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Amperometric detection of organic thiols at a tungsten wire electrode following their separation by liquid chromatography

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Abstract

The amperometric detection of thiols following their separation by reversed-phase chromatography and reaction into a post-column mercury carrier stream is shown to be a sensitive method when using a tungsten wire sensor as the working electrode in a three-electrode flow cell. Five organic thiols (cysteine, homocysteine, reduced glutathione, D,L-penicillamine and 2-mercaptopropionic acid) and thiourea could be separated within approx. 18 min. The analytical performance is comparable, and stability superior, to chemically modified electrodes previously reported.

Keywords: Tungsten wire electrode; Thiols; Cysteine; Homocysteine; Glutathione, reduced; Penicillamine; 2-Mercaptopropionic acid; Thiourea

1. Introduction

The assay of thiols has been of interest for many years due to their importance in clinical, pharmaceutical and environmental studies [1]. Accordingly, many methods have been reported for the determination of thiols, including spectrophotometry [2], gas chromatography [3], polarography [4] and radioimmunoassay [5]. Other methods which involve pre- and post-column reaction/derivatization have been employed, e.g., fluorimetry [6], ion-exchange chromatography [7] and high-performance liquid chromatography [8]. These methods either require complex sample manipulation, during which thiol degradation may occur, or suffer from sensitivity, specificity or productivity limitations.

The recent development of a high-performance liquid chromatographic (HPLC) method with electrochemical detection [9] has provided a simple, specific and sensitive method for thiols. A variety of electrochemical detectors for HPLC have been described. Their main advantage over the more commonly used LC detectors is their much higher sensitivity to electroactive compounds. Additionally, some selectivity is possible through the choice of electrode potential. However, most of the LC detection for thiols requires the use of mercury electrodes [4,9] rather than the more common and acceptable carbon electrode [10] as thiols have a high overpotential to electro-oxidation at ordinary carbon electrodes. Several chemically modified electrodes have been used for the detection of thiols with low sensitivity and long term instability during both experimental operation and storage. They also have a tedious and complex preparation procedure [10].

An amperometric sensor constructed with a work-

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ing metallic tungsten electrode has been reported for detecting thiols and proteins [11,12]. It is shown that good detection limits (in the range 0.1–2.1 mg ml^{-1}) and sensitivity are achieved when organic thiols are injected into a mercury(II) reagent solution buffered in 0.005 *M* sodium acetate at pH 4.7, at an operating voltage of −0.20 V.

In this paper, we have evaluated the applicability of a tungsten electrode as an electrochemical detector in the HPLC determination of thiols. The chromatographic variables studied include the effect of pH, ionic strength, flow-rate of mobile phase and organic modifier on the retention of several thiols. A scheme for the isocratic separation of six components of thiols is presented, and the optimized conditions have been applied to the determination of spiked urine, blood serum and sea water samples.

2. Experimental

2.1. Chromatography

Liquid chromatography with electrochemical detector utilizing a post column reaction with Hg^{2+}, with two reciprocating dual-piston pumps was used. A pre-column pump with flow-rate 0.3 ml min^{-1} was used for delivering the mobile phase. A post-column pump with flow-rate 1.0 ml min^{-1} was used for the post-column reaction.

Thiols were separated isocratically on a commercial, octadecylsilyl-modified silica (C_{18}) column (250×4.6 mm I.D., Zorbax ODS, DuPont, Wilmington, DE, USA) packed with 5-μm particles. The chromatograms were recorded with an Omniscribe chart recorder interfaced to a microcomputer for peak processing and calculation of calibration data.

2.2. Amperometric sensor

A PAR-174 polarograph was used with a flow through cell made in perspex, described previously by Hidayat et al. [12] with some modification. The internal diameter of one of the electrode compartments was widened to 4 mm in order to provide enough room for a silver–silver-chloride reference electrode. This compartment was filled with hot liquid 20% (w/v) agar (in 1 *M* KCl solution). After the agar had cooled, the Ag–AgCl reference electrode was inserted into the solid agar. The Ag–AgCl reference electrode was prepared by electrolysis at a silver wire electrode (1.0 mm diameter) in 1% KCl by application of a potential at +0.6 V for 5 min.

The working electrode was a tungsten wire of 1.0 mm in diameter (31.4 mm^2 in surface area), and the auxiliary electrode was 1.0 mm Pt wire. The electrodes were made from wires of 99% purity obtained from Johnson Matthey, UK.

2.3. Reagents

Solvents and reagents used in this study were all commercial analytical grade. Mobile phases and standard solutions were prepared with Milli-Q water (deionized distilled water passed through a carbon adsorption–ion-exchange system; Milli-Q, Millipore).

Mobile phase solutions were prepared by dilution of trichloroacetic acid (ACS certified grade, Fisher Scientific, Springfield, NJ, USA) with Milli-Q water to the desired concentration, followed by titration with base to obtain the desired pH (measured versus standard buffers with a glass electrode).

The post-column reaction used optimized conditions as determined in our previous work [12]. The post column buffer solution was a mixture of sodium dihydrogen phosphate (NaH_2PO_4) and phosphoric acid (reagent grade, Mallinckrodt, Paris, KY, USA) of 0.1 *M*, pH 3.1, containing 25 m*M* Hg^{2+}. The mobile phase and post column buffers were sonicated for 10 min before use to reduce the level of oxygen. All reagents were used without further purification.

Compounds used in this study included cysteine (Cys), homocysteine (h-Cys), reduced glutathione (GSH), 3-mercaptopropanoic acid (3-Mpa), penicillamine (Pen) and thiourea (Thi). All chemicals were purchased from Sigma (St. Louis, MO, USA) and were stored at 4°C. Aqueous thiol stock solutions (100 m*M*) were prepared using the mobile phase (1 h nitrogen sparge) and stored at 4°C. Autoxidation causes degradation of the solution after one week [14]. It was necessary to prepare dilute standard solutions immediately prior to HPLC separation.

2.4. Sample preparation

The blood-plasma was prepared in the Heart Research Institute Laboratory (Sydney, Australia). About 50 ml of fresh blood drawn with a syringe from a vein, was centrifuged at 550 *g* for 30 min. The clear solution was mixed with methanol and centrifuged at 550 *g* for 30 min. Equal amounts of clear solution, (plasma) were prepared, one for an unspiked sample and to the other was added thiol. The concentration of each thiol in a 20 μl injection was 75 ng Cys, 50 ng h-Cys, 75 ng GSH and 50 ng Pen. In order to prevent the rapid loss of the thiols in these samples, the standard solutions were made in mobile phase of pH 2.1. Both sets of sample plasma was filtered with 0.22 mm filters and centrifuged at 2500 *g*. The clear liquids were diluted with equal amount of mobile phase. After centrifugation at 2500 *g* for 5 min, an aliquot of the supernatant was subjected to HPLC.

Sea water was taken from a pool at Coogee Beach, (Sydney, Australia) and kept in an icebox during transport to the laboratory. Sea water and urine samples were spiked with thiol standards, and filtered with 0.22 mm filters. The clear liquids were diluted with equal amount of mobile phase, and stored at −5°C, before use.

2.5. Procedure

Initially, the applied potential vs. current of selected thiols (Cys, h-Cys, Glu, Pen, Thi) were recorded at various potentials ranging from +0.2 V to −1.3 V, using a tungsten working electrode with Ag–AgCl reference and Pt auxiliary electrode. The potential giving the highest change in current was used for further experiments. For the post-column reaction we used an optimized phosphate buffer previously achieved [12], viz.: 0.1 *M*, pH 3.1 and at flow-rate 1 ml min^{-1}.

Chromatographic parameters optimized were: (1) pH of the mobile-phase, (2) concentration of pairing ion, (3) concentration of organic modifier and (4) flow-rate. Finally, the method was evaluated by determining the recovery of added thiols to blood serum, urine and sea water samples.

3. Results and discussion

3.1. Choice of mobile phase

Various approaches have been used to separate a mixture of thiols including anion or cation-exchange, and reverse phase with or without an ion pairing reagent [15,16,18]. Anion-exchange has a low efficiency for separation of conjugate anions [15], whereas cation-exchange poorly retained neutral thiols [16]. Direct reverse phase without a pairing ion has the disadvantage that the hydrophilic compounds (e.g., cysteine, homocysteine) show very little retention [18]. Use of a pairing ion, such as alkyl sulfate–sulfonate improves the separation in reverse phase mode, but this approach requires relatively long mobile–stationary phase equilibration time [17].

In this study, we use the reverse phase approach with 3-chloroacetate (TCA) as an ion pairing reagent. The advantages of this mobile phase, as has been reported [9,18], are as follows: (1) it requires relatively short mobile–stationary phase equilibration time (elution of only 2–5 column volumes was required for column-mobile phase equilibration); (2) relatively high efficiency of separation for conjugate anions compared to other pairing ions; (3) applicability for separation of wide range of neutral and cationic hydrophilic thiols. The pK_a values of these thiols are typically 8 to 10 [19], and their retention increases with the size of the halogen used (F<Cl< Br) [18]. Depending on the kind of sample and type and amounts of thiols in the sample to be separated, a suitable choice of the 3-haloacetate may be made.

The results in the following sections suggest that reversed-phase with TCA as the ion pairing reagent is compatible with the post column reaction and sensor developed in this experiment. The optimization of conditions are presented in detail in the following sections.

3.2. Principle of detection

Individual thiols, after chromatographic separation with a C_{18} column, were detected by post-column complex reaction with 25 m*M* Hg^{2+} in 0.1 *M* phosphate buffer of pH 2.2. The complex reaction

reduces the Hg^{2+} activity in the solution, and thus reduces the cathodic current of the ion, which is detected amperometrically with a tungsten wire electrode.

3.3. Applied potential vs. current of selected thiols after HPLC separation

Previous reports on the indirect detection of thiols indicated that the type and condition of supporting electrolyte affected the reduction potential of Hg^{2+} ions [11,12]. Because the supporting electrolyte solution used in this study was different from the electrolytes previously used, it was required to determine the optimum working potential which produces the highest current changes due to addition of thiols. A mixture of five thiols (containing 50 ng Cys, 50 ng h-Cys, 100 ng GSH, 75 ng Pen, and 75 ng Thi) were injected into 0.05 *M* phosphate buffer of pH 2.2, 50 m*M* TCA–methanol (99:1) at flow-rate of 0.3 ml min^{-1}. The potential applied varied from 0.0 V to −0.5 V.

Fig. 1. shows that the potential which give the highest current change was −0.3 V; shifted 60 mV negative with respect to experiments reported before [12].

This result also confirmed that the mobile phase used in this study, after mixing with the post-column buffer solution, was compatible with the sensor (tungsten wire). A working potential of −0.3 V was therefore selected for further experiments.

3.4. Optimisation of separation

3.4.1. Effect of mobile phase pH on the retention of thiols

Since the separation is accomplished via ion pairing and the thiols have both anionic and cationic functionalities, the mobile phase pH is expected to have a profound effect on its behavior.

The effect of the mobile phase pH on the retention of 5 thiols (Cys, h-Cys, GSH, Pen and Thi) over the range of 2.2 to 6.0 is shown in Fig. 2. The selection of the mobile phase pH was aimed at reducing the capacity factor of the thiol which has the longest retention time, to below 10, but with a high enough value to extend the life of the column.

As shown in Fig. 2 as the pH decreased, the retention of each thiol increased. A possible explanation for this behavior is that the protonation of the carboxylate site enhances the ion pair formation. The three thiols (Cys, h-Cys and Glu) were not well resolved at pH values higher than 3.5.

MacCrehan and Shea [18] reported that the highest

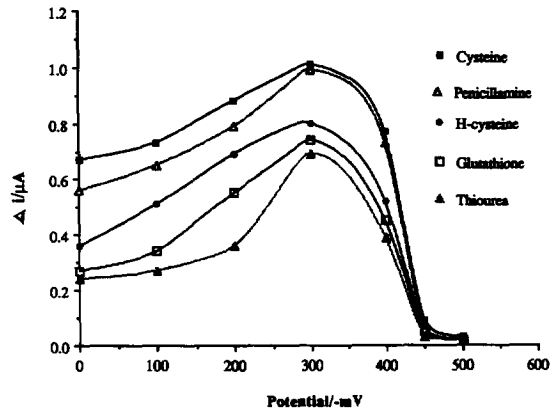

Fig. 1. Change in the reduction current of Hg^{2+} at a tungsten electrode with the passage of selected thiols after separation with HPLC. Column: C_{18}; mobile phase: 0.05 *M* TCA (pH 2.2)–methanol (99:1). Flow-rate: 0.3 ml min^{-1}. Postcolumn reaction: 0.1 *M* phosphate buffer containing 25 μ*M* Hg^{2+} of pH 3.1, at flow-rate 1.0 ml min^{-1}.

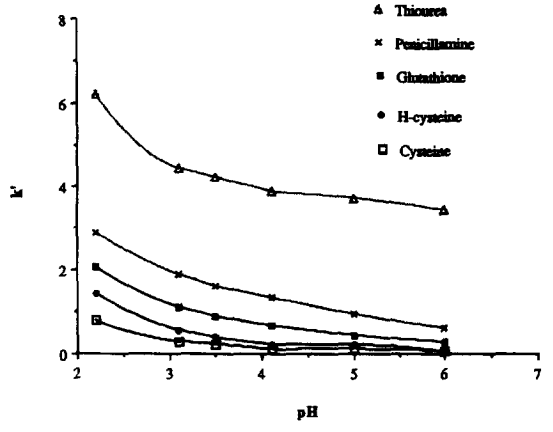

Fig. 2. Effect of mobile phase pH on the capacity factor of selected thiols. Column: C_{18}; mobile phase: 0.05 *M* TCA (pH 2.2)–methanol (99:1). Flow-rate: 0.3 ml min^{-1}. Postcolumn reaction: 0.1 *M* phosphate buffer containing 25 μ*M* Hg^{2+} of pH 3.1, at flow-rate 1.0 ml min^{-1}. Electrode: tungsten wire at −0.3 V (vs. Ag, AgCl)

retention of thiols studied was achieved at pH nearly 1.8. It should be noted, however, that utilization of a pH 2 mobile phase could accelerate column degradation; thus, for such conditions, the use of an acid resistant-C_{18} column is recommended.

The cysteine peak is well resolved from the homocysteine peak at pH 2.2, therefore this pH was suitable for separation and detection of thiol compounds in this method. In flow injection [12] with a low pH and high buffering power, high sensitivity was achieved, and the interferences due to pH changes were completely eliminated.

3.4.2. *Effect of organic modifier on the retention of thiols*

Increasing the organic modifier (methanol) from 1% to 3%, did not significantly affect the retention time and the peak resolution. However, without an organic modifier, a slight increase in the capacity factor was observed, the peak widths were broadened, and therefore the resolution was worse. A minimum amount of organic modifier in this study is needed to obtain a good resolution.

3.4.3. *Effect of pairing ion concentration on the retention of thiols*

The aim of optimizing the TCA concentration is to resolve the cysteine peak from the void peak as well as the homocysteine peak, with a minimum TCA concentration. A too high TCA concentration may result in low sensitivity, or may interfere with the electrode. Fig. 3 shows the change in k' with the TCA concentration. Increasing the concentration of TCA increased the retention time of five thiols tested. The best chromatogram was achieved with 50 m*M* TCA. At this concentration, the cysteine was separated from homocysteine, while keeping the capacity factor of thiourea below 10. Since the pH and ionic strength remain constant in this experiment, the increased retention of the thiol tested with TCA concentration must be a result of solvophobic ion interactions between the TCA anions and the analytes.

Any ionic interaction between the counter cation and the buffer anion should cause a decrease in retention of the net-cationic thiols, by decreasing the "free" anion available for cysteine pairing.

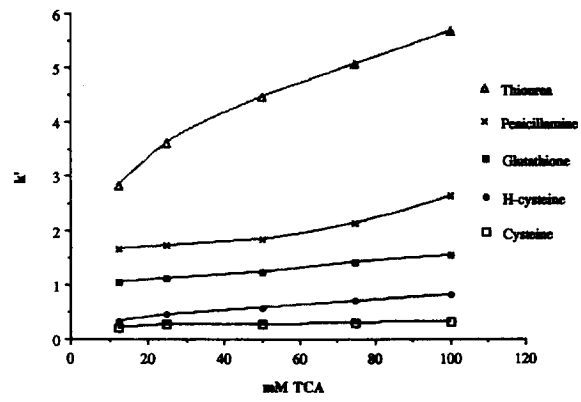

Fig. 3. Effect of pairing ion concentration on the capacity factor of selected thiols. Column: C_{18}; mobile phase: 0.05 *M* TCA (pH 2.2)–methanol (99:1). Flow-rate: 0.3 ml min^{-1}. Postcolumn reaction: 0.1 *M* phosphate buffer containing 25 μM Hg^{2+} of pH 3.1, at flow-rate 1.0 ml min^{-1}. Electrode: tungsten wire at −0.3 V (vs. Ag, AgCl)

3.4.4. *Effect of flow-rate*

Increasing the flow-rate from 0.1 to 1.0 ml min^{-1}, decreased retention time, and made the resolution worse. At a flow-rate above 0.75 ml min^{-1}, the three thiols (Cys, h-Cys and Glu) were not well resolved, while at flow-rate 0.1 ml min^{-1}, the k' of penicillamine reduced to 7.0, which implies that the k' for thiourea and neutral thiol compounds would be over 10.

3.5. *Optimisation of detection*

The performance of the tungsten wire electrode was evaluated with a mixture of the standard thiols that were well separated chromatographically with the optimum conditions. A typical chromatogram of a mixture of six biogenic thiols at optimum conditions is shown in Fig. 4 and the details of calibration are summarized in Table 1.

The detector response was linear (r^2>0.994) over the experimental range of 5–150 ng of injected thiols. The slopes ranged from 0.018 to 0.002 nA ng^{-1}. The average precision (R.S.D.) of 5 thiols, determined from 5 replicates, was 2.0%. None of the solutions determined exhibited a precision worse that 2.5% R.S.D.. The relative standard deviation for the chromatographic capacity factor was 2.7% R.S.D..

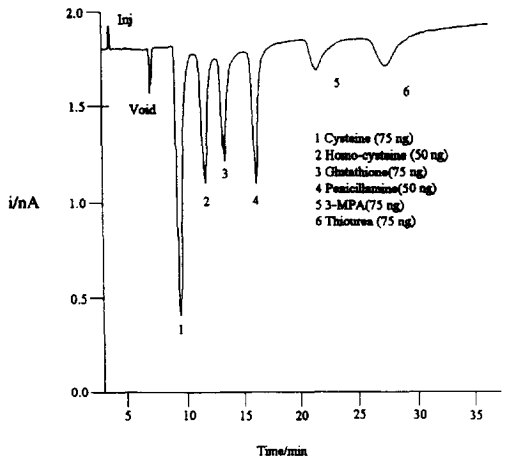

Fig. 4. Chromatogram of thiols under optimum conditions. Column: C_{18}; mobile phase: 0.05 *M* TCA (pH 2.2)–methanol (99:1). Flow-rate: 0.3 ml min^{-1}. Postcolumn reaction: 0.1 *M* phosphate buffer containing 25 μM Hg^{2+} of pH 3.1, at flow-rate 1.0 ml min^{-1}. Electrode: tungsten wire at −0.3 V (vs. Ag, AgCl)

The precisions of the retention times were better than 2.0% R.S.D., which is very good for HPLC methods.

The limit of detection (3 times the background noise) for standard solutions were in the picomole range, which corresponds to about 1.0 to 9.38 ng when a 20 μl sample injection is used. This is comparable with previous methods [13,18–21]. The observed detection limit in urine, blood plasma and sea water sample for cysteine, glutathione and penicillamine were about a factor of 2 higher because of interferences.

Table 1
HPLC calibration data of selected thiols with amperometric detection using a tungsten electrode

Analyte	Slope[a] (nA ng^{-1})	LOD[b] (ng)
Cysteine	0.018	1
Homocysteine	0.012	1
GSH	0.007	3
Penicillamine	0.011	1
Thiourea	0.002	9

Mobile phase: 0.05 *M* TCA (pH 2.2)–methanol (99:1). Other conditions as in Fig. 4.

[a] Amounts injected: 5–150 ng in 20 μl injection volume.

[b] LOD (limit of detection)=concentration that yields a current of three times the background noise.

3.5.1. Selectivity

The method is selective toward the sulfhydryl-containing amino acids in a mixture of amino acids. As previously reported [11,12], the tungsten electrode does not respond to Br^-, Co^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, NO_2^-, NO_3^-, Pb^{2+}, SCN^-, SO_4^{2-}, Sr^{2+} and Zn^{2+}. There was no response to L-leucine, D,L-alanine, D,L-histidine, L-aspargine, L-arginine, L-methionine glycine, lycine, aspartic acid and glutamic acid [12,22]. As a result of this selectivity, it is only necessary that the HPLC separate the sulphydryl compounds from each other and not necessarily from the other amino acids and peptides in the sample.

3.5.2. Stability

The stability of the tungsten wire electrode for thiol analysis has been studied in flow injection [12]. There was no reduction in peak height during continuous use for three months with no resurfacing of the electrode. However, it is always advisable to make the calibration curve of the standard on the same day as the analysis of the unknown samples.

Compared to chemically modified electrodes (CME), and mercury–gold amalgam electrodes [13], a tungsten electrode is more stable. A newly resurfaced gold-amalgam electrode may operate efficiently for only several minutes. Using a CME, after a period of use, the response is reduced because of the gradual leaching of the modifying chemicals from the CME surface. In fact, some decrease in response has been observed previously even for conventional carbon paste electrodes in LCEC upon long-term exposure to a binary mobile phase containing a small fraction of organic components.

The design of the Ag–AgCl reference electrode which uses a solid agar gel plug, may also contribute to the stability of this sensor. The solid agar gel prevents the diffusion of the thiols injected into the liquid chromatographic system through to the reference electrode. Retention times of this method are comparable to those achieved by Stamler and Loscalzo [14] with capillary zone electrophoresis sepa-

ration. As derivatization is not necessary, our method is more simple and is cheaper.

3.6. Performance and applications

3.6.1. Applications

Blood plasma, urine and sea water samples which had been prepared according to the procedure outlined in Section 2.4, were analysed by this method. Only cysteine was observed in the real samples with 8.5 ng ml^{-1} in urine, 12.0 ng ml^{-1} in blood plasma and 16.0 ng ml^{-1} in sea water. Other thiols missing from the chromatograms may have been completely oxidized or were undetectable [22,23]. Penicillamine, for example, is only detectable in patients on penicillamine therapy, who have penicillamine concentrations in urine, and whole blood in the range between 2.5 to 25 mg l^{-1}, and 0.7 to 1.8 mg l^{-1}, respectively.

To evaluate the applicability of the methods, the recovery of added thiols was studied. A mixture of five thiol standard solutions were added to the samples (Fig. 5). An average recovery of 93.8±3.5% was achieved (Table 2). The poorer recovery of thiols in blood samples, and penicillamine relative to the other samples, is due to the initial rate of loss of penicillamine as observed earlier [22,23].

The method may be used for the analysis of other thiols that fulfill two requirements: (1) they must show some affinity for the column packing and (2) they must react with Hg^{2+}. We suggest that this method may be applied, without, or with little, modification in mobile-phase pH for optimum separation, to inter alia environmental, pharmaceutical and biomedical samples, soil and plant and water samples and herbicides or pesticides containing thiols, in soil, water and plant samples.

3.6.2. Comparison with other electrodes

Thiols can be oxidized at various electrodes. At glassy carbon, cysteine and glutathione can be oxidized at +1.35 V and +1.1 V, respectively [9,18]. At a bare gold electrode, cysteine is oxidized at +0.9 V [18]. However, these oxidation potentials are high and many compounds may interfere, and therefore the analysis of thiols is not selective at these electrodes.

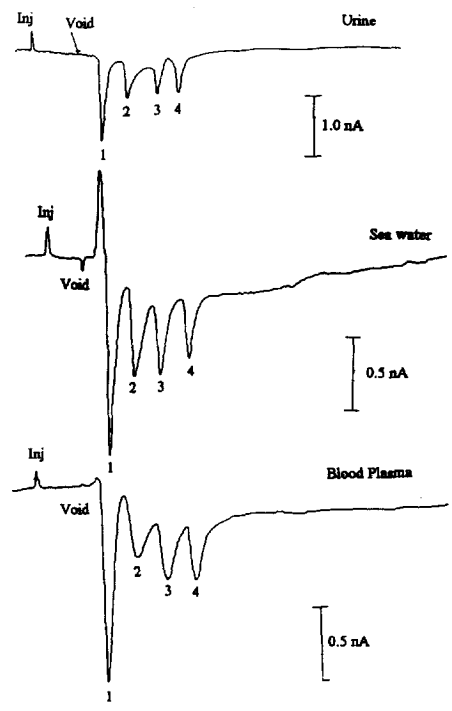

Fig. 5. Chromatograms of samples spiked with 1, 75 ng cysteine, 2, 50 ng homocysteine, 3, 75 ng glutathione, 4, 30 ng penicillamine. Chromatographic conditions as for Fig. 4.

Mercury–gold electrodes have been used for the analysis of biogenic thiols in sediment pore water samples [9,18]. It is a very sensitive electrode for thiols with relatively low working potential (+0.3 V) and so is highly selective; however the stability of the sensor is limited. The current changed by about 10% over one week of constant use [18]. A major structural change of the gold substrate occurs during the aging process, due to roughing and activation of the gold surface. This requires that the gold wire be resurfaced and reamalgamated at least every week. Stripping voltammetry using a HMDE or Hg-pool cell electrode, although it is a more sensitive method, requires several steps, which complicates the method if it is used in HPLC. The advantage of the tungsten electrode, as most metallic electrodes, is that it is easy to prepare and has relatively greater stability.

Table 2
Recovery of selected thiols added to urine, blood plasma and sea water samples

Samples	Recovery of added thiols (%)			
	Cysteine (75 mg)	H-cystine (50 mg)	Glutathione (75 mg)	Penicillamine (50 mg)
Urine	96.2	97.6	98.2	97.2
Sea water	94.5	100.0	98.0	89.2
Blood plasma	98.0	85.2	86.7	84.5

Optimum HPLC conditions were used for these analysis.

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