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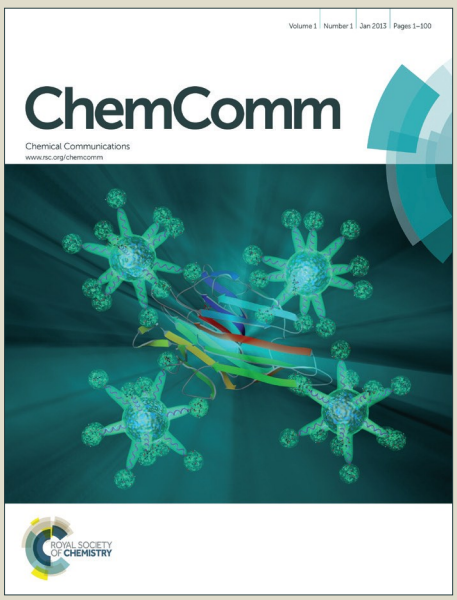

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An Accurate Mass Spectrometric Approach for the Simultaneous Comparison of GSH, Cys, and Hcy in L02 Cells and HepG2 Cells using New NPSP Isotope Probes

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A novel accurate method was developed for simultaneous quantitative comparison of GSH, Cys and Hcy in normal cells and cancer cells using new NPSP isotope probes based on LC/ESI-MS.

The relevance of oxidative stress in the genesis of various types of cancer has been well established.[1] Studies have also shown that more reducing substances in cancer cells than in normal cells are produced in response to the elevated reactive oxygenspecies (ROS) stress.[2] As important reducing substances, intracellular biothiols, such as glutathione (GSH), cysteine (Cys) and homocysteine (Hcy), play crucial roles in combating oxidative stress and maintaining redox homeostasis in both normal and cancer cells.[3] Besides, GSH, Cys, and Hcy are closely interrelated through the metabolic pathways in biological systems.[4] For example, Hcy can be converted into Cys by cystathionine and β–lyase; Cys is a synthetic precursor of GSH and the level of Cys is rate-limiting for the synthesis of GSH.[4a,5] Considering the important regulatory roles of GSH, Cys, and Hcy in cellular redox environment as well as their close interconvertible relationship, simultaneous quantitative analysis and comparison of GSH, Cys, and Hcy in normal and cancer cells is significant to better understand the relationship between redox environment and cancer pathogenesis and can provide valuable information for their therapeutic effects.

Until now, various methods have been reported for the determination of biothiols. However, due to the similar structure and reactivity of GSH, Cys, and Hcy, the extensively used UV[6], electrochemical assays[7], chemiluminescence method[8], and fluorescence spectroscopy[9] could not detect GSH, Cys, and Hcy simultaneously with effective discrimination from each other. Mass spectrometry (MS), which can provide the molecular weight and structural information of the targets with high specificity, is undoubtedly a preferred tool for the simultaneous discrimination and detection of multiple components.[10] But, there are issues when the MS is used for accurate quantitation. For example, the unstable ion signal[11] and random adsorption[12] of analytes may lead to large system error. Besides, limited to the ability due to high reactivity of free thiol group(-SH)[13] and the long response time of the existing probes to biothiols[14], oxidation of biothiols during the sample preparation and derivatization process is another grand challenge involved in accurate quantification of these species.

In this study, we developed a novel method for effective discrimination and accurate comparison of GSH, Cys, and Hcy in both normal and cancer cells using new N-(phenylseleno)phthalimide (NPSP) isotope probes based on liquid chromatography/electrospray ionization mass spectrometry (LC/ESI-MS) analysis. First, a new isotope probe NPSP-d_5 was designed and synthesized to construct a NPSP-d_0/NPSP-d_5 isotope probe pair (Scheme 1A). Biothiols in normal cells and cancer cells can be respectively tagged by NPSP-d_0 or NPSP-d_5 through the formation of a Se-S bond linkage (Scheme 1B). Next, the two types of cells are mixed in equal ratios, and a series of subsequent operations including cell homogenization, deproteination with acids and sample injection into LC/ESI-MS were conducted. The HPLC can effectively separate multiple components of complex biological system.[10b] The MS can achieve qualitative and quantitative analysis based on molecular weight and structural information as well as the signal intensity.[10] Using this strategy, GSH, Cys, and Hcy in human normal hepatocyte L02 cells and human hepatoma HepG2 cells can be quantitatively compared through the ion signal intensities of biothiols-NPSP-d_0 and biothiols-NPSP-d_5 at the same time (Scheme 1C). The results shows that more reducing biothiols are in HepG2 cells. This study exhibits that (1) concurrent sample preparation and analysis in the same LC/ESI-MS run were operated after mixing of two parallel samples labeled with NPSP isotope probes, leading to decreased quantification error and increased sample throughput; (2) complete derivatization of biothiols can be achieved within 10 s using the NPSP isotope probes, which reduces the autoxidation probability of biothiols and leads to a more reliable analysis; (3) simultaneous discrimination and accurate comparison of GSH, Cys, and Hcy in normal and cancer cells can be achieved, indicating the potential utility of this method in the future research of studying the role of biothiols in carcinogenesis.

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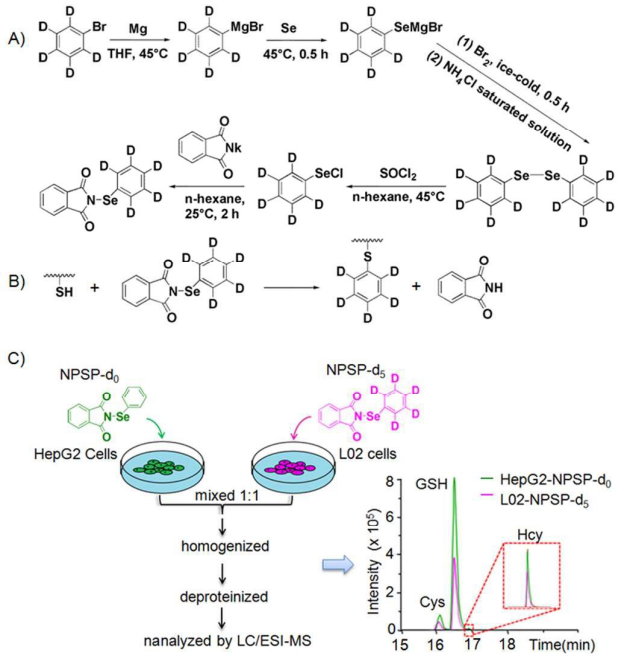
†Electronic Supplementary Information (ESI) available: Details of the general experimental section; Capability of NPSP isotope in avoiding oxidation of biothiols; MTT assay; Stability of biothiols-NPSP; Simultaneous comparison of GSH, Cys and Hcy in cells with converse labeling. See DOI:10.1039/x0xx00000x



Scheme 1 (A) Synthetic route for the isotope probe NPSP-d_5; (B) Reaction of NPSP-d_5 with biothiols; (C) Schematic illustration of the biothiols tagging strategy for HepG2 Cells and L02 cells with NPSP isotope probes for LC/ESI-MS quantitation.

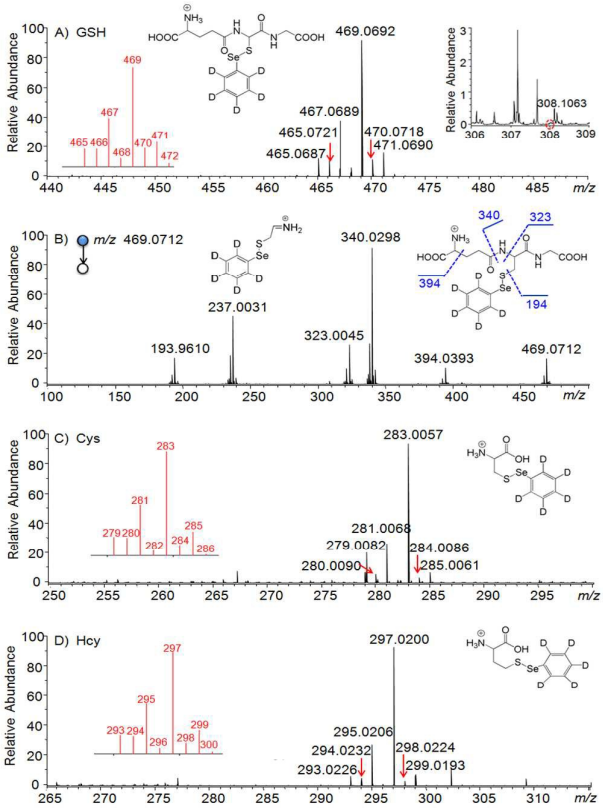


Fig. 1 ESI-MS spectra of NPSP-d_5 derivatized products of (A) GSH (C) Cys and (D) Hcy, (B) CID MS/MS spectrum of target ion (*m/z* 469). The insets of (A), (C), and (D) in red are the simulated isotope peak distribution of the GSH-NPSP-d_5 ion, Cys-NPSP-d_5 ion, and Hcy-NPSP-d_5 ion. ○ indicates no observation of the protonated GSH $[GSH+H]^+$ (*m/z* 308.09).

Isotope labeling techniques in combination with MS have been widely used as quantification tools to measure concentrations of natural compounds,[11b, 15] among which the well known and classical approaches include SILAC[16] (stable isotope labeling by amino acids in cell culture), ICAT[14, 17] (isotope-coded affinity tag), iTRAQ[18] (isobaric tag for relative and absolute quantitation), and isotope dilution method[19]. This strategy allows two samples to be labeled respectively and then analyzed in the same LC/MS run.[20] The accurate relative quantification can be realized by comparing the signal intensities of the same compounds labeled with heavy or light labeling.[11b, 12, 15b, 17, 20, 21] In our previous reports, it was shown that the selenamide reagent N-(phenylseleno)phthalimide (NPSP) can derivatize biothiols selectively for mass spectrometric analysis.[10a, 22] Here, a new isotope probe NPSP-d_5 was designed and synthesized to construct the NPSP-d_0/NPSP-d_5 isotope probe pair. To verify the reactions of NPSP-d_5 with biothiols, GSH was chosen as a test compound. Through the derivatization of NPSP-d_5, phenylselenenyl tag (Se-Ph-d_5) was added to the cysteine residue, which can survive collision-induced dissociation (CID) and are useful for structural confirmation of the product ions.[22] Figure 1 is the ESI-MS spectrum showing the reaction products. We can see that there was no longer a peak at *m/z* 308.09 in the spectrum corresponding to the protonated GSH, indicating the occurrence of complete derivatization of GSH by NPSP-d_5 (right inset of Figure 1A). Instead, a dominant peak at *m/z* 469.07 corresponding to the protonated GSH-NPSP-d_5 appeared. The characteristic selenium isotope distribution of the protonated GSH-NPSP-d_5 ion agrees well with the simulated isotope peak distribution of the corresponding ion (red inset of Figure 1A), which also verifies its assignment. Upon CID of *m/z* 469.07, backbone and side chain cleavages (the formation of *m/z* 193.96, 237.00, 323.00, 340.03, and 394.04) were observed in Figure 1B, further confirming the product ion structure and the newly formed Se-S bond in the GSH derivatized product. As comparison, we also made ESI-MS analysis for the reaction products of NPSP-d_0 with GSH (Figure S1, ESI†). A mass increase of 5 Da occurring for GSH-NPSP-d_5 compared with the GSH-NPSP-d_0 agrees with the replacement by five deuterium atoms. Similar results were obtained after the derivatization of Cys and Hcy by NPSP-d_5 (Figure S1C and S1D, ESI†). Thus, the synthesis of NPSP-d_5 isotope probe was further confirmed. In addition, our expermental results showed that only cysteine could be derivatized by NPSP-d_5 in the presence of 19 other natural amino acids (Figure S2, ESI†), demonstrating that NPSP-d_5 isotope probe has exclusive specificity toward the free-SH group.

A major problem encountered in accurate determination of GSH is the autoxidation of GSH into GSSG.[23] Some artificial oxidation occurs during the derivatization process and other sample pretreatment steps.[23, 24] Rapid reaction with free-SH groups will shorten the oxidation time and reduce the oxidation probability. NPSP selenylation reaction towards thiols can be completed in seconds, which is of benefit for the accurate analysis of GSH. We made comparisons of NPSP-d_5/NPSP-d_0 with two commonly used alkylating reagents NEM and IAM in derivatization of GSH. A 10-fold molar excess of NPSP-d_5/NPSP-d_0 could realize the complete derivatization of GSH in 10 s and the oxidation product GSSG was negligible, with total oxidation percentage 0.16% for NPSP-d_0 and 0.17% for NPSP-d_5 (Figure S3A, S3B and Table S1, ESI†). In stark contrast, 50-fold molar excess of NEM or IAM and a longer incubation time of 30 min were neeged for derivatizing GSH. However, much more oxidation product GSSG (the total oxidation percentage was 9.45% for NEM and 15.40% for IAM) and no completed derivatiziation of GSH by IAM were observed (Figure S3C,

S3D and Table S1, ESI†).This proved that NPSP isotope probes could effectively decrease unwanted GSH oxidation as a result of rapid derivatizing capability, thus allowing accurate quantification of biothiols.

ESI-MS has become one of the most powerful tools in chemical and biological research because analytes can be ionized softly and measured with high selectivity.[10b, 11b, 13, 25] However, there are quantitation issues when the analysis is dependent on ESI-MS, such as the unstable ion signal[11a] and random adsorption of analytes at the inner surfaces of ESI emitters[11b]. Besides the inaccuracy introduced by the ESI-MS process, sample preparation was also another major source of quantification error.[11b] To address these issues, a NPSP-d_0/NPSP-d_5 isotope probe pair was constructed for achieving accurate quantitative comparison of biothiols in two samples. To test the quantitative comparison capability of the isotope probe pair, two sets of GSH respectively labeled by NPSP-d_0 and NPSP-d_5 with 13 different concentration ratios (GSH labeled by NPSP-d_0/GSH labeled by NPSP-d_5 from 1:4 to 10:1) were first mixed and then analyzed by ESI-MS. Figure S4 A1-A5(ESI†) show MS spectra of mixed products. A correlation plots with signal intensity (peak area) ratios of the protonated GSH-NPSP-d_0 ([GSH-NPSP-d_0+H]$^+$, *m/z* 464) and the protonated GSH- NPSP-d_5 ([GSH-NPSP-d_5+H]$^+$, *m/z* 469) as y and actual sample concentration ratios as x was shown as Figure S4 B (ESI†). The peaks of *m/z* 464 and 469 were chosen for comparison because they are the highest peak of each product and do not overlap with other isotope peaks in the mixed samples. The signal intensity ratios agreed well with the actual sample concentration ratios in each mixed sample. The regression equation was y =1.0518 x + 0.0291, with a linear coefficient of 0.9994, indicating that the peak intensity ratio of NPSP-d_0/NPSP-d_5 labeled GSH can represent actual concentration proportion in the sample. Similar results were obtained for the analysis of Cys and Hcy (Figure S5, ESI†). Thus the quantitative comparison of biothiols could be achieved by the NPSP-d_0/NPSP-d_5 isotope probe pair.

As the components of the biological sample are complex, salts and small metabolites could suppressed the MS signal and are adverse for biothiol detection in cells. Therefore, a LC/ESI-MS method was developed to exclude the interference of complex components and achieve simultaneous quantitative analysis of GSH, Cys and Hcy. Two sets of mixture containing GSH, Cys and Hcy (each is 5 μM) were derivatized by NPSP-d_0 and NPSP-d_5, respectively, and then mixed in 1:1 ratio. GSH, Cys and Hcy could be effectively separated by the HPLC under optimal elution condition. LC/ESI-MS analysis showed that GSH, Cys and Hcy could be simultaneously detected and quantitatively compared (Figure 2). Reaction products GSH-NPSP-d_0/NPSP-d_5 (*m/z* 464/469), Cys-NPSP-d_0/NPSP-d_5 (*m/z* 278/283 and Hcy-NPSP-d_0/NPSP-d_5 (*m/z* 292/297) with different elute times of 16.0 min, 16.5 min and 17.0 min were identified based on their individual *m/z*. It turns out that the signal intensity ratios of GSH, Cys and Hcy product ions in the merged sample was 0.92 ± 0.059 : 1.05 ± 0.036 :1.00 ± 0.042 (fairly close to the mixing ratio of 1:1:1), illustrating the capability of the isotope probe pair to realize the simultaneous and quantitative comparison of GSH, Cys and Hcy in two samples. In addtion, NPSP probes showed strong anti-interference ability in quantitative comparisons of biothiols in the presence of other speices, such as the other natural amino acids, sugar, phospholipids, and metal ions (Figure S6, ESI†). The limit of detection (LOD) of GSH was as low as 2.51 × 10^{-13} mol (Figure S7, ESI†).

Due to the difference of redox environment between normal cells and cancer cells, research on reducing substances such as

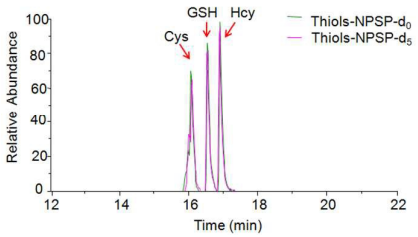


Fig. 2 Extracted ion chromatograms (EICs) of GSH, Cys and Hcy (each in 5 μM) derivatized by both NPSP-d_0 (green line) and NPSP-d_5 (pink line).

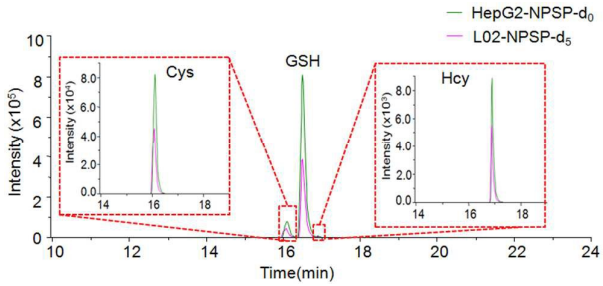


Fig. 3 EICs of the derivatized biothiols from normal L02 cells (pink line) and HepG2 cells (green line). HepG2 was labeled by NPSP-d_0 and L02 labeled by NPSP-d_5.

GSH, Cys and Hcy is significant for exploring the relationship between the biothiols and cancer pathogenesis. Studies have demonstrated that increase of GSH concentrations to resist greater ROS stress in cancerous cells occurs as compared to their corresponding normal cells.[2] In this study, the content of GSH, Cys and Hcy and in human normal hepatocyte L02 cells and human hepatoma HepG2 cells was accurately compared using the proposed NPSP isotope probes based on the LC/ESI-MS analysis. The NPSP isotope probes (6.0 mg/mL and 9.0 mg/mL) showed low cytotoxicity in living cells (Figure S8, ESI†). After equal amount of HepG2 cells labeled by NPSP-d_0 and L02 cells labeled by NPSP-d_5 were mixed, the mixed cell sample was homogenized, deproteinized with acids and detceted by LC/ESI-MS. The biothiols-NPSP were stable during the sample pretreatment process (Figure S9, ESI†). GSH-NPSP, Cys-NPSP and Hcy-NPSP were separated effectively from each other and identified by their individual *m/z* (Figure 3). The extracted ion chromatogram (EIC) peak areaes of GSH, Cys and Hcy labeled with NPSP-d_0 in HepG2 cells were 2.58 ± 0.084, 1.62 ± 0.056 and 1.58 ± 0.064 times of those labeled with NPSP-d_5 in L02 cells (Table S2, ESI†), respectively, meaning that the contents of GSH, Cys and Hcy were higher in HepG2 cells than in L02 cells. To check the result, we further incubated L02 cells with NPSP-d_0 and incubated HepG2 cells with NPSP-d_5 conversely. Similarly, the contents of Cys, Hcy and GSH in HepG2 cells were also higher than that in L02 cells, with peak areaes of GSH-NPSP-d_0, Cys-NPSP-d_0 and Hcy-NPSP-d_0 being 0.4 ± 0.013, 0.55 ± 0.020 and 0.68 ± 0.021 (Table S2, ESI†) times of those of GSH-NPSP-d_5, Cys-NPSP-d_5 and Hcy-NPSP-d_5 (Figure S10, ESI†).

In summary, we have developed a novel accurate method for simultaneous quantitative comparison of GSH, Cys and Hcy in normal cells and cancer cells using new NPSP isotope probes based on LC/ESI-MS. This method excludes the sources of quantification error introduced by sample preparation and mass spectrometric signal fluctuation. Quantititive derivatization of biothiols can be achieved within 10 s using the NPSP isotope probes, which can reduce the autoxidation of biothiols. Accurate quantitative comparison of biothiols in L02 cells and HepG2 cells showed that more reducing biothiols were in the HepG2 cells. The new NPSP

isotope probes have supplied an effective tool for the simultineous and accurate detection of multiple biothiols in different cells.

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