Urinary Isoxanthohumol Is a Specific and Accurate Biomarker of Beer Consumption¹–³

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Abstract

Biomarkers of food consumption are a powerful tool to obtain more objective measurements of dietary exposure and to monitor compliance in clinical trials. In this study, we evaluated the effectiveness of urinary isoxanthohumol (IX) excretion as an accurate biomarker of beer consumption. A dose-response clinical trial, a randomized, crossover clinical trial, and a cohort study were performed. In the dose-response trial, 41 young volunteers (males and females, aged 28 ± 3 y) consumed different doses of beer at night and a spot urine sample was collected the following morning. In the clinical trial, 33 males with high cardiovascular risk (aged 61 ± 7 y) randomly were administered 30 g of ethanol/d as gin or beer, or an equivalent amount of polyphenols as nonalcoholic beer for 4 wk. Additionally, a subsample of 46 volunteers from the PREDIMED (Prevención con Dieta Mediterránea) study (males and females, aged 63 ± 5 y) was also evaluated. Prenylflavonoids were quantified in urine samples by liquid chromatography coupled to mass spectrometry. IX urinary recovery increased linearly with the size of the beer dose in male volunteers. A significant increase in IX excretion (4.0 ± 1.6 µg/g creatinine) was found after consumption of beer and nonalcoholic beer for 4 wk (P < 0.001). Receiver operating characteristic curves showed that IX is able to discriminate between beer consumers and abstainers with a sensitivity of 67% and specificity of 100% (positive predictive value = 70%, negative predictive value = 100% in real-life conditions). IX in urine samples was found to be a specific and accurate biomarker of beer consumption and may be a powerful tool in epidemiologic studies. This trial was registered at the International Standard Randomized Controlled Trial registry as ISRCTN72996101 (study 1), ISRCTN95345245 (study 2), and ISRCTN35739639 (study 3). J. Nutr. 144: 484–488, 2014.

Introduction

In nutritional epidemiology, accurate assessment of dietary exposure is crucial for studying the effect of diet on health. Until now, the most common way of assessing the diet followed by participants in such studies has been by analyzing the results of self-reporting FFQs, but this method is liable to systematic bias because of factors such as age, gender, and social desirability and conflicts of interest.

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⁷ Abbreviations used: IX, isoxanthohumol; LC-MS/MS, liquid chromatography coupled with tandem mass spectrometry; MeOH, methanol; PREDIMED, Prevención con Dieta Mediterránea; ROC, receiver operating characteristic; SPE, solid-phase extraction; XN, xanthohumol; ⁸PN, ⁸-prenylnaringenin.

(IX), resulting in beer having larger amounts of IX than XN (8). Up to 48 h after ingestion (9), IX is converted into 8-prenyllaringenin (8PN) in the distal colon by an O-demethylation catalyzed by gut microbiota (9,10).

In a recent study (11), we developed a specific methodology to analyze urinary XN, IX, and 8PN. Moreover, in an acute study with 10 volunteers who consumed a single dose of beer (330 mL), IX was the only beer prenyllavonoid able to discriminate between beer and nonbeer consumers. Accordingly, to test the potential of IX as a biomarker of beer consumption, we embarked on 3 studies: a dose-response clinical trial, an intervention clinical trial, and a cohort study of a free-living population.

Materials and Methods

Reagents and materials

Methanol (MeOH), acetonitrile, and ammonium bicarbonate of HPLC grade were used (Sigma-Aldrich). HPLC-grade acetic acid, formic acid, and ammonium acetate were purchased from Panreac Quimica. Ultrapure water (MilliQ) was generated by the Millipore System. IX, 8PN, and XN (97–99% purity) were purchased from Enzo Life Science. Taxifolin (>90% purity) was obtained from Extrasynthese. Ascorbic acid was supplied by Sigma-Aldrich. Solid-phase extraction (SPE) cartridges Oasis MCX 96-well, 60-μm plates (30 mg) were obtained from Waters.

Identification and quantification of IX, 8PN, and XN in urine samples by SPE–liquid chromatography coupled to mass spectrometry

Prenyllavonoids in urine samples were quantified following the method previously described based on SPE and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (11). Briefly, 1 mL of urine samples was loaded into a 96-well plate Oasis MCX cartridge previously equilibrated with 1 mL of MeOH and 1 mL of 5-mM ammonium acetate buffer adjusted to pH 5.0. Cartridges were then washed with 1 mL of MeOH, 5-mM pH 5.0 ammonium acetate buffer (1:1, v/v) solution and eluted with 0.5 mL of MeOH (twice). The elute obtained was evaporated to dryness under a gentle stream of N₂, and the residue was reconstituted with 100 μL of 5-mM pH 7.0 ammonium bicarbonate buffer containing 20 mg/L of ascorbic acid.

The identification and quantification of IX, 8PN, and XN in urine samples was performed using an HP Agilent 1100 system equipped with a quaternary pump and a refrigerated auto sample coupled to an API 3000 triple-quadrupole mass spectrometer (AB Sciex) with a turbo ion spray source working in negative mode. Chromatographic separation was performed with a Luna C18 column, 50 mm × 2.0 mm i.d., 5 μm (Phenomenex), using 5-mM ammonium bicarbonate buffer adjusted to pH 7.0 as the aqueous mobile phase and acetonitrile and methanol as organic phases in a 1:1 proportion. All chromatographic conditions were previously described in detail (11). For the quantification of analytes in urine samples, the multiple reaction monitoring mode was used, monitoring 3 transitions: 353/119 (IX and XN), 339/219 (8PN), and 303/285 (taxifolin, internal standard). The MS/MS parameters are described elsewhere (11).

Taxifolin was used as the internal standard. All results were corrected for urinary creatinine, which was performed following the method adapted to the micrortiter 96-well plate (6).

Analysis of IX, XN, and 8PN in beer samples by LC-MS/MS

The beer foam was removed by ultrasonication and filtered through a 0.45-μm polytetrafluoroethylene filter. Then 20 μL of beer samples were injected into the LC-MS/MS system without any other pretreatment. The identification and quantification of analytes was carried out as previously described.

Study design

The suitability of beer prenyllavonoids for use as a specific and selective biomarker of beer consumption was evaluated in 2 different intervention clinical trials (studies 1 and 2) and in a cohort study (study 3).

Study 1. A dose-response, randomized, crossover clinical trial including 41 young, healthy volunteers (20 males and 21 females, aged 28 ± 3 y, mean BMI 22.3 ± 2.6 kg/m²) was performed. All participants drank 3 different doses of a previously selected beer (beer no. 1) the night before (2100 h), and a spot urine sample was collected in the morning (0800 h).

Beer no. 1 was selected from among 19 Spanish beers based on its prenyllavonoid content (data not shown). The doses of beer administered to male volunteers were 1 beer (330 mL), 2 beers (660 mL), or 3 beers (990 mL), whereas female volunteers drank 1 beer (330 mL), 1.5 beers (495 mL), or 2 beers (660 mL). Seven days before the first intervention (run-in period), participants were asked to refrain from consuming beer, nonalcoholic beer, or hop-derived products such as hop-based food supplements. A 4-d washout period was set between interventions. After the run-in and each washout period, a blank spot urine sample was also collected in the morning. Young (21–39 y), normal weight (BMI 18.5–24.9 kg/m²), nonsmoker participants were selected. Volunteers with a previous medical history of serious illness, alcoholism, or drug addiction, or those taking medication were excluded from the study. The different interventions were crossed and distributed randomly once per week for 3 wk.

Study 2. In a randomized, open, crossover, controlled clinical intervention trial, 33 males with high cardiovascular risk (aged 61 ± 7 y, mean BMI 29 ± 4 kg/m²) randomly were administered 30 g of ethanol in gin (92 mL) or beer (660 mL of beer no. 2), or an equivalent amount of polyphenols in nonalcoholic beer (990 mL/d) for 4 wk. The participants were recruited in the outpatient clinic of the Internal Medicine Department at the Hospital Clinic of Barcelona, Spain. All were moderate alcohol consumers (1–3 drinks/d) and had diabetes or ≥3 of the following cardiovascular risk factors: tobacco smoking, hypertension, plasma LDL cholesterol concentrations ≥160 mg/dL, plasma HDL cholesterol concentrations ≤35 mg/dL, overweight or obesity (BMI ≥25 kg/m²), and/or family history of premature coronary heart disease. Participants with documented coronary heart disease, stroke, or peripheral vascular disease, HIV infection, alcoholic liver disease, malnutrition, or neoplastic or acute infectious diseases were excluded from the study. Participants were asked not to drink alcoholic beverages 15 d before the first intervention (run-in period) and not to change their dietary pattern during the study. Diet and exercise were monitored after the run-in period and after each intervention period using a previously validated 7-d food record questionnaire (5 weekdays and 2 weekend days) (12) and the Minnesota Leisure Time Physical Activity Questionnaire, respectively. Twenty-four-hour urine collections were obtained the morning after the run-in period and after the last day of each intervention. In this study, 24-h urine collections rather than single spot urine samples were chosen to guarantee the detection of urinary prenyllavonoids, because volunteers were asked to drink the beer during the 2 main meals. All urine samples from the 2 clinical trials were coded randomly and stored at −80°C until analysis.

The beer, nonalcoholic beer, and gin were provided by the research team. Beer beverages were supplied by Centro de Información Cerveza y Salud through the Asociación de Cerveceros de España.

The study protocols of both studies were approved by the Ethics Committee of the University of Barcelona and the Institutional Review Board of Hospital Clinic, Barcelona, Spain. The volunteers were fully informed and gave written consent.

Study 3: Free-living population from the PREDIMED study cohort.

To demonstrate the suitability of the new biomarker, urine samples from the free-living participants of the PREDIMED (Prevención con Dieta Mediterránea) study were analyzed. The PREDIMED is a large, parallel-group, multicenter, controlled, randomized 5-y clinical trial aimed at assessing the effects of the Mediterranean diet on the primary prevention of cardiovascular disease. A subsample of 46 volunteers was selected at random from the cohort studied at the Hospital Clinic to analyze urinary excretion of prenyllavonoids: 32 males and 14 females with a mean age of 63 ± 5 y and a mean BMI of 29 ± 3 kg/m². At baseline, participants completed a 137-item validated FFQ (13) and the validated Spanish version of the Minnesota Leisure Time Physical Activity Questionnaire (14). Five volunteers reported no beer consumption, and 41 volunteers reported drinking intermittently or daily between 22 and 825 mL/d of beer.
beer. The detailed recruitment method, study protocol, and exclusion and inclusion criteria were previously described by Estruch et al. (15,16). Spot-urine samples from all participants were collected in the morning, coded, and stored at −80°C until analysis.

The IRB of the Hospital Clinic of Barcelona approved the study protocol and each participant signed an informed consent form.

Statistical analysis
Statistical analysis was performed using IBM SPSS Statistics (version 19; SPSS). Descriptive statistics (means ± SDs) were used for the baseline characteristics of the participants. Nonparametric tests were used in the statistical analysis of the data. A Wilcoxon test for related samples was performed to compare changes in response to each intervention in both clinical trials. A Mann-Whitney test for unrelated samples was used to compare responses according to gender. The female population was split into 2 according to IX excretion using the 50th percentile. A Pearson linear correlation was used to evaluate the relation between beer consumption and urinary excretion of prenyllflavonoids. A receiver operating characteristic (ROC) curve was assessed to calculate the sensitivity, specificity, accuracy, and the ability to discriminate between positive and negative results of the new biomarker proposed. The cutoff point providing optimal sensitivity and specificity for the identification of beer consumers was also calculated using the ROC curve. All statistical tests were 2-tailed and the level of significance was 0.05.

Results
Analysis of beer samples
The prenyllflavonoid concentrations found in the beer samples are shown in Table 1.

In the first study, male volunteers consumed 152 ± 23 μg, 304 ± 47 μg, and 456 ± 71 μg of IX, and 18 ± 2.4 μg, 36 ± 4.9 μg, and 55 ± 7.4 μg of 8PN in 330 mL, 660 mL, and 990 mL of beer, respectively. The intake by female volunteers was 152 ± 23 μg, 207 ± 32 μg, and 304 ± 47 μg of IX, and 18 ± 2.4 μg, 24 ± 3.3 μg, and 36 ± 4.9 μg of 8PN in 330 mL, 495 mL, and 660 mL of beer, respectively.

In the second intervention study, the daily intake of IX and 8PN was 364 ± 29 μg and 22 ± 3.5 μg, respectively, during the alcoholic beer intervention. During the nonalcoholic beer intervention, the daily intake of IX and 8PN was 184 ± 71 μg and 19 ± 2.3 μg, respectively. XN was not detected in any beer sample.

Clinical trials
Study 1. After consumption of the different volumes of beer, IX was found in the urine samples of all volunteers, whereas it was not detected after the run-in and washout periods. Urinary excretion of IX increased linearly with the dose size (Fig. 1) in male volunteers, showing a dose-response behavior. Regression analysis of mean IX excretion for the 3 doses in males showed a linear association (r = 0.85) (P < 0.001). However, although IX excretion in females also showed a linear relation (r = 0.89) (P < 0.001), a saturation behavior after the intake of only 1 beer (330 mL) was observed because no significant differences were observed among the 3 doses consumed. The excessively high SD in the female population showed that the females formed 2 groups based on IX excretion. Therefore, the female population was split into 2 according to IX excretion using the 50th percentile (>4.83 μg IX/g creatinine). One of the subgroups showed a dose response similar to that of the male population, whereas the other subgroup showed a saturation behavior after consuming 330 mL of beer (Fig. 1). Regression analysis including both genders at a beer dose of 0 mL, 330 mL, and 660 mL also showed a linear association (r = 0.83) (P < 0.001).

The mean urinary excretion in males was 2.4 ± 0.18, 3.3 ± 0.33, and 4.6 ± 0.45 μg/g creatinine after intake of 330 mL, 660 mL, and 990 mL of beer, respectively. In comparison, the mean IX excretion in females was higher at lower doses, 4.4 ± 0.31, 4.7 ± 0.4, and 4.9 ± 0.7 μg/g creatinine after the intake of 330 mL, 495 mL, and 660 mL of beer, respectively. Therefore, significant differences in urinary excretion of IX were observed between males and females (P < 0.001). 8PN was detected in urine samples but under the limit of quantification (<5 μg/L). XN was not detected in any urine sample from the volunteers.

Study 2. After a 4-wk intervention with alcoholic (660 mL/d) or nonalcoholic beer (990 mL/d), IX was found in all urine samples but was not detected in urine samples after the wash-out period and gin intervention. IX increased to 4.0 ± 1.6 μg/g creatinine after the beer intervention compared with the run-in and wash-out period (P < 0.001), and 4.2 ± 1.3 μg/g creatinine after nonalcoholic beer intake (P < 0.001).

PREDIMED population
In study 3, participants from the PREDIMED cohort who reported intermittent or daily beer consumption had significantly higher IX urinary concentrations than nonbeer consumers (P < 0.05). Beer consumers excreted 3.04 μg IX/g creatinine (95% CI: 1.81, 4.28 μg IX/g creatinine), whereas IX was not detected in urine samples from nonbeer consumers.

ROC curves
ROC curves (Fig. 1, Supplemental Fig. 1) were assessed using the data obtained from the 2 clinical intervention trials and the cohort study to evaluate the effectiveness of urinary IX measurement as a biomarker of beer intake.

Using the clinical intervention trial data, the AUC was 0.990 (95% CI: 0.98, 1.00). The optimal cutoff point was 0.48 μg IX/g creatinine, which allowed differentiation between beer and nonbeer consumption with a sensitivity of 98% and specificity of 96% (likelihood ratio = 29.96, positive predictive value = 99%, negative predictive value = 96%).

Using the data from the PREDIMED study, the cutoff point of 0.48 μg IX/g creatinine allowed discrimination between beer and nonbeer consumers with an AUC of 0.904 (95% CI: 0.797, 1.00), sensitivity of 67% (95% CI: 65, 69%), and specificity of 100%. In the free-living population, the sensitivity might be lower for different reasons: the beer dose was not established as in the clinical studies and thus beer consumption varied between 22 mL/d and 825 mL/d. In addition, both female and male volunteers were included. Despite these factors, ROC curves showed that IX is a good biomarker because the positive and negative predictive values were still high, 70% and 100%, respectively. Therefore, according to the traditional academic point system, the accuracy of the new biomarker was found to be excellent (AUC > 0.9).

Discussion
The development of potent new nutritional biomarkers is crucial for epidemiologic research because the more objective data they
Isoxanthohumol: a biomarker of beer consumption

Isoxanthohumol (IX) is a specific, sensitive, and accurate biomarker of beer intake, which allows for a more accurate assessment of food and nutrient intake of the participants evaluated. Nutritional biomarkers are also very useful in clinical trials for monitoring compliance with administered interventions. New biomarkers need to be validated in 2 stages: 1) in dose-response, controlled clinical trials to identify the range of intake in which the biomarker is reliable, and 2) in free-living populations to evaluate the suitability of the biomarker in a habitual diet. Moreover, an effective biomarker of food intake should be specific to the dietary component of interest, sensitively reflect changes in food intake by its concentration in the biofluid, provide a good correlation between excretion and exposure, and have a robust quantification method. Taken as a whole, the results of the current studies suggest that urinary IX excretion is an excellent biomarker of alcoholic and non-alcoholic beer intake because it fulfills all the aforementioned criteria. Unlike other polyphenol-based biomarkers (4–6), IX is specific to only 1 dietary component (beer), being found exclusively in hops, which makes it an ideal and highly sensitive biomarker of beer intake. In fact, a cutoff point of 0.48 mg IX/g creatinine allowed perfect discrimination between beer and nonbeer drinkers. AUCs obtained in both ROC curves, using the data from the clinical trials and PREDIMED cohort, were >0.9, which means that IX is a highly accurate biomarker of beer intake according to the traditional academic point system. Furthermore, IX proved to be a good biomarker of both alcoholic and non-alcoholic beer because no differences in IX urinary excretion were found between the intervention periods of both types of beer, even though the regular beer had a higher concentration of polyphenoloids.

In this study, urinary IX excretion was also investigated in a dose-response clinical trial. Interestingly, IX recovery in urine samples increased linearly with dose size in male volunteers, whereas a saturation behavior was observed after a single dose of 1 beer (330 mL) in 1 subgroup of the female population. Gender differences in urinary polyphenol recovery have been reported previously for isoflavones (20,21) and may be due to differences in absorption and metabolism. In general, female volunteers excreted twice as much IX after the consumption of 1 beer (330 mL) as male volunteers, yet in a subgroup of female volunteers, no differences in IX excretion were found after 1.5 and 2 beers, suggesting that in some women IX excretion may be saturated at 152 μg. In contrast, male and some of the female volunteers excreted IX linearly, depending on the size of the beer dose. Intestinal absorption of IX has not been previously studied, but if IX behaves similarly to XN, then an active efflux pump system is expected to be responsible for the uptake of IX (22). XN has shown high permeability in Caco-2 cells, and once inside the cell, 90% of XN remains trapped in the cytosol bound to a cytosolic protein in a process that is temperature-dependent and saturable (22). Therefore, a saturation of IX absorption, similar to XN, could be expected, and our results suggest the dose of saturation differs between genders.

In summary, urinary IX was validated as a biomarker of beer consumption. Measurement of urinary IX concentration proved to be a specific and accurate biomarker of beer intake, which might be expected because it is a component exclusive to beer in a regular diet. This new biomarker can be used to discriminate between nonbeer and beer consumers, and it may be a useful tool for monitoring participant compliance in intervention trials. However, IX might be less effective in distinguishing intermittent female beer consumers because IX urinary excretion in some women is not dose-dependent.

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**Literature Cited**


