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DETERMINATION OF N-ACETYL-L-CYSTEINE IN BIOLOGICAL FLUIDS

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SUMMARY

For determination of a drug in biological fluids, accuracy and sensitivity of detection of the adopted method are decisive parameters, but practical aspects such as time and necessary instrumentation are equally important. In the case of N-acetyl-L-cysteine, sample pretreatment can be kept at a minimum if sufficiently selective modes of derivatization, chromatography and detection are employed. Its concentration in serum is between 1 and 20 μmol/l; for quantitative analysis capillary gas chromatography and mass fragmentographic detection are employed. For urine with its ten-fold higher concentration the preferred method is reversed-phase high-performance liquid chromatography after thiol-selective derivatization.

INTRODUCTION

N-Acetyl-L-cysteine, an endogenous product of cysteine metabolism [1], is of considerable therapeutic importance as a mucolytic drug in the treatment of bronchitis [2]; it also protects against the hepatotoxicity of high doses of *p*-acetylaminophenol [3]. For parenteral nutrition N-acetyl-L-cysteine may serve as a substitute for cysteine, offering the advantages of higher solubility and stability. In this context appropriate analytical methods are required for monitoring its bioavailability and pharmacokinetics. Gas chromatography and liquid chromatography are complementary methods for the quantitative determination of N-acetyl-L-cysteine in plasma and urine.

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EXPERIMENTAL

Chemicals

4-Chloro-7-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl) was from Serva (Heidelberg, F.R.G.). N,4-Dimethyl-N-nitrosobenzenesulfonamide was from E. Merck (Darmstadt, F.R.G.). Nucleosil C_{18}, 7 μm, was purchased from Macherey and Nagel (Düren, F.R.G.). All other chemicals of reagent grade were from Merck.

Buffers

EDTA—citrate: 29.4 g of sodum citrate and 744 mg of EDTA are dissolved in 200 ml of distilled water; the buffer is adjusted to pH 8.3 with sodium hydroxide.

Phosphate: 17.8 g of $Na_2HPO_4 \cdot 2H_2O$ are dissolved in 200 ml of distilled water; the buffer is adjusted to pH 8.0 with phosphoric acid.

Instrumentation for high-performance liquid chromatography (HPLC)

The liquid chromatograph is a modular instrument consisting of a Spectra-Physics pump Model 740 B, a pressure monitor Model 714, and a Rheodyne Injector Model 7105. The eluate is monitored at 470 nm with a spectrophotometer from ERC, Model 7210; the signals are registered with a Servogor recorder Model RE 571, and integrated with a Hewlett-Packard integrator Model HP 3390. The column, 30 cm × 4.6 mm, is filled with Nucleosil C_{18}, 7 μm, by slurry packing. During chromatography the column is immersed into a water bath wit a constant temperature of 30° C.

Derivatization and HPLC

The oxidized form N,N′-diacetylcystine must be reduced in order to determine the overall resorption of the administered compound; this is effected by the addition of 10 μl of a solution of dithioerythritol (145 mg in 10 ml water) and 100 μl of phosphate buffer to 250 μl of urine or deproteinized serum. An aliquot of 250 μl is removed after 30 min and mixed with 250 μl of EDTA—citrate buffer and 50 μl of a solution of NBD-Cl (100 mg in 100 ml of methanol). After 20 min the sample is ready for chromatography. Weighed standards are dissolved in water and treated analogously. Aliquots of 20 μl are injected for HPLC. The mobile phase is aqueous disodium hydrogen phosphate (0.5%)—acetonitrile (70:30, v/v); the flow-rate is 1.6 ml/min. More than 100 samples can be processed before a column needs to be repacked.

Sample preparation and gas chromatography (GC)

Blood serum (0.5 ml) is mixed with 20 μl of a solution of N-acetyl-D-cysteine (250 nmol/ml in distilled water). One drop of ethanethiol is added and after 5 min the protein is precipitated by the addition of 1 ml of acetone. After centrifugation the supernatant is adjusted to pH 9 with a drop of aqueous ammonia (25%) and extracted with 1 ml of hexane. The aqueous phase is then concentrated to about half of its initial volume with a stream of nitrogen and under moderate heating. The aqueous solution is transferred to an exchange column, Dowex 1-X8, OH^-, 50—100 mesh, 40 × 5 mm. After 10 min the column is washed with 4 ml of water, then with 5.0 ml of hydrochloric acid

(0.1 mol/l). The last 1.0 ml containing the N-acetylcysteine is collected in a Reacti-Vial®. The solution is dried in a vacuum centrifuge. Diazomethane in 0.9 ml of diethyl ether and 0.1 ml of methanol and a drop of ethanethiol are added to the dry residue and the sample is treated by sonication for 2 min. After 30 min at room temperature the solvent is removed with a nitrogen stream, the residue is dissolved in 10 μl of toluene under sonication and the sample is centrifuged; 1 μl (for urine samples 0.1 μl) is injected for gas chromatography—mass spectrometry (GC—MS).

GC—MS conditions

The instrument is a Finnigan 4021 with an Incos data system. The samples are injected in the splitless mode onto a glass capillary, 25 m × 0.25 mm, deactivated with diphenyltetramethyldisilazane according to the method of Grob et al. [4] and coated with Chirasil-Val; the split is opened after 0.7 min. The injector temperature is 250°C; the carrier gas is helium; the inlet pressure 100 kPa. The temperature programme is 0.7 min isothermal at 60°C, then at a rate of 40°C/min to 145°C, at a rate of 5°C/min to 175°C, and at a rate of 40°C/min to 200°C, isothermal for 6 min. Interface and ion-source temperatures are 250°C; electron-impact ionization, electron energy 70 eV; scans from *m/z* 100 to *m/z* 210 in 0.95 sec, bottom time 0.05 sec, electron multiplier 1.6 kV. Mass fragmentograms are constructed monitoring *m/z* 132, the base peak in the mass spectrum of N-acetyl-S-methylcysteine methyl ester. Also suitable is chemical ionization using isobutane and mass fragmentography of *m/z* 192, the quasimolecular ion.

RESULTS AND DISCUSSION

For pharmacokinetic studies non-selective methods have been employed, such as utilization of radioactive tracers [5] or determination of total thiol content [6]. However, to gain detailed insight into the metabolic fate of N-acetyl-L-cysteine, selective methods are required; more reliable is HPLC [7] or GC [1]. The HPLC method is based upon prechromatographic derivatization of the thiol group with different N-substituted maleimides forming fluorescent derivatives; the detection limit for the pure compound is in the femtomole range. A drawback of both published procedures is the relatively complicated isolation of N-acetyl-L-cysteine. In spite of their high sensitivity, they are apparently not suitable for analysis of serum; no quantitative data have been given although these values are of great importance for pharmacokinetic studies.

The goal of this investigation was to establish routine methods for the quantitative analysis of N-acetyl-L-cysteine in serum and urine in its therapeutic concentration range in a fast and uncomplicated manner. The choice of a method is not only determined by the sensitivity of the actual analytical step, but also by more practical aspects such as cost and time required for the procedure including sample pretreatment, and the limit of detection of the respective compound in an actual sample, not of the pure standard.

Determinations of N-acetyl-L-cysteine in serum were anticipated to be difficult, due to its low concentration and the small sample volumes available.

The therapeutic concentration range is about 1—30 μmol/l. In urine it is present in concentrations between 10 and 200 μmol/l; therefore, determination of N-acetyl-L-cysteine in urine should be relatively unproblematic, provided that potentially interfering substances are removed during the clean-up, separated by chromatography or selectively suppressed by the detection mode.

Conversion of N-acetyl-L-cysteine to the S-NBD derivative renders the sample pretreatment for HPLC very simple; for determinations in the relatively high concentration range typical for urine this derivative proved to be suitable. Reaction with NBD-Cl is fast, and the yields are reproducible and quantitative [8]. The derivatives are stable, which is especially important for routine analysis of large numbers of samples.

The chromatogram of a standard and a urine sample of a patient treated with N-acetyl-L-cysteine is shown in Fig. 1. The lower limit of photometric detection at 470 nm is about 5 μmol/l in urine; it is likely that employment of a fluorescence detector would increase the sensitivity. The calibration line is linear, with a slope of 1.06 cm peak height vs. 1 μmol/l and a correlation coefficient of 0.9996. The same slope is obtained for the oxidized form N,N'-diacetyl-L,L-cystine, indicating that reduction with dithioerythritol is quantitative. Appealing is the simplicity and reproducibility of the described procedure. Pretreatment of the sample is minimal, and reduction and derivatization take less than 1 h. This facilitates the analysis of large numbers of samples.

Analysis of sera of different patients showed that individually various contaminants interfere with the quantitative determination by HPLC. In this case we reverted to the method of enantioner labelling [9] in combination with capillary GC. A chromatogram of a standard is shown in Fig. 2a. Initially it was intended to employ a flame-ionization detector for GC, but with the adopted sample pretreatment the chromatograms were not sufficiently clean (not shown). An alkali flame-ionization detector with its high sensitivity and selectivity for nitrogen-containing compounds affords considerably less-complex

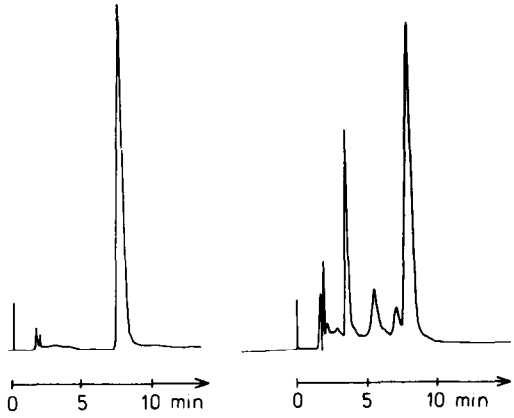

Fig. 1. HPLC of N-acetyl-S-NBD-cysteine on a column, 300 × 4.6 mm, packed with Nucleosil C_{18}, 7 μm. Mobile phase, aqueous disodium hydrogen phosphate (0.5%)—acetonitrile (70:30, v/v); flow-rate, 1.6 ml/min; photometric detection at 470 nm; injected amounts, 3.1 and 2.8 nmol. Left: aqueous solution of N-acetylcysteine, 153 μmol/l. Right: urine containing 140 μmol/l endogenous N-acetylcysteine.

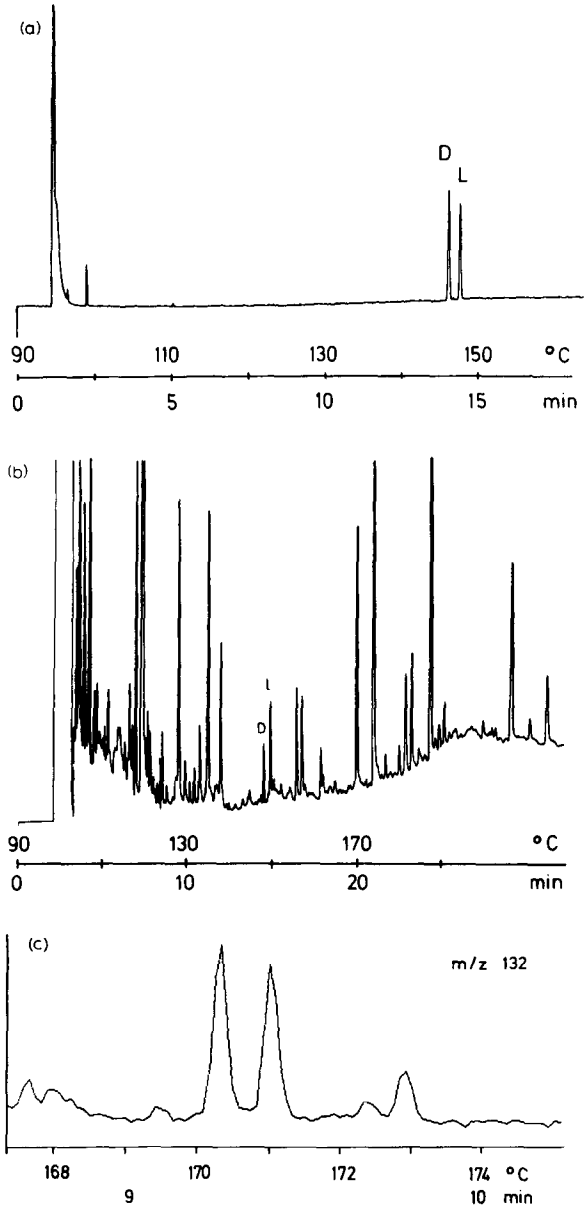

Fig. 2. (a) GC separation of the enantiomers (L and D) of N-acetyl-S-methylcysteine methyl ester on a capillary, 25 m × 0.25 mm, coated with Chirasil-Val; carrier gas is hydrogen, inlet pressure 50 kPa, temperature programme 4°C/min; split injection, split ratio 1:50; injector and detector temperature 250°C. (b) Determination of N-acetyl-L-cysteine by enantiomer labelling; separation on a glass capillary, 10.5 m × 0.1 mm, coated with Chirasil-Val; carrier gas hydrogen, inlet pressure 90 kPa; detection with a nitrogen-selective alkali flame-ionization detector. The concentration in this serum sample was determined as 12 μmol/l; amount after splitting 10 fmol. (c) Determination of N-acetyl-L-cysteine by enantiomer labelling and GC—MS (*m/z* 132) in a control serum spiked with 0.6 μmol/l N-acetyl-L-cysteine; 1.2 μmol/l N-acetyl-D-cysteine are added as internal standard.

chromatograms; however, for accurate quantitative analysis this still is deemed inappropriate due to the relatively noisy baseline and the appearance of a number of additional peaks (Fig. 2b). Logically, the next choice would have been a sulphur-selective flame-photometric detector, but we did not have such a device. Therefore, detection was done by mass fragmentography monitoring *m/z* 132 (Fig. 2c). The method is sufficiently sensitive to allow quantitative determinations in the concentration range expected for endogenous N-acetyl-L-cysteine, i.e. about 0.5 μmol/l. Control analyses were performed without addition of N-acetyl-D-cysteine to show that this isomer is not present endogenously.

A calibration graph was constructed with human blood serum spiked with increasing amounts of synthetic N-acetyl-L-cysteine. Between 0.5 and 30 μmol/l a straight line with a slope of 0.952, a correlation coefficient of 0.999 and an intercept of 0.43 μmol/l endogenous N-acetyl-L-cysteine were obtained. As the dosages applied in clinical studies lead to concentrations in serum of 1—20 μmol/l, the enantiomeric internal standard N-acetyl-D-cysteine is added to a concentration of 10 μmol/l. The great advantage of enantiomer labelling is that only sufficiently large signal-to-noise ratios of the peaks of both antipodes are required; the recovery over all steps of the analysis need not be known.

The described procedures have been employed in the assessment of the bioavailability of N-acetyl-L-cysteine. As an example the concentration—time curve in the blood of a male patient receiving an intravenous infusion of 1.6 mmol during the initial 6 h is shown in Fig. 3. The values determined with the less selective alkali flame-ionization detector are of similar magnitude.

The serum levels of N-acetyl-L-cysteine determined by HPLC are considerably lower. At first this was considered as an indication of binding to plasma proteins. As the clean-up for HPLC involves precipitation of plasma proteins, a significant portion of the drug would be removed with the precipitated protein. However, comparison of values obtained by enantiomer labelling with the addition of internal standard before and after precipitation of plasma

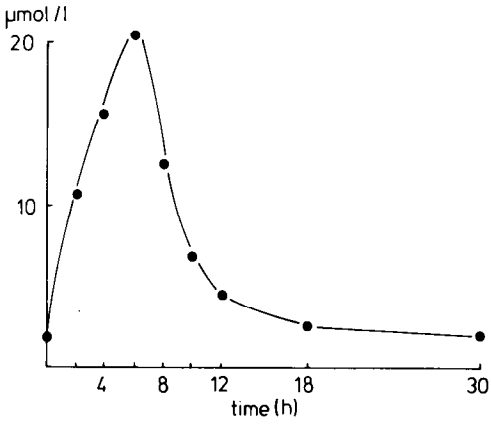

Fig. 3. Typical time dependence of the serum concentration of total N-acetyl-L-cysteine/cystine in a patient receiving 1.6 mmol of N-acetyl-L-cysteine by intravenous infusion during the initial 6 h.

proteins yielded identical values. The reason why HPLC affords lower values is currently being investigated.

In conclusion we want to emphasize that for routine determinations of drugs in biological fluids the method with the maximum sensitivity is not always the most suitable; in fact, the high selectivity of mass fragmentography, not so much its sensitivity, is crucial to achieve sufficiently accurate results. In addition, aspects such as time and cost are important from the point of view of practicability. The present study also shows that it may be necessary to employ different analytical procedures for the determination of the same compound in different types of samples.

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