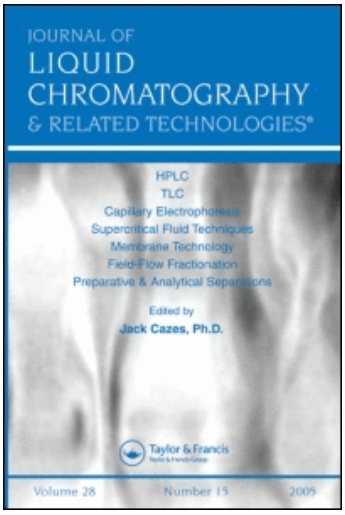


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# Determination of N-Acetylcysteine and Main Endogenous Thiols in Human Plasma by HPLC with Ultraviolet Detection in the Form of Their S-Quinolinium Derivatives

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# Determination of N-Acetylcysteine and Main Endogenous Thiols in Human Plasma by HPLC with Ultraviolet Detection in the Form of Their S-Quinolinium Derivatives

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**Abstract:** A new, sensitive, repeatable, and robust high performance liquid chromatography assay for the determination of total *N*-acetylcysteine, cysteine, cysteinylglycine, glutathione, and homocysteine in human plasma has been developed. The thiol concentrations were measured using precolumn specific derivatization with 2-chloro-1-methylquinolinium tetrafluoroborate, followed by high performance liquid chromatographic reversed phase separation with spectrophotometric detection. The oxidized and protein bound forms of thiols were converted into their reduced forms employing a reductive agent tris(2-carboxyethyl)phosphine. The chromatographic separation was accomplished in 12.5 min; the within run and between run imprecision were all less than 10%. The elution profile was as follows: 0–4 min, 11% B; 4–8 min, 11–30% B; 8–12 min, 30–11% B. The calibration graphs, obtained with the use of normal plasma spiked with growing amounts of analytes, were linear over the concentration ranges, covering most experimental and clinical cases (1–32 μM for homocysteine and glutathione, 1–320 μM for N-acetylcysteine and cysteine, and 1–64 μM for cysteinylglycine). For all analytes, recoveries between 91.2 and 108.6%, were observed.

**Keywords:** Determination, HPLC, N-acetylcysteine, Plasma thiols

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## INTRODUCTION

Thiols are a class of organic sulfur compounds characterized by the presence of sulfhydryl groups. In biological systems, thiols have numerous functions, including a central role in coordinating the antioxidant defense network. Plasma thiols, including cysteine, cysteinylglycine, glutathione, and homocysteine are being investigated as potential indicators of health status and disease risk.[1] N-acetylcysteine, an egzogenic aminothiol, is known to be a powerful mucolytic agent for the treatment of chronic bronchitis and other pulmonary diseases complicated by the production of viscous mucus.[2,3] N-acetylcysteine was also used in cancer chemotherapy[4] and as an antidote in paracetamol overdosage.[5] The properties of N-acetylcysteine as a protective agent in paracetamol over dosage and in chemotherapy depend on its function as a precursor amino acid for glutathione synthesis.[6] An important observation is that N-acetylcysteine causes a substantial decrease of plasma homocysteine (45%), cysteinylglycine (24%), and cysteine (11%) via increasing thiols urinary excretion.[7,8] Cysteine is a natural sulfur containing amino acid involved in a variety of important cellular functions, including detoxification, metabolism, and protein synthesis. Cystine, symmetrical disulphide of cysteine, is excreted through the kidneys and genetic mutations in the genes; encoding this transport system can cause cistinuria, characterized by excessive amounts of cystine, arginine, lysine, and ornithine in urine.[9] Although homocysteine is a normal metabolite, elevated levels of this thiol in plasma can be reasonably considered an independent risk factor for cardiovascular and neurodegenerative diseases, such as Alzheimer's.[10–14] Thiol oxidation and thiol disulphide equilibrium in particular, has an important role in many enzymatic and transport processes and hence, is a significant determinant of protein structure and function.[15] The facile oxidation of this compound leads to a variety of disulfides forms in vivo. These include low molecular mass symmetrical and unsymmetrical disulfides and disulfides with proteins, in human plasma especially with albumin. Reduced, free oxidized and protein bound forms of cysteine, homocysteine, glutathione, and cysteinylglycine comprise the plasma redox thiol status.[16] Oxidative stress defined as imbalance between oxidant production and antioxidant defense may lead to the oxidation of macromolecules and subsequent tissue damage.[17]

Tripeptide $\gamma$-glutamylcysteinylglycine, commonly known as glutathione, occupies a central place in the world of cellular thiols. Enhancing tissue glutathione reserve in mammals is a challenging task, particularly because glutathione is not available to most tissues when administered orally or injected intraperitoneally. The net amount of tissue glutathione depends primarily on two factors: (1) neosynthesis of glutathione, and (2) regeneration from its oxidized form.[18] Synthesis

of glutathione in the cell is rate limited by the availability of cysteine, which, in its reduced form, is highly unstable. Thus, one strategy to enhance intracellular glutathione is to improve cysteine availability within the cell. N-acetylcysteine may act to increase intracellular cysteine and subsequent glutathione levels through two mechanisms: (1) acting as an extracellular reducing agent, it can reduce cystine to the more easily transported cysteine, and (2) it can also be taken up into the cell by separate transport pathways and deacylated intracellulary to produce cysteine.[19] Additionally, N-acetylcysteine also acts by direct reaction between its reducing thiol group and reactive oxygen species protecting against oxidative damage in vitro and in vivo.[20] Since the response of a cell to oxidative stress typically involves alterations in thiol content,[21] plasma aminothiol concentrations are increasingly utilized for clinical and translational research involving oxidative stress,[17] and for routine clinical diagnosis and monitoring of various human diseases and metabolic disorders.[22,23] To assess the potential efficacy and toxicity associated with administration of N-acetylcysteine as a dietary supplement in addition to total parenteral nutrition and intravenous administration, assays capable of concurrently determining endogenous and exogenous aminothiols in human plasma are needed.

This report fills a need for a simple and reliable method to measure total N-acetylcysteine and simultaneous quantitation of its metabolically related main plasma aminothiols cysteine, cysteinylglycine, glutathione, and homocysteine. The method is based on the derivatization of analytes with 2-chloro-1-methylquinolinium tetrafluoroborate, separation of their 2-S-quinolinium derivatives by reversed phase HPLC, followed by detection and quantitation at 355 nm.



## EXPERIMENTAL

### Apparatus

The liquid chromatography equipment used for the analysis was made by Hewlett–Packard (1100 Series system, Waldbronn, Germany) and consisted of a quaternary pump, autosampler, thermostated column compartment, vacuum degasser, and diode array detector. For instrument control, data acquisition, and data analysis a Hewlett–Packard ChemStation for LC 3D system including single instrument Hewlett–Packard ChemStation software and Vectra color computer was used. UV spectra were recorded on a Hewlett–Packard HP 8453 diode array UV–Vis spectrophotometer. Water was purified using a Millipore Milli-QRG (Vienna, Austria) system.

## Chemicals and Reagents

L-Cysteine hydrochloride (CSH), DL-cystine (CSSC), glutathione (GSH), and oxidized glutathione (GSSG), DL-Homocysteine (HCSH), DL-homocystine (HCSSCH), cysteinylglycine (CGSH), and N-acetylcysteine (NACSH) were received from Sigma (St. Louis, MO, USA). 2-Chloro-1-methylquinolinium tetrafluoroborate (CMQT) was prepared in this laboratory according to the procedure described earlier.[24] Perchloric acid (PCA), ethylenediaminetetraacetic acid disodium salt (EDTA), sodium hydrogen phosphate heptahydrate ($Na_2HPO_4 \cdot 7H_2O$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4 \cdot 2H_2O$), and HPLC grade acetonitrile were from J.T. Baker (Deventer, The Netherlands). 3-Mercaptopropionic acid (3 MPA), 3,3′-dithiodipropionic acid (3,3′MPA) were purchased from Fluka (Buchs, Switzerland). Trichloroacetic acid (TCA) and tris(2-carboxyethyl)phosphine (TCEP) were from Merck (Darmstadt, Germany). All other reagents were HPLC or analytical reagent grade purchased from commercial sources. The pH of the buffers was adjusted by potentiometric titration. The titration system was calibrated with standard pH solutions. All reagents were tested and found to be stable for unattended analysis.



## Calibration Standards

Stock solutions of 10 mM N-acetylcysteine, cysteine, cysteinylglycine, glutathione, and homocysteine and their symmetrical disulfides needed in the method development procedure were prepared by dissolving appropriate amounts of the compounds in 2 mL of 0.1 M hydrochloric acid and diluting to the volume of 10 mL. These solutions were kept at 4°C for several days without noticeable change of the thiol content. For preparation of calibration standards, plasma samples were each placed in polypropylene tubes and spiked with the appropriate amount of working standard solutions of disulfides. Blood was collected from apparently healthy volunteers by venipuncture into tubes with EDTA. The tubes were placed on ice and processed within 20 min. After centrifugation at $4000 \times g$ at 4°C for 10 min, the clear plasma supernatant was collected and stored at −18°C until analysis.

## Assay Procedure

To 100 μL of plasma, 100 μL of phosphate buffer (pH 7.4, 0.2 M), 20 μL (0.1 mM) of 3,3′-dithiodipropionic acid (internal standard) and 10 μL of tris(2-carboxyethyl)phosphine phosphate buffer solution (0.25 M), were

added. The mixture was vortex mixed, followed by addition after 10 min of 10 μL of 0.1 M 2-chloro-1-methylquinolinium tetrafluoroborate. After 2 minutes, 100 μL of 3 M perchloric acid was added and the precipitated protein was separated by centrifugation for 10 min at 12000 × g. The supernatant was transferred to a vial, and 20 μL was injected into the chromatographic system.

### Chromatography

A 20 μL of the final analytical solution was injected onto a 150 × 4.6 mm, 5 μm ZORBAX SB $C_{18}$ column (Waldbronn, Germany). The mobile phase (flow rate, 1.2 mL $min^{-1}$; temperature, 25°C) consisted of 0.07 M trichloroacetic acid buffer (solution A), adjusted to pH 1.65 with lithium hydroxide solution of the same concentration, and acetonitrile (solution B). The elution profile was as follows: 0–4 min, 11% B; 4–8 min, 11–30% B; 8–12 min, 30–11% B. Identification of peaks was based on comparison of retention times and diode–array spectra, taken at a real time of analysis, with the corresponding set of data obtained by analyzing authentic compounds.



### Calibration

Calibration curves for plasma, total N-acetylcysteine, and metabolically related thiols were constructed by processing 100 μL of calibration standard samples of plasma, spiked with appropriate disulfides. The concentration ranges of glutathione and homocysteine were from 1.0 to 32 μM, for cysteine and N-acetylcysteine from 1 to 320 μM, and for cysteinylglycine from 1.0 to 64 μM of plasma. The calibration ranges can be easily extended up if needed.

## RESULTS AND DISCUSSION

Determination of thiols is difficult because they are highly reactive and easily oxidized during sample preparation. In addition, most thiols lack the structural properties necessary for the production of signals compatible with common HPLC detectors, such as UV absorbance and fluorescence. Therefore, the analyst must resort to derivatization for signal enhancement and labile sulphydryl group blocking if fluorescence or UV-Vis detection methods are employed. Numerous methods for the determination of thiols have been reported, including HPLC,[25–27] gas chromatography-mass spectrometry,[28] and capillary electrophoresis.[29]

To determine thiol content in human plasma, we exploit 2-Chloro-1-methylquinolinium tetrafluoroborate (CMQT) as the derivatization reagent, which rapidly and specifically reacts with −SH group of thiol compounds to form stable thioether linkage.[24,25] 2-S-quinolinium derivatives possess well defined absorption maximum at 355 nm whereas absorption maximum of CMQT occurs at 328 nm. This bathochromic shift is analytically advantageous because, thanks to this phenomenon, we avoid a huge chromatographic signal of the derivatization reagent excess.

## The pH Effect on Derivatization of Investigated Thiols

Because of the great susceptibility to oxidation, and for analytical UV signal enhancement, a derivatization reaction of investigated thiols was employed. The most important condition of successful derivatization is reaction pH control. The pH effect on the derivatization yield with CMQT in the range between pH 5 and 9 was studied. Reaction was quenched after 2 and 20 min by acidifying the mixture. In all cases, derivatization reaction was complete and 2-S-quinolinium derivatives were stable if reaction was carried out at pH 7 (Figures 1a and b). Actually, under these conditions, the derivatization reaction mixture is ready to be chromatographed just after mixing the substrates. The yield of derivatization reaction for all analytes and internal standard (3-mercaptopropionic acid) remained constant in the pH range 7–9, if a short (2 min) reaction time was used. When the longer reaction time was employed (20 min) at pH 9, substantial yield decrease of homocysteine derivative was observed, most likely because of thioether bond hydrolysis. When reaction in slightly acidic conditions (pH 5) was carried out, derivatization reaction required significantly longer time (Figures 1a and b).

## Optimization of Chromatographic Conditions

In order to establish optimum RP-HPLC conditions for separation of 2-S-quinolinium derivatives of N-acetylcysteine, cysteine, cysteinylglycine, glutathione, homocysteine, 3-mercaptopropionic acid, and derivatization reagent excess, several parameters of chromatographic conditions, such as pH and concentration of the TCA buffer, organic modifier concentration, mobile phase flow rate, and temperature were tested. Preliminary experiments for method development for plasma thiols determination were carried out in standard water solution. The TCA buffer was tested over the pH range of 1.5–3.5. Resolution between neighboring peaks within pH ranges 1.5–1.7, 2.2–2.4, and 3.1–3.5 for all analytes was

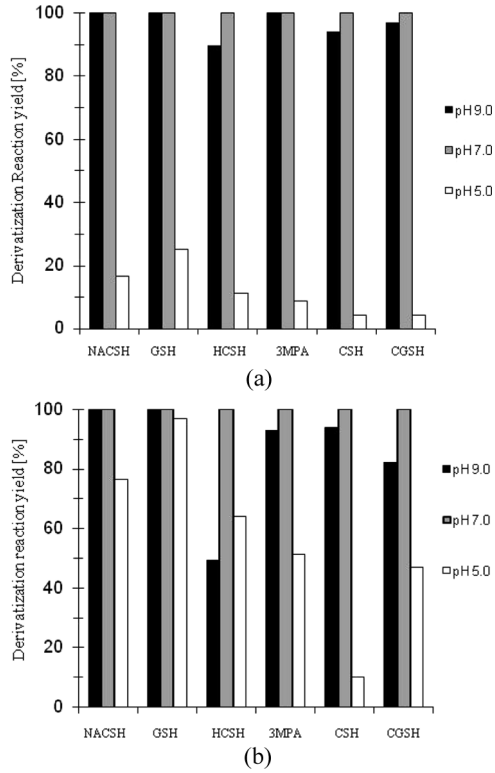


***Figure 1.*** The pH effect on derivatization reaction yield of thiols for 2 min (a) and 20 min (b) reaction time. Other derivatization conditions: 0.2 M phosphate buffer, 7-fold molar CMQT excess.



satisfying. For N-acetylcysteine, glutathione, and homocysteine coelution at pH 2.5–3.0 and pH 1.8–2.1 was observed (Figure 2). Effect of TCA buffer concentration on peak heights, retention factors, and resolutions was also determined (data not shown). In general, retention factors and resolution values increased with the TCA buffer concentration decrease. The peak heights for all of the analytes showed an increasing trend with pH decrease. As expected, retention factors for all analytes were increased significantly with the mobile phase pH decrease and TCA concentration growth. This retention behavior can be explained by the growing concentration of TCA, which acts as an ion pairing reagent for all positively charged molecules. Moreover, the pH decrease leads to positive charge growth of aminothiol moieties, because of progressive protonation of their amino groups, which in turn increased the net positive charge of the derivative as a whole. Increased acetonitrile content in

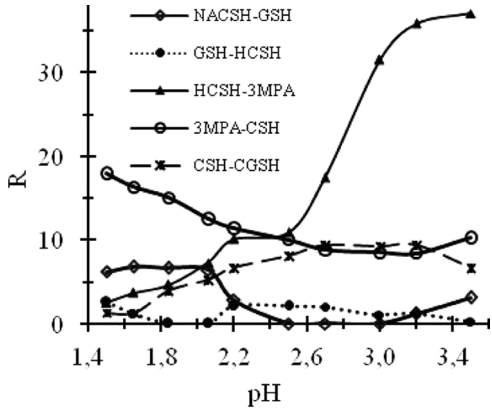


***Figure 2.*** Effect of the TCA buffer pH on resolution. Other chromatographic conditions: TCA concentration 0.07 M, flow rate 1.2 mL $min^{-1}$, temperature 25°C.



mobile phase produced a decrease in all retention factors. The peak heights of all analytes and internal standard were positively correlated to the acetonitrile mobile phase concentration. Finally, the chromatographic conditions used for subsequent analysis of plasma samples were as follows: the mobile phase consisting of 0.07 M trichloroacetic acid buffer (solution A), adjusted to pH 1.65 with lithium hydroxide solution of the same concentration, and acetonitrile (solution B), flow rate, 1.2 mL $min^{-1}$, temperature, 25°C. Under these conditions and the gradient profile shown in Experimental Section, CMQT-derivatives of the analytes and internal standard eluted after 7.4 min (RSD, 1.1%, n = 5) for N-acetylcysteine, 8.1 min (RSD, 0.7%, n = 5) for glutathione, 8.3 min (RSD, 0.5%, n = 5) for homocysteine, 8.5 min (RSD, 1.0%, n = 5) for 3-mercaptopropionic acid, 9.8 min (RSD, 0.3%, n = 5) for cysteine and 10 min (RSD, 0.7%, n = 5) for cysteinylglycine (Figure 3).

## Internal Standard Approach

In the case of biological samples with complicated matrices, like body fluids, when the analytical procedure consists of several steps, inclusion of an internal standard is desirable. The use of an internal standard in HPLC has distinct advantages, such as minimalizing the contribution of sample preparation including reduction and derivatization reactions, injection variation, and peak broadening caused by deterioration of the analytical column, to the final results. From amongst the disulphide candidate internal standard tested (data not shown), 3,3′-dithiodipropionic acid (3,3′MPA)

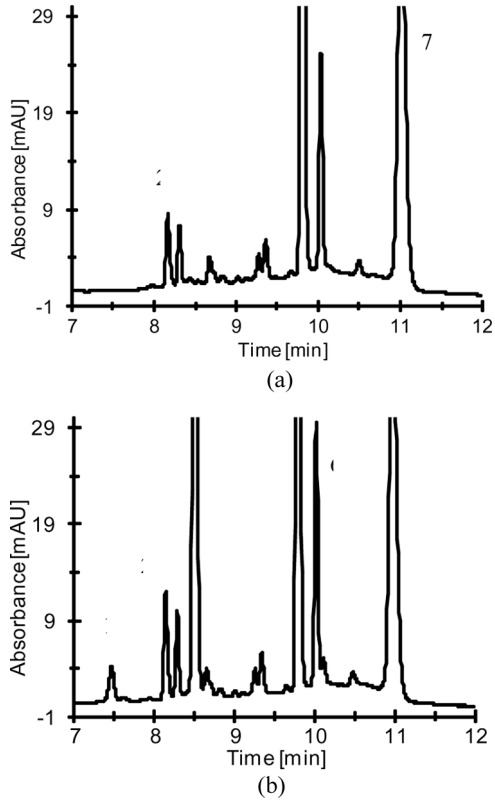


*Figure 3.* Typical HPLC chromatogram of human plasma (a) and plasma spiked (b) (4 μM for N–acetylcysteine, cysteinylglycine, glutathione and homocysteine, 20 μM for 3-mercaptopropionic acid and 40 μM for cysteine). Peaks: 1, N-acetylcysteine; 2, glutathione; 3, homocysteine; 4, 3-mercaptopropionic acid (IS); 5, cysteine; 6, cysteinylglycine 2-S-quinolinium derivatives; 7, excess of CMQT. Experimental details as described in the text.

was chosen. 3,3′MPA undergoes all steps of the analytical procedure including reduction and derivatization, possesses similar chemical and chromatographic properties, therefore greatly fulfills the requirements for a good internal standard.

## Search for Reducing Reagent

Because of high affinity to oxidation, low molecular mass thiols exist in human plasma mostly as symmetrical, unsymmetrical, and protein bound disulfides. More than 90% of plasma thiols stored are protein bound, and

of the remainder the majority is in the form of disulfides.[30] Thus, determination of total thiol content must comprise a disulphide bond disruption step. A reducing agent is necessary both for the reduction of the sulfide bonds and to keep the thiol in a reduced form until the start of derivatization. In addition, the reducing agent must be compatible with the thiol specific derivatization agent. Thiols such as 2-mercaptoethanol and dithiothreitol are not suitable in our assay because they consume CMQT and may produce additional derivatives, finally interfering with the analytes. Some HPLC methods exploit sodium borohydride (NaBH4)[25] and tri-n-butylphosphine (TNBP)[31,32] as reduction reagents. Use of sodium borohydride is very inconvenient and may lead to problems with the derivatization reaction because of pH control difficulties. Moreover, sodium borohydride decomposition reaction during sample preparation is concomitant with significant sample foam. TNBP has a highly disagreeable odor and is poorly soluble in water so that it must be dissolved in methanol or dimethylformamide which constrains its routine use.

Thus, for simultaneous determination of NACSH, CSH, CGSH, GSH, and HCSH in plasma samples, nonvolatile, stable, and soluble in water tris(2-carboxyethyl)phosphine was used.



## Validation Data

Validation parameters linearity, inaccuracy, imprecision, recovery, and lower limit of detection and quantification were tested according to the literature recommendations.[33,34]

### Linearity

A seven-point calibration plot was constructed using triplicate of the final analytical solution prepared according to the recommended procedure (Assay procedure, Calibration). The peak height ratios of thiol-CMQT derivatives to that of internal standard were plotted versus analyte concentrations and the curves were fitted by least-square linear regression analysis. The equations for the linear regression for N-acetylcysteine, glutathione, homocysteine, cysteine, and cysteinylglycine, were $y = 0.0051x + 0.0076$, $r = 0.9989$; $y = 0.0221x + 0.0381$, $r = 0.9992$; $y = 0.0085x + 0.0467$, $r = 0.9996$; $y = 0.0134x + 1.4843$, $r = 0.9994$; $y = 0.0303x + 0.1915$, $r = 0.9987$, respectively. Standard errors for the slope, for N-acetylcysteine, glutathione, homocysteine, cysteine, and cysteinylglycine, were 0.0000247, 0.000144, 0.000043, 0.000102, and 0.000163 mAU, respectively. Standard errors for the intercept were as follows: 0.003387, 0.002015, 0.000607, 0.01389, and 0.004513 $\text{mAU}/\text{mol} \times \text{L}$, respectively.

Inaccuracy, Imprecision, and Recovery

Intra-run imprecision and inaccuracy for the assay of total NACSH, CSH, CGSH, GSH, and HCSH as 2-S-quinolinium derivative expressed in RSD and mean relative error ($E_{rel}$), were determined for three concentrations representing the whole range of the calibration curve (Table 1). The absolute recovery was determined by the addition of the appropriate disulfides to the plasma samples of known concentration of endogenous total thiols from an equivalent plasma matrix. After addition of internal standard, the samples then proceeded through the procedure described in section Assay procedure.

Lower Limits of Detection and Quantitation

The limits of detection were assessed as the minimum detectable quantity of appropriate thiol that could be detected without interference from the baseline noise (signal-to-noise ratio of 3:1 for LLD and 6:1 for LLQ). The detection (LLD) and quantitation (LLQ) limits for N-acetylcysteine, cysteine, cysteinylglycine, glutathione, and homocysteine were 250 and 500, 100, and 200, 150, and 300, 200, and 400, and 200 and 400, nM of plasma, respectively.



***Table 1.*** Validation data

| Thiol | Added (μM) | Found (μM) | Recovery (%) | RSD* (imprecision) (%) | $E^*_{rel}$ (inaccuracy) (%) |
|---|---|---|---|---|---|
| NACSH | 1 | 1.02 | 102.4 | 8.06 | 2.45 |
| | 160 | 158.2 | 98.9 | 2.63 | −1.10 |
| | 320 | 322.3 | 100.7 | 0.52 | 0.73 |
| GSH | 1 | 1.08 | 108.3 | 13.6 | 8.32 |
| | 16 | 15.9 | 99.9 | 3.56 | −0.12 |
| | 32 | 31.9 | 99.7 | 1.32 | −0.25 |
| HCSH | 1 | 0.99 | 99.0 | 9.84 | −0.95 |
| | 16 | 16.3 | 101.7 | 3.63 | 1.72 |
| | 32 | 31.9 | 99.8 | 4.00 | −0.19 |
| CSH | 1 | 1.08 | 108.6 | 3.35 | 8.60 |
| | 160 | 156.1 | 97.6 | 0.47 | −2.40 |
| | 320 | 322.1 | 100.7 | 1.31 | 0.66 |
| CGSH | 1 | 0.91 | 91.2 | 9.7 | −8.75 |
| | 32 | 32.7 | 102.1 | 1.18 | 2.14 |
| | 64 | 63.8 | 99.6 | 1.34 | −0.35 |

*n = 3.

## CONCLUSION

A selective, sensitive, and simple HPLC method with spectrophotometric detection for simultaneous determination of N-acetylcysteine, cysteine, and related aminothiols in human plasma has been developed. The proposed method incorporates a technique previously elaborated in our laboratory utilizing the UV derivatizing reagent 2-chloro-1-methylquinolinium tetrafluoroborate. CMQT is an ultraviolet thiol specific tagging reagent and reacts rapidly with thiols via the nucleophilic displacement of the chlorine atom by sulfur contained in the thiol molecule, thereby producing the stable thioether. The 2-S-quinolinium derivatives of N-acetylcysteine, glutathione, homocysteine, cysteine, and cysteinylglycine are separated from each other, internal standard, other plasma components, and the excess of the derivatization reagent by ion pair reversed phase liquid chromatography with ultraviolet detection.

In comparison to earlier publications,[25] the method reported here has several advantages: (1) in contrast to sodium borohydride, tris(2-carboxyethyl)phosphine is more stable and, thus, is more suitable for routine use; (2) eluted under strongly acidic conditions, well separated 2-S-quinolinium derivatives peaks are higher, resulting in lower LOD and LOQ; (3) in terms of pH conditions, sample and mobile phase show great compatibility, so retention times of all analytes have very good repeatability. Because the method requires neither extraction nor preconcentration, it is not time consuming, laborious, or expensive. The whole unattended HPLC instrument acquisition time is 12 min. Its accuracy, precision, and recovery are well within the criteria[33,34] for biological sample analysis. We believe that this method fulfils experimental and clinical requirements for routine determination of N-acetylcysteine and main endogenous thiols in plasma. It is noteworthy that the implementation of plasma analysis for thiols with the use of this method is facilitated, because equipment for HPLC-UV analysis is often a part of the existing, standard instrumentation in hospital laboratories and staff is usually well experienced in its use. The UV detector is known for its stability and low demand in terms of maintenance. To the best of our knowledge, an HPLC-UV method for total content of N-acetylcysteine and four main thiols in plasma was not described so far.



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