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METHODOLOGIES FOR THE ANALYSIS OF REDUCED AND OXIDIZED N-ACETYLCYSTEINE IN BIOLOGICAL SYSTEMS

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ABSTRACT

A rapid and sensitive assay is described for both reduced and oxidized N-acetylcysteine in biological matrices. Free, reduced N-acetylcysteine is derivatized, along with any endogenous free thiols, *in situ* by treatment of the samples with the membrane-permeable, thiol-reactive agent monobromobimane. The N-acetylcysteine-monobromobimane adduct thus formed is analysed by high performance liquid chromatography with fluorescence detection. Oxidized N-acetylcysteine is released from disulfides by *in situ* treatment of the samples with dithiothreitol, rendering the total N-acetylcysteine content of the system available for derivatization and analysis. The conditions of derivatization ensured 100 per cent recovery of N-acetylcysteine as the monobromobimane adduct, and calibration curves were linear over the range 0·1 μM to 1·0 mM N-acetylcysteine. The precision of the assay procedures was 97 per cent over this range. These assay procedures have been applied to studies of the pharmacokinetics of N-acetylcysteine following single oral and intravenous administrations of the drug to a single human volunteer.

KEY WORDS Analysis N-acetylcysteine Pharmacokinetics

INTRODUCTION

N-acetyl-L-cysteine (NAC) is an extremely useful therapeutic agent which was originally indicated solely as a mucolyticum in the treatment of a variety of obstructive broncho-pulmonary disorders,[1] but which is becoming increasingly important in the treatment of paracetamol overdose[2] and as an adjunctive agent in cancer chemotherapy.[3]

Despite its increasing use, knowledge of the disposition of this compound in biological systems is poor and this is almost certainly due to the lack of suitable specific analytical techniques for NAC in biological milieux.

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Early pharmacokinetic and dispositional studies were often performed using the radiolabelled drug with radiometric quantitation, paying little attention to the contribution of potential metabolites to these measurements.[4,5] More specific chromatographic techniques for NAC have been few and often involved complex sample preparation and/or chromatography. Early work by Sheffner *et al.* described a simple thin layer method for NAC in tissue extracts.[6] Plasma and urinary total NAC levels have been quantitated by high performance liquid chromatography (h.p.l.c.) using ion-pair h.p.l.c.[7] The method involves pre-column derivatization of NAC with dinitrofluorobenzene following sample reduction. Plasma NAC levels have also been analysed by a coupled ion-exchange extraction – gas chromatographic technique.[8] N-acetylcysteine has been identified in control human urine by a gas chromatographic technique[9] and more recently with an ion-pair h.p.l.c. method involving extraction of thiols from urine by ion-exchange chromatography following sample reduction and with subsequent pre-column derivatization with a fluorophore maleimide.[10]

Generally, the investigation of sulfhydryl components of living systems presents special analytical problems, due, mainly, to their relatively facile oxidation to disulfide species. The effective analysis of NAC in such system also presents additional problems due to the potential interaction of NAC with endogenous biochemical processes either by metabolic degradation and/or by thiol–disulfide exchange reactions. Thus, any comprehensive analytical technique for NAC in biological systems must account for these problems.

In recent work we have detailed the development of techniques for the simultaneous analysis of endogenous reduced and oxidized, low molecular weight and protein sulfhydryl components of biological systems, based centrally upon the use of the membrane-permeable, thiol-reactive agent monobromobimane (mBBr).[11] We now report the extension of these studies to the analysis of NAC in biological systems, in the presence of endogenous sulfhydryl components.

MATERIALS AND METHODS

Compounds

[^{14}C]-N-acetyl-L-cysteine (sp. act. 7 mCi/mmol) was purchased from Inveresk Research International, Musselburgh, Scotland. N-acetyl-L-cysteine was purchased in greater than 99 per cent purity form Sigma Chemical Company, St Louis, USA. N,N′-diacetylcystine was generated in aqueous solutions containing 10^{-4} M $CuCl_2$ by continuous oxygenation. N-acetylcysteine in granulated form (Fabrol®, 200 mg, Ciba-Geigy, Gothenburg, Sweden) and injectable (Fluimucil®, 300 mg in 3 ml, Zambon Pharmaceutica, Bresso, Italy) were the kind gift of Mr B. Mellander,

Ciba-Geigy, Gothenburg, Sweden. All other materials were as detailed previously.[11]

Human volunteer study

A single healthy male volunteer (age 26 yr, weight 94 kg) who had given informed consent in writing, took part in an oral/i.v. cross-over dosing study with NAC. The volunteer received on separate occasions one week apart, single administrations of 200 mg NAC orally and intravenously by slow infusion over a four minute period. Blood samples (5 ml) were withdrawn through an indwelling cannula immediately prior to and at regular intervals after dosing in each case. Samples were collected into heparinized tubes and plasma separated by centrifugation at 1500 × g for 5 minutes at 4° C. Packed cells were resuspended to the original haematocrit with isotonic saline. Samples were analysed as soon as possible to minimize artefacts due to oxidation. Urine samples were also collected for the duration of the experiments.

Assay of pharmaceutical formulations

The NAC contents of the oral and intravenous formulations used were assayed, following dissolution (of the granules) and dilution to give test samples of approximately 100 μM NAC.

Assay procedures

The content of free NAC in biological samples was determined by *in situ* derivatizations with mBBr as described previously.[11] Bound NAC present in labile disulfides within samples was also determined by *in situ* derivatization with mBBr following reduction of samples with dithiothreitol (DTT).[11]

Calibration curves for NAC were constructed at regular intervals by spiking known amounts of standard into Krebs buffer pH 7·4 containing 25 mM Hepes.

Controls to determine the absolute recovery of NAC during the derivatization procedure were performed using the ^{14}C-labelled material. The precision of the assay was determined, in a blind fashion, by assay of solutions of containing known amounts of the compound.

The h.p.l.c. of the NAC–mBBR adduct was essentially as described previously,[11] except that the elution buffer additionally contained 0·25 per cent (v/v) perchloric acid. There is one cautionary note however; the adducts formed between mBBr, NAC and other aminothiols are charged and sensitive both to pH fluctuations and column ageing. Thus the retention time of the mBBr–thiol adducts in general, and that of the NAC–mBBR adduct particularly, tend both to decrease with column age and, most importantly, increase with decreasing pH. Thus, some slight alterations in running buffer pH may be necessary in order to ensure optimal separation of the

NAC–mBBr adduct and those of other aminothiols, particularly when beginning a new column.

Pharmacokinetic analysis

Intravenous plasma pharmacokinetic parameters were derived by a computer programme assuming two-compartment open kinetics. Terms were also corrected for the duration of the infusion.[12]

RESULTS

Separation of the mBBR–NAC adduct

The h.p.l.c. system used in this study afforded rapid separation of the mBBr–NAC adduct (r.t., 7·0 min) formed during *in situ* derivatisation of biological samples with mBBR. This adduct peak was well separated from both the reagent hydrolysis peaks (Figure 1(a)) and the mBBr adduct peaks of cysteine and GSH (Figure 1(b)). Figure 1(c) also shows a trace obtained

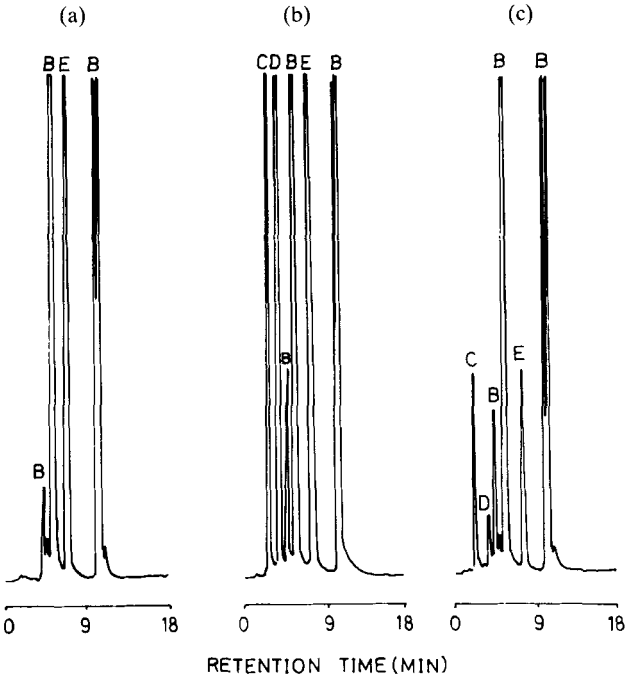

Figure 1. Typical h.p.l.c. traces obtained from the analysis of NAC standards and NAC in plasma with mBBr. Trace (a) = NAC (100 μM); trace (b) = NAC, cysteine, GSH (all 100 μM); trace (c) = plasma from 10 min after infusion of NAC (200 mg) to a human volunteer. Peak C = cysteine–mBBr adduct (r.t. 2·3 min); peak D = GSH–mBBr adduct (r.t. 3·5 min); peaks B = reagent peaks (r.ts 4·6 and 5·6 min); peak E = NAC–mBBr adduct (r.t. 7·4 min). Integrator attenuation 2^5mV F.s.d.

from the derivatization of plasma obtained 10 min after infusion of NAC (200 mg) to a volunteer.

Dithiothreitol reduction of oxidized NAC

The effectiveness of the DTT treatment used in these experiments to liberate NAC from oxidized sulfhydryl components of biological samples was tested using standard samples of NAC disulfide. Figures 2(a) and 2(b) indicate typical h.p.l.c. traces obtained from the derivatization of a sample containing 50 μM NAC disulfide and a similar sample treated with DTT prior to derivatization, respectively. Stoichiometric recovery of NAC was achieved under these conditions and this recovery extended up to concentrations as much as 1 mM NAC disulfide. Figure 2(c) shows a similar trace obtained following reduction of a plasma sample obtained 10 min after infusion of NAC (200 mg) to a volunteer.

Absolute recovery of NAC

The recovery of NAC from Krebs buffer as the mBBr adduct under the derivatization conditions used was 102 ± 3 per cent and 99 ± 2 per cent at

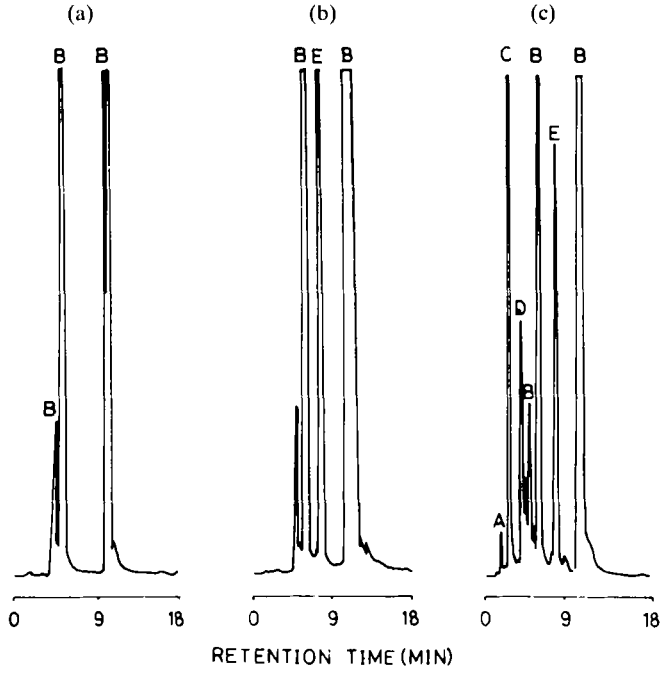

Figure 2. Some typical h.p.l.c. traces obtained from the prederivatization DTT reduction of NAC standards and NAC-containing plasma with DTT. Trace (a) = NAC disulfide (50 μM); trace (b) = NAC disulfide (50 μM) treated with DTT (5 mM) prior to derivatization; trace (c) = plasma obtained 10 min after infusion of NAC (200 mg) to a volunteer, pretreated with DTT as above. Peak assignment as for Figure 1

1 μM NAC and 1 mM NAC, respectively ($n = 4$). This recovery was unaffected by spiking NAC into plasma and assaying immediately.

Calibration curves

Calibration curves were constructed at regular intervals, typically over the range 1 μM to 1 mM NAC. Curves constructed of integrated peak areas vs. NAC concentration were linear over the full range and passed through the origin (Figure 3). The slope of the line is similar to that for the corresponding analysis of GSH.[11]

Sensitivity, reproducibility, and precision

The absolute limit of the detection of NAC, using the relationship, limit of detection = $2(N/S)A$, where N represents noise, S represents the signal and A is the injected amount, was calculated as 10 pmol/sample. However, a working limit of sensitivity was set at 50 pmol/sample (5 pmol on-column).

The same-day reproducibilities of assay were ±4, ±5 and ±3 per cent at 1 μM, 10 μM and 1 mM NAC, respectively ($n = 4$). The corresponding day-to-day variation was not greater than these values. The precision of the assay was checked by assaying, in a 'blind fashion', samples of Krebs buffer and blank plasma spiked with known concentrations of NAC. Table 1 shows results of such controls and indicates a mean precision of assay of NAC over the range 10 μM to 1 mM of 97 per cent.

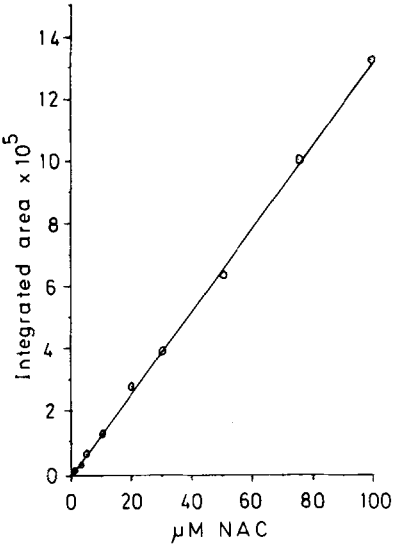

Figure 3. A typical calibration curve for the analysis of NAC in biological systems over the range 1 to 100 μM. Equation of the line: $y = 12{\cdot}9\,x$. Correlation coefficient of the line: 0·997

Table 1. The precision of assay of NAC using monobromobimane

Actual amount of NAC/sample (nmol)	Assayed* amount (nmol)		Percentage deviation of assay from standard (to n.s.f.)	
	Krebs	Plasma†		
60·0	60·6	59·3	1·0	1·0
5·0	4·7	4·6	6·0	8·0
15·0	14·7	15·3	2·0	2·0
100·0	102·9	100·1	3·0	0·0
1·0	1·0	1·1	0·0	7·0
35·0	35·6	34·0	2·0	3·0
75·0	77·9	77·1	4·0	3·0
0·0	0·0	0·0	0·0	0·0
20·0	20·0	20·7	0·0	4·0
80·0	82·8	81·8	4·0	2·0
3·0	3·2	2·9	7·0	3·0
8·0	8·6	7·1	7·0	11·0
		$\bar{X}$	3·0	4·0
		SD	3·0	3·0

* Assayed blind.
† Assayed immediately after addition.

The pharmacokinetics of NAC following separate oral and intravenous administration to a healthy volunteer

An infusion of NAC (200 mg) over a four minute period to a healthy volunteer resulted in a peak free NAC plasma concentration of 76 nmol/ml immediately after the infusion period (Figure 4(A)). Following this, the plasma free NAC concentration fell rapidly in a biphasic manner, becoming undetectable between two and two and a half hours after the infusion period. Treatment of the plasma samples with DTT prior to derivatization resulted in the liberation of additional NAC from labile disulfides. A peak total NAC concentration of 80 nmol/ml was achieved two minutes after the infusion period, with the concentration declining biphasically, but less rapidly than free NAC, to a level of 3 nmol/ml six hours after the infusion period.

By subtraction of the free NAC concentration from that of total NAC at each time point, an estimate of NAC present in labile disulfides in plasma at each time point may be obtained. Figure 4(A) indicates that the peak concentration of NAC (30 nmol/ml) present in labile disulfides within plasma was achieved ten minutes after the infusion period and that the concentration declines steadily thereafter. Pharmacokinetic parameters derived from the free and total NAC plasma concentration–time curves are shown in Table 2.

Following oral administration of NAC (200 mg), free NAC could not be

Table 2. Pharmacokinetic parameters derived from the assay of free and total NAC in plasma following the intravenous administration of NAC (200 mg) to a volunteer

Parameter	Units	Free NAC	Total NAC
A	nmol ml^{-1}	109·28	73·51
α	min^{-1}	0·12	0·098
$T_{½\alpha}$	min	5·60	7·10
B	nmol ml^{-1}	10·19	37·52
β	min^{-1}	0·018	0·0063
$T_{½\beta}$	min	37·7	108·6
V_β	ml kg^{-1}	593·00	262·00
V_{ss}	ml kg^{-1}	307·00	264·00
Cl	ml min^{-1}	1001·30	197·40
Cl	L h^{-1} kg^{-1}	0·63	0·13
AUC (0–∞)	nmol ml^{-1} min	1224·70	6222·60

detected above the working limit of sensitivity in the plasma at any time point. In contrast, however, treatment of some plasma samples with DTT prior to derivatization was shown to liberate small amounts of NAC from labile disulfides. The peak total plasma level of NAC of 3·4 nmol/ml was achieved 90 min after dosing, with levels declining linearily therafter, becoming undetectable between four and five hours after dosing (Figure 4(B)). The area under the total NAC concentration–time curve was 0·41 μmol min ml^{-1}.

N-acetylcysteine could not be detected in red blood cells at any time point following either oral or intravenous infusion.

Following intravenous administration of NAC the urinary excretions of free and total NAC totalled 0·6 and 23 per cent of the dose, respectively, over the six hour collection period, with the majority of these (0·4 and 21 per cent, respectively) recovered during the initial three hours. In contrast, following oral administration of NAC, neither free nor oxidized NAC could be detected in urine above background control levels.

Assay of pharmaceutical preparations

Assay of the oral and intravenous NAC formulations used in this study revealed them to possess within 2 per cent of the stated content. Thus, the NAC content of the oral granulated formulation was 197·6 mg and that of the intravenous formulation 300·6 mg.

DISCUSSION

The methodologies described here allow for the rapid analysis of both reduced and oxidized NAC in most biological systems. Although these

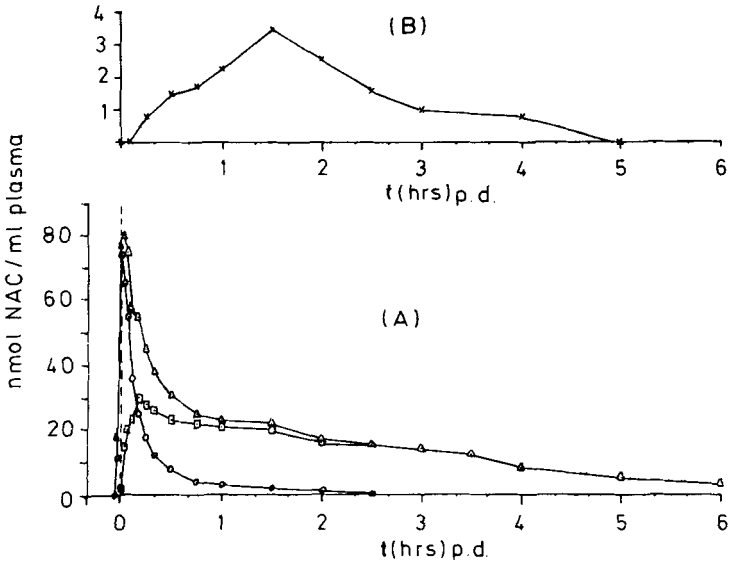

Figure 4. Plasma level vs. time curves of NAC following the (A) oral and (B) intravenous administration of NAC (200 mg) to a volunteer. Intravenous administration: free NAC ○—○; total NAC following reduction △—△; labile disulfides □—□. Oral administration: no free NAC; total NAC following reduction ×—×

procedures offer similar assay sensitivity to other published procedures,[9,10] the use of monobromobimane in the pre-column derivatization of NAC offers several important advantages to these. First, the membrane permeability of mBBr and its reactivity towards free thiols in solution allow rapid *in situ* derivatization of NAC, thus, eliminating the risk of oxidation artefacts during sample work-up. Secondly, by a simple pre-derivatization step involving *in situ* reduction of the sample with DTT, it is possible to assay the total NAC content of the sample and thus estimate NAC present in labile disulfides within the sample. Finally, we have previously demonstrated that under the conditions of derivatization employed, endogenous thiol components of biological systems are also amenable to co-analysis with NAC.

Until now, owing to the lack of appropriate analytical techniques, there has been a lack of comprehensive information on the disposition of NAC in man. When NAC is administered intravenously, this study shows that plasma levels of the free, reduced drug follow a two-compartment open model characterized by rapid distribution and clearance. The total body clearance of NAC of approximately twice the renal blood flow in healthy humans and the extremely low recovery of free NAC in the urine (< 1 per cent of the dose), strongly suggest that NAC undergoes rapid and extensive metabolism when given intravenously.

One possibility is that NAC, as a typical thiol, may undergo both oxidation

and/or disulfide exchange reactions to yield NAC containing disulfides. Following the intravenous administration of NAC in this experiment, the reduction of plasma samples to liberate NAC, and other low molecular weight thiols, from disulfide species demonstrated that total NAC plasma levels also follow a two-compartment open model. In contrast to that of the free drug, this model is characterized by more prolonged α and β phases and a much reduced clearance. From analysis of the kinetics of formation of NAC-containing disulfides and the concurrent analysis of the levels and redox status of cysteine and GSH in these plasma samples (data not shown), it is likely that this route of metabolism of NAC is governed not by chemical oxidation, but by thiol–disulfide exchange reactions between NAC and endogenous low molecular weight disulfides.

Despite this, only 20 per cent of the dose was recovered as NAC-containing disulfides in the urine during the collection period, indicating that other metabolic events play the major role in the clearance of intravenously administered NAC. It is probable that these involve deacetylation of NAC to cysteine and subsequent metabolism of this endogenous amino acid.

Following oral administration of NAC, the free drug could not be detected in plasma at any time point, with only small quantities of NAC liberated from disulfides by reduction. Additionally, excretion of NAC could not be detected in urine above control background levels.

A comparison of the oral and intravenous areas under the curve (AUCs) for the free drug indicated that free NAC is not bioavailable from this conventional oral formulation. In contrast to this, the absolute bioavailability of NAC, as determined by comparison of the corresponding total NAC curves, is 6·9 per cent.

This poor oral bioavailability of NAC may be due to incomplete release of the drug from the formulation. However, this is unlikely as there was no difficulty in assaying the NAC content of the granule formulation used. NAC may also be poorly absorbed from an oral dose. However, again this appears unlikely as pharmacokinetic experiments involving oral dosing of [^{35}S]-NAC to humans demonstrated high bioavailability of the radioactivity.[5] The final and most likely explanation for this is that rapid and extensive metabolism of NAC occurs when it is administered orally, involving a high first-pass metabolic clearance in the gut and/or liver. We are at present unable to estimate this first-pass loss, as it is not possible to estimate the percentage of the drug absorbed in this experiment.

These techniques are at present being applied to further pharmacokinetic experiments in humans and to attempts to delineate potential metabolic processes which control the disposition of NAC in man.

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