A Simple HPLC–UV Method for Simultaneous Determination of Cysteine and Cysteinylglycine in Biological Fluids

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Summary. A new and simple method based on high-performance liquid chromatography with ultraviolet detection (HPLC-UV) for the determination of cysteine (Cys) and cysteinylglycine (CysGly) in plasma and urine has been developed. The method involves reduction of disulfide bonds with tris(2-carboxyethyl)phosphine, derivatization of the analytes with 2-chloro-1-methylquinolinium tetrafluoroborate, and separation on Aeris PEPTIDE XB-C18 column (150 mm × 4.6 mm, 3.6 µm, Phenomenex) with UV detection at 355 nm. The calibration lines, obtained with human plasma and urine spiked with Cys-Gly and Cys, were linear in the range of 2.5–50 µmol L⁻¹ and 20–300 µmol L⁻¹, respectively. The intra- and inter-day precision values of the method, expressed as a relative standard deviation, were 0.25–11.1% and 0.71–12.3%, respectively. The analytical recovery varied from 89.7 to 112.3%. The LOQs for total Cys and CysGly were 1.5 pmol and 2.3 pmol in peak, respectively. The method was successfully applied to samples donated by apparently healthy individuals. Concentrations of Cys and CysGly in human plasma from 18 subjects varied from 141.6 to 217.8 µmol L⁻¹ and from 21.1 to 50.9 µmol L⁻¹, respectively. Their concentrations in urine samples (n = 14) ranged from 137.3 to 426.8 µmol L⁻¹ and from 1.6 to 4.9 µmol L⁻¹, respectively.

Key Words: cysteine, cysteinylglycine, high-performance liquid chromatography, plasma and urine, ultraviolet detection

Introduction

Thiols play an important role in metabolic processes of all living organisms, and analytical control of these compounds is very important in order to understand their physiological and pathological functions. Cysteine (Cys), a product of metabolic transsulfuration pathway, constitutes a fundamental substrate for glutathione biosynthesis whose enzymatic degradation leads to cysteinylglycine (CysGly) formation. The imbalance in physiological levels of Cys and CysGly is closely related to some disorders including cystinosis, cystinuria, rheumatoid arthritis, lung cancer, and chronic kidney dis...
Ubiquitous natural occurrence of thiols in biological systems, their biochemical importance, and linkage with many disorders have promoted ample studies towards possibility of their determination in biological samples.

It is well established that sample preparation and optimization of separation conditions are two crucial steps for all analytical methods. The choice of the way of sample preparation and chromatographic conditions directly influences the quality of the results of an analytical measurement and thereby time as well as total cost of the analysis. Plasma and urine belong to the most frequently analyzed human biofluids. Unfortunately, the determination of thiol compounds in biological samples still remains perplexing. The main challenge lies in their physicochemical properties. Aside from the great susceptibility to oxidation, which can occur before or during analytical process, most thiols lack the structural properties necessary for the generation of signals compatible with common high-performance liquid chromatography (HPLC) detectors such as ultraviolet (UV) absorbance and fluorescence. Therefore, the analyst must resort to derivatization for signal enhancement and labile sulfhydryl group blocking if fluorescence or UV–vis detection methods are employed. Moreover, low concentration of biologically important thiols in real matrices, high polarity, and good solubility in water make their extraction very troublesome. Among a variety of assays designed to determination of thiols in biological fluids, most of them depend on derivatization followed by chromatographic separation and ultraviolet [7–9] or fluorescence detection [10–15]. Most of chromatographic procedures dedicated to hydrophilic thiols measurements exploit reversed phase (RP)-HPLC and buffered eluents containing ion-pairing reagents, such as alkyl sulphonates or trichloroacetic acid [7–20]. Unfortunately, such approach requires a long equilibration time of a chromatographic column and significantly increases the total costs of analysis.

2-Chloro-1-methylquinolinium tetrafluoroborate (CMQT) was previously used for the determination of biologically important thiols in different matrices including plasma [9, 13], defined media [12], saliva [16], plant tissues [17], and urine [20]. All of these methods allow determination of thiols in the only one matrix. Here, we describe a simple HPLC-UV-based method for simultaneous determination of Cys and CysGly, which is applicable to human plasma and urine. The assay is based on reduction of disulfide bonds with tris(2-carboxyethyl)phosphine (TCEP), derivatization of thiols with CMQT followed by liquid chromatography separation, and UV detection at 355 nm. Importantly, presented assay enables significant reduction of
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hazardous chemicals consumption and shortening the analysis time, without compromising separation.

Materials and Methods

Chemicals and Reagents

Cysteine (Cys), cysteinylglycine (CysGly), homocysteine (Hcy), glutathione (GSH), and their symmetrical disulfides, tris(2-carboxyethyl)phosphine hydrochloride (TCEP) and sodium phosphate dibasic heptahydrate (Na₂HPO₄×7H₂O), were purchased from Sigma-Aldrich Company (St. Louis, MO, USA). Acetonitrile (MeCN), methanol (MeOH), ethanol (EtOH) HPLC gradient grade, perchloric acid (PCA), and sodium dihydrogen phosphate monohydrate (NaH₂PO₄×H₂O) were obtained from J.T. Baker (Deventer, Netherlands). 2-Chloro-1-methylquinolinium tetrafluoroborate (CMQT) was prepared in our laboratory as described previously [18]. All reagents were tested and found to be stable for unattended analysis.

Instrumentation

All chromatographic analyses were performed with a 1220 Infinity LC system (Agilent Technologies, Waldbronn, Germany) equipped with binary pump integrated with two-channel degasser, autosampler, column oven and diode-array detector (DAD) controlled by OpenLAB CDS ChemStation software. Analytes were separated on Aeris PEPTIDE XB-C18 column (150 mm × 4.6 mm, 3.6 µm) from Phenomenex (Torrance, CA, USA). Water was purified using Milli-QRG system (Millipore, Vienna, Austria). For pH measurement, an HI 221 (Hanna Instruments, Woonsocket, RI, USA) pH meter was used. The precipitated proteins were removed from sample by centrifugation using a Hettich Mikro 200R centrifuge with fast cool function (Hettich Zentrifugen, Tuttlingen, Germany).

Biological Samples Collection and Pretreatment

The first morning urine samples were collected anonymously from healthy volunteers in different age and sex. The donors were not supplemented with analytes before sample collection. Blood was collected by venipuncture
from apparently healthy subjects in a fasting state to the K3 EDTA Vacutainer tubes, cooled on ice and centrifuged at 800×g for 15 min at room temperature within 30 min after collection. The plasma supernatant was stored at −80 °C or analyzed within 2 h. Both plasma and urine samples were prepared according to modified procedure published previously [19]. A 24 μL of appropriate biological fluid was diluted with 24 μL of 0.2 mol L⁻¹ pH 7.8 phosphate buffer and treated with 2 μL of 0.25 mol L⁻¹ TCEP solution for 15 min in order to reduce disulfide bonds. Next, 2 μL of 0.1 mol L⁻¹ CMQT solution was added and the reaction mixture was vigorously mixed before keeping it for 3 min at room temperature. Then, 4 μL of 3 mol L⁻¹ PCA was added and precipitated proteins (for plasma samples) were removed by centrifugation at 12,000×g for 10 min at 4 °C. Then, 1 μL of supernatant was injected onto HPLC column.

The investigation was performed after approval by the Ethical Committee of the University of Łódź.

**Chromatographic Conditions**

The chromatographic separation of the 2-S-quinolinium derivatives of Cys and CysGly was accomplished using isocratic elution with mobile phase containing H₂O (90%) and EtOH (10%) (v/v) at a flow rate of 1.5 mL min⁻¹. The 2-S-quinolinium derivatives of thiols were detected at 355 nm, and separations were performed at room temperature. Identification of peaks was based on comparison of retention times and diode-array spectra, taken at real time of analysis, with the corresponding set at data obtained by analyzing authentic compounds.

**Stock Solutions of Reagents and Standards**

Stock solutions of 0.25 mol L⁻¹ TCEP and 0.1 mol L⁻¹ CMQT were prepared by dissolving appropriate amounts of these compounds in 1 mL of deionized water. Stock solutions of 0.05 mol L⁻¹ of Cys and CysGly as well as their oxidized forms were prepared by dissolving appropriate amounts of these compounds in 1 mL of 0.5 mol L⁻¹ HCl. Solutions were kept at 4 °C for several days without noticeable change of the analyte content. The working solutions were prepared daily by appropriate dilutions with deionized water.
Calibration

For preparation of calibration standards for the determination of total Cys and CysGly, aliquots of 24 µL human plasma or urine samples from apparently healthy donors were spiked with the increasing amount of working standard solutions of oxidized Cys and CysGly to provide a final concentration of exogenous Cys and CysGly of 20, 50, 100, 200, and 300 nmol mL$^{-1}$ and 0.5, 1, 5, 20, 30, and 50 nmol mL$^{-1}$, respectively. Then, samples were processed according to the procedure described in section “Biological samples collection and pretreatment.” The calibration standards were prepared in three replicates. The peak height values of Cys-CMQT and CysGly-CMQT were plotted versus analyte concentration, and the curves were fitted by least-square linear regression analysis.

Results and Discussion

Sample Preparation

It must be emphasized that a successful biological sample analysis cannot be achieved without an appropriate, convenient, and reliable sample preparation methodology. Due to a large number of individual compounds in plasma and urine samples, leading to difficulty in resolving the analytes of interest, low concentrations of exogenous or endogenous compounds of interest resulting in detection difficulties, and conjugation of analytes to protein and/or low-molecular-mass components of the analyzed mixture, a total thiol assays must usually comprise disulfide bond reduction followed by chemical derivatization. In the present study, for simultaneous determination of total Cys and CysGly, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), which reacts with all disulfides at room temperature, was used. Our earlier study has shown that TCEP reduces all dimer and mixed disulfides, efficiently making them accessible to derivatization reagent [9, 17, 19]. In the present methodology, for signal enhancement and labile sulfhydryl group protection, we exploited CMQT, a highly reactive and thiol specific UV derivatization reagent [18]. CMQT widely used for UV derivatization of hydrophilic thiols in their HPLC analysis in physiological fluids [7–9, 18, 19] reacts rapidly and quantitatively with hydrophilic thiols in slightly alkaline water solution to form stable S-quinolinium derivatives (Fig. 1). The derivatization scheme takes advantage of great susceptibility of the quinolinium molecule at position 2 to nucleophilic displacement and the high nucleo-
philicity of the thiol group. It has been earlier shown that the reaction is completed within 1–5 min at room temperature [9, 18]. In general, derivatization of Cys and CysGly is useful to increase their hydrophobicity, detectability in UV region, as well as chemical stability.

Fig. 1. Chemical derivatization reaction equation of Cys and CysGly with CMQT

**Separation**

Biologically important thiols are typically determined by a RP-HPLC technique with different modes of detection, mainly UV [7–9, 12, 20] and fluorescence [10, 11, 13–15]. Acetonitrile (MeCN) containing mixtures are by far the most frequently used mobile phases in HPLC. Most of chromatographic procedures for total thiols content are based on reversed phase mode and exploit MeCN in a mixture with trifluoroacetic acid (TFA) [10], trichloroacetic acid (TCA) [9, 12, 16, 20], or acetic acid [21]. MeCN possesses unique properties such as dissolving a wide range of solutes, low acidity, minimal chemical reactivity, and UV and FLD compatibility. Nevertheless, due to its toxicity, reducing MeCN consumption is the first option in greening LC, but the principles in doing so also apply to other mobile phase additives [22]. Some of mobile phase ingredients, e.g., buffer components or ion-pairing reagents, exhibit corrosive or cytotoxic properties and are persistent in the environment. Thus, the removal from eluents of constituents such as MeCN, TCA, TFA, or some surfactants becomes advantageous.

It is commonly known that the choice of chromatographic parameters directly affects quality of the separation. In our approach for chromatographic conditions optimization, a standard mixture of 2-S-quinolinium derivatives of Cys and CysGly was used. In order to optimize the separation conditions, different mobile phase compositions were tested. The con-
centrations of MeCN, MeOH, and EtOH in mobile phase studied ranged from 5 to 30%. The type and amount of organic modifier were adjusted independently to achieve optimal separation of Cys and CysGly S-quinolinium derivatives from each other and other plasma/urine endogenous thiols, which are known to react with CMQT [9]. As expected, the organic modifier increase was associated with a decrease in capacity factor and an increase in peak heights (data not shown). For all solvents, similar relationships were obtained. To avoid column deterioration, lower concentrations of the organic modifier were not tested. Contents of the organic solvent exceeding 30% resulted in a weak separation and elution of the analytes at the front of the chromatogram.

The method optimization also involved changes of the flow rate to ensure that the method can efficiently separate all sample components. To reduce the run time, flow rates of 1.2, 1.5, 1.75, 2, and 3 mL min\(^{-1}\) were assayed. The flow rate of 1.5 mL min\(^{-1}\) was selected to show lower retention times (~2 min) as well as good resolution. Higher flow rates were discarded.

Fig. 2. HPLC analyses of human plasma and urine for total Cys and CysGly, after reduction with TCEP and derivatization with CMQT. Chromatographic conditions: mobile phase EtOH–H\(_2\)O (10:90, \(v/v\)), flow rate 1.5 mL min\(^{-1}\), temperature 25 °C
to exceed the maximum operating pressure for the column or peaks overlapping (data not shown). After thorough study of the above chromatographic variables, the optimal separation conditions for plasma and urine sample were chosen. Figure 2 shows the final result of the procedure. Exemplary separation profiles obtained with the use of MeCN are also depicted in Fig. 3. Finally, for plasma and urine analysis, we decided to use the mixture of EtOH and water (10:90%, v/v) (section “Chromatographic conditions”). Interestingly, similar satisfactory results were obtained when ordinary alcohol, purchased in the supermarket, was used (Fig. 4). Under recommended conditions, all analytes elute within 2.5 min (Fig. 2) in contrast to earlier published methodologies enabling Cys determination, for which total separation time was 7 min [15, 20] and 6 min [12]. The short whole unattended instrument acquisition time enables analysis of 570 plasma or urine samples for 24 h. Making allowances for the content of EtOH in mobile phase (10%), such approach significantly reduces its consumption to 220 mL per diem.

Fig. 3. Representative chromatograms of human plasma and urine samples, after reduction with TCEP and derivatization with CMQT. Chromatographic conditions: mobile phase MeCN–H2O (12:88, v/v), flow rate 2 mL min⁻¹, temperature 25 °C.
**Validation of the Method**

The method was validated according to the guidelines for biological sample analysis [23]. Peaks of the analytes were identified by comparison of their retention times as well as spectra with those of authentic standards. The repeatability of the measurements was tested by injecting standard solution in ten replicates. The relative standard deviation values for retention time of Cys and CysGly were 0.31% and 0.73%, respectively.
Specificity of the method

Specificity of the method, defined as the ability to assess unequivocally the analyte in the presence of components which may be expected to be present in the sample, was also evaluated. Since Hcy and GSH are present in biological fluids and are derivatized with CMQT under the same conditions as Cys or Cys-Gly, the mixture containing all derivatized compounds was chromatographed under recommended conditions (section “Chromatographic conditions”). It has been established that 2-S-quinolinium derivatives of Hcy as well as GSH were not retained and eluted at the front of the chromatogram.

Precision and accuracy

Precision is expressed in terms of relative standard deviation, whereas accuracy as the percentage of analyte recovery calculated by expressing the mean measured amount as percentage of added amount. Accuracy was calculated with the use of a formula:

\[
\text{Accuracy (\%)} = \frac{\text{measured amount} - \text{endogenous content}}{\text{added amount}} \times 100\%
\]

<table>
<thead>
<tr>
<th>Table I. Accuracy and precision</th>
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<tbody>
<tr>
<td>Thiol</td>
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<td></td>
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<tr>
<td>Plasma</td>
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<tr>
<td>CysGly</td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>50</td>
</tr>
<tr>
<td>Cys</td>
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<tr>
<td>20</td>
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<tr>
<td>100</td>
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<tr>
<td>300</td>
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<tr>
<td>Urine</td>
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<tr>
<td>CysGly</td>
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<tr>
<td>1</td>
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<td>20</td>
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<tr>
<td>Cys</td>
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<td>20</td>
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<tr>
<td>100</td>
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<td>300</td>
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Three concentrations representing the entire range of the calibration curves were studied: one near the lower limit of quantitation, one near the center, and one near the upper boundary of the standard curve. Measured concentrations were assessed by the application of calibration curves obtained on that occasion. Detailed data concerning intra- and inter-day precision and accuracy are given in Table I.

**Linearity of the method**

Standard addition method was used for calibration of the method. The linearity of the method was assayed using six-point calibration plots, and at each concentration, three replicates were assayed, independently for plasma and urine. Calibration data including regression equations are shown in Table II. Validation parameters of all fitted calibration curves were satisfactory.

**Table II. Calibration data**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Regression equation</th>
<th>Linear range (nmol mL$^{-1}$)</th>
<th>$R^2$</th>
<th>LOQ$^a$</th>
<th>LOD$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CysGly</td>
<td>$y = 0.208x + 0.62$</td>
<td>1–50</td>
<td>0.9973</td>
<td>2.3</td>
<td>0.8</td>
</tr>
<tr>
<td>Cys</td>
<td>$y = 0.0262x + 7.36$</td>
<td>20–300</td>
<td>0.9971</td>
<td>1.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Urine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CysGly</td>
<td>$y = 0.099x + 0.20$</td>
<td>1–50</td>
<td>0.9930</td>
<td>2.3</td>
<td>0.7</td>
</tr>
<tr>
<td>Cys</td>
<td>$y = 0.115x + 11.6$</td>
<td>20–300</td>
<td>0.9997</td>
<td>1.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

**Low limit of quantification**

The limit of quantification (LOQ) defined as the concentration that produces a detector signal that could be clearly distinguished from the baseline (larger than nine times the baseline noise) for Cys and CysGly were 1.5 pmol and 2.3 pmol in peak, respectively. These values were determined by spiking a proxy matrix (0.9% NaCl in pH 7.4 10 mmol L$^{-1}$ phosphate buffer) with decreasing concentrations of analyte until signal to noise ratio of 9:1. The LOQs achieved with this assay, e.g., for Cys, were significantly lower in comparison with previously published studies, 2.4 pmol [10], 4 pmol [20], 14.4 pmol [12], 10 pmol [16], 20 pmol [21], and 15.4 pmol [24], and allowed the analysis of all the samples studied.
Real Sample Analysis

When the analytical procedure had been validated, it was applied to the analysis of 18 plasma and 14 urine samples derived from apparently healthy volunteers. Average concentrations of total Cys in plasma and urine were 181.8 ± 25.7 and 246.0 ± 79.8 μmol L⁻¹, respectively. Average total CysGly content in plasma and urine was 29.99 ± 7.98 and 2.09 ± 1.26 μmol L⁻¹, respectively. These results are consistent with those reported earlier [9, 20, 25, 26], and this indicates that elaborated assay can be successfully used in large populations. It is safe for the operator and environmentally friendly, meeting the needs of the biological sample analysis.

Conclusions

The development of analytical methods for determination of biologically important thiols has still attracted a lot of interest. An attractive HPLC–UV-based procedure that minimalizes the use of harmful chemicals to determine Cys and CysGly has been developed. The proposed high-throughput method is cheap, accurate, precise, and sensitive, and it is suitable for the analysis of plasma and urine for total Cys and CysGly. The great advantage is that the method exploits neither toxic solvents nor buffers and ion-pairing reagents in mobile phase. A major advantage that also evidently arises is the development of a single HPLC method that is applicable to different matrices and very short (2.5 min) separation time, without losing efficiency. The LOQs achieved with this methodology are lower in comparison with other methods. Via this simple yet efficient method, 570 samples per 24 h can be analyzed using less than 220 mL of EtOH. The quickness in the analytical time and the low cost of our proposed method make it a reliable tool for analytical laboratories or research groups when an elevated number of samples must be analyzed daily. The estimated validation parameters are more than sufficient to allow the analytical method to be used for monitoring of the total form of both thiols. With minor modifications, our assay can be adapted for the determination of the redox status of Cys and CysGly in biological samples.
Acknowledgment

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Conflict of Interest

The authors have declared no conflict of interest.

Abbreviations

CMQT: 2-chloro-1-methylquinolinium tetrafluoroborate, Cys: cysteine, CysGly: cysteinylglycine, TCEP: tris(2-carboxyethyl)phosphine hydrochloride

References