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LC-MS/MS Quantification of Sulforaphane and Indole-3-carbinol Metabolites in Human Plasma and Urine after Dietary Intake of Selenium-Fortified Broccoli

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ABSTRACT: This study aimed at developing a sensitive LC-MS/MS method for the quantification of sulforaphane (SFN) and indole-3-carbinol metabolites in plasma and urine after dietary intake of regular and selenium-fertilized broccoli using stable isotope dilution analysis. In a three-armed, placebo-controlled, randomized human intervention study with 76 healthy volunteers, 200 g of regular (485 μg of total glucosinolates and <0.01 μg of selenium per gram fresh weight) or selenium-fertilized broccoli (589 μg of total glucosinolates and 0.25 μg of selenium per gram fresh weight) was administered daily for 4 weeks. Glucoraphanin and glucobrassicin metabolites quantified in plasma and urine were SFN−glutathione, SFN−cysteine, SFN−cysteinylglycine, SFN−acetylcysteine, and indole-3-carboxaldehyde, indole-3-carboxylic acid, and ascorbigen, respectively. Dietary intake of selenium-fertilized broccoli increased serum selenium concentration analyzed by means of atomic absorption spectroscopy by up to 25% ($p < 0.001$), but affected neither glucosinolate concentrations in broccoli nor their metabolite concentrations in plasma and urine compared to regular broccoli.

KEYWORDS: broccoli, glucosinolates, LC-MS/MS, glucobrassicin metabolites, glucoraphanin metabolites, sulforaphane, indole-3-carbinol, selenium

INTRODUCTION

Numerous human studies have shown an association between a diet rich in cruciferous vegetables (e.g., broccoli, cabbage, and Brussels sprouts) and a decreased risk for prostate cancer.[1,2] This chemopreventive activity of cruciferous vegetables is attributed to their contents in glucosinolates[3] and probably selenium (Se).[4]

Among the glucosinolates, glucoraphanin and glucobrassicin represent the quantitatively dominating groups in broccoli. After dietary intake, glucoraphanin and glucobrassicin undergo a myrosinase-catalyzed hydrolysis, yielding sulforaphane (SFN) and indole-3-carbinol (I3-C), respectively. Once absorbed in the gastrointestinal tract, SFN and I3-C can be further metabolized into a broad spectrum of metabolites.[5−7] SFN is primarily metabolized through the mercapturic acid pathway, whereby its glutathione conjugate (SFN−GSH) is formed intracellularly and is sequentially degraded to form cysteinylglycine (SFN−Cys-Gly), cysteine (SFN−Cys), and *N*-acetylcysteine (SFN−NAC) conjugates in the upper intestinal lumen.[5−7]

Glucobrassicin metabolites are also generated through the enzymatic activity of the myrosinase that releases indole-3-acetonitrile (I3-ACN) under acidic conditions.[8] At neutral pH, the myrosinase catalyzes the formation of I3-C via the unstable intermediate indole-3-methyl isothiocyanate.[9] I3-C might undergo oxidative metabolization, yielding the corresponding aldehyde and/or carboxylic acid (I3-CAL, I3-CA).[10] Numerous in vitro studies using the free compound I3-C also showed its oligomerization into dimers, linear and cyclic trimers, and higher oligomers at acidic pH values as they occur in the stomach.[9,11] Furthermore, I3-C has also been demonstrated to react with L-ascorbic acid to form ascorbigen (ASG) in vitro.[12]

Glucoraphanin metabolites in biological samples are commonly quantified by analytical methods based on HPLC coupled to UV or mass detection.[13−15] Recently, Egner et al. reported a very sensitive and well-validated method for quantification of SFN and its mercapturic acid pathway conjugates in urine samples using a stable isotope dilution assay (SIDA) after bolus administration of broccoli infusions containing 175 mg of glucoraphanin to healthy volunteers.[16]

Glucobrassicin metabolites after dietary intake have been quantified by means of less sensitive analytical methods that require higher doses for reliable quantification. In one of the previously reported studies, ASG was quantified in rat plasma after dietary administration of approximately 117 mg of ASG/kg of body weight for 7 days using HPLC-MS.[17] Reed et al.[18] administered amounts of 400−1200 mg of I3-C to human subjects and were able to quantify only one of the glucobrassicin metabolites, 3,3′-diindolylmethane (DIM), by means of HPLC-MS.[18] We aimed at elucidating whether other metabolites are formed in vivo after dietary intake of glucobrassicin-containing

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broccoli that might require more sensitive analytical techniques with lower limits of detection. Therefore, we developed a sensitive LC-MS/MS method for quantification of low amounts of glucosinolate metabolites in plasma and urine after habitual dietary intake of regular and selenium-fertilized broccoli using SIDA. In addition, we investigated the influence of selenium fertilization on glucosinolate concentrations as Se fertilization has been shown to dose dependently decrease glucosinolates in various cultivars such as broccoli.[19] Thus, dietary intake of selenium-fertilized broccoli with low amounts of glucosinolates might result in lower glucosinolate metabolite concentrations in plasma and urine compared to regular broccoli.

In this study, we applied a previously published protocol for Se fertilization that is not likely to negatively affect glucosinolate concentrations in the plants[20] but might help to improve the nutritional status of selenium after dietary intake of the Se-fertilized broccoli. With the LC-MS/MS methods developed here, we were able to quantify glucoraphanin (SFN, SFN−GSH, SFN−Cys-Gly, SFN−Cys, SFN−NAC) and glucobrassicin metabolites (ASG, I3-CAL, I3-CA) in plasma and urine samples of healthy volunteers after dietary administration of regular and moderately Se-fertilized broccoli in a meal representative amount of 200 g.

MATERIALS AND METHODS

Chemicals and Materials. All chemicals were of LiChrosolv or p.a. quality if not described otherwise. Acetone, acetonitrile, chloroform, citric acid, formic acid (98−100%), dichloromethane (freshly distilled), diethyl ether, ethyl acetate, L-glutathione (reduced), hydrochloric acid (32%), methanol, potassium phthalimide, sodium carbonate, sodium chloride, sodium hydrogen carbonate, sodium hydroxide, sodium sulfate (anhydrous), sulfuric acid (95−97%), and *tert*-butyl methyl ether were from Merck (Darmstadt, Germany). *N*-Acetyl-L-cysteine, L-ascorbic acid, hydrobromic acid (48%), 3-chloroperbenzoic acid, L-cysteine, L-cysteinylglycine, hydrazine monohydrate (≥98%; purum quality), indole-3-acetonitrile, indole-3-carbinol, indole-3-carboxaldehyde (I3-CAL), indole-3-carboxylic acid (I3-CA), 4-methoxyindole (99%), sodium thiomethoxide, 1,1′-thiocarbonyldi-2(1*H*)-pyridone, and trifluoroacetic acid (≥98%; purum quality) were obtained from Sigma-Aldrich (Taufkirchen, Germany). $^{13}C_6$-L-Ascorbic acid (98 atom % ^{13}C) was provided by Omicron Biochemicals (South Bend, IN), whereas 3,3′-diindolylmethane and DL-sulforaphane were from LKT Laboratories (St. Paul, MN). Deuterium oxide (100%), dimethyl sulfoxide-d_6 (+0.03% TMS), acetonitrile-d_3, and d_8-tetrahydrofuran (99.5% D) were bought from Eurisotop (Saint-Aubin, France). Disodium hydrogen phosphate was purchased from Honeywell Riedel-de Haën (Seelze, Germany). C_{18} solid phase cartridges (1 mL, 100 mg) were provided by Supelco (Taufkirchen, Germany).

Broccoli Products. In a large-scale field cultivation experiment, broccoli (cultivar Ironman; Frankenthal, Germany) was grown under regular conditions without selenium fertilization (Se−) and with 2 mg per plant of foliar selenate application (Se+) as described previously.[20] Harvested broccoli florets were immediately transported to a nearby located vegetable-processing company. After washing, regular and selenium-fertilized broccoli were prepared by blanching at 94 °C for 120 s under industrial conditions and immediately frozen (Rheintal Tiefkühlkost, Bobenheim-Roxheim, Germany). Se concentrations of blanched broccoli products were analyzed by ICP-MS (Phytolab, Vestenbergsgreuth, Germany). Individual glucosinolate concentrations were analyzed by HPLC-DAD-MS according to the EU official HPLC-MS/MS method, using glucotropaeolin (320 μg) as internal standard.[21] Intra- and interday precisions were below 6%, and the recovery of the internal standard was 91%.

Chemical Synthesis and Isolation of Analytical and Internal Standards. Glucoraphanin mercapturic acid pathway metabolites (SFN−GSH, SFN−Cys-Gly, SFN−Cys, SFN−NAC) were synthesized according to the method of Kassahun et al. with slight modifications.[5] Briefly, free DL-SFN and the respective reactants (GSH, Cys-Gly, Cys, *N*-acetylcysteine) were dissolved in sodium hydrogen carbonate buffer (0.5 mol/L; pH 10) and stirred at room temperature (RT) under nitrogen for 2 h. Then, the reaction was stopped by adjusting the pH to 5 using 32% hydrochloric acid (HCl), and metabolites were purified by semipreparative RP-HPLC-DAD/UV at 200−400 nm/210 and 280 nm using an Aqua C_{18} column (250 × 10 mm; 12.5 nm; 5 μm) as stationary phase that was equipped with a guard column (C_{18}, 4.0 × 3.0 mm; both from Phenomenex, Aschaffenburg, Germany). Flow rates were set at 2.5 mL/min for all unlabeled glucoraphanin metabolites and d_8-SFN-NAC, at 3.0 mL/min for d_8-SFN, and at 2.8 mL/min for ASG and $^{13}C_6$-ASG. Mobile phases consisted of 0.1% formic acid in water (A) and acetonitrile (B; ACN). The gradient for purification of SFN−GSH started with 100% A, arrived to 30% A within 20 min and to 0% A within 25 min, as described previously,[22] whereas isolation of SFN−Cys-Gly was performed using 100% A as initial value, decreasing to 70% A within 7 min and to 30% A within 16 min to finally reach 0% A within 17 min. The gradient for SFN−Cys started with 100% A, arrived to 74% A within 2 min, to 72% A within 20 min, and to 65% A within 22 min, before decreasing to 0% A within 23 min. For SFN−NAC, the gradient started with 100% A, decreased to 70% A within 7 min and to 30% A within 16 min to finally reach 0% A within 17 min. Collected fractions were dried under nitrogen, and stock solutions in 0.1% formic acid in water were prepared and stored at −80 °C with protection from direct light.

Octadeutero-SFN synthesis was performed according to a procedure that has previously been described for the synthesis of DL-SFN, starting with the educt 1,4-dibromobutane.[23] In our case, the synthesis has been slightly modified concerning the amount and type of educt used: We used octadeuterated 1,4-dibromobutane, which was synthesized according to a method described for synthesis of the corresponding unlabeled compound.[24] The product received (crude d_8-SFN; 2.26×10^{-4} mol) was a yellow oil. Sample cleanup was performed by means of semipreparative RP-HPLC-DAD using an Aqua C_{18} HPLC column (5 μm, 12.5 nm, 250 × 10 mm) equipped with a guard column (C_{18}, 4.0 × 2.0 mm, both columns purchased from Phenomenex) as stationary phase as well as 0.1% formic acid in water (A) and methanol (B) as mobile phases, respectively. A flow rate of 3 mL/min was used, and the gradient started with 100% A, arrived to 34% A within 20 min and to 0% A within 23 min, at which it was kept until 28 min. The signal eluting after 21.4 min was collected and the combined phases were freeze-dried subsequently.

Synthesis of d_8-SFN-NAC was performed using d_8-SFN (14.3 mg; 77.3 μmol) and *N*-acetyl-L-cysteine (160 mg; 980 μmol) according to the procedure applied for the respective unlabeled standard. The compound was purified by means of semipreparative RP-HPLC using the same mobile phases and gradient as for SFN-NAC.

For quantification of glucobrassicin metabolites, ASG was synthesized from L-ascorbic acid and I3-C according to the method of Kiss and Neukom.[12] Briefly, a suspension of L-ascorbic acid (289.2 μmol) and I3-C (344.9 μmol) in McIlvaine buffer[25] was stirred at room temperature under nitrogen for 1 h and filtered subsequently. The resulting filtrate was extracted with 10 mL of diethyl ether four times, and the aqueous phase was then extracted three times with 10 mL of ethyl acetate, respectively. The resulting crude product (22.3 mg) was dissolved in 22.3 mL of water and ASG isolated by means of semipreparative RP-HPLC-DAD, with mobile phases consisting of 0.1% formic acid in water (A) and ACN (B). The gradient for purification of ASG started with an initial value of 100% A, which was kept for 5 min. Then, it was decreased

to 55% A within 6 min, to 42% A within 11 min, to 41% A within 16 min, and to 35% A within 17 min to finally reach 0% A within 18 min, at which the gradient was kept for another 5 min. $^{13}C_6$-ASG was synthesized by using L-$^{13}C_6$-ascorbic acid as an educt. Further synthesizing procedures are identical to those used for the synthesis of the unlabeled ASG. The resulting crude product (13.0 mg) was dissolved in 13.0 mL of water, and $^{13}C_6$-ASG was purified by means of semipreparative RP-HPLC using the same mobile phases and gradient as described for ASG. Mass spectra demonstrated an increase in the molecular mass of 6 g/mol in both cases (data not shown).

Spectroscopic Data of Synthesized Standards. After synthesis of the analytical and internal standards, purity and structure were confirmed by RP-HPLC, direct electrospray MS, and NMR experiments. For MS analyses, samples were dissolved in 0.1% formic acid in water and directly injected into the ion source of an ion trap mass spectrometer (LCQ Classic, Thermo Electron Corp., Waltham, MA). An electrospray ionization with positive polarity at a flow rate of 10 μL/min, a sheath gas flow rate of 60 arbitrary units, an auxiliary gas flow of 0 arbitrary units, a source voltage of 4.5 kV, a capillary temperature of 200 °C, and a capillary voltage of 17 V, respectively, was set with a scan range of 50−1500 amu. NMR spectroscopies (^{1}H, ^{13}C, DQF-COSY, HMQC, and HMBC) were performed at 298 K using an AMX 400 spectrometer (Bruker, Rheinstetten, Germany) after the synthesized adducts had been dissolved in D_2O, DMSO-d_6, or ACN-d_3. Analyses were performed with the computer program WIN-NMR (version 6.0; Bruker) and X-WIN-NMR (version 3.1; Bruker). Chemical shifts are reported relative to residual H_2O (δ 4.65) or, in case of the other two solvents, relative to the TMS standard. Chemical structures of all adducts were identified considering the parameters reported below for each compound.

DL-SFN−GSH: HPLC elution time, 13.08 min; yield, 41% (2.78 mg; 5.74 μmol; white solid); LC-ESI(+)-MS, m/z 485 (100; $[M + H]^+$), 507 (18; $[M + Na]^+$); ^{1}H NMR and COSY (400 MHz, D_2O), δ 1.76 [m, 4 H, CH, C(3) and C(4)], 2.09 [m, 2 H, CH, C(10)], 2.45 [t, J = 7.6 Hz, 2 H, CH, C(9)], 2.64 [s, 3 H, CH, C(1)], 2.87 [m, 2 H, CH, C(2)], 3.52 and 3.84 [dd, 1 H, CH, C(6) and dd, 1H, CH, C(6′)], 3.72 [m, 2 and 1 H, CH, C(11) and C(5)], 3.90 [s, 2 H, CH, C(8)], 4.75 [t, J = 6.6 Hz, 1 H, CH, C(7)]; ^{13}C NMR (400 MHz; D_2O; HMBC, HMQC), δ 19.75 [CH, C(3)], 26.44 [CH, C(4)], 26.44 [CH, C(10)], 31.66 [CH, C(9)], 35.95 [CH, C(6)], 36.49 [CH, C(1)], 41.98 [CH, C(8)], 46.93 [CH, C(5)], 52.56 [CH, C(2)], 53.23 [CH, C(7)], 54.16 [CH, C(11)], 172.46 [C, C(b)], 173.89 [C, C(e)], 174.11 [C, C(a)], 174.99 [C, C(d)], 197.23 [C, C(c)].

DL-SFN−Cys-Gly: HPLC elution time, 11.29 min; yield, 48% (3.83 mg; 10.85 μmol; white solid); LC-ESI(+)-MS, m/z 356 (100; $[M + H]^+$), 711 (28; $[2M + H]^+$); ^{1}H NMR and COSY (400 MHz, DMSO-d_6, TMS), δ 1.66 [m, 4 H, CH, C(3) and C(4)], 2.52 [s, 3 H, CH, C(1)], 2.66 and 2.77 [m, 2 H, CH, C(2)], 3.34 and 3.51 [dd, 1 H, CH, C(6) and dd, 1 H, CH, C(6′)], 3.61 [t, 3 H, CH, C(5) and C(7)], 3.74 [s, 2 H, CH, C(8)]; ^{13}C NMR (400 MHz; DMSO-d_6, TMS, HMBC, HMQC), δ 20.36 [CH, C(3)], 27.40 [CH, C(4)], 38.89 [CH, C(1)], 39.88 [CH, C(6)], 41.86 [CH, C(8)], 46.93 [CH, C(5)], 53.47 [CH, C(2)], 54.59 [CH, C(7)], 172.10 [C, C(c)], 172.94 [C, C(b)], 196.53 [C, C(a)].

DL-SFN−Cys: HPLC elution time, 11.29 min; yield, 37% (3.36 mg; 11.28 μmol; white solid); LC-ESI(+)-MS, m/z 299 (100; $[M + H]^+$), 321 (64; $[M + Na]^+$), 619 (78; $[2M + Na]^+$), 635 (55; $[2M + K]^+$); ^{1}H NMR and COSY (400 MHz, DMSO-d_6, TMS), δ 1.61 [m, 4 H, CH, C(3) and C(4)], 2.52 [s, 3 H, CH, C(1)], 2.68 and 2.77 [m, 2 H, CH, C(2)], 2.98 and 2.92 [dd, 1 H, CH, C(6) and dd, 1 H, CH, C(6′)], 2.87 [m, 2 H, CH, C(2)], 3.52 and 3.84 [dd, 1 H, CH, C(6) and dd, 1H, CH, C(6′)], 3.42 [t, 2 H, CH, C(5)], 5.01 [t, 1 H, CH, C(7)]; ^{13}C NMR (400 MHz; DMSO-d_6, TMS, HMBC, HMQC), δ 20.20 [CH, C(3)], 27.23 [CH, C(6)], 28.52 [CH, C(4)], 38.73 [CH, C(1)], 43.77 [CH, C(5)], 53.50 [CH, C(2)], 58.54 [CH, C(7)], 172.46 [C, C(a) and C(b)].

DL-SFN−NAC: HPLC elution time, 12.71 min; yield, 67% (1.55 mg; 4.56 μmol; white solid); LC-ESI(+)-MS, m/z 341 (93; $[M + H]^+$), 363 (100; $[M + Na]^+$); ^{1}H NMR and COSY (400 MHz, D_2O), δ 1.71 [m, 4 H, CH, C(3) and C(4)], 1.90 [s, 3 H, CH, C(8)], 2.59 [s, 3 H, CH, C(1)], 2.82 [m, 2 H, CH, C(2)], 3.49 and 3.84 [dd, 1 H, CH, C(6) and dd, 1 H, CH, C(6′)], 3.66 [t, J = 6.5 Hz, 2 H, CH, C(5)], 4.63 [t, 1 H, CH, C(7)]; ^{13}C NMR (400 MHz; D_2O, HMBC, HMQC), δ 19.61 [CH, C(3)], 21.74 [CH, C(8)], 26.45 [CH, C(4)], 35.64 [CH, C(6)], 36.42 [CH, C(1)], 46.74 [CH, C(5)], 52.34 [CH, C(2)], 53.13 [CH, C(7)], 174.11 [C, C(b)], 174.53 [C, C(c)], 197.36 [C, C(a)].

d_8-SFN: HPLC elution time, 21.70 min; total yield, 0.12% (0.042 g; 2.26×10^{-4} mol; viscous yellow oil); LC-ESI(+)-MS, m/z 186 (16; $[M + H]^+$), 208 (9; $[M + Na]^+$), 224 (100; $[M + K]^+$); ^{1}H NMR and COSY (400 MHz, ACN-d_3), δ 2.52 [s, 3 H, CH, C(1)]; ^{13}C NMR (400 MHz; ACN-d_3, HMBC, HMQC), δ 38.00 [CH, C(1)].

d_8-SFN−NAC: HPLC elution time, 12.74 min; yield, 66% (17.75 mg; 51.02 μmol; white solid); LC-ESI(+)-MS, m/z 349 (25; $[M + H]^+$), 371 (74; $[M + Na]^+$), 718 (100; $[2M + Na]^+$), 735 (12; $[2M + K]^+$); ^{1}H NMR and COSY (400 MHz, D_2O), δ 1.91 [s, 3 H, CH, C(8)], 2.60 [s, 3 H, CH, C(1)], 3.50 and 3.85 [dd, 1 H, CH, C(6) and dd, 1 H, CH, C(6′)], 4.62 [t, 1 H, CH, C(7)]; ^{13}C NMR (400 MHz; D_2O, HMBC, HMQC), δ 22.10 [CH, C(8)], 36.15 [CH, C(6)], 36.73 [CH, C(1)], 53.13 [CH, C(7)], 173.11 [C, C(b)], 174.53 [C, C(c)], 196.80 [C, C(a)].

DL-SFN (commercially available standard; measured for comparison): HPLC elution time, 21.51 min; viscous yellow oil; LC-ESI(+)-MS, m/z 178 (100; $[M + H]^+$), 200 (60; $[M + Na]^+$); ^{1}H NMR and COSY (400 MHz, DMSO-d_6, TMS), δ 1.74 [m, 4 H, CH, C(3) and C(4)], 2.53 [s, 3 H, CH, C(1)], 2.69 and 2.80 [m, 2 H, CH, C(2)], 3.74 [t, J = 6.0 Hz, 2 H, CH, C(5)]; ^{13}C NMR (400 MHz; DMSO-d_6, TMS, HMBC, HMQC), δ 19.91 [CH, C(3)], 29.09 [CH, C(4)], 37.89 [CH, C(1)], 44.92 [CH, C(5)], 52.73 [CH, C(2)], 128.36 [C, C(a)].

ASG: HPLC elution time, 13.20 min; yield, 6.7% (5.9 mg; 19.3 μmol; white amorphous solid); LC-ESI(+)-MS, m/z 306 (11; $[M + H]^+$), 328 (47; $[M + Na]^+$), 344 (6; $[M + K]^+$), 633 (100; $[2M + Na]^+$), 649 (9; $[2M + K]^+$); ^{1}H NMR and COSY (400 MHz, DMSO-d_6, TMS), δ 3.10/3.25 [m, 2 H, CH, C(5)], 3.80 [d, 2 H, CH, C(7)], 4.00 [d, 1 H, CH, C(9)], 4.10 [m, 1 H, CH, C(8)], 6.90 [m, 1 H, CH, C(3)], 7.05 [m, 1 H, CH, C(2)], 7.15 [s, 1 H, CH, C(6)], 7.30 [d, 1 H, CH, C(1), 7.50 [d, 1 H, CH, C(4)]; ^{13}C NMR (400 MHz; DMSO-d_6, TMS, HMBC, HMQC), δ 31.20 [CH, C(5)], 75.00 [CH, C(7) and C(8)], 79.90 [C, C(b)], 86.50 [CH, C(9)], 106.10 [C, C(c)], 109.80 [C, C(d)], 111.00 [CH, C(1)], 117.10 [CH, C(3)], 117.90 [CH, C(4)], 121.70 [CH, C(2)], 124.90 [CH, C(6)], 128.40 [C, C(e)], 137.50 [C, C(f)], 177.60 [C, C(a)].

$^{13}C_6$-ASG: HPLC elution time, 12.96 min; yield, 5.83% (2.5 mg; 8.0 μmol; white amorphous solid); LC-ESI(+)-MS, m/z 312 (4; $[M + H]^+$), 334 (14; $[M + Na]^+$), 623 (2; $[2M + H]^+$), 645 (100; $[2M + Na]^+$), 661 (7; $[2M + K]^+$). Due to the low recovery, no NMR experiments were performed in this case. Structure and purity were controlled by means of mass analysis and fragmentation patterns of the metabolites using direct injection technique into the ion source of a mass spectrometer (LCQ) with electrospray ionization in the positive mode.

Design of the Human Intervention Study. A controlled and randomized human intervention study was performed at the Max Rubner-Institute in Karlsruhe, in accordance with the Declaration of Helsinki and with the approval by the Ethical Board of the Medical Association of the state of Baden-Württemberg, Germany. All participants gave their written consent. Inclusion criteria were male, nonsmoker, and age above 50 years (range of 50−82 years). Exclusion criteria were history or current serious diseases such as cardiovascular disease, cancer, diabetes, disorders of the gastrointestinal tract, chronic inflammatory diseases, and infections, as well as food allergies, regular supplement intake, alcohol abuse, and/or drug addiction. A clinical checkup included total blood count, analysis of lipid and glucose metabolism, and

liver and kidney function to exclude serious hidden diseases. After completion of the medical history and physical examination, a total of 76 healthy volunteers were recruited and randomly assigned to three study groups.

After a 1 week washout phase without glucosinolate intake, subjects were advised to consume a daily dose of regular blanched broccoli (n = 25; 200 g/day), selenium-fertilized blanched broccoli (n = 25; 200 g/day), or placebo (n = 26) for 4 weeks. Regular blanched broccoli served as a broccoli matrix control. White soft gel "placebo" capsules containing starch served as a broccoli independent time control. During the entire study, volunteers were advised to refrain from food and food products containing reasonable amounts of glucosinolates. A list with appropriate products was provided (e.g., various cabbage species, collard, kale, radish, horseradish, mustard, etc.). Once a week, the participants were provided with the respective broccoli or placebo and were advised to prepare the blanched broccoli by standard cooking along with the main dish. Compliance was achieved by regular phone calls and by reminding volunteers during their weekly visits at the study center to adhere to the study conditions. Blood and spot urine samples were collected in the morning of day 1 before intervention and in the morning of day 28, the day after the last broccoli meal had been taken. On both days we collected first morning urine.

Fasting blood samples were taken in supine position into serum and ethylenediaminetetraacetic acid (EDTA) tubes (Monovette, Sarstedt, Nümbrecht, Germany). Serum samples were allowed to clot for at least 30 min, whereas EDTA samples were immediately placed on ice before centrifugation at 1800g and 4 °C for 15 min. Urine samples designated for glucoraphanin metabolite analysis were stabilized with ascorbic acid. Plasma and urine samples were stored at −80 °C until analysis. Standard laboratory measurements of clinical chemistry (data not shown) and serum selenium (atomic absorption spectroscopy) were performed by a local accredited commercial laboratory (Medizinisches Versorgungszentrum Labor Prof. Seelig, Karlsruhe, Germany).

Preparation of Plasma and Urine Samples for LC-MS/MS Analysis. *Glucoraphanin Metabolites.* Plasma (500 μL) and urine samples (25 μL for the major metabolites SFN−NAC and SFN; 400 μL for the minor metabolites SFN−GSH, SFN−Cys-Gly, and SFN−Cys) for the analysis of glucoraphanin metabolites were prepared by spiking the samples with d_8-SFN (0.00156 μg for plasma; 0.00039−0.013 μg for urine) and d_8-SFN-NAC (0.00039 μg for plasma; 0.00078−0.075 μg for urine) as internal standards and homogenization for 1 min. Proteins were then precipitated using 100 μL of precooled (4 °C) trifluoroacetic acid. After homogenization for 1 min, samples were centrifuged at 13400g and 4 °C for 6 min, and the supernatant was subsequently loaded onto Discovery C_{18} solid phase extraction cartridges (100 mg, 1 mL; Supelco, Taufkirchen, Germany), which had been conditioned with methanol (4 mL) and equilibrated with formic acid (0.1% in water; 4 mL). After a washing step with methanol/water (5:95; v/v; 2 mL), the metabolites were eluted with ACN/water (90:10; v/v; 4 mL), evaporated to dryness under a nitrogen stream, and redissolved in 100 μL (plasma) or 200 μL (urine) of formic acid (0.1% in water). Ten microliters of each sample was injected into the HPLC-ESI(+)-MS/MS system. A mobile phase gradient starting from 100% aqueous formic acid (0.1% formic acid in water), decreasing to flux 100% of the 0.1% formic acid in acetonitrile phase within 15 min, and remaining at the organic phase for 5 min was used.

Glucobrassicin Metabolites. Isolation of glucobrassicin metabolites from plasma and urine was performed following a modified liquid−liquid extraction procedure reported by Anderton et al., who analyzed samples by means of HPLC using 4-methoxyindole as internal standard.[10] However, we decided to perform our quantification by means of LC-MS/MS. After addition of the internal standards 4-methoxyindole (4-Met; 0.005−0.02 μg abs) and $^{13}C_6$-ASG (0.00125 μg abs) to 900 μL of plasma and homogenization for 1 min, proteins were precipitated using 3 mL of ice-cold ACN, followed by homogenization for 1 min and centrifugation at 1700g and 4 °C for 6 min. The resulting supernatant was extracted twice with 4 mL of *tert*-butyl methyl ether for 2 min each time. After both extraction steps, phases were separated by centrifugation at 1700g and 4 °C for 6 min, respectively. The organic phases were combined, dehydrated with sodium sulfate, evaporated to dryness under a nitrogen stream, and redissolved in 200 μL of water/ACN (1:1; v/v) prior to injection of 10 μL into an HPLC-ESI(+) tandem mass spectrometric system. Isolation of glucobrassicin metabolites from urine was performed similarly, using 200 μL of urine (0.000625−0.005 μg of $^{13}C_6$-ASG and 0.005−0.06 μg of 4-Met) but skipping the protein precipitation and the dehydration step with sodium sulfate. Besides, extraction with *tert*-butyl methyl ether was performed twice and, after evaporation of the collected organic phases to dryness, the samples were redissolved in 100 μL of water/ACN (1:1; v/v). A mobile phase gradient starting from 100% aqueous formic acid (0.1% formic acid in water) for 5 min, decreasing to flux 100% of the 0.1% formic acid in ACN within 30 min, and remaining at the organic phase for 5 min was used.

Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS). HPLC-MS/MS analyses were carried out on a Finnigan Surveyor Plus HPLC system (Thermo Electron Corp.), equipped with a triple-quadrupole mass spectrometer (TSQ Quantum Discovery; Thermo Electron Corp.). For glucoraphanin metabolites, electrospray ionization in positive mode and selected reaction monitoring (SRM) with a spray voltage of 3.5 kV, a sheath gas pressure of 35 arb, an auxiliary gas pressure of 15 arb, and a capillary temperature of 320 °C were selected. For glucobrassicin metabolites, electrospray ionization in positive mode and SRM with a spray voltage of 4.06 kV, a sheath gas pressure of 50 arb, an auxiliary gas pressure of 20 arb, and a capillary temperature of 280 °C were used. Capillary offset was 35 V, whereas scan time and scan width were 0.2 s and 0.7 amu, respectively, for glucoraphanin and glucobrassicin metabolites. All analyses were performed on an Aqua C_{18} column (5 μm, 12.5 nm, 150 × 2 mm) as stationary phase equipped with a guard column (C_{18}, 4.0 × 2.0 mm; both from Phenomenex). The mobile phase consisted of 0.1% formic acid in water (A) and 0.1% formic acid in ACN (B). It was operated with a flow rate of 0.2 mL/min, and the injection volume was 10 μL. For SFN and its mercapturic acid pathway conjugates, the gradient started with 100% A, arrived to 0% A within 15 min, and was kept at 0% A until 20 min. The UV detector wavelength was set at λ = 210 nm. For glucobrassicin metabolites, the gradient started with 100% A, at which it was kept for 5 min before decreasing to 0% A within 30 min, at which it was kept until 35 min. The effluent was monitored at λ = 280 nm. Quantification of all metabolites in human plasma and urine samples was performed by means of SIDA in the cases of SFN, SFN−NAC, and ASG or by the internal standard method for SFN−GSH, SFN−Cys-Gly, SFN−Cys, I3-CAL, and I3-CA. Commercially available 4-methoxyindole was used as internal standard for quantification of I3-CA and I3-CAL, whereas in the cases of SFN−GSH, SFN−Cys-Gly, and SFN−Cys we used d_8-SFN-NAC as internal standard. The fragment ion with highest intensity was used for quantification, whereas the second ion only served to confirm the precursor ion. Ideal collision energies for every single compound were determined by direct injection (flow injection analysis) of 10−50 μg/mL of the synthesized and purified standards into the ion source of the mass spectrometer.

Statistical Analysis. Unless otherwise stated, data are expressed as the mean value of 25 (regular and Se-fertilized blanched broccoli) or 26 (placebo) volunteer sample analyses ± the standard error of the mean (SEM). Differences among groups were calculated by analysis of variance (one-way ANOVA) using SigmaStat Software Inc. (version 3.5, Point Richmond, CA). When a significant effect (p < 0.05) was found, Dunn's method post hoc test was used to determine differences between means.

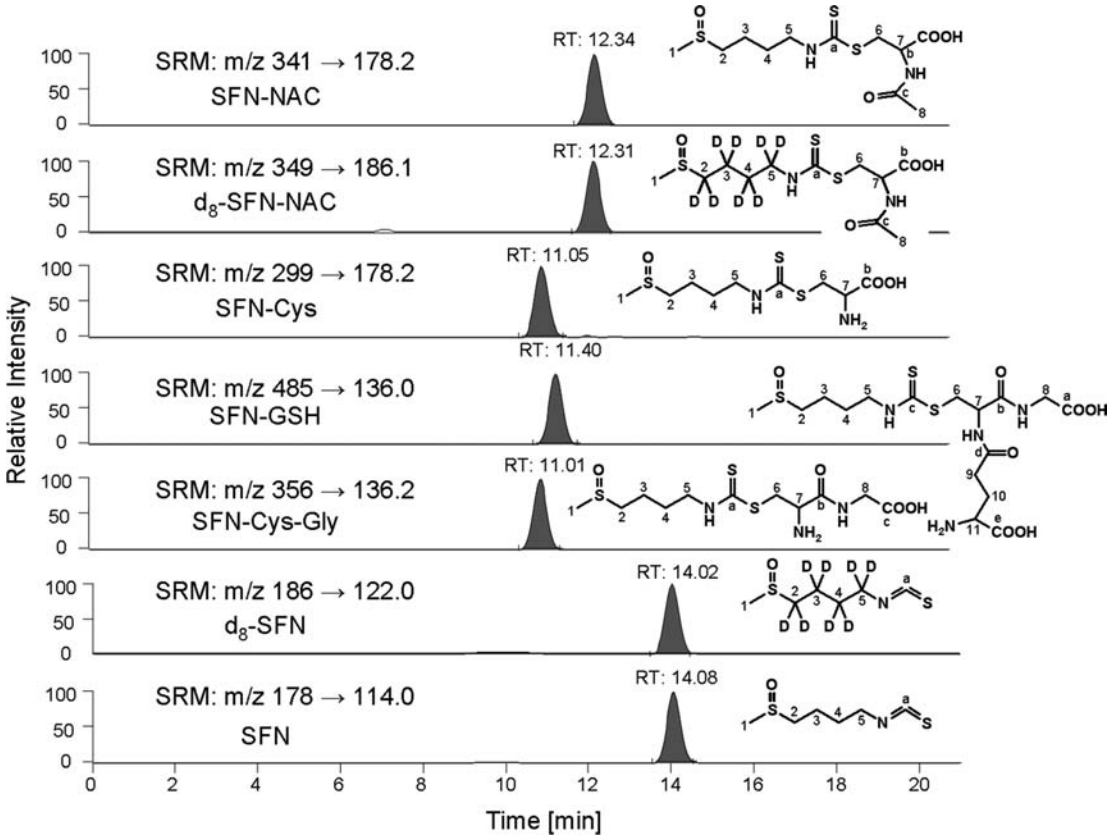

Figure 1. RP-HPLC-ESI(+)-MS/MS chromatograms and structures of glucoraphanin pathway metabolites quantified and their octadeuterated internal standards.

RESULTS AND DISCUSSION

We aimed at developing a RP-HPLC method, coupled to ESI(+) tandem mass spectrometry, to allow for the sensitive, specific, and precise quantification of low amounts of glucosinolate metabolites in plasma and urine after habitual dietary intake of regular and selenium-fertilized broccoli using SIDA. Figures 1 and 2 show SRM chromatograms for the glucoraphanin and glucobrassicin metabolites investigated and their internal standards, indicating the mass transition from precursor ion to product ions used for quantification. Further elucidation of the chemical structures was performed by NMR experiments. Figure 3 shows exemplary DQF-COSY spectra for SFN−NAC (Figure 3A) and d_8-SFN-NAC (Figure 3B). The respective arbitrary numbering of the NMR signals refers to the numbering given in Figures 1 and 2.

Intraday precision values for all compounds quantified were below 10% for most of the compounds (Table 1). Noteworthy for SFN, SFN−NAC, and ASG, the relative standard deviation values were much lower compared to the other metabolites, which emphasizes the advantage of SIDA over the use of structurally different and unlabeled internal standards.

Limits of detection (LOD) and limits of quantification (LOQ) are presented in Table 1. These results are comparable to those from another recently published study by Egner et al., who demonstrated LOD values ranging from 1 to 6 pg in urine samples,[16] whereas we found values between 0.7 and 10.9 pg in urine and plasma. In comparison to the cyclocondensation method that is widely used for the quantification of total glucoraphanin metabolites and reported to reach LOD values of 100 nmol/L,[13] the sensitivity of the here presented methods was by a factor of about 30−500 higher.

Analytical recoveries for glucoraphanin metabolites from plasma/urine were 99/103% (free SFN), 75/89% (SFN−GSH), 102/91% (SFN−Cys-Gly), 75/73% (SFN−Cys), and 96/98% (SFN−NAC), whereas those for glucobrassicin metabolites were 95/98% (ASG), 91/94% (DIM), 64/67% (I3-C), 81/89% (I3-CA), 102/104% (I3-CAL), and 99/91% (I3-ACN).

After the analytical procedures for quantification of the glucobrassicin and glucoraphanin metabolites were established and validated, plasma and urine samples of healthy volunteers who participated in a 4 week intervention study with regular or Se-fertilized broccoli were analyzed. All subjects complied with the study protocol, and none of them reported any illness or discomfort during the study. Clinical routine parameters before and after the study (total blood count, lipid and glucose metabolism, liver and kidney function) were within the physiological range and not affected by the dietary intervention regime (data not shown). The study participants did not differ in age, BMI, standard parameters of clinical chemistry (Table 2), and initial serum Se concentrations (mean serum selenium concentrations of 87.7 ± 13 and $90.2 \pm 10\ \mu g/L$ within the study groups who were administered regular and selenium-fertilized broccoli, respectively).

Selenium contents of the Se-fertilized broccoli administered were about 25 times higher than those of the regular broccoli (Table 3). Furthermore, the total glucosinolate content of selenium-fertilized broccoli was about 20% higher compared to the nonfertilized product, which is in agreement with results obtained

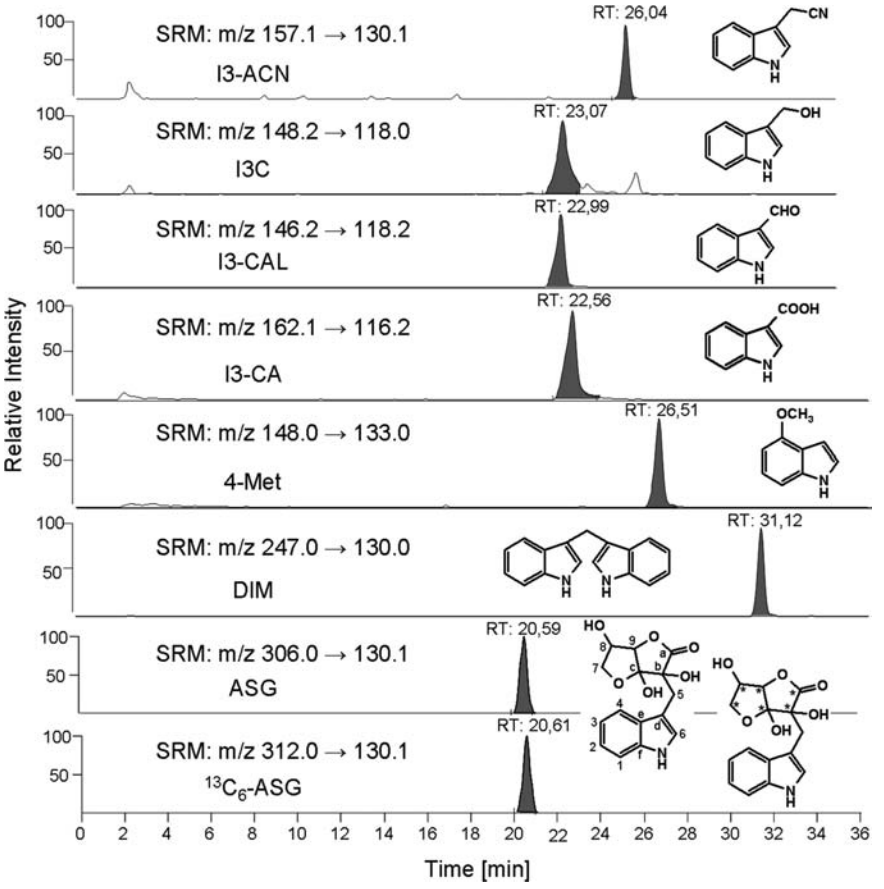

Figure 2. RP-HPLC-ESI(+)-MS/MS chromatograms and structures of glucobrassicin metabolites quantified and their internal standards.

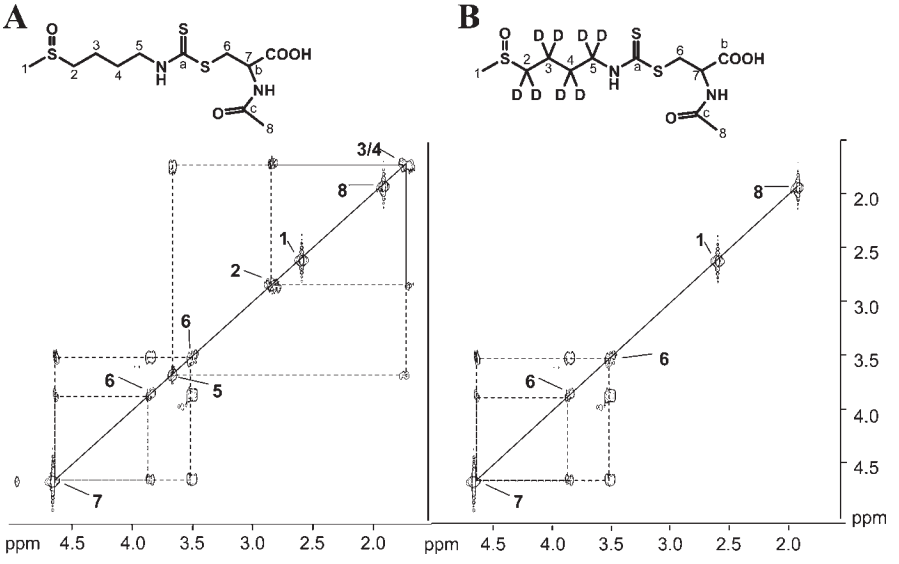

Figure 3. DQF-COSY spectra of isolated SFN−NAC (A) and d_8-SFN−NAC (B) standards.

previously.[20] However, the cellular mechanisms by which Se fertilization increases total glucosinolates need to be clarified in future studies. We conclude that moderate selenium fertilization of broccoli (0.25 μg/g fresh weight) does not negatively affect glucosinolate formation.

Dietary intake of the Se-fertilized broccoli for 4 weeks increased serum Se concentration by up to 25% ($p < 0.001$) compared to regular broccoli intake, resulting in a mean serum level of 101 ± 12 μg/L. This is in line with results from human intervention studies using Se-biofertilized foods. When participants consumed a Se-biofertilized biscuit (containing approximately 89 μg of Se) in increasing amounts over 24 weeks (one biscuit per day for the first 8 weeks and up to three biscuits per

Table 1. Limits of Detection (LOD) and Quantification (LOQ) of Glucoraphanin and Glucobrassicin Metabolites Analyzed in Human Plasma and Urine as well as Intraday and Interday Precisions of Glucosinolate Metabolites[a]

	LOQ/LOD (pg)		intraday precision[b] (%)		interday precision[b] (%)	
metabolite/MW	plasma	urine	plasma	urine	plasma	urine
SFN/177	3.7/1.2	16/5.3	4.2	5.7	3.6	4.7
SFN−GSH/484	1.9/0.5	25/8.2	9.5	8.2	10.8	7.1
SFN−Cys-Gly/355	4.3/1.4	2.1/0.7	5.1	4.8	6.4	9.5
SFN−Cys/298	1.2/0.3	30/10	8.4	7.9	15.9	14.9
SFN−NAC/340	4.1/1.4	33/11	3.5	2.0	3.6	2.0
I3-C/147	13/4.4	26/8.8	nd[c]	nd	nd	nd
I3-CAL/145	6.7/2.2	17/5.8	2.8	9.7	15.7	19.6
I3-CA/161	4.8/1.6	21/6.9	6.7	10.1	13.8	13.1
I3-ACN/156	4.7/1.6	23/7.8	nd	nd	nd	nd
DIM/246	7.4/2.5	37/12	nd	nd	nd	nd
ASG/305	0.6/0.3	4.3/1.5	3.4	0.6	3.2	0.7

[a] Analyses were performed by HPLC injections of 10 μL of samples as described under Materials and Methods. [b] Expressed as relative standard deviation of replicate analyses (%). [c] nd, not detectable.

Table 2. Baseline Characteristics of Study Participants before Intervention[a]

	Se−	Se+	placebo
n	25	25	26
age (years)	62.6 ± 7.4	63.6 ± 6.4	61.8 ± 7.9
BMI (kg/m^2)	27.4 ± 4.2	27.8 ± 3.5	26.8 ± 2.8
glucose (mg/dL)	96.4 ± 15	103 ± 23	96.0 ± 18
triacylglycerol (mg/dL)	105 ± 86	137 ± 111	131 ± 74
cholesterol (mg/dL)	218 ± 42	236 ± 46	228 ± 39

[a] Groups: Se−, regular broccoli; Se+, selenium-fertilized broccoli. Data are given as the mean ± SD.

Table 3. Selenium and Glucosinolate Contents of Blanched Regular (Se−) and Selenium-Fertilized (Se+) Broccoli

	μg/g fresh weight	
	regular (Se−)	fertilized (Se+)
selenium	<0.01	0.25
glucoiberin	29.1	45.7
glucoraphanin	133	226
4-hydroxyglucobrassicin	22.9	24.8
glucobrassicin	111	107
4-methoxyglucobrassicin	35.4	32.0
neo-glucobrassicin	153	154
total glucosinolates	485	589

day for the last 8 weeks), a rise in plasma Se concentrations from 122 to 192 μg/L was demonstrated.[26] When subjects were supplemented with 480 μg of Se/day using Se-biofertilized milk for 1 week, serum Se increased from 107 to 154 μg/L.[27]

Besides an increase of serum Se concentrations, we detected a rise of all glucoraphanin metabolites synthesized in plasma samples drawn from volunteers after consumption of regular and Se-fertilized blanched broccoli (Table 4). Overall, mean plasma concentrations of glucoraphanin metabolites were very low after administration of the regular blanched broccoli (26.7 mg of glucoraphanin/day) compared to results from Hanlon et al., who detected about 69 nmol/L (SFN) after administration of liquidized broccoli containing 3.9 mg of SFN/day.[28] Maximum values presented in our study here were 4.6 and 4.8 nmol/L for SFN and SFN−NAC, respectively. A reason for these comparatively low plasma concentrations of glucoraphanin metabolites could be the long time that had passed between the last broccoli administration on day 27 and the blood and urine collection on the morning of day 28 of the intervention. This hypothesis is supported by results presented by Gasper et al.,[29] who demonstrated peak concentrations of glucoraphanin metabolites about 1.5 h postdose, with baseline values reached about 24 h after broccoli consumption. Another reason for the low plasma concentrations of glucoraphanin metabolites presented here might have been low contents of glucosinolate in the blanched broccoli. In general, we do not assume a significant impact of the blanching process applied in our study (94 °C for 120 s) on the glucosinolate concentrations in blanched broccoli (485 μg/g fresh weight (FW); Table 3) because the results are comparable to those analyzed in raw broccoli.[20] However, we hypothesize at least a partial inactivation of the myrosinase due to the blanching process[30,31] and some leaching of glucosinolates into the cooking water during household preparation because subjects were advised to prepare the blanched broccoli by standard cooking with the main dish.

Administration of Se-fertilized broccoli resulted in slightly lower mean plasma concentrations for all mercapturic acid pathway metabolites compared to that of regular, non-Se-fertilized broccoli, although the level of statistical significance was not reached for any of the metabolites (Table 4).

Notably, plasma SFN−Cys significantly increased after intervention in the placebo group. We may also speculate that SFN−Cys is a rather stable plasma glucosinolate metabolite compared to others because it was detectable before the intervention in all three groups. The washout period of 1 week might have been too short and could have contributed to the detection of SFN−Cys in preinterventional volunteers. However, this numerical increase may be of minor importance because its concentration is rather low and does not seem to be related to the

Table 4. Concentrations of Glucoraphanin Metabolites in Human Plasma and Urine before and after Dietary Administration of Placebo, Blanched Broccoli (Se−), or Blanched Selenium-Fertilized Broccoli (Se+)[a]

		plasma (nmol/L)		urine (nmol/mmol creatinine)	
intervention	metabolite	pre-intervention	post-intervention	pre-intervention	post-intervention
placebo	SFN	<LOD*	<LOD*	1.49 ± 0.95a	0.05 ± 0.03b
	SFN−GSH	<LOD*	<LOD*	<LOD*	<LOD*
	SFN−Cys-Gly	<LOD*	<LOD*	0.91 ± 0.12a	0.58 ± 0.12b
	SFN−Cys	0.02 ± 0.02a	0.23 ± 0.08b	0.25 ± 0.11	0.20 ± 0.12
	SFN−NAC	<LOD*	<LOD*	6.12 ± 2.81a	0.79 ± 0.41b
broccoli (Se−)	SFN	<LOD*a	4.63 ± 1.71b#	0.37 ± 0.12a	66.0 ± 18.9b#
	SFN−GSH	<LOD*	0.08 ± 0.06	<LOD*	<LOD*
	SFN−Cys-Gly	<LOD*a	1.86 ± 0.67b#	0.85 ± 0.08a	1.48 ± 0.23b#
	SFN−Cys	0.04 ± 0.03a	0.47 ± 0.12b	0.32 ± 0.19a	23.1 ± 6.23b#
	SFN−NAC	<LOD*a	4.82 ± 0.74b#	5.19 ± 2.74a	370 ± 75.2b#
broccoli (Se+)	SFN	<LOD*a	4.00 ± 1.30b#	0.18 ± 0.11a	35.5 ± 11.7b#
	SFN−GSH	<LOD*	0.02 ± 0.02	<LOD*	<LOD*
	SFN−Cys-Gly	<LOD*a	1.36 ± 0.59b#	1.09 ± 0.13	1.14 ± 0.16#
	SFN−Cys	0.16 ± 0.05	0.36 ± 0.10	0.09 ± 0.06a	20.4 ± 7.00b#
	SFN−NAC	<LOD*a	3.62 ± 0.90b#	2.05 ± 0.51a	305 ± 77.4b#

[a] Data are presented as the mean value (n = 25−26) ± SEM. Different letters indicate statistically significant differences ($p < 0.05$) within the experimental groups pre- and post-intervention; # indicates statistically significant differences versus placebo; no statistical differences were obtained for mean metabolite values among the broccoli intervention groups (Se+ vs Se−); * for LOD, see Table 1.

broccoli consumption in our intervention. These speculations are supported by our results obtained from the urine samples analyzed. Although we were able to detect glucosinolate metabolites in all groups before intervention, all glucosinolate metabolites except SFN−Cys decreased in the placebo group during the intervention period, but increased after broccoli consumption.

Glucoraphanin metabolite concentrations in urine samples were also very low, reaching, for example, a mean value of 370 nmol/mmol creatinine for the quantitatively dominating metabolite SFN−NAC after administration of regular blanched broccoli (Table 4). Egner et al.[16] quantified about 4751 nmol SFN−NAC/mmol creatinine after administration of a hot water infusion prepared from broccoli sprouts (400 μmol of glucoraphanin) to healthy volunteers.

Urine samples from the human intervention trial presented here tended to contain ($p > 0.05$) higher mean concentrations for all quantified metabolites after administration of the regular broccoli compared to the respective samples from volunteers who had eaten the Se-fertilized broccoli, which is in agreement with the results obtained from the plasma analysis. However, the mean x-fold increase of SFN, SFN−Cys, and SFN−NAC after intervention with selenium-fertilized (Se+) broccoli is higher than that after intervention with regular (Se−) broccoli (Figure 4A), although glucoraphanin metabolite concentrations in plasma and urine were not significantly different. Considering the higher concentration of glucoraphanin in the Se-fertilized broccoli, which was 226 μg/g FW compared to 133 μg/g FW in the regular broccoli (Table 3), we conclude that a dietary intake of Se-fertilized broccoli of up to 0.25 μg Se/g FW does not negatively affect glucoraphanin metabolites formed in vivo. Previous findings show a reduction of SFN contents in the plant from 239 to 41 μmol/L due to Se treatment from 0 to 10000 ppm.[19] Charron et al. reported a 90% reduction of SFN from 0.4 to 0.04 μmol/g after treatment of *Brassica oleracea* with 1 mg/L Na_2SeO_4, yielding a Se accumulation of 270.7 μg/g in shoots.[32] In our study, the selenium enrichment of broccoli was achieved by spraying 2 mg of selenate on one broccoli sprout, yielding 0.25 μg Se/g FW. The protocol for Se fortification applied in our study does not negatively affect glucosinolate concentrations[20] and does not, in turn, decrease glucoraphanin metabolites formed in vivo after dietary intake of the Se-fertilized broccoli.

Glucobrassicin metabolites identified in the plasma samples of our study comprised I3-CAL, I3-CA, and ASG, with I3-CAL being the quantitatively dominating metabolite (Table 5). Neither free I3-C nor I3-ACN or DIM could be detected above the LOD (3 nmol/L for I3-C; 1 nmol/L for I3-ACN and DIM) in any of the samples analyzed.

In vitro studies with synthetic glucobrassicin proved I3-ACN to be the only product at pH 3, whereas at pH 7, which is the physiological pH of broccoli plant tissue, only DIM (>98%) and free I3-C (<2%) were detected.[33] These findings might explain the absence of I3-ACN in our study after the dietary intervention. For the concentrations of I3-C below the LOD in our study, we hypothesize the instability of this metabolite under acidic conditions as they occur in the human stomach. This assumption is in agreement with results reported by Reed et al.,[18] who conducted a human intervention trial in which bolus doses of 400−1200 mg of I3-C were administered. Herein, no free I3-C, but DIM, was quantified as I3-C metabolite, which is not consistent with our results as we could not detect DIM in any of the samples. This discrepancy can be explained by the high doses given by Reed et al.,[24] of which the highest exceeded the dose in our study by a factor of about 18.

Next to the lower glucobrassicin doses in our study, matrix effects might also have contributed to the different metabolite spectrum. Whereas Reed at al. administered free I3-C,[18] the

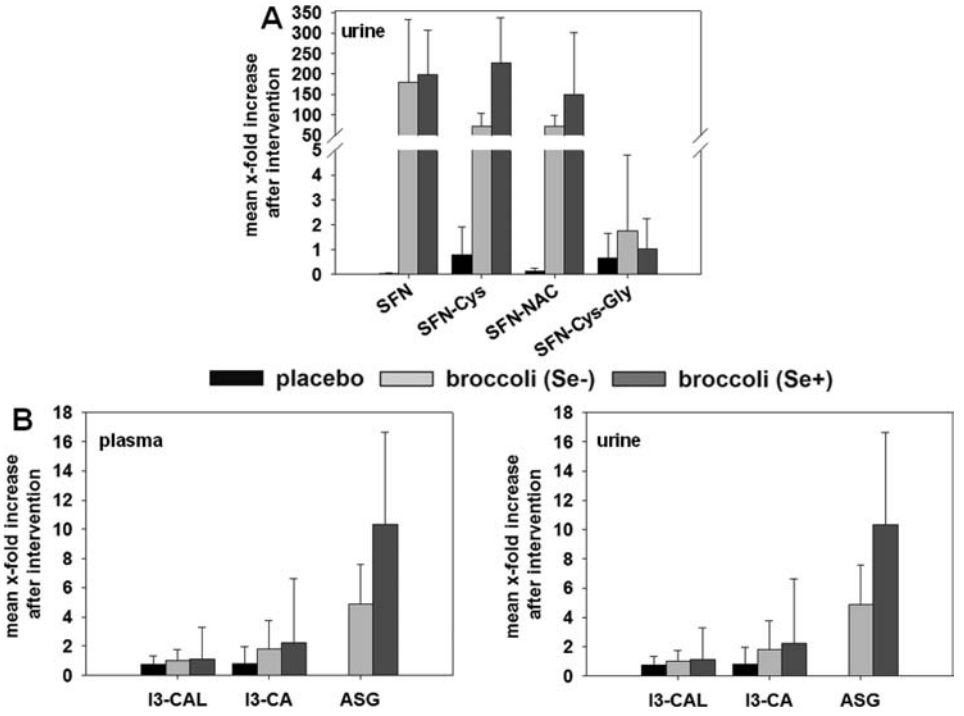

Figure 4. Mean *x*-fold increase of glucoraphanin (A) and glucobrassicin (B) metabolites after the respective intervention with placebo, blanched (Se−) broccoli or blanched selenium-fertilized (Se+) broccoli in human urine and plasma. Data are presented as *x*-fold increase of mean values (n = 25−26). For statistical analysis, see Tables 4 and 5.

Table 5. Concentrations of Glucobrassicin Metabolites in Human Plasma and Urine before and after Dietary Administration of Placebo, Blanched Broccoli (Se−), or Blanched Selenium-Fertilized Broccoli (Se+)[a]

		plasma (nmol/L)		urine (nmol/mmol creatinine)	
intervention	metabolite	pre-intervention	post-intervention	pre-intervention	post-intervention
placebo	I3-CAL	106 ± 8.91a	77.5 ± 5.58b	123 ± 23.0	196 ± 51.5
	I3-CA	37.6 ± 3.18	30.2 ± 3.65	86.5 ± 26.9	198 ± 82.2
	ASG	0.01 ± 0.01	<LOD*	0.13 ± 0.07a	0.59 ± 0.26b
broccoli (Se−)	I3-CAL	120 ± 12.2	125 ± 8.94#	101 ± 13.01a	165 ± 23.9b
	I3-CA	40.1 ± 4.30a	73.4 ± 8.37b#	140 ± 46.1	199 ± 47.9
	ASG	0.03 ± 0.02a	0.15 ± 0.06b#	0.22 ± 0.05a	1.71 ± 0.29b#
broccoli (Se+)	I3-CAL	106 ± 7.61	120 ± 16.6#	118 ± 18.5a	228 ± 40.8b
	I3-CA	35.6 ± 3.21a	78.5 ± 14.2b#	73.3 ± 16.0a	218 ± 39.9b
	ASG	0.02 ± 0.01a	0.16 ± 0.07b#	0.16 ± 0.04a	1.56 ± 0.23b#

[a] Data are presented as the mean value (n = 25−26) ± SEM. Different letters indicate statistically significant differences (p < 0.05) within the experimental groups pre- and post-intervention; # indicates statistically significant differences versus placebo; no statistical differences were obtained for mean metabolite values among the broccoli intervention groups (Se+ vs Se−); * for LOD, see Table 1.

broccoli of our study contained free L-ascorbic acid (840 mg L-ascorbic acid/kg broccoli), which might have favored the formation of ASG over that of I3-C.[12]

Another reason why we could not detect I3-C might be the characteristic pharmacokinetics of this compound. Reed at al. showed that peak plasma concentrations of I3-C were reached 3.0 ± 1.2 and 2.0 ± 0 h postdose after administration of 400 or 1000 mg of I3-C to human volunteers, respectively.[18] Therefore, we assume the I3-C concentrations below the LOD found in our study are at least in part due to the fact that plasma and urine clearance might have occurred prior to sample collection. This hypothesis is also supported by findings from Anderton et al.,[10] showing that I3-C oxidation in vivo results in the formation of I3-CA and I3-CAL, two metabolites that were also quantified in our study. The observation that I3-CAL, I3-CA, and ASG in plasma decreased in the placebo group after intervention but increased after broccoli consumption might be explained by our study conditions, because we advised all volunteers to refrain from glucosinolate-containing foods.

In urine samples (Table 5), the only metabolites quantified were I3-CAL, I3-CA, and ASG, whereas I3-C, I3-ACN, and DIM

could not be detected above the LODs of 8.8 pg (I3-C), 7.8 pg (I3-ACN), and 12 pg (DIM). Like for the results presented for the glucoraphanin metabolites, mean values of these three glucobrassicin metabolites did not differ significantly among the intervention groups, although the mean *x*-fold increase of all detected glucobrassicin metabolites in plasma and urine was higher after the intervention with blanched selenium-fertilized broccoli (Se+) compared to the intervention with placebo and blanched broccoli (Se−) (Figure 4B). We explain these findings with comparable contents of glucobrassicin in the administered broccoli preparations, ranging from 107 to 111 μg/g FW for Se-fertilized and regular, non Se-fortified broccoli, respectively (Table 3). The result that we detected for I3-CAL and I3-CA in both plasma and urine samples of volunteers of the placebo group led us to the assumption of a possible ubiquity of those metabolites in vivo, which is not associated with broccoli consumption. The presence of I3-CA in the urine of healthy volunteers as well as its occurrence as an intermediate in the tryptophan metabolism has been demonstrated previously.[34] Also, I3-CAL has been identified in the urine of phenylketonuria patients and in urine from rats to which D-[2-^{14}C]-tryptophan was administered by intraperitoneal injection as well as in adrenal tumor tissue and human pancreas and spleen.[34−36] These findings support our hypothesis that the presence of the glucobrassicin metabolites I3-CA and I3-CAL in the plasma and urine is not associated with only the dietary intake of cruciferous vegetables. However, our results indicate that ASG and the glucoraphanin metabolites reliably serve as short-term markers for the intake of cruciferous vegetables. We do not hypothesize their use as long-term markers because our results and also data from Reed et al.[18] do not point to a possible bioaccumulation of these metabolites in vivo.

In summary, a very sensitive LC-MS/MS method for the detection of glucosinolate metabolites has been established. Applying the here developed method, it could be shown for the first time that even low concentrations of glucosinolate metabolites were quantified in plasma and urine after dietary intake of regular and selenium-fertilized broccoli in a meal representative amount of 200 g. In addition, neither glucosinolate concentrations in Se-fertilized broccoli nor glucosinolate metabolite concentrations in plasma and urine after dietary intake of the Se-fertilized broccoli were negatively affected by moderate selenium fertilization, whereas serum selenium was markedly increased.

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ABBREVIATIONS USED

ACN, acetonitrile; ASG, ascorbigen; EDTA, ethylenediaminetetraacetic acid; FW, fresh weight; DIM, 3,3′-diindolylmethane; d_8-SFN, octadeuterosulforaphane; d_8-SFN-NAC, octadeuterosulforaphane-*N*-acetylcysteine; 4-Met, 4-methoxyindole; I3-ACN, indole-3-acetonitrile; I3-ACN, indole-3-acetonitrile; I3-C, indole-3-carbinol; I3-CA, indole-3-carboxylic acid; I3-CAL, indole-3-carboxaldehyde; LOD, limit of detection; LOQ, limit of quantification; Se, selenium; SFN, sulforaphane; SFN−GSH, sulforaphane−glutathione conjugate; SFN−Cys-Gly, sulforaphane−cysteinylglycine conjugate; SFN−Cys, sulforaphane−cysteine conjugate; SFN−NAC, sulforaphane-*N*-acetylcysteine; SIDA, stable isotope dilution assay; SRM, selected reaction monitoring.

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