01 Jan 2006

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Recommended Citation

W. Wu et al., "Separation and Quantification of N-acetylcysteine-amide (NACA) by HPLC with Fluorescence Detection," *Biomedical Chromatography*, John Wiley & Sons, Jan 2006.
The definitive version is available at https://doi.org/10.1002/bmc.583

BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **20**: 415–422 (2006)
Published online 15 September 2005 in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/bmc.583

ORIGINAL RESEARCH

Separation and quantification of *N*-acetyl-L-cysteine and *N*-acetyl-cysteine-amide by HPLC with fluorescence detection

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Received 2 June 2005; revised 20 June 2005; accepted 21 July 2005

ABSTRACT: *N*-acetyl-L-cysteine (NAC) is a well-known antioxidant that is capable of facilitating glutathione (GSH) biosynthesis and replenishing intracellular GSH under oxidatively challenging circumstances. *N*-acetyl-cysteine-amide (NACA), the amide form of NAC, is a newly designed and synthesized thiol-containing compound which is believed to be more lipophilic and permeable through cell membranes than NAC. The metabolic and antioxidant effects of these compounds *in vitro* and *in vivo* are under investigation. However, an analytical method that can separate and quantify both compounds simultaneously is not yet available, to the best of our knowledge. Because of their structural similarities, the two compounds are difficult to separate using earlier HPLC methods which were designed for NAC quantification. Therefore, the goal of this work was to develop an HPLC method with fluorescence detection for simultaneous quantification of NAC and NACA in biological blood and tissue samples. A gradient HPLC program with fluorescence detection (λ_{ex} = 330 nm, λ_{em} = 376 nm) using *N*-(1-pyrenyl)maleimide (NPM) as the derivatizing agent was developed. The calibration curves were linear over a concentration range of 25–5000 nM ($r^2 > 0.997$). The coefficients of variation for within-run precision and between-run precision ranged from 0.67 to 5.23% and for accuracy ranged from 0.98 to 10.54%; the percentage relative recovery ranged from 94.5 to 102.8%. This new method provides satisfactory separation of NAC and NACA, along with other biological thiols, in 20 min with a 5 nM limit of detection (LOD) per 5 μL injection volume.

KEYWORDS: *N*-acetylcysteine amide; *N*-acetylcysteine; thiols; NPM; HPLC

INTRODUCTION

N-acetyl-L-cysteine (NAC) is a well-known thiol-containing antioxidant that has been used in the clinics for multiple uses for more than 50 years (Kelly, 1998; Holdiness, 1991; Parcell, 2002; Ziment, 1986; Flanagan, 1987), and it is the only approved antidote for acetaminophen intoxication. The evidence from both *in vitro* and *in vivo* studies indicates that NAC is capable of facilitating intracellular glutathione (GSH) biosynthesis by reducing extracellular cystine to cysteine (Issels *et al.*, 1988), or by supplying sulfhydryl (-SH) groups that can stimulate GSH synthesis and enhance gluathione-*S*-transferase activity (Nakata *et al.*, 1996; De Vries and De Flora, 1993; De Flora *et al.*, 1985). NAC is also a potent free radical scavenger as a result of its nucleophilic actions on reactive oxygen species (Aruoma *et al.*, 1989). Therefore, NAC treatment or supplementation can be considered for conditions of GSH depletion and free radical formations during oxidative stress. NAC has also been used as a metal chelating agent for several toxic metals such as boron, chromium, cobalt, cadmium, arsenic, gold and lead (Barnner *et al.*, 1986; Llobet *et al.*, 1986; Patrick, 2003; Ottenwalder and Simon, 1987). Some studies have also demonstrated that NAC can act as a radioprotective agent against much oxidative damage induced by UV, ionizing radiation, and gamma rays (Neal *et al.*, 2003; Murley *et al.*, 2004; Morley *et al.*, 2003; Sridharan and Shyamaladevi, 2002; Pajonk *et al.*, 2002).

N-acetyl-cysteine-amide (NACA), the amide form of NAC, is a newly designed and synthesized thiol-containing compound. NACA is believed to cross the cell membrane more easily due to its high lipophilicity. One recent study provided evidence that NACA had more efficient membrane permeation than NAC and could replenish intracellular GSH in red blood cells (Grinberg *et al.*, 2005). This compound was also shown to cross the blood–brain barrier, scavenge free-radicals, chelate copper, and protect red blood cells from oxidative stress (Atlas *et al.*, 1999; Offen *et al.*, 2004).

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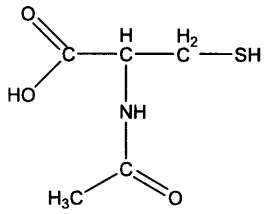
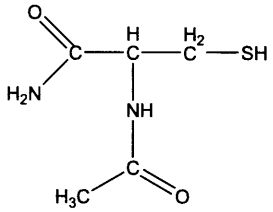
Abbreviations used: CYS, cysteine; GSH, glutathione; HCYS, homocysteine; NAC, *N*-acetyl-L-cysteine; NACA, *N*-acetyl-L-cysteine amide; NPM, *N*-(1-pyrenyl)maleimide.

Contract/grant sponsor: NIEHS; Contract/grant number: 1R15ESO12167-01A1.
Contract/grant sponsor: Environmental Research Center for Emerging Contaminants, University of Missouri-Rolla.



NAC



NACA

Figure 1. Structures of NAC and NACA.

The metabolic and antioxidant effects of these compounds *in vitro* and *in vivo* are under investigation. However, an analytical method that can simultaneously separate and quantify both compounds along with other biological thiols, such as glutathione, cysteine (CYS) and homocysteine (HCYS), is not yet available, to the best of our knowledge. Because of the structural similarities between these two compounds (Fig. 1), they are difficult to separate using earlier HPLC methods that were designed for NAC quantification in biological samples (Winters *et al.*, 1995; Ercal *et al.*, 1996; Baeyens *et al.*, 1988; Cotgreave and Moldeus, 1987).

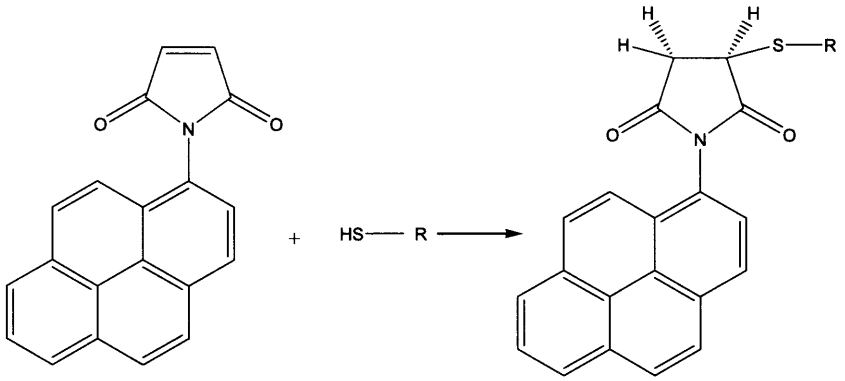
N-(1-pyrenyl)maleimide (NPM) is an aromatic compound that can selectively react with sulfhydryl groups and form a fluorescent adduct with a high extinction coefficient (Fig. 2). It has been used by our research group as the fluorescent derivatizing agent for many thiol compounds (Winters *et al.*, 1995; Ercal *et al.*, 1996; Yusof *et al.*, 2000). The goal of this study was to develop an HPLC method for the separation and simultaneous quantification of NAC and NACA with fluorescence detection using NPM as the derivatizing agent. Biological thiols, such as glutathione, cysteine and homocysteine, can also be determined simultaneously using this method.

EXPERIMENTAL

Reagents and chemicals. Acetonitrile, acetic acid and *o*-phosphoric acid (all HPLC grade) were purchased from Fisher Scientific (Houston, TX, USA). NAC, GSH, CYS, HCYS and NPM were purchased from Sigma (St Louis, MO, USA). NACA was provided by Novia Pharmaceuticals Ltd (Haifa Bay, Israel).

Animals. All experiments were performed with adult Sprague–Dawley rats, each weighing about 250 g, that were purchased from Charles River Laboratories (Wilmington, MA, USA). They were kept in a temperature-controlled (25°C) room equipped to maintain a 12 h light–dark cycle. Standard rat chow (Purina rat chow) and tap-water were given *ad libitum*. NACA 500 mg/kg body weight was orally administrated to the rats after overnight fasting. Thirty minutes later after dosing, rats were anesthetized and sacrificed according to the University of Missouri Animal Care Regulations. Plasma and tissue samples were collected and then kept at −70°C for later analysis, or minced and homogenized on ice and derivatized by NPM immediately.

HPLC system. The HPLC system (Thermo Electron Corporation) consisted of a Finnigan™ SpectraSYSTEM SCM1000 Vacuum Membrane Degasser, a Finnigan™ SpectraSYSTEM P2000 Gradient Pump, a Finnigan™ SpectraSYSTEM AS3000 autosampler and a Finnigan™ SpectraSYSTEM FL3000 fluorescence detector (λ_{ex} = 330 nm and λ_{em} = 376 nm). The injection volume was 5 µL for all the plasma and tissue samples. The HPLC column was a Reliasil ODS-1 C_{18} column (5 µm packing material) with 250 × 4.6 mm i.d. (Column Engineering, Ontario, CA, USA). Two



NPM thiol NPM-thiol adduct

Figure 2. Formation of fluorescent NPM–thiol adduct.

Table 1. Gradient program of mobile phases used in the separation

Time (min)	Mobile phase A (%)	Mobile phase B (%)	Flow rate (mL/min)
0.0	100		0.7
6.0	100		0.7
6.1		100	1.7
13.0		100	1.7
13.1	100		0.7
20.0	100		0.7

Mobile phase A: 0.05% acetic acid in 70:30 acetonitrile–HPLC-H_2O (v:v).
Mobile phase B: 0.4% o-phosphoric acid in 70:30 acetonitrile–HPLC-H_2O (v:v).

mobile phases were used in the gradient separation program: mobile phase A was composed of 0.05% acetic acid in 70:30 acetonitrile–HPLC-H_2O (v:v) and mobile phase B was composed of 0.4% *o*-phosphoric acid in 70:30 acetonitrile–HPLC-H_2O (v:v). The gradient program (Table 1) was used for optimal separation and quantification of NAC, NACA and other biological thiols.

Preparation of calibration solution. For calibration studies, a calibration standard stock solution of NAC and NACA (1 mM) was prepared by accurately weighing out 0.0016 g of both NACA and NAC and dissolving them to 10 mL of serine-borate buffer (100 mM Tris buffer containing 10 mM borate and 5 mM serine with 1 mM diethylenetriaminepentaacetic acid; pH = 7.0; Neal *et al.*, 1997). This stock mixture solution was further diluted in serine-borate buffer to prepare the diluted calibration standard working solutions (100 and 10 μM). Varying volumes of these working solutions were added into plasma or tissue homogenates and to prepare the final plasma or tissue calibration standard solutions in varying concentrations, as 25, 125, 250, 500, 1250, 2500 and 5000 nM.

Sample preparation and derivatization. Plasma was obtained by centrifugating blood samples for 10 min at 1000 ***g***. Tissue samples, ranging from 0.2 to 0.5 g, were minced and homogenized in 1 mL serine-borate buffer as described above. L-Serine, in the presence of borate, can inhibit γ-glutamyl transpeptidase (GGT) which exists in some biological tissues such as the liver and kidneys, and can hydrolyze GSH; therefore, it is used to prevent the loss of GSH during the process of homogenization. Two-hundred and fifty microliters of plasma or tissue homogenates were added to a 750 μL 1 mM NPM solution and incubated at room temperature for 5 min; then 10 μL of 2 M HCl were added to stop the reaction. The final pH of the solution should be less than 2.0, which is important for stabilizing the derivatives. Derivatized plasma or tissue samples were filtered through 0.45 μm nylon filters (Advantec MFS Inc., Dulin, CA, USA) and injected directly onto the HPLC system.

Protein assay. The Bradford method (Bradford, 1976) was used to determine the protein contents of the tissue samples and the protein content determined in each tissue sample was used to normalize the thiol concentration data, so that a reasonable comparison could be achieved between different treatment groups. The Coomassie blue dye working solution was made by diluting concentrated Coomassie blue solution (Bio-Rad Laboratories, Hercules, CA, USA) with distilled water (1:4, v/v). A standard curve was constructed using 0.05 mL bovine serum albumin (BSA), ranging from 0.25 to 1.0 mg/mL, as the serial standard solutions with the addition of 2.5 mL of the Coomassie blue working solution. The homogenated tissue samples were subjected to appropriate dilutions and 0.05 mL of each sample was added to 2.5 mL of the same Coommasie blue working solution that had been used in the standard curve construction. The mixtures were then incubated at room temperature for at least 5 min and the absorbance was measured at 595 nm by a spectrophotometer.

METHOD VALIDATION

Calibration curves

Calibration curves (Fig. 3) were plotted using integrated peak areas vs standard thiol concentrations (25, 125, 500, 1250, 2500 and 5000 nM). Linearity in each curve was achieved over a concentration range of 25–5000 nM ($r^2 > 0.997$).

Accuracy, precision and recovery

Accuracy was determined by analyzing seven replicates of plasma or tissue samples containing 25, 125, 500 and 1250 nM of NAC and NACA, respectively. The mean values for the seven replicates at each concentration in each matrix were calculated and the relative deviation of mean value from the true value served as the measure of accuracy.

Within-run precision was determined by analyzing seven replicate NAC- or NACA-spiked control plasma samples or tissue samples at concentration levels ranging from 25 to 1250 nM in one analytical run, and comparing the NAC or NACA concentrations calculated from the peak areas of the seven replicates in each matrix. [The average calibration curves used in the calculations were, for NAC, $y = 30.857x - 1626.5$ ($r^2 = 0.999$); for NACA, $y = 29.331x + 437.36$ ($r^2 = 0.999$)]. Between-run precision was obtained by derivatizing seven replicate NAC- or NACA-spiked control plasma samples or tissue samples at concentration levels ranging from 25 to 1250 nM in three different analytical runs and comparing the NAC or NACA concentrations calculated from the peak areas of the seven replicates in each matrix. The coefficients of variations were calculated in each matrix and served as the measure of precision.

Relative recovery was determined by spiking the liver, kidney, lung, brain and plasma samples with 25, 125, 500 and 1250 nM of NAC and NACA in three

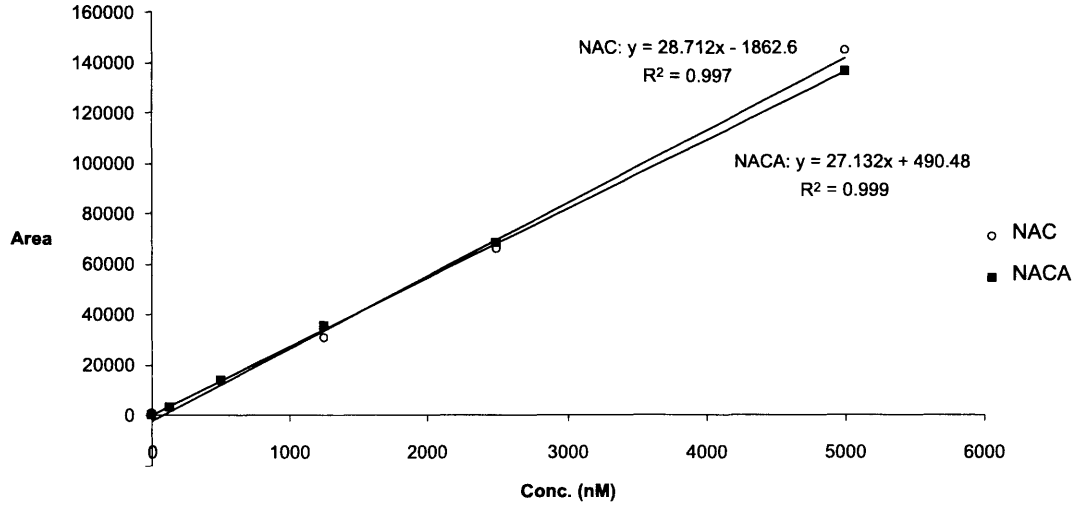


Figure 3. Calibration curves of NAC and NACA in plasma matrix.

Table 2. Between-run and within-run precision, accuracy and relative recovery of three samples spiked with NAC and NACA (25, 125, 500 and 1250 nM) in sample matrices and standards

Sample matrix	Plasma	Kidney	Lung	Brain	Liver	Standard
Between-run precision (n = 7)	2.06–4.89%	1.56–3.93%	2.78–5.23%	1.57–4.38%	1.35–2.45%	0.67–3.22%
Within-run precision (n = 7)	1.94–3.58%	1.47–4.29%	2.27–4.94%	2.18–3.88%	1.65–3.07%	0.79–3.27%
Accuracy (n = 7)	1.58–6.16%	2.24–8.27%	2.77–10.54%	3.25–9.28%	1.24–7.58%	0.98–4.25%
Percentage relative recovery (n = 3)	99.1 ± 5.7	98.4 ± 4.9	94.5 ± 8.2	96.4 ± 4.7	102.8 ± 3.3	N/A

Percentage relative recovery is reported as the average relative recovery (±standard deviation) of the samples spiked with 25, 125, 500 and 1250 nM NAC and NACA in each sample matrix. N/A = not applicable.

replicates. Recoveries were calculated by comparing the analytical results for those three spiked samples with the un-spiked pure standards at the above-mentioned four concentrations that represent the 100% recovery.

The coefficients of variation (CV) for between-run and within-run precision, the relative deviation for accuracy, and relative recovery of the samples spiked with NAC and NACA (25, 125, 500 and 1250 nM) in the sample matrix and standards are shown in Table 2. The CVs for within-run precision and between-run precision ranged from 0.67 to 5.23% and the relative deviations for accuracy ranged from 0.98 to 10.54%; the percentage relative recovery ranged from 94.5 to 102.8%.

Sensitivity and autosample stability

The lower limit of quantitation (LLOQ) was the concentration of NAC or NACA when its peak area was 10 times that of the peak area of the blank (signal-to-noise = 10). The LLOQ of both NAC and NACA by this method was found to be 25 nM. The detection limit determined by this technique was found to be 5 nM (signal-to-noise = 3) with 5 μL injection volume.

The autosample stability was measured by determining the seven replicates of derivatized NAC- and NACA-spiked plasma or tissue samples at three concentrations (125, 500 and 1250 nM) kept in HPLC autosampler vials and stored at room temperature after 0, 6, 12 or 24 h of sample derivatization or stored at 4°C after 1, 3, 6 or 12 days of sample derivatization. The relative standard deviations were found to be less than 10% when the derivatized samples were stored at room temperature and less than 15% when the derivatized samples were stored at 4°C at those varying concentrations in each sample matrix.

Investigation of interferences

Figure 4 shows a chromatogram of a derivatized standard mixture (1250 nM) of NAC, NACA, GSH, CYS and HCYS in a liver matrix with NPM derivatization. The retention time of NAC was 4.84 min and the retention time of NACA was 5.25 min. As seen in Fig. 4, the aforementioned biological thiols do not interfere with the detection of NAC and NACA when this method is used.

RESULTS

This study examined the derivatization of NAC and NACA with NPM and their separation by the gradient

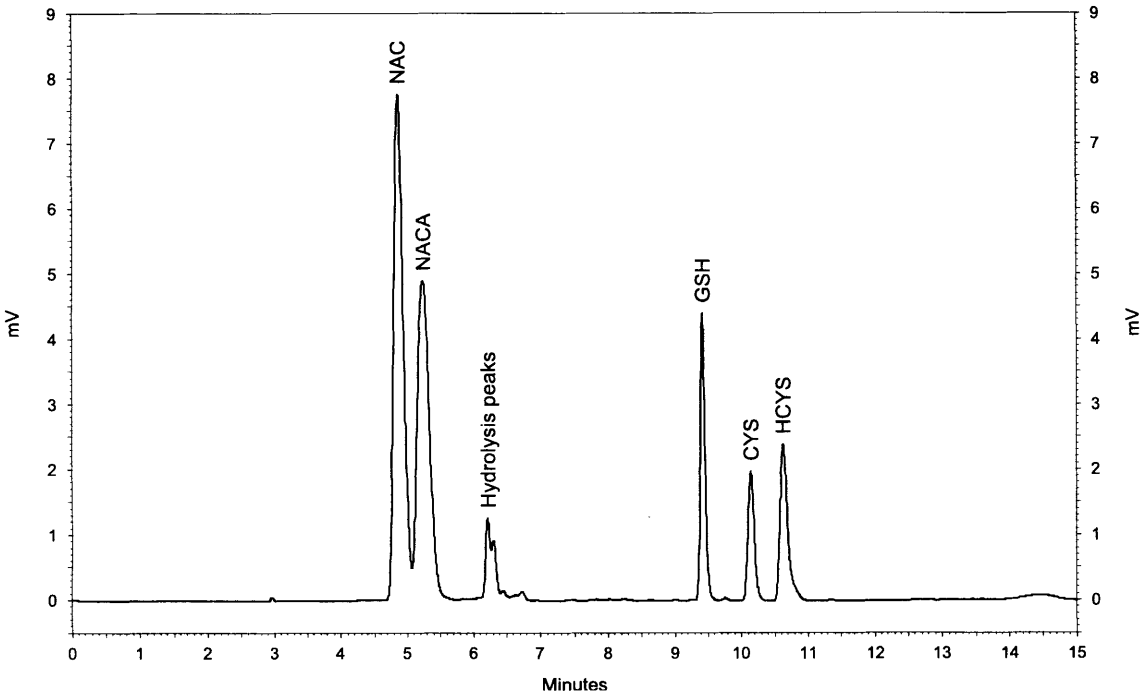


Figure 4. Chromatogram of a derivatized standard mixture (1250 nM) of NAC (4.835 min), NACA (5.250 min), GSH (9.475 min), CYS (10.197 min) and HCYS (10.703 min) in a liver matrix. Separation conditions: an ODS-1 C_{18} column (5 μm packing material) with 250 × 4.6 mm (i.d.) was used for the separation. The gradient program of mobile phases was described in the Experimental section. The NPM derivatives were measured using a fluorescence detector (λ_{ex} = 330 nm and λ_{em} = 376 nm).

Table 3. Thiol levels in biological samples after 30 min oral administration of 500 mg/kg body weight NACA

		Thiol levels (mean ± SD)			
	Samples	NAC	NACA	GSH	CYS
Plasma	Control (n = 3)	ND	ND	6.96 ± 3.02	16.67 ± 4.03
	NACA-treated (n = 3)	84.55 ± 6.73	15.07 ± 5.08	22.59 ± 2.39	158.82 ± 30.76
Kidney	Control (n = 3)	ND	ND	1.82 ± 0.23	29.72 ± 1.90
	NACA-treated (n = 3)	11.01 ± 0.01	0.01 ± 0.004	2.35 ± 0.68	44.02 ± 2.05
Lung	Control (n = 3)	ND	ND	6.76 ± 1.38	0.51 ± 0.25
	NACA-treated (n = 3)	0.24 ± 0.02	0.01 ± 0.002	6.63 ± 1.45	5.05 ± 1.46
Brain	Control (n = 3)	0.01 ± 0.01	ND	10.18 ± 0.63	1.51 ± 0.34
	NACA-treated (n = 3)	0.07 ± 0.02	0.05 ± 0.01	10.97 ± 0.98	1.66 ± 0.05
Liver	Control (n = 3)	ND	ND	13.55 ± 1.85	2.66 ± 0.39
	NACA-treated (n = 3)	0.02 ± 0.01	0.04 ± 0.002	31.57 ± 2.19	5.84 ± 1.77

Calibration curves used in the above calculation: GSH, $y = 10.271x + 233.39$; CYS, $y = 10.089x + 253.65$.
Unit for plasma samples is μM; unit for tissue samples is nmol/mg protein. ND = not detectable. SD = standard deviation.

HPLC system. Figure 5(a) shows the chromatogram of a control plasma sample from Sprague–Dawley rats given phosphate-buffered saline solution only. As seen in Fig. 5(a), there is no NACA peak in this chromatogram. Figure 5(b) shows the chromatogram of the plasma sample obtained from an animal that was administrated 500 mg/kg body weight of NACA and sacrificed 30 min thereafter. Both NAC and NACA peaks appeared right before the NPM hydrolysis peaks. Figure 6 shows the chromatograms of lung samples obtained from Sprague–Dawley rats. The chromatogram of a control lung sample (a) shows no peaks at the retention times of these two compounds (NAC and NACA), while the chromatogram of the lung sample from a rat given NACA (b) had a significant NAC peak at 4.8 min and a smaller NACA peak at 5.2 min.

The levels of these important biological thiols in samples of plasma, liver, lung, kidney and brain tissues are reported in Table 3. The blood and tissue samples were collected after 30 min oral administration of 500 mg/kg body weight NACA. The levels of the thiols in the tissue samples were calculated by using the thiol concentrations (nM) of homogenated tissue solutions over the protein content in the solutions (mg protein/

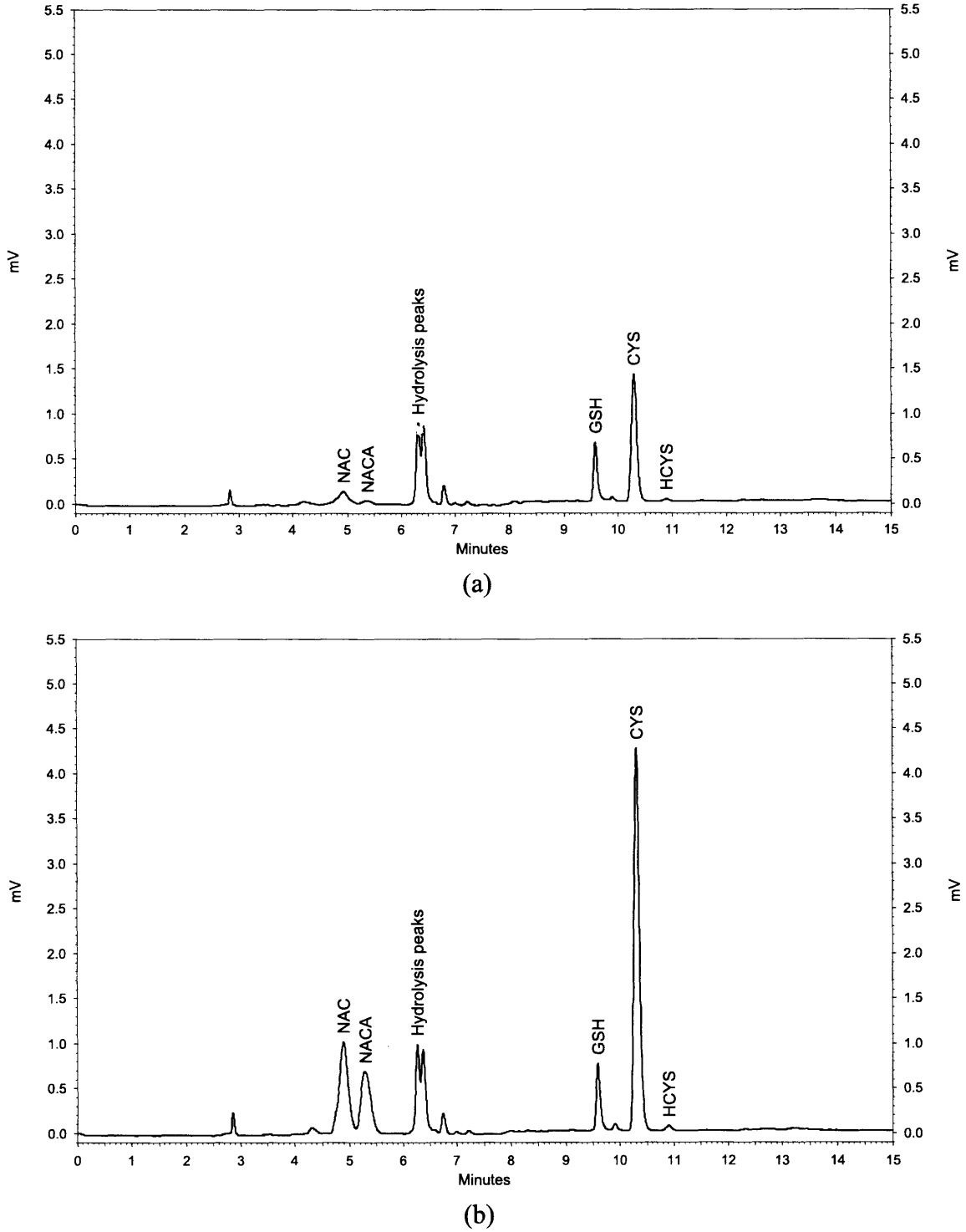


Figure 5. Chromatograms of plasma samples obtained from Sprague–Dawley rats. Separation conditions are the same as those in Fig. 4. (a) Control plasma sample obtained from an animal administrated with phosphate-buffered saline solution only (no NACA peak). (b) Plasma sample obtained from an animal sacrificed 30 min after the administration of 500 mg/kg body weight NACA.

mL). The concentration of NACA in plasma samples was significant after 30 min oral administration of 500 mg/kg NACA, compared with the levels of NACA in control samples that had received only phosphate buffered saline solution. It was also noticed that the concentrations of other biological thiols (such as GSH in the plasma and liver samples and CYS in plasma, kidney, lung and liver samples) increased after NACA administration, indicating that NACA can replenish GSH and CYS in biological systems.

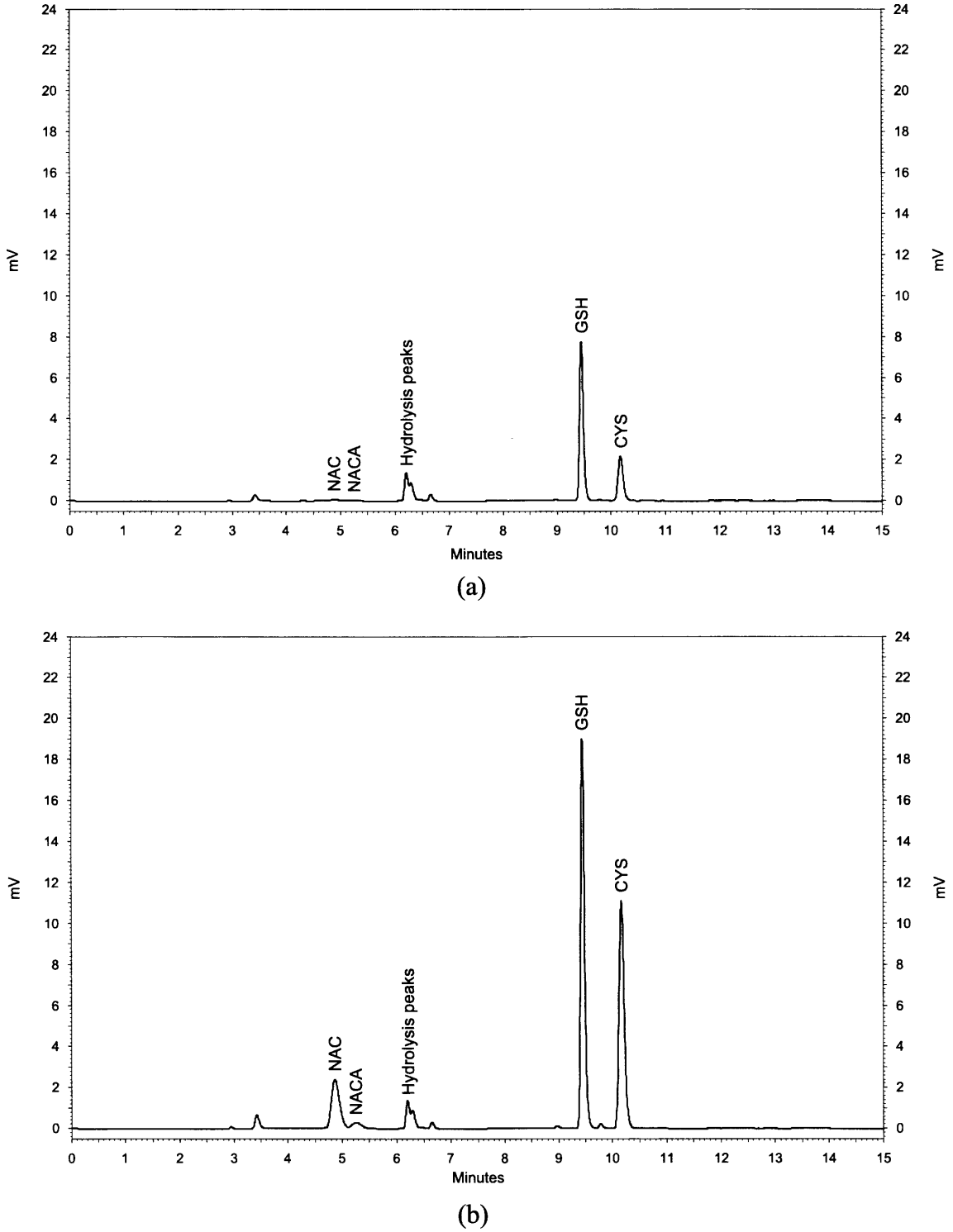


Figure 6. Chromatograms of lung samples obtained from Sprague–Dawley rats. Separation conditions are the same as those in Fig. 4. (a) Chromatogram of the control lung sample showing no NAC or NACA peaks. (b) Chromatogram of the lung sample taken 30 min after oral administration of 500 mg/kg body weight NACA.

CONCLUSION

A new HPLC method, using NPM as the derivatizing agent, has been developed for separating and quantifying NAC and NACA. High concentrations of biological thiols (GSH, CYS and HCYS) do not interfere with the determination of NAC and NACA. The application of this method to biological samples that included plasma,

liver, lung, kidney and brain tissues provided satisfactory results.

This assay provides a sensitive, rapid and simple method for analyzing NAC, NACA, GSH, CYS and HCYS simultaneously in biological samples. It is also a useful tool for studying the metabolic mechanisms and various effects of NAC and NACA in biological environments.

Acknowledgments

This work was supported by grants 1R15ESO12167-01A1 from NIEHS, and Environmental Research Center for Emerging Contaminants at the University of Missouri-Rolla. We thank Barbara Harris and Julie Breckenridge for their careful editing of our manuscript. We also thank Dr Avi Livnat and Novia Pharmaceuticals Ltd for providing NACA.

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