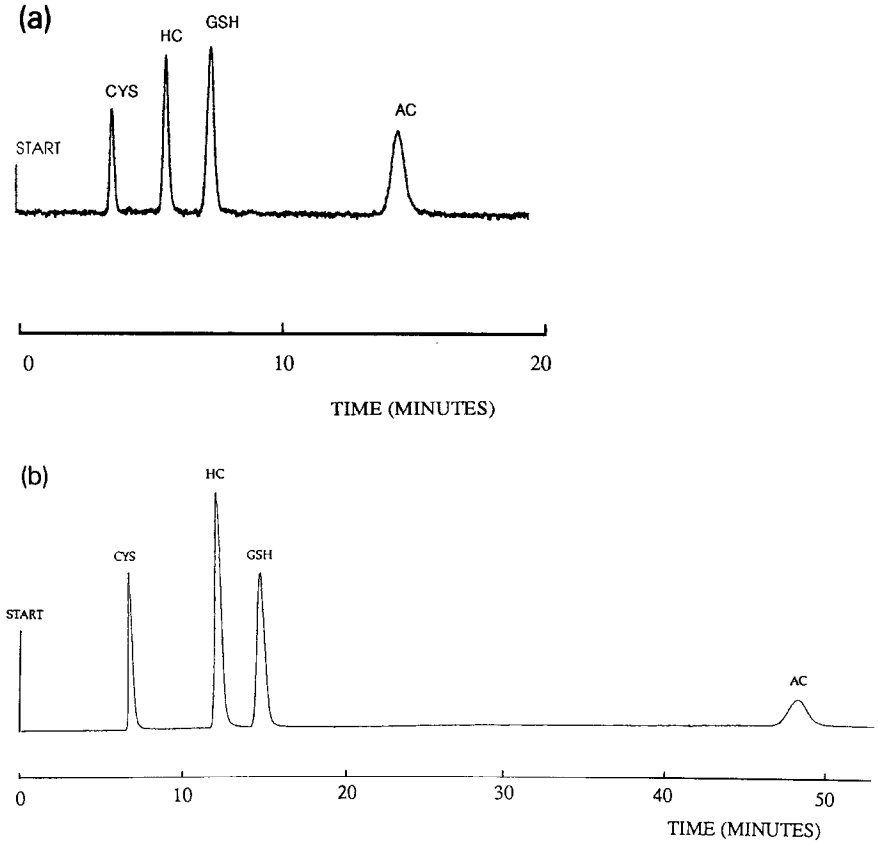

Fig. 1. Micro-LC separation of (A) SBD- and (B) ABD-thiol derivatives. CYS = cysteine; HC = homocysteine; GSH = glutathione; AC = acetylcysteine. Concentrations between 1 and 10 μg ml^{-1}. Column, 250 × 0.32 mm I.D., fused silica, 5-μm Nucleosil C_{18}; mobile phase, 0.15 *M* H_3PO_4–CH_3CN (90:10, v/v); flow-rate, 5 μl min^{-1}; detection, λ_{ex} = 380 nm, λ_{em} = 510 nm.

fluorescence detection. The detection limits for the SBD derivatives were 39, 7.4, 14 and 40 pg per injection (signal-to-noise ratio = 2), respectively, and those for the ABD derivatives were 77, 19, 24 and 29 pg per injection, respectively. The relative standard deviation of the peak areas was 1.86% (n = 10). The SBD derivatives generally yielded higher fluorescence signals than the ABD derivatives under identical conditions. Similar results were obtained from the calibration graphs (fluorescence intensity *versus* thiol concentration) for SBD–cysteine and ABD–cysteine as representative thiol derivatives. Linearity was observed in the range 0–3000 pg per injection (Fig. 2). In comparison with conventional HPLC in combination with concentration-based detectors, the sensitivity is increased in micro-LC as sample dilution is reduced when the same mass of sample is injected into a column of smaller I.D.[11].

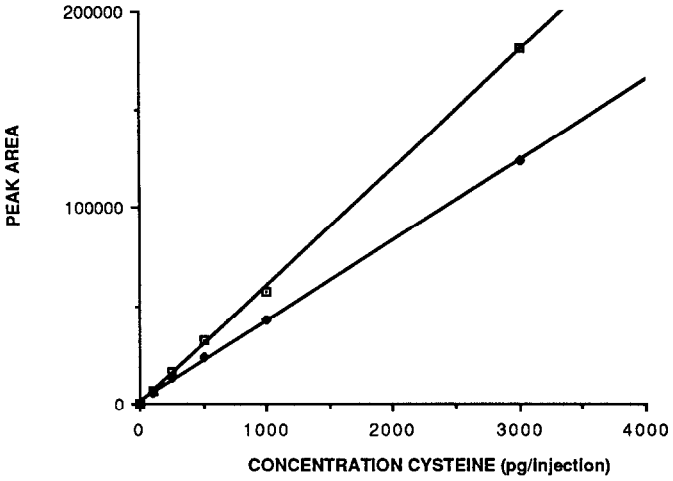

Fig. 2. Calibration graphs (0–3000 pg per 60-nl injection) for SBD- and ABD-cysteine. □ = SBD-cysteine; $y = 483.34 + 59.973x$, $R = 1.00$; ◆ = ABD-cysteine; $y = 2078.3818 + 41.0241x$, $R = 1.00$.

Isocratic and gradient chromatographic behaviour of other thiols

Various other thiol compounds of biological and pharmacological importance were derivatized with SBD-F and ABD-F. However, by using the recommended mobile phase, 0.15 *M* H_3PO_4–CH_3CN (90:10, v/v), most of the thiol derivatives showed inconveniently high retention times (>60 min). With a higher acetonitrile content [0.15 *M* H_3PO_4–CH_3CN (70:30, v/v)], all the derivatives were eluted in less than 10 min, giving the retention times and detection limits shown in Table I.

Gradient elution of these thiol derivatives resulted in a more efficient elution of the latter and also of the originally assayed thiol compounds. Fig. 3 shows the micro-LC gradient separation of several thiol compounds with various polarities, derivatized with both fluorogenic reagents. Blank runs gave stable baselines. It is advisable to follow with an isocratic elution just after the gradient elution to compensate for the volume delay. Eluent sonication and helium flushing for 20–30 min prior to chromatography is likewise advisable. An initial gradient elution trial from 0.15 *M* H_3PO_4–CH_3CN (100:0) to (50:50) over 15 min was initially tried. As expected, gradient elution allowed the elution of all the SBD- and ABD-thiol derivatives. However, certain thiol derivatives gave overlapping peaks for both types of derivatives. Considering the results obtained from the isocratic elution, a gradient elution from 0.15 *M* H_3PO_4–CH_3CN (95:5) to (70:30) was subsequently tried; the chromatogram obtained for the SBD derivatives is shown in Fig. 3A. As observed, the derivatives from a mixture of nine thiol compounds were clearly resolved in less than 30 min.

With the ABD derivatives a small addition of acetonitrile was required to allow a better elution of all the thiol derivatives, as their retention times are slightly higher than those of the corresponding SBD derivatives. Fig. 3B shows the chromatogram of eleven ABD derivatives separated with gradient elution from 0.15 *M* H_3PO_4–CH_3CN (95:5) to (65:35) over 20 min.

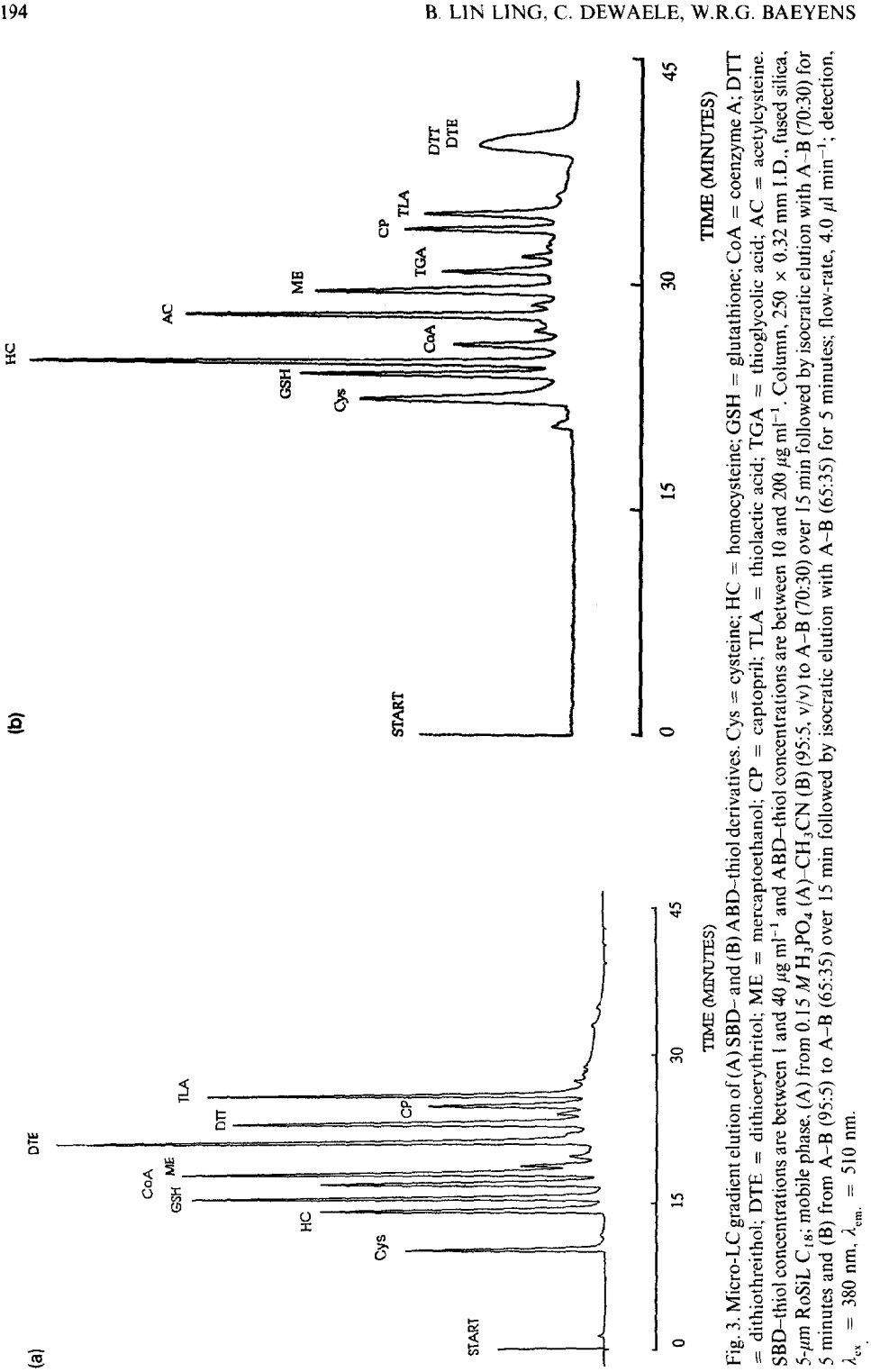

Fig. 3. Micro-LC gradient elution of (A) SBD- and (B) ABD-thiol derivatives. Cys = cysteine; HC = homocysteine; GSH = glutathione; CoA = coenzyme A; DTT = dithiothreithol; DTE = dithioerythritol; ME = mercaptoethanol; CP = captopril; TLA = thiolactic acid; TGA = thioglycolic acid; AC = acetylcysteine. SBD–thiol concentrations are between 1 and 40 μg ml^{-1} and ABD–thiol concentrations are between 10 and 200 μg ml^{-1}. Column, 250 × 0.32 mm I.D., fused silica, 5-μm RoSiL C_{18}; mobile phase, (A) from 0.15 *M* H_3PO_4 (A)–CH_3CN (B) (95:5, v/v) to A–B (70:30) over 15 min followed by isocratic elution with A–B (70:30) for 5 minutes and (B) from A–B (95:5) to A–B (65:35) over 15 min followed by isocratic elution with A–B (65:35) for 5 minutes; flow-rate, 4.0 μl min^{-1}; detection, $\lambda_{ex.}$ = 380 nm, $\lambda_{em.}$ = 510 nm.

TABLE I

RETENTION TIMES AND DETECTION LIMITS OF SBD- AND ABD–THIOL DERIVATIVES

Column, 250 × 0.32 mm I.D., fused silica, 5-μm RoSiL C_{18}; mobile phase, 0.15 *M* H_3PO_4–CH_3CN (70:30, v/v); flow-rate, 5 μl min^{-1}; detection, $\lambda_{ex.}$ = 380 nm, $\lambda_{em.}$ = 510 nm.

Thiol	*Retention time (min)*	*Detection limit (pg per 60-nl injection)*
SBD derivatives		
Acetylcysteine	3.90	39
Captopril	4.45	55
Cysteine	3.75	40
Coenzyme A	3.95	30
Dithioerythritol	4.02	58
Dithiothreitol	4.05	39
Glutathione	3.90	14
Homocysteine	4.80	7
Mercaptoethanol	3.95	8
Thioglycolic acid	4.30	30
Thiolactic acid	4.55	35
ABD derivatives		
Acetylcysteine	5.80	29
Captopril	9.45	102
Cysteine	5.10	77
Coenzyme A	5.80	31
Dithioerythritol	14.25	68
Dithiothreitol	12.95	130
Glutathione	5.30	24
Homocysteine	5.70	19
Mercaptoethanol	6.85	10
Thioglycolic acid	7.55	49
Thiolactic acid	10.45	93

Higher resolution was achieved by using longer columns (Fig. 4). However, direct comparison with the shorter columns is difficult owing to the different gradient profiles. The inconvenience of the long duration of analysis (>2 h) and the obvious dilution effect when using these long columns reduce their practical use.

Application of the described method to the determination of glutathione and cysteine in biological samples (blood, plasma) and to the analysis of pharmaceutical compounds (drug quality control) is currently under investigation. Fig. 5A shows the preliminary isocratic chromatogram obtained from human whole blood treated with trichloroacetic acid and derivatized with SBD-F[18]. Fig. 5B shows the analysis of a glutathione-spiked human whole blood sample. Disposable 4-mm syringe filters (pore size 0.45 μm; Alltech) were used to inject clear particle-free derivatized sample solutions. Further research on this subject will be reported elsewhere.

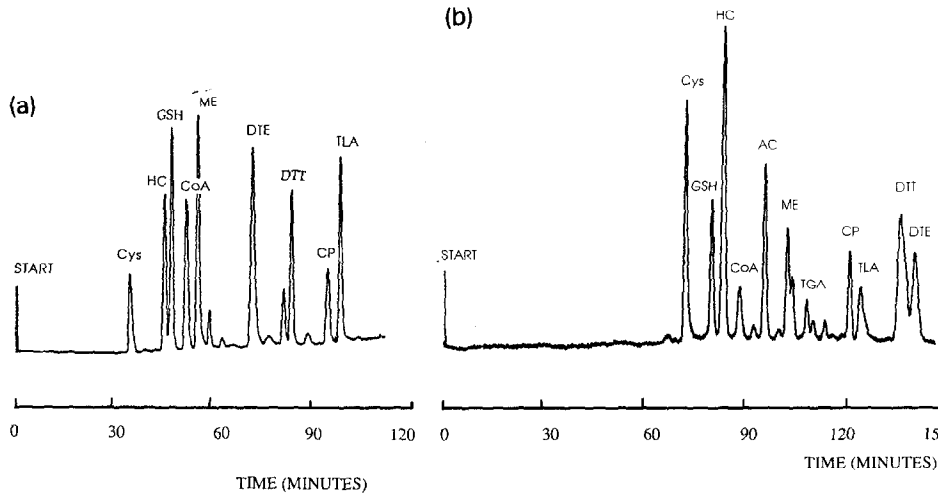

Fig. 4. Micro-LC gradient elution of (A) SBD– and (B) ABD–thiol derivatives on a long fused-silica capillary column (1000 × 0.32 mm I.D.), RoSiL C_{18}, 5 μm. The same compounds as in Fig. 3 were are analysed under similar experimental conditions, except for the flow-rate (2.0 $\mu l\ min^{-1}$) and the mobile phase: (A) from A–B (95:5, v/v) to A–B (70:30) over 90 min followed by isocratic elution with A–B (70:30) for 60 min; (B) from A–B (95:5) to A–B (65:35) over 90 min followed by isocratic elution with A–B (65:35) for 60 min.

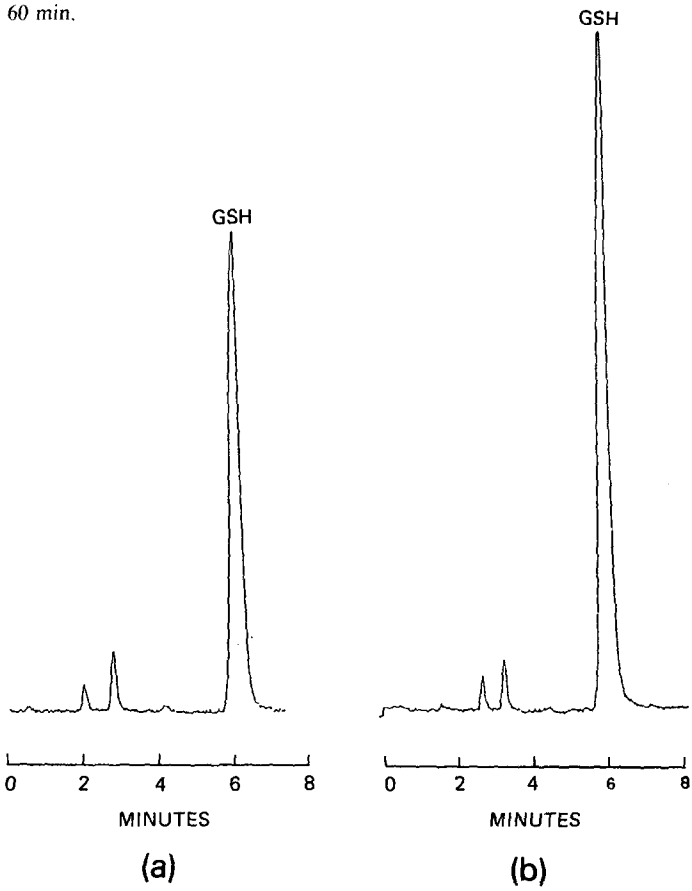

Fig. 5. Chromatogram obtained from human whole blood treated with trichloroacetic acid and derivatized with SBD-F[17]. (A) Sample as such and (B) sample spiked with 75 $\mu g\ ml^{-1}$ of glutathione added to the supernatant. Column, 250 × 0.32 mm I.D., fused silica, 5-μm RoSiL C_{18}; mobile phase, 0.15 M H_3PO_4–CH_3CN (90:10, v/v), isocratic; flow-rate, 5 $\mu l\ min^{-1}$; detection, $\lambda_{ex.}$ = 380 nm, $\lambda_{em.}$ = 510 nm.

CONCLUSIONS

The combined use of the micro-LC separation technique with selective and sensitive fluorescence detection allows the specific determination of trace amounts of various thiol compounds of biological and pharmacological interest. Conventional LC equipment may be successfully adapted to capillary columns, providing a suitable analytical method for the determination of thiols in different matrices. Gradient reversed-phase chromatography provided the separation of several fluorescent SBD– and ABD–thiol derivatives covering a wide range of polarities. The application of the system to the identification of glutathione in human whole blood was achieved.

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