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ORIGINAL CONTRIBUTION

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Urinary excretion of total isothiocyanates from cruciferous vegetables shows high dose–response relationship and may be a useful biomarker for isothiocyanate exposure

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Abstract *Background* Isothiocyanates (ITCs), hydrolysis products from glucosinolates, are a family of biologically active compounds originating from cruciferous vegetables. Many ITCs are assumed to have cancer preventive effects and to further evaluate these potential health effects, reliable biomarkers of ITC exposure are needed. *Aim of the study* In this study we investigated the ability of urinary ITC excretion to reflect a low or high daily intake of cruciferous vegetables. *Methods* The design was a controlled human crossover study ($n = 6$). Subjects consumed a self-restricted glucosinolate-free diet 48 h before the study-day where a basic diet supplemented with 80 or 350 g of mixed cruciferous vegetables was consumed. All urine was collected in intervals during the 48 h period after ingestion of the cruciferous vegetables. Total ITC in the cruciferous mixture and total ITC and their metabolites in urine was quantified as the cyclocondensation product of 1,2-bezenedithiol by high performance liquid chromatography. *Results* The total urinary excretion of ITCs correlated significantly with the two doses of ITC from diets with high or low cruciferous content ($r_s = 0.90$, $P < 0.01$). The fraction of urinary ITC excreted was 69.02 ± 11.57% and 74.53 ± 8.39% of the amounts ingested for 80 and 350 g cruciferous vegetables, respectively. *Conclusion* The results in this study indicate that the urinary excretion of ITCs, measured by use of the cyclocondesation reaction, is a useful and precise tool that may be used as a biomarker of ITC exposure in population based studies.

Key words isothiocyanates – urinary biomarker – cruciferous vegetables – biokinetic

Introduction

A diet rich in cruciferous vegetables is thought to reduce the risk of many common cancers [14]. In contrast to other plants, cruciferous plants are characterized by the presence of glucosinolates. When the plant material are chewed or damaged, glucosinolates are hydrolysed by the intracellular enzyme myrosinase (thioglycoside, EC 3.2.3.1) or later by the same enzymatic process conducted by microbes in the human gut, to form isothiocyanates (ITCs) [2]. ITCs have been shown to have anticarcinogenic effects, and they appear to act as blocking agents by modifying the metabolism of carcinogenic compounds through

their influence on biotransformation enzymes [11], including phase 2 enzymes that protect animal cells against oxidative stress and carcinogenesis [12]. The anticartinogenic action of ITCs has been supported by epidemiological studies [3, 7], demonstrating a protective effect of the human breast and lung. Other epidemiological studies have, however, failed to find significant beneficial effects [13]. In epidemiological studies consumption of cruciferous vegetable is usually measured by food-frequency questionnaire or other self-reported methods with high risk of subjective bias. Therefore more reliable measuring methods are necessary and a dietary biomarker represents an objective alternative. A method, the cyclocondensation assay, has been developed to measure total ITC and their metabolites (dithiocarbamates) [15] taking advantage of the quantitative reaction of these compounds with 1,2-benzenedithiol producing 1,3-benzodithione-2-thiole (Fig. 1). This assay provides the opportunity of measuring total ITC equivalents (= all ITCs and their metabolites) in different biological fluids, thereby making the dietary exposure to total ITCs measurable. So far, this biomarker has only been used to measure intake of high amounts of a single type of cruciferous vegetable or pure ITCs, but to enable the use of this biomarker in future epidemiological studies it has to be validated, whether it can measure lower and more realistic intakes of different types of cruciferous vegetables. According to the European Prospective Investigation into Cancer and Nutrition (EPIC) study [5] the average portion size of a single portion of cruciferous vegetables is 80 g where in average 80% is eaten boiled or steamed and 20% is eaten raw. In the present study, we therefore investigated whether ITC metabolites can be measured in urine after an intake of only 80 g of mixed cruciferous vegetables where 80% was served steamed and 20% was raw. A fundamental quality of a biomarker in studies of diet-disease associations is that the biomarker can reflect dose–response relationship and thereby link the measured biomarker with the true dietary intake. For that reason, we also included a dose of 350 g cruciferous vegetables prepared similarly to the 80 g to investigate the dynamic range of the biomarker.

Materials and methods

Subjects

Six healthy, non-smoking subjects, two males and four females, participated in the study. Subjects were 26–30 years of age, with BMI from 19.0 to 28.7 and were recruited from universities in Copenhagen. Exclusion criteria included smoking, regularly intake of medicine, intake of dietary supplementation <14 days before study start, >200 ml pure alcohol/week, pregnancy and major weight chances during the last 3 months. Subjects received oral and written information about the study, were instructed to report if any discrepancy appeared according to the protocol and gave their written consent. The study was approved by the local Research Ethics Committee of Copenhagen and Frederiksberg (J.nr. KF 01-161/01).

Study design and diet

The study was a randomised, crossover design with two intervention periods, each lasting 5 days and separated by a washout period of at least 2 weeks with habitual diet. Days 1 and 2 were a run-in period where the subjects were instructed to avoid all kinds of fruit and vegetables as well as coffee, tea and chocolate. On day 3 the subjects were provided a strictly controlled basic diet with either 80 or 350 g mixed cruciferous vegetables as supplement to the breakfast. On this day subjects had to consume breakfast and lunch at the Department of Human Nutrition and a snack and dinner was provided to consume at home. The basic diet was glucosinolate-free and based on bread, milk, cream cheese, eggs, meat, potatoes and biscuits. Women's diet contained 10 MJ whereas men were provided a diet with 12 MJ. The energy distribution was 48.6 E% from carbohydrate, 16.3 E% from protein and

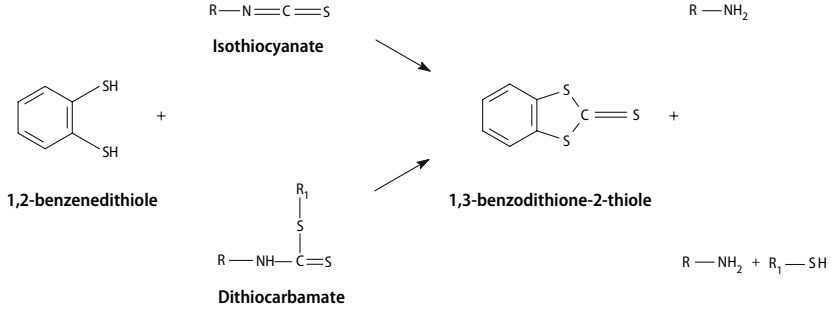

Fig. 1 Cyclocondensation reaction of ITC and an ITC metabolite (dithiocarbamate) with 1,2-benzenedithiol forming 1,3-benzodithione-2-thiole

35.1 E% from fat (calculations based on Dankost, the Danish Institute for Food and Veterinary Research [9]). The difference in energy intake between the high and low dose of cruciferous vegetables was balanced with other food items to obtain the same energy- and macronutrient level. The composition of the mixed cruciferous vegetables is shown in Table 1. The food that needed preparation was prepared in advance and stored at −18°C. The different cruciferous vegetables were each from the same cultivar and from the same producer, purchased from the local greengrocery. Cauliflower, white cabbage and Brussels sprouts were cut into pieces and steamed in an industrial furnace for 4 min. An oil/vinegar marinade and raw sliced Chinese cabbage were added to the mixture, after it had cooled to room temperature, and it was stored frozen (−20°C). On the afternoon before the day of the intervention (day 2), the vegetables were transferred from −20°C to +5°C. On the morning of day 3, red cabbage (stored whole at +5°C throughout the study period, one head used at each intervention day) was finely cut into longitudinal wedges, providing equal amount of stem and leaf, and added to the thawed cruciferous vegetable mixture prior to providing the mixture to the subjects. On days 4 and 5 subjects were instructed to follow a self-restricted diet without any fruits, vegetables, coffee, tea or chocolate.

Sample collection

A urine sample was collected after 12 h fasting in the morning of day 3. Samples were subsequently collected in intervals between 0–2, 2–4, 4–6, 6–8, 8–12, 12–24 and 24–48 h after consumption of the cruciferous vegetables. The urine was collected in 2500 ml or 500 ml plastic bottles containing 2.5 or 0.5 g L-ascorbic acid, respectively, as stabilizing agent. Samples were stored at −80°C until analysis.

Analysis of ITC equivalents in urine and cruciferous vegetables

Urine samples were blinded by technicians and analysed in randomised order. ITC and their metabolites were determined in urine and in the cruciferous vegetables by the cyclocondensation assay as described in a previous study from our laboratory [6]. Samples of fresh red cabbage were frozen separately from each of the study days and were added and blended with the cruciferous mixture (after this had thawed at 5°C overnight) before analysing [6].

Calibration and standards

Standards of 1,3-benzodithione-2-thiole and the *N*-acetyl-L-cystein conjugate of sulforaphane (SFN–NAC) were synthesised in our laboratory [6]. Quantification of the cyclocondensation product in urine samples was based on a calibration curve achieved by spiking blank urine samples with 1,3-benzodithione-2-thiole (six levels from 0.1 to 50 μmol/l, triplicate data). Reproducibility of the method was controlled by analysing 50 μl of a pure standard of 10 μg/ml 1,3-benzodithione-2-thiole dissolved in methanol and blank urine spiked with two levels of SFN-NAC (1.25 and 2.5 μmol/l) carried through the cyclocondensation reaction. The limit of quantification of 1,3-benzodithione-2-thiole was determined as 5 pmol in urine, based on the calibration curve.

Table 1 Proportion of the different cruciferous vegetables in the diet and total content of ITCs in the supplementation

Cruciferous vegetables	80 g dose	350 g dose
Cauliflower (g)	38	168
White cabbage (g)	17	146
Red cabbage (g)	10	45
Brussels sprouts (g)	8	35
Chinese cabbage (g)	7	28
Total ITCs[a] (μmol)	51.1 ± 3.5	223.7 ± 23.1

[a]Mean ± SD of all cruciferous vegetables on the different trial days

Statistical analysis

All determinations are expressed as the mean ± SD. Coefficient of variation (CV) was determined as (SD/mean) × 100%. Data were not normally distributed and nonparametric tests were used in the statistical analysis. Wilcoxon Signed Rank test was performed to compare 48 h urinary ITC excretion at the different intake levels of cruciferous vegetables. Spearman correlations were used to examine associations between intake of 0, 80 and 350 g cruciferous vegetable and excretion of urinary ITC metabolites. Statistical analysis was performed by SigmaStat Version 2.0.

Results

Subjects

Subject's compliance with the study protocol was high, although, subject 4276 made an error by collecting the urine from the interval 8 to 12 h and 12 to 24 h in the same container after the 80 g cruciferous supplementation. The baseline values of ITC in the urine ranged from non-detectable to 0.23 μmol with a mean of 0.02 ± 0.02 μmol before the 80 g supplementation and from 0.01 to 0.42 μmol with a mean of 0.15 ± 0.15 μmol before the supplementation with 350 g.

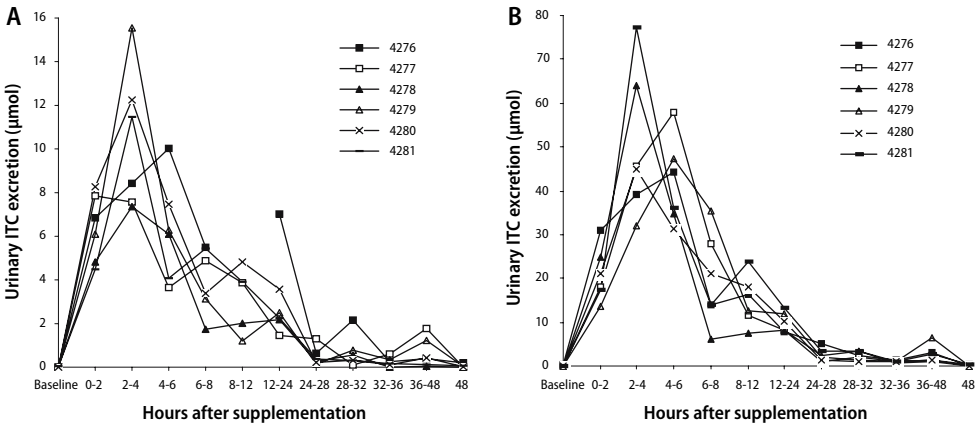

Fig. 2 Urinary excretion of ITC equivalents from six subjects after ingestion of (**A**) 80 g mixed cruciferous vegetables (the gap in the curve of subject 4276 is due to a missing urine collection) or (**B**) 350 g mixed cruciferous vegetables. It should be noted that the *x*-axis is not linear but reflects the sampling times, which differed in length

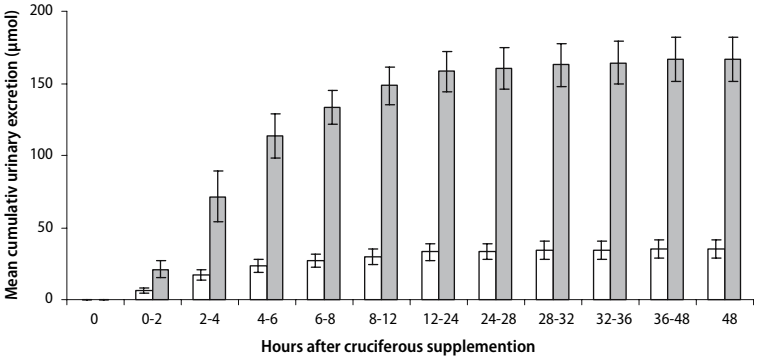

Fig. 3 Mean cumulative urinary ITC excretion after ingestion of 80 g (white bar) or 350 g (grey bar) cruciferous vegetables, respectively, error bars: SD for all individuals at a given time interval. Note that the *x*-axis is not linear but reflects the sampling times

Analysis of urinary excretion of total ITC equivalents

Total ITC equivalents were measured in urine after the cyclocondensation reaction with 1,2-benzenedithiol, forming 1,3-benzodithione-2-thiole. Recovery in blank urine samples spiked with the included standards was 83.2 ± 5.6%. The total amount of urinary excreted ITC equivalents in every time-interval after consumption of either 80 or 350 g of cruciferous vegetables is presented in Fig. 2A, B. The maximum excretion of ITC equivalents was observed between 2 and 6 h after treatment with both doses. In average the ITC equivalent excretion was 35.08 ± 6.13 µmol after ingestion of the 80 g dose increasing to 166.88 ± 15.19 µmol after intake of the 350 g dose. This significant difference ($P < 0.05$) between the total 48 h urinary ITC excretion corresponds to a 4.74-fold increase in the urinary excretion reflecting the 4.38-fold difference in the two cruciferous intake levels (Fig. 3). The measured amounts of ITCs in the two cruciferous doses are shown in Table 1. The ITC content of the cruciferous vegetables was measured from each trial day and the correlation between the amount of ITCs consumed and the 48 h urinary ITC equivalent excretion was found to be high ($r_s = 0.90$, $P < 0.001$, correlation calculated by baseline, 80 and 350 g levels of ITC equivalent excretion). This significant correlation was somewhat stronger after 24 h urine collection ($r_s = 0.93$, $P < 0.001$) and significant correlations between the ITC intake and urinary ITC equivalent excretion were observed for all urine collection periods. The fraction of the ITC dose excreted during 48 h ranged from 53.6% to 85.7% (mean ± SD: 69.02 ± 11.57%) for the 80 g dose and 62.6–82.7% (mean ± SD: 74.53 ± 8.39%) for the 350 g dose. The coefficient of variation (CV%) between subjects was, as expected, quite similar for the two doses; 16.8% and 11.3% for the low and high dose, respectively.

Discussion

Our finding of a high correlation between differences in low or high cruciferous consumption and urinary ITC equivalent excretion, demonstrates a highly significant dose-response relationship between intake of cruciferous vegetables and ITC equivalent excretion in human urine.

Baseline values before both supplementations indicated that 48 h with a glucosinolate free diet were sufficient to excrete nearly all ITC metabolites. The

ITC equivalent excretion after supplementation also revealed that 48 h is enough to eliminate all ITC metabolites from the body.

In the present study, the mean excretion of ITCs in the 48 h urine, as percentage of the dose, ranged from 69.0 ± 11.6% to 74.5 ± 8.4% for the low and high dose, respectively. The rest of the consumed ITCs may not be converted from glucosinolates to ITCs and thereby excreted unchanged in faeces. This lack of conversion is possibly due to insufficient myrosinase activity in the cruciferous vegetables and in the gastrointestinal tract. Furthermore, other hydrolyses products like nitrils can be formed from glucosinolates depending on the conditions under which the glucosinolates are degradated by myrosinase [2], but ITCs and their metabolites account for the major part of the glucosinolate metabolites generated.

The cruciferous mixture used in our study was prepared according to typical European ways of preparation and this involved that most of the cruciferous vegetables were steamed. Glucosinolates are relatively heat resistant, but the myrosinase enzyme is very susceptibly to heat [1, 8] and the 4 min steaming of 80% of the cruciferous vegetables may have decreased the myrosinase activity [1]. Nevertheless, the ITC excretion in our study was quite high compared to other studies with steamed/boiled cruciferous vegetables [1, 4]. This may be explained by our quite short preparation time and the use of a mixture of raw and steamed vegetables, whereby undegraded myrosinase from the raw vegetables may assist in the conversion of glucosinolates to ITCs in the steamed vegetables, when the mixture is chewed and eaten.

In this present study, the average inter-individual variation in ITC equivalent excretion was rather low (CV, 14%) compared to our previous study with broccoli consumption, where high variation was found between individuals [6]. Since there are a number of factors that may affect the ITC metabolism *in vivo* (mastication, gut microflora, GSH-genotype) a higher inter-individual variation could have been expected in this study as well. Generally, the few studies conducted in this area (including this one) have small subject numbers and conclusions on inter- and intra-individual variation should thus be interpreted with caution [1, 4, 10, 11].

Despite the excellent performance of urinary ITC equivalents excretion as a biomarker of cruciferous vegetables in this study, the biomarker may not reflect the true individual intake in epidemiological studies. A lot of factors influence on the content of ITCs in the cruciferous vegetable (species, cultivar, growth conditions, post-harvest conditions and cooking habits) and additionally, uptake of ITCs is also affected by individual metabolic activity, and gut microflora [5]. These factors may cause under or overestimation of the association of the true intake of cruciferous vegetables. Using the urinary ITC equivalent excretion as a biomarker for the *exposure* to ITCs circumvents these interfering factors and the cyclocondensations assay may therefore especially serve as a reliable urinary biomarker of ITC exposure. Moreover, assessment of human exposure to ITCs is especially relevant when evaluating studies of cruciferous vegetables cancer preventive effects. However, collections of 48 h urine samples will be inconvenient in larger studies. Since ITC and their metabolites are relatively rapidly excreted, spot urine samples may be inadequate, but collections of, e.g. 12 h urine samples from 12.00 a.m. to 12.00 p.m. may be suitable to reflect the daily exposure of dietary ITCs.

In conclusion, the present study shows that urinary excretion of ITC equivalents, measured by use of the cyclocondesation reaction, is a useful and precise biomarker for ITC exposure. The biomarker can reliably measure ITC excretion after intake of low and realistic amounts of mixed cruciferous vegetables and this qualifies it as a biomarker of ITC exposure, that may be used in future case control or cohort studies, evaluating ITCs cancer preventive properties.

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