Journal of Chromatography, 385 (1987) 343–356
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 1065

DETERMINATION OF N-ACETYLCYSTEINE, INTACT AND OXIDIZED, IN PLASMA BY COLUMN LIQUID CHROMATOGRAPHY AND POST-COLUMN DERIVATIZATION

MARGARETA JOHANSSON*
Department of Research and Development, ACO Läkemedel AB, Box 3026, S-171 03 Solna (Sweden)
and
DOUGLAS WESTERLUND
Department of Analytical Pharmaceutical Chemistry, Uppsala University Biomedical Center, P.O. Box 574, S-751 23 Uppsala (Sweden)

SUMMARY

N-Acetylcysteine in plasma may exist as intact N-acetylcysteine (NAC) or be oxidized to disulphides, either as a dimer or mixed with other thiol-containing compounds. To prevent oxidation of NAC, whole blood was immediately centrifuged after collection and the plasma proteins were precipitated with perchloric acid. NAC was measured by direct injection of the supernatant into the chromatographic system and the oxidized forms, coupled to small sulphides (ONACS), were determined after reductive cleavage of all NAC disulphides in the supernatant with dithiothreitol before injection. The total plasma concentration of the compound, *i.e.*, including the fraction coupled to proteins (ONACP), was assayed after an initial reduction of the disulphide linkages in plasma. After subsequent precipitation of proteins, the supernatant was directly injected. The chromatographic system was a reversed-phase column (C_{18}) with an acidic mobile phase. After a fast (<6 s) post-column reaction with pyrenemaleimide, NAC was detected by fluorimetry. The contribution to the band broadening by the reactor was about 10%. The limit of quantification of NAC in plasma was 240 n*M*, with an intra-assay precision of 14%.

INTRODUCTION

N-Acetylcysteine (NAC) is an important mucolytic drug[1], and when administered intravenously it is an effective antidote in the treatment of paracetamol poisoning[2].

Thiols are sensitive to oxidation, and in plasma NAC may be oxidized to disulphides, either as a dimer or mixed with cysteine or glutathione. Further, proteins such as albumin contain a free thiol group that may bind NAC by a disulphide linkage. In order to determine the NAC concentration correctly, it is important to avoid oxidation during sample collection, storage and work-up.

0021-9673/87/$03.50

In previously described methods for the determination of NAC in plasma, either the total concentration of NAC (*e.g.*, NAC, ONACS and ONACP)[3,4] or the concentration of its oxidized form, coupled to small sulphides (*e.g.*, NAC and ONACS)[5], has been quantified. These methods have been based on liquid chromatography[3–5] or gas chromatography[6,7]. The preparation of the sample includes reduction of the disulphides, extensive purification and pre-column derivatization, *i.e.*, very laborious procedures. However, a rapid liquid chromatographic method with electrochemical detection of NAC has recently been described[8].

This paper presents a simple, rapid method for the determination of the concentration of NAC, the concentration of the oxidized form, coupled to small sulphides, and the total concentration of NAC. The method includes careful sample handling, separation by reversed-phase chromatography and post-column derivatization with pyrenemaleimide.

EXPERIMENTAL

Chemicals and reagents

NAC was obtained from Diamalt (Munich, F.R.G.), dithiothreitol from Sigma (St. Louis, MO, U.S.A.), acetonitrile (HPLC quality), methanol (HPLC quality), perchloric acid (Suprapur quality) and buffer substances (analytical-reagent grade) from Merck (Darmstadt, F.R.G.) and pyrenemaleimide from Fluka (Buchs, Switzerland).

Apparatus

The liquid chromatograph (Fig. 1) consisted of a double Milton-Roy minipump with a pulse damper (Laboratory Data Control, Riviera Beach, FL, U.S.A.), a Waters Wisp 710 B automatic injector equipped with a cooling unit (Waters Assoc., Milford, MA, U.S.A.), a Waters nitrile guard column (4.0 × 4.6 mm I.D.) and a Microspher C_{18} analytical column (100 × 4.6 mm I.D.), 3 μm (Chrompack, Middelburg, The Netherlands). The outlet of the column was connected by a low dead volume T-coupling (Swagelok, Solon, OH, U.S.A.) to a Milton-Roy minipump for the addition of boron buffer. The third limb of the T-piece was connected to another T-piece for adding pyrenemaleimide with a Model B 94 pump (Eldex, Menlo Park, CA, U.S.A.). The third limb of the last T-piece was connected to a Model RF-530 fluorescence detector (Shimadzu, Kyoto, Japan) via a knitted PTFE capillary (4.2 m × 0.3 mm I.D.) for the post-column reaction. The derivative formed was measured

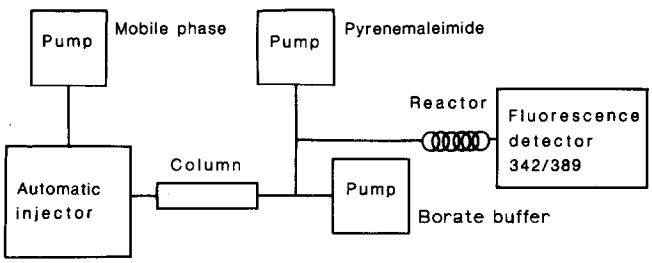

Fig. 1. Scheme of the liquid chromatographic system.

at 389 nm after excitation at 342 nm. The Milton-Roy pump used for buffer delivery and the Eldex pump were pulse-damped by stainless-steel columns (150 × 4.6 mm I.D.) packed with glass beads and connected by a T-coupling. The integrator was a Shimadzu C-R3A.

Chromatographic system

NAC was eluted from the Microspher C_{18} column with a mobile phase consisting of $1 \cdot 10^{-4}$ *M* EDTA in 1% (v/v) acetonitrile in phosphate buffer (pH 2) of ionic strength (μ) 0.01. The flow-rate was 1.0 ml/min. Alkalinization of the mobile phase to pH 10 was performed with 0.05 *M* borate buffer (pH 11) at 1 ml/min before $5 \cdot 10^{-5}$ *M* pyrenemaleimide in acetonitrile was added at 0.2 ml/min.

Pre-column derivatization

The derivatization was studied at pH 9 and 10 (carbonate buffer) and pH 11 (phosphate buffer) in batch experiments at 25.0 ± 1°C. To 5.00 ml of the appropriate buffer (μ = 0.1), 1.00 ml of a freshly prepared solution of $6.1 \cdot 10^{-6}$ *M* NAC in water and 600 μl of $3.5 \cdot 10^{-4}$ *M* pyrenemaleimide in acetonitrile were added. The formation of the derivative was followed by injection of the mixtures into the liquid chromatographic (LC) system described below.

The reaction rate was determined at 25.0 ± 0.1°C with different concentrations of pyrenemaleimide. To 5.00 ml of a carbonate buffer (pH 10; μ = 0.1), 1.00 ml of a freshly prepared aqueous solution of $7.4 \cdot 10^{-6}$ *M* NAC and 600 μl of $13.6 \cdot 10^{-6}$–$339.4 \cdot 10^{-6}$ *M* pyrenemaleimide in acetonitrile were added. The reaction was stopped by mixing 200 μl of the reaction solution with 1.80 ml of phosphate buffer (pH 5; μ = 1.0) and the amount of the derivative formed was determined by injection of 100 μl of the mixture into the LC system described below.

The chromatographic system consisted of a Hypersil ODS column (150 × 4.6 mm I.D.), 5 μm (Shandon, Runcorn, U.K.), with a mobile phase consisting of 40% (v/v) methanol in phosphate buffer (pH 7.0; μ = 0.01). The derivative formed was analysed at 389 nm after excitation at 342 nm.

Post-column derivatization

The optimization of the reaction conditions was studied in a flow-injection analysis system. PTFE capillaries (I.D. 0.5 mm) of different lengths (5–1000 cm) were knitted as described by Engelhardt and Neue[9]. Peak heights and peak areas were measured after the injection of 150 μl of $6.1 \cdot 10^{-6}$ *M* NAC in 0.01 *M* hydrochloric acid into the reagent stream. The contributions to the band broadening by the different parts of the flow-injection analysis system were studied by direct injection of the derivative formed in batch experiments. The PTFE capillary was 4.2 m × 0.3 mm I.D. A Waters M-440 detector was used with the integrator output connected to a Servogor recorder (BBC Goertz, Vienna, Austria). The time constant of the detector was 25 ms and the recorder time constant was 500 ms.

Preparation of N,N'-diacetylcystine

NAC was oxidized to N,N'-diacetylcystine after dissolution in water and further dilution to $2.5 \cdot 10^{-4}$ *M* with carbonate buffer (pH 10; μ = 0.1). After briefly sparging with oxygen, the solution was left overnight at room temperature.

Reduction of N,N'-diacetylcystine with dithiothreitol

N,N'-Diacetylcystine was reduced by mixing 2.00 ml of the $2.5 \cdot 10^{-4}$ *M* solution, 3.00 ml of the appropriate buffer of $\mu = 1.0$ (pH 7 and 12 phosphate buffers and pH 9 and 10 carbonate buffers) and 0.5 ml of a $1.3 \cdot 10^{-2}$ *M* dithiothreitol solution.

The latter was prepared in freshly boiled water and then chilled under nitrogen. The reduction was stopped at different times by mixing the buffer solution with an equivalent volume of mobile phase or 1 *M* phosphoric acid. NAC, N,N'-diacetylcystine and oxidized and reduced dithiothreitol were separated on a Nucleosil C_{18} column (250 × 4.6 mm I.D.), 5 μm (Machery, Nagel & Co., Düren, F.R.G.) The mobile phase consisted of 7% (v/v) methanol and 0.5% (v/v) triethylamine in 0.1 *M* phosphoric acid (pH 2.3). The compounds were detected at 216 nm with a Spectro Monitor III variable-wavelength detector (Laboratory Data Control).

Blood sample collection

Venous blood samples were collected in chilled EDTA Vacutainer tubes (Becton Dickinson, Grenoble, France) and were immediately centrifuged at 4°C. After centrifugation, the plasma proteins in 1.00 ml of plasma were directly precipitated with 0.2 ml of 2.3 *M* perchloric acid containing $7 \cdot 10^{-3}$ *M* dithiothreitol. The supernatant was stored at −70°C. An aliquot of untreated plasma was stored at −20°C.

Determination of NAC

The perchloric acid supernatant was thawed and mixed and 100 μl were injected into the column.

Determination of oxidized NAC coupled to small sulphides (ONACS)

To 250 μl of the perchloric acid supernatant, 125 μl of phosphate buffer (pH 12; $\mu = 2.0$), 70 μl of 1 *M* sodium hydroxide and 50 μl of freshly prepared 0.03 *M* dithiothreitol were added. The mixture was left at room temperature for 20 min, then acidified with 15 μl of perchloric acid and 100 μl were injected into the column.

Determination of total NAC (NAC, ONACS and ONACP)

To 500 μl of plasma were added 375 μl of 0.1 *M* sodium hydroxide and 100 μl of 0.03 *M* dithiothreitol. The mixture was left at room temperature for 20 min, then the proteins were precipitated with 250 μl of 1.7 *M* perchloric acid. After centrifugation, 100 μl were injected into the column.

RESULTS AND DISCUSSION

Chromatography

The use of LC for the determination of thiols has recently been reviewed[10]. The reversed-phase mode has been preferred to the ion-exchange mode because of its better chromatographic performance and the high degree of flexibility. In reversed-phase LC the content of the organic solvent, the kind and concentration of the ion-pairing reagent and the ionic strength of the eluent can be varied[11].

In this study, one ion-exchange column, Nucleosil 5 SB (5 μm), and two reversed-phase columns, Nucleosil C_{18} (5 μm) and Microspher C_{18} (3 μm), were tested initially. As expected, the last column exhibited the highest efficiency, with 47 000

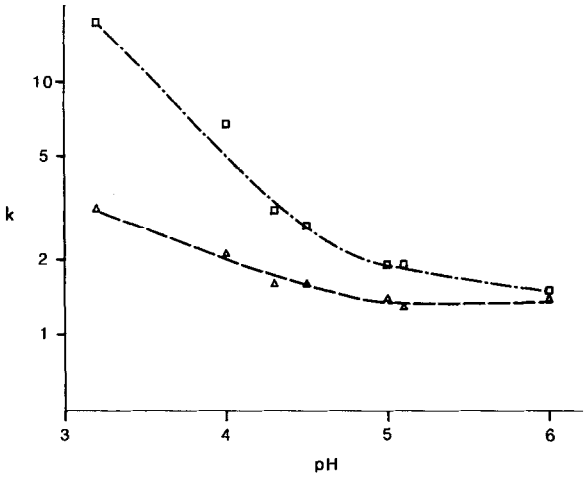

Fig. 2. Influence of pH on the retention of NAC (△) and N,N'-diacetylcystine (□). Column: Nucleosil C_{18}. 5 μm (250 × 4.6 mm I.D.). Mobile phase, phosphate buffer (μ = 0.01). Detection wavelength, 216 nm.

plates/m [reduced plate height (h) = 7.1], followed by the Nucleosil C_{18} column with 20 000 plates/m (h = 10.0) and then the ion-exchange column with 13 000 plates/m (h = 15.4). The mobile phases for the reversed-phase columns were acidic phosphate buffers with 7% methanol (Nucleosil) or 1% acetonitrile (Microspher) as organic solvent, and for the ion-exchange column phosphate buffer (pH 7; μ = 0.05). Addition of $1 \cdot 10^{-4}$ M EDTA to the mobile phase improved the efficiency of only the Microspher C_{18} column, while the Nucleosil C_{18} column was unaffected. The beneficial effect of EDTA may be due to complex formation with metal ions on the silica surface. The phosphate buffer used at higher ionic strength with the Nucleosil C_{18} column may function as a complexing agent[12–14], as the addition of EDTA did not further improve the peak symmetry. Improvement of peak shapes by addition of EDTA has been observed previously in the analysis of both thiols[15] and catecholamines[16].

Complications due to the adsorption of thiols and displacement by other thiols have been reported to occur on Hypersil ODS and Spherisorb ODS columns[15]. However, this effect was not observed with NAC on the Microspher C_{18} column. Addition of dithiothreitol to the mobile phase has been shown to improve the peak shape and reproducibility in the analysis of mercaptopurine on a LiChrosorb 10 RP-18 column[17].

The retention of the compounds was dependent on pH (Fig. 2). A low pH was used in the mobile phase because of the good selectivity and reduced rate of disulphide formation[18,19]. The presence of methanol prolonged the lifetime of the Nucleosil C_{18} column. To achieve both good selectivity and high sensitivity, Microspher C_{18} was chosen as the solid support for the bioanalytical method.

Derivatization

Derivatization of thiols has recently been reviewed[20]. They have been deri-

vatized with different substituted benzofurazans[21–24], dansylaziridine[25], bimanes[26,27], *o*-phthalaldehyde[28,29] and N-substituted maleimides[30–40]. Both pre-column[21,22,24–27,29–40] and post-column derivatization[23,28] have been used. However, when the preparation of the plasma sample includes reduction of the disulphide linkages with dithiothreitol, post-column derivatization is preferable. With pre-column derivatization a high excess of reagent is necessary, unless NAC is carefully isolated before derivatization.

Bimane and dansylaziridine are unsuitable reagents for post-column derivatization, as the reagents themselves are fluorescent[40]. Maleimides have previously been used only for pre-column derivatization, but as the derivative is reported to be unstable[41], they would seem more suitable for post-column derivatization.

The reaction between the thiol and the maleimide consists in a nucleophilic addition of the thiol to the activated carbon–carbon double bond. The derivative formed is sensitive to hydrolysis at the NC=O positions in the maleimide ring (Fig. 3). The rate of hydrolysis is faster with an electron-attracting substituent at the N-position, while an electron-donating substituent stabilizes the derivative[41].

In this study, both pre- and post-column techniques were used for investigating the derivatization of NAC with pyrenemaleimide. In the batchwise experiments, the reaction products were determined by LC and fluorimetric detection with direct injection of the reaction solution, whereas the optimal conditions for post-column derivatization were investigated by flow-injection analysis. A remarkably large difference in the reaction rates was found between the batch and the flow-injection analysis experiments.

The pre-column derivatization of NAC with pyrenemaleimide was investigated

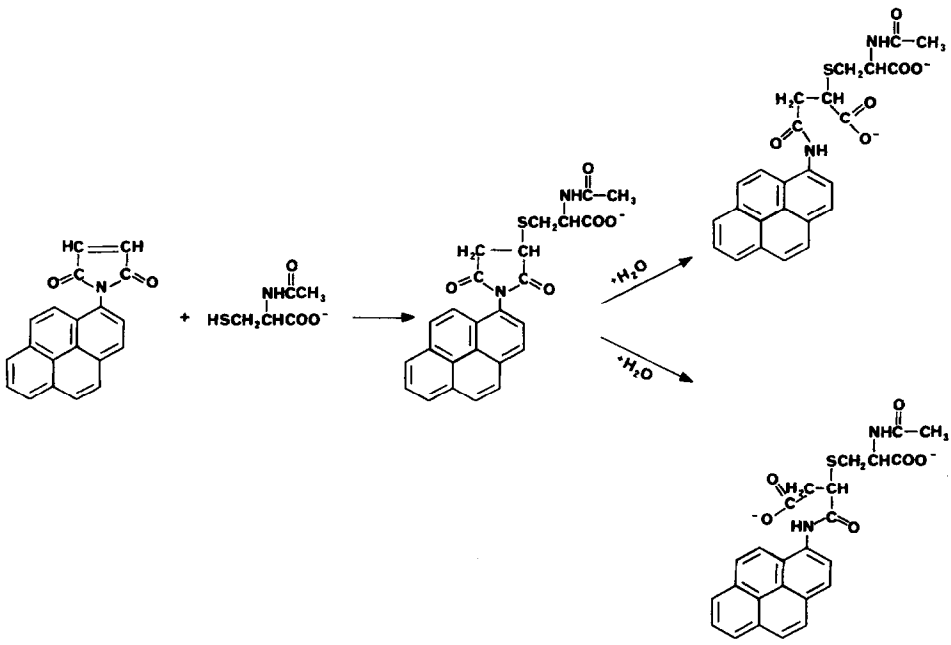

Fig. 3. Reaction scheme for the derivatization of NAC with pyrenemaleimide and for the hydrolysis of the derivative.

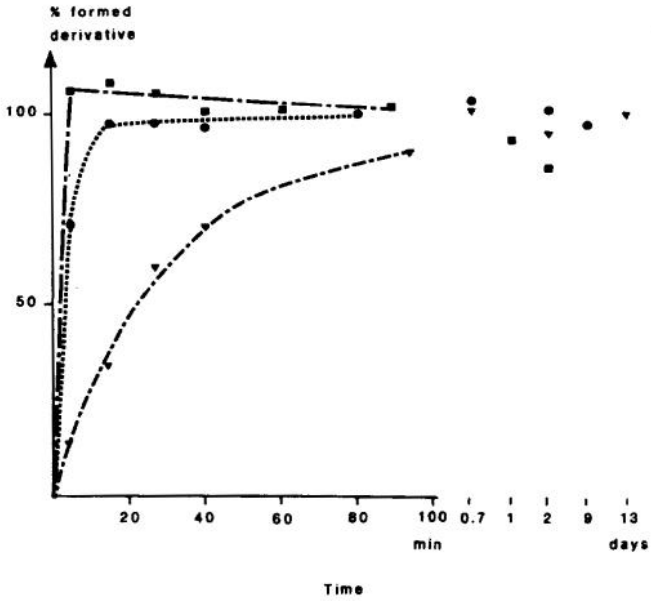

Fig. 4. Influence of pH on the formation and stability of the pyrenemaleimide derivative NAC. NAC concentration, $0.9 \cdot 10^{-6}$ *M*; pyrenemaleimide concentration, $31.8 \cdot 10^{-6}$ *M*. ▼, pH 9; ●, pH 10; ■, pH 11.

at pH 9, 10 and 11. The reaction rate increases with increasing pH (Fig. 4). The reaction is apparently completed after *ca.* 10–15 min at pH 10. The stability of the derivative was surprisingly good; at pH 10 the derivative was stable for at least nine days. The reaction rate was independent of the excess of pyrenemaleimide. Variation in the pyrenemaleimide concentration from 1.1 to 27.5 times higher than that of NAC gave the same reaction rate at pH 10. This indicates that the second-order rate constant is so high that an almost 30-fold difference in the reagent concentration has no influence on the reaction rate.

The optimal reaction time for the post-column derivatization was investigated by using knitted capillaries of I.D. 0.5 mm but with different lengths in a flow-injection analysis system (Fig. 5). The peak area reached a plateau after about 5.5 s, when the pH was 10.2. The reaction time was considerably shorter than that in the batch experiments. It is improbable that the conditions in the flow system would accelerate the reaction 100-fold compared with batchwise reactions. The most likely interpretation of this enormous difference in reaction rates is that the measurements in the batch experiments were performed on the product obtained after hydrolysis of the first-formed derivative (*cf.*, Fig. 3). The pH dependence of the reaction rate as illustrated in Fig. 4 may then be interpreted as an increase in hydrolysis with increasing pH. This seems reasonable, but the rate of the initial reaction (the nucleophilic attack of the thiol on the activated carbon) should also increase with increasing pH. The thiolate ion would be expected to be more reactive than the uncharged thiol, and the pK_a value of the thiol group of NAC is 9.65[18].

The band-broadening contributions of the different parts of the flow-injection system were determined by injection of the derivative formed in the batch experi-

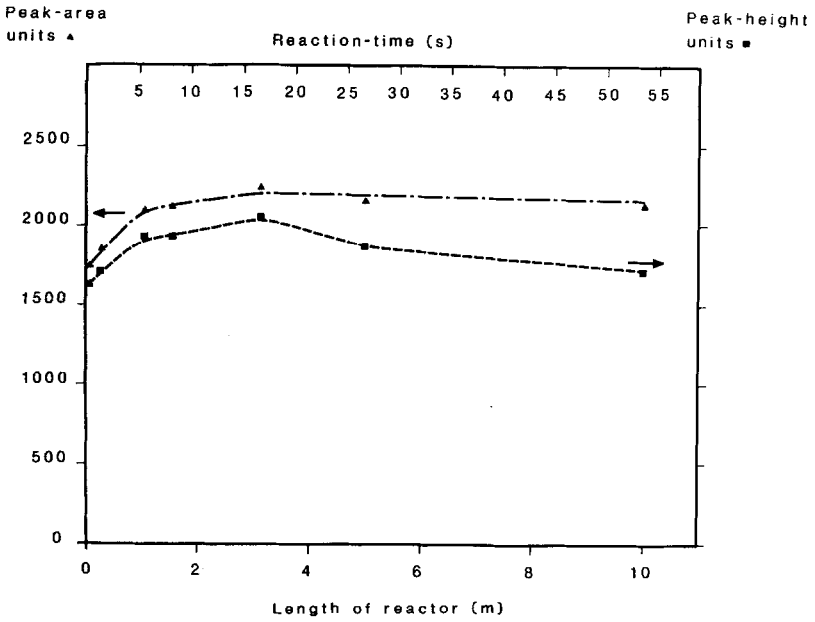

Fig. 5. Influence of the length of the knitted PTFE capillary (reaction time) on the peak area (▲) and the peak height (■) of the fluorescence signal in a flow-injection system. Conditions: mobile phase, 1% (v/v) acetonitrile in phosphate buffer (pH 2; μ = 0.01), flow-rate 1.0 ml/min; buffer, 0.05 *M* borate (pH 11.0), flow-rate, 1.0 ml/min; reagent, $5 \cdot 10^{-5}$ *M* pyrenemaleimide, flow-rate 0.2 ml/min; injection volume, 150 μl of $6 \cdot 10^{-6}$ *M* NAC; detection, fluorescence (342/389 nm).

ments. The magnitude of the dispersion in the reactor may depend on the properties of the analyte, *i.e.*, the diffusion coefficient and interactions with the different interfaces in the reactor.

The reaction product from the batch experiments probably corresponds to the hydrolysed compound (see Fig. 3). It bears a closer relationship to the reaction product in question than any other readily available compound, and it was therefore used to study the extra-column dispersion effects. The contribution by the column was determined by injection of NAC into the whole system (Table I). The contribution by the post-column derivatization system to band broadening was less than 12%. The PTFE capillary was knitted to reduce the dispersion[9]. The use of T-connectors

TABLE I

SOURCES OF BAND BROADENING

Parameter	*Mixing device*	*PTFE capillary**	*Column*	*Residuals***	*Total*
σ^2 (μl^2)	480	840	9640	72	11 032
σ^2 (%)	4.3	7.6	87.4	0.7	
σ (μl)	22	29	98	8.5	105

* The knitted PTFE capillary measured 4.2 m × 0.3 mm I.D.

** Residuals consisted of the injector, detector and recorder.

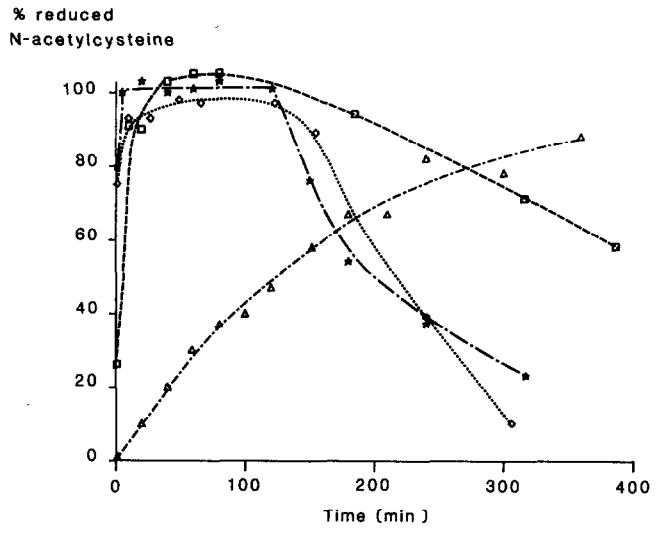

Fig. 6. Influence of pH on the reduction of N,N'-diacetylcystine in buffer solutions. N,N-diacetylcystine concentration, $0.9 \cdot 10^{-4}$ *M*; dithiothreitol concentration, $1.2 \cdot 10^{-3}$ *M*. △, pH 7.0; □, pH 9.2; ◇, pH 9.9; ★, pH 11.9.

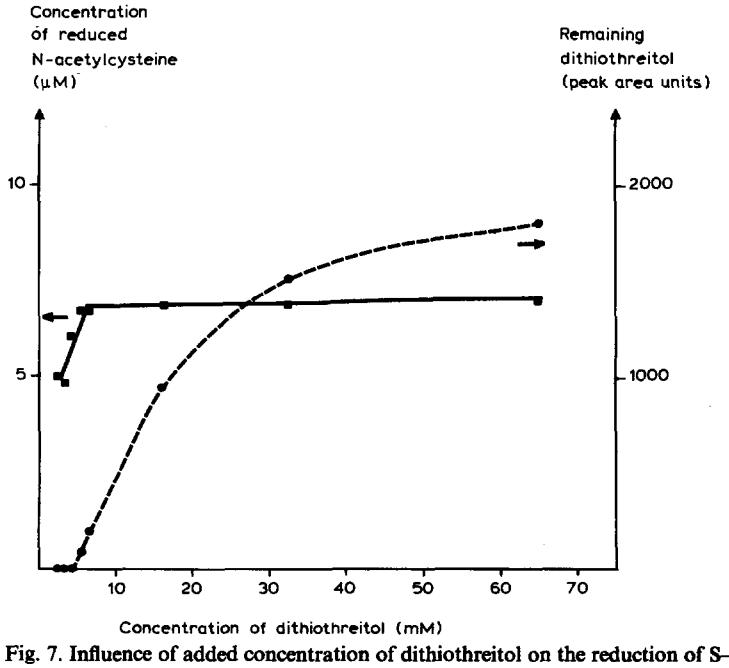

Fig. 7. Influence of added concentration of dithiothreitol on the reduction of S–S-linked NAC in plasma. ■, Reduced NAC concentration; ●, remaining dithiothreitol.

with a low dead volume decreased the peak width by a factor of 2 compared with ordinary T-connectors. A further decrease of the peak width by a factor of about 2 was achieved by decreasing the internal diameter of the PTFE capillary from 0.5 to 0.3 mm.

Reduction of the disulphides

Dithiothreitol[3,4,42–44], tributylphosphine[45–48], potassium cyanide[49] and thiopropyl-Sepharose[5,6,39] have been used to reduce disulphides. A high water solubility and convenient sample preparation were the reasons for using dithiothreitol in this method.

The reduction of N,N′-diacetylcystine with dithiothreitol was investigated in buffer solutions of pH 7, 9, 10 and 12 (Fig. 6). Because the rate of reduction of N,N′-diacetylcystine was faster at higher pH, a pH of 12 was used in this method. A concentration of dithiothreitol of more than 5.5 m*M* was necessary in order to reach a plateau of the reduced N-acetylcysteine concentration in plasma (Fig. 7). An incubation time of 20 min was used in this method. The remaining dithiothreitol in plasma was detected in the chromatographic system (Fig. 8), which was used to ensure that the added amount of dithiothreitol was adequate. The required amount of dithiotreitol varied between different plasma samples, owing to individual variations in endogenous thiols and in the metal and oxygen contents. A chromatogram obtained from the determination of the total concentration of NAC is shown in Fig. 8.

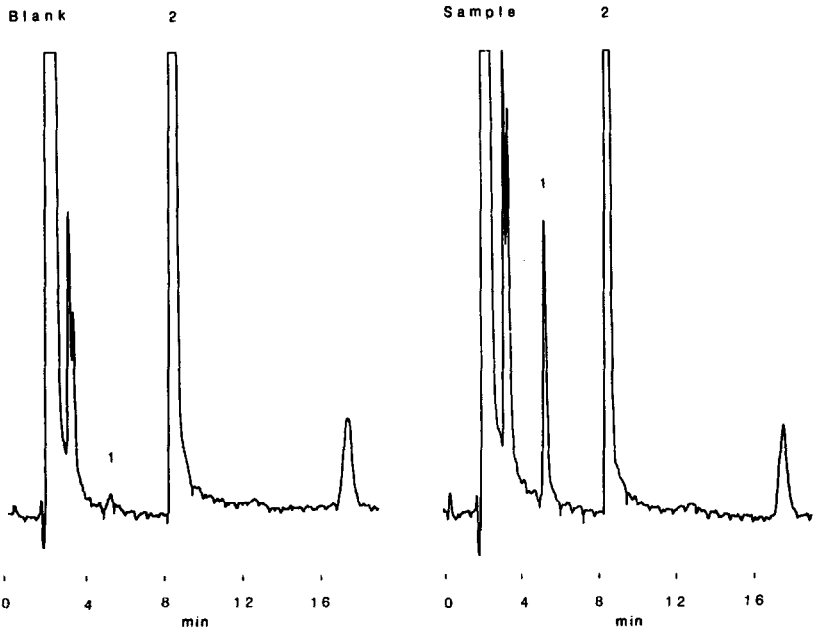

Fig. 8. Plasma blank and sample chromatograms from the determination of the total concentration of NAC. Analyses were performed as described in the text. The plasma sample contained 6.5 μM NAC, ONACS and ONACP. 1 = NAC; 2 = dithiothreitol.

Sample handling and sample stability

As the thiols are easily oxidized, the sample handling procedure must be carefully controlled. Fifty percent of NAC was oxidized within 90 min and 160 min, respectively, in two human whole-blood samples, stored in ice–water. Because a low pH reduces the rate of oxidation, centrifugation of the whole blood at 4°C was immediately followed by an acidic protein precipitation, centrifugation and freezing of the supernatant. Less than 5% and 8%, respectively, of NAC was oxidized in the two human whole blood specimens, when centrifugation and protein precipitation were performed within 10 min. Protein precipitation with perchloric acid resulted in a 60% higher chromatographic efficiency than protein precipitation with trichloroacetic acid.

Addition of dithiothreitol ($7 \cdot 10^{-3}$ *M*) to perchloric acid increased the absolute recovery of NAC from 80% to 100% in both water and plasma samples, indicating that dithiothreitol has an antioxidizing effect. No reduction of N,N′-diacetylcystine by dithiothreitol was observed at this low pH. NAC in the supernatant from the protein precipitation was stable at −70°C for at least two months, whereas at −20°C 13% degradation occurred within 6 days. The plasma samples for the determination of the total concentration of NAC were stable at −20°C for at least three months. In the automatic injector chilled to 4°C the prepared samples were stable for at least 9 h.

Quantitative determinations

The detection limit, defined as a signal-to-noise ratio of 3, was 10 pmol. It was not possible to increase the sensitivity by injecting volumes larger than 100 µl, as the peak width increased with the volume injected because of a low enrichment effect

TABLE II

INTRA-ASSAY PRECISION AND ABSOLUTE RECOVERIES OF THE DIFFERENT FORMS OF NAC

Compound	*Concentration (µM)*		*Absolute recovery (%)**	*R.S.D.** (%)*	*n*
	Added	*Found*			
NAC	0.24	0.26	107	14.4***	8
	2.85	2.94	103	3.4	8
	5.64	5.49	97	3.4	8
ONACS	0	0.33	–	17.5***	8
	0.60	1.06	105	6.1	8
	4.28	4.48	95	6.6	8
	8.83	8.99	97	4.0	7
Total NAC	0	0.31	–	14.9	8
	1.36	1.79	100	4.8	8
	4.03	4.42	99	5.8	8
	7.95	8.44	101	1.7	8

* Absolute recovery $= \dfrac{\text{determined concentration} - \text{endogenous concentration}}{\text{added concentration}}$.

** Relative standard deviation.

*** Peak-height measurements.

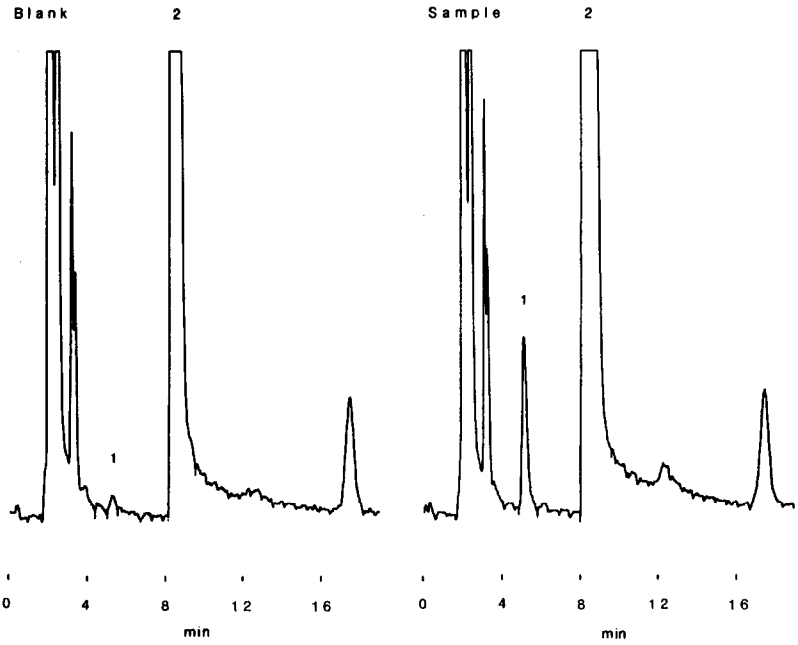

Fig. 9. Plasma blank and sample chromatograms from determination of the concentration of oxidized NAC coupled to small disulphides. The analyses were performed as described in the text. The plasma sample contained 4.9 μM NAC and ONACS. 1 = NAC; 2 = dithiothreitol.

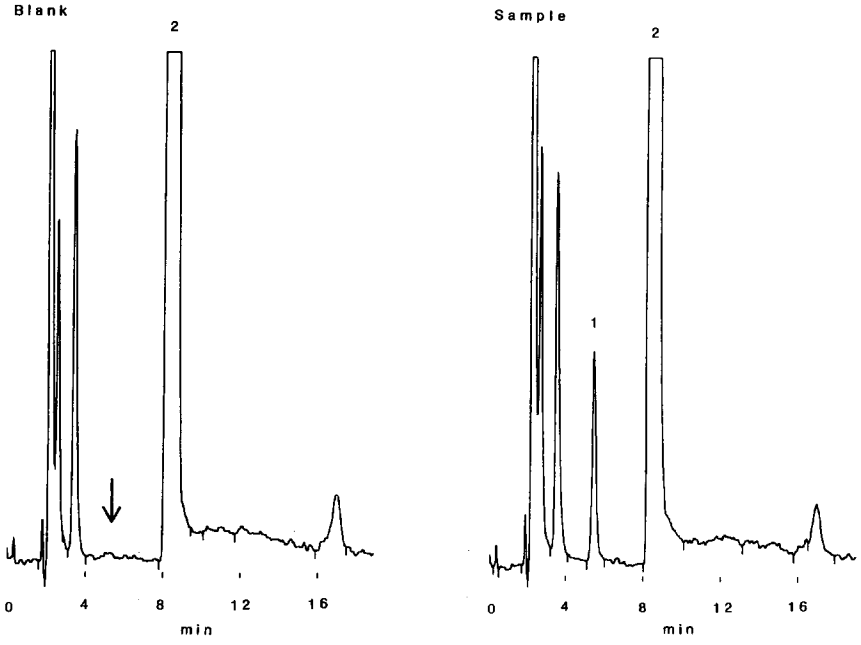

Fig. 10. Plasma blank and sample chromatograms from the determination of the concentration of NAC. The analyses were performed as described in the text. The plasma sample contained 2.3 μM NAC. 1 = NAC; 2 = dithiothreitol.

with the sample dissolved in perchloric acid. The limit of determination of NAC was 240 n*M*.

The precision and absolute recovery of NAC and of the total concentration of NAC were determined by addition of NAC to blank plasma, and the precision and recovery of the ONACS concentration by addition of N,N'-diacetylcystine (Table II). The absolute recoveries were compared by direct injection of NAC dissolved in 0.34 *M* perchloric acid containing $6 \cdot 10^{-4}$ *M* dithiothreitol. The inter-assay precision of the total concentration of NAC was 6.8% (relative standard deviation) at a level of 5.6 μM ($n = 14$). An endogenous concentration of about 0.30 μM of ONACS was found. This concentration is in the same range as previously reported for ONACS[5]. Representative blank and sample chromatograms from the determination of the concentration of the oxidized form (ONACS) and of the concentration of NAC are shown in Figs. 9 and 10, respectively. The endogenous thiols cysteine and glutathione are eluted with the front.

ACKNOWLEDGEMENTS

We are grateful to Solveig Axelsson for skilful technical assistance and to Björn Lindeke (ACO Läkemedel AB) for comments on the manuscript.

REFERENCES

1 L. Bonanomi and A. Gazzaniga, *Therapiewoche*, 30 (1980) 1926.
2 G. B. Corcoran, W. J. Racz and J. R. Mitchell, *Pharmacologist*, 20 (1978) 259.
3 P. A. Lewis, A. J. Woodward and J. Maddock, *Pharm. Sci.*, 73 (1984) 996.
4 P. A. Lewis, A. J. Woodward and J. Maddock, *J. Chromatogr.*, 327 (1985) 261.
5 B. Kågedal, M. Källberg and J. Mårtensson, *J. Chromatogr.*, 311 (1984) 170.
6 L. R. Morgan, M. R. Holdiness and L. E. Gillen, *Semin. Oncol.*, 10 (1983) 56.
7 H. Frank, D. Thiel and K. Langer, *J. Chromatogr.*, 309 (1984) 261.
8 O. H. Drummer, N. Christophidis, J. D. Horowitz and W. J. Louis, *J. Chromatogr.*, 374 (1986) 251.
9 H. Engelhardt and U. D. Neue, *Chromatographia*, 15 (1982) 403.
10 D. Perett and S. R. Rudge, *J. Pharm. Biomed. Anal.*, 3 (1985) 3.
11 L. A. Allison, J. Keddington and R. E. Shoup, *J. Liq. Chromatogr.*, 6 (1983) 1785.
12 M. Verzele and C. Dewaele, *J. Chromatogr.*, 217 (1981) 399.
13 M. Verzele, *LC Mag.*, 1 (1983) 217.
14 M. Verzele and C. Dewaele, *Chromatographia*, 18 (1984) 84.
15 D. Perett and S. R. Rudge, *J. Chromatogr.*, 294 (1984) 380.
16 P.-O. Edlund and D. Westerlund, *J. Pharm. Biomed. Anal.*, 2 (1984) 315.
17 R. Jonkers, B. Oosterhuis, R. ten Berge and C. van Boxtel, *J. Chromatogr.*, 233 (1982) 249.
18 L. Bonanomi and A. Gazzaniga, *Eur. J. Respir. Dis.*, 61 (1980) 45.
19 C. A. Mairesse-Ducarmois, G. J. Patriarche and J. L. Vandenbalk, *J. Pharm. Belg.*, 31 (1976) 169.
20 K. Imai, T. Toyo'oka and H. Miyano, *Analyst (London)*, 109 (1984) 1365.
21 T. Toyo'oka and K. Imai, *Anal. Chem.*, 56 (1984) 2461.
22 T. Toyo'oka and K. Imai, *J. Chromatogr.*, 282 (1983) 495.
23 Y. Watanabe and K. Imai, *Anal. Chem.*, 55 (1983) 1786.
24 J. Andrews, P. Ghosh, B. Ternai and M. Whitehouse, *Biochim. Biophys. Arch*, 214 (1982) 386.
25 E. Lankmayr, K. Budna, K. Müller and F. Nachtmann, *Fresenius Z. Anal. Chem.*, 295 (1979) 371.
26 R. Fahey, G. Newton, R. Dorian and E. Kosower, *Anal. Biochem.*, 111 (1981) 357.
27 G. Newton, R. Dorian and R. Fahey, *Anal. Biochem.*, 114 (1981) 383.
28 H. Nakamura and Z. Tamura, *Anal. Chem.*, 53 (1981) 2190.
29 K. Mopper and D. Delmas, *Anal. Chem.*, 56 (1984) 2557.
30 C.-W. Wu, L. Yarbrough and F. Wu, *Biochemistry*, 15 (1976) 2863.

31 K. Shimada, M. Tanaka and T. Nambara, *J. Chromatogr.*, 227 (1982) 445.
32 K. Shimada, M. Tanaka and T. Nambara, *Anal. Chim. Acta*, 147 (1983) 375.
33 H. Takahashi, Y. Nara, H. Meguro and K. Tuzimura, *Agric. Biol. Chem.*, 43 (1979) 1439.
34 K. Yamamoto, T. Sekine and Y. Kanaoka, *Anal. Biochem.*, 79 (1977) 83.
35 M. Machida, T. Takahashi, K. Itoh, T. Sekine and Y. Kanaoka, *Chem. Pharm. Bull.*, 26 (1978) 596.
36 T. Sekine, K. Kato, K. Takamore, M. Machida and Y. Kanaoka, *Biochim. Biophys. Acta*, 354 (1974) 139.
37 B. Jarrott, A. Anderson, R. Hooper and W. Louis, *J. Pharm. Sci.*, 70 (1981) 665.
38 J. Miners, I. Fearnley, K. Smith, D. Birkett, P. Brooks and M. Whitehouse, *J. Chromatogr.*, 275 (1983) 89.
39 B. Kågedal and M. Källberg, *J. Chromatogr.*, 229 (1982) 409.
40 N. Kosower, G. Newton, E. Kosower and H. Ranney, *Biochim. Biophys. Acta*, 622 (1980) 201.
41 S. Matsui and H. Aida, *J. Chem. Soc.*, (1978) 1277.
42 W. Cleland, *Biochemistry*, 3 (1964) 480.
43 J. Eyem, J. Sjödahl and J. Sjöquist, *Anal. Biochem.*, 74 (1976) 359.
44 M. Abounassif and T. Jefferies, *J. Pharm. Biomed. Anal.*, 1 (1983) 65.
45 R. Humphrey and J. Potter, *Anal. Chem.*, 37 (1965) 164.
46 A. Kirkpatrick and J. A. McClaren, *Anal. Biochem.*, 56 (1973) 137.
47 U. Rüegg and J. Rudinger, *Methods Enzymol.*, 47 (1977) 111.
48 J. A. McClaren and B. Sweetman, *Aust. J. Chem.*, 19 (1966) 2355.
49 H. Takahashi, Y. Nara, T. Yoshida, K. Tuzimura and H. Meguro, *Agric. Biol. Chem.*, 45 (1981) 79.