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Determination of N-acetylcysteine in human plasma by gas chromatography–mass spectrometry

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ABSTRACT

An automatic mass spectrometric method for the quantitation of N-acetylcysteine (NAC) in human plasma has been developed. NAC was extracted from plasma with ethyl acetate and derivatized in two steps with 2-praponol and pentafluoropropionic anhydride. The volatile derivative obtained was ideal for gas chromatographic–mass spectrometric analysis. Data obtained by analysing the plasma of healthy volunteers to whom 600 mg of NAC had been orally given are reported.

INTRODUCTION

The widest application of N-acetylcysteine (NAC) is in the treatment of obstructive bronchitis [1]. However, NAC has been shown to be the ideal precursor of glutathione, an antagonist to the toxic effects of paracetamol abuse, and to prevent bladder haemorrhage caused by the accumulation of acrolein during treatment with cyclophosphamide [2]. It has been suggested that NAC could have a role as a free radical scavenger, and this has opened new treatment perspectives for the drug.

The setting up of a more specific analytical method than those presently used was therefore considered to be of primary importance. Such a method could give added impetus to clinical studies.

Several high-performance liquid chromatographic (HPLC) procedures have already been described [3,4] for the analysis, via the preparation of fluorescent derivatives, of compounds containing sulphydryl groups in biological fluids. Nevertheless, their specificity is poor and long analysis times are required to allow the analytical column to elute all the components. These procedures are thus time-consuming and unsuitable for routine work; futhermore, it is difficult to make them automatic. An HPLC method with electrochemical detection was recently described for NAC [5], which avoided the need for derivatization. This procedure, therefore, seems to be the simplest method available. In our opinion the mercury/gold electrode used by the authors is far too easily contaminated, causing a rapid loss of sensitivity. For these reasons, we avoided using it for routine work. The contrary holds true for the mass spectrometric method described here:

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it is very specific and is easy to realize, the resulting analytical procedure being simple and easy to automate.

EXPERIMENTAL

NAC and N-propionylcysteine (NPC) were synthesized by the Medicinal Chemistry Department of Zambon Group. Perfluoropropionic anhydride (PFPA) was purchased from Supelchem (Milan, Italy). Dithiothreitol (DTT), EDTA and sodium dithionite were obtained from Merck (Milan, Italy). All solvents were from RPE Carlo Erba (Rodano, Italy).

The analyses were carried out on a 25 m × 0.32 mm I.D. fused-silica capillary column coated with cross-linked HP5 stationary phase (film thickness 0.17 mm), purchased from Hewlett-Packard (Cernusco Sul Naviglio, Italy).

Chromatographic system

A Carlo Erba Mega 5160 gas chromatograph equipped with an automatic on-column injector was used.

Helium was used as the carrier gas at a flow-rate of 1 ml/min. The sample was injected at an initial column temperature of 60°C. The column was programmed at 50°C/min to 150°C, then at 15°C/min to 260°C. The final temperature was maintained for 2 min. The retention times of derivatized NAC and NPC were 4.0 and 4.5 min, respectively.

Mass spectrometry

A Finnigan Mat 8222 double-focusing inverse geometry mass spectrometer was used. The whole analytical procedure (*i.e.* injection, analysis run and quantitative calculations) was computer-controlled.

The conditions were as follows: ionization technique, electron impact; ionization energy, 70 eV; accelerating voltage, 3 kV; filament current, 3 mA; resolution, 3000; transfer line temperature, 280°C.

The monitored ions (nominal mass) were m/z 360 and 392 for the NAC derivative (arising from the losses of C_2F_5 and $COO^iC_3H_7$ from the molecular ion, respectively), and m/z 346 and 406 for NPC derivative (arising from the losses of $COOC_2F_5$ and $COO^iC_3H_7$ from the molecular ion, respectively).

Plasma preparation and storage

Fresh samples of heparinized blood, collected in plastic tubes containing 200 μl of 0.02 *M* EDTA, 50 μl of 7 m*M* sodium dithionite and 50 μl of 7 m*M* DTT, were centrifuged at 5000 *g* at 10°C for 5 min. The plasma was separated and divided into two aliquots each of 1 ml. The first aliquot was immediately frozen with dry ice and stored at −20°C until analysis. This was the sample used for the determination of total NAC (free NAC plus NAC bound to plasma thiols and proteins). The second aliquot had 200 μl of 0.4 *M* $HClO_4$ added immediately and

was centrifuged at 10 000 *g* at 0°C for 15 min. The supernatant was frozen with dry ice and stored at –20°C until its analysis for free NAC.

Sample preparation for total NAC analysis

To 0.5 ml of plasma, 1 mg of DTT, dissolved in 1 ml of 0.1 *M* phosphate buffer (pH 7.4), and 1 μg of NPC, as internal standard, were added. The samples were incubated at 37°C for 1 h to reduce disulphide bonds to sulphydryl groups and then immediately extracted.

Sample preparation for free NAC analysis

To 1 ml of plasma held at 0°C, 100 μg of DTT, dissolved in 1 ml of 0.1 *M* phosphate buffer (pH 7.4), and 0.5 μg of NPC, as internal standard, were added. The sample was then immediately extracted.

Extraction and derivatization of NAC and NPC

To 1 ml of plasma held at 0°C, 100 μg of DTT, dissolved in 1 ml of 0.1 *M* was added. The proteins were precipitated by centrifuging at 4°C at 2000 *g* for 20 min, and 200 μl of 2 *M* HCl and 6 ml of ethyl acetate were added to the supernatant. NAC and NPC were extracted by gentle mixing with a rotating stirrer for 15 min. The organic and aqueous phases were separated by centrifuging at 1000 *g* for 5 min. The organic phases were then transferred to conical glass tubes and dried under a stream of nitrogen at 30°C.

The dry residues were then subjected to the two-step derivatization. In the first step, the residue was treated with 100 μl of 2-propanol, saturated with HCl, at 60°C for 20 min. At the end of the reaction any excess reagent was removed by a stream of nitrogen. In this step the isopropylic ester of the thiazoline-carboxylic acid is formed by cyclization of NAC and NPC, accompanied by water loss. The second step was treatment with ethyl acetate–PFPA (3:1) at 60°C for 20 min. Again, excess reagent was removed by a stream of nitrogen. In this step the thiazoline ring opens and two perfluoroacyl groups are introduced, leading to two high-molecular-mass derivatives (MW_{NAC} = 479, MW_{NPC} = 493).

After the second step the dry residues were suspended in 50 μl of ethyl acetate, and 1 μl was then injected into the GC–MS system.

RESULTS

The two-step derivatization led to the formation of two single compounds of NAC and NPC, which gave rise to symmetric GC peaks. Moreover, as shown in the two mass spectra reported in Fig. 1, their MS characteristics were very good, *i.e.* high molecular masses and spectra rich with high-mass fragment ions.

Not only did the monitored NAC and NPC fragment ions, *i.e.* *m/z* 360 and 392 and *m/z* 346 and 406, respectively, result in spectra being free of any interference but the resulting ion chromatograms were shown to be almost free of background

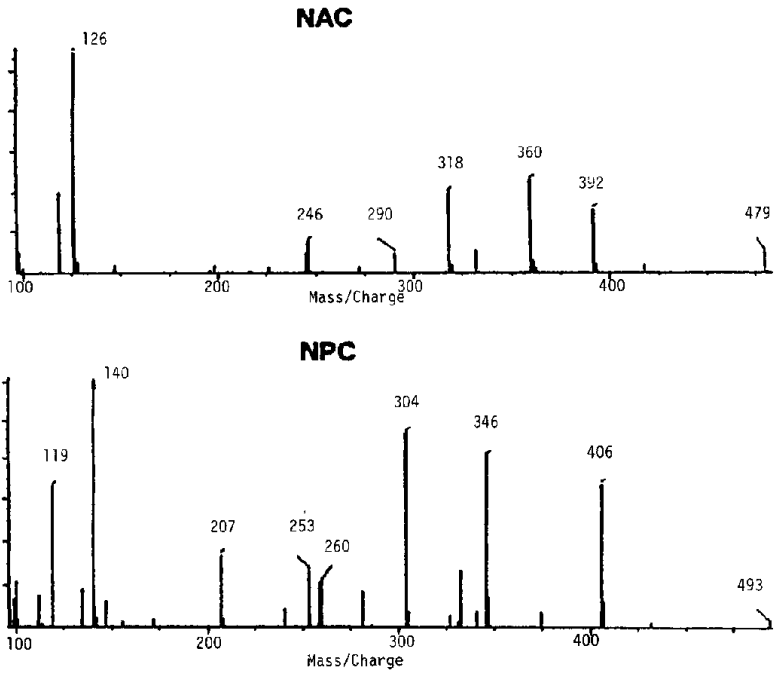

Fig. 1. Mass spectra obtained from injecting 50 ng of NAC and NPC into the GC–MS system, following reaction with 2-propanol saturated with HCl and PFPA.

noise; the technique therefore showed great sensitivity. Fig. 2 shows the chromatograms of a basal plasma sample (A) and of a sample of plasma from a healthy volunteer (B) treated orally with 600 mg of NAC, in which the concentration of NAC was found to be 0.31 μg/ml.

Identification of NAC in plasma

The identification of NAC in plasma was based on the measurement of the ion-density ratio of the selected ions at *m/z* 392 and 360. The mass spectrum of the peak scanned for derivatized NAC in a human plasma sample was identical with that of the standard compound and had the same retention time.

Linearity and sensitivity

Calibration curves were constructed by adding known amounts of NAC to human basal plasma and by analysing them as described above. Typical standard curves were produced in the concentration range 25–200 ng/ml for the dosage of free NAC, and 0.1–2.0 μg/ml for total NAC, and were expressed by the equations:

$$y = 0.051x + 0.007 \quad (r^2 = 0.99981)$$
$$y = 3.28x + 0.021 \quad (r^2 = 0.99997)$$

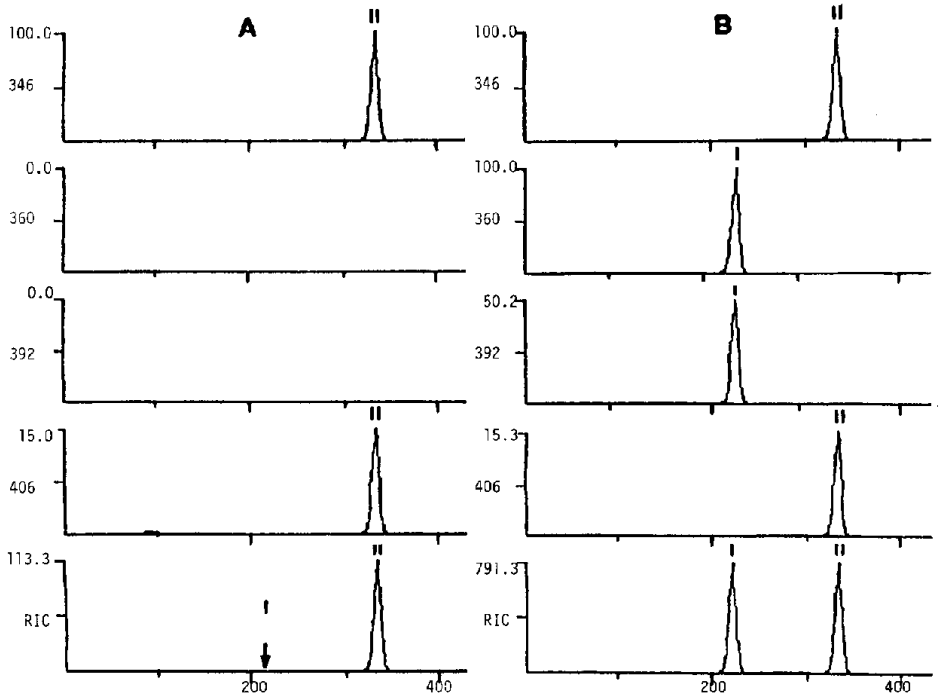

Fig. 2. Mass fragmentographic profiles of NAC (I) and NPC (II): (A) basal plasma extract containing 0.5 μg/ml NPC as internal standard; (B) plasma from a patient given 600 mg of NAC orally.

Recovery and precision

The recovery of the whole procedure was checked at each point of the calibration curves used to quantitate free NAC and total NAC by comparing the area value of NAC extracted from plasma with that obtained by analysing external standards with the same concentration. The overall mean recovery of the method was 85.0 ± 1.0% (n = 10).

The intra-assay precision was calculated by replicate analysis of plasma samples obtained from healthy volunteers given 600 mg of NAC. For free NAC the coefficient of variation (C.V.) was 6.5% (n = 6); for total NAC the C.V. was 5.0% (n = 6). Both coefficients were calculated at the lowest concentration point found.

Plasma levels

Figs. 3 and 4 show the plasma levels of NAC in six healthy volunteers orally administered 600 mg of NAC. The mean peak concentrations found were 0.68 mg/l and 3.1 mg/l for free and total NAC, respectively.

The absorption of the drug was very rapid and was followed by a two-exponent elimination phase. The mean half-lives of the elimination phase were 0.41 and 0.59 h for free and total NAC, respectively.

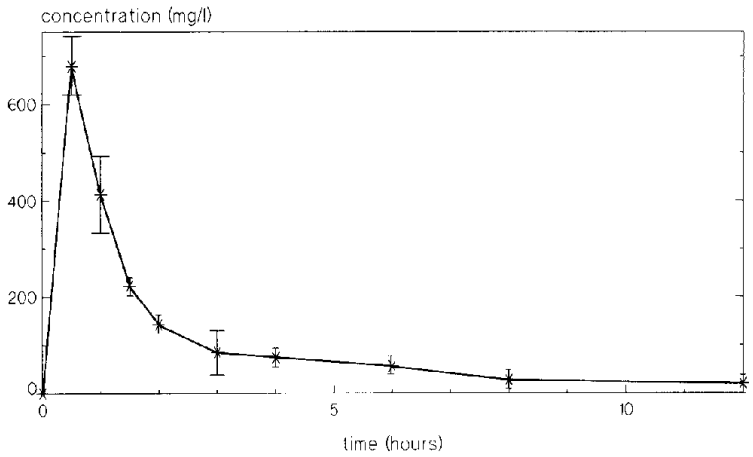

Fig. 3. Mean plasma levels of free NAC in six healthy volunteers after oral administration of 600 mg of NAC. Data are expressed as mg/l.

These data are in good agreement with those reported in previous studies [6,10], demonstrating the accuracy of this automatic procedure.

DISCUSSION

Many different HPLC methods using fluorimetric or electrochemical detection have been used to quantitate NAC in plasma. It is, however, difficult to make such procedures automatic. Where sensitivity is concerned, maleimide- and monobromobimane-based derivatives are suitable for NAC analysis in plasma.

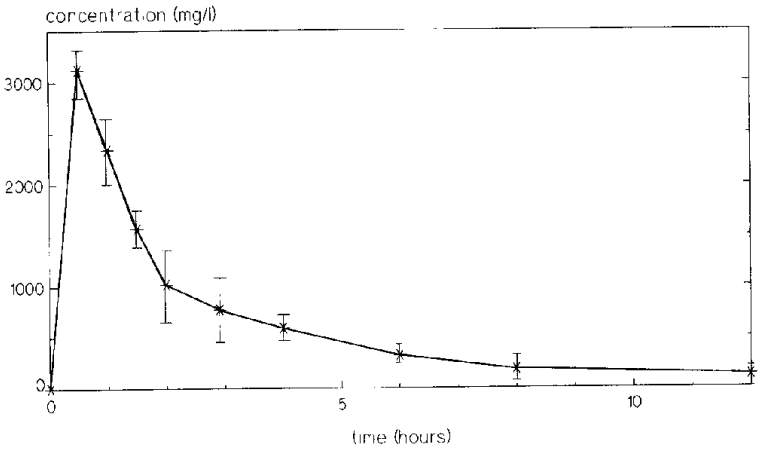

Fig. 4. Mean plasma levels of total NAC in six healthy volunteers after oral administration of 600 mg of NAC. Data are expressed as mg/l.

Unfortunately, their specificity is poor and reaction with other thiols present in plasma leads to the formation of several compounds with high retention times, making the time of analysis longer than 40 min; moreover, ghost peaks sometimes disturb the analysis. Furthermore, the pressure in the column tends to rise rapidly and thus cause the computer-controlled sequence to stop; this means that the samples must be reanalysed.

Using an electrochemical detector equipped with a mercury/gold electrode allows one to avoid the derivatization step, shortening the time of analysis. The gold/mercury cell is, however, quite sensitive to contamination and, as a consequence, rapidly loses its performance in terms of both sensitivity and reproducibility.

The mass fragmentographic procedure, on the other hand, is both simple and specific. Its sensitivity and reliability make it most useful for the routine determination of free and total NAC concentrations in plasma following the administration of NAC at dosage levels as low as 200 mg.

It is easy to make the procedure automatic, and the data system then fully controls the injection of the sample into the gas chromatograph, the analysis and the calculation. This automation allows up to 30 samples to be analysed daily and significantly reduces the overall cost of the analysis.

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