A Urinary Biomarker for Uptake of Dietary Isothiocyanates in Humans

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### Abstract
Isothiocyanates (ITCs) are a family of biologically active compounds that are distributed widely in cruciferous vegetables. Although studies in rodents have shown that these compounds are effective and versatile inhibitors of tumorigenesis, the role of dietary ITCs in the protection against human cancers remains to be established. A prerequisite of human studies is to develop an uptake biomarker for dietary ITCs. In this study, we describe a rapid high-performance liquid chromatography-based assay to measure the total ITC level in human urine. This assay is based on a previously described reaction of ITCs or their thiol conjugates with 1,2-benzenedithiol to yield a cyclocondensation product, 1,3-benzodithiole-2-thione, which then can be quantified by reverse phase high-performance liquid chromatography with UV detection. This new assay was validated by analyzing urine samples from 14 subjects who had consumed a known amount of watercress or brown mustard in a controlled experiment. The N-acetylcysteine conjugates of phenethyl ITC and allyl ITC from watercress and brown mustard, respectively, were quantified and compared with the results obtained from the current assay. Results of the two methods were highly correlated ($r = 0.978$), indicating the specificity of this new assay for dietary ITCs. The feasibility of this assay for population-based studies was examined using stored urine samples collected from nine participants of a prospective cohort study in Shanghai, China, who indicated that they were daily consumers of dark green vegetables. There was a 10-fold variation in urinary ITC contents among these samples, ranging from 0.7 to 7.0 μmol/g creatinine. These results show the potential use of this uptake biomarker in epidemiological studies to identify the role of dietary ITCs in modifying cancer risks in humans.

### Introduction
Epidemiological studies have shown that diets rich in vegetables and fruits are associated with low cancer risk (1, 2). Although there are a number of substances in vegetables and fruits that may contribute to the protective effects, the exact nature of these compounds or components and the mechanisms by which they exert the effects remain, to a large degree, to be investigated. A better understanding of the identities of these compounds and their biological activities will have important implications toward the long-term goal of reducing the incidence of human cancers.

Studies from this and other laboratories have shown that ITCs, a major family of nonnutrient compounds in cruciferous vegetables, are effective and versatile inhibitors against tumorigenesis in various animal models. For example, benzyl ITC is effective against lung and forestomach tumorigenesis in mice treated with polycyclic aromatic hydrocarbons and liver tumorigenesis induced by N-nitrosodiethylyamine in rats (3–5). It also inhibits methylazoxymethane-induced colon tumorigenesis in rats (6). Phenethyl ITC inhibits nitrosamine-induced tumorigenesis in a variety of tissues including mouse lung and rat lung and esophagus (7, 8). Besides natural ITCs, some of the synthetic ITCs also show remarkable efficacy in the inhibition of tumorigenesis (9, 10).

### Results of the assay
In vitro and in vivo studies indicate that inhibition of cytochrome P-450s and induction of phase II enzymes are the likely mechanisms of ITCs in tumor inhibition (11–13). Structure-activity relationship studies have shown that the -N==C=='S functional group in ITCs is critical for such tumor inhibitory activities (14). It is highly likely that other natural ITCs, not yet identified or studied for their biological activity, could also exert inhibitory effects against tumorigenesis by these and other related mechanisms.

The results of animal studies suggest that dietary ITCs may play a role in protecting humans against cancer development. A prerequisite to studying the potential of dietary ITCs in modifying human cancer risks is to develop markers of their uptake. We and others have shown previously that dietary ITCs are metabolized initially by conjugation with glutathione in the mercapturic acid pathway, resulting in the excretion of the mercapturic acids or dithiocarbamates as major urinary metabolites in rodents and humans (15–17). We have developed assays to quantify urinary metabolites of phenethyl ITC or allyl ITC in individuals who have consumed meals containing watercress or brown mustard, respectively (18, 19). These studies demonstrated intake-dependent excretion of the ITC conjugates in urine. However, the utility of these biomarkers in epidemiological studies is limited, because they are applicable only to selected populations whose diets are rich in these ITCs. A useful biomarker of more general applications in human studies would be one that reflects total ITC intake.

Zhang et al. (20) described a spectroscopic assay using a
cyclocondensation product, 1,3-benzodithiole-2-thione, derived from reaction of ITC and 1,2-benzenedithiol, to quantify the total amount of ITCs in vegetables. Based on this reaction, we developed a convenient HPLC-based assay to quantify total ITC in human urine. Cyclic condensation reaction occurs with ITC conjugates due to the reversible reaction of dithiocarbamates to release ITCs (21–23). In this study, we validated this biomarker with urine samples collected from subjects who had consumed a controlled diet containing watercress or brown mustard. We also evaluated the potential application of this biomarker in population-based studies with a small set of urine samples collected from participants of a cohort study in Shanghai, China.

Materials and Methods

Instruments. An HPLC system consisting of two Shimadzu LC-10AS pumps, a SPD-10AV UV-VIS detector, a SIL-10A auto injector, and a SCL-10A system controller (Shimadzu Scientific Instruments, Inc., Columbia, MD) was used in batch analysis. An Eppendorf CH-30 column heater and a TC-50 temperature controller (Eppendorf Scientific, Inc., Madison, WI) were used to keep the column temperature constant for overnight batch analyses. An HPLC system (Waters Associates, Milford, MA) equipped with an automatic gradient controller, two model 501 pumps, and a Waters 990 photodiode array detector was used in the initial study and to obtain the UV spectra of the cyclocondensation product. A water bath shaker Julabo UC-8A (Julabo USA, Kutztown, PA) was used in all incubations. Centrifugation was done by using a Savant Speed-Vac SVC-100H (Savant Instruments, Inc., Holbrook, NY). Mass spectra were obtained from a Hewlett-Packard 5988A mass spectrometer (Hewlett-Packard Co., Wilmington, DE). NMR spectra were recorded on a Bruker AM 360 MHz NMR spectrometer (Bruker Instruments, Inc., Billerica, MA).

Chemicals. Phenethyl ITC was purchased from Aldrich Chemical Co., (Milwaukee, WI). 1,2-Benzenedithiol was obtained from Lancaster Synthesis, Inc. (Windham, NH). 6-Phenyhexyl ITC and ITC-NAC conjugates were prepared and characterized in this laboratory previously (12, 24). All other chemicals are reagent grade from commercial sources.

Preparation of 1,3-Benzodithiole-2-thione. The synthesis has been described previously (20, 25). Briefly, to a solution containing 1,2-benzenedithiol (71 mg, 0.5 mmol) in 10 ml of methanol, 74 mg of methyl ITC in 5 ml of methanol were added dropwise over 10 min while stirring under N2. The reaction mixture was stirred for 5 h at room temperature. After removing the solvent under vacuum, a light yellow solid was obtained. It was purified on a silica gel column eluted with methylene chloride. TLC (Uniplate, Silica Gel GHLF, 250 µm; Analtech, Inc., Newark, DE) shows a single spot at Rf = 0.38 in hexane/methylene chloride (4:1). A single peak was observed on a HPLC chromatogram monitored at 365 nm with purity >99% based on integration (the HPLC conditions are described below). Mass spectra showed a molecular ion at 184 (calculated 184). 1H-NMR in DMSO-d6, showed: δ 7.52 (m, 2H) δ 7.87 (m, 2H).

Urinary Specimens. Human urine specimens used in this study were collected from previous studies (19, 26). Six urine specimens were collected from two participants at different time periods from 0–2, 4–8, 8–12, and 12–24 h after consumption of 20 g of brown mustard in a controlled diet (19). Eight 24-h urine specimens were collected over 3 days from two participants who consumed 56.8 g of fresh watercress in each meal, three meals a day, for 3 consecutive days in a controlled diet (26). All urine samples were stored at −20°C before analysis. We also used stored urine collected from nine participants of a prospective male-only cohort study in Shanghai, China, which was accrued during 1986–1989 (27). These subjects indicated, during the baseline interview, that they were daily consumers of dark green vegetables. The urine specimens have been kept frozen at −20°C until analysis. Under this storage condition, our study showed that the ITC conjugates are stable for at least 2 years.

Analyses of Urinary Allyl ITC-NAC and Phenethyl ITC-NAC. A 1-ml urine sample was centrifuged (3000 rpm) in a 1.5-ml microcentrifuge tube for 20 min. Three aliquots of supernatant (50 µl each) were analyzed by an HPLC system in conjunction with a C18 reverse phase column (Prodigy 5 ODS 3, 150 × 4.6 mm; Phenomenex, Torrance, CA) and a C18 guard column. The flow rate of the mobile phase was set at 1 ml/min. The chromatogram was recorded at 254 nm, and UV spectra were obtained by a Waters 990 photodiode array detector. In the analysis of urine samples containing allyl ITC-NAC, the mobile phase was 90% of phosphate buffer (0.1 M, pH 3.0) and 10% of CH3CN. In the analysis of urine samples containing phenethyl ITC-NAC, the mobile phase consisted of 70% phosphate buffer (0.1 M, pH 3.0) and 30% CH3CN. Quantification was done with calibration curves of allyl ITC-NAC and phenethyl ITC-NAC standard solutions.

HPLC Analysis of ITC Conjugates in Urine as the Cyclocondensation Product. Urine specimens were centrifuged (3000 rpm) for 20 min, and supernatants were used in the analysis. A 2-propanol solution of 1,2-benzenedithiol (10 mm, degassed), 600 µl, phosphate buffer (0.1 M, pH 8.5, degassed), 500 µl, urine sample, 100 µl were mixed in a 2-ml Chromacol autosampler vial with a screw cap (Chromacol, Inc., Trumbull, CT). Triplicate samples were processed for each urine specimen. Triplicates of phenethyl ITC-NAC standard solutions (20 and 100 µM) were used to obtain an average conversion rate for each batch analysis. In a negative control sample, a urine specimen was replaced by the same amount of deionized water. Sample vials were incubated in a water bath shaker at 65°C for 24 h. They were allowed to cool down to room temperature and centrifuged (3000 rpm) for 20 min. Sample vials were loaded into the auto injector for HPLC analysis. The consistency of the analysis was monitored in the beginning, middle, and at the end of each batch using multiple samples of the standard dissolved in 2-propanol and water mixture. A standard curve was constructed using a series of 1,3-benzodithiole-2-thione solutions from 0.01 to 20 µM in 2-propanol/water in the same batch of the urine samples (Fig. 1).

A Phenomenex Bondclone C18 column (150 × 3.9 mm) together with a guard column C18 (30 × 3.9; Phenomenex) was used for all analyses. The mobile phase consists of methanol (70%) and H2O (30%) running at a flow rate of 1.75 ml/min with a sample injection volume of 20 µl and the UV detection wavelength at 365 nm. Under this condition, the cyclocondensation product, 1,3-benzodithiole-2-thione, eluted approximately at 3 min (Fig. 2). The total concentrations of ITCs and their conjugates in urine specimens were calculated by using the standard curve and were expressed as µM of ITC equivalent, because the exact composition of the total ITCs is not revealed in this analysis. The average concentration of ITC equivalent

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* Portions of this work was presented at the eighty-seventh Annual Meeting of the American Association for Cancer Research, April 20–24, 1996.
and its standard deviation for each urine specimen were calculated from triplicate experiments. The interday or intraday precision was determined by assaying a urine sample once a day for 5 consecutive days or during 1 day in 3–5-h intervals.

Results

Assay Development

Selection of Organic Solvent for the Cyclocondensation Reaction. In our study of the reaction of 1,2-benzenedithiol and ITC conjugates in a mixture of methanol and phosphate buffer, we noticed that it produced precipitate upon cooling to room temperature, which caused precipitation of the cyclocondensation product from the reaction mixture. This problem significantly reduced the accuracy of the quantitative HPLC analysis. The same problem was encountered with other organic solvents that are compatible with reversed-phase HPLC analysis, e.g., acetonitrile, ethanol, 1-butanol, dimethylformamide, and tetrahydrofuran. The precipitation appeared to be associated with the oxidation of 1,2-benzenedithiol in the reaction mixture because it occurred upon leaving the 1,2-benzenedithiol solution at room temperature overnight. On the other hand, the reaction using 2-propanol as solvent did not generate precipitate and allowed direct analysis of the reaction mixture. Thus, degassed 2-propanol was used in the cyclocondensation reaction throughout this study.

Determination of Reaction Time and Conversion Rate. Using the reaction conditions described in “Materials and Methods,” phenethyl ITC-NAC or 6-phenylhexyl ITC-NAC solution (0.2 mM) in 100 µl of H₂Ο and 1,2-benzenedithiol solution (10 mM) in 600 µl of 2-propanol were mixed in a 7-ml glass vial by vortexing. The mixture was incubated at 65°C in a water bath shaker. Aliquots of the reaction mixture, 20 µl, were analyzed by the HPLC system at 30, 60, 90, 120, 150, and 180 min. Results showed that reactions were completed within 30 min with conversion rates of 94 and 110%, respectively. A 2-h reaction time was adopted for the analysis of urine samples to ensure the completion of cyclocondensation reactions. In actual batch analysis of urine samples, the conversion rates (recoveries) based on phenethyl ITC-NAC standard solutions fell typically in the range of 100–106%.

Assay Validation

This assay showed a high degree of interday and intraday precision with a variation coefficient of 1.4 and 0.5%, respectively. To validate this biomarker, we analyzed six urine samples containing NAC conjugate of allyl-ITC as a result of ingesting brown mustard and eight urine samples containing NAC conjugate of phenethyl-ITC from consumption of watercress (see “Materials and Methods”). A method for the analysis of specific urinary allyl-ITC-NAC was used for the analysis of both conjugates (19). We also measured the cyclic condensation product in these samples after reaction with 1,2-benzenedithiol using the current assay. Under our detection conditions, the limit of detection for the cyclic dithiol thione was 0.2 pmol, whereas that for the NAC conjugate of phenethyl ITC was 0.5 pmol. There was no detectable amount of ITC conjugates in the control urine samples collected before the vegetable diet using either assays. Results obtained from both methods are shown in Table 1. Both methods generated relatively small standard deviation.
Application of Assay Using Field Samples

As an initial effort to test the general applicability of this biomarker in urine specimens obtained from epidemiological studies, we analyzed nine urine samples collected during 1986 to 1989 from a male-only cohort study in Shanghai, China (27). Relatively low amounts of total ITCs were present in these samples as compared with those found in samples collected in the brown mustard or watercress studies. Table 2 shows that a wide range of concentration, from 0.3 to 11 μM, was found in these samples. After correction for creatinine level, a 10-fold range was still evident among these samples. It should be mentioned that the assays for phenethyl ITC- and allyl ITC-NAC were unable to detect these specific conjugates in the urine samples.

Discussion

In this study, we have demonstrated that 1,3-benzodithiole-2-thione, a cyclic condensation product of the reaction between 1,2-benzenedithiol and ITC, is a potential uptake biomarker for dietary ITCs in humans. We found a tight correlation of this biomarker with the mercapturic acids of allyl ITC and phenethyl ITC in urine samples collected from individuals who had consumed known amounts of mustard and watercress, respectively. Because both methods measure the content of ITC-NAC conjugates, one would expect that, within experimental error, identical results should be obtained from two methods. However, allyl-ITC or phenethyl-ITC are not the only ITCs in these sources would also contribute to the formation of the cyclocondensation product. Thus, a somewhat greater value would be expected for the cyclocondensation product. This trend was, however, not always observed in all samples, because the opposite trend occurred in 6 of 14 samples. These discrepancies were likely caused by experimental errors associated with the two methods.

This urinary biomarker, although lacking specificity for individual ITC, affords a better detectability than the specific ITC mercapturic acid metabolites. This is illustrated in our attempts to analyze the allyl or phenethyl ITC conjugate in the urine samples obtained from the Shanghai cohort study. We were unable to detect these specific conjugates in the urine samples obtained from this study, probably due to infrequent consumption of vegetables rich in these ITCs. Other important features of this assay are its speed and high reproducibility (<5% intraassay variability). The assay requires no sample extractions and involves only an initial centrifugation of urine samples and reaction with 1,2-benzenedithiol, followed by a second centrifugation before HPLC analysis. The assay procedure is illustrated in Fig. 4.
believe that the amounts detected accurately reflect the total reversible reaction. The equilibrium of the ITC conjugate with primarily from the mercapturic acids of ITCs in the urine via a specific conjugates and the biomarker in this validation study consuming a watercress or a mustard diet. Although the sample pounds often present in human urine, such as nitrile, thiocyanate, and disulfiram (28). Although these compounds are occurs with other thionyl compounds such as thiourea, carbon disulfide, and disulfiram (28). Although these compounds are unlikely to be present in human urine, we cannot completely rule out the possibilities. We have found that several compounds often present in human urine, such as nitrile, thiocyanate, and isocyanate, did not interfere with the assay. Furthermore, the specificity of the assay for ITCs and dithiocarbamates is evident from the study using the urine samples collected after consuming a watercress or a mustard diet. Although the sample size is relatively small, the close agreement between the specific conjugates and the biomarker in this validation study supports the notion that the cyclic thione derivative comes primarily from the mercapturic acids of ITCs in the urine via a reversible reaction. The equilibrium of the ITC conjugate comes with free ITC and thiol has been reported (21, 29). Because the reaction conditions used in the assay were established to quantitatively convert ITC conjugates to the cyclic product, we believe that the amounts detected accurately reflect the total urinary content of ITC conjugates.

To demonstrate the feasibility of this assay for its application in population-based studies, we assayed stored urine specimens accrued approximately 10 years ago from participants of a prospective cohort study in Shanghai who were daily consumers of dark green vegetables. Although all nine urine samples were positive for total ITC, a wide range in the amount of total ITCs in these samples was found. It is not known whether there was a loss due to long-term storage. However, the observation that the ITC conjugate levels in these samples were comparable to those found in the 2-year-old urine samples from a Singapore cohort, a population with daily consumption of cruciferous vegetables, provides indirect evidence that the ITC conjugates are relatively stable upon storage at −20°C. These results suggest that this assay is applicable to samples of long-term storage. The wide range of values illustrates its potential as a biomarker for dietary ITC uptake in epidemiological studies.

Evidence from rodent studies indicates that ITCs exert inhibitory activity against tumorigenesis by inhibiting cytochrome P-450 enzymes or by stimulating phase II enzymes or both (11–13). Recently, studies have shown that changes in these enzyme activities could also occur in humans upon consumption of cruciferous vegetables (26, 30). These alterations in enzyme activities will result in a decrease of carcinogen activation and increase of detoxification, both of which underlie inhibition of chemical carcinogenesis. These results, taken together, suggest that ITCs are among the active ingredients in cruciferous vegetables that are responsible for their protective effect against cancer. Whether dietary ITCs are important in lowering cancer risk in humans remains a challenging question in the field of nutritional carcinogenesis. The application of the biomarker described in this study to population-based investigations may help us better understand the role of these compounds in the protection against human cancers.

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Fig. 4. Steps involved in the analysis of cyclocondensation product in human urine as an uptake biomarker of total ITC.

The cyclocondensation reaction of 1,2-benzenedithiol also occurs with other thionyl compounds such as thiourea, carbon disulfide, and disulfiram (28). Although these compounds are unlikely to be present in human urine, we cannot completely rule out the possibilities. We have found that several compounds often present in human urine, such as nitrile, thiocyanate, and isocyanate, did not interfere with the assay. Furthermore, the specificity of the assay for ITCs and dithiocarbamates is evident from the study using the urine samples collected after consuming a watercress or a mustard diet. Although the sample size is relatively small, the close agreement between the specific conjugates and the biomarker in this validation study supports the notion that the cyclic thione derivative comes primarily from the mercapturic acids of ITCs in the urine via a reversible reaction. The equilibrium of the ITC conjugate comes with free ITC and thiol has been reported (21, 29). Because the reaction conditions used in the assay were established to quantitatively convert ITC conjugates to the cyclic product, we believe that the amounts detected accurately reflect the total urinary content of ITC conjugates.

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