Journal of Chromatography, 311 (1984) 170—175
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2237

Note

Determination of non-protein-bound N-acetylcysteine in plasma by high-performance liquid chromatography

BERTIL KÅGEDAL*, MAGNUS KÄLLBERG and JOHANNES MÅRTENSSON

Department of Clinical Chemistry, University Hospital, S-581 85 Linköping (Sweden)

(First received March 30th, 1984; revised manuscript received June 7th, 1984)

N-Acetylcysteine has been identified as a normal constituent in urine [1] and its excretion has been determined by both gas—liquid chromatography (GLC) [2] and high-performance liquid chromatography (HPLC) [3]. The compound has not been detected in plasma except after drug intake. As a mucolytic agent it has been used for inhalation treatment for a long time [4], and it has also been documented as an effective oral drug in chronic bronchitis [5,6]. The compound is a precursor for the in vivo synthesis of glutathione, and it therefore exerts a beneficial effect in paracetamol (acetaminophen) poisoning [7].

If only disulphide interchange is considered, N-acetylcysteine may be found as a free thiol, as a low-molecular-weight disulphide, and as a disulphide with SH-containing proteins. The present paper describes a HPLC method for the determination of non-protein-bound N-acetylcysteine in plasma suitable for pharmacokinetic studies. After precipitation of plasma proteins, low-molecular-weight disulphides in the supernatant are reduced, and liberated N-acetylcysteine is purified using an organomercurial adsorbent and a cation exchanger. Derivatization is then done with N-(7-dimethylamino-4-methyl-3-coumarinyl)-maleimide (DACM), and quantitation is accomplished by fluorometric detection after separation of the N-acetylcysteine derivative from other acid thiol derivatives by reversed-phase ion-pair HPLC.

EXPERIMENTAL

Materials

N-Acetylcysteine was obtained from Sigma (St. Louis, MO, U.S.A.) and N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM) was from Fluka (Buchs, Switzerland). Stock solutions of these compounds were prepared

0378-4347/84/$03.00

as described previously [3]. Thiopropyl-Sepharose 6B (TPS) was from Pharmacia (Uppsala, Sweden). It was used in its free thiol form and contained 20 μmol of thiol per ml of gel suspension. The organomercurial adsorbent *p*-acetoxymercurianiline-Sepharose 4B (PAMAS) was prepared according to the description of Sluyterman and Wijdenes [8], and the strong cation exchanger AG 50W-X8, 100—200 mesh, was from Bio-Rad Labs. (Richmond, CA, U.S.A.).

High-performance liquid chromatography

HPLC of the fluorescent DACM derivatives of N-acetylcysteine was performed with tetramethylammonium as ion-pairing reagent as previously described [3]. We employed a Supelcosil LC-8 (250 × 4.6 mm, particle size 5 μm) column from Supelco (Bellefonte, PA, U.S.A.), and as detector we used a Fluoromonitor III filter fluorometer from LDC (Riviera Beach, FL, U.S.A.). The detector was operated with a mercury lamp, a 360-nm excitation filter and a 418—700 nm emission filter. An RP-8 (particle size 10 μm) column, 30 × 4.6 mm (RP-GU) from Brownlee Labs. (Santa Clara, CA, U.S.A.) was connected to the injector instead of the sample loop. Once the N-acetylcysteine derivative had passed this loop column, the injector was switched to the load position again. Late peaks were thereby trapped in the loop column and could be removed by backward flushing with the same mobile phase as in the analytical column. Backward flushing was accomplished with a mini HPLC pump (Eldex E-120-S-2, Eldex Labs., Menlo Park, CA, U.S.A.).

Procedure

Venous blood was taken in vacuum tubes containing K_3EDTA (Vacutainer), and plasma was obtained by centrifugation. A 1.0-ml aliquot of each plasma sample was precipitated with 0.2 ml of trichloroacetic acid, 200 g/l. After vortex mixing and standing for 15 min the samples were centrifuged, and 0.6-ml aliquots of the clear supernatants were transferred to new tubes and mixed with 0.4 ml of 20 mmol/l Na_2EDTA and 1.0 ml of sodium borate buffer, pH 10.0 (prepared from boric acid, 0.24 mol/l final volume). Then 1.0 ml of TPS suspension was added and the tubes were placed in an end-over-end mixer for 30 min. Then 1.0 ml of 4.0 mol/l acetic acid was added to the tubes. After centrifugation, 3.0 ml of the supernatant were transferred to a 1.3 × 0.7 cm column of PAMAS which retains the thiols. Washing was done with 2 × 1 ml of water and elution was effected by 3.0 ml of 10 mmol/l cysteine hydrochloride. The eluate was transferred to a 2.5 × 0.5 cm AG 50W-X8 (H^+) column, which retains the cysteine and other positively charged thiols. The effluent was collected in a tube containing 0.2 ml of 0.18 mol/l Na_2EDTA together with a further 1-ml washing with 10 mmol/l hydrochloric acid. From the effluent, 2.0 ml were transferred to a new tube, 0.2 ml of 0.1 mol/l sodium hydroxide was added for neutralization, and then 3 ml of 50 mmol/l carbonate buffer containing 10 mmol/l Na_2EDTA pH 9.0 were added. Finally, 0.5 ml of 20 μmol/l DACM in acetone, was added. After mixing, the tubes were placed in a water bath 37°C, usually overnight. Standard was derivatized by mixing 50 μl of N-acetylcysteine working solution (10 μmol/l) with 5.0 ml of the carbonate buffer used in the derivatization of the plasma samples. Then 0.5 ml of 20 μmol/l DACM was added, and after mixing the tubes were placed in the water bath.

Plasma concentrations after oral intake

Seven healthy subjects aged 21 to 40 years received 400 mg of N-acetylcysteine (Fabrol®, Ciba-Geigy, V. Frölunda, Sweden). The drug was dissolved in about 100 ml of water and was given orally to the subjects after overnight fasting. Venous blood samples were taken from a catheter placed in a cubital vein, at the times shown.

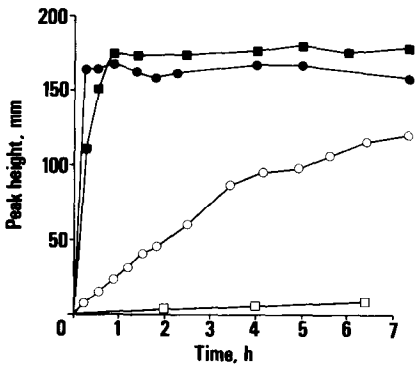
RESULTS

Derivatization

In the previous studies on urinary thiols [3] the derivatization was carried out by incubation at 37°C for 20 h, but preliminary experiments indicated that it should be possible to shorten this time. Fig. 1 shows the effect of pH on the development of the N-acetylcysteine derivative with DACM. It can be seen that the fluorescence developed most rapidly at high pH (9.7) and was completed within less than half an hour. At lower pH ($\leqslant 7.6$) the fluorescent product developed more slowly. Maximal fluorescence was obtained at pH 9.0, and this pH was therefore chosen in the final method.

Reduction of low-molecular-weight disulphides

Preliminary experiments showed that protein-free extracts of plasma taken at about 1 h after N-acetylcysteine ingestion contained N-acetylcysteine mainly as disulphide. The N-acetylcysteine could be liberated by reduction with thiopropyl-Sepharose 6B in alkaline medium. We found that transferring the extracts to a borate buffer pH 10 was more suitable than adjusting the pH with strong ammonia [2,3]. We also found higher N-acetylcysteine values when reduction with the thiopropyl-Sepharose 6B was performed in this buffer. Fig. 2 shows that with the chosen amount of thiopropyl-Sepharose 6B, the reduction was completed in 20—30 min. A reduction time of 30 min was therefore chosen in the final method.



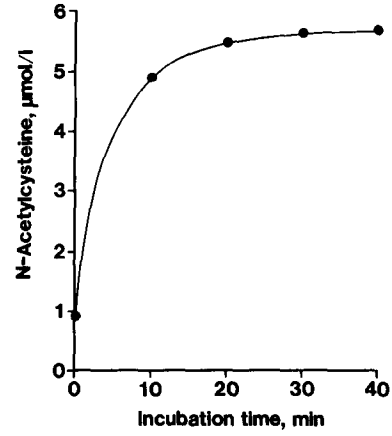


Fig. 1. Effect of pH on the development of the fluorescent DACM derivative of N-acetylcysteine. Temperature 37°C. □, pH 6.0; ○, pH 7.6; ■, pH 9.0; ●, pH 9.7.

Fig. 2. Liberation of free N-acetylcysteine from supernatant of plasma by reduction with thiopropyl-Sepharose 6B.

High-performance liquid chromatography

Ion-pair HPLC [3] was adopted for DACM derivatives of plasma thiols. In addition to the peaks from the N-acetylcysteine derivatives there were also other peaks, some of them with long retention times. Fig. 3 shows that, by use of the column switching technique described, late peaks could be eliminated by trapping in the loop column. This substantially increased the possible number of injections that could be done in a working day.

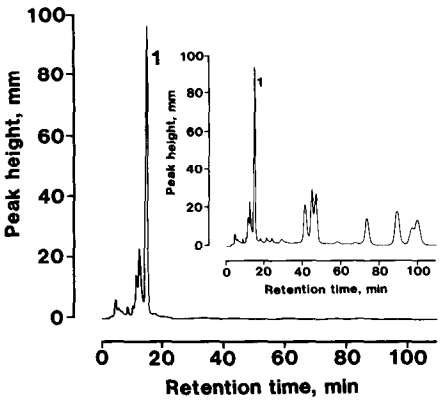


Fig. 3. Chromatogram of acid plasma thiols by HPLC, before and after introduction of the column-switching technique. The N-acetylcysteine peak is designated 1.

Precision

Plasma samples obtained at different times after intake of N-acetylcysteine were used for precision studies. From duplicate determinations the imprecision (coefficient of variation) was calculated as 5.7—6.5% for different ranges of measurement (Table I). From the standard deviation (S.D.) obtained for the lower range the detection limit (2 S.D.) was calculated as 0.14 μmol/l, which was quite satisfactory.

TABLE I

PRECISION OF THE METHOD CALCULATED FROM DUPLICATE DETERMINATIONS

No. of duplicates	Range (μmol/l)	Mean (μmol/l)	S.D. (μmol/l)	C.V. (%)
28	0.2—2.0	1.09	0.07	6.5
42	2.0—10	4.49	0.25	5.7

Recovery

We added N-acetylcysteine to plasma samples to increase the concentration by 1.0 μmol/l (Table II). The increase found by analysis before and after addition showed a quantitative recovery.

Application to pharmacokinetic studies

Fig. 4 shows the plasma concentrations obtained in seven subjects after ingestion of 400 mg of N-acetylcysteine. As can be seen, the maximum value, ranging from 2.6 to 18.4 μmol/l (mean 7.4 μmol/l), was obtained at 25—45

TABLE II

ANALYTICAL RECOVERY OF N-ACETYLCYSTEINE ADDED TO PLASMA SAMPLES, CORRESPONDING TO AN INCREASE IN CONCENTRATION OF 1.0 μmol/l

Initially present (μmol/l)	After addition (μmol/l)	Increase found (μmol/l)	Recovery (%)
0.22	1.34	1.12	112
0.25	1.19	0.94	94
0.28	1.27	0.99	99
0.30	1.27	0.97	97
		Mean ± S.D.	101 ± 8

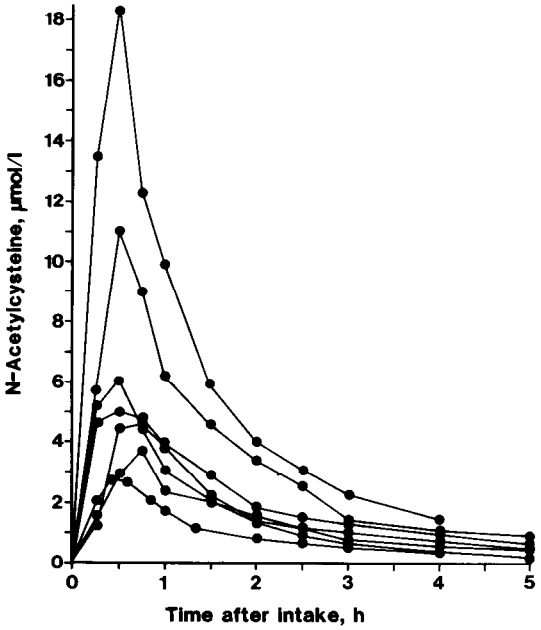


Fig. 4. Non-protein-bound plasma N-acetylcysteine concentration after oral intake of 400 mg of N-acetylcysteine (Fabrol).

min after intake. The half-life of elimination ($t_{1/2}$) for N-acetylcysteine was calculated from the 1.5—5-hour segment of the log plasma concentration versus time curve, and was found to vary between 1.1 and 2.3 h, with a mean of 1.7 h.

DISCUSSION

In an earlier paper we described an HPLC system which was capable of resolving N-acetylcysteine and mercaptoacetate as their derivatives with N-(1-pyrene)maleimide (PM) and DACM. The applicability of the method was shown for the determination of urinary thiols using PM. However, the sensitivity for the thiols was about ten-fold higher with DACM than with PM. With DACM we have now obtained a highly sensitive method for the determination of N-acetylcysteine in plasma, both for study of the high concentrations after intake and for study of its lower concentration during elimination.

Pharmacokinetic data for N-acetylcysteine after peroral intake are scanty. Rodenstein et al. [9] gave 100 mg of ^{35}S-labelled N-acetylcysteine to five patients with heart and pulmonary diseases. Calculated from the radioactivity determinations given in the paper, a maximal plasma concentration of 21—39 μmol/l was obtained after 2—3 h. A substantial amount of radioactivity still remained in the plasma after 24 h. The free N-acetylcysteine concentration in plasma after oral intake of 400 mg of N-acetylcysteine was estimated by Maddock [10], who found maximal plasma concentrations of 0—3.6 μmol/l. No data were given regarding the method used, and therefore the results are difficult to evaluate. Recently, Morgan et al. [11] published a GLC method for plasma using the principles developed by Hannestad and Sörbo [2]. No protein precipitation was performed before reduction, and therefore their method should estimate the total concentration in plasma. They gave 250 mg of N-acetylcysteine in gelatin capsules per m^2 body surface to five subjects with bronchogenic carcinomas. Assuming a standard body surface of 1.73 m^2 for their patients this corresponds to a mean dose of 433 mg which was close to what we gave. They obtained a mean maximal N-acetylcysteine concentration of 10.7 μmol/l, i.e. a mean value only slightly higher than our mean value for non-protein-bound N-acetylcysteine. Their maximum values were obtained at a mean of 0.72 h after intake, and the mean $t_{½}$ was 2.1 h, again values similar to ours. This in comparison with our data may indicate that the protein binding of N-acetylcysteine by disulphide bridges is low. However, direct measurements must be done in order to estimate the protein binding of N-acetylcysteine in plasma.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swedish Medical Research Council, Project No. B84-13X-05644-05A, and Ciba-Geigy Läkemedel AB, Västra Frölunda, Sweden.

REFERENCES

1 H. Kodama, T. Ikegami and T. Araki, Physiol. Chem. Phys., 6 (1974) 87—89.
2 U. Hannestad and B. Sörbo, Clin. Chim. Acta, 95 (1979) 189—200.
3 B. Kågedal and M. Källberg, J. Chromatogr., 229 (1982) 409—415.
4 S. Chodosh, W. Baigelman, T.C. Medici and K. Einstein, Curr. Ther. Res., 17 (1975) 319—334.
5 Multicenter Study Group, Eur. J. Resp. Dis., 61 (Suppl. 111) (1980) 91—108.
6 G. Boman, U. Bäcker, S. Larsson, B. Melander and L. Wåhlinder, Eur. J. Resp. Dis., 64 (1983) 405—415.
7 B.H. Rumac and R.G. Peterson, Pediatrics, 62 (Suppl.) (1978) 898—903.
8 L.A. Æ. Sluyterman and J. Wijdenes, Biochim. Biophys. Acta, 200 (1970) 593—595.
9 D. Rodenstein, A. De Coster and A. Gazzaniga, Clin. Pharmacokin., 3 (1978) 247—254.
10 J. Maddock, Eur. J. Resp. Dis., 61 (Suppl. 111) (1980) 52—58.
11 W. Morgan, M.R. Holdiness and L.E. Gillen, Semin. Oncol., 10 (Suppl. 1) (1983) 56—61.