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REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF MERCAPTOACETATE AND N-ACETYLCYSTEINE AFTER DERIVATIZATION WITH N-(1-PYRENE)MALEIMIDE AND N-(7-DIMETHYLAMINO-4-METHYL-3-COUMARINYL)MALEIMIDE

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SUMMARY

We have developed a high-performance liquid chromatographic system capable of resolving mercaptoacetate and N-acetylcysteine as their N-(1-pyrene)maleimide (PM) and N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM) derivatives. Good resolution was obtained by ion pairing with tetramethylammonium hydroxide and chromatography on reversed phase. The detection limits for the thiols were about 50 fmol as their DACM derivatives and about 400 fmol as their PM derivatives. The method is illustrated by chromatography of urinary thiols which indicates that the derivatization and chromatography procedures should be well applicable in bioanalytical work.

INTRODUCTION

Earlier methods for thiol determination such as the nitroprusside method, and iodometric, amperometric, spectrophotometric and colorimetric methods require several milliliters of sample and have detection limits of around 10 μmol/l [1]. Recently, Hannestad and Sörbo [2] published a gas chromatographic method for the determination of 3-mercaptolactate, mercaptoacetate and N-acetylcysteine in urine. The thiols were determined with a sensitivity of about 3 μmol/l using 5 ml of urine. When these thiols are to be determined in serum, for example, a much higher sensitivity is required. An attempt to estimate N-acetylcysteine in serum after oral ingestion of 400 mg of substance was performed by Maddock [3]. He used a high-performance liquid chromatographic (HPLC) method but did not give any chromatographic data. The method had a sensitivity of 1 μmol/l, which, however, was not sufficient to

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quantitate N-acetylcysteine in all sera. Recently, high sensitivity for the determination of biologically interesting thiols has been obtained by HPLC of the fluorescent sulfhydryl derivatives with N-(9-acridinyl)maleimide [4], with monobromobimane [5] and in HPLC methods with electrochemical detection [6].

The maleimide derivatives (for review see ref. 7) are by themselves not fluorescent, but they react with thiol-containing substances giving highly fluorescent compounds. These reagents have mainly been used as fluorescent probes in the study of thiol-containing proteins and enzymes [7]. However, they should also be applicable for the labeling of low-molecular-weight sulfhydryl compounds in chromatographic separation as recently shown [4] for HPLC of N-(9-acridinyl)maleimide derivatives with glutathione, homocysteine, coenzyme A and cysteine. Two maleimides, N-(1-pyrene)maleimide (PM) [8,9] and N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide (DACM) [10], are commercially available but to our knowledge their potential use in HPLC methods for low-molecular-weight thiol-containing substances has not been explored. This paper describes the ion-pair HPLC separation of mercaptoacetate and N-acetylcysteine derivatives obtained with these two maleimides.

EXPERIMENTAL

Materials

Mercaptoacetic acid was obtained from E. Merck (Darmstadt, G.F.R.) and N-acetylcysteine was from Sigma (St. Louis, MO, U.S.A.). N-(1-Pyrene)maleimide and N-(7-dimethylamino-4-methyl-3-coumarinyl)maleimide were from Fluka (Buchs, Switzerland). Tetramethylammonium hydroxide (10% in water) was from Merck. All other reagents were of reagent grade or better. The analytical column was a LiChrosorb RP-8, 5 μm (250 × 4.0 mm) from Merck.

Apparatus

The apparatus we used was a Constametric III pump from LDC (Riviera Beach, FL, U.S.A.), a Rheodyne (Berkeley, CA, U.S.A.) Model 7125 sample injector with a 100-μl sample loop, and a Perkin-Elmer (Beaconsfield, Great Britain) Model 3000 spectrofluorometer with an LC-cell accessory. With chromatography of PM derivatives the fluorometer was set at 342 nm excitation (band width 15 nm) and 396 nm emission (band width 20 nm), and with DACM derivatives the corresponding wavelengths were 400 and 480 nm.

Derivatization and chromatography of pure thiols

Stock solution of PM, 1 mmol/l, was prepared in ethanol—acetone (1:1). DACM, 0.5 mmol/l, was dissolved in acetone. The solutions were stored at 4°C and before use the reagents were diluted to a concentration of 20 μmol/l in ethanol—acetone and acetone, respectively.

Stock solutions (1 mmol/l) of mercaptoacetic acid and N-acetylcysteine were prepared in hydrochloric acid, 10 mmol/l, containing disodium EDTA, 2 mmol/l. These solutions were stored in the refrigerator at 4°C and were stable as controlled by thiol determination with the 5,5'-dithiobis-(2-nitrobenzoic acid) method [11]. Dilutions to the concentrations 100, 75, 50, 25 and 10

μmol/l were prepared freshly in the hydrochloric acid containing disodium EDTA and immediately before derivatization these thiol solutions were diluted 1:101 with carbonate buffer, 50 mmol/l, containing disodium EDTA, 10 mmol/l, pH 9.0.

The derivatization was performed in tubes with PTFE-lined screw-caps by reacting 5.05 ml of thiol solution of an appropriate concentration (less than 1 μmol/l) with 0.5 ml of PM or DACM, 20 μmol/l, which gave a final maleimide concentration of 1.8 μmol/l. After mixing, the tubes were placed in a water-bath at 37°C for approximately 20 h. The addition products initially formed will hydrolyze and the final derivatives are ready for chromatography. The derivatives obtained were stable at room temperature for at least four days. The solutions were diluted 1:5 with the mobile phase and 100-μl samples were then loop-injected. The mobile phase was prepared from sodium phosphate buffer, 2 mmol/l, to which tetramethylammonium hydroxide (TMA) was added, and the pH adjusted to 7.4 with hydrochloric acid (6 mol/l). The amount of TMA added was calculated to give a final concentration of 10 mmol/l in the mobile phase after mixing the solution with methanol to give an appropriate methanol concentration; for details, see Results. All mobile phases were filtered through a 0.5-μm cellulose acetate filter, type EH (Millipore Corp., Bedford, MA, U.S.A.) and were thereby also deaerated.

Application for urinary thiols

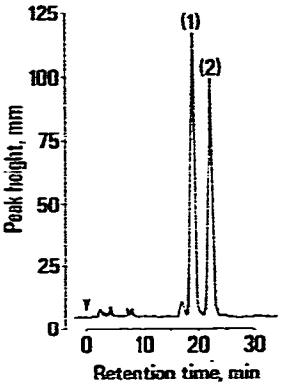
Urinary thiols were purified according to the description of Hannestad and Sörbo [2]. Thus, from freshly collected urine a 5-ml aliquot, mixed with EDTA and adjusted to pH 9.8—10.0, was reacted with thiopropyl-Sepharose, acidified with acetic acid, and after centrifugation the thiols were obtained in free form. From the supernatant, an amount of less than 2 μmol of thiols as measured according to Rootwelt [11] was adsorbed on an organomercurial adsorbent, *p*-acetoxymercurianiline—Sepharose 4-B. After washing, the thiols were eluted with cysteine. The eluate was then applied on a small cation-exchange chromatography column (AG 50 W) which retained the cysteine. The thiols of interest were obtained in the first hydrochloric acid eluate (4.0 ml) and 0.2 ml of disodium EDTA, 0.18 mol/l, was added. From this eluate, containing less than 0.5 mmol/l thiols, 250 μl were neutralized with 25 μl of sodium hydroxide, 0.1 mol/l. Then 5 ml of carbonate buffer, 50 mmol/l, containing disodium EDTA, 10 mmol/l, pH 9.0, were added, followed by 0.5 ml of maleimide, 0.5 mmol/l. This amount of maleimide was chosen in order to obtain an excess of maleimide in relation to the thiols. Hydrolysis and chromatography were performed as described above.

RESULTS

Chromatography

PM derivatives of mercaptoacetate and N-acetylcysteine each gave a single peak, as shown in Fig. 1.

The DACM derivative of mercaptoacetate gave a single peak, but N-acetylcysteine in addition to a major peak also gave a small peak with a shorter retention time. However, by choosing an appropriate methanol concentration in the mobile phase, these peaks could be baseline separated (Fig. 2).



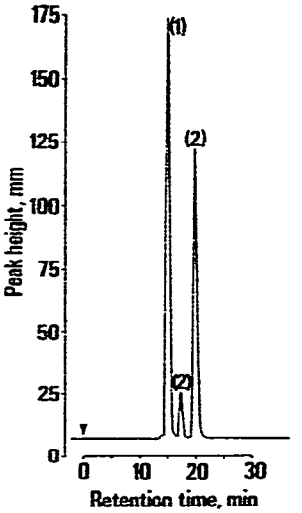


Fig. 1. Chromatographic separation of PM derivatives of 9 pmol mercaptoacetate (1) and 9 pmol N-acetylcysteine (2). Mobile phase: sodium phosphate buffer (2 mmol/l, containing TMA, pH 7.4)—methanol (70 : 30, v/v). Final TMA concentration 10 mmol/l. Flow-rate 0.75 ml/min, pressure 22 MPa, ambient temperature.

Fig. 2. Chromatographic separation of DACM derivatives of 9 pmol mercaptoacetate (1) and 9 pmol N-acetylcysteine (2). Mobile phase: sodium phosphate buffer (2 mmol/l, containing TMA, pH 7.4)—methanol (85 : 15, v/v). Final TMA concentration 10 mmol/l. Flow-rate 0.75 ml/min, pressure 18 MPa, ambient temperature.

Sensitivity and linearity

Both with mercaptoacetate and N-acetylcysteine the DACM derivatives gave higher detector responses than PM derivatives when peak heights were compared. For both types of fluorophores the sensitivity was high, and the lowest detection limit was obtained for the DACM derivatives (Table I).

Linearity of the analytical procedure, including the derivatization step and detector response, was evaluated for both derivatization reagents using injected amounts of 1.8—18 pmol. Linearity was found for the range tested (Fig. 3).

Chromatography of urinary thiols

To test the utility of the method for thiol determinations with the maleimides, a clean-up procedure [2] for urine was chosen which gives an extract containing acid and neutral thiols. Fig. 4 shows such a urinary chromatogram obtained with PM. Two major peaks corresponding to mercaptoacetate and N-acetylcysteine were found together with a number of hitherto unidentified peaks. Similar chromatograms were obtained with DACM. Reagent peaks with longer retention times were observed with the procedure for urinary thiols, causing interference with the PM derivatives. However, with the procedure for pure thiols only insignificant reagent peaks were obtained due to the lesser amount of maleimide used.

The precision of the method for urinary mercaptoacetate and N-acetylcysteine was estimated from duplicate analysis of urinary samples from sixteen

TABLE I

COMPARISON OF SENSITIVITIES OBTAINED WITH PM AND DACM DERIVATIZATION PRODUCTS

	Amount injected (pmol)	Peak height (mm)	Noise (mm)	Detection limit* (fmol)
PM derivatives				
Mercaptoacetate	1.8	49	4.3	320
N-Acetylcysteine	1.8	41	4.3	380
DACM derivatives				
Mercaptoacetate	1.8	160	1.7	38
N-acetylcysteine	1.8	115	1.7	53

*Detection limit was calculated as $\frac{2N}{S} \cdot A$, where N = noise, S = signal (peak height), and A = amount of substance injected.

healthy subjects. The range for the mercaptoacetate concentration was 3.5—9.5 μmol/l and for N-acetylcysteine it was 13.8—44.1 μmol/l. From the mean of 6.4 μmol/l and S.D. of 0.30 μmol/l (calculated from duplicate analysis) a coefficient of variation (C.V.) of 4.7% was obtained for mercaptoacetate. The mean value for N-acetylcysteine was 30.7 μmol/l (S.D. = 0.70 μmol/l) and the C.V. was 2.3%. The inter-assay variability was estimated from repeated analysis of a urinary sample during three weeks (n = 12). The results were for mercaptoacetate 5.9 ± 0.62 μmol/l (mean ± S.D.), C.V. = 10.5%, and for N-acetylcysteine the results were 35.3 ± 2.54 (mean ± S.D.), C.V. = 7.2%.

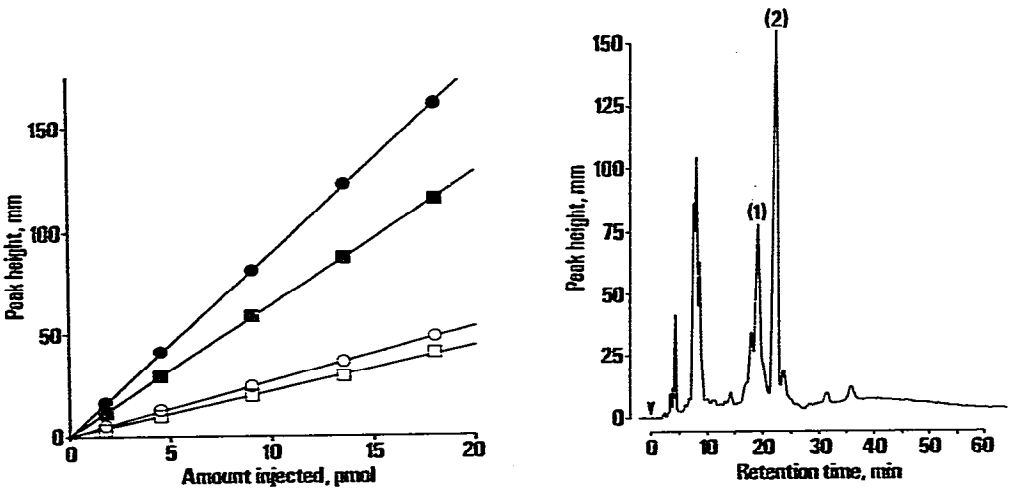


Fig. 3. Standard curves for mercaptoacetate (○, ●) and N-acetylcysteine (□, ■) derivatives with PM (open symbols) and DACM (filled symbols).

Fig. 4. Chromatography of urinary thiols. Chromatographic conditions are the same as in Fig. 1. Peaks 1 and 2 correspond to mercaptoacetate and N-acetylcysteine, respectively.

DISCUSSION

A number of maleimides which react with thiols have recently been synthesized [7]. When such a reagent is coupled to the thiol it will afterwards hydrolyze giving a carboxylic acid, and thus the products with mercaptoacetate and N-acetylcysteine will contain two carboxyl groups. We thus thought that these derivatives would be suitable for ion-pair chromatography. These expectations were fulfilled, and we have shown here that the substances could be well separated, both as the PM and the DACM derivatives. With the use of DACM two peaks appeared for N-acetylcysteine. This may be a drawback when this derivatization is used in the analysis of complex biological fluids. We considered the possibility that an impurity of the substance was responsible for the extra peak by analysing N-acetylcysteine from different sources. Identical results were obtained. Furthermore, corresponding peaks were found in urinary chromatograms with DACM. There is a possibility that the hydrolysis of maleimide adduct occurs in two different positions, giving rise to two different products. Such possibilities have been discussed both for derivatives with PM [9], DACM [12] and N-(9-acridinyl)maleimide [4].

In comparison with earlier thiol determinations [1], the sensitivity of the chromatographic—fluorometric detection of the derivatives was extremely high, with a detection limit of about 50 fmol for the DACM derivatives. These results also compare well with the detection limit of 2.5 pmol obtained for some monothiols in serum as described by Takahashi et al. [4]. They used N-(9-acridinyl)maleimide in a reversed-phase HPLC method. Recently, Newton et al. [5] published an HPLC method for analysis of biological thiols using the fluorogenic reagent monobromobimane for derivatization. They found a sensitivity of 2 pmol for cysteine which is in the same range.

It was previously shown [2] that the procedure used for clean-up of urine gave extracts containing both mercaptolactate, mercaptoacetate and N-acetylcysteine. With the present method the latter two thiols always gave well-defined peaks, but with mercaptolactate we obtained a broad peak with a retention time longer than that of N-acetylcysteine. The corresponding peak from urine may be observed in Fig. 4 with a retention time between 30 and 55 min. This chromatographic behaviour of mercaptolactate may depend on the interaction of its hydroxyl group with the silica matrix of the column.

As presented here the chromatographic method gave promising resolution and sensitivity for determination of urinary mercaptoacetate and N-acetylcysteine. The precision of the method is satisfactory for urinary mercaptoacetate and N-acetylcysteine in healthy subjects, and the values agree closely with those found in healthy subjects by gas chromatography [2]. Further studies, however, are needed in order to eliminate reagent peaks and to obtain satisfactory results for mercaptolactate.

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