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Simultaneous Quantitation of Multiple Biological Thiols Using Reactive Ionization and Derivatization with Charged Mass Tags

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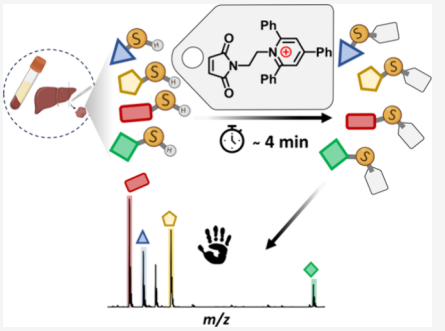
ABSTRACT: The biologically important thiols (cysteine, homocysteine, N-acetyl cysteine, and glutathione) are key species in redox homeostasis, and there is a clinical need to measure them rapidly, accurately, and simultaneously at low levels in complex biofluids. The solution to the challenge presented here is based on a new derivatizing reagent that combines a thiol-selective unit to optimize the chemical transformation and a precharged pyridinium unit chosen to maximize sensitivity in mass spectrometry. Derivatization is performed simultaneously with ionization ("reactive ionization"), and mass spectrometry is used to record and characterize the thiol reaction products. The method is applicable over the concentration range from 1 μM to 10 mM and is demonstrated for 25 blood serum, 1 plasma, and 3 types of tissue samples. The experiment is characterized by limited sample preparation (<4 min) and short analysis time (<1 min). High precision and accuracy (both better than 8%) are validated using independent HPLC-MS analysis. Cystine−cysteine redox homeostasis can be monitored by introducing an additional reduction step, and the accuracy and precision of these results are also validated by HPLC-MS.



INTRODUCTION

Specificity in detection and accurate quantitation of free biological thiols are imperative given their characteristic redox properties and nucleophilicity, which influence diverse physiological processes. Abnormal levels of low-molecular-weight endogenous thiols, including cysteine (Cys), homocysteine (hCys), and glutathione (GSH), can alter metabolic pathways and lead to disease.[1−6] For example, thiol equilibration between the reduced free thiol and the oxidized disulfide form helps to maintain redox homeostasis in biological systems. These equilibria are responsible for shielding cells from oxidative damage by neutralizing oxidizing species.[7−10] Monitoring the redox homeostasis of thiols in biological systems is significant in diagnostics to assess oxidative stress. Furthermore, GSH is produced from Cys, which is synthesized from hCys via the transsulfuration pathway.[11,12] So Cys and hCys are precursors of GSH, and variations in Cys and hCys from normal levels are responsible for disease generation. For example, abnormal Cys levels are closely related to neurotoxicity, slow growth, edema, liver damage, and muscle weakness and have an association with lung cancer.[1] Atypical hCys levels have been implicated in cardiovascular diseases, Alzheimer's disease, and osteoporosis.[4,13,14] The interconnection among Cys, hCys, and GSH in biological processes highlights the need for simultaneous quantitation of these three free biothiols.

On the other hand, exogenous thiols such as N-acetylcysteine (NCys) have reducing properties, resulting in their extensive use as antioxidants in addressing human health concerns.[15−18] While NCys is used to prevent acetaminophen poisoning, overdoses of NCys can lead to hemolysis, thrombocytopenia, and acute renal failure.[19] Given that the clinical symptoms of acetaminophen overdose and NCys overdose are similar, it is critically important to measure NCys content in biofluids and tissues with adequate molecular specificity.[19] Thus, the simultaneous measurement of exogenous NCys with the three endogenous interrelated thiols (Cys, hCys, and GSH) along with monitoring oxidative stress in a wide range of biological samples has important clinical applications. Moreover, the usual clinical chemistry requirements of speed, low unit cost, and small sample sizes can all be applied to these measurements.

To date, a significant number of analytical methods involving prior separation have been developed to target biothiols with high specificity. Fluorescence measurements alone or in combination with high-performance liquid chromatography (HPLC) represent a common approach. Fluorescence spectroscopy can be used alone provided multiple fluorometric probes are available, but a major limitation is limited selectivity among the thiols, causing higher values for certain thiols and, in some cases, requiring separation prior to an analysis.[20,21] Over the years, many

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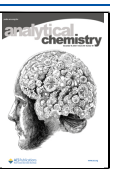

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HPLC-based analytical methods have been developed, some achieving the very low limit of detection (LOD) of 100 fM.[21−23] HPLC coupled with mass spectrometry (MS) is considered the gold standard for many biological sample diagnostics, and while extremely useful, the prolonged separation time makes it unsuitable for point-of-care (POC) clinical or intraoperative diagnostics. Other methods, such as capillary electrophoresis,[24,25] voltammetry,[26] and spectrophotometry,[27,28] have been developed to address the detection and quantitation of specific thiols, but all require prior separation and are not applicable to the simultaneous quantitation of multiple thiols.

MS alone is a powerful bioanalytical technique, especially for rapid POC analysis including intraoperative diagnostics.[29−35] Tandem MS (MS/MS) is well known to allow the fragmentation of specific precursor ions, thereby providing diagnostic structural information to confirm particular analytes of interest in complex mixtures. Direct analysis of metabolites in biological matrixes by single-stage MS is challenging due to the low ionization efficiency. However, this problem can be addressed by the use of precharged derivatizing reagents in the course of reactive ionization. We show here that this approach offers high ionization efficiency, limited sample preparation, and highly sensitive quantitation in complex biological mixtures using simple single-stage MS and without the requirement for LC-based separation.[36−38] Moreover, the high ionization efficiency of the precharged moiety reduces the need for deuterated or other isotopically labeled standards for quantitation during simultaneous analysis of multiple analytes even in complex mixtures. Previous studies have shown multiple strategies for labeling biological thiols for MS analysis; they include selenylation,[39,40] isotopic labels,[41−44] isobaric tags,[45] tandem mass tags (TMT),[46] and precharged probes.[47−49] Maleimide-based mass tags are especially advantageous owing to their fast and specific reactivity toward free biological thiols; this has led to their application in tissue imaging, peptide analysis, and quantitative proteomics.[42,47,48,50,51] However, simultaneous and direct quantitative analysis, viz., without prior separation, of multiple free biothiols from biological samples using maleimide-based precharged probes by MS has not been reported previously.

In this work, we developed a simple and rapid method for quantitatively discriminating four low-molecular-weight biothiols simultaneously. The method is applicable to biofluids and tissue samples and covers a concentration range from 1 μM to 10 mM. This approach uses a precharged maleimide as a derivatizing reagent, as this reacts specifically with thiols. The resulting products provide signals at characteristic m/z values for each thiol, enabling relative quantitation of multiple thiols in a single MS scan. The application of this method is demonstrated in blood serum and mouse blood plasma as well as in various tissues, suggesting its potentially wide applicability in clinical analysis. High precision is observed, and the accuracy of this simple method was confirmed by HPLC-MS. By introduction of an additional reduction step, the total cystine content in biological samples was measured easily, demonstrating the capability to track the redox homeostasis of Cys and its oxidized form, cystine.

EXPERIMENTAL SECTION

Material and Reagents. All four standard thiols (Cys, hCys, NCys, and GSH), tetrakis(decyl) ammonium bromide (TDAB), and the chemicals used for the Katritzky reaction (synthesis of the precharged maleimide) were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile (ACN) and water were obtained from Fisher Scientific, and ethanol (EtOH) was acquired from Decon Laboratories (Sherman Oaks, CA).

Synthesis of the Derivatizing Reagent. To a solution of 1-(2-aminoethyl)maleimide hydrochloride (500 mg) and 2,4,6-triphenylpyrylium tetrafluoroborate (1.120 g) in 10 mL of ACN was added 0.47 mL of triethyl amine (TEA) at 0 °C, dropwise. The reaction was stirred at room temperature for 12 h. The resulting mixture was concentrated in vacuo and then recrystallized from EtOH to precipitate TEA·HCl. After filtration, the filtrate was concentrated in vacuo. The residue was purified using aluminum oxide column chromatography (dichloromethane:MeOH = 9:1) to afford thiosuccinimide **5** (1.023 g, 76% yield). MS and NMR analyses were conducted for its characterization (Supporting Information, Figures S1, S2, and S5a).

Serum Collection and Mouse Model for Tissue Analysis. The human blood serum samples were collected in accordance with Institutional Review Board (IRB no. BNK09-138) approvals for Hoosier Cancer Research Network, Inc. It is noted that this set of blood serum samples was acquired four years ago, sampled and analyzed on multiple occasions, and repeatedly frozen−thawed. The mouse tissue samples were obtained from C57BL/6 female mice fresh carcasses discarded after oocyte retrieval under another IACUC approved study (IACUC #1111000314) at the Purdue Transgenic and Genome Editing Facility.

Ionization and MS Parameters. Nanoelectrospray ionization (nESI) MS analysis was performed using a Thermo LTQ linear ion trap mass spectrometer (ThermoFisher Scientific, San Jose, CA). Spray emitters with 5 μm inner diameter tips were pulled from borosilicate glass capillaries (1.5 mm o.d., 0.86 mm i.d., and 10 cm length) using a micropipet tip puller. This equipment and the needed supplies were purchased from Sutter Instruments (Novato, CA). A spray voltage of +1.5 kV was applied to record the mass spectra. The distance between the spray tip and inlet was maintained at 5 mm, unless otherwise noted. Other instrumental parameters used for MS analysis were the capillary temperature of 275 °C, capillary voltage of 15 V, and tube lens of 65 V. MS/MS was used to confirm the identity of the products.

Thiol Measurement by Reactive MS. To analyze individual thiols and to establish calibration curves, they were derivatized using a solution of reagent **5** (1.1 mM) prepared in a mixed solvent (ACN-EtOH 2:15 by volume). Standard solutions of each thiol were prepared in aqueous solution. For simultaneous quantitation of multiple thiols, additional standard solutions were prepared by mixing the previously prepared thiol standard solutions to achieve a total thiol concentration of 10 mM. A solution of TDAB (**10**) was prepared in ACN for use as an internal standard (IS). All standard solutions were further diluted with EtOH as needed to reach the desired concentrations. The reactions between thiols and **5** were conducted by mixing thiol standard solutions or thiol samples with maleide **5** solution in a 1:1 ratio. Acetic acid (HAc) 1% was added to the reaction mixtures to maintain the pH at 5.0 or less. After 1 min, the reaction mixtures were mixed with **10** (concentration adjusted due to the three times higher ionization efficiency of **10** compared to that of **6**; Figure 2) in a 1:1 volumetric ratio. These samples were then subjected to MS analysis. For further simplification, a premixed

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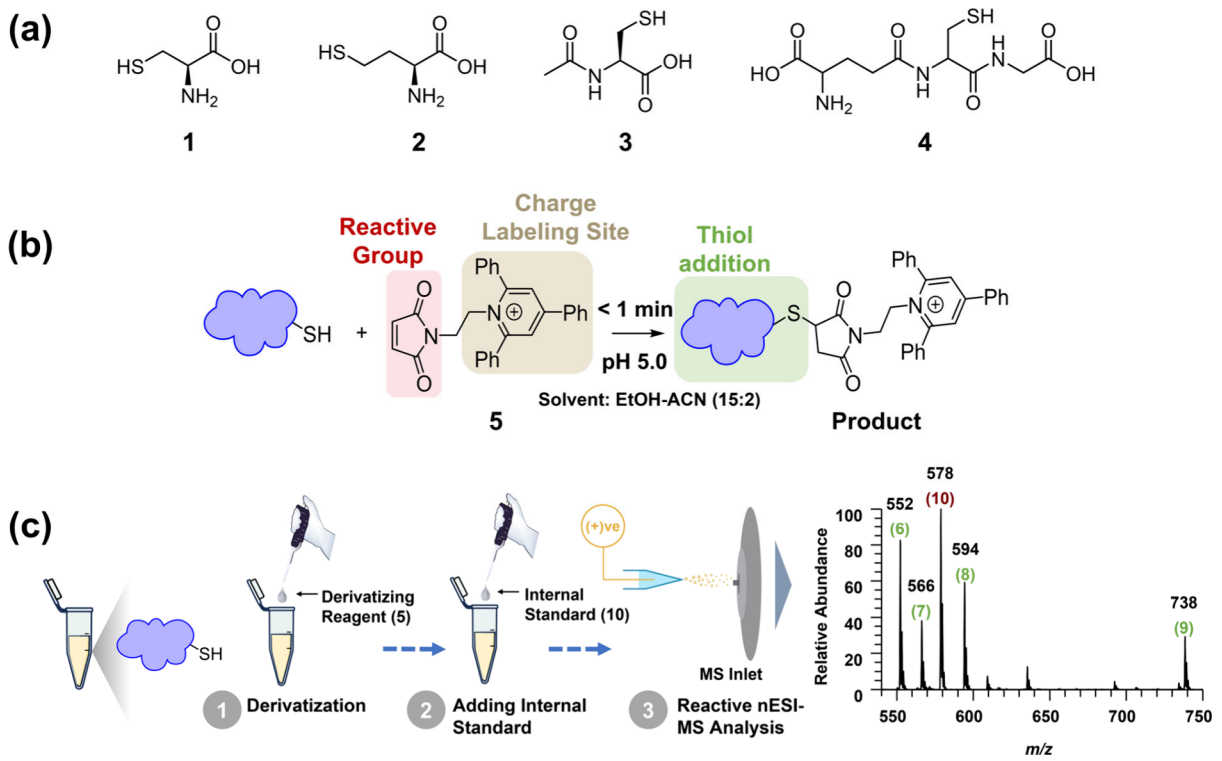

Figure 1. Free thiol measurement using chemical derivatization. (a) Chemical structure of low-molecular-weight free thiols used in this study: **1** Cys, **2** hCys, **3** NCys, and **4** GSH. (b) Chemical reaction of mass tag **5** with a free thiol group, forming thiosuccinimide as a product. The precharged nature of the pyridinium moiety contributes to the high ionization efficiency of derivatizing reagent **5** and its products **6**–**9** in MS analysis. (c) Schematic representation of the thiol mixture (**1**–**4**) measurement using nESI-MS analysis in positive mode to observe products **6**–**9** (m/z 552, m/z 566, m/z 594, and m/z 738) and IS, **10** (m/z 578).

solution of **5** and **10** can be mixed with **1** for this quantitation (Supporting Information, Figure S8). The quantitative performance of this variant was further tested with paper spray ionization (Supporting Information, Figure S9). Note that the minor difference in the reagent addition sequence does not decrease the performance. In the case of biofluids, 25 human blood serum samples and 1 mouse plasma sample were analyzed to quantify free Cys (**1**). Methanol was used to precipitate protein, and the supernatant solution was then mixed with a 10 μM IS (**10**) solution in a 1:1 ratio and subsequently subjected to MS analysis.

HPLC-MS Analysis. The HPLC-MS analysis is adapted from the reported method.[52,53] N-Ethyl maleimide (NEM) was used to derivatize Cys, hCys, and GSH in the biological sample matrix. Details of the sample pretreatment procedure are given in the Supporting Information (pp S5 and S6). An Agilent 1290 Infinity II high-performance liquid chromatography (LC) system coupled to an Agilent 6470 series triple quadruple mass spectrometer was used to analyze Cys-NEM, hCys-NEM, GSH-NEM, and cysteine in each sample. A Waters Acquity UPLC HSS T3 (2.1 mm × 100 mm, 1.8 μm) column (Waters Corporation Milford, MA) was used for LC separation. HPLC-grade water with 0.1% formic acid (v/v) was used as mobile phase A while HPLC grade acetonitrile with 0.1% formic acid (v/v) was used as mobile phase B. The LC gradient was as follows: time 0 min, 2% B; time 1 min, 2% B; time 8 min, 25% B; time 9 min, 95% B; time 15 min, 2% B. The flow rate was 0.3 mL/min. Multiple reaction monitoring (MRM) was used for the MS analysis in the HPLC-MS experiment. Data were acquired in positive electrospray ionization (ESI) mode. (For further details about separation and MRM profiling, see the Supporting Information, pp S5 and S6 and Table S1).

RESULTS AND DISCUSSION

Development of Derivatizing Reagent, Reaction Conditions, and Quantitation Method. The introduction of a precharged moiety by derivatizing analytes of interest enhances the ionization efficiency and offers significant advantages for quantitation using single-stage MS analysis.[36−38] Here, a new derivatizing reagent **5** was designed and constructed by incorporating a precharged pyridinium group (Figure 1, the charge-labeled site) to significantly enhance its ionization efficiency and by using a maleimide moiety for specific reaction with thiol groups (Figure 1, the reactive group).[54−56] The specificity was confirmed by testing 11 free amino acids with various functional groups in their side chains, namely, serine (−OH), lysine ($-NH_2$), aspartic acid (−COOH), tyrosine (phenol), tryptophan (indole), histidine (imidazole), arginine (guanidinium), glycine (−H), methionine ($-SCH_3$), hCys (−SH), and Cys (−SH). The results clearly demonstrate that **5** reacts only with free thiols Cys and hCys (Supporting Information, Figure S3).

The quantitative performance, including the establishment of calibration curves, linear regression, and relative standard deviations (RSD), was examined in the case of Cys **1** using TDAB **10** as the IS due to its precharged nature and the fact that its mass (m/z 578) is similar to that of the product **6** (m/z 552). Under optimized conditions (pH 5.0 and solvent EtOH:ACN in 15:2, Supporting Information Figure S4), this maleimide−thiol addition was complete within 1 min. Note that excess **5** was required to completely convert all of **1** to **6**.

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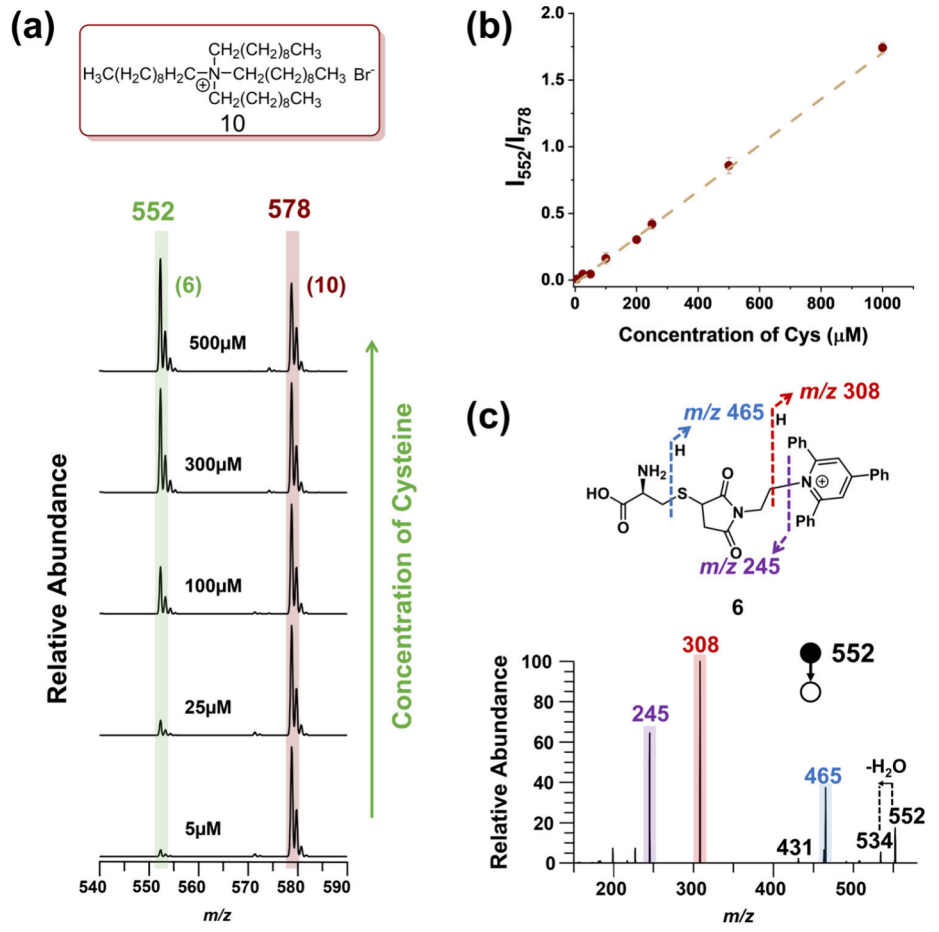

Figure 2. Quantitation of cysteine. (a) nESI mass spectra for quantitation of cysteine at different concentrations. (b) Calibration curve for cysteine solutions with eight different concentrations by plotting the ion ratio of m/z 552 and m/z 578. Three technical replicates are used to produce the calibration curve. (c) Product ion MS/MS of **6** (m/z 552) used to confirm the structure.

Table 1. Calibration Equations, Linear Regressions, and Relative Standard Deviations (RSDs) for Biothiols at Different Concentrations

Range conc.	1 μM−100 μM[a]		100 μM−1000 μM[b]		1 mM−10 mM[c]	
Analyte	Calibration Equation	R^2	Calibration Equation	R^2	Calibration Equation	R^2
Cys	Y = 0.009x − 0.007	0.996	Y = 0.002x − 0.028	0.999	Y = 0.055x + 0.124	0.987
hCys	Y = 0.006x + 0.002	0.989	Y = 0.001x − 0.004	0.999	Y = 0.055x + 0.112	0.998
NCys	Y = 0.009x + 0.219	0.997	Y = 0.001x + 0.046	0.993	Y = 0.213x + 0.207	0.984
GSH	Y = 0.004x − 0.008	0.997	Y = 0.001x − 0.019	0.994	Y = 0.026x + 0.082	0.967

[a]10 μM of IS was used and RSD < 6%. [b]50 μM of IS was used and RSD < 6%. [c]100 μM of IS was used and RSD < 6%

The nature of the product **6** was confirmed by MS/MS analysis through a consideration of the observed fragmentations which include water loss (forming m/z 534), backbone cleavage (m/z 465, m/z 308, and m/z 245), and reversion to **5** (m/z 431) (Figure 2c). This diagnostic fragmentation pattern of product **6** was confirmed in each complex biological mixture examined. An eight-point calibration curve was built by using triplicate measurements at each concentration. The concentrations covered the range from 1 μM to 1000 μM **1** (Figure 2b), the range within which biothiols are normally present in biological samples.[57]

Simultaneous Quantitation of Multiple Thiols. Given that different thiols produce distinctive reaction products (with different intensities and m/z values), reactive ionization provides the potential for the simultaneous quantitation of multiple thiols. To test this capability, four low-molecular-weight biothiols—Cys, hCys, NCys, and GSH—were used. Five-point calibration curves were built in triplicate for each analyte in a mixed sample over the concentration range of 1 to 100 μM, with R^2 values showing excellent linear regression (Supporting Information, Figure S6). To extend the concentration range, higher concentrations of IS were used, thus allowing the calibration of thiol solutions over the ranges of 100 μM to 1000 μM (Supporting Information, Figure S7) and 1 mM to 10 mM (Table 1). MS/MS was used to confirm the identity of the analytes of interest from their unique fragmentation patterns (Supporting Information Figure S5 for MS/MS analysis of **5** and all reaction products **7**−**9**). Notably, this method features a short analysis time (of around 1 min) and high precision in each test (RSD within 6%). This method has implemented single-stage MS scan-based quantitation for simplicity and broader applicability. However, tandem MS

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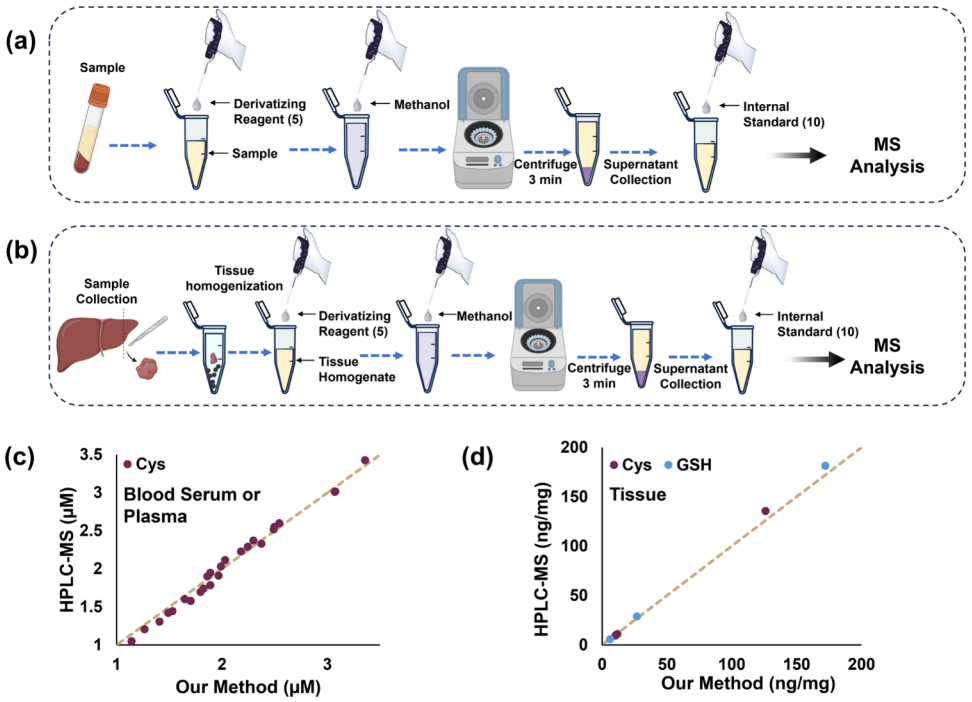

Figure 3. Simultaneous measurement of Cys and GSH in biological samples by derivatization coupled with MS analysis. Workflow for (a) blood serum or plasma and (b) tissue. Comparison of reactive ionization and HPLC-MS for (c) blood serum or plasma and (d) tissue samples. Closely similar values are seen with errors of less than 9%. Cys and GSH were measured simultaneously (ng/mg) in tissue; data for Cys (red) and GSH (blue). Broken lines in parts c and (d) are autocorrelations, X = Y.

(MS/MS)-based analysis can provide a better quantitative performance.

In cases of extreme physiological conditions (e.g., one or more analytes having orders of magnitude higher concentrations than the others), high signals from the more concentrated analytes may suppress signals of lower-concentration analytes and thus affect simultaneous quantitation by MS. To estimate the magnitude of this effect, standard solutions were prepared with one component (Cys or GSH to mimic a common condition in blood serum or tissue samples) having a 2 orders of magnitude higher concentration than the other three and then used to re-establish calibration curves (Supporting Information, Tables S2 and S3). Both cases show good linear regression with high precision (RSD < 8%). The orders of magnitude higher concentration of one analyte affects the sensitivity of the other three, which results in higher RSD values (from RSD < 6% to RSD < 8%) especially in the case of a higher concentration of Cys **1** where the derivatized product **6** has a higher ionization efficiency than products of hCys (**7**) and GSH (**9**). Furthermore, in biological samples, Cys and GSH concentrations can both be very high relative to the other two. To study this additional situation, standard solutions with Cys and GSH 2 orders of magnitude higher in concentration were used to build the calibration curves (Supporting Information, Table S4) which again showed excellent linear regression and high precision (RSD < 10%).

Measurement of Cys in Biofluids and Validation of Accuracy by HPLC-MS. To explore the applicability of this method in the case of complex biofluids, we utilized human blood serum and mouse plasma. The derivatized samples were subjected to MS analysis (Figure 3a), and the presence of **6** (reaction product from Cys) MS/MS experiments were employed to verify the characteristic fragmentations of its signal at m/z 552. Accuracy assessment of the method involved comparison with HPLC-MS in all cases (Figure 3c). The results showed closely similar values from both methods, with errors below 9%, validating the high accuracy of our simple method. It is notable that HPLC-MS requires approximately 20 min to analyze each sample, whereas the new approach involves only 4 min of sample preparation and less than 1 min for analysis, highlighting its potential applicability in rapid clinical analysis.

Measurement of Cys and GSH in Tissue Samples and Validation of Accuracy by HPLC-MS. Tissue samples are an important biological matrix, and multiple ambient ionization MS, MS-imaging, and MS/MS-based methods have been developed to assist surgeons in making decision during surgery.[29,58−64] Considering this, we applied our method to multiple mouse tissue samples, including brain, kidney, and lung tissues. Again, sample preparation was minimal. After the reaction between the tissue homogenate and the derivatizing reagent **5**, the reaction mixture was mixed with 10 μM **10** in a 1:1 volumetric ratio and was directly subjected to MS analysis (Figure 3b). Good agreement between the two methods was observed for the simultaneous analysis of Cys and GSH in tissue samples (Figure 3d). This demonstrates the applicability of the reactive ionization method in complex tissue analysis, opening possibilities for investigating cellular mechanisms involving free thiols.

Measurement of Cystine by Integrating Derivatization with an Additional Reduction Step. An imbalance in the metabolism of ROS causes oxidative stress, which can eventually lead to cell death. Cys-cystine redox homeostasis plays a vital role in neutralizing these ROS.[65,66] In this process for biological redox signaling and control, Cys is oxidized to cystine via disulfide bond formation, whereas cystine is reduced to Cys by breaking the disulfide (Figure 4a). By introducing an additional reduction step into our method,

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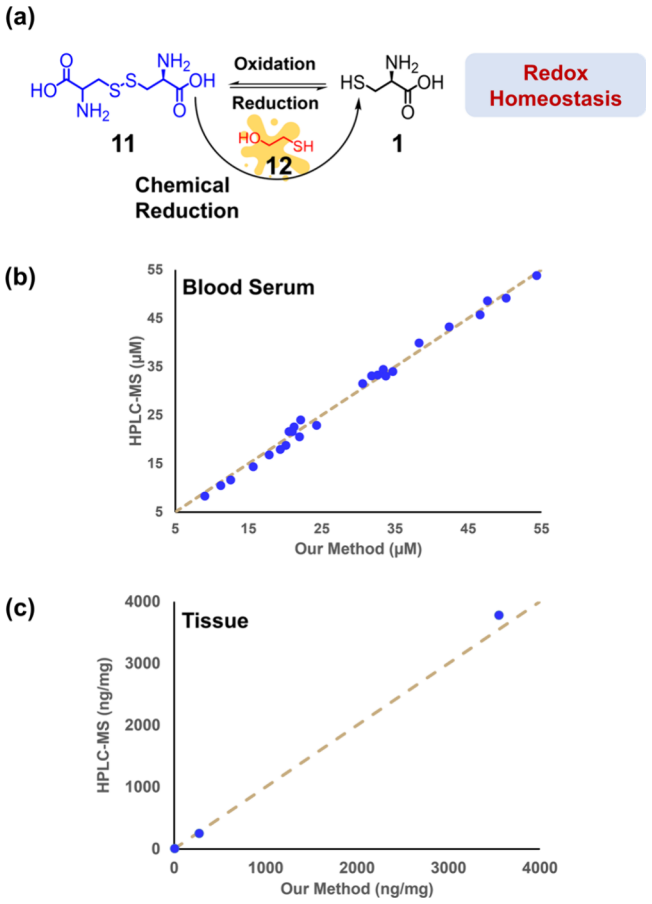

Figure 4. Measurement of cystine **11** by reduction to Cys **1**. (a) Schematic illustration of redox homeostasis and the chemical reduction of cystine **11** by 2-mercaptoethanol **12**. Comparison of the present method of cystine analysis with HLPC-MS in (b) blood serum and (c) tissue. Broken lines are autocorrelations, X = Y.

cystine can be easily measured in biofluids and tissue homogenates. This might be applied to monitor Cys-cystine redox homeostasis in cellular processes in the future. 2-Mercaptoethanol was used as a reducing agent for the rapid reduction of disulfide bonds.[67] After the original Cys in these biological samples was quantified, the same samples were treated with 2-mercaptoethanol to reduce cystine to Cys. The same analytical process was then used to quantify the total amount of Cys after the reduction. The concentration of cystine was determined by subtracting the original amount of Cys (before reduction) from the total amount of Cys (after reduction). To evaluate the accuracy of our approach, a comparison with HPLC-MS was carried out. Twenty-five blood serum samples and three different tissue samples were analyzed using both methods in parallel. The results showed closely similar values, with a maximum error percentage of 9% (Figure 4b and Figure 4c).

CONCLUSIONS

A fast and simple MS-based method has been developed to quantify thiols over a wide concentration range (1 μM to 10 mM) across various biological matrixes, including blood sera, plasma, and tissue. This approach utilizes a novel precharged maleimide-based derivatizing reagent specifically designed to capture biological thiols from complex matrixes. The precharged nature of the probe generates a high signal, eliminating the need for separation steps. This method achieves accuracy comparable to that of HPLC-MS while requiring minimal sample preparation. Additionally, it is suitable for monitoring the redox homeostasis of Cys-cystine. By enabling precise thiol quantification in biological samples with minimal sample preparation and rapid analysis, this method has the potential for POC applications where quick results are essential.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.4c03807.

NMR and mass spectrometry characterization of the derivatizing reagent, tandem mass spectrometry of thiol reaction products, calibration curves for the measurements, and method details for HPLC-MS (PDF)

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Author Contributions

‖M.S. and L.Q. contributed equally to this work.

Author Contributions

The project was initiated by R.G.C., who directed the work. M.S. and L.Q. developed the quantitation method. M.S. performed the biological experiments and analyzed the MS data. L.Q. synthesized the mass tag. Y.H-.H. acquired the HPLC-MS data. M.S. and L.Q. contributed experimental data, and all authors contributed to writing the manuscript. All authors have approval to the final version of the manuscript.

Notes

Biosafety Level Information: All biological samples were handled following BSL 2 protocols.

The authors declare no competing financial interest.

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