Amino Acids (2009) 37:395–400
DOI 10.1007/s00726-008-0167-x

ORIGINAL ARTICLE

Simultaneous detection of *N*-acetyl-L-cysteine and physiological low molecular mass thiols in plasma by capillary electrophoresis

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Received: 20 March 2008 / Accepted: 28 July 2008 / Published online: 11 August 2008

Abstract *N*-acetyl-L-cysteine (NAC) is a therapeutic drug widely used as mucolytic agent in the treatment of respiratory diseases. Recently it has been proposed that NAC administration may modify the plasma levels of low molecular weight thiols (LMW) like cysteine, homocysteine and glutathione, though it has been still debated if their plasma concentration increases or decreases during the therapy. Therefore research calls for methods able to analyze simultaneously NAC and the other plasma LMW thiols in order to evaluate if NAC is able to modify plasma thiols concentration and in particular to reduce homocysteine levels in hyperhomocysteinemia. In this paper we present a new capillary electrophoresis method that allows a baseline separation of plasma NAC from the physiological thiols. The proposed method has been utilized to measure the drug and the physiological LMW thiols in NAC administered chronic obstructive broncho-pneumopathy (COPB) disease patients.

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Keywords NAC · LMW thiols · Capillary electrophoresis

Abbreviations

CE	Capillary electrophoresis
LIF	Laser induced fluorescence
NAC	*N*-acetylcysteine
5-IAF	5-Iodoacetamidofluorescein
SSA	5-Sulphosalicylic acid
TBP	Tri-*n*-butylphosphine
Cys	Cysteine
Cysgly	Cysteinylglycine
GSH	Glutathione
Glucys	Glutamylcysteine
Hcy	Homocysteine
COPB	Chronic obstructive broncho-pneumopathy

Introduction

N-acetyl-L-cysteine (NAC) is a thiol-containing drug that is frequently used as mucolytic agent (Boban et al. 1983) and for the treatment of acetaminophen hepatotoxicity (Prescott et al. 1979). NAC has also been used as metal chelating drug for several toxic metals such as boron, chromium, cobalt, cadmium, arsenic, gold and lead (Barnner et al. 1986; Ottenwälder and Simon 1987). Some studies have also demonstrated that NAC can act as a radioprotective agent against oxidative damage induced by UV, ionizing radiation, and gamma rays (Spotheim-Maurizot et al. 1993; Murley et al. 2004). Recently, its antioxidant/radical-scavenging activity (Särnstrand et al. 1995; Moldéus et al. 1986) has been the subject of considerable attention, in terms of extending its therapeutic applications also

considering that the drug has been shown to be relatively well tolerated and without serious side effects (Prescott et al. 1977; Smilkstein et al. 1988; Sheiner et al. 1992).

It has been supposed that the mucolytic effect of NAC may be mediated by the splicing of disulphide bonds between mucus macromolecules (Ziment 1986; Hultberg et al. 1994). Another effect of *N*-acetyl-L-cysteine may be an interference with the metabolism of thiol compounds in plasma. The presence of a sulphydryl group in NAC explains its tendency to bind to cysteine and other endogenous sulphydryl-containing compounds. It also displaces cysteine or other thiols from their protein binding sites, forming various mixed disulphides, including protein low molecular-weight disulphides (Hultberg et al. 1994; Wiklund et al. 1996). It has been suggested that the low molecular weight mixed disulphides thus formed with NAC have a high renal clearance, which may lead to a more efficient elimination of thiols, and particularly Hcy, from plasma (Ventura et al. 1999). But, on the other side, NAC is metabolized in cysteine, which is an important precursor of Hcy, GSH and consequently also of Cys-Gly and Glu-Cys (Olsson et al. 1988; Nakata et al. 1996; De Vries and De Flora 1993; De Flora et al. 1985), thus its administration may lead to increasing levels of thiols in plasma. Therefore the final effect of this molecule on plasma thiol status may be due to the balance between the renal elimination and biochemical synthesis during the therapy. The modification of LMW thiols plasma concentration may have important consequences. The increase in total plasma Hcy and Cys concentrations is associated with the development of vascular disease (Ozkan et al. 2002; McCully 1969). Hcy and Cys can promote atherogenesis through their toxic effect on endothelial function, vascular smooth muscle cell activation and hemostatic activation (Perla-Kajan et al. 2007; Hajjar 2001; D'Angelo and Selhub 1997). In contrast, GSH and Cys-Gly have important antioxidant and likely anti-atherogenic properties (Morrison et al. 1999; Lapenna et al. 1998). At now no definitive results have been proposed on the role of NAC administration on the physiological LMW thiols levels perhaps for the difficulties to measure NAC and all the physiological low molecular weight thiols simultaneously. Some HPLC methods have been described for NAC quantification in biological samples (Ercal et al. 1996; Baeyens et al. 1988; Cotgreave and Moldeus 1987; Tsikas et al. 1998) but an analytical method which can simultaneously separate and quantify *N*-acetyl-L-cysteine and other biological LMW thiols, such as glutathione, cysteine and homocysteine, glutamylcysteine and cysteynilglycine is not available yet, to the best of our knowledge. Moreover, the capillary electrophoresis method previously reported has been applied exclusively to pharmaceutical quality control but not to plasma samples (Jaworska et al. 1999; Dette and Wätzig 1994). Thus, we describe the first capillary electrophoretic method for the quantification of NAC in plasma samples and in general the first assay able to simultaneously measure all physiological thiols and *N*-acetyl-L-cysteine.

Materials and methods

Chemicals

NAC, Homocysteine, Cysteine, Cysteinylglycine, Glutathione, Glutamylcysteine, Na_3PO_4, H_3BO_3, N-methyl-D-glucamine, DMF (N,N-dymethilphormamide), TBP (tri-*n*-butylphosphine), NaOH, 5-IAF (5-iodoacetamidofluorescein), SSA (5-sulphosalicylic acid), were obtained from Sigma (St. Louis, USA). Membrane filters (0.45 μm), obtained from Millipore (Bedford, USA), were used to filter all buffer solution before CE analysis. The water used for the experiments was MilliQ grade.

Sample collection and total plasma thiols determination

Blood was collected by venipuncture into evacuated tubes containing EDTA, and immediately centrifuged at 3000×*g* at 4°C for 2 min. Plasma was aliquoted and then stored at −80°C, analyses were performed within 1 week. 200 μL of plasma sample were mixed with 20 μL of 10%v/v TBP in DMF for 10 min to reduce disulfide bonds. Plasma proteins were then precipitated by adding 200 μL of 5-sulphosalicylic acid (6%) and removed by centrifugation (2000×*g* at 4°C for 5 min). Supernatant (100 μL) was derivatized by adding 100 μL of 300 mmol/L sodium phosphate buffer pH 12.5 and 25 μL of 4 mmol/L of 5-IAF. After 15 min at room temperature, derivatized samples were diluted 100 fold in water and analyzed by capillary electrophoresis.

Capillary electrophoresis

The P/ACE 5510 capillary electrophoresis (CE) system equipped with laser induced fluorescence was used (Beckman instruments, CA, USA). The P/ACE 5510 system was fitted with a 30 kV power supply with a current limit of 250 μA. The dimension of the uncoated fused-silica capillary was 75 μm ID and 67 cm length (50 cm to the detection window). Analysis was performed applying 14 nL of sample under nitrogen pressure (0.5 psi) for 2 s using a 20 mmol/L sodium phosphate, 16 mmol/L boric acid as electrolyte solution with 75 mmol/L N-methyl-D-glucamine, pH 11.4. The separating conditions (28 kV, 180 μA at normal polarity) were reached in 20 s and held at a constant voltage for 10 min. All separations were carried out at 40°C. After each run no rinse was necessary; capillary was equilibrated with 1 min of run buffer.

Results

We have recently described a capillary electrophoresis method for the quantification of Cys-Gly, Hcy, Cys, GSH and Glu-Cys by using 5-IAF as selective thiol reagent (Zinellu et al. 2003, 2008), in an uncoated fused-silica capillary of 75 μm ID ×57 cm length using 5 mmol/L sodium phosphate, 4 mmol/L sodium borate as run buffer in presence of 75 mmol/L N-methyl-D-glucamine at pH 11.4. The attempt to utilize these conditions for NAC detection failed since *N*-acetyl-L-cysteine overlapped homocysteine peak even using a 67 cm long capillary. To resolve the peak we decided to increase the separation times.

Since the property of N-methyl-D-glucamine is to mask the silanol groups on the uncoated capillary surface causing a decrease of the electroendoosmotic flux, longer separation times could be obtained by incrementing its concentration in the run buffer. But as described in our previous paper, a concentration of this compound over 75 mmol/L resulted in a peak broadening and in a loss of resolution (33). Therefore we chose to extend the run time by increasing the concentration of the run buffer electrolyte. As described in Fig. 1a, the resolution between peaks improved by increasing the buffer sodium phosphate (maintaining constant the phosphate/borate ratio 5/4) and a complete separation of analytes (Rs > 1) was obtained at phosphate concentrations of 20 mmol/L. Even if these conditions induced a rise of migration times (Fig. 1b) the increase of the run buffer concentration also yielded a significant sensitivity improvement demonstrated by the increment of about 30% of NAC peak respect to the peak obtained by the electrophoretical conditions adopted in the previous work (Fig. 1c). As shown in Fig. 2, a baseline resolution between all peaks was achieved when a 20 mmol/L sodium phosphate, 16 mmol/L boric acid with 75 mmol/L N-methyl-D-glucamine, at pH 11.4 run buffer were used, with a run time shorter than 10 min. To assess the suitable derivatization times of the reaction between NAC and 5-IAF, 100 μmol/L of drug were mixed with 4 mmol/L of 5-IAF in a 300 mmol/L sodium phosphate buffer, pH 12.5 and incubated for 120 min at room temperature. In these conditions the reaction reached the plateau in 10 min (data not shown), similarly to the derivatization times of physiological thiols described in our previous work (33).

Calibration curves for a standard water solution of thiols, obtained by five replicates, showed good correlation coefficients ($r = 0.999$ for all thiols) ensuring a linear response over the concentrations tested (Table 1). Precision tests, performed after repeated injection of the same sample, indicated a good repeatability of the method both for migration times (CV < 0.7%) and areas (CV < 2.1%). Within-run precision (intraassay) of the method was

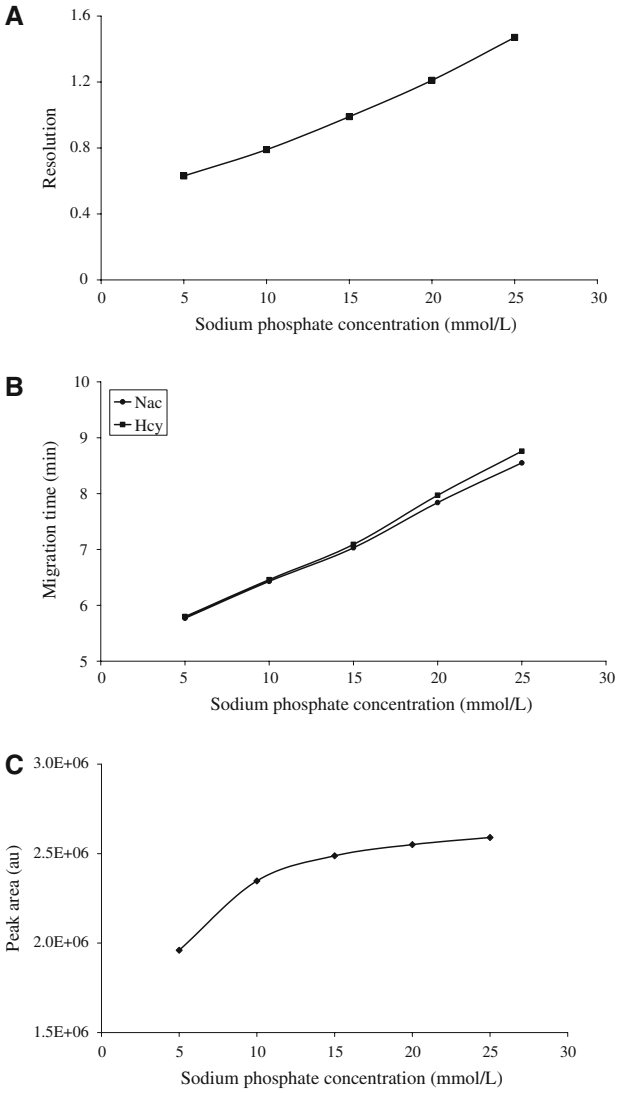

Fig. 1 Effect of concentration of phosphate/borate run buffer on resolution of NAC and Hcy peak (**a**), migration time (**b**) and height (**c**) of NAC. Electrophoretic conditions: uncoated silica capillary 67 cm × 75 μm ID, cartridge temperature 15°C, voltage 28 kV. The ratio phosphate/borate (5/4) has been maintained constant

evaluated by measuring ten times the same biological sample, independently prepared, in the same sample set, while between-run (interassay) precision was determined by measuring the same biological sample on ten consecutive days. A good reproducibility of intra-assay and interassay tests was obtained (CV < 3.5 and 5.2%, respectively) as reported in Table 1. Thiols recovery was determined by adding pure standards to plasma samples. The analytical recoveries, evaluated at four different concentrations for every thiol, were between 98.2 and 101.7% (Table 1). The LOD of NAC, calculated by 14-nL injections of a known solution of standards (after 100-fold water dilution) was 200 pmol/L, corresponding to an injected quantity of about 2.5 amol, with an S/N of 3.

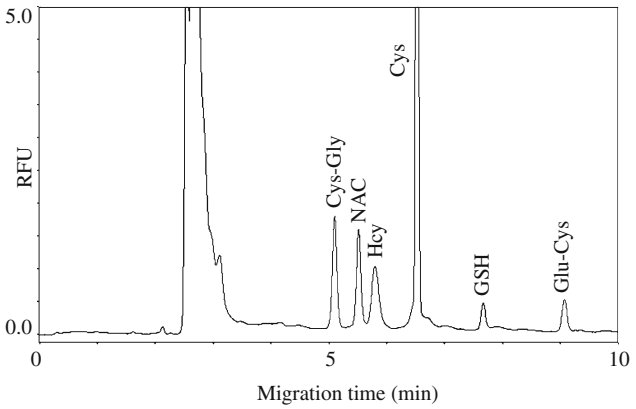

Fig. 2 Electropherogram of a thiols standard mix obtained under the optimized conditions. Cys-Gly 20 μmol/L, NAC 16 μmol/L, Hcy 12 μmol/L, Cys 100 μmol/L, GSH 4 μmol/L, Glu-Cys 4 μmol/L

The new capillary electrophoresis method was applied to the determination of plasma NAC from COBP patients treated with an oral dose of 600 mg/day of drug. The electropherograms obtained before and post NAC administration of a COBP patient are reported in Fig. 3a and b. Table 2 shows plasma thiol mean values obtained from three patients affected from COBP before the beginning of therapy, after 2 h of the first and second day NAC administration. As previously reported (15), after the first NAC administration cysteine and homocysteine plasma levels decreased, and for the first time we showed that also Cys-Gly, GSH and Glu-Cys concentration were lowered. Interestingly, we observed that at the second day treatment some LMW thiols already raised, as GSH, Cys and mainly Cys-Gly that increase at higher levels than baseline values.

Concluding remarks

NAC is a therapeutic drug widely used as a mucolytic agent in the treatment of respiratory diseases. Recently it has been proposed that a single NAC intravenous administration induces an efficient and rapid decrease of the main

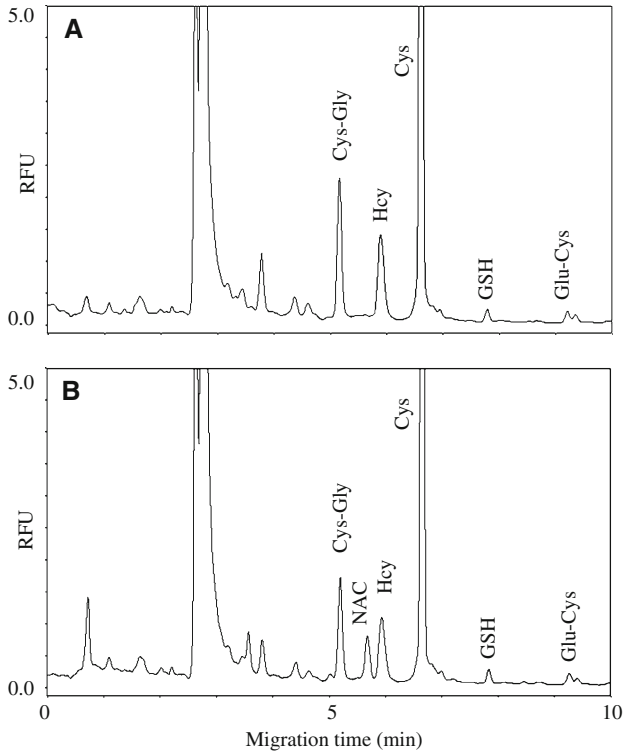

Fig. 3 Representative electropherograms of thiols from plasma sample before (**a**) and post (**b**) 600 mg NAC oral administration

circulating plasma LMW thiols Cys and Hcy (Bolme et al. 1988; Nakata et al. 1996; De Vries and De Flora 1993; De Flora et al. 1985). This effect seems particularly relevant for Hcy and may be partly explained by the displacement by NAC of thiols from their plasma binding sites (plasma proteins) and by the formation, in large excess of NAC-Cys and NAC-Hcy mixed disulphides, which in turn undergoes a higher renal clearance, resulting in an increase of urinary excretion (Ventura et al. 1999). This effect may represent the start of an alternative approach in the treatment of hyperhomocysteinaemic conditions also considering that the drug has been shown to be relatively well tolerated and without serious side effects. However, considering the fact that NAC is catabolized in cysteine that is also the

Table 1 Calibration curves, analytical recovery and reproducibility of the new method

Thiols	Calibration curves (μmol/L)				Analytical recovery (%)	Method reproducibility (μmol/L)	
	Range	Slope	Intercept	*r*	Recovery mean (CV)	Intraassay mean (CV)	Interassay mean (CV)
CysGly	5–80	2.73	5.88	0.999	98.7 (2.71)	23.34 (3.3)	23.67 (4.7)
NAC	0.5–16	3.51	−6.91	0.999	98.2 (2.53)	4.17 (3.3)	4.02 (5.1)
Hcy	2–48	3.40	6.67	0.999	101.7 (3.18)	12.47 (3.5)	12.72 (4.6)
Cys	20–400	3.11	−5.61	0.999	100.7 (2.78)	235 (3.1)	231 (4.9)
GSH	1–6	2.43	−1.35	0.999	98.6 (3.46)	3.61 (3.0)	3.80 (5.2)
GluCys	1–6	2.92	1.78	0.999	99.0 (3.61)	2.83 (3.2)	2.82 (5.1)

Table 2 Plasma thiol values obtained from three patients affected from BPCO before the beginning of therapy, after 2 h of the first and second day of NAC administration

	Cys-Gly	NAC	Hcy	Cys	GSH	Glu-Cys
Basal	23.2 ± 1.2	0	14.1 ± 1.4	200 ± 18	1.73 ± 0.84	1.69 ± 0.58
1st day	15.4 ± 4.1	4.1 ± 1.1	10.4 ± 1.9	178 ± 16	1.38 ± 1.01	1.41 ± 0.32
2nd day	30.6 ± 4.5	2.8 ± 1.4	11.0 ± 0.6	190 ± 29	1.59 ± 0.66	1.13 ± 0.19

Concentration are expressed as μmol/L

precursor of Hcy, GSH, Glu-Cys and Cys-Gly, a protracted drug administration may induce an increase in the plasma LMW thiols concentration. At now, even if NAC is a well known drug its effects on Cys, GSH and Hcy levels are still debated, while its effects on Glu-Cys and Cys-Gly are still almost unknown. By this work we describe the first capillary electrophoresis method for NAC measurement together with the other physiological thiols. We demonstrate the method applicability on patients affected by COBP under NAC therapy, also showing for the first time that the NAC treatment reduces the thiols plasma levels after the first administration, but already at the second day therapy thiols tend to increase thus confirming that the effect of NAC on LMW plasma thiols concentration is very complex. Therefore, owing to the good reproducibility, sensitivity, the user-friendliness and the rapidity of both pre-analytical and analytical steps, the assay may be an important tool in the evaluation of the NAC ability to modify plasma concentration of physiological thiols.

Acknowledgments This study was supported by the "Fondazione Banco di Sardegna – Sassari – Italy" and by the "Ministero dell'Università e della Ricerca" Italy. The manuscript language revision by Mrs Maria Antonietta Meloni is greatly appreciated.

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