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DEVELOPMENT AND VALIDATION OF AN HPLC METHOD WITH POST-COLUMN DERIVATISATION FOR ASSAY OF *N*-ACETYLCYSTEINE IN PLASMA

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SUMMARY

A quantitative reversed-phase HPLC method has been developed that enables determination of both low endogenous and high therapeutic concentrations of *N*-acetylcysteine (NAC) in plasma. The compound is detected fluorimetrically after derivatisation with *ortho*-phthalaldehyde in the presence of a primary amine.

Validation of the method revealed injection and method repeatability were good. The linear range was adequate and the limit of quantification was between 0.4 and 0.6 μM. Recovery of *N*-acetylcysteine from plasma samples was also acceptable.

This method was applied to plasma samples from patients with a clinical septic shock who had received very high doses of *N*-acetylcysteine. Six samples were taken at different times after administration of *N*-acetylcysteine. The blood-concentration profiles obtained indicate the method is suitable for following the evolution of NAC in plasma under these conditions and can therefore be used for pharmacokinetic profiling.

INTRODUCTION

N-Acetylcysteine (NAC) (Fig. 1) is mainly used as a mucolyticum in bronchitis or pulmonary diseases. By depolymerising mucopolysaccharides it reduces the viscosity of pulmonary secretions [1]. Besides its mucolytic effect it also has anti-oxidant and anti-inflammatory effects, is used as an antidote in paracetamol poisoning [2–4], and can also be administered as adjunctive therapy to patients suffering from clinical septic shock,

although the benefit of adjunctive therapy with NAC remains controversial [5,6]. It is, therefore, important to be able to monitor the pharmacokinetic profile of NAC in plasma. NAC occurs in plasma in its intact form and in its oxidised forms. The oxidised forms can couple with small sulphides or to proteins [7].

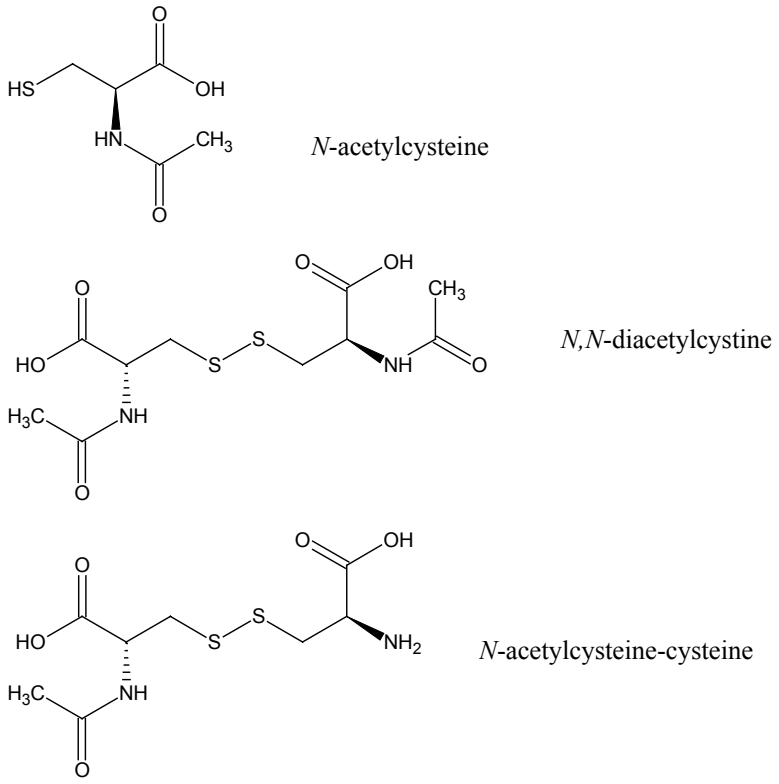
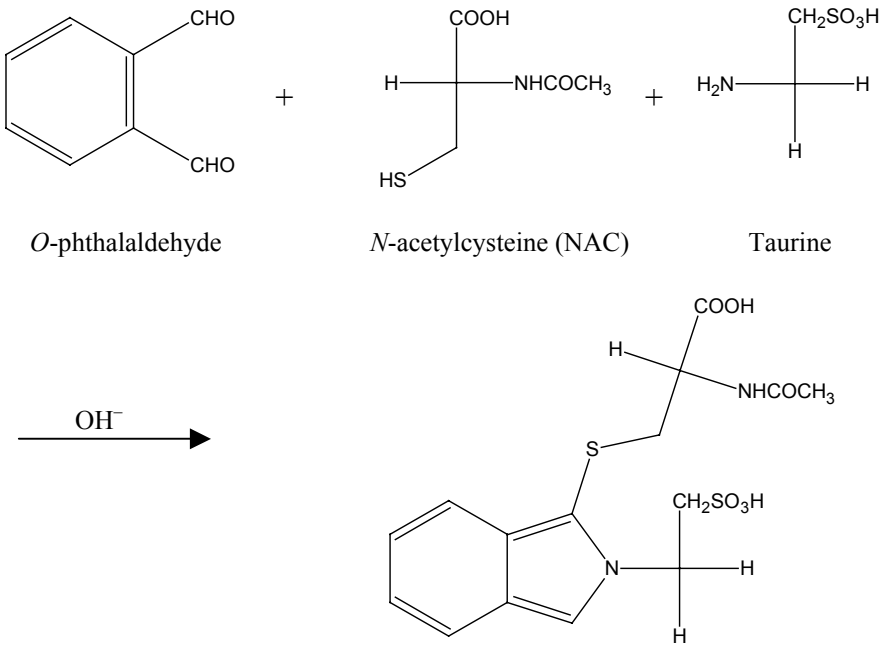


Fig. 1

Structure of *N*-acetylcysteine and some of its oxidised forms

NAC has been determined by a variety of methods, e.g. gas chromatography [8] and high-performance liquid chromatography (HPLC) [9–13]. NAC concentrations during its use as additional therapy in cases of clinical septic shock have not yet been investigated. A method of analysis that enables accurate quantification of NAC under these conditions was therefore needed. Determination of total plasma NAC concentrations was the aim of this study.

An HPLC method was developed that enabled determination of both low baseline concentrations of NAC of endogenous origin and the high therapeutic concentration levels occurring after infusion, to enable monitoring of the pharmacokinetics of the compound. A previously developed HPLC method with post-column derivatisation and fluorimetric detection for determination of total NAC concentrations [13] was used as the starting point. Alterations to this method, for example reduction of the amount of reagent and use of a different post-column reaction, were found necessary to work with small sample volumes (100 µL here compared with 500 µL in previous work [13]) and potentially high plasma concentrations. Fluorimetric detection was retained because of its high sensitivity. Because NAC itself cannot be detected fluorimetrically, post-column derivatisation was still needed. The latter entailed reaction of *ortho*-phthalaldehyde (OPA) with a primary amine (taurine in this work) and a thiol group-containing molecule (NAC) in a basic environment [14–18]. The reaction is displayed in Fig. 2.



O-phthalaldehyde *N*-acetylcysteine (NAC) Taurine

Fig. 2

Chemical reaction performed for assay of *N*-acetylcysteine

EXPERIMENTAL

Apparatus

HPLC was performed with a Merck–Hitachi (Tokyo, Japan) L-6000 pump (pump 1), a Rheodyne (Cotati, CA, USA) manual injector with 100-µL injection loop, a 50 mm × 4.6 mm i.d., 10 µm particle, LiChrosorb RP-18 guard column (Merck, Darmstadt, Germany), and a 250 mm × 4.6 mm i.d., 5 µm particle, LiChrosorb RP-18 analytical column (Merck) (Fig. 3). The outlet of the column was connected by a low dead volume T-coupling (Swagelok, Solon, OH, USA) to another Merck–Hitachi L-6000 pump (pump 2) to enable addition of *o*-phthalaldehyde (OPA) in borate buffer (BB). The third limb of the T-piece was connected to a second T-coupling to enable addition of taurine (TAU) in borate buffer by means of a third Merck–Hitachi L-6000 pump (pump 3). The third limb of this last T-coupling was connected to a Merck–Hitachi F-1050 fluorescence detector via a stainless steel reaction coil (8 m × 0.25 mm i.d.) to accommodate the derivatisation reaction. The pumps for delivery of the post-column derivatisation reagents were pulse-damped by means of 30 mm × 4.6 mm i.d. stainless steel columns packed with LiChrosorb RP-18, 10 µm particle, placed between the pump and the T-couplings. A Merck–Hitachi D-2500 chromato-integrator was used for data acquisition and treatment.

Chemicals and Reagents

N-Acetylcysteine was purchased from Sigma (Steinheim, Germany). Dithiotreitol (Sigma) was used to reduce the oxidised NAC, which is coupled as the disulphide. $HClO_4$ (Sigma) was used to precipitate the plasma proteins. *N*-(1-pyrenyl)maleimide (PMI) was initially tested in the post-column derivatisation reaction, as described elsewhere [13]. OPA (Sigma) and TAU (Fluka, Buchs, Switzerland) were used in the post-column reaction that was finally selected.

To prepare carbonate buffer appropriate quantities of sodium carbonate (Na_2CO_3) and sodium bicarbonate ($NaHCO_3$) (Merck) were mixed. For phosphate buffers sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$), disodium hydrogen phosphate ($Na_2HPO_4.2H_2O$), and trisodium phosphate ($Na_3PO_4.12H_2O$) from Merck were used. Borate buffer was prepared by dissolving sodium tetraborate ($Na_2B_4O_7$) (Merck) and adjusting the pH with sodium hydroxide (NaOH) (Carlo Erba, Val de Reuil, France). Methanol (MeOH) and acetonitrile (ACN) were HPLC grade from BDH (Poole, UK). Water for solutions was made in-house with a Milli-Q system (Millipore, Milford, MA, USA).

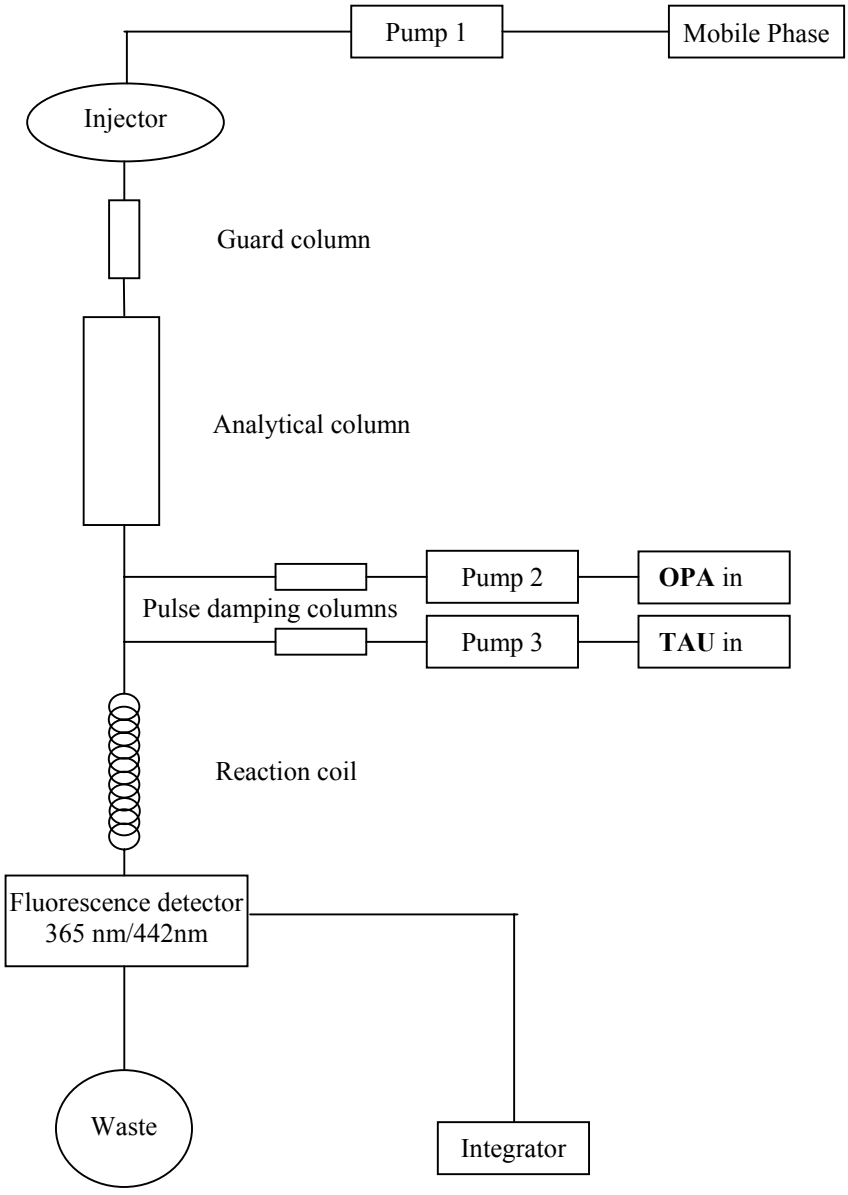


Fig. 3

Instrumentation. OPA, ortho-phthalaldehyde; TAU, taurine; BB, borate buffer

Solutions and Chromatographic Conditions

The mobile phase was 1% acetonitrile (ACN) in phosphate buffer pH 3.0, ionic strength (μ) 0.01. The flow rate was 1.0 mL min^{-1}.

The derivatisation reagents were 10^{-3} M OPA in 0.05 M borate buffer pH 11 and 10^{-3} M TAU in 0.05 M borate buffer pH 11. For these reagents the flow rates were 0.5 mL min^{-1}. The reaction product was measured at 442 nm after excitation at 365 nm.

Sample Pretreatment

NaOH (0.1 M, 150 μL) and dithiotreitol (DTT; 0.05 M, 40 μL) were added to 200 μL sample or standard to release oxidised NAC from its bound forms. The solution was homogenised and placed in a warm water bath at 37°C for 30 min. $HClO_4$ (1.7 M, 100 μL) was then added to precipitate the proteins. The mixture was vortex mixed, centrifuged at 3000 rpm for 10 min, and the supernatant was immediately transferred to a new vial. The injection volume was 100 μL for both standards and samples; occasionally samples were diluted with Milli-Q water if the concentration of NAC was outside the linear range.

Standards for Quantitative Analysis

Standard solutions were spiked plasma standards prepared by diluting ten times more concentrated aqueous NAC standards with plasma. The concentrations prepared were 5×10^{-7}, 1×10^{-6}, 3×10^{-6}, and 5×10^{-6} M. Blanks were water diluted tenfold with plasma.

Spiked Samples

Spiked plasma samples used for method development and validation were prepared by spiking plasma with aqueous NAC standards as explained above and leaving the NAC to bind to plasma proteins and small sulphides for at least 5 h at room temperature or under refrigeration. They were then frozen at −18°C until analysis.

Samples of Patient Plasma

Patients were included in the study within 4 h of diagnosis of septic shock. They received NAC as a 150 mg kg^{-1} bolus followed by continuous infusion of 50 mg kg^{-1} for 4 h. Blood samples were taken at baseline and 2, 4, 6, 24, and 48 h after the start of infusion. The plasma

samples were diluted two or four times, depending on the expected NAC content, before sample pretreatment.

RESULTS AND DISCUSSION

Development and Optimisation

Initially, post-column derivatisation of NAC with pyrenemaleimide (PMI; 5×10^{-6} M in acetonitrile, 0.2 mL min^{-1}) in a basic environment (0.05 M borate buffer, pH 11, 1.0 mL min^{-1}) was tested, using fluorimetric detection with λ_{ex} = 342 nm and λ_{em} = 389 nm [13]. The reaction was found to be too slow and furnished peaks which were too small. Increasing the length of the reaction coil from 4 to 8 m did not lead to sufficient improvement, neither did changing the buffer (from borate to carbonate), the ionic strength of the buffers, the flow rates of the reagents, or the concentration of PMI.

Post-column reaction with OPA as derivatisation reagent and TAU as primary amine was therefore introduced. This reaction is, for example, also used for determination, by flow-injection analysis, of primary amines and of secondary amines of amides, after transformation to primary amines [18,19]. The thiol group needed for the reaction is provided by NAC (Fig. 1). Fluorimetric detection was performed at λ_{ex} = 365 nm and λ_{em} = 442 nm.

Table I

Peak heights and precision for on-line reaction using different coil lengths

Coil length (m)	Concn NAC (M)	Height (n = 6)	*RSD* (%)
4.2	10^{-6}	158968	1.06
	10^{-7}	13157	4.26
	10^{-8}	nd[a]	–
8	10^{-6}	217903	0.94
	10^{-7}	26910	5.41
	10^{-8}	7328	17.78
10	10^{-6}	208443	0.73
	10^{-7}	13972	3.84
	10^{-8}	nd	–

[a]Not determined

On-line reaction with OPA and taurine (experimental set-up without guard and analytical columns) enabled easy detection of 10^{-8} M NAC in aqueous solution. To evaluate the time needed for the on-line reaction, reaction coils 4.2, 8, and 10 m long were tested. The results can be found in Table I. An 8-m length yielded the highest peaks, indicating the best compromise between reaction time and the diffusion in the reaction coil, and was therefore used throughout the remaining experiments. The repeatability for 10^{-8} M and 10^{-7} M concentrations of NAC was bad (Table I), because variations among injections were high.

When the analytical column was introduced into the system the retention time of NAC was approximately 9.5 min when using the mobile phase reported elsewhere [13], i.e. phosphate buffer, pH 2.0, $\mu = 0.01$, containing 10^{-4} M EDTA and 1% (*v*/*v*) ACN. Peak areas for the 10^{-6} M solution from the on-line reaction and from the system with the column were comparable.

For sample pretreatment a reaction time of 30 min at 37°C [20] was found to give higher NAC peak areas than 20 min at room temperature [13] for a one-day old aqueous NAC mixture in which formation of the dimer is expected. A concentration of 0.03 M DTT [13] was found to be too low for some plasma samples – the NAC peak was smaller than expected and those from excess DTT almost disappeared. Concentrations above 0.05 M gave rise to additional peaks (in blank plasma also) some of which eluted close to the NAC peak and interfered with its quantitative determination. A DTT concentration of 0.05 M was therefore chosen for further experiments.

The precision of determinations in plasma (repeatability and time-variable intermediate precision) in the linear range were bad when using the mobile phase reported elsewhere [13], because DTT gives rise to several interfering peaks (reaction products) after the sample pretreatment step. One of these peaks co-eluted with the NAC peak, although did not always occur. This peak was best observed in aqueous solutions of DTT undergoing sample pretreatment. In plasma this peak varied between samples and caused the bad precision that was observed. The mobile phase was therefore changed to enable separation of these peaks. Solvent strength (ACN concentration), selectivity (MeOH as organic modifier), and mobile phase buffer pH were changed, and EDTA was removed. Table II shows the results obtained from the different mobile phases tested to solve this problem. In the first instance mobile phase containing 2.5% ACN, buffer pH 3.0, and $\mu = 0.01$ was preferred, because analysis time could then be

Table II

Retention times (min) for peaks eluting near the retention time of NAC, measured in different solutions using several mobile phases

% ACN, buffer pH 2.0, $\mu = 0.01$			% MeOH, buffer pH 2.0, $\mu = 0.01$			% ACN, buffer pH 3.0, $\mu = 0.01$		
10%	5%	2.5%	12.3%	6.15%	3.1%	5%	2.5%	1%
Blank + DTT								
4.48	5.66					3.94	4.20	4.79
4.84	6.05	7.34	5.40	6.85	8.07	6.10	7.33	8.68
10^{-6} M NAC								
4.42	5.64	7.02	5.18	6.68	7.96	4.70	5.61	6.66
Mixture NAC + DDT								
4.44	5.64	7.03	5.19			3.90	4.19	4.80
4.84	6.04	7.31	5.36	6.73	8.00	4.67	5.59	6.64

limited to 20 min and the NAC peak was separated from the DDT reaction products. In analysis of plasma samples, however, NAC eluted too much in the tail of the elution peak, so mobile phase containing 1% ACN, buffer pH 3.0, and $\mu = 0.01$ was finally used. The run time with this mobile phase was approximately 30 min. Representative chromatograms obtained from blank plasma and from a plasma sample spiked with 5×10^{-6} M NAC are given in Fig. 4.

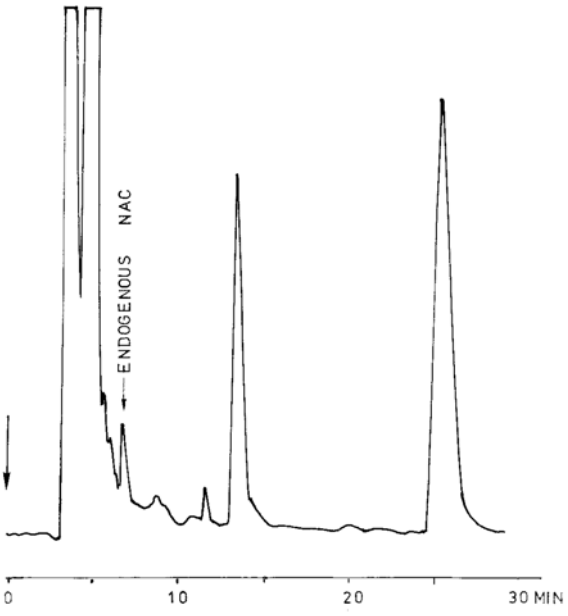
Method Validation

Repeatability

Relative standard deviations were determined for the on-line reaction (without guard and analytical columns) and for replicate injection (with guard and analytical column) of aqueous NAC solutions. The results can be seen in Table III. As already mentioned, the repeatability of the on-line reaction was bad for 10^{-7} and 10^{-8} M NAC solutions. It can be stated that 10^{-8} M can be regarded as the detection limit for the on-line reaction.

With regard to the repeatability of injections when the column was introduced, acceptable variance was observed for 5×10^{-7} and 10^{-6} M concentrations. Four different additional concentrations (2.5×10^{-7}, 7.5×10^{-7}, 2.5×10^{-6}, and 5×10^{-6} M) of aqueous NAC standards were measured at this stage and visual evaluation indicated linearity in this range; linearity studies are discussed more thoroughly in the next section.

(a).



(b).

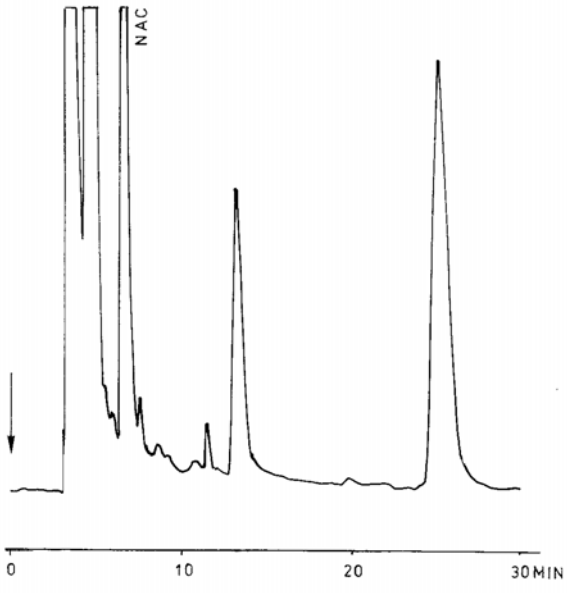


Fig. 4

Chromatograms obtained from (a) blank plasma and (b) plasma spiked with 5×10^{-6} M NAC

Table III

Repeatability results for: (a) the on-line reaction (without guard and analytical columns); (b) injection (with columns) of aqueous NAC solutions; and (c) injection and complete method for plasma sample

Concn NAC (M)	Area (n = 6)	Height (n = 6)
	RSD (%)	*RSD* (%)
On-line reaction		
5×10^{-6}	1.00	0.66
1×10^{-6}	1.60	0.99
1×10^{-7}	4.55	2.37
1×10^{-8}	16.43	16.58
Injection (with columns) of aqueous NAC solutions		
5×10^{-7}	2.47	0.86
1×10^{-6}	1.91	2.10
Injection of plasma sample		
1×10^{-6}	2.25	1.94
Complete method		
1×10^{-6}	2.93	1.73

When the repeatability of injections of plasma samples was first investigated it was seen that peak area decreased as a function of time (Fig. 5), probably because of adsorption of NAC by the protein sediment in the plasma. It was observed that this adsorption increased (peak areas decreased) with increasing contact time between supernatant and sediment. In the method we therefore chose to separate the supernatant from the sediment immediately after centrifugation. When repeatability was re-tested (Table III) the repeatability for injections of the plasma sample and for the complete method were comparable with those obtained for aqueous NAC samples. For comparison it can be mentioned that the repeatability (inter-assay precision) of the method described in Ref. [13] was 6.8% for a concentration of 5.6×10^{-6} M. It can therefore be concluded that the repeatability of this new method is substantially better than that described previously.

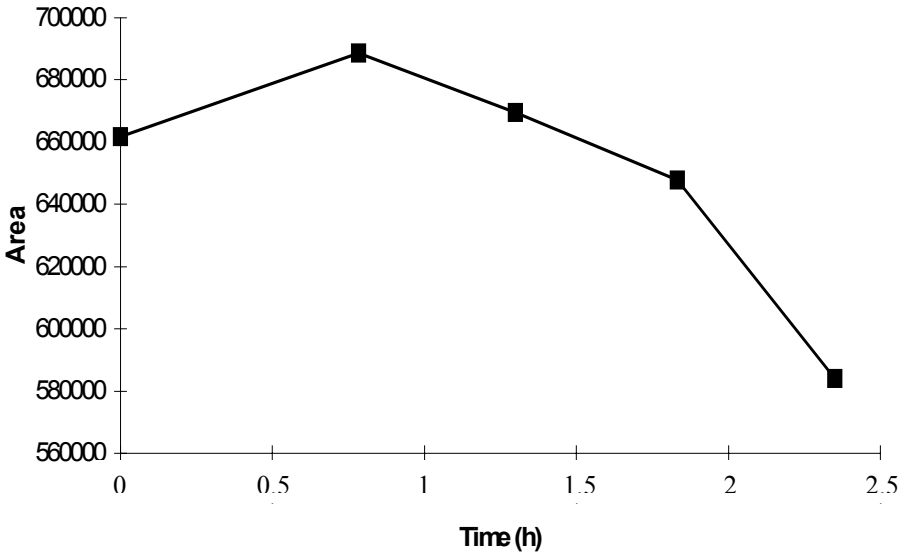


Fig. 5

Decrease of peak area as a function of time when the supernatant was not separated from the sediment

Linearity

Linearity was examined in the ranges 10^{-6}–10^{-5} M, as in Ref. [13], and 10^{-7}–10^{-6} M. The method was found to be linear for concentrations ranging from 10^{-7} to 5×10^{-6} M, although heteroscedasticity of the results was observed in the linear range. It is therefore recommended that weighted least-squares regression is used to estimate the calibration lines, to increase the reliability of the estimates. A linear relationship was found between concentration and standard deviation; this enables use of the variances for different standards in the weighted regression [21].

Limit of Quantification, LOQ

Table IV shows that the *LOQ* is between 0.4 and 0.6 μM; this was confirmed by applying the criteria described in Ref. [22]. Recovery of NAC from plasma samples was acceptable above the *LOQ*.

Recovery from Samples Containing Large Concentrations of NAC

Recovery was determined for samples spiked at a concentration above the highest in the calibration plot. This test was performed because

Table IV

Determination of *LOQ*, recovery, and the between-day precision for different concentrations

Concentration (μM)		*n*	Recovery (%)	*RSD* (%)
Added	Found			
0.1	0.130	6	129.6	53.4
0.2	0.153	6	76.3	25.2
0.4	0.408	6	102.0	21.8
0.6	0.590	6	98.2	5.2
1	0.921	6	92.1	4.0
3	3.42	6	113.8	10.2
4	4.09	6	102.3	10.5

concentrations up to 2.7×10^{-3} M were found in samples from patients and no information was available about recovery at these concentrations. Samples in the range 1×10^{-5} to 5×10^{-3} M were examined. The results are displayed in Table V. By analogy with treatment of samples from patients, these samples also were diluted two- or fourfold before sample pretreatment. Otherwise there would not have been sufficient DTT to release NAC from its bound forms. Further dilution to a concentration in the calibration range was performed before injection, as already described. For this concentration range recoveries from plasma were acceptable. The results in Tables IV and V indicate that the method enables determination of both low endogenous NAC concentrations and the high plasma NAC concentrations obtained after infusion.

Table V

Recovery and precision for high plasma concentrations of NAC

Concentration added (μM)	Total dilution factor	*n*	Recovery (%)	*RSD* (%)
10	4	6	107.5	13.4
50	20	6	105.9	11.5
100	40	6	103.4	11.7
500	200	6	83.8	12.2
1000	400	6	90.5	13.7
5000	2000	6	84.3	18.3

Analysis of Plasma Samples from Patients

Plasma samples from 22 patients were examined. Twelve patients received an infusion of NAC whereas ten others served as a control group. For each patient, samples were taken at six different times – at baseline and 2, 4, 6, 24, and 48 h after the start of infusion. Only the total NAC concentration was determined, because the samples consisted of untreated plasma (to determine the different forms of NAC occurring in plasma special sampling is required). Concentrations were calculated from a calibration curve between 5×10^{-7} and 5×10^{-6} M. The concentrations found in the plasma of the 22 patients are shown in Fig. 6. The concentrations of NAC in the ten control samples remained at baseline whereas for the 12 patients who received NAC the blood concentration profile as a function of time could be determined. Medical interpretation of these profiles is discussed elsewhere [5,23].

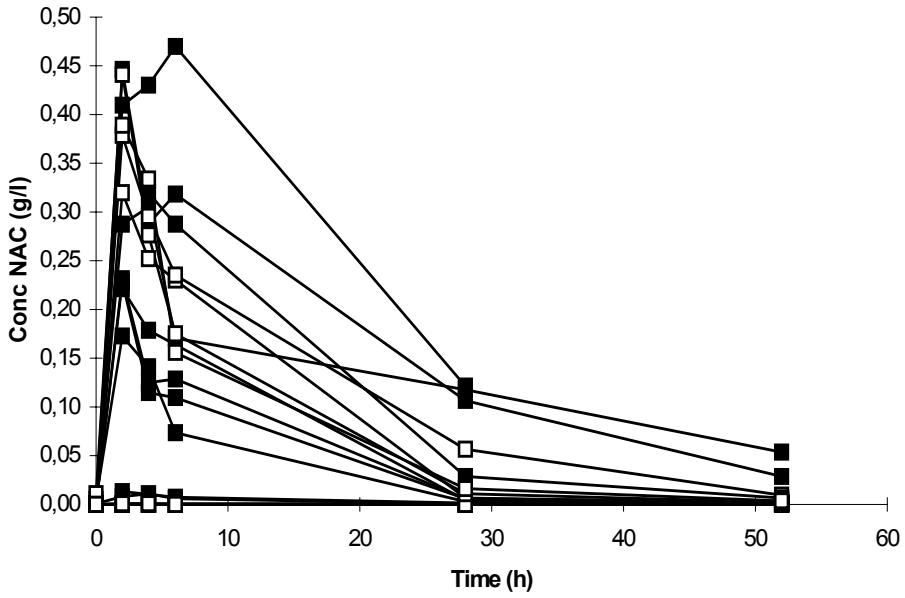


Fig. 6

Concentrations of NAC found in the plasma of the 22 patients

CONCLUSION

A previously developed HPLC method has been used as the starting point in the optimization of a quantitative method for assay of total concentrations of NAC in plasma. Modifications were needed to enable work with smaller sample volumes. It was also necessary to change the post-column reaction and mobile phase composition.

Validation of the method revealed precision was good. A linear range with a limit of quantification between 0.4 and 0.6 μM was found, which made it possible to use the method for quantitative determinations. Recovery from plasma samples was acceptable. The procedure could also be applied, after dilution, for concentrations outside the linear range, because recoveries were comparable with those in the linear range. This also means the method can be used to determine both low endogenous plasma concentrations and the high concentrations obtained after infusion of *N*-acetylcysteine.

The method was applied to plasma from patients treated with NAC for clinical septic shock. The method was found to be useful for pharmacokinetic profiling of NAC.

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