Supporting Information

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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Contents:

1. General Experimental Sections

Materials. Glutathione (GSH, the reduced form, MW 307.3 Da), cysteine (Cys, MW 121.0 Da), homocysteine (Hcy, MW 135.0 Da), two alkylating reagents 2-iodoacetamide (IAM) and N-ethylmalemide (NEM), HPLC-grade formic acid (FA), anhydrous acetonitrile (ACN), LC/MS-grade water, trichloroacetic acid (TCA), phenylselenyl chloride, and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were all purchased from Sigma-Aldrich (St. Louis, MO, USA). The 20 naturel amino acids except cysteine, sugar (glucose), and phospholipids (lecithin, cephalin,) were all purchased from Sangon Biotech Co., Ltd. (Shanghai, China). N-(Phenylseleno)phthalimide (NPSP-d_0, MW 302.2 Da) was purchased from Alfa Aesar (Heysham, UK). Deionized water was obtained using a Sartorius Arium 611 VF system (Sartorius AG, Germany) to a resistivity of 18.2 M Ω·cm.

Instrument. The ^{1}H NMR spectra were collected in $CDCl_3$ at 25 °C on a Bruker Avance 300 spectrometer, with tetramethylsilane as an internal reference. Chemical shifts are reported in the standard notation of parts per million. Absorption bands in NMR spectra are listed as singlet (s), doublet (d), triplet (t), or multiplet (m). MaXis ESI-Quadrupole-Time of flight-mass spectrometer (Bruker Daltonic Inc., Bremen, Germany) and Agilent series 1100 HPLC system (Agilent Technologies, Massy, France) equipped with an autosampler, a binary pump and an Acclaim C18 column (120Å, 4.6 × 250 mm, 5 μm, Dionex Technologies) were employed to obtain and process high-resolution data. The MTT analysis was recorded on a TRITURUS microplate reader.

Synthesis and characterization of NPSP-d_5. Synthesis of NPSP-d_5 is outlined in Scheme 1A. All reactions were carried out under nitrogen with standard vacuum-line techniques. Drying of solvents was carried out by distillation under N_2 with sodium (for tetrahydrofuran, THF), CaH_2 (for CH_2Cl_2 and hexane) and Mg (for methanol). As depicted in Scheme 1A, the synthesis of NPSP-d_5 includes three steps. First, the intermediate product **1** (diphenyldiselenide-d_{10}) was obtained from bromobenzene-d_5 according to the reported method in literature.[1] Second, the intermediate product **2**

(benzeneselenenylchloride-d_5) was synthesized as follows: $SOCl_2$ (73 μL, 1.00 mmol) was added to the solution of diphenyl diselenide-d_{10} (0.322g, 1.00 mmol) in n-hexane (15 mL), and the reaction mixture was stirred at 45 °C for 1 h; The resulting solution was concentrated to give a powder, which was recrystallized from drying and degassing hexane to give saffron yellow needle crystals. Third, degassed hexane (15 mL) was added into the mixed powder of intermediate product **2** (0.372 g, 1.90 mmol) and potassium phthalimide (0.300 g, 1.62 mmol); The desired product NPSP-d_5 was obtained after the mixture stirred at 25 °C for 2 hours, filtered to remove insoluble solid materials, concentrated on a rotary evaporator, collected by filtration and washed thoroughly with dry hexane. NPSP-d_5 was purified by recrystallizing from CH_2Cl_2-hexane.[2] ^{1}H-NMR (300 MHz, $CDCl_3$): δ 7.87 (2H, m), 7.78 (2H, m).

Biothiol derivatization. All the samples were freshly prepared just before use. GSH, Cys and Hcy were prepared in ACN/H_2O (1:1 by volume) solution for reactions with NPSP isotope probes or in CH_3COONH_4-$NH_3\cdot H_2O$ buffer (10 mM, pH 8.0) for reactions with NEM and IAM, then diluted to desired concentrations. Concentrated NPSP-d_0, NPSP-d_5, IAM and NEM were prepared in anhydrous ACN as stock solutions. For the reactions of biothiols with NPSP-d_0 or NPSP-d_5, a small amount of concentrated NPSP-d_0 or NPSP-d_5 was added into the boithiol samples in ACN/H_2O, in 10-fold molar excess, and vibrated for 10 s. For the reactions of biothiols with NEM or IAM, a small amount of concentrated NEM or IAM was added into boithiols samples in CH_3COONH_4- $NH_3\cdot H_2O$ buffer, with 50-fold molar excess, vibrated for 30 s, incubated for 30 min at 37 °C in the dark. All the resulting products were added with FA (1% by volume) prior to the ESI-MS test.

Cell Culture and Treatment. L02 cells and Hep G2 cells were obtained from the American Type Culture Collection (Manassas, VA). The L02 cells and Hep G2 cells were respectively cultured with 1640 and DMEM medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Hyclone, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA) and incubated at 37 °C in a

humidified atmosphere of 5% CO_2 and 95% air. The cells were collected by trypsin digestion and centrifuged at 1000 rpm for 5 min in culture medium, washed twice with PBS buffer (0.0067 M, pH 7.4, Hyclone, USA), and then resuspended in 1 mL PBS buffer with cell density of 5×10^6 cells/mL. The cell density was determined by hemocytometer.

Cell incubation with probes. NPSP-d_0 (6 mg/mL) and NPSP-d_5 (6 mg/mL) freshly prepared in anhydrous ACN were added to the L02 cells and HepG2 cell suspensions, respectively. The cells were incubated at 37 °C for 15 min for completed biothiol derivatization. Upon labeling, the two samples are mixed in 1:1 ratio, homogenized for 10 min, centrifuged at 14000 rpm for 10 min and the supernatant was collected. After the supernatant was acidified by 60% TCA (typically 100 μL of TCA60 for 1 mL of supernatant)[3] for 10 min, the resulted solution was centrifuged at 14000 rpm for 2 min and supernatant was collected. Unless specified otherwise, all of the operation was at 0 °C, in order to avoid the oxidation of biothiols in biological samples. The resulted biological sample was diluted before analyzed by LC/ESI-MS.

Detection with LC/ESI-MS. For LC/ESI-MS system, gradient elution was used. The mobile phase was composed of solvent A (0.1% FA in water) and solvent B (ACN).The injection volume was 6 μL. As flow rate, 1 mL/min was selected. Before the analysis of biological samples, the reversed-phase C18 column (120Å, 4.6 × 250 mm, 5 μm, Dionex Technologies) was pre-equilibrated with solvent A for 20 min. Optimum separation for biological samples was achieved in gradient condition as follows (expressed as the proportion of eluent B): 0-10 min, 12% solvent B; 10-20 min, linear gradient of eluent B from 12% to100%; 20-25 min, flush the column with 100% solvent B for 5 min; 25-26 min, linear gradient of eluent B from 100% to12%; then re-equilibrate the system to the initial isocratic conditions. The auto-sampler syringe was washed with solvent A. The fluent was split to about 1/3 by a “Tee” mixer and then flowed to the commercial ESI source for MS analysis to address the issue of incompatibility in sample volume between HPLC and ESI-MS.

The system of ESI-MS was operated in the positive ion mode. A high voltage of +4.5 kV was applied to the spray probe. The nebulizing gas pressure (N_2) was kept at 0.4 bar and the flow rate for the drying gas (N_2) was 4.0 L/min. The source temperature (dry temperature) was 180 °C and the sample injection flow rate was 3 μL/min. Collision-induced dissociation (CID) was used for further structural confirmation of the product ions and N_2 was used as the collision gas. Data acquisition was performed using the Data analysis software (version 4.0, Bruker Daltonic Inc., Bremen, Germany)

2. Reaction of NPSP-d_0 with GSH

To make comparison with the reaction of NPSP-d_5 and GSH, the products of GSH derivatized by NPSP-d_0 were analyzed by ESI-MS. Similar results were obtained with GSH-NPSP-d_5, with GSH completely derivatized by NPSP-d_0 in 10 s (no longer a peak at *m/z* 308.09 corresponding to GSH, top inset of Figure S1 A). The isotope peak distribution of GSH-NPSP-d_0 and daughter ions of it upon CID agrees well with the simulated isotope peak distribution of the corresponding ion.

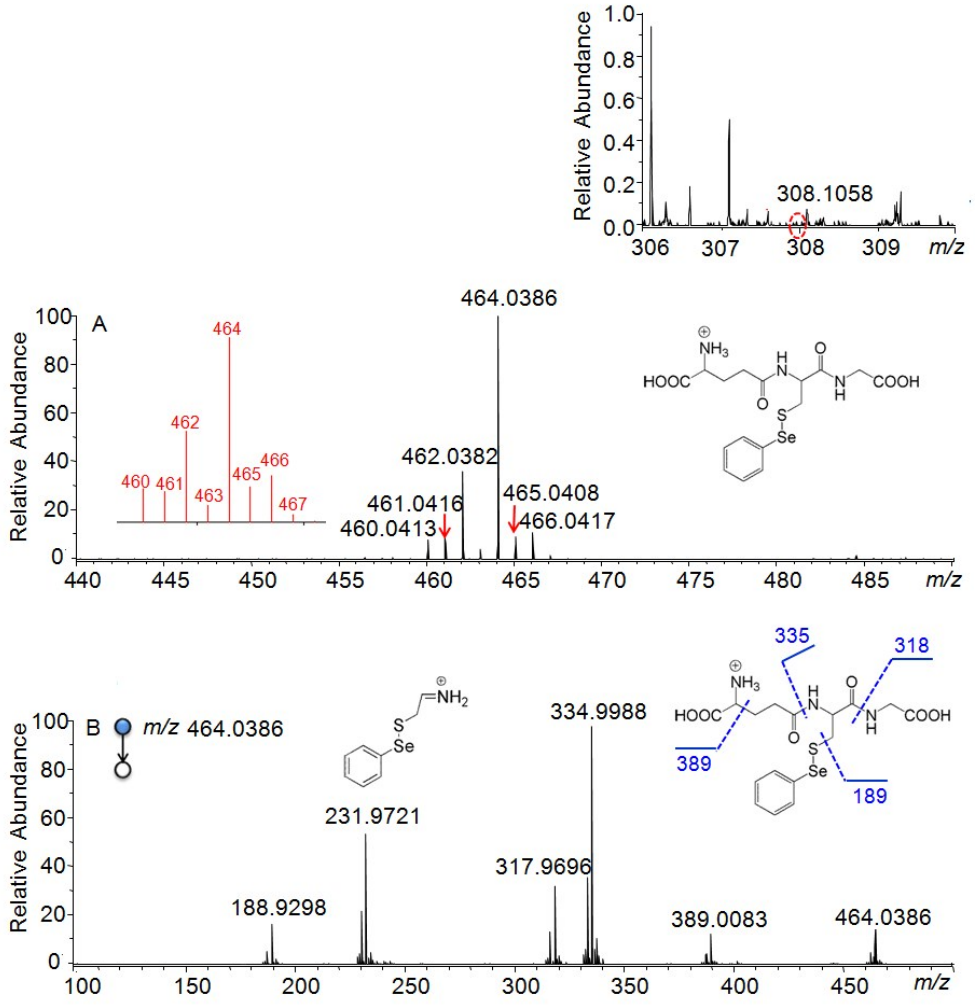


Figure S1. ESI-MS spectrum of the GSH products derivatized by NPSP-d_0, (B) CID MS/MS spectrum of the target ion (*m/z* 464).The red inset of (A) in red is the simulated isotope peak distribution of the GSH-NPSP-d_0 ion.

3. Selectivity of NPSP isotope probes

To test the selectively of the new isotope probes, the reaction products of NPSP-d_5 (or NPSP-d_0) with cysteine (5 μM) in the presence of 19 other natural amino acids (5 μM for each) was analyzed by ESI-MS. The result (Figure S2) shows that only cysteine with a free-SH group can be derivatized by NPSP-d_5 with a mass shift of 161 Da and characteristic selenium isotope distribution. Similar results were obtained for NPSP-d_0 in the selectivity test (data was not shown). Combining Figure 1B with Figure S2 demonstrates that NPSP isotope probes have exclusive specificity toward the free-SH group.

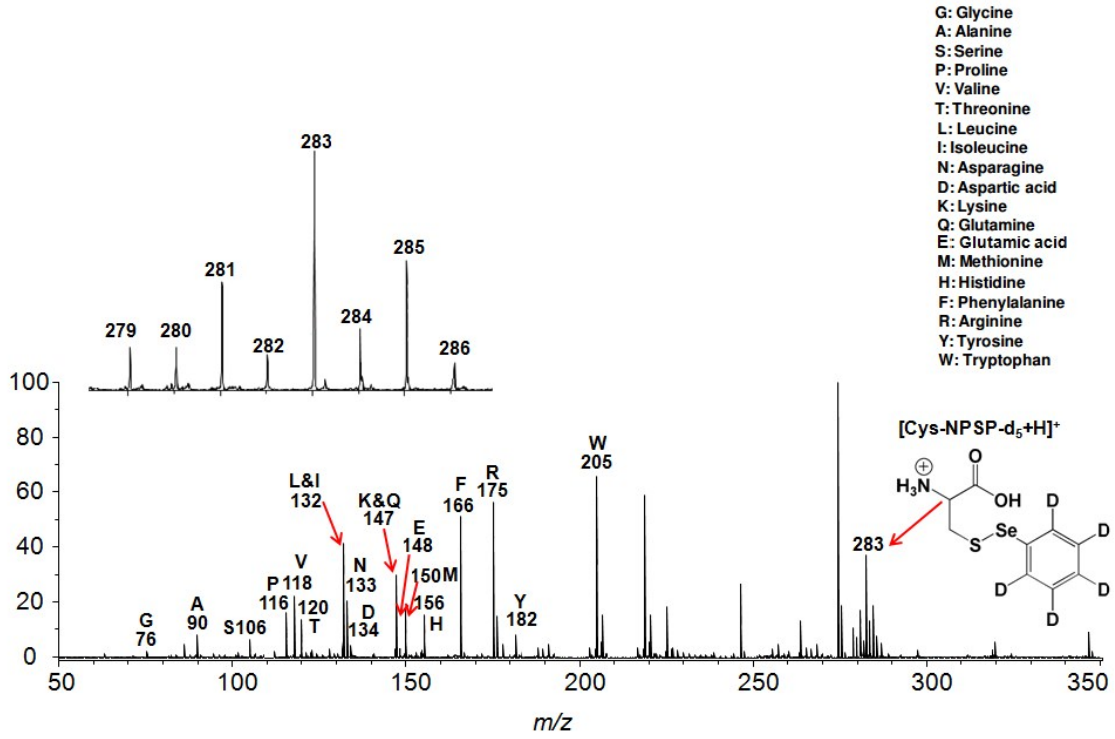
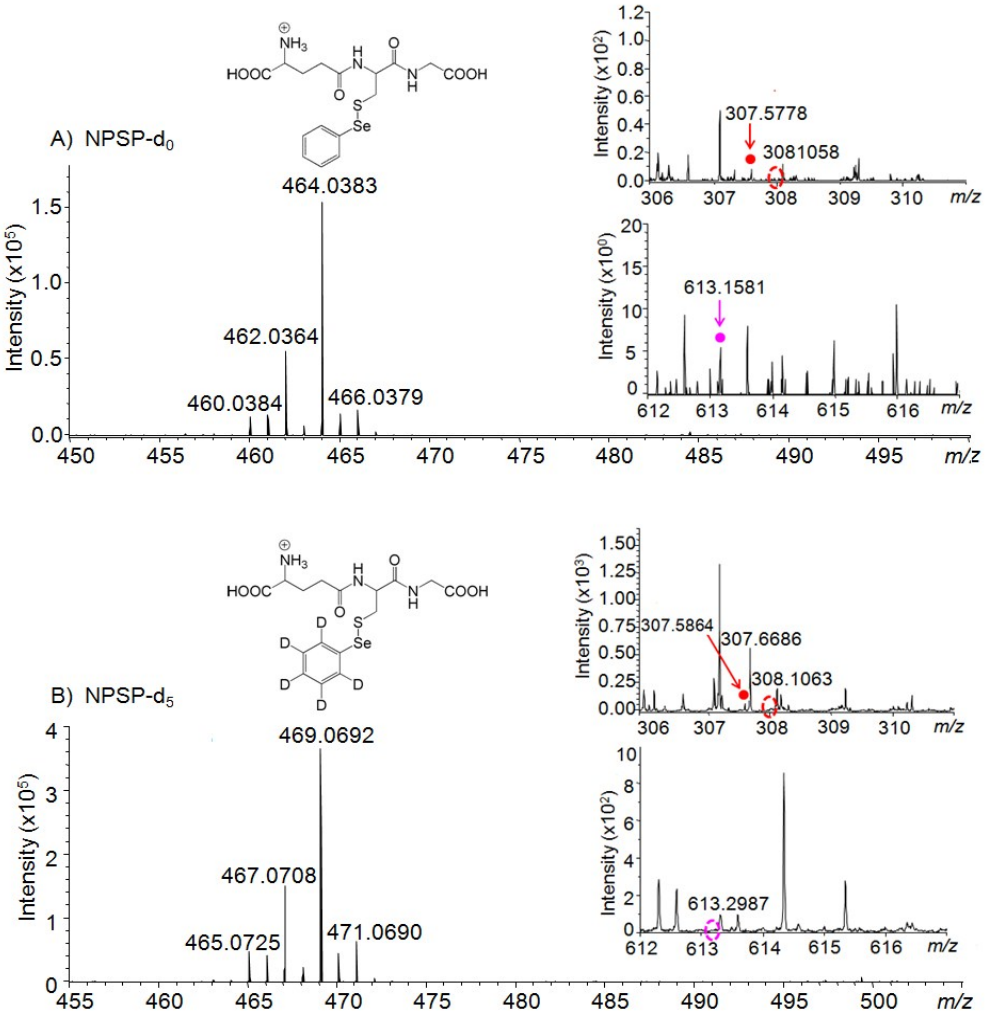


Figure S2. ESI-MS spectrum showing the selection of NPSP-d_5 with cysteine (5 μM) in the presence of 19 other natural amino acids (5 μM for each) in ACN/H_2O (1:1 by volume) containing 1% FA. The inset shows the zoomed-in peak distribution of the protonated Cys-NPSP-d_5.

4. Capability of NPSP isotope probes in avoiding the oxidation of biothiols

To test the capability of NPSP isotope probes in avoiding oxidation of biothiols, comparisons of NPSP-d_0/NPSP-d_5 with two commonly used alkylating reagents NEM and IAM in derivatizing GSH were made. It was shown that there was no or negligible oxidation product GSSG found for reactions of NPSP-d_0/NPSP-d_5 with GSH. While much more oxidation product GSSG was seen in reactions of NEM and IAM with GSH. So, NPSP isotopes probes can effectively avoiding the oxidation of biothiols.



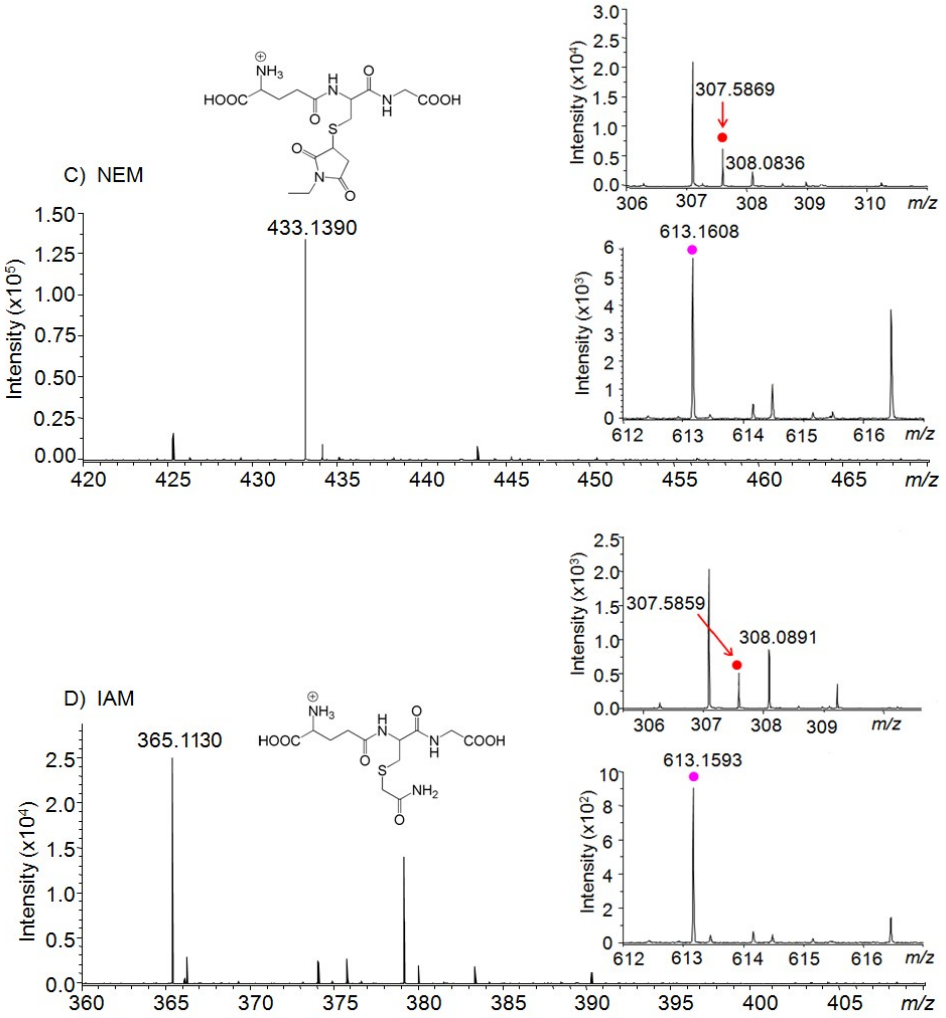


Figure S3. ESI-MS spectra of the derivatized products of GSH by (A) NPSP-d_0, (B) NPSP-d_5, (C) NEM, and (D) IAM. The right insets (top) and (bottom), are zoomed-in spectra of (A), (B), (C), and (D) in the range of *m/z* 306～312 and in the range of *m/z* 612～618.

● indicates the singly charged GSH oxidation product $[GSSG+H]^+$ (*m/z* 613.16);

● indicates the doubly charged GSH oxidation product $[GSSG+2H]^{2+}$ (*m/z* 307.58);

○ indicates no observation of the protonated GSH $[GSH+H]^+$ (*m/z* 308.09);

○ indicates no observation of the singly charged GSH oxidation product $[GSSG+2H]^{2+}$ (*m/z* 307.58).

Table S1. Percentages of GSH oxidation during the derivatization process using

different derivatizing reagents.

Reagent	$Intensity_{307.58}$ /$Intensity_{TM}$	$Intensity_{613.15}$ /$Intensity_{TM}$	Total pencentage
NPSP-d_0	0.12%	0.04%	0.16%
NPSP-d_5	0.15%	0.02%	0.17%
NEM	4.30%	5.15%	9.45%
IAM	11.2%	4.20%	15.40%

TM (target material): the desired derivatized products of GSH by four reagents.

The derivatized products of GSH by derivatizing reagents NPSP-d_0, NPSP-d_5, NEM, and IAM are $[GSH\text{-}NPSP\text{-}d_0+H]^+$ (*m/z* 464.04), $[GSH\text{-}NPSP\text{-}d_5+H]^+$ (*m/z* 469.07) $[GSH\text{-}NME+H]^+$ (*m/z* 433.14), and $[GSH\text{-}IAM+H]^+$ (*m/z* 365.11), respectively.

5. Quantitative comparison of biothiols using NPSP isotope pair

To test the quantitative comparison capability of NPSP isotope pair in GSH, Cys and Hcy, derivatizing products biothiols-NPSP-d_0 and biothiols-NPSP-d_5 were mixed in the different ratios and analyzed by ESI-MS. All the detected signal intensity ratios agreed well with actual sample mixing concentration ratios, showing good quantitative comparison capability of NPSP isotope pair.

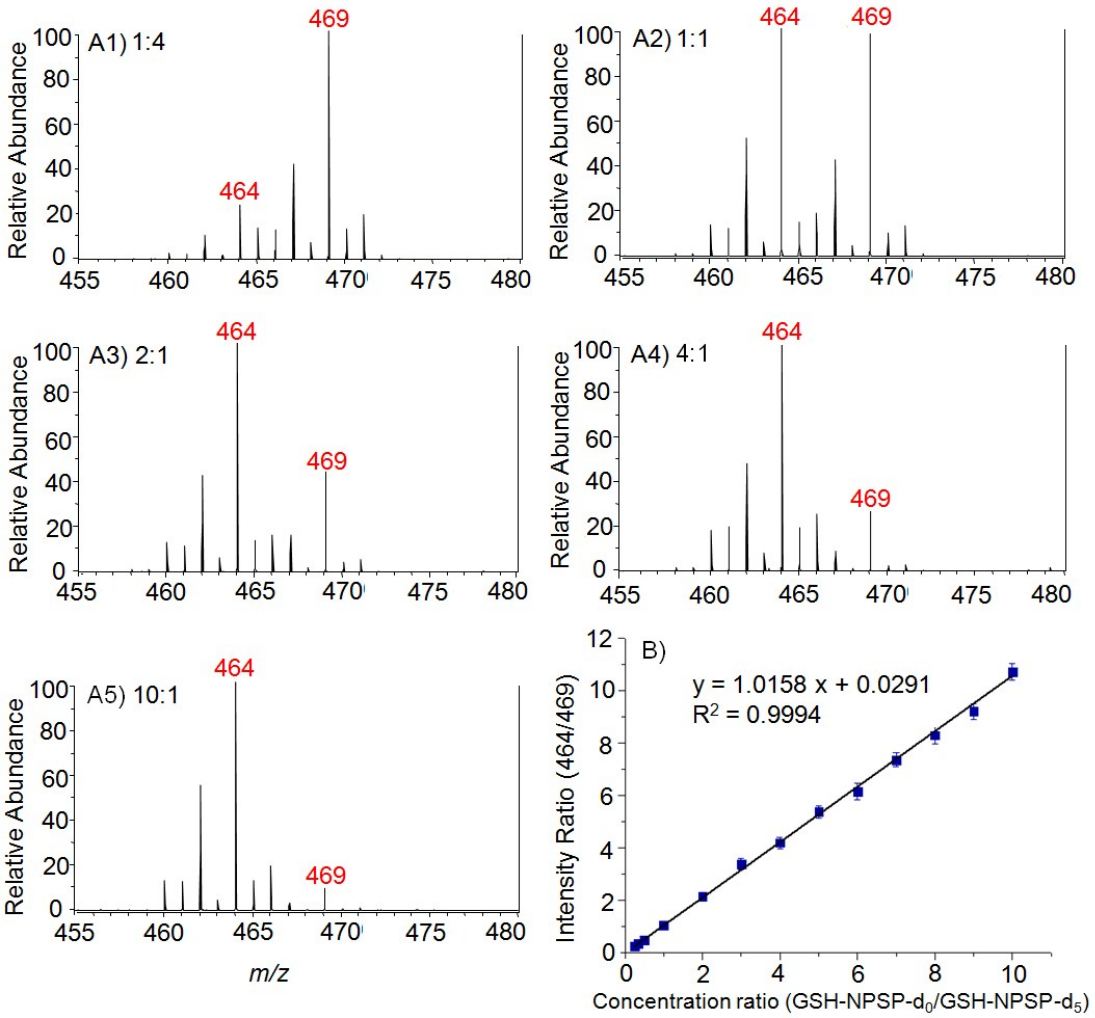


Figure S4. ESI-MS relative quantification for GSH labeling by NPSP isotope probes. A1 to A5: MS spectra of the NPSP-d_0 and NPSP-d_5 derivatized GSH mixed in the ratios of 1:4, 1:1, 2:1, 4:1 and 10:1. (B) Calibration curve. The data were obtained from triplicate meaurements.

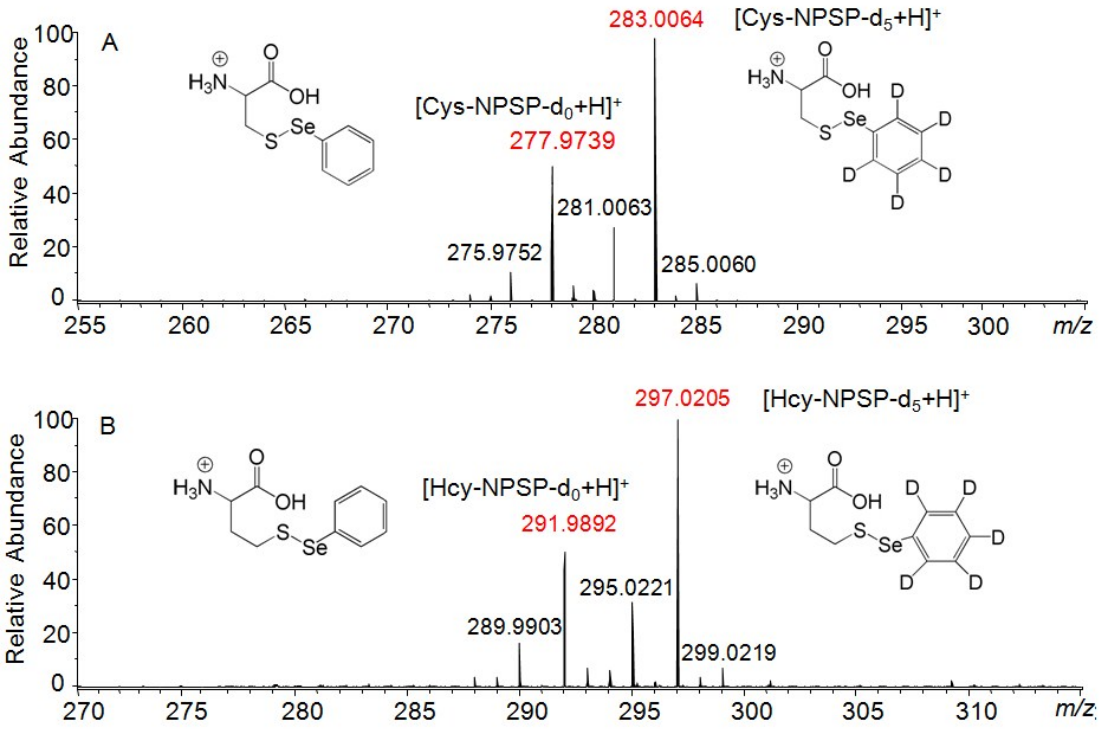


Figure S5. ESI-MS spectra of (A) the mixture of Cys-NPSP-d_0 and Cys-NPSP-d_5, (B) the mixture of Hcy-NPSP-d_0 and Hcy-NPSP-d_5 mixed in the ratio of 1:2. All the intensity ratios of $[\text{Cys-NPSP-d}_0\text{+H}]^+/[\text{Cys-NPSP-d}_5\text{+H}]^+$ (0.48 ± 0.0252) and $[\text{Hcy-NPSP-d}_0\text{+H}]^+/[\text{Hcy-NPSP-d}_0\text{+H}]^+$ (0.51 ± 0.0436) match well with the mixing concentration ratios.

6. Anti-disturbance ability of NPSP isotope probes

To test the anti-disturbance ability of the new isotope probes in quantitative comparison of biothiols, two sets of mixture containing GSH, Cys and Hcy (each in 5 μM) were derivatized by NPSP-d_0 and NPSP-d_5, respectively, and mixed in 1:1 ratio. then added with other species such as 19 other natural amino acids (5 μM for each), sugar (glucose, 5 mM), phospholipids (lecithin, cephalin, 5 mM for each) and metal ions (K^+, Na^+, Ca^{2+}, Mg^{2+}, 5 mM for each; Zn^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+}, 2.5 mM for each) and analyzed by LC/ESI-MS. The signal intensity of GSH, Cys and Hcy in the merged sample was 0.90:1.06: 1.00 (Figure 5S), which was quite consistent with the results of Figure 3. In addition, biothiols-NPSP-d_0 and biothiols-NPSP-d_5 display similar abundances (Figure 5S). This demonstrates that sugar, phospholipids and metal ions do not interfere with the quantitative comparison of biothiols in our experiments and the new NPSP isotope probes have strong anti-disturbance ability.

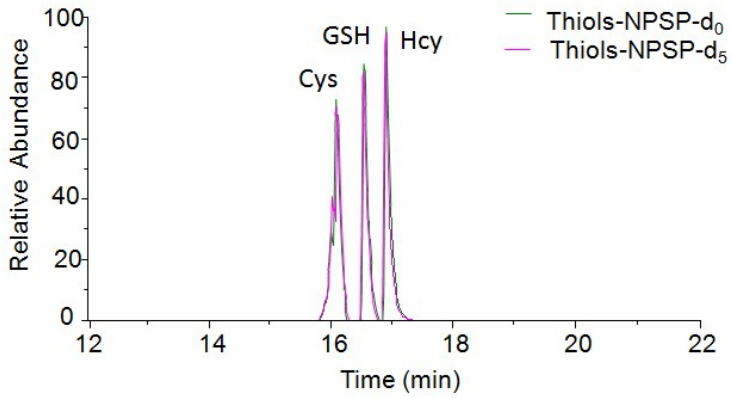


Figure S6. Extracted ion chromatograms (EICs) of GSH , Cys and Hcy (each in 5 μM) derivatized by both NPSP-d_0 (green line) and NPSP-d_5 (pink line) and added with 19 other natural amino acids (5 μM for each), sugar (glucose, 5 mM), phospholipids (lecithin, cephalin, 5 mM for each) and metal ions (K^+, Na^+, Ca^{2+}, Mg^{2+}, 5 mM for each; Zn^{2+}, Cu^{2+}, Fe^{2+}, Cd^{2+}, 2.5 mM for each)

7. Sensitivity of NPSP isotope probes

To test the sensitivity of the new isotope probes, GSH with 8 different concentrations (20 nM, 100 nM, 200 nM, 300 nM, 500 nM, 1μM, 2μM and 20 μM) were respectively added with 10-fold molar excess of NPSP-d_5 and the products were analyzed by LC-ESI/MS. The plot of the peak area (*m/z* 469) versus the concentration showed linearity in the range from 200 nM to 20 μM with regression equation y =1075.12 x + 58.30 and R^2 value 0.9997. The limit of detection (LOD) calculated based on standard devitation S of the blank samples and slope of the calibration was 41.9 nM (or 2.51×10^{-13} mol for the injected sample amount).

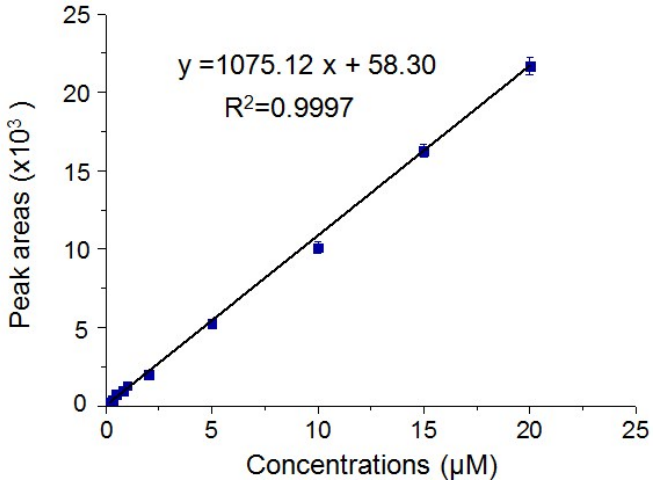


Figure S7. Calibration curve of GSH-NPSP-d_5 based on peak area (y) of extracted ion chromatograms (EICs) in LC/ESI-MS versus GSH-NPSP-d_5 concentration (x). All the values are reported as mean values from triplicate measurements (n=3), and the error bars in the represent for standard deviation (SD).

8. Cyotoxicity assay of NPSP isotope probes

To evaluate the potential toxicity of NPSP isotope probes to L02 and HepG2 cells, MTT assay was performed. First, L02 and HepG2 cells (10^6 cells/mL) were seeded in 96-well microliter plates to a total volume of 100 μL per well. The plates were incubated for 24 h at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Then L02 and HepG2 cells were incubated with NPSP isotope probes (6 mg/mL and 9 mg/mL) for 15 min, respectively. And 20 μL of MTT solution (5 mg/mL, phosphate-buffered saline PBS) was added to each well and incubated for 4 h, the remaining MTT solution was removed, DMSO (150 μL) was added to each well to dissolve the formazan crystals. The cell viability was measured at 490 nm in a Triturus microplate reader. The results showed that NPSP isotope probes had low cytotoxicity under experimental concentration.

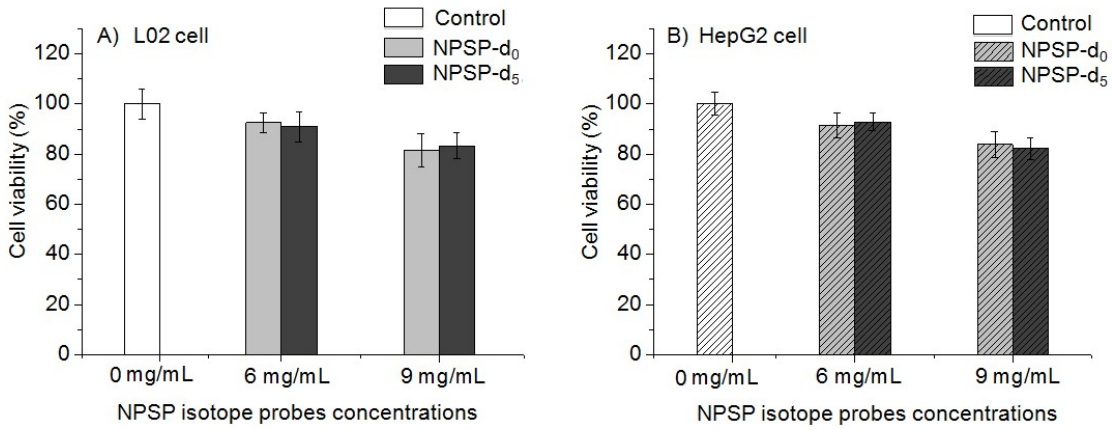


Figure S8. MTT assay of L02 (A) and HepG2 (B) cells incubated with NPSP isotope probes (0 mg/mL, 6 mg/mL and 9 mg/mL) for 15 min. Blank bar represents for control; light grey bar represents for cells incubated with NPSP-d_0; dark bar represents for cells incubated with NPSP-d_5.

9. Stability of biothiols-NPSP

To evaluate the stability of biothiols-NPSP during the sample pretreatment process including homogenization, deproteination with acids, simulation experiment was done in the absence of cells. Biothiols GSH, Cys and Hcy were all 20 μM, each of them was derivatized by NPSP-d_0 and NPSP-d_5 respectively. In order to explore the stability of biothiols-NPSP, all of the derivative products were divided into two: one was analyzed by ESI-MS without no pretreatment ($Intensity_{untreated}$); the another one was pretreated with homogenization and deproteination as did in the biological sample pretreatment process, then analyzed by ESI-MS ($Intensity_{treated}$). The relative abundance was the ratio of $Intensity_{treated}/Intensity_{untreated}$. There was no obvious signal intensity differences between biothiols-NPSP samples treated and untreated, suggesting biothiols-NPSP samples were stable during the biological sample pretreatment.

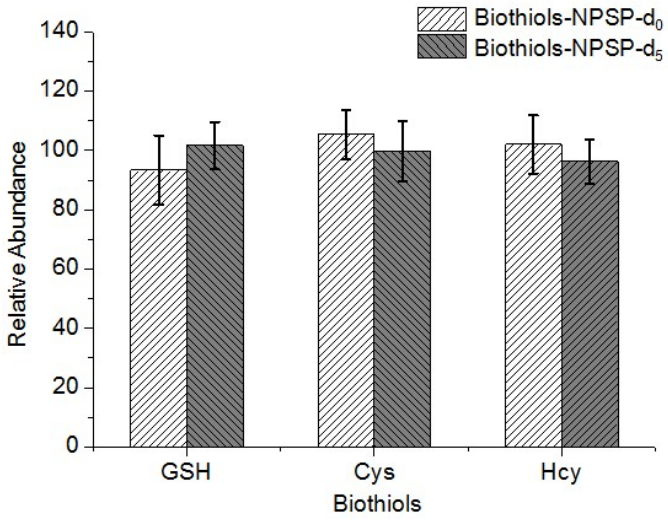


Figure S9. The stability of biothiols-NPSP during the sample pretreatment process. Light grey bar represents for derivative products biothiols-NPSP-d_0; dark bar represents for derivative products biothiols-NPSP-d_5.

10. Simultaneous comparison of GSH, Cys and Hcy in cells with converse labeling

To check the results obtained in the experiment of HepG2 cells labeled by NPSP-d_0 and L02 cells labeled by NPSP-d_5, we further employed converse labeling strategy for cells: L02 cells labeled by NPSP-d_0 and HepG2 cells labeled by NPSP-d_5. Similar results were obtained, suggesting higher GSH, Cys and Hcy in HepG2 cells than in L02 cells.

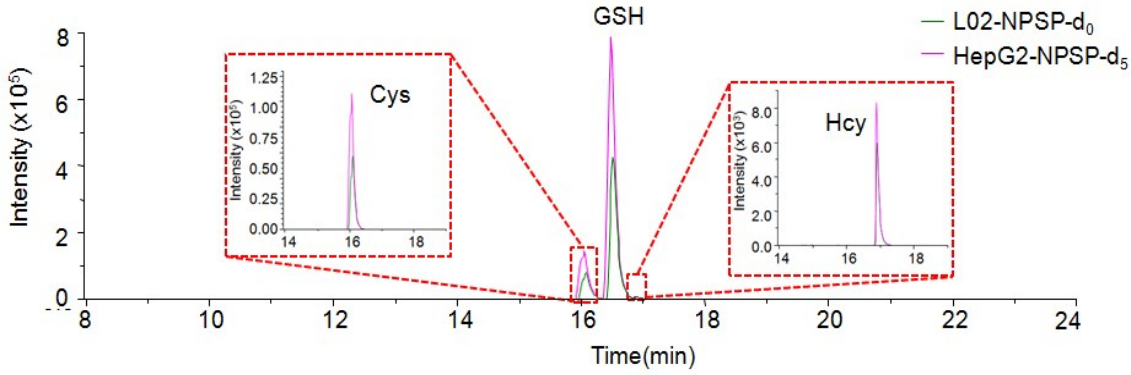


Figure S10. EICs of the derivatized biothiols from normal L02 cells (green line) and HepG2 cells (pink line). HepG2 labeled by NPSP-d_5 and L02 labeled by NPSP-d_0.

Table S2. Ratios of GSH, Cys and Hcy contents in HepG2 cells and L02 cells measured by the extracted ion chromatogram (EIC) peak areas. All the values were reported as mean values ± SD (n=3).

Biothiols	HepG2-NPSP-d_0/L02-NPSP-d_5	L02-NPSP-d_0/HepG2-NPSP-d_5
GSH	2.58±0.084	0.41±0.013
Cys	1.62±0.056	0.55±0.020
Hcy	1.58±0.064	0.68±0.021

References:

1. H. J. Reich , M. L. C., and P. S. Clark, Reagents for synthesis of organoselenium compounds: diphenyl diselenide and benzeneselenyl chloride. *Organic Syntheses,* 1979, **59**.
2. K. C. NrcolAou, N. A. P. a. D. A. C., N-(Phenylseleno)phthalimide (NPSP). *Tetrahedron,* 1985**, 41**, 4835-4841.
3. Giustarini, D.; Dalle-Donne, I.; Milzani, A.; Fanti, P.; Rossi, R., Analysis of GSH and GSSG after

derivatization with N-ethylmaleimide. *Nature protocols,* 2013, **8** , 1660-1669.