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High-performance liquid chromatography assay for *N*-acetylcysteine in biological samples following derivatization with *N*-(1-pyrenyl)maleimide

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Abstract

N-Acetylcysteine is a thiol antioxidant with expanding clinical importance. A sensitive, rapid method for determining reduced *N*-acetylcysteine (NAC) concentration in biological samples has been developed which uses a modified reversed-phase high-performance liquid chromatography (HPLC) technique in conjunction with the derivatizing agent *N*-(1-pyrenyl)maleimide (NPM). The NAC–NPM adduct was analyzed by HPLC with fluorescence detection. The calibration curve for NAC was linear over the range 8–2500 n*M* and the coefficient of variation obtained for the within-run precision and the between-run precision for 0.5 m*M* NAC was 1.5% and 2.7%, respectively. Relative recovery of NAC from biological materials ranged between 86% and 96% and the limit of quantitation from biological samples was 32 n*M*. These results suggest practical advantages relative to other widely-accepted methods of NAC measurement.

Keywords: *N*-Acetylcysteine; *N*-(1-Pyrenyl)maleimide

1. Introduction

Clinical applications for the thiol antioxidant NAC have expanded in recent years as the importance of oxidative mechanisms to various mammalian disease processes has become better understood. Direct relationships exist between depletion of L-γ-glutamyl-L-cysteinylglycine (GSH), the principal intracellular thiol, and immunosupression, xenobiotic-mediated hepatotoxicity, apoptic cell death, and respiratory dysfunction [1]. NAC's ability to indirectly replenish GSH is well-known and appears to stem from both its capacity to act as precursor to intracellular cysteine and its tendency to increase the activity of enzymes like GSSG reductase [2]. NAC's impact on GSH metabolism, combined with a low toxicity profile with a forty year history of safe adult and pediatric administration, have led researchers to investigate its role in illnesses as diverse as acquired immune deficiency syndrome (AIDS), adult respira-

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tory distress syndrome (ARDS), and heavy-metal poisoning [3]. Consequently, the need for a method that can make accurate, convenient measurements of NAC has intensified.

Several methods have been proposed for the measurement of NAC, but they have practical limitations in terms of sensitivity or time-consuming procedures. Prior investigations conducted in our laboratory resulted in the development of a new HPLC method for measuring biological thiols which incorporated the derivatizing agent *N*-(1-pyrenyl)maleimide [4]. NPM readily reacts with free sulfhydryl groups to form fluorescent derivatives (Fig. 1). This method afforded substantial improvements in rapidity of analysis, sensitivity, stability and ease of use when compared to existing methods for GSH, cysteine and homocysteine quantitation in biological samples. The purpose of the current study is to extend our previous work with biological thiols in order to establish the utility of NPM derivatization for the measurement of NAC. Preliminary pharmacokinetic studies in Sprague–Dawley rats were included to demonstrate the potential relevancy of this approach in biological materials.

2. Experimental

2.1. Reagents and chemicals

Acetonitrile, methanol, acetic acid, water, phosphoric acid (all HPLC grade), dithiothreitol (DTT), disodium EDTA, and hydrogen peroxide (H_2O_2) were purchased from Fisher (St. Louis, MO, USA). NPM was obtained from Aldrich (Milwaukee, WI, USA) and NAC and cysteine were purchased from Sigma (St. Louis, MO, USA).

2.2. Animals

All experiments were performed with adult male Sprague–Dawley rats weighing 200–250 g that were purchased from Charles River Labs (Wilmington, MA, USA). The rats were housed in stainless steel cages in a temperature controlled (25°C) room equipped to maintain a 12 h light–dark cycle and allowed standard rat chow (Purina rat chow) and water ad libitum. Six rats were anaesthetized with metofane obtained from Pitman–More (Mundelein, IL, USA) and intraperitoneal injections of NAC (500 mg/kg) were administered. After 15 min had elapsed, the animals were reanaesthetized with metofane and blood samples were collected via intracardiac puncture. The animals were then sacrificed and samples of liver, brain and kidney were obtained. The liver and kidney samples were trimmed of extraparenchymal tissue, then liver, brain and kidney samples were minced and homogenized on ice. Immediately after homogenization, samples were derivatized with NPM for HPLC analysis.

Urine was collected 30 min after NAC administration directly into vials containing phosphoric acid

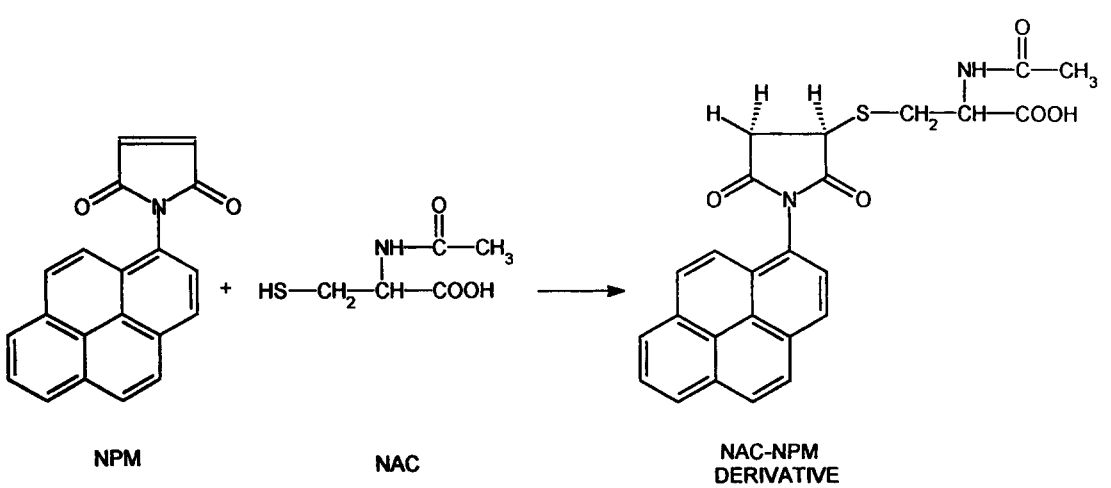

Fig. 1. Formation of fluorescent NAC–NPM adduct.

and EDTA to avoid oxidation of thiols. Urine samples were kept at −70°C until derivatization with NPM.

2.3. HPLC system

The HPLC system (Shimadzu) consisted of of a Model LC-10A pump, a Rheodyne injection valve with a 20-μl filling loop and a Model RF 535 fluorometer operating at an excitation wavelength of 330 nm and an emission wavelength of 380 nm. The HPLC column (Astec, Whippany, NJ, USA) is 100× 4.6 mm I.D. and was packed with 3 μm particles of C_{18} packing material. Quantitation of the peaks from the HPLC system was performed with a Chromatopac Model CR601 integrator (Shimadzu). The mobile phase was water–acetonitrile (50:50, v/v) and was adjusted to a pH of 3.75 through addition of 0.1 ml of acetic and phosphoric acids. The NPM derivatives were eluted from the column isocratically at a flow-rate of 0.45 ml/min.

2.4. Assay procedures

2.4.1. Calibration and NAC relative recovery

A calibration curve representative of the quantities of NAC commonly found in biological samples was constructed by injecting NPM-derivatized standards in a volume of 100 μl. Linearity was obtained over the full range 8–2500 n*M* (r=0.999). Relative recovery was determined by spiking serum with five biologically relevant concentrations of NAC and comparing the results to those obtained from aqueous samples supplemented with the same concentrations.

2.4.2. Dithiothreitol (DTT) reductions

Oxidized NAC (NAC_2) was obtained by reacting 0.5 ml H_2O_2 (3%) with 5 ml reduced NAC (1 m*M*) at room temperature. After 5 min had elapsed, a sample was derivatized with NPM and injected onto the HPLC system to confirm the absence of an NAC peak. Newly oxidized NAC was then treated with 9:1 (v/v) DTT (50 m*M*) and the mixture was incubated at 37°C for 30 min. The resulting reduced NAC was also derivatized with NPM and subjected to HPLC analysis. Reduced NAC and total NAC can thus be determined by this method.

2.4.3. Sample derivatization

Serum, urine and homogenates of liver, kidney and brain tissue from Sprague–Dawley rats were derivatized with NPM, which reacts with free sulfhydryl groups to form fluorescent derivatives. Acetonitrile (250 μl) and 500 μl of 1.5 m*M* NPM solution in acetonitrile was added to diluted samples (250 μl). The resulting solution was mixed and then incubated at room temperature for five min. At the conclusion of the incubation period, 10 μl of 50% (v/v) acetic acid was added to stop the reaction. Filtration through a 0.2-μm acrodisc was performed and the derivatized samples were injected onto a 3 μm C_{18} column in a reversed-phase HPLC system.

2.5. Protein determination

The Bradford method was used to determine the protein content of the cell samples [5]. Concentrated Coomassie Blue (Bio-Rad) was diluted 1:5 (v/v) with distilled water. A 5-ml volume of the diluted reagent was added to 0.1 ml of a bovine serum albumin standard solution which contained 10–100 μg from a 1 mg/ml stock solution. The mixture was incubated at 37°C for 5–10 min and the absorbance was measured at 595 nm. The homogenized samples were subjected to appropriate dilutions (1:100 for liver, 1:100 for kidney and 1:20 for brain) and 0.1 ml of each sample was used for the protein assay.

3. Results

In situ derivatization of biological samples with NPM produced an NPM–NAC adduct which was rapidly separated by the HPLC system employed for this study. The chromatogram pictured in Fig. 2A confirms that adequate separation of the NPM–NAC adduct from both hydrolysis and NPM–cysteine (NPM–cys) peaks. Chromatograms prepared from kidney (Fig. 2B) and liver (Fig. 2C) tissue samples further illustrate the well-defined separation of the NPM–NAC and NPM–cys adduct peaks achieved by this method. Retention times for NAC and cys were 4.78 and 5.35 min, respectively.

3.1. Calibration curves

A calibration curve was constructed by plotting

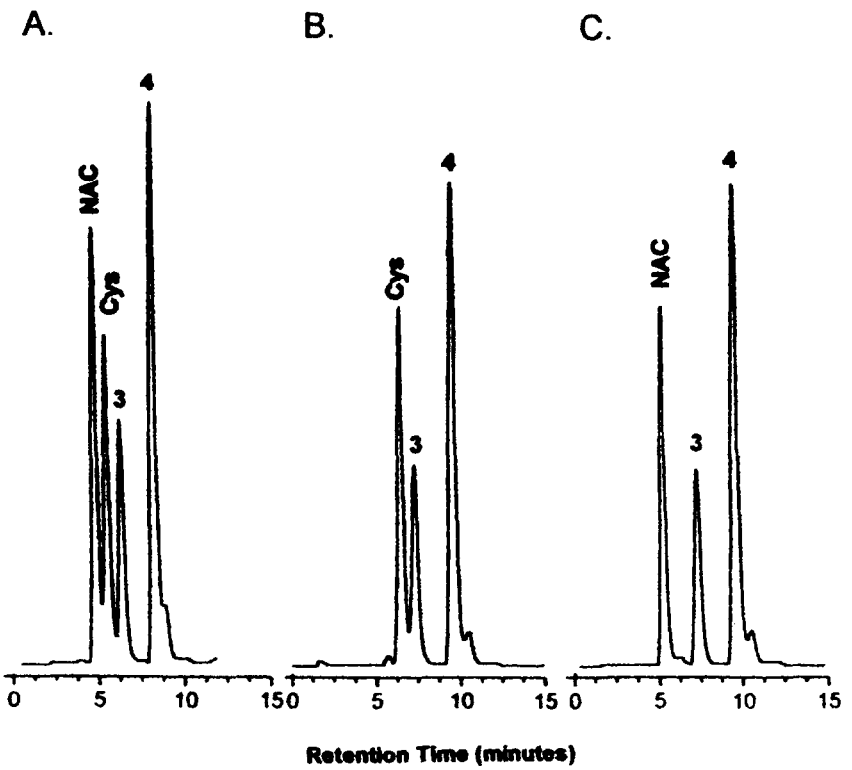

Fig. 2. (A) Standard chromatogram containing peaks from both the NPM–NAC (500 n*M*) and NPM–cys adducts. (B) Chromatogram obtained from kidney tissue (no NAC peak). (C) Chromatogram showing 425 n*M* NAC peak from liver tissue. Peaks 3 and 4 labelled in each chromatogram represent NPM-derivatized hydrolysis products.

integrated peak areas versus NAC concentrations at regular intervals. Linearity was displayed for NAC concentrations ranging from 8 to 2500 n*M*. The regression line (r=0.999) passed through the origin.

3.2. Sensitivity, stability, reproducibility and relative recovery

The lower detection level for NAC was established at 8 pmol/sample (0.2 pmol on-column). Tissue, serum and urine samples derivatized with NPM and maintained at 4°C remained stable for at least two weeks. The coefficient of variation (C.V.) for within-run precision was 1.5%, while the C.V. for between-run precision was 2.7%. Relative recovery experiments were performed by spiking serum samples with known concentrations of NAC (Fig. 3) and comparing the results to chromatograms from a standard curve using the same NAC concentration. The mean relative recovery of five separate experiments was 90.6%.

3.3. Results of biological sample analysis

Table 1 shows NAC concentrations in samples of serum, liver, and brain tissue taken from Sprague–Dawley rats. The rats were sacrificed and the samples were obtained 15 min after receiving intraperitoneal injections of 500 mg/kg NAC. The concentration of NAC in serum samples was higher than in tissue homogenates.

3.4. NAC_2 results

NAC_2 is also found in biological samples [3]. In order to obtain an accurate representation of total NAC, the NAC_2 present in the sample must be reduced by DTT prior to derivatization with NPM. A chromatogram showing the large NPM–NAC adduct

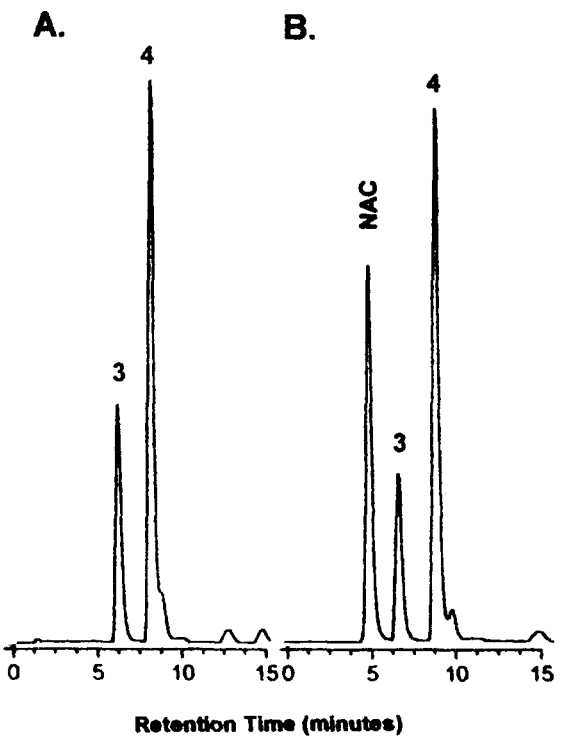

Fig. 3. (A) Chromatogram of serum before spiking with NAC. (B) Chromatogram of serum spiked with 500 n*M* NAC.

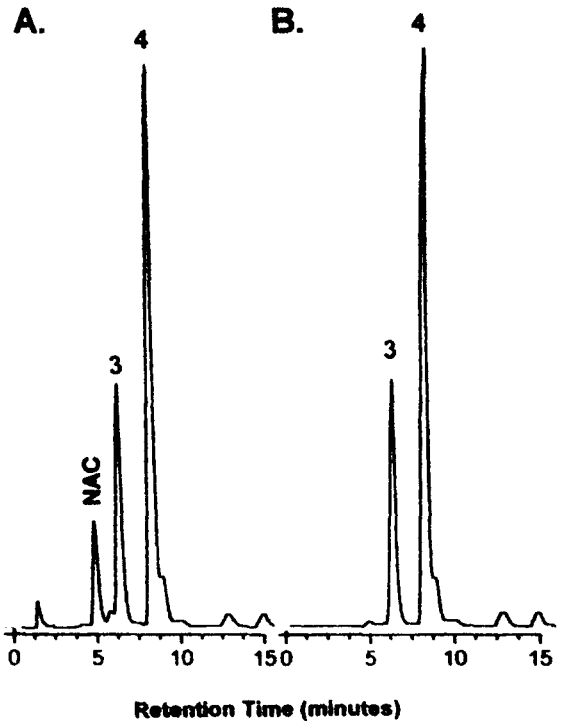

Fig. 4. (A) Chromatogram showing serum sample treated with DTT and then derivatized with NPM. Calculated NAC concentration was 125 n*M*. (B) Chromatogram of serum sample subsequently derivatized with NPM.

peak in serum treated with DTT is pictured in Fig. 4A. The appreciably smaller NPM–NAC adduct peak in serum not treated with DTT is shown in Fig. 4B.

4. Discussion

There are several other published methods which have been applied to the measurement of NAC or derivatives of NAC. A combination of HPLC with mass spectroscopy was used to detect nitrosourea metabolite derivatives of NAC [6]. This was effective for its purpose, but was apparently complex in terms of both procedure and required equipment. Stenken et al. [7] described a HPLC method utilizing electrochemical detection. While it had advantages in

Table 1
NAC in tissues 15 min after intraperitoneal injection of 500 mg/kg NAC

Sample	NAC concentration	
	Mean±S.D. (n=3)	Control
Serum	2.69±1.05 μmol/ml	ND
Liver	37.83±9.75 nmol/mg protein	ND
Kidney	ND	ND
Brain	8.40±2.08 nmol/mg protein	4.00±1.52 nmol/mg protein

After derivatization with NPM, the NAC–NPM adducts formed were measured by HPLC and sample NAC concentrations were compared to known standards.
ND=Not detectable.

terms of sensitivity and the ability to detect both reduced and oxidized forms of thiols in a single analysis, it required preparation of Au–Hg amalgam electrodes on approximately a weekly basis. Several studies have focused on HPLC with fluorometric detection of derivatives of NAC. Gabard and Mascher [8] utilized HPLC with post-column derivatization with *o*-phthalaldehyde. This method was sensitive to 6 n*M*, and was therefore appropriate to measure NAC in biological systems, but their handling methods required reduction of samples to determine total NAC and did not readily yield separate reliable measurements of reduced and oxidized thiols. Lewis et al. [9] published a method involving extraction and back-extraction steps followed by pre-column derivatization with 2,4-dinitrofluorobenzene. There were apparently problems with variability of relative recovery which necessitated the use of *N*-acetylpenicillamine as an internal standard. Moldeus and Cotgreave [3] employed pre-column derivatization with monobromobimane to assay NAC; however, this assay reaction must be carried out in the dark. Kagedal and Kallberg [10] previously reported an NPM pre-column derivatization method in which the reaction was carried out in aqueous solutions at pH 9, but a 20-h incubation period was required. It is increasingly apparent that despite the current availability of methods to assay NAC, all have some practical limitations.

The NAC assay described in the present study may prove useful to the extent that it can be run under ambient conditions, requires only about 18 min per sample to run, is susceptible to automation of assay being an HPLC method, shows linearity of the assay from 8 to 2500 n*M*, has low coefficients of variation for reproducibility, and gives 90% or greater relative recovery of NAC from biological materials. Measurement of total NAC by reduction of the samples is another advantage of this technique. Application of the present assay to biological materials including serum, liver, kidney, and brain samples has been demonstrated.

Acknowledgments

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