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Determination of *N*-acetylcysteine in human plasma by liquid chromatography coupled to tandem mass spectrometry

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Abstract

An analytical method for the determination of total *N*-acetylcysteine in human plasma has been developed, validated and applied to the analysis of samples from a phase I clinical trial. The analytical method consists of plasma digestion with dithiothreitol in order to reduce all the oxidized forms of *N*-acetylcysteine, and extraction with ethyl acetate followed by determination of levels by an LC–MS–MS method. The intra- and inter-assay precision and accuracy of this technique were good and the limit of quantitation was 50 ng/ml of plasma. The concentration working range was established between 50 ng/ml and 1000 ng/ml. This method has been used in the analysis of approximately 800 human plasma samples from a clinical study with 24 volunteers; the precision of the quality controls was in the range 8.7 to 13.4% and the accuracy was in the range −5.9 to 8.5%, expressed as the RSD and the relative error, respectively. © 2000 Elsevier Science B.V. All rights reserved.

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1. Introduction

N-Acetylcysteine (NAC) is useful as a mucolytic agent for the treatment of chronic bronchitis and other pulmonary diseases complicated by the production of viscous mucus [1,2]. It is also used as an antidote to paracetamol (acetaminophen) poisoning [3] and has been found to be effective in the prevention of cardiotoxicity by doxorubicin and haemorrhagic cystitis from oxazaphosphorines [4]. Since NAC is present in plasma as a free thiol and in various oxidized forms (disulfides) resulting from reaction with other thiols (another NAC, cysteine, glutathione or proteins), the pharmacokinetics of this compound must account for all those forms. For this purpose, a reductive cleavage of the disulfides in plasma is made before the extraction and instrumental analysis steps. Several analytical methods have been proposed for the measurement of NAC in plasma using gas chromatography (GC) [5,6], GC–mass spectrometry (MS) [7] or high-performance liquid chromatography (HPLC) [8–11]. All these methods use time-consuming extraction or long derivatization steps that hamper the pharmacokinetic studies of this compound. An LC–MS–MS method using atmospheric pressure chemical ionization has been reported but it was developed to be used in aqueous solutions [12].

In this paper we describe a method for simplifying and speeding up the assay of NAC in plasma using an LC–MS–MS method. After a reductive cleavage of the disulfides in plasma, NAC is extracted with

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acidic ethyl acetate and analyzed by LC–MS–MS. This method has been used to compare NAC plasma levels in a clinical study with 24 healthy volunteers after administration of single (200 mg) oral doses in two different formulations, one associated with paracetamol and chlorpheniramine and another one without any association.

2. Experimental

2.1. Materials and reagents

Both NAC and *N*-acetylserine (NAS), a tentative internal standard were supplied by Sigma >99% (Steinheim, Germany). HPLC-grade acetonitrile was from Panreac (Montcada i Reixac, Spain). HPLC-grade ethyl acetate came from Merck (Darmstadt, Germany). Water was purified by a Milli-Q system from Millipore (Bedford, MA, USA) until achieving a resistivity of 18 MΩ cm. Hydrochloric acid 37% GR, 2-mercaptoethanol 99% and 1,4-dithiotheritol (DTT) were from Merck.

Four types of biological samples were involved in this study: blank samples, standard samples that were used to obtain the calibration curves, quality control or replicate samples that were used in the calculation of the intra- and the inter-assay accuracy and precision in the validation as well as in the stability studies, and the samples obtained from the volunteers of the clinical trial. Quality control samples were also used to monitor the accuracy and precision in the analysis of the samples from the clinical trial.

To prepare the standard and the quality control samples, blank heparinized human plasma was obtained from untreated healthy volunteers (0.75 mg sodium heparinate/ml blood, using an aqueous 5% sodium heparinate solution). Blank plasma was obtained by centrifugation of blood at 2000 *g* for 15 min at 4°C. Blank plasma obtained from every untreated volunteer was pooled and frozen at −20°C to be used during the study in the preparation of standards and quality controls.

2.2. Preparation of standard and quality control samples

A standard stock solution (2000 μg/ml) was prepared by dissolving 20.0 mg of NAC in 10 ml of water–acetonitrile (80:20). A serial dilution scheme in the same solvent was applied to obtain the suitable range of calibration standard solutions of 10, 5, 2, 0.5, 0.2 and 0.1 μg/ml. NAS stock solution (200 μg/ml) was prepared by dissolving 20.0 mg of *N*-acetylserine in 100 ml of water–acetonitrile (80:20) and the working NAS solution (20 μg/ml) was prepared by one 1:10 dilution with the same solvent as the former solution.

Calibration curves in the validation study were prepared as follows. Seven 0.5-ml aliquots of untreated human plasma were transferred into 5-ml polypropylene tubes and spiked with 50 μl of each standard solution. Effective concentrations of NAC in plasma samples were 10, 20, 50, 100, 200, 500 and 1000 ng/ml. One plasma blank with only 100 μl of water was also prepared for each calibration curve. Quality controls employed in the calculation of the accuracy and precision in the validation study were prepared in the same way as the calibration standards, with nominal concentrations of 10, 20, 50, 100 and 500 ng/ml.

When the real samples were analyzed, calibration curves were obtained using calibration standards of nominal plasma concentrations of NAC different from the standards used in the validation. Nominal concentrations were: 50, 75, 100, 200, 500 and 1000 ng/ml. Also, quality control concentrations used in the sample analysis from the clinical trial were: 75, 200 and 500 ng/ml.

2.3. Sample preparation

Calibration curves, quality controls and real samples were treated as follows: 0.5 ml of a DTT solution (1 mg/ml in phosphate buffer at pH 7.4) was added to each plasma sample and after 1 h incubation at 37°C, the samples were transferred to 10-ml polypropylene tubes and spiked with 50 μl of working NAS solution, except for the blanks. Following the addition of 300 μl of 2 *M* HCl to each tube, an extraction with 4 ml of ethyl acetate saturated with 2-mercaptoethanol was carried out by shaking the tubes for 10 min. The tubes were then centrifuged at 2000 *g* for 15 min and the organic layer separated and then transferred to microcentrifuge polypropylene tubes for evaporation in a rota-

tional vacuum concentrator (Alpha-RVC Christ, Osterode am Harz, Germany), first at 50°C and 100 mbar for 35 min and, in a second cycle, at 25 mbar for 5 min maintaining the same temperature. The residue was resuspended in 125 μl acetonitrile, the vortex shaken and sonicated for 10 min and 125 μl of 10 m*M* of a 2-mercatoethanol solution in water to prevent reoxidation of the NAC was added to the acetonitrile resuspended residue. The resuspended sample was transferred to a clean low-volume autosampler vial and 50 μl was injected into the LC–MS–MS system.

2.4. Method validation

Duplicate calibration curves and replicates of the quality controls (QCs) were analyzed. The peak areas generated by the ion chromatograms of NAC were obtained. As NAS recovery was extremely low (2%), this compound was discarded as internal standard, and the calibration curves constructed using the peak areas of NAC instead of the peak area ratios as usual. Therefore, although NAS has been added to the samples, it will not be considered in the validation and the analysis of real samples. Weighted least squares (weighing factor=concentration^{-2}) was used to fit response of data versus the effective concentration to the equation: area response=slope·concentration±intercept. The weighing factor was used to correct the heteroscedascity of the responses, and the weighing factor=concentration^{-2} chosen according to the constant relative standard deviation (RSD) values observed for the peak areas of the concentrations studied. The back calculated values of the concentration were also statistically evaluated. Calibration curve equations were used to calculate the concentrations of NAC in the samples and the QCs from their peak areas.

The intra-assay precision and accuracy were determined by analyzing a set of QC samples ($n=6$) at each of the five levels, 10, 20, 50, 100 and 500 ng/ml, using a calibration curve obtained from duplicate standards. The inter-assay precision and accuracy studies were also carried out by analyzing QC samples ($n=2$) at the above five concentrations for a further three times using the same kind of calibrating curves.

2.5. Recovery

Absolute recoveries of NAC and NAS from human plasma were calculated at 200 and 500 ng/ml by comparing the area ratios of the plasma samples with reference samples. To reproduce exactly the same LC–MS–MS conditions for all plasma samples, reference samples were analyzed in the presence of plasma extracts. For reference samples of the 200 ng/ml effective concentration, blank plasma samples were prepared and processed as described above and resuspended with 50 μl of the NAC calibration standard solution of 2 μg/ml, 50 μl of the working internal standard solution, 105 μl of acetonitrile and 45 μl of a 28 m*M* solution of 2-mercaptoethanol. In the case of reference samples with a 500 ng/ml effective concentration, a 5 μg/ml calibration standard solution was used. Replicate plasma samples were prepared as the calibration standards of 200 and 500 ng/ml effective concentration, the number of replicates for each concentration being six.

2.6. Stability

Stability of NAC under the experimental conditions was investigated by comparison of the mean areas obtained in each case with the mean areas from a control set of replicates (time zero). The concentration of the spiked plasma samples was 50 ng/ml and 500 ng/ml, except in the case of stability under autosampler conditions. The number of replicates employed for each determination was five. Results were expressed for each concentration level as the percentage of the initial control concentration (C_0) which is referred to as 100%. Stability was studied for the dry residue after 24 and 72 h at 4°C, for up to three freeze–thaw cycles and for one and three months storage below −20°C. Stability under autosampler conditions was studied by reanalyzing the samples of the intra-day validation after 24 h at room temperature.

2.7. LC–MS–MS

LC was performed using two Applied Biosystems (San Jose, CA, USA) SF-400 pumps and a Gilson 233XL (Villiers-le-Bel, France) autosampler. The LC

system was coupled to an API 365 PESciex (Concord, Canada) turboionspray tandem mass spectrometer. The separation was achieved on a 5-μm Kromasil C_{18} column (50×4.6 mm I.D.). Elution was achieved at room temperature with acetonitrile–water (70:30) as the mobile phase. The LC system was operated isocratically at 1 ml/min. The column eluent was split and approximately 200 μl/min were introduced in the mass spectrometer. The turboionspray temperature was set at 350°C, using nitrogen (5.0 grade) at 7 l/min as the auxiliary gas and zero grade air as the nebulizing gas at a pressure of 90 p.s.i. (1 p.s.i.=6894.76 Pa). The ionspray voltage was set at 5000 V and the orifice voltage was set at −10 V. The instrument was programmed for a scan dwell time of 500 ms. The responses of NAC and the internal standard were measured in the negative ion mode using the MRM (multiple reaction monitoring) technique. The protonated molecular ion of NAC (*m/z* 162) was dissociated by collision with nitrogen at a gas thickness of $1.56 \cdot 10^{15}$ molecules cm^2 and under an energy of 12 eV. The mass spectrometer was set to selectively monitor parent to daughter fragments of *m/z* 162>84 for NAC. Data were acquired and analyzed using the MassChrom 1.0 software package, running on a Power Macintosh 7300/200 computer.

3. Results and discussion

3.1. Mass spectra

LC–MS–MS for the determination of NAC and NAS in human plasma was investigated. Negative electrospray mass spectra of NAC and NAS show intense $[M-H]^-$ ions at *m/z* 162 and 146, respectively. When these ions undergo fragmentation in the collision cell, the product ion mass spectra shown in Fig. 1a and b are generated. Under the experimental conditions employed, NAC shows two low-mass intense product ions at *m/z* 84 and 32, and NAS a main product ion at *m/z* 116. Although the product ion at *m/z* 84 was not the most intense one, the 162>84 reaction was used to obtain the NAC responses instead of the 162>32 reaction because the former showed higher specificity due to the higher mass of the product ion. The formation of the product ion at *m/z* 84 can be explained by a consecutive decarboxylation and loss of the thiol group of the molecular ion. The intense fragment ion at *m/z* 116 obtained for NAS can be produced by a loss of formaldehyde from the molecular ion. Because the monitored reactions for these compounds involved very low mass, negative ion detection was used to increase the specificity of the analytical method.

3.2. Specificity

NAC is present in the plasma at a concentration around 13 ng/ml as an endogenous compound [8]. For this reason, ion chromatograms from blank plasma samples showed an interference peak at the same retention time of NAC that is approximately 1 min (see Fig. 2a). The area of this peak was constant through the blanks studied in the validation and in volunteers involved in the clinical study before receiving the drug. When an estimation of the NAC concentration corresponding to these blanks was done, a value of approximately 11 ng/ml was obtained that is very consistent with the previously published concentration [8]. The comparison of the ion chromatograms corresponding to the blank plasma and the standard of 50 ng/ml (limit of quantitation, LOQ) (see Fig. 2a and c) shows that the influence of the endogenous NAC in the range of concentrations studied is low.

Interference of NAS in the ion profile of NAC (162>84) was also investigated because both compounds had the same retention time. No interference was expected studying the product ion mass spectra of both compounds because although both NAC and NAS have a common product ion of *m/z* 84, only NAC has the correct precursor ion at *m/z* 162. Fig. 2b shows the ion chromatogram corresponding to the NAC transition (162>84) of a blank sample spiked with NAS at a 2 μg/ml level. Although the interference peak was a little higher than in the case of the blank plasma, due possibly to a certain cross talk in the mass spectrometer, its influence on the LOQ standard was low enough as demonstrated in the accuracy and precision of the back calculated values shown in Tables 1, 2 and 3 that meet the acceptability criteria for the validation of a bioanalytical method [13]. The intercept value higher than zero in

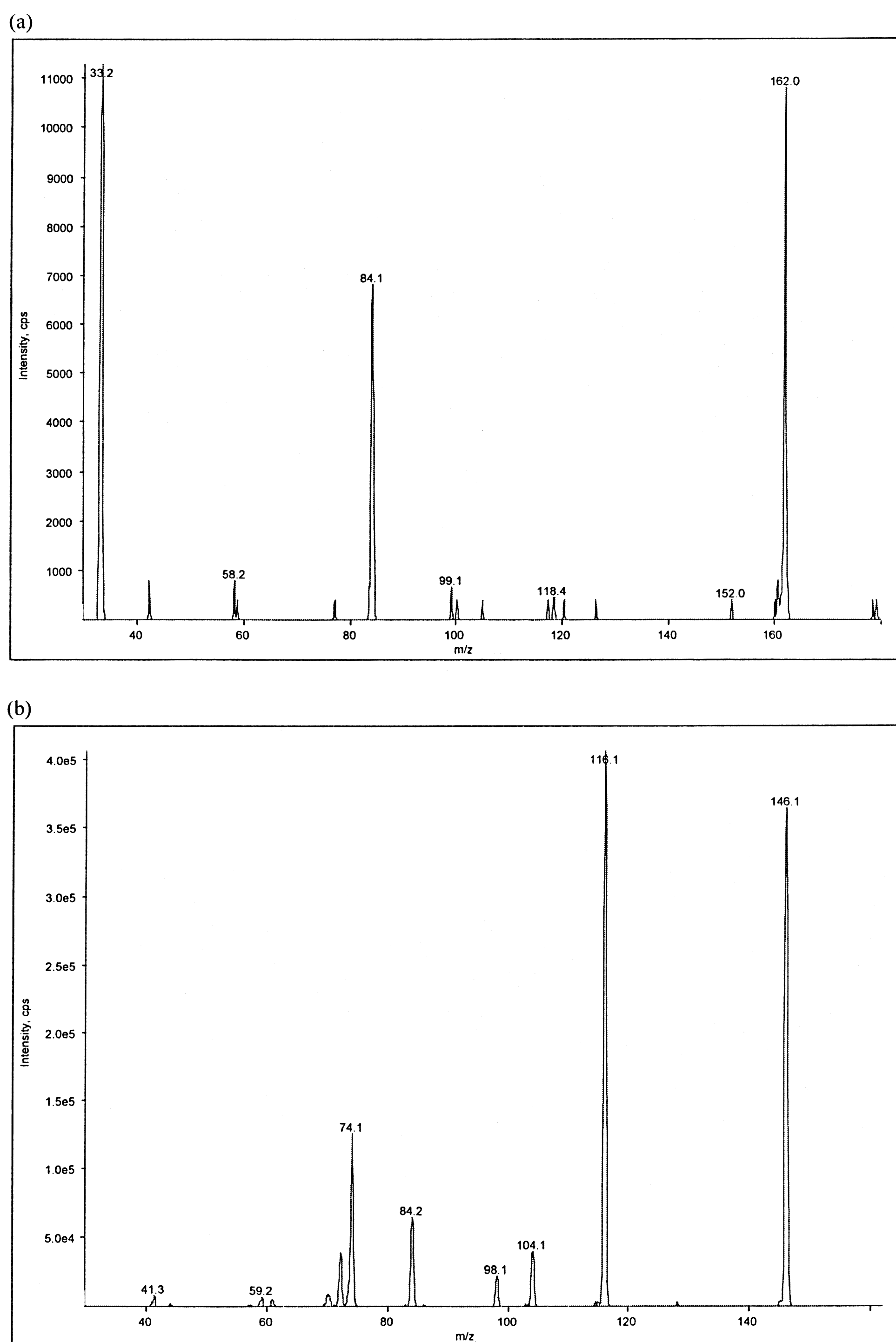


Fig. 1. (a) Product ions mass spectrum of *N*-acetylcysteine. (b) Product ions mass spectrum of *N*-acetylserine.

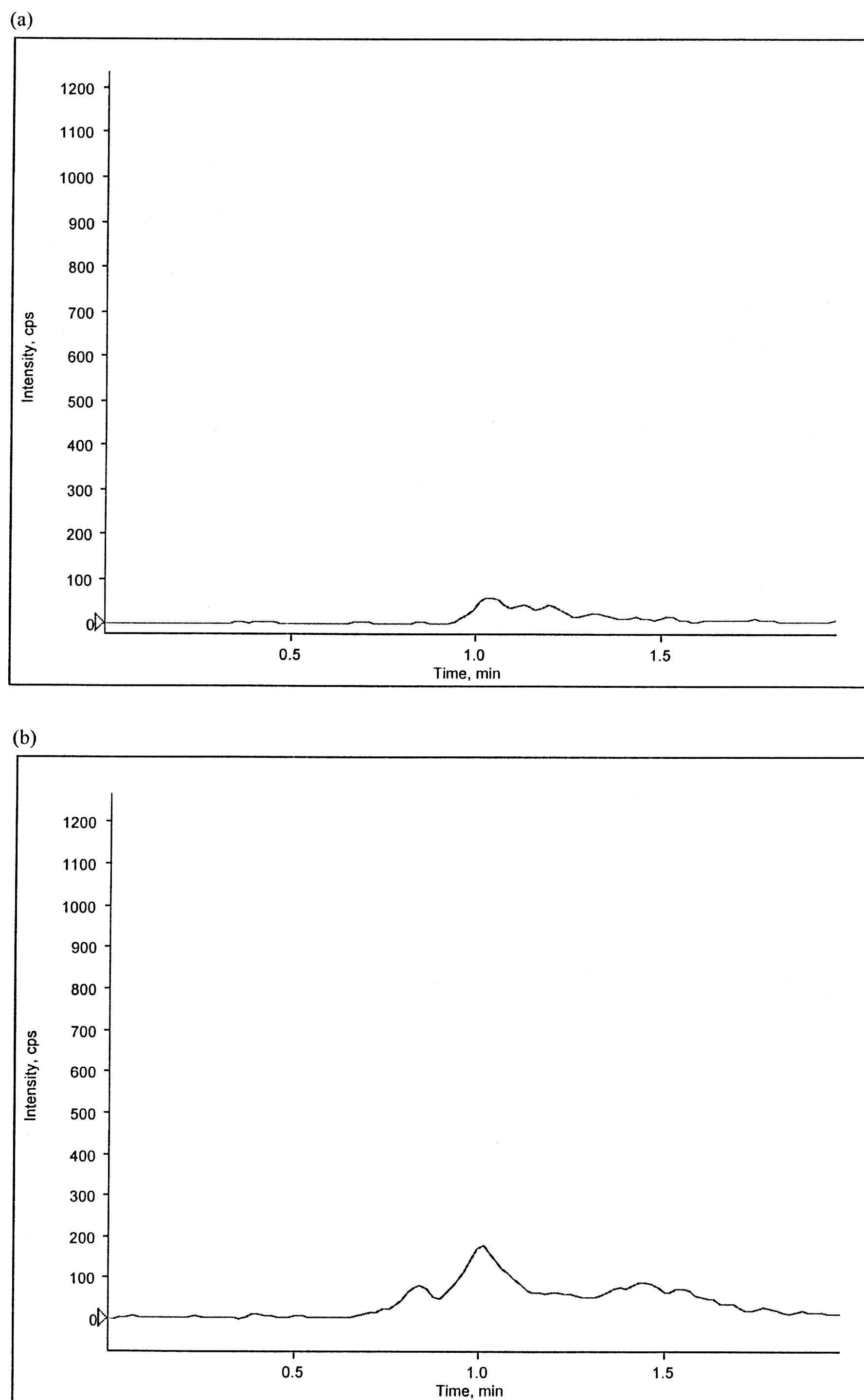
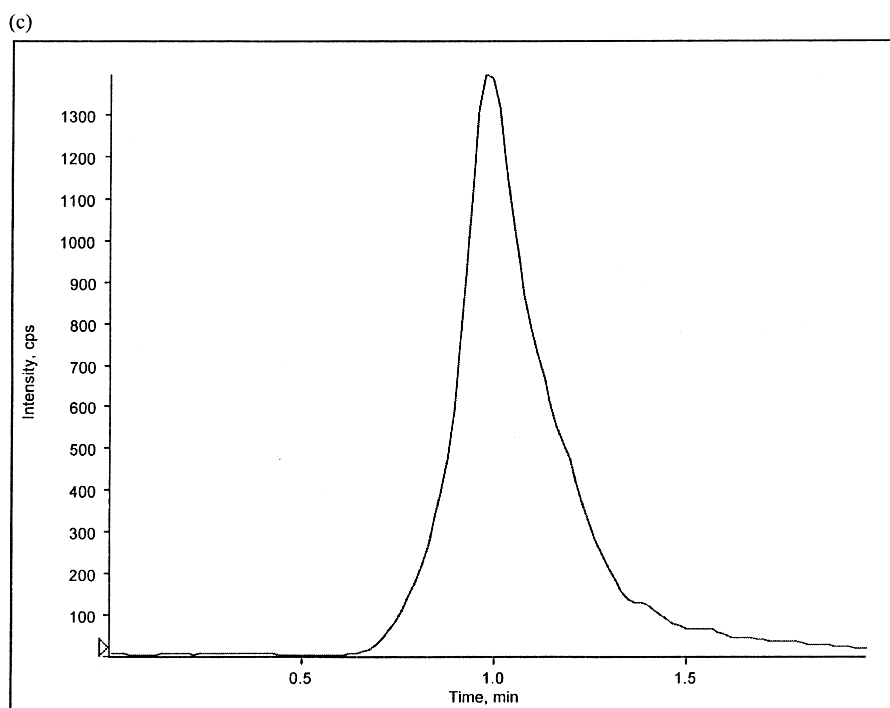


Fig. 2. Representative MRM chromatograms of *N*-acetylcysteine in human plasma: (a) blank human plasma, (b) blank human plasma containing 2 μg/ml of *N*-acetylserine (c) blank human plasma containing 50 ng/ml of *N*-acetylcysteine and (d) a sample from a volunteer (289.6 ng/ml) 0.5 h after oral administration of 200 mg of *N*-acetylcysteine.

(c)



(d)

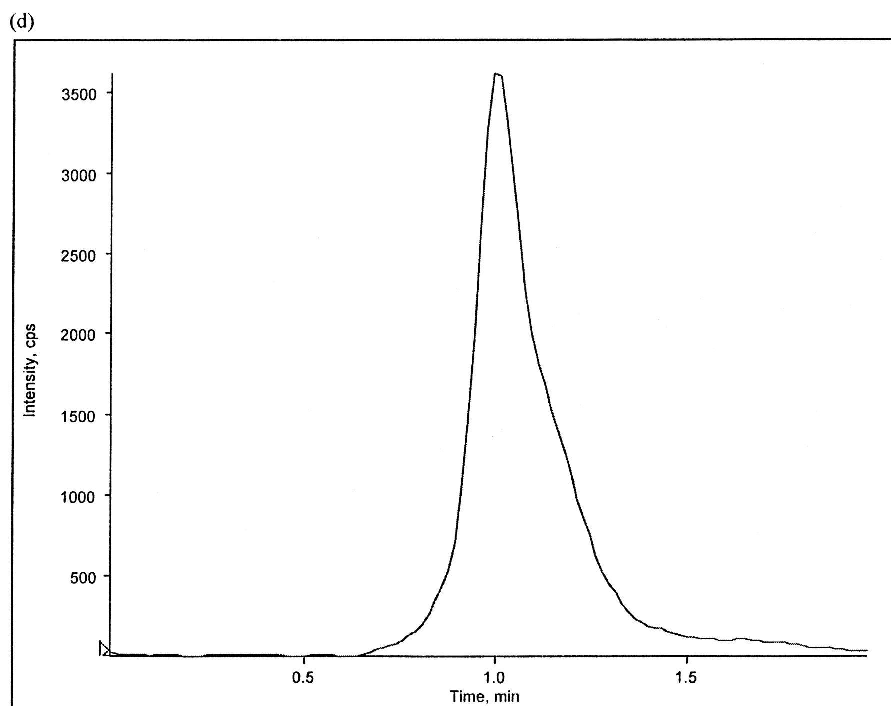


Fig. 2. (*continued*)

Table 1
Calibration curve statistics for *N*-acetylcysteine (back calculated concentrations)

Effective concentration (ng/ml)	Mean calculated concentration (ng/ml)	SD (ng/ml)	RSD (%)	RE (%)	*n*
50	50.15	1.80	3.58	0.31	7
100	100.41	5.93	5.91	0.41	8
200	195.88	10.10	5.16	−2.06	8
500	497.27	20.46	4.11	−0.55	8
1000	1019.28	59.94	5.88	1.93	8

Table 2
Intra-assay precision and accuracy for *N*-acetylcysteine

Effective concentration (ng/ml)	Mean calculated concentration (ng/ml)	SD (ng/ml)	RSD (%)	RE (%)	*n*
50	55.08	7.78	14.12	10.15	6
100	98.86	10.17	10.28	−1.14	6
500	490.84	14.08	2.87	−1.83	6

the calibration curve (see Section 3.3) corrects the contribution of the small interference of the endogenous NAC and the internal standard.

3.3. Validation results

Table 1 shows the accuracy and precision of the back calculates corresponding to eight standard curves. The assay was linear in the concentration range of 50–1000 ng/ml, the lower calibration points of 10 and 20 ng/ml being strongly affected by the basal levels of NAC. Precision was between 3.6% and 5.9% and the relative error was in the range of −2.1% and 1.9%. The mean slope was 113.7 ±28.4 (n=8), the mean intercept was 9603±2829 (n=8) and correlation coefficients (r) were between 0.9917 and 0.9993.

The precision and accuracy of this method were checked by calculating the intra- and the inter-assay variation at three concentrations (50, 100 and 500 ng/ml; concentrations of 10 and 20 ng/ml were not considered because they were outside the working range). Tables 2 and 3 show the accuracy and precision intra- and inter-assay, respectively. RSDs were in the range of 2.9% and 15.4% (LOQ) and relative errors (REs) were between −4.8% and 10.2%. The LOQ was established as 50 ng/ml, its accuracy and precision values being 10.2% and 15.4%, respectively. These results indicate that the method was reliable within the analytical range in spite of the absence of an internal standard.

The extraction recovery at 200 and 500 ng/ml was established by comparing the peak areas of NAC in plasma samples with the peak areas of reference samples. The NAC recoveries were 47% at 200 ng/ml and 43% at 500 ng/ml. As mentioned previously, NAC recovery was 2.5% at 2 μg/ml.

3.4. Stability

Dry residue and resuspended plasma extracts of NAC were found to be stable for 72 h at 4°C and for

Table 3
Inter-assay precision and accuracy for *N*-acetylcysteine

Effective concentration (ng/ml)	Mean calculated concentration (ng/ml)	SD (ng/ml)	RSD (%)	RE (%)	*n*
50	50.22	7.73	15.40	0.43	6
100	95.21	7.23	7.59	−4.79	6
500	496.25	44.17	8.90	−0.75	6

Table 4
Summary of stability studies for *N*-acetylcysteine

Stability study	% of C_0	
	Low concentration (50 ng/ml)	High concentration (500 ng/ml)
Autosampler conditions 24 h[a]	100.0	100.4
Dry residue 24 h	100.0	90.08
Dry residue 72 h	107.3	104.5
Freeze–thaw 1 cycle	103.6	125.6
Freeze–thaw 2 cycles	111.5	120.8
Freeze–thaw 3 cycles	114.5	138.4
One month in plasma below −20°C	152.4	106.9
Three months in plasma below −20°C	155.3	121.6

[a] Low concentration was 100 ng/ml.

24 h in the autosampler at room temperature respectively. The effect of the freeze and thaw cycles was studied, and a significant overestimation of the NAC concentration appeared after the first cycle, showing a certain tendency to increase with the number of cycles. This same effect was observed in the long term stability studies of NAC in plasma stored at below −20°C (see Table 4). The fact that the overestimation was observed both in the freeze–thaw cycles and in the long term stability in plasma below −20°C suggested that this phenomenon was probably due to the freeze–thaw process. In the long term stability study, samples were frozen and thawed once while the reference samples were processed immediately after preparation and did not show this overestimation effect. In the case of the analysis of real samples, this effect has been avoided by preparing a batch of calibration curves and quality controls that was stored together with the samples below −70°C. In this way, real samples, calibration curves and quality controls underwent the same number or freeze and thaw cycles. The values obtained in the analysis of the quality controls during the analysis of the real samples (see Table 5) indicated that the measured values were not overestimated.

3.5. Application

This validated analytical method has been used in the determination of around 800 plasma samples from a phase I clinical trial in which 24 healthy volunteers received 200 mg of NAC. The quality of the analytical data was evaluated by the calculation of precision and accuracy of the quality controls used during the study. Precision was in the range 8.7 to 13.4% and accuracy in the range −5.9 to 8.5%, expressed as RSD and RE, respectively (see Table 5). Fig. 2d shows the NAC transition (162>84) from a sample from a volunteer (289.6 ng/ml), 0.5 h after oral administration of 200 mg NAC.

4. Conclusions

The use of the LC–MS–MS allows for accurate, precise and reliable measurement of *N*-acetylcysteine

Table 5
Quality control precision and accuracy for *N*-acetylcysteine in real samples

Effective concentration (ng/ml)	Mean calculated concentration (ng/ml)	SD (ng/ml)	RSD (%)	RE (%)	*n*
75	70.59	9.46	13.40	−5.88	33
200	206.50	19.97	9.67	3.25	34
500	542.46	47.20	8.70	8.49	34

concentrations in human plasma. Validation results as well as quality control accuracy and precision in the analysis of real plasma samples demonstrated that the analytical method is rugged enough to be used for the determination of NAC in plasma samples from a clinical trial involving 24 volunteers, although no internal standard was used in the assay. NAS was tested as internal standard but it was not suitable due to its low recovery (2%). Even though the internal standard was not used in the quantitation, it was added in the analysis of the plasma samples from the clinical trial because the validation of the assay was carried out with it. As it has been demonstrated its interference over the quantitation of NAC was not significant. The assay method is specific due to the inherent selectivity of tandem mass spectrometry. Short run times were obtained by the use of the MS–MS technique: 90 samples that include plasma from four volunteers, calibration curves and QCs were analyzed in less than 4 h. In spite of the preparation procedure is the limiting factor to speed up the analysis of NAC, it is simple involving an extraction of the incubated plasma with ethyl acetate (plasma incubation with a reductive reagent to reduce all the oxidized forms of the NAC must be carried out whatever the sample treatment employed). This method avoids long derivatization steps pre- or post-column used in HPLC–UV or –fluorescence based methods and GC or GC–MS methods. An improvement in the sample preparation can be achieved by the use of an automated extraction method. The method described here is an example of the use of the LC–MS–MS technique in the direct analysis of small and ionic molecules in biological fluids. The low molecular mass of these kinds of molecules force one to use low mass ions in the multiple reaction experiment losing part of the high specificity typical of the MS–MS technique when used in molecules of higher molecular mass, especially in dirty matrix-like biological fluids. This effect has been compensated by the use of negative ions that introduced additional specificity to the analysis. Also reversed-phase chromatography of these small and ionic molecules is difficult and sometimes must be carried out using mobile phases incompatible with the mass spectrometer. The specificity obtained in this method allows one to simplify the chromatography of NAC because lower separation is required. To analyze NAC by LC–MS–MS requires a good relationship between all factors that have influence in the analysis: sample preparation, liquid chromatography and mass spectrometry conditions.

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