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Analysis of cysteine and *N*-acetylcysteine in human plasma by high-performance liquid chromatography at the basal state and after oral administration of *N*-acetylcysteine

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Abstract

A high-performance liquid chromatographic method for the determination of free reduced cysteine and *N*-acetylcysteine in human plasma at the basal state and after oral administration of *N*-acetylcysteine is described. The method is based on acid-catalysed conversion of plasma thiols to the corresponding *S*-nitroso derivatives by excess of nitrite and their subsequent cation-pairing RP-HPLC with detection at 333 nm. Recovery rates of cysteine and *N*-acetylcysteine added to human plasma were 94.6 and 99.6%, respectively. Inter- and intra-day precision were below 6%. In healthy humans ($n=5$), free reduced cysteine was determined to be (mean±S.E.) 10.0±0.96 μ*M*. No *N*-acetylcysteine was detected in plasma of these subjects above the limit of detection (e.g. 170 n*M*). The method was successfully applied to a pharmacokinetic study on orally administered *N*-acetylcysteine to healthy volunteers. © 1998 Elsevier Science B.V.

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1. Introduction

Low molecular weight (LMW), sulfhydryl-group(s) containing compounds such as cysteine (Cys), homocysteine, cysteinylglycine (Cysgly) and reduced glutathione (GSH) are involved in a variety of biochemical processes. Free reduced Cys is the most abundant LMW thiol in human plasma [1]. *N*-Acetylcysteine (NAC) is a widely used mucolytic drug [2]. In humans, orally administered *N*-acetylcysteine is mainly metabolized to Cys [3,4]. Endogenous NAC seems to be exclusively present in human urine from *N*-acetylation of Cys in the kidney [5].

A variety of analytical approaches has been developed for the analysis of thiols including Cys in biological fluids [1,6–15]. The majority of these methods uses a derivatization procedure in order to obtain compounds suitable for detection by UV, fluorometry or gas chromatography (reviewed in [16,17]). Saville has published a spectrophotometric assay method for the quantitation of thiols utilizing the derivatization of thiols with nitrous acid to the corresponding *S*-nitroso derivatives [18]. Since that time several works have been reported that used this approach to measure LMW and protein SH groups (reviewed in [16]). However, because of a lack in

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selectivity none of these methods have been applied to the quantitative determination of thiols in plasma or in mixtures of thiols in aqueous solutions.

The aim of the present work was to establish the utility of the *S*-nitrosylation with nitrous acid for the determination of Cys and NAC in human plasma by reversed-phase high-performance liquid chromatography (RP-HPLC) and UV detection. The potential relevancy of this approach for the simultaneous measurement in human plasma of Cys and NAC from orally administered *N*-acetylcysteine is demonstrated by performing a successful preliminary pharmacokinetic study in healthy volunteers.

2. Experimental

2.1. Chemicals

GSH, Cysgly and D,L-homocysteine were purchased from Sigma (Munich, Germany). Cys, *N*-acetyl-L-cysteine (NAC), 1-octanesulfonic acid and diethylenetriaminepentaacetic acid (DTPA) were obtained from Aldrich (Steinheim, Germany). Sodium nitrite was bought from Fluka (Neu-Ulm, Germany). Acetonitrile was obtained from J.T. Baker (Deventer, The Netherlands). 5-Sulfosalicylic acid was purchased from Merck (Darmstadt, Germany).

2.2. Standards

Standard solutions of reduced thiols (each 10 m*M*) were prepared in 50 m*M* phosphate buffer, pH 7.4, subdivided into 50 μl aliquots, and stored at −20°C until use. Aliquots from these solutions or from their dilutions in the same buffer were added to plasma samples or were derivatized as described below.

2.3. Sample collection and derivatization procedure

Blood from healthy normal fasted volunteers was collected in tubes containing EDTA as an anticoagulant. The monovettes were put on ice and immediately centrifuged at 2°C (1500 *g*, 5 min). 500 μl aliquots of plasma were treated with 5 μl of a 100 m*M* aqueous solution of DTPA, 50 μl of a 10 m*M* aqueous solution of nitrite followed by 50 μl of a 50 wt.% solution of 5-sulfosalicylic acid and 50 μl of 5 *M* HCl. For quantitative measurements the samples were allowed to stand at room temperature for 20 min for maximum derivatization. The suspensions were then centrifuged (1500 *g*, 5 min) at 2°C, and 200 μl aliquots of the supernatants were injected into the HPLC system or stored at −20°C until analysis. The same procedure was used for derivatization of thiols in buffer solutions with the exception that no centrifugation was performed. Standard solutions of *S*-nitroso derivatives were also prepared by incubation of 100 μl aliquots from a thiol solution in phosphate buffer with 100 μl of a 10 m*M* solution of sodium nitrite in distilled water and with 10 μl of a 5 *M* HCl solution.

2.4. Reversed-phase high-performance liquid chromatography

RP-HPLC analyses were performed on a Pharmacia LKB solvent delivery system (Freiburg, Germany) consisting of a gradient pump model 2249 and of a low pressure mixer. The system was connected with an UV/visible LDC Spectromonitor model 1204D (LDC Analytical, Gelnhausen, Germany) and a Shimadzu integrator model C-R3A (Kyoto, Japan). The analytical column (250×4.6 mm I.D.) was packed with 5 μm particles size Nucleosil material from Macherey-Nagel (Düren, Germany). The mobile phase consisted of sodium dihydrogenphosphate and the cation-pairing agent 1-octanesulfonic acid, each 10 m*M*, in acetonitrile–water (9:91, v/v). The pH was adjusted to 2.0 by orthophosphoric acid. Isocratic elutions were performed at a flow-rate of 1.5 ml/min. The effluent was monitored at 333 n*M*. After 25 min of analysis retained compounds were eluted from the column for 10 min with acetonitrile–water (50:50, v/v), at a flow-rate of 1.5 ml/min.

2.5. Standard curves

Standard curves were prepared by derivatization and RP-HPLC analyses of mixtures of NAC and Cys in phosphate buffer containing each thiol at concentrations of 0, 0.62, 1.25, 2.5, 5, 7.5, 10, 15 and 30

μ*M*. Linear regression analysis between the peak area observed for each thiol (*y*) and the concentration of the thiol used (*x*) resulted in linear responses with the following regression equations: $y=160+6046x$ ($r^2=0.997$) for NAC and $y=-28+5345x$ ($r^2=1.000$) for Cys. These standard curves were used to calculate the recovery rates of Cys and NAC from plasma and the accuracy of the method. Inter-day reproducibility of the standard curves was studied by generating standard curves in buffer as described above on four consecutive days. The slopes of the standard curves were determined as (mean±S.D.): 6070±242 for NAC and 5200±280 for Cys.

2.6. Recovery and precision studies

A series of equal aliquots of buffered solutions of NAC and Cys were combined. Plasma stored on ice was treated in duplicate with these mixtures in order to obtain added concentrations of 2.5, 5.0, 7.5, 10, 15 and 20 μ*M* of each thiol. By using standard curves in aqueous solutions, basal plasma values and those from addition of the thiols were calculated. Mean recovery rates of the thiols from plasma were calculated by linear regression analysis between the found net concentrations of exogenously added thiols (*y*) vs. the added thiol concentrations (*x*).

Intra-day precision of the assay was determined by four-fold analysis of a pooled plasma sample from a healthy volunteer with and without external addition of thiols. Inter-day precision was determined by analysis on four consecutive days of a pooled plasma sample from a healthy volunteer with and without external addition of thiols.

2.7. Pharmacokinetic study

Five healthy volunteers (two females, three males; age: 28.8±6.2 years; weight: 79±23.5 kg, both mean±S.D.) fasted over night for 12 h were orally administered each a 600 mg *N*-acetylcysteine effervescent tablet dissolved in 100 ml of mineral water. Five min before, 60 and 90 min after administration of the tablet blood was taken, treated as described above and samples were analyzed by RP-HPLC.

3. Results

Fig. 1A–C show typical chromatograms from RP-HPLC analyses of thiols in plasma samples of a healthy volunteer. Fig. 1D shows a chromatogram from the RP-HPLC analysis of a mixture of synthetic NAC, Cys, Cysgly and GSH in aqueous solution using the same derivatization procedure as for plasma samples. The retention times of the *S*-nitroso derivatives of NAC, Cys, Cysgly and GSH in human samples ($n=14$) were determined to be (mean±S.D., min) 6.36±0.12, 10.24±0.18, 21.3±0.21 and 23.71±0.23, respectively. The *S*-nitroso derivative of synthetic D,L-homocysteine eluted at 32.88±0.25 min (mean±S.D., $n=3$) when isocratic elution was performed. The compound eluted at a retention time of about 24.8 min could not be identified. Cysgly, GSH and D,L-homocysteine were used as standards in the study because these thiols endogenously occur in human plasma.

By using the derivatization procedure described here no reduced thiols could be measured by the method of Ellman [19] in the supernatants of nitrite- and acid-treated plasma samples or in their similarly treated aqueous solutions. The use of high molar excess of nitrite over thiols was necessary because nitrite rapidly reacts also with sulfhydryl and other functionalities of proteins and of other plasma constituents under the derivatization conditions [20]. Storage of the reaction products at room temperature rapidly led to a decrease in the concentration of Cys and NAC in the absence of DTPA (data not shown). The chelating agent DTPA was found to highly increase the stability of Cys and NAC at room temperature: No changes in the concentrations of all *S*-nitroso derivatives were measured in samples stored at room temperature for up to 24 h in the presence of 1 m*M* DTPA (data not shown). No dependence of the derivatization time and the stability on the thiol concentration was observed in the range investigated (data not shown).

Data from recovery and accuracy experiments are summarized in Table 1. Linear standard curves were obtained for analyses of NAC and Cys in plasma spiked with up to 20 μ*M* of each thiol. Linear regression analysis between mean concentrations measured (*y*) and those added (*x*) resulted in the following regression equations: $y=0.02+0.996x$

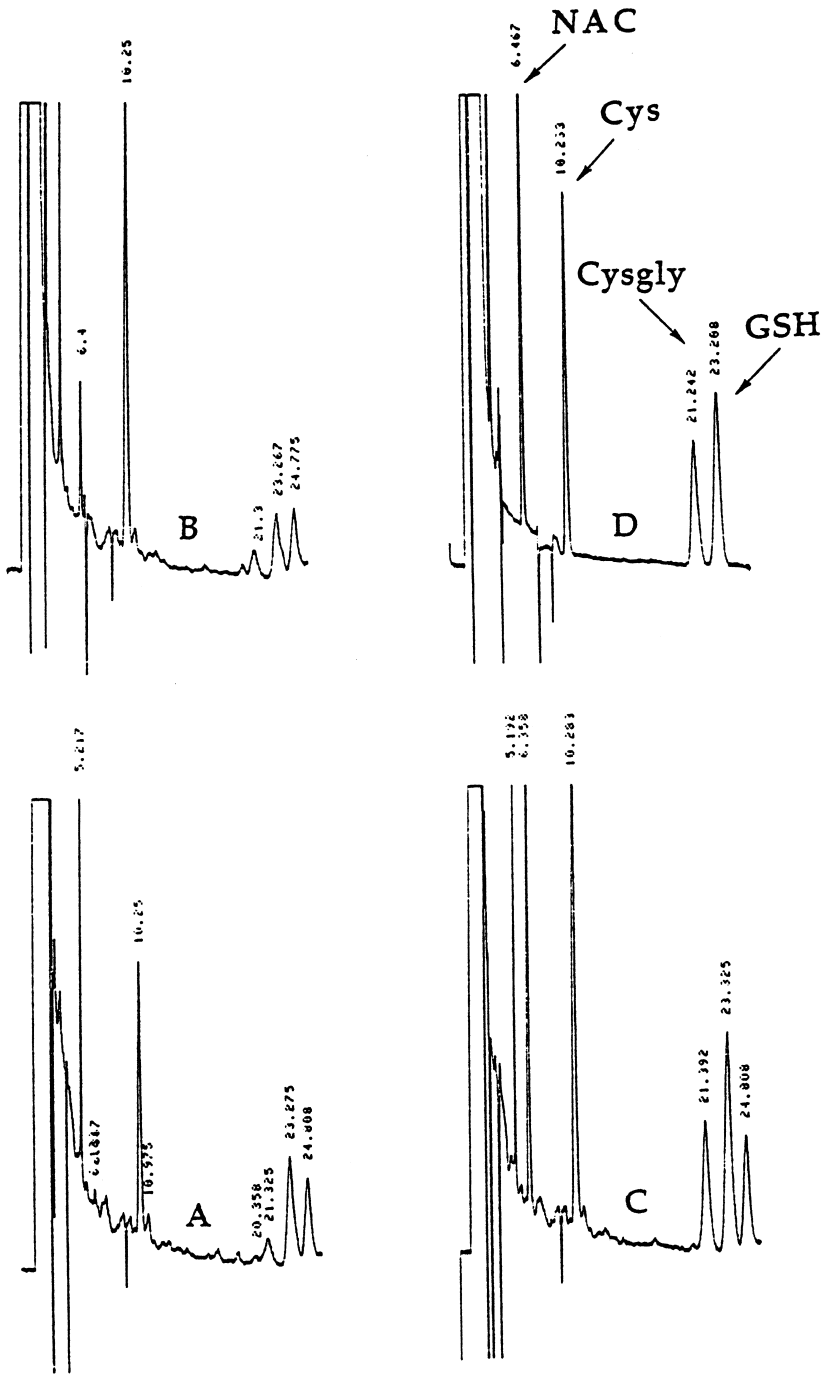

Fig. 1. Representative chromatograms from RP-HPLC analyses of plasma samples of a healthy volunteer. (A) Unspiked plasma (basal); (B) plasma 90 min after oral administration of 600 mg *N*-acetylcysteine effervescent tablet (ACC® long, HEXAL PHARMA GmbH, Holzkirchen, Germany); (C) basal plasma spiked each with 10 μ*M* of *N*-acetylcysteine (NAC), cysteine (Cys), cysteinylglycine (Cysgly) and glutathione (GSH); and (D) a reference mixture containing each 10 μ*M* of NAC, Cys, Cysgly and GSH in 50 m*M* phosphate buffer (pH 7.4). NAC, Cys, Cysgly and GSH were analyzed as their corresponding *S*-nitroso derivatives as described in Section 2.

Table 1
Accuracy of the method for the determination of NAC and Cys in human plasma

Thiol added (μ*M*)	Thiol found (μ*M*, mean±S.D.)		R.S.D. (%)	
	NAC	Cys	NAC	Cys
0	N.D.	7.3±0.28	N.A.	3.8
2.5	2.3±0.14	10.7±0.57	6.1	5.3
5.0	5.3±0.21	11.1±0.42	4.0	3.8
7.5	7.8±0.32	14.2±1.13	4.1	7.9
10	9.7±0.35	18.7±0.81	3.6	4.3
15	14.6±0.28	21.0±1.41	1.9	6.7
20	20.2±0.49	26.5±0.71	2.4	2.7
mean±S.D.			3.7±1.5	4.9±1.8

N.D., not detectable; N.A., not applicable.

(r^2=0.998) for NAC and y=7.53+0.946x (r^2=0.979) for Cys. The slopes of these regression equations give the mean recovery rates which amounted to 99.6% and 94.6%, respectively. The values for R.S.D shown in Table 1 indicate that basal concentrations of Cys can accurately be determined. Also, NAC added to human plasma can accurately be determined in the whole concentration range.

Intra-day R.S.D. of the method for Cys (7.5 μ*M*) in a unspiked human plasma sample was 2.6%. The corresponding values for a plasma sample spiked each with 10 μ*M* of NAC and Cys were 3.2% and 2.6%, respectively. Inter-day R.S.D. of the method for Cys (8.9 μ*M*) in a unspiked human plasma sample was 5.3%. The corresponding values for a plasma sample spiked each with 10 μ*M* of NAC and Cys were 4.1% and 3.8%, respectively.

Limits of detection of the method for assaying NAC and Cys were determined by analysing in triplicate aqueous solutions of these compounds in the concentration range 100 n*M* to 2000 n*M*. The following detection limits (signal-to-noise ratio of 3:1) were calculated: 170 n*M* (R.S.D. 12%) for NAC and 350 n*M* (R.S.D. 20%) for Cys.

Oral administration of a 600 mg *N*-acetylcysteine effervescent tablet to healthy volunteers resulted in the appearance of free reduced *N*-acetylcysteine in the plasma at a mean concentration of 4.6 μ*M* after 60 min, and of 2.5 μ*M* after 90 min of administration (Fig. 2). Before administration, NAC could not be detected in these volunteers at concentrations exceeding the detection limit of the method for NAC. The identity of plasma NAC derived from orally administered *N*-acetylcysteine effervescent tablet was established by a previously described GC–MS method [21]. The major metabolite of orally administered *N*-acetylcysteine in the volunteers was found to be Cys which reached a mean maximum concentration of 18.6 μ*M*. Thirty min later the mean concentration of Cys was 17.4 μ*M*. The highest plasma concentration of NAC (8.1 μ*M*) and Cys (21.2 μ*M*) 60 min after administration have been seen in the volunteer receiving the highest dose of *N*-acetylcysteine per kg body weight (e.g. 10 mg/kg).

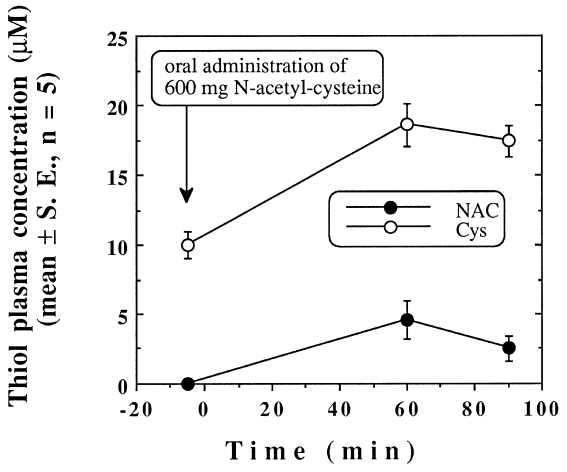

Fig. 2. Concentration–time curves for NAC and Cys before and after oral administration of 600 mg *N*-acetylcysteine effervescent tablets to healthy volunteers. The arrow indicates the time point of administration.

4. Discussion

The present work demonstrates that acid-catalysed *S*-nitrosylation of reduced free thiols in human plasma and subsequent cation-pairing RP-HPLC of the resulting *S*-nitroso derivatives and UV detection at 333 nm represent a useful method to quantitate accurately and precisely the reduced forms of free Cys and NAC in human plasma. We demonstrated that this method is useful to simultaneously determine these thiols in human plasma at the basal state and after oral administration of the drug *N*-acetylcysteine. Our findings with respect to basal levels of Cys [1] and NAC as well as their levels after oral administration of the drug *N*-acetylcysteine are in agreement with data from previous pharmacokinetic studies on orally administered *N*-acetylcysteine [3,4].

Because of the low molar absorption coefficient of the *S*-nitroso group ($\varepsilon \approx 700\ M^{-1}\ cm^{-1}$) our method is less sensitive than other techniques that use for example fluorescence [1,15] or electrochemical detection [9,17]. At present, the most suited and sensitive method for the determination of physiological LMW thiols in plasma seems to be the use of monobromobimane fluorescent labelling and HPLC as described by Mansoor et al. [1]. The detection limit of this method has been reported to be <2 pmol of a thiol. In our method we could detect about 170 n*M* of NAC which corresponds to an injected amount of approximately 34 pmol. Recently, Ercal et al. have reported a HPLC assay for NAC in biological fluids of the rat following derivatization with *N*-(1-pyrenyl)maleimide [15]. The limit of quantitation of this method has been reported as 32 n*M*. However, the limit of detection of our method is satisfactory for precise and accurate quantitation of Cys, the most abundant free reduced thiol in human plasma at the basal state, and of NAC following administration of *N*-acetylcysteine to humans. The main fields of application of our method should be the quantitative determination of Cys in human plasma in different clinical situations, and of Cys and NAC in pharmacokinetic studies on *N*-acetylcysteine in humans. Simultaneous quantitative protein precipitation and *S*-nitrosylation and high stability of the *S*-nitroso derivatives should provide a general use of the method especially in terms of automation.

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