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Quantification of Sulforaphane Mercapturic Acid Pathway Conjugates in Human Urine by High-Performance Liquid Chromatography and Isotope-Dilution Tandem Mass Spectrometry

Patricia A. Egner[*,†], **Thomas W. Kensler**[†], **Jian-Guo Chen**[‡], **Stephen J. Gange**[§], **John D. Groopman**[†], and **Marlin D. Friesen**[†]
Departments of Environmental Health Sciences and Epidemiology, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland 21205, and Qidong Liver Cancer Institute, Qidong, 226200 People's Republic of China

Abstract

We report validation of the first high-pressure liquid chromatography isotope-dilution mass spectrometry method to measure sulforaphane (SFN) and its glutathione-derived conjugates in human urine. As epidemiological evidence continues to mount that the consumption of a diet rich in cruciferous vegetables may reduce the risk of certain cancers, the development of analytical methodologies to accurately measure isothiocyanates (ITCs) and their subsequent metabolic products becomes paramount. SFN, the principal ITC produced by broccoli, is an effective chemopreventive agent with multiple modes of action. SFN and SFN conjugates have often been measured collectively utilizing a cyclocondensation assay with 1,2-benzenedithiol. More recently, some of the major SFN conjugates have been determined using mass spectrometry. Here, triple-quadrupole mass spectrometry has been coupled with the use of stable isotope-labeled internal standards of D8-SFN and all four D8-SFN mercapturic acid pathway conjugates to provide an accurate, precise, sensitive, and specific method for analysis of these compounds. Using urine samples collected during an earlier intervention with broccoli sprouts, the concentrations of SFN, SFN-cysteine, and the mercapturic acid SFN-*N*-acetylcysteine were sufficiently high such that as little as 50 nL of urine was required for analysis. Although each study participant received an equivalent dose of broccoli sprout preparation, the interindividual conversion of the precursor glucosinolate to SFN varied over 100-fold. These 98 urines provided an ideal sample set for examining the robustness of the assay. The mean urinary concentrations ± standard deviations in overnight voids following ingestion of the first dose were 4.7 ± 5.1, 0.03 ± 0.05, 0.06 ± 0.06, 18 ± 15, and 42 ± 23 nmol/mg creatinine for SFN, SFN-glutathione, SFN-cysteine-glycine, SFN-cysteine, and SFN-*N*-acetylcysteine, respectively. This method determines SFN and all four SFN glutathione-derived metabolites with minimal sample preparation and will be extremely useful in understanding the role of SFN-rich foods in preventing cancer and other chronic diseases.

Introduction

Epidemiological studies have reported that the consumption of a diet rich in cruciferous vegetables reduces the risk of cancer (1) as well as many chronic degenerative diseases (2).

[*]To whom correspondence should be addressed. pegner@jhsph.edu.
[†]Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health.
[‡]Qidong Liver Cancer Institute.
[§]Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health.

Cruciferous vegetables such as broccoli, cauliflower, cabbage, watercress, and Brussels sprouts contain a number of biologically active constituents including glucosinolates that can be degraded to isothiocyanates (ITCs)[1] and indoles (3). Glucosinolates are hydrolyzed to ITCs through the action of the plant enzyme myrosinase or to a lesser degree by microbes in the human gut (4). Several ITCs, including sulforaphane (SFN; 1-isothiocy-anato-4-methylsulfinylbutane), the principal isothiocyanate contained in broccoli (5), are effective chemopreventive agents in animals (6, 7). Efficacy is attributed to the activation of cytoprotective enzymes through Nrf2 signaling, induction of apoptosis, and inhibition of cytochrome P450s and histone deacetylases (8–10). Although only a few human clinical trials have begun to examine the role of SFN as a preventive agent, an inverse association was found for the excretion of SFN metabolites and both aflatoxin-N^7-guanine DNA adducts and *trans*,*anti*-phenanthrene tetraol, a metabolite of the combustion product phenanthrene (11).

Studies in humans and experimental animals have shown that ITCs are metabolized primarily to mercapturic acids (12, 13). ITCs are initially conjugated to glutathione (GSH) by glutathione transferases and metabolized sequentially to form cysteinyl-glycine (CysGly), cysteine (Cys), and, finally, *N*-acetylcysteine conjugates (NAC). ITCs and conjugates can be collectively measured by a cyclocondensation reaction of deconjugated ITCs with 1,2-benzenedithiol yielding 1,3-benzenedithiol-2-thione, which is readily quantifiable by high-pressure liquid chromatography (HPLC) coupled to UV detection (14). This method has proven to be a valuable tool for examining the total amount of ITCs in plant extracts (14) or the collective levels of ITCs and metabolites in plasma, urine, and tissue (15, 16); however, it does not allow a distinction to be made between different ITCs or ITC conjugates. In addition, analyte specificity of the cyclocondensation reaction can be problematic because 1,2-benzenedithiol also reacts with dithiocarbamates, carbon disulfide, substituted thioureas, disulfiram, and chemicals used in the rubber industry (14). More recent methods for measuring SFN and SFN metabolites directly have relied on HPLC coupled with UV (17) or mass spectrometric (MS) detection (12, 18–20). Although these reports have provided a platform for further examination, they may require cumbersome sample preparation (12), may only measure SFN-NAC (18), use other biological matrices for validation (19), or use *N*-acetyl-*S*-(*N*-butylthiocar-bamoyl)-L-Cys as an internal standard (20). Here, we report a sensitive and specific HPLC/isotope dilution mass spectrometric method to quantify SFN and the four glutathione-derived conjugates in human urine. Stable isotope-labeled internal standards for each of the four conjugates were synthesized from D8-SFN, purified by HPLC, and characterized by MS. Utilizing human urine samples collected in a previously reported broccoli sprout intervention in China (11), we demonstrate the specificity, sensitivity, and versatility of this method. As this method quantitatively measures the individual SFN -conjugates and SFN, it will be extremely useful in clinical trials seeking to elucidate the role of SFN-rich foods in the prevention of disease as well as for examining determinants of interindividual variation in SFN pharmacokinetics.

Experimental Procedures

Study Population

A clinical trial evaluating the chemopreventive activity of broccoli sprouts was conducted in 2003 as a collaborative study between the Johns Hopkins Bloomberg School of Public Health and the Qidong Liver Cancer Institute. Study design and consent forms were approved by the Institute Review Boards monitoring human studies at both institutions:

[1]Abbreviations: ITC, isothiocyanates; SFN, sulforaphane, 1-isothiocy-anato-4-methylsulfinylbutane; CysGly, cysteineglycine; Cys, cysteine; NAC, *N*-acetylcysteine; HPLC, high-pressure liquid chromatography; MS, mass spectrometry; D8-SFN, 1-isothiocyanato-4-methylsulfinyl(1,1,2,2,3,3,4,4-2H8)butane; MS/MS, tandem mass spectrometry.

Details of the study are reported in Kensler et al. (11). Briefly, individuals participating in a randomized clinical trial drank a hot water infusion prepared from 3 day old broccoli sprouts. This preparation contained 400 μmol of glucoraphanin, the primary glucosinolate found in broccoli sprouts and the precursor for SFN. Because the clinical trial utilized a hot water infusion that inactivated myrosinase found in the plant, the bioavailability of SFN was solely dependent on the hydrolysis of glucoraphanin by microbes present in the gut (16, 21). Overnight urine voids collected approximately 12 h after drinking the first broccoli sprout infusion were used to determine the levels of SFN and its conjugates by the isotope dilution tandem MS methodology described herein. In the earlier report (11), total ITC concentrations (as assessed using the cyclocondensation assay) were determined from urines collected at later time points in the study. For our current evaluation, we chose the first overnight urine collection for analysis because these samples had not been previously thawed and refrozen.

Chemicals

SFN and custom-synthesized D8-SFN [1-isothio-cyanato-4-methylsulfinyl(1,1,2,2,3,3,4,4-2H8)butane] were purchased from LKT Laboratories (St. Paul, MN). Glutathione (GSH), cysteineglycine (CysGly), Cys, and NAC were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of analytical/reagent grade or higher. Creatinine levels were determined by Hagerstown Medical Laboratory, Inc. (Hagerstown, MD) using a kinetic alkaline picrate (Jaffee reaction) method measured on a Dade-Behring Dimension analyzer.

Preparation of Standards and Internal Standards

SFN-GSH, SFN-CysGly, SFN-Cys, and SFN-NAC were synthesized independently using both SFN and D8-SFN according to the method of Kassahun et al. (12) with a slight modification. At the completion of the 3 h reaction, the aqueous layer was acidified with 1 M HCl to pH 3, and a large excess of ice-cold acetonitrile was added, thereby precipitating the SFN conjugates. Following centrifugation at 13000*g* for 5 min, the solvent layer containing unconjugated SFN was removed. The aqueous fraction containing the SFN conjugate was subsequently purified by reverse-phase HPLC using a Luna C18 (2) 4.6 mm × 250 mm column (Phenomenex, Torrance, CA) and a Waters 996 photodiode array detector monitoring 200–300 nm. Chromatographic separation of the SFN conjugates and SFN was achieved using a linear gradient of 1% acetic acid/water and 1% acetic acid/acetonitrile over 40 min. Purity and structural confirmation of the conjugates were confirmed by HPLC and direct electro-spray MS prior to combining aliquots. Collected HPLC fractions were dried using a Savant Speed Vac (Savant Instruments, Inc., Farmington NJ) over several days until a constant weight was obtained. Each purified, dried conjugate was dissolved in 5% acetonitrile/1% acetic acid/water (initial MS mobile phase) and scanned using a Beckman DU 800 spectrophotometer (Beckman Coulter, Inc., Somerset, NJ). Millimolar absorption coefficients (mM^{-1} cm^{-1}) for SFN-GSH, SFN-CysGly, SFN-Cys, and SFN-NAC were determined to be 7.46, 8.2, 7.02, and 5.9, respectively. An absorption coefficient of 8.0 mM^{-1} cm^{-1} was previously reported for SFN-GSH (13). The molar extinction of 0.90 mM^{-1} cm^{-1} for SFN and D8-SFN was obtained from the Merck Index (22). Solutions were stored at −80 °C at pH 3 and protected from direct light.

Isolation of SFN and Its GSH-Derived Conjugates from Urine

Urine samples were thawed and centrifuged, and aliquots were removed for SFN conjugate and creatinine analyses. Urine samples designated for MS analysis were immediately acidified to pH ~ 3 in either the initial HPLC/MS mobile phase [water/acetonitrile/acetic acid (95/5/0.1, v/v/v)] or in 10 mM ammonium acetate buffer to prevent degradation.

For the determination of SFN-Cys, SFN-NAC, and SFN, 10 μL of urine was diluted 1–50 in the initial HPLC/MS mobile phase (Scheme 1a). Ten microliters of this solution was then transferred to a silanized glass injection insert, such that an equivalent of 0.2 μL of urine was used for each analysis. This sample was then spiked with 50 μL of initial HPLC/MS mobile phase containing 1 ng each of the three stable isotope-labeled internal standards: D8-SFN, D8-SFN-Cys, and D8-SFN-NAC. This solution was then brought to 100 μL by the addition of 40 μL of initial HPLC/MS mobile phase. For two urine samples with SFN-Cys concentrations below 15 pg/0.2 μL urine, the analysis was repeated with an equivalent of 1 μL of urine. For 14 urine samples with SFN-NAC concentrations greater than 4000 pg/0.2 μL, the analysis was repeated with an equivalent of 0.05 μL of urine.

For the determination of SFN-GSH and SFN-CysGly (Scheme 1b), which were present in concentrations more than 100-fold lower than SFN-NAC, 50 μL of urine was diluted with 950 μL of 10 mM ammonium acetate buffer (pH 3). This sample was then spiked with 50 μL of initial HPLC/MS mobile phase containing 1 ng each of the two stable isotope-labeled internal standards: D8-SFN-GSH and D8-SFN-CysGly. This solution was applied to a 3 mg Varian Bond-Elut LRC C-18 SPE column (Varian, Inc., Walnut Creek, CA) that had been preconditioned with 3 mL of methanol followed by 3 mL of water. SFN-GSH and SFN-CysGly were eluted with 4 mL of water/methanol/ acetic acid (50/50/0.1, v/v/v). Eluants were taken nearly to dryness in amber vials under nitrogen and redissolved in 100 μL of initial HPLC/MS mobile phase prior to injection of 10 μL.

HPLC-MS/MS Analysis of SFN and Its GSH-Derived Conjugates

Analysis of the SFN and SFN conjugates was carried out on a Thermo-Finnigan TSQ Quantum Ultra triple quadrupole mass spectrometer coupled to a Thermo-Finnigan Surveyor Plus HPLC and autoinjector (ThermoElectron Corporation, San Jose, CA). Samples were maintained at 10 °C before injection of 10 μL aliquots. Chromatographic separation was carried out at 35 °C at a flow rate of 100 μL/min on a 1 mm × 150 mm × 5 μm Luna C18(2) microbore HPLC column (Phenomenex, Torrance, CA). Mobile phase compositions were as follows (v/v/v): (A) water/acetonitrile/acetic acid (95/5/0.1) and (B) acetonitrile/acetic acid (100/0.1). The initial composition was held at 100% A for 3 min and then ramped to 60% B at 14 min. The column was then re-equilibrated for 9 min at initial conditions. The column flow was diverted away from the electrospray ionization (ESI) ion source except for the time period from 3 to 15 min.

Positive ESI-MS/MS was conducted with the capillary temperature set at 220 °C, the sheath gas at 49 arbitrary units, and the spray voltage at 5 kV. The same HPLC conditions were used for analysis of the two groups of metabolites. MS/MS transitions and collision energies for analysis of higher concentration analytes (SFN-Cys, SFN-NAC, and SFN) are given in Scheme 1a. In all transitions, a resolution of ±0.01 amu was chosen. The corresponding selected reaction monitoring chromatogram for these analytes in a typical urine sample is given in Figure 1a. MS/MS transitions and collision energies for analysis of lower concentration analytes (SFN-CysGly and SFN-GSH) are given in Scheme 1b. All transitions were resolved at ±0.01 amu. For SFN-GSH and D8-SFN-GSH, the sum of the two transitions monitored was used for quantitation. Identification of SFN-GSH was dependent on the presence of the two peaks monitored with the expected area ratio. The corresponding selected reaction monitoring chromatogram for these analytes for the same urine sample is shown in Figure 1b.

Results

Sensitivity and range of analysis for the HPLC-MS/MS analysis of SFN and four SFN conjugates in urine are given in Table 1. Limits of detection ranged from 1 to 6 pg,

depending upon the analyte. Linear 11-point isotopic dilution standard curves for each analyte ($r^2 > 0.98$) were generated by duplicate injection of 10 μL mixtures of D8-SFN-GSH, D8-SFN-CysGly, D8-SFN-Cys, D8-SFN-NAC, and D8-SFN (each at 1000 pg/ 100 μL) with varying concentrations of each of the five nonlabeled standards (4000, 2000, 1000, 500, 250, 125, 63, 31, 16, 8, and 0 pg/100 μL). Peak areas were fitted using the method of least-squares with a $1/X$ weighting factor. Limits of determination and range of analysis for the method in urine (Table 1) depended on the analyte and the amount of urine used for analysis.

Validation of the method for the analysis of SFN and SFN conjugates is shown in Tables 2 and 3. Accuracy was determined by using urine from a study participant who had received the placebo beverage in the clinical trial. The absence of any endogenous SFN or SFN metabolites in the urine sample was confirmed prior to use. Accuracy was evaluated by spiking the blank urine with the five SFN analytes at known levels and comparing the concentrations measured in the samples. All four concentration levels were examined in replicate ($n = 5$). The accuracy ranged from 70 to 101% and was worst for lower concentrations of SFN-GSH (Table 2).

Intraday reproducibility was evaluated in urine samples from three study participants who excreted high, medium, or low amounts of SFN and SFN conjugates. Concentrations were determined in five independent analyses, and the % coefficient of variation was less than 12% for all compounds at the three concentrations tested (Table 3).

The LC-MS/MS method described above was used to analyze 98 urines collected over a 12 h period after drinking a broccoli sprout infusion containing 400 μmol of the SFN precursor glucoraphanin. Selected reaction monitoring chromatograms for a typical urine sample are given in Figure 1a,b. The urinary concentrations in these samples of SFN and SFN conjugates corrected for creatinine excretion are summarized in Table 4. Sensitivity was very dependent on the particular SFN conjugate quantified. Levels of SFN, SFN-NAC, and SFN-Cys were orders of magnitude higher in urine than levels of SFN-GSH and SFN-CysGly, such that different size aliquots of each urine sample were analyzed separately. The wide ranges reported for each analyte reflect interindividual differences in SFN bioavailability and/or factors affecting biotransformation and excretion.

Discussion

Evidence promoting the health benefits of vegetable consumption continues to mount with an emphasis on the role of crucifers. This family of vegetables is of particular interest because it is exceptionally rich in glucosinolates, precursors to ITCs. Numerous epidemiological studies have shown that dietary intake of ITCs is inversely associated with cancer risk (1, 2). Therefore, analytical methodologies examining specific ITCs and their metabolic products become increasingly important in understanding the multiple anticarcinogenic mechanisms through which cruciferous vegetables may act (10, 24). For example, SFN-Cys is thought to be the most potent inhibitor of histone deacetylase among SFN and its metabolites (9). Although urinary levels of total ITC equivalents may be an excellent biomarker of exposure to ITCs in general, cancer preventative potency varies widely for individual ITCs (7, 10, 24, 25). As a consequence, we focused on developing a method to measure SFN and SFN conjugates because SFN is the most potent anticarcinogenic ITC reported and is easily consumed in cruciferous vegetables such as broccoli.

In this report, we present an isotope dilution MS/MS method utilizing D8-SFN and D8-SFN conjugates as internal standards to quantify the GSH conjugation products of SFN in urine.

Urines previously collected in a broccoli sprout intervention study (11) were used to demonstrate the validity, accuracy, and sensitivity of the method. In the broccoli sprouts intervention, urines of study participants collected after 5, 9, 10, and 12 days of intervention were previously analyzed by the cyclocondensation assay for the total ITC equivalents excreted (11). Although ITC excretion rates were observed to vary widely between individuals, excretion rates were relatively constant over time for each individual. As the urine samples examined in the previous study had been frozen and rethawed several times, we chose to analyze a set of heretofore unthawed urines that were collected for roughly 12 h overnight after participants drank the first dose of broccoli sprout infusion. In our analyses, we attempted to diminish any degradation of SFN conjugates by quick-thawing these urines and keeping them on ice, maintaining all sample processing in an acidic environment and minimizing light exposure throughout the analyses.

As shown in Table 2, the SFN mercapturic acid pathway conjugates isotope dilution MS/MS assay is very sensitive and has a wide dynamic range for detection of SFN and its metabolites. For SFN-GSH and SFN-CysGly, 50 μL of urine was used for analysis. However, as SFN-Cys, SFN-NAC, and SFN urine concentrations were much higher, only 0.2 μL and in a few cases 0.05 μL of urine was required for determination of these three compounds, and solid-phase column cleanup was not required (Scheme 1a,b). In particular, the SFN-Cys and SFN-NAC concentrations in urine are spread over a large dynamic range, recapitulating the high interindividual variability in excretion rates as previously reported (11).

With the exception of SFN-GSH, accuracy and reproducibility were excellent and well within desired limits (Tables 2 and 3). The determination of the SFN-GSH conjugate proved somewhat problematic due to the very low concentration in the urine, but reanalysis of a larger aliquot of urine enabled accurate measurement. We also examined the impact of *GSTM1* or *GSTT1* polymorphisms on the rate of SFN metabolite excretion but found no significant effect (data not shown), as also reported earlier based upon analysis of samples collected at later time points and analyzed by the cyclocondensation assay (11).

The average urinary concentrations of each SFN conjugate and SFN are summarized in Table 4. In agreement with previous reports, SFN-NAC was the primary metabolite of SFN excreted in urine comprising 64.9% of the total products; SFN-Cys and SFN represented 27.6 and 7.2% of the total, respectively. The proportional distribution of metabolites compares well with SFN-urinary profiles as published by Al Janobi et al. (20). In the current analysis, the average rate of SFN and SFN-GSH-derived conjugates excreted was 41.0 μmol/12 h. Using the cyclocondensation method, Kensler et al. reported a total dithiocarbamate excretion rate of 42.1 μmol/12 h for all participants receiving the broccoli sprouts preparation averaged over the urine samples analyzed at later time points (days 5, 9, 10, and 12) in the same clinical trial (11). The concordance of the total SFN metabolite levels for the two methods indicates that the LC-MS/MS method utilizing D8-SFN internal standards gives reliable results. In addition, it provides sensitive, reliable, and accurate measurements for individual metabolites while greatly minimizing or eliminating sample preparation. Application of this method will now be expanded to examine the multifaceted actions of SFN as a chemopreventive agent.

Acknowledgments

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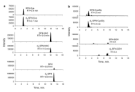
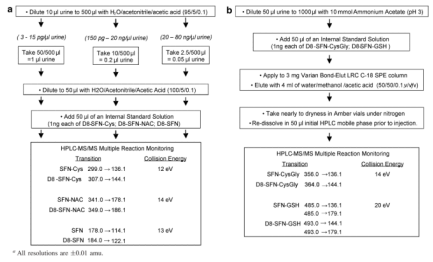


Figure 1.
(a) Selected ion mass chromatograms for the analysis of urine sample #152 for SFN-Cys, SFN-NAC, and SFN. (b) Selected ion mass chromatograms for the analysis of urine sample #152 for SFN-CysGly and SFN-GSH.



Scheme 1.

(a) Tandem Mass Spectrometry (MS/MS) Transitions and Collision Energies for Analysis of the Higher Concentration Analytes SFN-Cys, SFN-NAC, and SFN[a] and (b) MS/MS Transitions and Collision Energies for Analysis of Lower Concentration Analytes SFN-CysGly and SFN-GSH[a]

[a] All resolutions are ±0.01 amu.

Table 1

Sensitivity and Range of Analysis for the HPLC-MS/MS Analysis of SFN Conjugates in Urine

compound	limit of detection (pg)	range of sample volumes used (μL of urine)	limit of determination (ng/mL urine)	range of analysis (ng/mL urine)
SFN	1	1–0.05	5–100	5–80000
SFN-GSH	6	50	0.6	0.6–80
SFN-Cys-Gly	1	50	0.1	0.1–80
SFN-Cys	6	1–0.05	30–600	30–80000
SFN-NAC	3	1–0.05	15–300	15–80000

Table 2

Accuracy for the Measurement of SFN and Four SFN Conjugates at Four Concentrations in Human Urine

	ng/mL urine (% of spike recovered)			
SFN	632 (101)	1248 (100)	2513 (101)	5044 (101)
SFN-GSH	1.7 (70)	3.5 (71)	8 (83)	20 (99)
SFN-Cys-Gly	2.3 (94)	4.6 (93)	10 (96)	20 (100)
SFN-Cys	564 (90)	1132 (91)	2337 (93)	4777 (96)
SFN-NAC	549 (88)	1154 (92)	2345 (94)	4869 (97)

Table 3

Intraday Reproducibility for the Measurement of SFN and Four SFN Conjugates at Three Concentrations in Human Urine

	ng/mL (% coefficient of variation)		
SFN	250 (9)	558 (4)	3390 (3)
SFN-GSH	3.9 (12)	6.4 (4)	28 (6)
SFN-CysGly	0.24 (8)	8.5 (6)	19 (4)
SFN-Cys	163 (9)	1312 (7)	5152 (7)
SFN-NAC	1892 (8)	9756 (6)	22580 (6)

Table 4

Urinary Levels of SFN and SFN Conjugates (nmol/mg Creatinine) in Overnight Voids from 98 Individuals Following Early Evening Ingestion of a Broccoli Sprout Infusion Containing 400 μmol of Glucoraphanin

	nmol/mg		
metabolite	**mean**	**SD**	**range**
SFN	4.7	5.1	0.6–24
SFN-GSH	0.03	0.05	0.002–0.3
SFN-CysGly	0.06	0.06	0.0007–0.3
SFN-Cys	18	15	0.1–74
SFN-NAC	42	23	3.3–120
total	64	37	4.2–175