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Quantitative and comparative liquid chromatography-electrospray ionization-mass spectrometry analyses of hydrogen sulfide and thiol metabolites derivaitized with 2-iodoacetanilide isotopologues

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ABSTRACT

Hydrogen sulfide (H_2S), previously known as a toxic gas, is now recognized as a gasotransmitter along with nitric oxide and carbon monoxide. However, only few methods are available for quantitative determination of H_2S in biological samples. 2-Iodoacetanilide (2-IAN), a thiol-reacting agent, has been used to tag the reduced cysteine residues of proteins for quantitative proteomics and for detection of cysteine oxidation modification. In this article, we proposed a new method for quantitative analyses of H_2S and thiol metabolites using the procedure of pre-column 2-IAN derivatization coupled with liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI–MS). $^{13}C_6$-Labeled and label-free 2-IAN efficiently react with H_2S and thiol compounds at pH 9.5 and 65 °C. The derivatives exhibit excellent stability at alkaline conditions, high resolution on reverse phase liquid chromatography and great sensitivity for ESI–MS detection. The measurement of H_2S, L-cysteine, glutathione, and DL-homocysteine derivatives was validated using $^{13}C_6$-labeled standard in LC-ESI–MS analyses and exhibited 10 nM–1 μM linear ranges for DL-homocysteine and glutathione and 1 nM–1 μM linear ranges for L-cysteine and H_2S. In addition, the sequence of derivatization and extraction of metabolites is important in the quantification of thiol metabolites suggesting the presence of matrix effects. Most importantly, labeling with 2-IAN and $^{13}C_6$-2-IAN isotopologues could achieve quantitative and matched sample comparative analyses with minimal bias using our extraction and labeling procedures before LC–MS analysis.

1. Introduction

Hydrogen sulfide (H_2S), previously known as a toxic gas, is now recognized as a gaseous signaling molecule or gasotransmitter along with nitric oxide and carbon monoxide [1,2]. Critical evidence for the physiological roles of H_2S includes (1) endogenous H_2S can be detected in the brain [3–5], (2) endogenous H_2S is synthesized in cells from homocysteine and cysteine through cystathionine β-synthase [6] and cystathionine γ-lyase [7,8] and from beta-mercaptopyruvate through mercaptopyruvate sulfur transferase [9], (3) treatment of H_2S or H_2S donors alleviates the symptoms of renal damage [10,11] and cardiac infarction [12], (4) H_2S is a mediator of dietary restriction-mediated stress resistance and longevity [13]. Moreover, H_2S scavenges oxidants to produce anti-oxidant effects [14,15] and most importantly causes protein S-sulfhydration at the cysteine residues [16,17] to mediate a variety of cellular responses. Surprisingly, 10–25% of liver proteins are sulfhydrated under physiological conditions [18] indicating the effects of H_2S are prevalent in cells.

H_2S can be measured by direct detection with commercially available sulfide ion-selective electrodes, spectrophotometric determination after derivatization [19], polarographic sensors [20], gas chromatography [21,22], and HPLC-fluorimetric detection following derivatization with monobromobimane (mBBr) or dibromobimane [23–25], and HPLC-mass detection following derivatization with mBBr or mBBr isomer [26,27]. Especially, mBBr

Abbreviations: DAS, diacetanilide sulfide; EIC, extracted ion chromatograms; ESI, electrospray ionization; GILISA, Global Isotope-Labeled Internal Standard Addition; GSH, glutathione; GSIST, Group Specific Internal Standard Technology; 2-IAN, 2-iodoacetanilide; LOD, limit of detection; mBBr, monobromobimane; PAG, DL-propargylglycine; TOF, time-of-flight.

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derivatization of H_2S at alkaline conditions results in near complete formation of sulfide dibimane (SDB) product which can be detected by HPLC-electrospray ionization mass spectrometry (ESI–MS) with much higher sensitivity and specificity than other methods. In addition, stable isotope labeled SDB have been synthesized and used as an internal standard for quantitative measurement of H_2S [28]. However, two drawbacks associated with the mBBr derivatization methods are (1) the product SDB is not stable at alkaline conditions [5] and (2) the stable isotope labeled SDB are not commercially available.

A thiol-reacting agent, 2-iodoacetanilide (2-IAN), has been used to tag the reduced cysteine residues of proteins for quantitative proteomics [29] and for detection of cysteine oxidation modification [30]. We showed in this communication that 2-IAN and $^{13}C_6$-2-IAN isotopologues can be used to derivatize thiol metabolites resulting in metabolite derivative isotopologues. This novel stable isotope-coded derivatization method can be applied to quantitative analysis of thiol metabolites such as H_2S, cysteine, homocysteine and glutathione with liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI–MS).

2. Materials and methods

2.1. Materials

Sodium sulfide nonahydrate (Alfa Aesar), ^{13}C labeled (ring-$^{13}C_6$) and unlabeled 2-iodoacetanilide (Cambridge Isotope Laboratories, Inc.), DL-propargylglycine and L-cysteine (Sigma), DL-homocysteine (Santa Cruz Biotechnology), L-glutathione reduced (Fluka), and Acquity UPLC® BEH C_{18} 1.7 μm column, 2.1 × 100 mm (Waters) were purchased from the companies in parentheses. Methanol and acetonitrile of LC–MS grade were purchased from J.T. Baker. Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine sera were purchased from Invitrogen. HeLa and HepG2 cells were obtained originally from the American Type Culture Collection.

2.2. 2-IAN derivatization of free sulfide and thiol-containing metabolites

The standard reaction was performed in 50 μl of 20 mM sodium carbonate, pH 9.5, containing mixture of thiol-containing compounds and 0.2 mM 2-IAN or $^{13}C_6$-2-IAN at 65 °C for 2 h. Equal volume of 2% formic acid was then added to the product to terminate the reaction and stabilize the derivatives. 2-IAN or $^{13}C_6$-2-IAN was dissolved in acetonitrile as 0.1 M stock instead of DMSO to avoid the high background signals. Calibration standards were prepared from the reaction of 20 nM, 40 nM, 400 nM, 2 μM, 4 μM, 20 μM, and 40 μM Na_2S with 0.2 mM 2-IAN. For metabolite samples, 50 μl of reaction solution (20 mM sodium carbonate, pH 9.5 containing 0.2 mM 2-IAN) was added to an aliquot of vacuum-dried metabolite extracts followed by vigorous vortexing to dissolve the contents. The mixture was kept at 65 °C for 2 h and 50 μl of 2% formic acid was then added to terminate the reaction.

2.3. LC-ESI–MS analysis

An ultra-performance liquid chromatography (UPLC) system (Ultimate 3000 RSLC, Dionex) was connected to a quadrupole time-of-flight (QTOF)-MS detector with an electrospray ionization (ESI) interface (maXis HUR-QToF system, Bruker Daltonics). Samples were transferred into insert vials and kept in the autosampler with temperature set at 4 °C. Chromatography was performed at 35 °C with reversed-phase liquid chromatography on a BEH C_{18} column. The elution started from 99% mobile phase A (0.1% formic acid in ultrapure water) and 1% mobile phase B (0.1% formic acid in methanol), held at 1% B for 0.5 min, raised to 90% B in 7.5 min, held at 90% B for 1.5 min, and then lowered to 1% B in 0.5 min. The column was then equilibrated by pumping 1% B for 3 min until the next run. The flow rate was set at 0.2 ml/min during the entire chromatography. LC-ESI–MS data were acquired under the following ESI–MS settings: capillary voltage was 4500 V in positive ion mode; dry gas flow, heated with a temperature at 190 °C, maintained at 8 l/min; nebulizer gas was adjusted to 1.4 bar; mass detection range was set from m/z 100–1000.

2.4. Data process

LC–MS data were acquired by HyStar and micrOTOF control software (Bruker Daltonics). DataAnalysis and TargetAnalysis software (Bruker Daltonics) were used for processing LC–MS data. Target metabolites were identified by matching the theoretical m/z value and isotope pattern calculated from the chemical formula, and each identified metabolite signal was verified with individual derivative of standard. The quantitative value was obtained from the area of peak in each extracted ion chromatogram as signal response.

2.5. Cell culture

HepG2 cells were grew in Dulbecco's Modified Eagle Medium (DMEM) high glucose, containing 10% FBS and kept within an incubator supplying 5% CO_2 in atmosphere at 37 °C. The cells were rinsed with 2 ml of PBS for three times and harvested after draining the excessive PBS. The harvested cells were immediately subjected to metabolite extraction or stored at −70 °C.

2.6. Sample preparation of cellular metabolites

Harvested cells with a number of 2×10^6 were added with 200 μl of ultrapure water and disrupted by sonication for 5 s, twice. After centrifugation at 14,000×g for 10 min, 150 μl of the supernatant was transferred to a new tube and 450 μl of acetonitrile was added. After vigorous vortex, the mixture was centrifuged at 14,000×g for 10 min and 200 μl of the supernatant of each sample was aliquoted as water extract (WE) and vacuum-dried. Therefore, each aliquot of the dried cellular metabolites was obtained from about 5×10^5 cells. Similarly, 2×10^6 cell pellet was extracted with 200 μl of 2% formic acid or 200 μl of 2% ammonium hydroxide. Each portion was subjected to precipitation with the addition of acetonitrile and vacuum-drying following the same process in which water extract was obtained. The resulting extract was named formic acid extract (FE) or ammonium hydroxide extract (AE), respectively. The dried metabolite samples were stored at −20 °C or subjected to derivatization immediately.

2.7. Differential comparison in samples labeled with 2-IAN isotopologues

HepG2 cells were allowed to grow to the number of approximately 2×10^6 and treated with 2 mM Na_2S or 6 mM DL-propargylglycine (PAG) for 1 h at 37 °C. The cells were harvested and cell lysate was treated as described above. Aliquots of dried cellular metabolites from untreated cells were dissolved in 50 μl of 0.2 mM 2-IAN reaction solution and those from PAG- or Na_2S-treated cells were dissolved in 50 μl of 0.2 mM $^{13}C_6$-2-IAN reaction solution with vigorous vortexing to dissolve the contents. After incubation at 65 °C for 2 h, 50 μl of 2% formic acid was added to each sample. 2-IAN derivatives from the untreated cells were mixed with an equal volume of $^{13}C_6$-2-IAN derivatives from PAG or Na_2S-treated cells before subjecting to LC–MS analysis.

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