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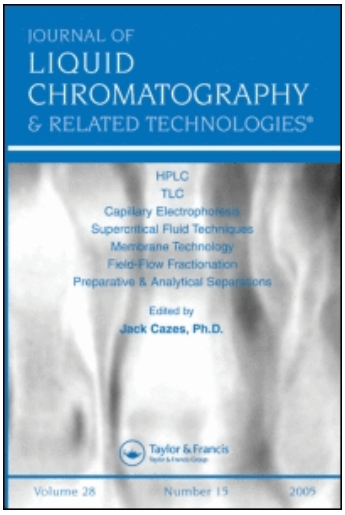

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DETERMINATION OF CYSTEINE AND N-ACETYLCYSTEINE IN URINE BY LIQUID CHROMATOGRAPHY WITH INDIRECT AMPEROMETRIC DETECTION

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ABSTRACT

Cysteine (CYS) and N-acetylcysteine (NAC) can be determined by liquid chromatography with indirect amperometric detection with iodine as oxidizing reagent. The linear range of detection can be adapted to the analytical requirements by adaptation of the generating current. With a generating current of 25 μA detection limits were found of 2 ng for CYS and 1.5 ng for NAC. Detector responses were linear up to 110 and 80 ng injected, respectively, with correlation coefficients better than 0.9998. The precision of the analysis, as assessed by repeated injection of a standard solution, was 0.6 %. After reduction of the sulphur compounds with dithiothreitol (DTT), urine samples were analyzed without further pretreatment using an ion-pair separation system. Endogenous levels of CYS and NAC in urine could be determined easily. The application to serum samples is also demonstrated.

INTRODUCTION

Sulfhydryl compounds (thiols) play an important role in several biological processes. For clinical and pharmaceutical analysis

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many methods have been developed to determine these compounds in biological fluids and tissues (1). In most cases a method including a separation by high-performance liquid chromatography is used (2). Still, a generally accepted method with adequate sensitivity, precision and reliability is not yet available. Since many biologically important thiols do not absorb UV light, pre- or post-column derivatization procedures are often used for detection (3-9). An alternative detection mode is amperometry with mercury-film electrodes (10,11). Due to the involvement of the electrode material itself in the electrochemical reaction, detection can be performed at a selective potential. With a dual-electrode detector both the reduced and the oxidized form of the thiols can be detected (12-15). Disadvantageous aspects of this detection method are the experimental cumbersomeness of the electrode preparation, electrode poisoning and the often unsatisfactory reproducibility of the measurements.

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Previous studies (16-20) have shown that indirect amperometric detection can be successfully applied for the determination of oxidizable compounds with unfavourable electrochemical characteristics such as sulphur compounds. The principle of indirect electrochemical detection is that an oxidizing reagent, bromine or iodine, is generated electrochemically in the column effluent, while its concentration is monitored amperometrically downstream. Compounds eluting from the column which react with the reagent cause a decrease in amperometric response in proportion to their concentration.

In a preliminary study (18) it was shown that thiols can be detected by indirect amperometry with iodine as the reagent. The method is superior to the mercury-film electrode detector in respect to reproducibility, and the sensitivity is adequate for the determination of N-acetylcysteine in human serum at therapeutic concentration levels (21). In the work presented here, the applicability of the method is studied for the determination of the endogenous concentrations of cysteine and N-acetylcysteine in urine.

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EXPERIMENTAL

Apparatus

The chromatographic equipment consisted of a Gynkotek (Germering, FRG) Model 300C pump with an extra home-made pulse damper, operated at a flow rate of 1.8 ml min^{-1}, and a Rheodyne injection valve with a 30 μl loop. Two different columns (A and B), 150 x 4.6 mm i.d., slurry packed with Hypersil ODS (5 μm), have been used. Small differences in retention behaviour of the two columns were observed.

A KOBRA generating cell (Lamers en Pleuger, 's-Hertogenbosch, The Netherlands), which has a double thin-layer construction with the electrode compartments separated by an ion-exchange membrane (22), was used for the generation of iodine in the column effluent. The current source for the generating current was a

Metrohm (Herisau, Switzerland) E585 or a UCS6-2F from Elektronica (Amsterdam, the Netherlands). The downstream detector was an AMOR amperometric detector (Spark, Emmen, The Netherlands) with a glassy carbon working electrode and an in situ Ag/AgI reference electrode. The PTFE reaction coil between generating cell and detector cell provided a reaction time of 6 s at a flow rate of 1.8 ml/min.

Experiments were conducted at room temperature (20 ± 2°C).

Chemicals and Solutions

N-Acetylcysteine (NAC) and sodium octyl sulfate were obtained from Aldrich-Chemie. Glutathione (reduced, GSH) and dithiothreitol (DTT) were obtained from Janssen Chimica. Cysteine (CYS) was purchased from Merck. The other reagents were of analytical grade purity.

Unless stated otherwise the mobile phase consisted of a 0.03 mol l^{-1} phosphate buffer (pH 2.0) with 3 mmol l^{-1} sodium octyl sulfate, 1 mmol l^{-1} potassium iodide and 2 % (v/v) methanol.

Sample Preparation

Urine samples were filtered through a 0.8 μm Millipore filter. To 1 ml of a filtered sample, 80 μl of a 0.1 mol l^{-1} DTT solution and 400 μl of a 0.25 mol l^{-1} sodium phosphate buffer of pH 8.0 were added and the mixture was shaken. After 30 min an aliquot of 250 μl was taken and mixed with 1.5 ml of water before injection.

Serum samples were prepared as proposed by Johansson (8). To 400 μl of serum in a 1 ml capped vial, 80 μl of a 0.4 mol l^{-1}

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sodium hydroxide solution and 300 μl of a 0.4 mol l^{-1} DTT solution were added. The mixture was left at room temperature for 20 min, then 200 μl of 1.7 mol l^{-1} perchloric acid was added. After shaking for 10 s, the sample was centrifuges for 3 min at 14000 rpm. The supernatant was injected directly or diluted with water before injection.

RESULTS AND DISCUSSION

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Optimization of Separation and Detection

Since glutathione (GSH) may interfere in the analysis of biological samples other than urine, and dithiothreitol (DTT) is used for the reduction of CYS and NAC present in the oxidized form in the samples, the separation of CYS, NAC, GSH and DTT was studied. A reversed-phase system was used with an acidic mobile phase and octyl sulfate as an ion-pairing reagent for CYS and GSH. The selectivity of the separation depended on the pH and the octyl sulfate concentration of the mobile phase as shown in Figure 1. Increasing the methanol or the buffer concentration resulted in a general decrease of the retention times of the four sulfhydryl compounds studied. The optimal mobile phase contained 0.03 mol l^{-1} phosphate buffer of pH 2.0, 3 mmol l^{-1} octyl sulfate, 1 mmol l^{-1} potassium iodide and 2 % (v/v) methanol. The retention times of NAC, DTT, CYS and GSH were 2.1, 3.8, 6.7 and 9.8 min, respectively, with a flow rate of 1.8 ml min^{-1} (Figure 2).

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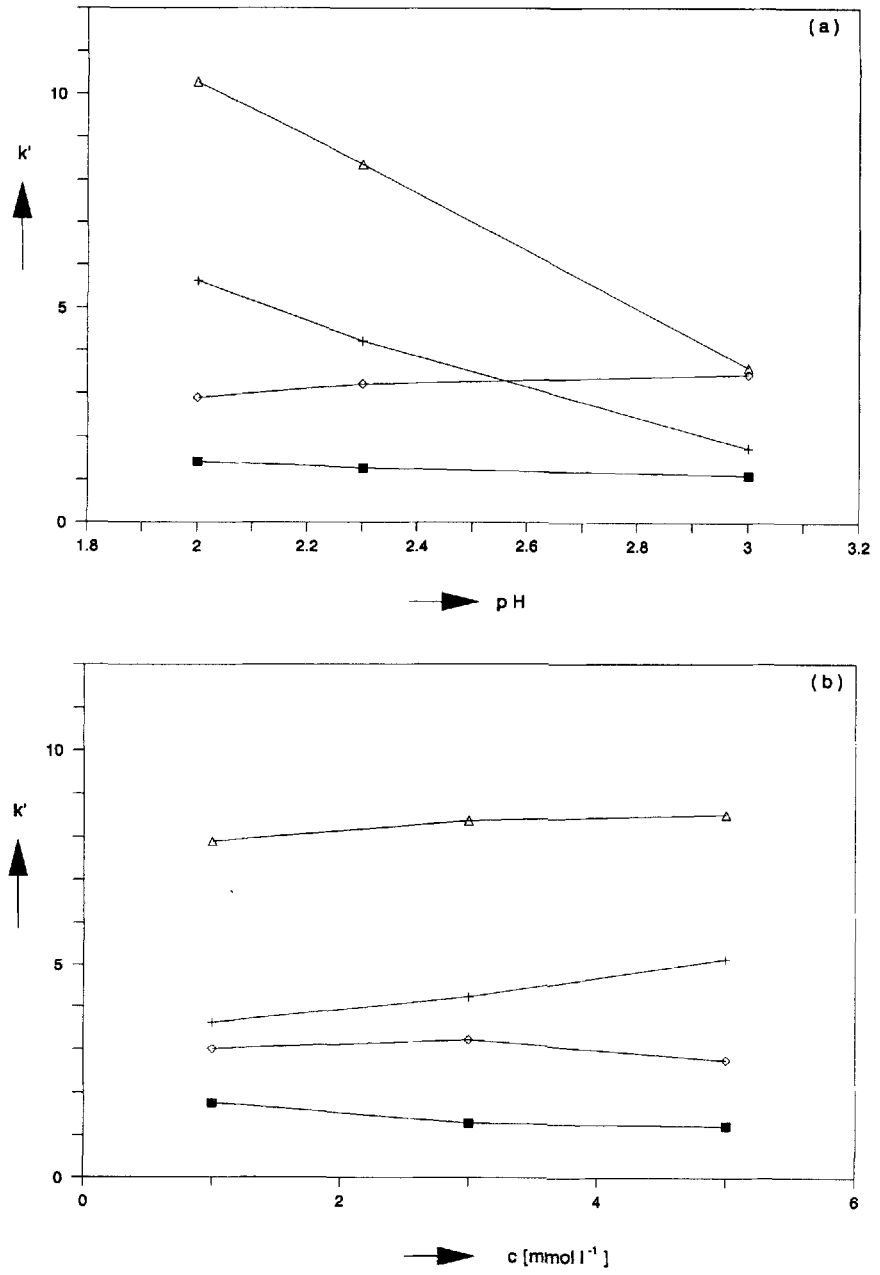

FIGURE 1. Effect of the mobile phase composition on capacity factors. Mobile phase with 0.03 mol l^{-1} phosphate buffer and 2 % (v/v) methanol. Column A. (a) Effect of the pH; 3 mmol l^{-1} octyl sulfate. (b) Effect of the octyl sulfate concentration; pH = 2.3. CYS (+), NAC (■), GSH (△) and DTT (◇).

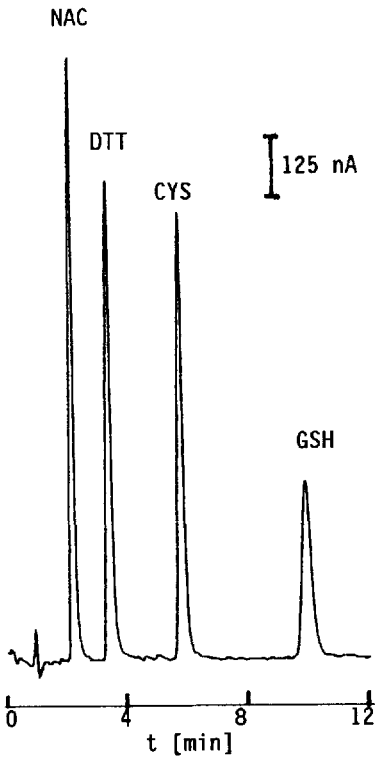

FIGURE 2. Chromatogram of a standard solution of CYS (80 ng), NAC (80 ng), GSH (240 ng) and DTT (260 ng). Mobile phase with 0.03 mol l^{-1} phosphate buffer (pH = 2.0), 3 mmol l^{-1} octyl sulfate and 2 % (v/v) methanol. Column A.

Irreversible adsorption of the thiols on the ODS stationary phase as reported previously (23) was not observed.

To optimize the indirect amperometric detection, the hydrodynamic voltammogram was measured of the iodine produced in the mobile phase. A current plateau was observed between detection potentials of +0.05 to +0.45 V *vs.* Ag/AgI. A plot of the detection baseline-current *vs.* the generating current in the range 0 - 100

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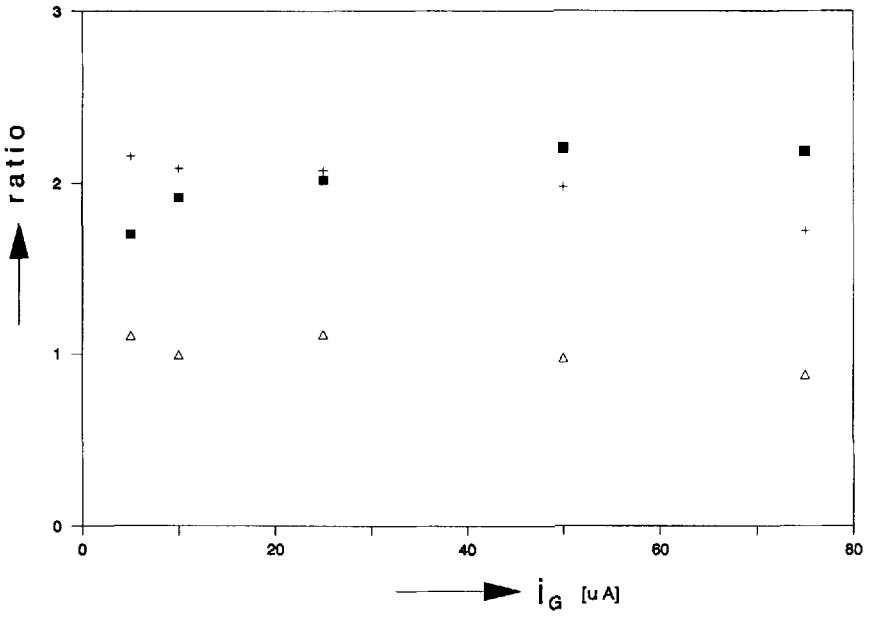

FIGURE 3. The influence of the generating current on the stoichiometric ratio (iodine : sulfhydryl) of the reaction of CYS (+), NAC (■) and GSH (△). Reaction time 6 s. Experimental conditions as in FIGURE 2.

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μA was linear with a slope of 0.058 and passed through the origin. This indicates that iodine is produced from iodide in the mobile phase with 100 % current efficiency.

To study the stoichiometry and kinetics of the reaction of iodine with the sulfhydryl compounds, peak areas were measured with different generating currents in the range 10 - 75 μA. From the peak areas the stoichiometric ratio, the number of iodine molecules reacting with one sulfhydryl molecule, was calculated by applying Faraday's Law. The results are given in Figure 3. The

TABLE 1

Sensitivities, Limits of Detection and Upper Limits of Linearity with a Generating Current of 25 μA.

Compound	Sensitivity [nA ng^{-1}]	LOD[a] [ng]	Upper Limit [ng]
CYS	12.1	2.0	110
NAC	16.3	1.5	80
GSH	1.6	15	800

[a]: limit of detection, signal : noise = 3

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stoichiometry of the reaction of CYS and NAC (2 : 1) is different from that of GSH (1 : 1). From the fact that the peak areas do not increase with the generating current it can be concluded that the reaction is virtually complete in the hold-up time of the reaction coil (6 s).

Sensitivity, Linearity and Precision

Calibration of the system was carried out with a detection potential of +0.15 V and a generating current of 25 μA. In Table 1 the observed sensitivities, detection limits and upper limits of the linear range for CYS, NAC and GSH are given. The lower sensitivity for GSH is due to its lower stoichiometric ratio and its longer retention time.

Linear responses were obtained up to peak heights of about 90 % of the baseline current value. In this current range

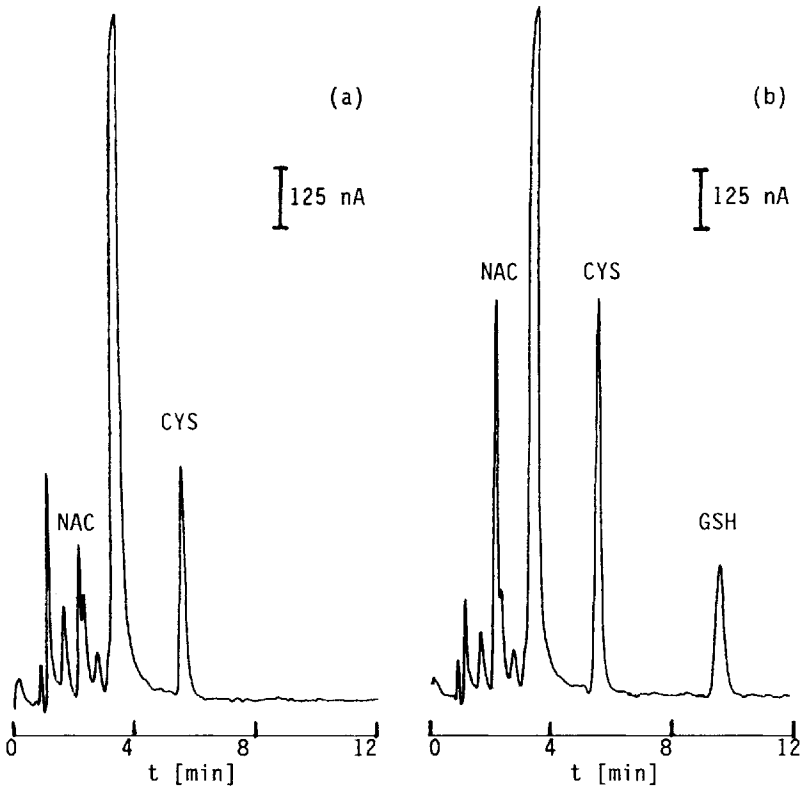

FIGURE 4. Chromatograms of urine samples. (a) Blank urine. (b) Urine spiked with 8 μg ml^{-1} CYS, 8 μg ml^{-1} NAC and 50 μg ml^{-1} GSH. Conditions as in FIGURE 2.

correlation coefficients of the calibration plots were > 0.9998 for the three compounds. However, the linear range can be changed by a change of the generating current i_G. Since the noise measured is proportional to the generating current, and the sensitivity is independent of i_G (see Figure 1), detection limits change also proportional with i_G. In this way a dynamic range of almost two

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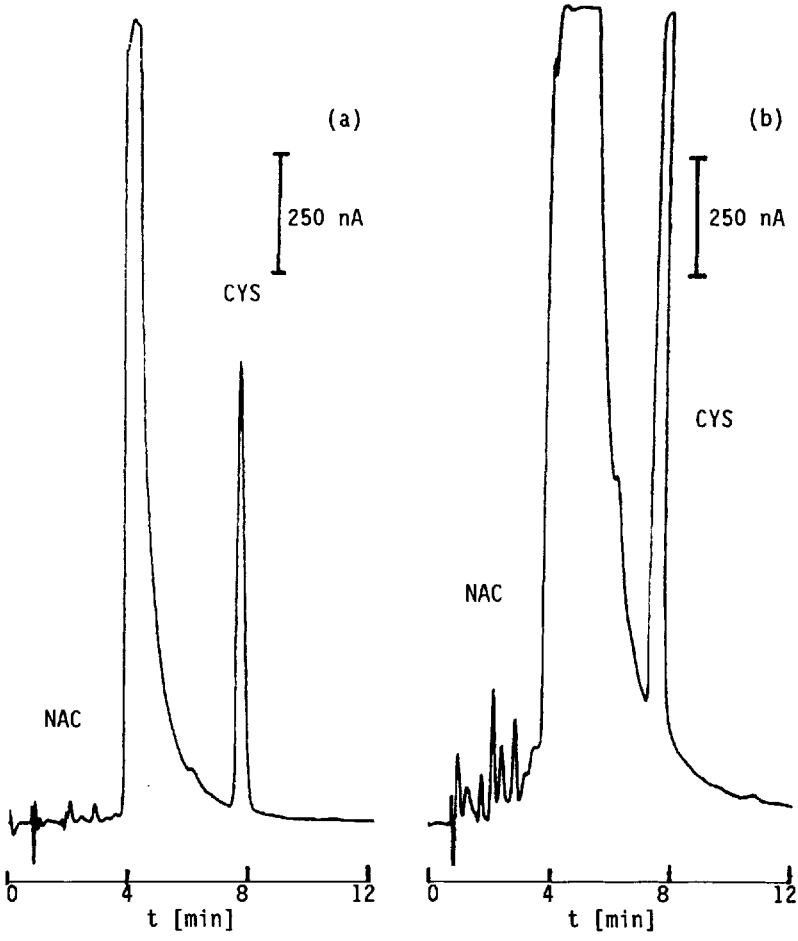

FIGURE 5. Chromatograms of a serum sample. (a) Supernatant diluted with water (1 + 4) before injection; (b) Direct injection of the supernatant. Column B. Other conditions as in FIGURE 2.

orders of magnitude can be maintained, while the system can be adapted to the expected concentration range of the samples.

The precision was assessed by seven successive injections of a standard solution of CYS (2.6 $\mu g\ ml^{-1}$), NAC (2.6 $\mu g\ ml^{-1}$) and GSH (8 $\mu g\ ml^{-1}$). The relative standard deviations of the peak heights were 0.6 %, 0.6 % and 2.0 %, respectively.

Application to Urine and Serum Samples

As has been shown before (8), the rate of reduction of disulfides by dithiothreitol is higher at a high pH. When only a DTT solution was added to a urine sample, the amount of CYS and NAC recovered reached a maximum after a 3 hours incubation time. However, when a buffer of pH 8.0 was added together with the DTT solution, an incubation time of 30 min was sufficient.

The recovery of the sulfhydryl compounds in the reduced form from urine was studied with the standard addition method. Different concentrations of CYS and NAC, covering the entire linear range of the method, were added to 7 aliquots of a urine sample before the DTT treatment. For CYS the recovery was 105.0 $\pm$ 3.2 %, and for NAC a recovery of 93.3 $\pm$ 2.6 % was found. These results may indicate that part of the NAC is hydrolysed during the DTT treatment in basic solution. Endogenous concentrations of 5.6 $\mu g\ ml^{-1}$ of NAC and 15.1 $\mu g\ ml^{-1}$ of CYS were found in this particular urine sample. These values are in the normal range as reported in the literature (24). As expected endogenous urinary GSH was not detected. Figure 4 shows representative chromatograms of blank and spiked urine samples.

Figure 5 shows that is possible to apply the method presented to the analysis of serum samples. However, due to the large difference in the endogenous serum concentrations of CYS and NAC, the two compounds have to be determined in different chromatographic runs, with different dilutions of the sample. Endogenous (total) concentrations found in this particular serum sample were 32.6 $\mu g\ ml^{-1}$ of CYS and 1.3 $\mu g\ ml^{-1}$ of NAC.

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REFERENCES

1. Russo, A., Detection and Quantitation of Biological Sulfhydryls, in Methods of Biochemical Analysis, Vol 33, Glick, D., ed., Wiley, New York, 1988, p. 165.

2. Perrett, D. and S.R.Rudge, Determination of Thiols and Related Compounds using High-performance Liquid Chromatography, J.Pharm.Biomed.Anal., 3, 3, 1985.

3. Beales, D., Finch, R. and McLean, A.E.M., Determination of Penicillamine and Other Thiols by Combined High-performance Liquid Chromatography and Post-column Reaction with Ellman's Reagent: Application to Human Urine, J.Chromatogr., 226, 498, 1981.

4. Frank, H. and Thiel, D., Determination of N-Acetylcysteine in Biological Fluids, J.Chromatogr., 309, 261, 1984.

5. Kagedal, B., Kallberg, M. and Martensson, J., Determination of Non-protein-bound N-Acetylcysteine in Plasma by High-performance Liquid Chromatography, J.Chromatogr., 311, 170, 1984.

6. Imai, K., Toyo'oka, T. and Miyano, H., Fluorigenic Reagents for Primary and Secondary Amines and Thiols in High-performance Liquid Chromatography, Analyst (London), 109, 1365, 1984.

7. Lewis, P.A., Woodward, A.J. and Maddock, J., Improved Method for the Determination of N-Acetylcysteine in Human Plasma by High-performance Liquid Chromatography, J.Chromatogr., 327, 261, 1985.

8. Johansson, M. and Westerlund, D., Determination of N-Acetylcysteine, Intact and Oxidized, in Plasma by Column Liquid Chromatography and Post-column Derivatization, J.Chromatogr., 385, 343, 1987.

9. Imai, K. and Toyo'oka, T., Design and Choice of Suitable Labelling Reagents for Liquid Chromatography, J.Chromatogr. Library, Vol. 39A, Elsevier, Amsterdam, 1989, p. 209.

10. Rabenstein, D.L. and Saetre, R., Mercury-based Electrochemical Detector for Liquid Chromatography for the Detection of Glutathione and Other Sulfur-containing Compounds, Anal. Chem., 49, 1036, 1977.

11. Saetre, R. and Rabenstein, D.L., Determination of Penicillamine in Blood and Urine by High Performance Liquid Chromatography, Anal. Chem., 50, 276, 1978.

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12. Allison, L.A., Keddington, J. and Shoup, R.E., Liquid Chromatographic Behaviour of Biological Thiols and the Corresponding Disulfides, J.Liquid Chrom., 6, 1785, 1983.

13. Allison, L.A. and Shoup, R.E., Dual Electrode Liquid Chromatography Detector for Thiols and Disulfides, Anal. Chem., 55, 8, 1983.

14. Lunte, S.M. and Kissinger, P.T., Detection of Thiols and Disulfides in Liver Samples Using Liquid Chromatography / Electrochemistry, J.Liquid Chrom., 8, 691, 1985.

15. Dupuy, D. and Szabo, S., Measurement of Tissue Sulfhydryls and Disufides in Tissue Proteine and Nonprotein Fractions by High Performance Liquid Chromatography using Electrochemical Detection, J. Liquid Chrom., 10, 107, 1987.

16. Kok, W.Th., Halvax, J.J., Voogt, W.H., Brinkman, U.A.Th. and Frei, R.W., Detection of Thioethers of Pharmaceutical Importance by Liquid Chromatography with On-line Generated Bromine, Anal. Chem, 57, 2580, 1985.

17. Isaksson, K., Lindquist, J. and Lundstrom, K., Reaction Detection for Liquid Chromatography with Electrochemical Generation and Detection of Excess Bromine, J.Chromatogr., 324, 333, 1985.

18. Kok, W.Th., Halvax, J.J. and Frei, R.W., Comparison of Electrochemical Detection Methods in Liquid Chromatography for the Determination of N-Acetylcysteine in Plasma, J.Chromatogr., 352, 27, 1986.

19. Debets, A.J.J., van de Straat, R., Voogt, W.H., Vos, H., Vermeulen, N.P. and Frei, R.W., Simultaneous Determination of Glutathione, Glutathione Disulfide, Paracetamol and its Sulfur-containing Metabolites using HPLC and Electrochemical Detection with On-line Generated Bromine, J.Pharm.Biomed.Anal., 6, 329, 1988.

20. Fabre, H. and Kok, W.Th., Determination of Cephalosporins and Decomposition Products by Liquid Chromatography with Indirect Electrochemical Detection, Anal.Chem., 60, 136, 1988.

21. Timmer, J.G., Vyth, A., Kok, W.Th., de Jong, J.M.B.V. and Jager, W.A.D., N-Acetylcysteine Therapy for Patients with Amyotrophic Lateral Sclerosis, Pharm.Weekblad, 7, 87, 1985.

22. Kok, W.Th., Brinkman, U.A.Th. and Frei, R.W., On-line Electrochemical Reagent Production for Detection in Liquid Chromatography and Continuous Flow Systems, Anal.Chim.Acta, 162, 19, 1984.

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23. Perrett, D. and Rudge, S.R., Problems Associated with the High-performance Liquid Chromatography of Thiols, J.Chromatogr., 294, 380, 1984.

24. Wissenschaftliche Tabellen Geigy, 8th ed., Ciba-Geigy AG, Basel, Switzerland, 1977.

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