Development of a Urinary Riboflavin Adherence Marker for a Wheat Bran Fiber Community Intervention Trial

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Abstract
Development of a reliable marker of adherence to high-fiber diets is essential for accurately assessing dietary fiber intake in community interventions and clinical trials. The objective of this study was to evaluate the feasibility of using a riboflavin tracer incorporated into wheat bran cereal to determine fiber intake and compare results to the more traditional methodology of measuring stool weight. The inpatient phase of the study established that the excretion of urinary riboflavin was highly correlated with the dose of the riboflavin-spiked wheat bran cereal (r = 0.95, P < 0.005) and could be used as a biomarker to validate fiber supplement intake. The outpatient clinical intervention included a group of seven African-American men and women, who were asked to incorporate 1/2 cup of wheat bran cereal (11.6 g of dietary fiber) into their daily diet for a 6-week period. The cereal was spiked with a 28-mg dose of riboflavin. Baseline measurements of urinary riboflavin and stool weight were compared to postintervention levels. Comparison of pre- and postintervention measures of riboflavin excretion showed a significant increase (0.8 ± 0.1 versus 6.0 ± 0.6 mg/day, P < 0.02), which confirmed a high level of adherence to the dietary intervention. Although wet stool weights at baseline were significantly lower than postintervention (106 ± 20 versus 146 ± 23 g/day; P < 0.03), differences in dry stool weights did not reach significant levels (28 ± 4 versus 33 ± 5 g/day, P < 0.30). Furthermore, pre- and poststool measurements overlapped and could not provide definitive data on participant adherence. These results indicate that the riboflavin tracer was a more sensitive biomarker of wheat bran fiber supplementation than stool weight and provided an accurate method for validating adherence to the dietary intervention. A riboflavin marker provides a valid technique for adherence assessment in large-scale community trials, in which collection of 3-day fecal samples is not a manageable option.

Introduction
Effective cancer control trials are contingent on maintaining sufficient numbers of subjects that adhere to experimental protocols (1). The inability to accurately assess participant adherence in outpatient nutritional interventions presents a major obstacle in studies attempting to evaluate the role of diet in cancer prevention. Correct interpretation of results is dependent on high levels of adherence by participants. Commonly, this parameter is measured entirely by self-report of dietary intake. Biological markers, when available, are used to confirm the accuracy of self-report. For example, urinary isoflavone content has been used to validate soy consumption (2). Self-reports of adherence have been shown to be more precise when participants are aware that accuracy is being cross-checked with biological markers (3, 4). Unfortunately, in most cases, satisfactory biomarkers are not available. Prior to this study, investigators have been unable to provide precise adherence data for interventions that increase insoluble dietary fiber intake. Innovative ideas are needed to allow investigators to validate participant adherence.

It is well established that increased bran and cellulose consumption increases stool weight (5, 6), but this parameter has not proven to be a sensitive measure of dietary fiber intake (7, 8). Due to the high variability of daily fecal weights within subjects and between subjects, 3 or 4 days of stool collection is necessary to detect over 75% excretion of a stool marker during wheat bran supplementation (7). The feasibility of measuring daily fecal weights for this length of time in large-scale community interventions is limited due to the level of commitment required from and great inconvenience to participants.

This study investigated the use of a riboflavin-spiked wheat bran cereal as a marker to assess adherence in an outpatient clinical trial. Riboflavin was chosen as a tracer because it is safe and stable under normal conditions and excess intake is readily excreted in the urine (9). The accuracy of the methodology was validated in the dose response inpatient phase of the study. The plausibility of using riboflavin as a biomarker in a community setting was also evaluated.

Subjects and Methods

Subjects
Eligible subjects were healthy men and women, ages 45–65 years, recruited from the housekeeping and grounds staff of the University of North Carolina at Chapel Hill. Eligibility was determined at baseline on the basis of the absence of serious

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comorbidities (e.g., non-insulin-dependent diabetes) and adequate nutritional status (e.g., dietary intake within normal ranges). Participants underwent a general physical examination and dietary intake assessment using the Arizona Food Frequency Questionnaire (10). The dietary exclusion criteria were dietary fiber intake of 30.0 g or more per day and the use of vitamin supplements containing riboflavin. All subjects signed informed consent forms according to federal and University of North Carolina Human Subjects Committee guidelines.

**Study Design**

**Inpatient Dose Response Study.** Six healthy volunteers (two men and four women) took part in the study. Each participant was admitted to the General Clinic Research Center at approximately 6 a.m. after an overnight fast and at least a week on a specific diet of wheat bran cereal (not labeled with riboflavin). A catheter was placed in an arm vein, and a blood sample was taken. The participant emptied his or her bladder before consuming or her assigned dose of riboflavin-spiked wheat bran cereal (38% or 10.6 mg of riboflavin; 75% or 21 mg of riboflavin; or 100% or 28 mg of riboflavin). A full, 100% dose of cereal weighed 35 g and contained 11.6 g of wheat bran fiber. Blood and urine samples were then collected every 30 min up to the 4-h time point and every hour up to the 8-h time point. All urine was collected thereafter until the 24-h collection was completed. Results from this phase of the study provided the basis for using a riboflavin-spiked wheat bran cereal in a work site intervention trial aimed at increasing fiber consumption in a minority population and developing an exact adherence marker (11).

**Outpatient Adherence Study.** Seven African-Americans (three men and four women) participated in this study. Baseline dietary assessment and sample collection were completed prior to dietary intervention. Each participant provided a 24-h urine collection, 3-day stool samples, and a blood sample, taken following an overnight fast. A run-in period of 2-2 weeks allowed participants to work up to a full dose of fiber (35 g of wheat bran cereal containing 28 mg of riboflavin marker). Weekly visits to the clinic site were required for the first 3 weeks of the study to provide intake instructions based on calendar self-report and to intervene if adherence was marginal (<75% of dose) or poor (<50% of dose; Ref. 12). After successfully completing 6 weeks of intervention at levels of 75% or more, postintervention blood, urine, and fecal samples were collected, and a final physical examination was carried out.

**Urine Samples.** Collected urine was placed in dark 3-liter bottles containing 5 ml of acetic acid as a preservative. Samples were refrigerated by participants and brought to the General Clinic Research Center. Volumes of samples were measured, acidified to pH 3-4, and stored frozen (-20°C) until analysis. Urinary creatinine excretion was measured to confirm completeness of collection. Samples deemed incomplete were not used, and a repeat collection was obtained. Urine riboflavin content was determined by a modified method of Chastain and McCormick (13). All extractions and analyses were carried out under red light because riboflavin is particularly sensitive to decomposition in extracted solutions. Frozen urine samples were defrosted, and duplicate 2.5-ml samples and riboflavin standards were saturated with ammonium sulfate. Urine was then centrifuged to remove solids. Supernatants were extracted twice with 80% (w/w) aqueous phenol to extract flavins. Phenol extractions were pooled, and 1.5 ml of water was added. Diethyl ether (water-saturated) was then used to remove the phenol from the aqueous solution. Total flavin content of the aqueous extracts was determined by the fluorescence at 450-nm excitation/525-nm emission in a fluorescence spectrophotometer (Hitachi model U-2000). A pool control was included in each batch of samples. Analyses were repeated if control values were not within the range of two SDs of the mean. Reported recovery in this multiple extraction procedure is in the 90% range (14).

**Fecal Samples.** Three-day fecal samples were collected and placed in sealed plastic bags and frozen until the participant brought the specimens to the clinic site, generally on day 4. Daily samples were weighed, homogenized, and freeze-dried to determine wet and dry weights.

**Statistical Analysis**

Statistical analyses were carried out with the SAS Version 6.11 statistical package (SAS Institute, Inc., Cary, NC). The Wilcoxon rank sum test was chosen because most parameters were not normally distributed due to small sample size. All distributions are reported as mean ± SE. Statistical significance was taken as P < 0.05.

**Results**

Three levels of riboflavin were tested in the inpatient phase of this study (Fig. 1). Participants ate one of three weighed doses of riboflavin incorporated into wheat bran cereal. Twenty-four-h urinary riboflavin excretion was well correlated to dose (r = 0.95, P < 0.005, 95% confidence interval = 0.62-0.999). This provided evidence that urinary riboflavin did indeed reflect daily intake in a dose-dependent manner.

In the outpatient phase of the study, daily fecal weights of participants varied considerably. Individual participants had markedly different fecal weight patterns, with great intersubject variability. Fig. 2 illustrates both the high intraindividual and interindividual variability over the 3-day collection period at baseline and following 6 weeks of dietary intervention. Mean daily wet stool weight (Table 1) significantly increased by 27% (106 ± 20 versus 146 ± 23 g/day, P < 0.03). A trend toward increased daily dry stool weight (15% increase from 28 ± 4 to 33 ± 5 g/day, P < 0.30) was also observed. Increased dry weight change correlated with increased wet weight (r = 0.92) and water content of samples both pre- and postintervention was approximately 75%.

**Fig. 1.** Twenty-four-h urinary riboflavin excretion following consumption of a 10.6-, 21-, or 28-mg dose of riboflavin-spiked wheat bran fiber cereal. Results are expressed as mean ± SE with an overall correlation of r = 0.95, P < 0.005.
At baseline, all participants were found to have sufficient riboflavin status, on the basis of urinary excretion 120 μg/day or more (15). Twenty-four- and stool measures following a 6-week intervention with 11.6 g/day wheat bran fiber*.

The relationship between wet stool weight and riboflavin excretion is illustrated in Fig. 3. The change in fecal weights correlated moderately to the change in urinary riboflavin levels (r = 0.57, P < 0.18), indicating that they were measuring similar phenomena (Fig. 3A). In Fig. 3B, the data show considerable variation in both wet stool weight and riboflavin values, but only riboflavin consistently showed a clear separation of pre- and postintervention values. Baseline levels of riboflavin ranged from 0.47 to 1.27 mg/day and average stool weights ranged from 44 to 137 g/day. Post stool weights ranged from 51 to 246 g/day, with several measurements overlapping the preintervention observations. In contrast, all postintervention measurements of riboflavin (4.0–7.5 mg/day) were markedly higher than baseline levels, demonstrating that participants had adhered to the intervention.

Discussion

Self-report of dietary intake is common practice in nutritional studies. Reliability of this methodology is questionable, and alternative assessments for adherence to high-fiber diets are assumed to be more accurate. Various strategies are used in attempts to verify self-report calendars (12, 16), but biomarkers that can be easily measured have not been developed. This study illustrates that a riboflavin tracer, incorporated into a high-fiber cereal, elevated urinary riboflavin levels in a dose-dependent manner. In addition, it could be used to validate self-report of adherence to a high wheat bran fiber dietary intervention.

The use of fecal weight to confirm supplemental fiber intake produced data with high intrasubject and intersubject variability, along with overlap of pre- and postintervention measurements, problems documented by other researchers (7, 8). The results reported here reflect the difficulty in using 3-day stool output to determine participant adherence to a high-fiber intervention. Recruitment is particularly challenging when stool collection is involved. Participants working outside the home find 72-h stool collections difficult. Few people are willing to attempt it, and specimens are usable only if collected and stored as instructed. These obstacles result in studies with small sample sizes. Furthermore, there is no easy method to validate completeness of outpatient stool collections. In contrast, completeness of 24-h urine collections can be validated by cross-checking with creatinine levels, acceptable norms of which have been established.

As anticipated in the outpatient phase of the study, preintervention/postintervention differences in riboflavin excretion and wet stool weights showed a moderate correlation (r = 0.57). Urinary riboflavin excretion showed an extremely strong correlation with dose (r = 0.95; Fig. 1). Therefore, it can be concluded that the change in riboflavin provided a more precise

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measure of the supplement intake than the change in stool weight. It is important to note that posttreatment stools and urine contain riboflavin and fiber from both the supplement and other foods in the diet. Typical of the range in the general population, for some participants, the addition of 11.6 g/day of insoluble fiber was not large in comparison to baseline fiber intake (range, 6.1–27.0 g/day). Riboflavin supplementation resulted in approximately a 10-fold increase in riboflavin intake above baseline levels for all participants. Thus, riboflavin had a better chance to be sensitive to supplement adherence and showed a clear separation between pre- and postintervention levels in urine samples. The riboflavin marker performed well scientifically, and urine samples were considerably easier for participants to collect and handle than stool samples. Overall, the use of 24-h urinary riboflavin as an adherence marker provided a novel and precise method to assess participant adherence to a dietary intervention.

Our urinary riboflavin excretion levels were strikingly similar to those recently reported by Zempleni et al. (17), who studied the pharmacokinetics of orally administered riboflavin supplements (20–60 mg). Despite the fact that our supplement was incorporated into a foodstuff, the reproducibility of the data reinforced our belief in the accuracy of the methodology. Use of a riboflavin marker in clinical trials requires careful taste monitoring for bitterness. Participants must be informed that riboflavin will cause yellow coloring in food and urine. In addition, consistency in riboflavin content in the formulated cereal requires quality control assessments. To minimize riboflavin deterioration, care must be taken to keep cereal and urine samples in the dark and at optimal storage temperatures. Orally administered riboflavin is rapidly absorbed, and >90% is excreted within 24 h of intake (17). Therefore, the use of a riboflavin marker is limited to verifying the level of dietary adherence in the previous 24 h. Nevertheless, we conclude that a riboflavin tracer as a biomarker for fiber intake in a community study is more feasible and sensitive than previously used methods.

The methodology developed in this study can easily be adapted to similar intervention protocols. With appropriate pilot studies, riboflavin can be incorporated into foods or added to pills as an internal marker. This approach provides a noninvasive, precise method for measuring adherence that can validate self-report.

References
Development of a urinary riboflavin adherence marker for a wheat bran fiber community intervention trial.
