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Detection of N-acetylcysteine, cysteine and their disulfides in urine by liquid chromatography with a dual-electrode amperometric detector

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Abstract: A method for the determination of N-acetylcysteine, cysteine and their disulfides in urine is described. The thiols and disulfides are separated by reversed-phase ion-pair chromatography with octyl sodium sulfate as the ion-pairing reagent and detected with a dual-electrode amperometric detector using Au/Hg amalgam electrodes. Both the thiols and disulfides are detected with this system. In addition, dimers and mixed disulfides can be detected individually.

Keywords: *N-Acetylcysteine; cysteine; liquid chromatography/electrochemistry; dual-electrode detection.*

Introduction

N-Acetylcysteine (NAC) is an endogenous product of cysteine metabolism [1], a mucolytic drug [2], and a chemoprotective agent for paracetamol poisoning [3, 4]. Cysteine (CYS) is an endogenous precursor of glutathione [5] and a metabolite of NAC [6, 7]. These amino acids occur both as the reduced thiol form and as oxidized disulfides. In addition, mixed disulfides formed by oxidative coupling of non-identical thiols are possible.

In order to study the effect of various drugs on the natural concentrations of these amino acids and the pharmacokinetics of NAC dosing, a method capable of determining both the oxidized and reduced forms of both compounds as well as potential mixed disulfides is needed. Previously described methods for the determination of NAC and CYS in plasma and urine have typically involved liquid chromatography using either pre- or postcolumn derivatization of the free thiol [8–12] or gas chromatography with detection by mass spectrometry [13–14]. For all of these methods, in order to determine disulfides the sample must first be reduced (usually with dithiothreitol) to free thiols. The amount of disulfide is determined by comparison of the amount of thiol detected with and without the reduction step. In addition to requiring time-consuming multiple analyses, none of these methods can determine which disulfides are present. In particular, dimers cannot be distinguished from mixed disulfides. More recently, electrochemical techniques capable of detecting both thiols and disulfides have been described [15–19]. Crawhall and Kalant [20] have described a method for post-column reduction of the disulfides to thiols followed by derivatization of the free thiol and detection by UV absorption. Using this method, the detection limit for cystine was only 0.3 nmol, nearly three orders of magnitude higher than can be achieved by electrochemical detection.

In this paper, a method to determine NAC, CYS and their disulfides is presented. This method is based on reversed-phase, ion-pair chromatography with dual-electrode electrochemical detection. Both thiols and disulfides can be detected with a single chromatographic injection using the dual-electrode detector. Chromatographic conditions are described which resolve NAC, CYS and their disulfides from other urine components. This is an extension of our earlier work on the detection of glutathione [16, 17]. Electrochemical preparation of disulfides from thiols is also described.

Experimental

Chemicals and reagents

NAC, CYS, cystine (CYS_2) and N-ethylmaleimide (NEM) were obtained from Sigma

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(St Louis, Missouri, USA). Sodium octyl sulfate was purchased from Eastman Kodak (Rochester, New York, USA). All other compounds were reagent grade or better and used as received. Solutions were prepared with water from a Syborn–Barnstead NanoPure (Boston, Massachusetts, USA) water purification system.

Apparatus

The liquid chromatograph was a Bioanalytical Systems, Inc. (BAS, West Lafayette, Indiana, USA) LC-400 system with dual LC-4B amperometric detectors. The mobile-phase reservoir was from Rainin (Woburn, Massachusetts, USA) and allowed deoxygenation of the mobile phase with argon. A 20 μl injection loop was used for all experiments. Dual Au/Hg amalgam electrodes were used for detection.

The Au/Hg amalgam electrodes were prepared as previously described [15]. A clean Au electrode was polished with 6 μm diamond polish, rinsed with methanol and water, and then further polished with 1 μm alumina polish. The polished electrode was rinsed with distilled water and sonicated for at least 2 min. A drop of triple distilled mercury was applied to the polished Au electrode for 1 min. The excess mercury was carefully removed and the Au/Hg amalgam allowed to equilibrate for at least 8 h (typically overnight). The resulting Au/Hg electrode was stable for several weeks. The primary determinant of electrode life was the amount of thiol injected into the system. Higher concentration samples shortened electrode life by stripping mercury from the electrode. For the experiments described here, electrodes typically lasted for at least a week of continuous use. To avoid any delays from electrode preparation, two cells were always maintained, one in use and the second already equilibrated as the Au/Hg amalgam for rapid changeover.

The electrochemical flow cell for synthetic applications was prepared according to the previously described design [21]. The flow cell was attached to a Sage 355 syringe pump (Orion, Cambridge, Massachusetts, USA) and controlled by a BAS CV-37 voltammograph.

Chromatographic system

The separation was accomplished on an ODS Hypersil (5 μm, 15 cm × 4.6 mm) column. The mobile phase was sodium phosphate (0.025 M, pH 2.5) containing octyl sodium sulfate (3 mM) ≃ methanol (99:1, v/v). The mobile phase was deoxygenated by purging with argon prior to use.

The dual-electrode thin-layer electrochemical cell was used in the series configuration. The upstream electrode was operated at −1.0 V vs an Ag/AgCl reference electrode to reduce any disulfides to the corresponding thiols. The downstream electrode was operated at +0.15 V vs Ag/AgCl for the detection of thiols.

Preparation of disulfides

Disulfides were initially prepared by addition of 0.5 ml 3% H_2O_2 to 5 ml of 1 mM solution of the thiol. Mixed disulfides were prepared in the same manner except a solution equimolar in the two thiols was used. In the preparation of mixed disulfides a mixture of the mixed disulfides and the two dimers resulted. While this procedure was useful for the preparation of disulfides for qualitative purposes, the yield was typically less than 50%. The major source of loss appeared to be further oxidation of the thiol to the sulfate and sulfoxide.

To achieve a quantitative oxidation of the thiols to the disulfides the synthetic electrochemical flow cell was used to more precisely control the extent of oxidation. A 1 mM solution of the thiol (or thiol mixture for preparation of mixed disulfides) was pumped through the cell at 1 ml min^{-1}. A potential of 0.8 V vs Ag/AgCl was applied to the cell. At this potential thiols are oxidized to disulfides on a single pass through the flow cell. Because of the precise control of the oxidation potential, no oxidation beyond the disulfide occurred. Standard disulfide solutions could therefore be prepared from thiol solutions of known concentrations.

Sample preparation

Urine was collected from human volunteers directly into vials containing sufficient phosphoric acid and EDTA to make the sample approximately 0.1 mM EDTA and pH 2. The acid made the sample thiols harder to oxidize, while the EDTA complexed metals which catalyze the oxidation of thiols. Without these precautions, much of the thiol is lost through air oxidation prior to analysis. The acidified sample was filtered through a 0.2 μm filter prior to chromatographic analysis. For experiments using NEM, 100 μl of 0.24 M NEM was

added to a 3 ml aliquot of the acidified sample. This sample was allowed to sit for 5 min and then filtered before injection onto the chromatographic column.

Results and Discussion

Optimization of chromatography for separation of thiols

Ion-pair reversed-phase chromatographic systems have been used for the separation of various thiols [15–17, 19]. NAC and CYS are much more hydrophilic than other commonly studied thiols and the chromatographic systems developed for these thiols are inadequate for the determination of NAC and CYS. Typically, NAC and CYS have very short retention times and are often obscured by early eluting endogenic compounds. For this work, a more acidic buffer, pH 2.5, was used to protonate the carboxylic acid groups of the amino acids fully and decrease their polarity. In addition, more ion-pairing agent, 3 mM octyl sodium sulfate, was used to increase the retention of the analytes. This system provided sufficient retention that both NAC and CYS were well removed from the void. Resolution of all compounds was achieved using this chromatographic system (Fig. 1).

As expected, NAC was retained less than CYS but still well removed from the early eluting material. Also as expected, the mixed disulfide, NAC·CYS, eluted between the corresponding dimers, N-acetylcystine (NAC_2) and cystine (CYC_2). A weakness of the system was that the other more hydrophobic thiols and disulfides had excessively long retention times. The disulfides of glutathione and homocysteine, for example, have retention times of several hours under these conditions. Because of the slow equilibration of the ion-pair system, this limitation cannot be overcome by a gradient elution approach.

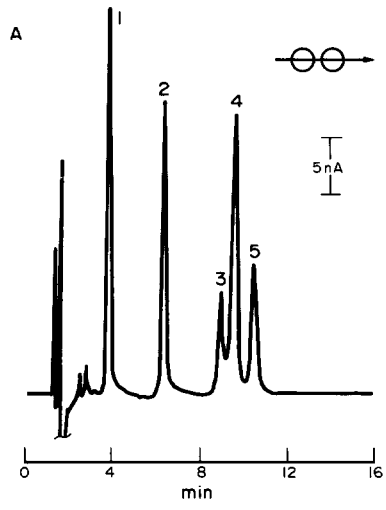

Figure 1
Separation of the thiols and disulfides. Peak identities: Peak 1, NAC; Peak 2, CYS; Peak 3, $(NAC)_2$; Peak 4, NAC·CYS; Peak 5, $(CYS)_2$. Stationary phase, ODS Hypersil; mobile phase, sodium phosphate (0.025 M, pH 2.5) containing 3 mM sodium octyl sulfate – methanol (99:1, v/v). Flow rate, 1 ml min^{-1}.

Detection of thiols and disulfides

Electrochemistry provides a powerful technique for the detection of thiols and disulfides. Unlike other detection schemes, it detects both thiols and disulfides individually in a single chromatographic experiment. Thiols form a complex with mercury which is very easily oxidized. Using a Au/Hg (gold/mercury amalgam) electrode, thiols are oxidized at +0.15 V vs an Ag/AgCl reference electrode. Disulfides are not readily detected directly, but can be reduced to the corresponding thiols post-column in the electrochemical cell by use of a dual-electrode detector. The two working electrodes are used in the series configuration with the upstream electrode operated at −1.0 V vs Ag/AgCl and the downstream electrode operated to detect thiols (Fig. 2). Only the current response at the downstream electrode is recorded: the upstream electrode is in essence a post-column reactor. Dual-electrode electrochemical detection of several thiols and disulfides is shown in Fig. 3A.

Only thiols are detected when the upstream electrode is turned off (Fig. 3C). This provides a convenient method of determining which peaks in a complex chromatogram correspond to disulfides. Thiols react with NEM to give a

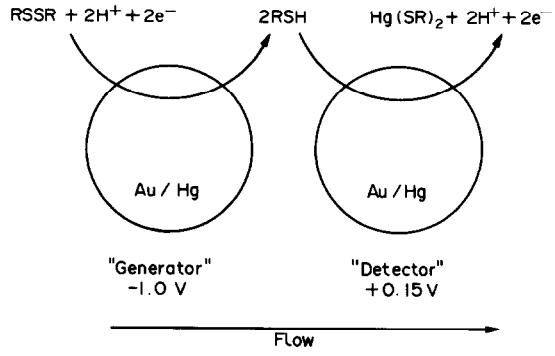

Figure 2
Dual-electrode cell for the detection of thiols and disulfides.

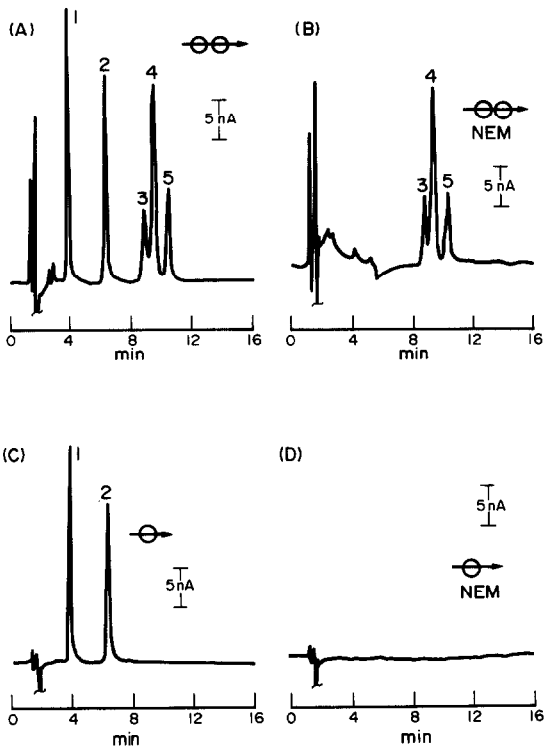

Figure 3
Use of NEM and dual-electrode detection for the determination of NAC, CYS and their disulfides. Peak identities and chromatographic conditions as in Fig. 1: A, dual-electrode detection (both thiols and disulfides detected); B, dual-electrode detection with addition of NEM (only disulfides detected); C, single-electrode detection (only thiols detected); D, single-electrode detection with addition of NEM (neither thiols nor disulfides detected).

product that is not detected electrochemically [17, 22, 23]. If an aliquot of the sample is reacted with NEM prior to chromatographic analysis, the thiol peaks are no longer present (Fig. 3B). This technique provides a convenient method of identifying the thiols in the sample. Finally, if the sample is treated with NEM and the upstream electrode is turned off, neither thiols nor disulfides will be detected (Fig. 3D). Any chromatographic peaks from such a sample must be from a non-thiol/disulfide interferent. While this detection method is very selective for thiols and disulfides, compounds which strongly chelate mercury will give a response. Commonly observed examples are EDTA and high concentrations of chloride. Fortunately, these compounds elute in the void of the chromatographic system for the separation of thiols and disulfides.

This method provided excellent detection for all thiols and disulfides studied. Detection limits of 500 fmol injected were achieved for NAC and CYS while detection limits of 1 pmol injected were achieved for the disulfides. Excellent linearity was found for all compounds (Table 1).

Analysis of urine samples

Typical chromatograms from a urine sample are shown in Fig. 4. The major thiol/disulfide compounds found were cystine and cysteine. NAC was present in small amounts as both the disulfide and free thiol. In addition, the mixed disulfide of CYS and NAC was detected. Urine from four volunteers (1 female and 3 male) was analyzed; average thiol levels were 0.39 μmol/l NAC, 11 μmol/l CYS, 0.71 μmol/l $(NAC)_2$, 9.5 μmol/l $(CYS)_2$, and 0.98 μmol/l NAC·CYS. The addition of NEM to the sample removed CYS and NAC but did not effect many other peaks in the urine chromatogram (Fig. 4B). By comparing dual-electrode detection (Fig. 4A) with single electrode detection (Fig. 4C), it can be seen that most thiols occur in urine predominantly in their oxidized, disulfide form. The addition of NEM to the urine sample and used a single electrode illustrates the selectivity of this method for thiols and disulfides. Only a few peaks near the void were detected under these conditions (Fig. 4D). These early non-thiol/disulfide peaks are probably due to halides (e.g. Cl^-) in the urine and to EDTA added to stabilize the sample.

Table 1
Representative standard curves for NAC, CYS and their disulfides

Compound	Slope (pmol nA^{-1})	Intercept (pmol)	r^2
NAC	4.28	0.04	0.9995
CYS	4.98	0.05	0.9994
$(NAC)_2$	10.4	0.17	0.9996
NAC·CYS	12.2	0.25	0.9994
$(CYS)_2$	14.0	0.24	0.9997

Curves were obtained from 1 to 400 pmol injected for each compound ($N = 6$).

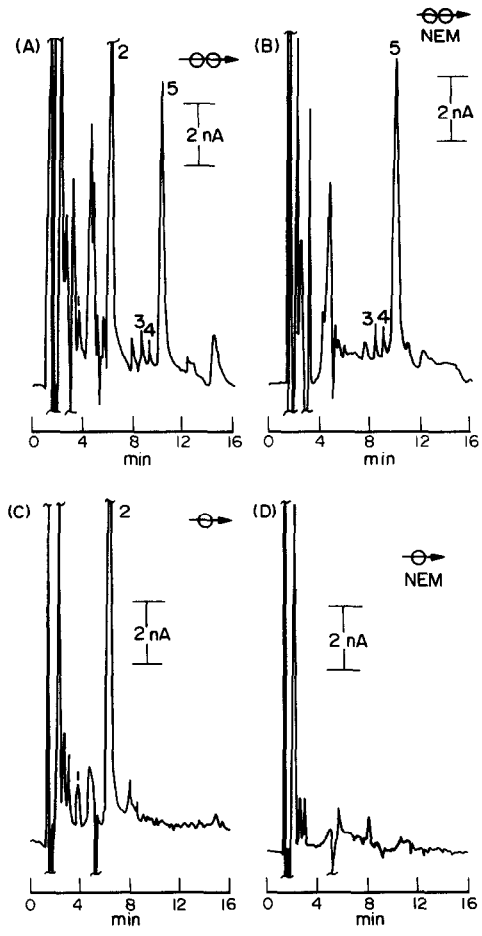

Figure 4
Detection of NAC, CYS and their disulfides in urine. Peak identities and chromatographic conditions as in Fig. 1. Detection conditions as in Fig. 3.

Conclusions

Dual-electrode detection with Au/Hg amalgam electrodes coupled to reversed-phase ion-pair chromatography provided a selective and sensitive method of detecting NAC, CYS and their disulfides in complex biological samples. A particular advantage over previous methods of analysis was that electrochemical detection provided for the simultaneous determination of thiols and disulfides with a single chromatographic injection. Using liquid chromatography with electrochemical detection it was possible to separate and detect both dimers and mixed disulfides. The use of NEM in combination with and without the upstream electrode provided a convenient method for distinguishing between thiols, disulfides and possible interferents. Finally, manipulation of the reversed-phase ion-pair chromatographic system provided excellent resolution of all analytes from the chromatographic void and each other. This method was demonstrated by detection of NAC, CYS and their disulfides in urine, but is applicable to any sample.

References

[1] H. Kodama, T. Ikegami and T. Araki, *Physiol. Chem. Phys.* **6**, 87–89 (1974).
[2] M. Aylward, J. Maddock and P.M. Dewland, *Eur. J. Respir. Dis.* **61**(Suppl. III), 81–89 (1980).
[3] L.F. Prescott, J. Park, A. Ballantyne, P. Adriaenssens and A.T. Proudfoot, *Lancet* **2**, 432–437 (1977).
[4] L.F. Prescott, R.N. Illingworth, J.A.J.H. Critchley, M.J. Stewart, R.D. Adams and A.T. Proudfoot, *Br. Med. J.* **2**, 1097–1100 (1979).
[5] A.L. Lehninger, in *Biochemistry*, p. 715. Worth, New York (1975).
[6] A.L. Sheffner, E.M. Medler, K.R. Bailey, D.G. Gallo, A.J. Mueller and H.P. Sarett, *Biochem. Pharmacol.* **15**, 1523–1535 (1966).
[7] D. Rodenstein, A. De Coster and A. Gazzaniga, *Clin. Pharmacokinet.* **3**, 247–254 (1978).
[8] B. Kagedal, M. Kallberg and J. Martensson, *J. Chromatogr.* **311**, 170–175 (1984).
[9] P.A. Lewis, A.J. Woodward and J. Maddock, *J. Chromatogr.* **327**, 261–267 (1985).
[10] K. Nakashima, C. Umekawa, H. Yoshida, S. Nakatsuji and S. Akiyama, *J. Chromatogr.* **414**, 11–17 (1987).
[11] M. Johansson and D. Westerlund, *J. Chromatogr.* **385**, 343–356 (1987).
[12] M. Johansson and S. Lenngren, *J. Chromatogr.* **432**, 65–74 (1988).
[13] U. Hannestad and B. Sorbo, *Clin. Chim. Acta* **95**, 189–200 (1979).
[14] H. Frank, D. Thiel and K. Langer, *J. Chromatogr.* **309**, 261–267 (1984).
[15] L.A. Allison and R.E. Shoup, *Anal. Chem.* **55**, 8–12 (1983).
[16] S.M. Lunte and P.T. Kissinger, *J. Chromatogr.* **317**, 579–588 (1984).
[17] S.M. Lunte and P.T. Kissinger, *J. Liq. Chromatogr.* **8**, 691–706 (1985).
[18] W.Th. Kok, J.J. Halvax and R.W. Frei, *J. Chromatogr.* **352**, 27–33 (1986).
[19] J.P. Richie, Jr and C.A. Lang, *Anal. Biochem.* **163**, 9–15 (1987).
[20] J.C. Crawhall and D. Kalant, *Anal. Biochem.* **172**, 479–483 (1988).
[21] D.J. Miner and P.T. Kissinger, *Biochem. Pharmacol.* **28**, 3285–3290 (1979).
[22] E. Roberts and G. Rouser, *Anal. Chem.* **30**, 1291–1292 (1958).
[23] N.M. Alexander, *Anal. Chem.* **30**, 1292–1294 (1958).

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