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Riboflavin as an independent and accurate biomarker for adherence in a randomized double-blind and placebo-controlled clinical trial

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Abstract

Background—Medication adherence is critical for success of clinical trials.

Objective—To assess oral riboflavin is an adherence marker.

Methods—Riboflavin was incorporated into active treatment and placebo pills for a clinical trial lasting for 2 years.

Results—The accuracy (area under the receiver operating curve) of urinary riboflavin was 0.91 as a binary classifier of adherence, and was similar or better than for two active study ingredients daidzein (0.92) and genistein (0.87) (all $P < 0.0001$). Decreased adherence over time was similar in the two study groups.

Conclusion—Riboflavin is an accurate and useful biomarker for study pill ingestion.

Keywords

biomarker; medication compliance; adherence; clinical trial; riboflavin; daidzein; genistein; receiver operating curve; sensitivity; specificity; accuracy

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Authors' contributions

V-MSR: Had overall responsibility for design conduct and interpretation of analyses of riboflavin daidzein and genistein and contributed to study design and manuscript preparation and has given final approval of the version to be published

FN: Contributed to study management including sample and data acquisition quality-control laboratory analyses statistical analyses interpretation of the data preparation the manuscript and has given approval of the version to be published

KEA: Contributed to conception and design of the study interpretation of study findings and manuscript preparation had overall responsibility for clinical aspects of the study and approved the version to be published

YFK HJ and NWC: Had overall responsibility for design and conduct of statistical model analyses and interpretation of the data contributed to manuscript preparation and approved the version to be published

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Introduction

Adherence to medication ingestion is crucial for the success of clinical trials (Vrijens et al., 2012, Czobor and Skolnick, 2011) and is also important for therapeutic efficacy in clinical practice (Cutler and Everett, 2010). Low adherence in clinical trials can lead to not achieving study outcomes, underestimating effect sizes and inadequate safety assessments (Demarkwahnefried et al., 1997, Demers, 1997, Vrijens and Urquhart, 2014), and may substantially increase the cost and reduce the success of drug development. Among patients with chronic medical conditions, adherence has been reported to range from 35 to 97% in clinical trials (Divi et al., 1997, Dickinson et al., 1988, Dhar et al., 1990), and from 43 to 78 percent in clinical practice (Waeber et al., 1999, Claxton et al., 2001, Cramer, 2003). Side effects of drugs, such as those commonly seen with cancer chemotherapeutic agents (Daniell, 1997, Dees et al., 1997) and compromised mental status (Carroll et al., 2004) can contribute to low adherence. Additional examples are found at the iAdherence web site (<http://www.iadherence.org>), as reviewed by Blaschke et al. (Blaschke, 2012). Three-way interactions among providers, patients, and the health-care system have been found to increase adherence (Osterberg and Blaschke, 2005). Therefore, objective measures remain important to document adequate adherence in clinical research and practice.

Indirect methods for monitoring adherence, such as self-reports, pill counting, and electronic surveys, are challenging and generally have low sensitivity and specificity (Douek et al., 1998, Domagalski et al., 1992, Kastrissios and Blaschke, 1997, Draper et al., 1997). Electronic monitoring devices that record accessing medication containers can enhance assessment of trial efficacy (Vrijens et al., 2005). More direct methods include directly supervising therapy, measuring a medication component or its metabolites in blood or urine, or adding an independent and unrelated marker substance to the test pills and then measuring the marker in urine or blood (Drane et al., 1980, Osterberg and Blaschke, 2005, Ho et al., 2009, Ramanujam et al., 2011). However, measurement of many intervention substances (e.g. antibody-based therapeutic agents) in urine or blood, can be analytically challenging, and is also difficult for compounds with short half-lives or large inter-individual differences in metabolism and disposition (e.g., soy isoflavones). Water soluble compounds that are excreted within one day, such as riboflavin (Ramanujam et al., 2011), lithium (von Houwelingen et al., 1987, Donahoo et al., 2004), *p*-aminobenzoic acid (Bingham and Cummings, 1983, Roberts et al., 1990), or radioactive chromium (Wolen et al., 1984) have been added to study diets or study pills and assayed in serum or urine as measures of adherence. We chose to use riboflavin because of its better known chronic toxicity and also it produces a bright-yellow color in urine that may serve as a visual signal of adherence for study participants. Riboflavin (vitamin B₂) has been used as an easily measured marker for assessing adherence in clinical research. When administered in doses up to 50 mg/day, riboflavin is rapidly absorbed and excreted in urine and has minimal adverse effects (Powers, 2003). It may have some beneficial therapeutic effects including decreased circulating levels of homocysteine, which may reduce risk for cardiovascular disease (Switzer et al., 1997, Hustad et al., 2000, Powers, 2003), and prevention of interval headaches (mild migraine or tension headaches) in children (Bruijn et al., 2010, Wilson et al., 2012), which may be a limitation for its use in some therapeutic trials.

Prior studies of riboflavin as a tracer for monitoring adherence have focused primarily on improving analytical methods for real-time monitoring of adherence (Fuller et al., 1984, Young et al., 1984, Fuller et al., 1986, Kapur et al., 1991, Hungerbuhler et al., 1995, Thilothammal et al., 1995, Baros et al., 2007, Ramanujam et al., 2011). We previously studied pharmacokinetics of riboflavin in healthy subjects given multiple daily supervised oral doses of riboflavin. We measured riboflavin in urine collected continuously for 12 hours before dosing, for 24 hours during each of the 4 days of dosing, and for 24 hours after the last dose in a supervised, outpatient clinical research setting (Ramanujam et al., 2011). Modification of the analytical method also enabled rapid on-site assessment of adherence at the time of study visits. We found that riboflavin concentrations, even in a single void sample collected 24 hrs after an oral dose of 30 mg of riboflavin, exceeded 1.0 µg/mL, and were higher than pretreatment levels (Ramanujam et al., 2011).

The purpose of this study was to validate the use of riboflavin for assessing adherence in a clinical trial in an ambulatory setting. The study involved unsupervised daily ingestion of a study pill that contained soy isoflavones as the active ingredients and riboflavin as an adherence marker for 5 days per week for up to 24 months. Because riboflavin is ubiquitous in foods and dietary supplements, we estimated basal levels of urinary riboflavin and devised a cut-off point for riboflavin concentration for assessing adherence. We also compared the performance of riboflavin as an adherence tracer against two active study pill ingredients that, in previous studies, displayed large inter-individual differences in metabolism (Lu et al., 1996, Ren et al., 2001) to illustrate the usefulness of having an adherence marker independent from treatment ingredients.

Materials and methods

Study design

This randomized, double-blind, placebo-controlled with two parallel arms was approved by the Institutional Review Board of the University of Texas Medical Branch (UTMB). Written informed consent was obtained from all subjects. The primary purpose was to study the biological effects of soy isoflavones in women, and those trial results will be reported separately. A secondary purpose was to validate riboflavin as a biomarker of adherence to daily ingestion of study pills in both placebo and active treatment arms of the study, as reported here. Trial registration: www.clinicaltrials.gov and the identifier is NCT00204490.

The study subjects were 197 premenopausal women between 30 and 42 years of age with regular monthly menstrual cycles. Four baseline visits consisted of two visits during each of two separate luteal phases not more than 6 months apart, after which 197 qualified subjects were randomized to treatment with isoflavone or placebo pills. Each placebo pill contained a 246 mg carbohydrate filler (90% maltodextrin and 10% Sethness caramel color). Each isoflavone pill contained 246 mg Nova Soy, which consisted of soy glycones (daidzin, genistin, and glycitin) and aglycones (daidzein, genistein, and glycitein) at a 9:1 molar ratio; the total aglycone equivalent amounts were 30 mg daidzein, 30 mg genistein, and 8.3 mg glycitein. Both the placebo pill and isoflavone pill also contained 15 mg riboflavin, 60 mg sorbitol, 3 mg magnesium stearate, and 676 mg dicalcium phosphate to give a final tablet weight of 1000 mg. Both pills were identical in appearance, designed by Dr. Brent

Flickinger, and generously provided for this study at no cost by Archer Daniel Midland Co. (Decatur, IL).

Subjects were randomized in blocks of six using the PLAN procedure in SAS[®] (SAS Version 9.3, SAS Institute, Inc. Cary, NC), which assured equal sizes of the study groups. All subjects, research staff, and investigators were blinded to the treatment assignments, which were known only to research pharmacists who dispensed supplies of the study pills but were not involved in other aspects of the trial. At each study visit subjects were given a three month supply of study pills in blister packs. Each blister for daily dosing contained two study pills and one prenatal vitamin pill (Rugby Prenavite Prenatal Formula, Swanson Health Products, Duluth, GA) that met the required daily intake of vitamins and minerals. Subjects were instructed to avoid vitamin supplements not provided by the study, and to take the pills contained in one blister pack daily for 5 days per week for up to 2 years.

Administration of study pills began on the second day of the menstrual bleeding following the fourth baseline visit. Study visits then occurred at 3-month intervals and between 20 to 24 days after onset of menstrual bleeding. Before each visit the subjects collected urine for 12 hours overnight in a 3 liter, light-protected container containing 1 gm sodium azide and 10 mL glycerol as preservatives. Aliquots of urine were stored at -20°C until analyzed for riboflavin by a high performance liquid chromatography (HPLC) with fluorescence detection (Chen et al., 2005, Ramanujam et al., 2011), and for daidzein and genistein by a previously described gas chromatography-flame ionization detection (GC-FID) method (Lu et al., 1995).

All baseline and treatment phase urine samples were analyzed for riboflavin, daidzein, and genistein by one of the investigators who was blinded to treatment assignment. Riboflavin results were used in discussions with the study participants regarding their adherence to ingestion of study pills, whereas the isoflavone results remained blinded to the subjects and members of the study team until the trial ended and data were ready for analysis.

Statistical analyses

The performance of a diagnostic test is often measured by the area under the curve (AUC) of the receiver operating curve (ROC). For ROC analyses, the true positive rate (sensitivity) is plotted as a function of the false positive rate (1-specificity) for different cut-off points of the measured parameter. Urinary concentrations of riboflavin, daidzein, and genistein ($\mu\text{g/mL}$) from subjects assigned to the isoflavone group were used to construct and compare the ROCs for these three analytes. Subjects in this arm of the study received study-provided pills containing isoflavones and riboflavin only during the intervention phase. Therefore, urinary levels at baseline were considered as reference of “truth” for no exposure to the study-provided isoflavone pills (i.e., the variable for exposure to isoflavone study pill was zero at baseline) and those during the intervention phase were “truth” of positive exposure to riboflavin and isoflavones (i.e. the variable for exposure to isoflavone pills was equal to 1). Urine samples from placebo subjects could not be included in pair-wise comparisons of the ROCs for these three substances, because they only were given riboflavin but not isoflavones.

For assessment of yes/no adherence, cut-off points (decision thresholds) for concentrations ($\mu\text{g/mL}$) of all three analytes were estimated by three different methods. Two approaches, close to '0,1' and Youden index methods (YODEN, 1950), gave equal weight to sensitivity and specificity and imposed no ethical, cost, and prevalence constraints, while the third approach, 'pre-test probability, cost ratio', allowed incorporation of the effect of costs and/or the prevalences for cut-off selection (Metz, 1978, Zweig and Campbell, 1993). When the cost ratio equals to one (cost of false positive and false negative is the same) and the prevalence is assumed to be 0.5 ('flip of the coin'), the cut-off point of pre-test probability=0.5 and cost ratio=1 will be equivalent to the Youden index.

For assessing the effects of treatments on adherence across study visits, urinary levels of riboflavin from all supplement-phase samples of the control and isoflavone groups were included. Baseline samples, which were collected before starting the study pills, were not used, because they did not involve ingestion of study pills. Adherence prior to each study visit for each subject was first categorized as yes or no based on whether the urinary level of riboflavin for that submitted urine sample was equal to or greater than, or less than the cut-off point, respectively. Overall distribution of adherence by group assignment was calculated from all available intervention phase samples. To account for variation within subjects on observations across study visits, the generalized estimating equations (GEEs) method was used to evaluate the adherence (binary dependent variable) in both the placebo and isoflavone treatment groups over the supplement phase of the study. A GEE allows us to achieve a marginal model for weak distribution assumptions for binary outcomes without correct specification of correlation structure. The model included treatment assignment (intention-to-treat), follow-up visits (up to 8 visits), and an interaction term between treatment assignment and follow-up visit. Analyses were performed using the built-in ROC function in SigmaPlot version 12.3 for Windows (Systat Software, Inc., San Jose, CA) and SAS Version 9.3. (SAS Institute, Inc. Cary, NC).

Results

In this double-blind, placebo controlled, randomized study with two parallel arms, all subjects ingested two study pills, each containing 15 mg of riboflavin, per day for 5 consecutive days each week for up to 2 years. Because no pills were taken for 2 days each week, collection of single void urine samples could occur up to 72 hrs after the last oral dosing. Subjects were asked not to take vitamin supplements other than the one provided by the study. Of 406 women screened, 197 were eligible and entered the intervention phase of the study and were randomly assigned to either the isoflavone (N=99) or placebo groups (N=98). Thirty-one randomized subjects (15 subjects from the placebo and 16 subjects from the isoflavone groups) did not return for the intervention phase follow-up visits and were not included in the analysis. Baseline and intervention urine samples were available from 166 subjects (83 in each group) for measurement of riboflavin and isoflavones.

Examples of urinary levels of riboflavin, daidzein, and genistein at each study visit (4 at baseline visits and, depending on the length of study participation, up to 8 intervention visits) from subjects with adherence ranging from low to high are shown in Figure 1. In the placebo group (Figure 1A–1C), only riboflavin increased above baseline visits (indicated by

study visit numbers = 0) during the intervention phase (indicated by study visit numbers >0). Placebo subjects with low, medium, and high compliance had one, four, and eight urine samples positive for riboflavin, but, as expected, no increases in either of the isoflavones over baseline values (Figures 1A, 1B and 1C, respectively). In the isoflavone group (Fig. 1D–1F), all urine samples during the intervention phase that were positive for riboflavin also contained higher levels of daidzein and genistein compared to baseline. However, varying degrees of adherence were also found in subjects with two, three, and eight samples positive for high levels of all three analytes indicating low, medium, and high compliance (Figures 1D, 1E and 1F, respectively). Riboflavin levels that were similar to baseline levels in either treatment group indicated that subjects did not ingest the study pills. For example, the subject in Figure 1A did not take placebo pills within 24 hrs of scheduled visit numbers 1, 2 and 4–7 and the subject in Figure 1D did not take isoflavone pills within 24 hrs before scheduled visit numbers 2 and 4–8.

The performance of riboflavin as a compliance tracer was analyzed from the AUC of the ROC constructed for riboflavin, daidzein, and genistein, using data from subjects in the isoflavone group. For these ROC analyses, a new categorical variable for a gold standard of truth was created. Urine samples during baseline were assigned to truth of no exposure to study pills, while urine samples during intervention phase were assigned to truth of positive exposure to study pills. ROC analyses are shown in Figure 2 and Table 1. AUCs of ROCs for all three analytes were >0.5 (represented by the 45 degree diagonal line in Fig. 2), and all P-values were <0.0001 (95% CI in Table 1).

ROC analyses indicate that all three analytes served as good biomarkers for discriminating adherence (yes vs. no) to medications in the isoflavone intervention group. Application of the method of DeLong et al. (DeLong et al., 1988) for calculating the Standard Error of the AUC and the difference between two AUCs showed no significant differences between the ROCs of riboflavin and daidzein (−0.005, 95%CI: −0.027 to 0.017, ChiSquare: 0.19, and P=0.66). There were significant differences between ROC areas for daidzein and genistein (0.045, 95%CI: 0.028 to 0.061, ChiSquare: 26.8, P<0.0001) and between riboflavin and genistein (0.040, 95%CI: 0.015 to 0.064, ChiSquare: 10.2, P=0.001). These results show that riboflavin and daidzein have similar measures of accuracy in binary classification of whether or not a subject ingested isoflavone study pills. While genistein also performs well in binary classification of adherence, it does not perform as well as that of daidzein or riboflavin in adherence classification.

To perform binary classification of adherence, decision thresholds (i.e., cut-off points) of urinary concentrations were developed. For cut-off points between 1.16 to 1.42 µg/mL of riboflavin in urine, specificity is about 0.9 and sensitivity is about 0.8. When cut-off points were decreased to 0.8 µg/mL of riboflavin, sensitivity increased to ~0.9 but the specificity decreased to 0.75. Youden indices, which represent thresholds with maximized specificity and sensitivity, were 1.42 µg/mL for riboflavin, 2.06 µg/mL for daidzein, and 2.20 µg/mL for genistein (Table 1).

To compare adherence between the two groups in this trial, we chose the riboflavin cut-off point of 1.42 µg/ml (Youden index, Table 1), which minimizes false positive classification

(i.e., erroneous classification of non-adherent subjects as adherent) more than false negative classification (i.e., erroneous classification of adherent subjects as non-adherent). Overall adherence rates, which included all intervention phase visits, were similar between the isoflavone and placebo groups (76.8% vs. 78.7%, $P=0.49$). The analyses with GEEs, which assess within-subject variation during the intervention phase, indicated that the adherence rates decreased significantly with each intervention phase visit (OR: 0.91, 95% CI: 0.84 – 0.99) and to a similar degree in the isoflavone and control groups ($P=0.49$). Changing cut-off points affected adherence rates but did not affect the results of comparisons between the two groups (results not shown).

A graph showing urinary excretion of riboflavin, daidzein, and genistein was generated for each subject, as shown by the examples in Figure 1. After visual inspection of graphical displays of all 197 randomized subjects, we found examples of urinary profiles of all three analytes from some subjects that were inconsistent with their random assignment codes. This suggested that errors had sometimes occurred in dispensing of study pills (four examples are shown in Figure 3). Soy-enriched products and isoflavone-rich foods (e.g. legumes) are ubiquitous in the diet, and for this reason we used a very conservative cut-off for urinary daidzein of 2.5 $\mu\text{g/mL}$ (specificity 0.99). We identified twenty placebo subjects with urine samples that tested positive for both daidzein and genistein (indicated by arrows in Figure 3) and had high levels of riboflavin as well (3 examples are shown in Figure 3A–C). For subjects randomized to the isoflavone group, using a riboflavin cut-off $>3.0 \mu\text{g/mL}$ (specificity of adherence in ingestion of the study pills of ~ 0.99) and a daidzein level $<2.06 \mu\text{g/mL}$ (below cut-off, Table 1), four were found to have received one batch of placebo study pills (see example in Fig. 3D). Arrows in all panels indicate such likely errors in pill dispensing. Treatment assignment could not be confirmed by urinary riboflavin and isoflavone results for three subjects because their urine samples tested negative for all three analytes, indicating non-adherence to ingestion of study pills.

Discussion

We showed previously that after a supervised oral dose of 30 mg of riboflavin given to subjects observed in a clinical research unit, this substance was readily detected in single void urine samples for at least 24 hours and that riboflavin did not affect the time courses and peak levels of daidzein and genistein (Ramanujam et al., 2011). The present study of unsupervised ingestion of study pills in ambulatory subjects is the first proof-of-concept study showing that riboflavin use has multiple beneficial applications in a RCT. Firstly, adherence to daily ingestion could be directly assessed in every subject. Secondly, variable adherence between and within subjects was documented (Figure 1). Thirdly, the area of ROC plots showed that riboflavin has a performance accuracy of 0.91 (Figure 2) as a binary classifier of adherence. Its accuracy in discriminating yes/no adherence (Figure 2) was comparable to or better than that for the two active study ingredients, daidzein and genistein (areas of ROC plots = 0.92 and 0.87, respectively). Fourthly, the method was able to detect decreased adherence over time during the treatment phase, which lasted for up to 2 years, and that this decrease in adherence was balanced between the two randomized treatment groups. Fifthly and unexpectedly, it served as a quality control measure in this RCT, by detecting errors in dispensing of active treatment or placebo pills (Fig. 3).

We evaluated the *accuracy* and *usefulness* of riboflavin as an adherence measure. *Accuracy*, or performance, of a diagnostic test is traditionally assessed by the area under a ROC, and not only by the specificity and sensitivity of an arbitrary cut-off decision threshold from a ROC (Metz, 1978, Zweig and Campbell, 1993). The accuracy of a ROC analysis is dependent on the truth references chosen for both positive truth and negative truth of exposure. Two different forms of a gold standard of truth for adherence were used for ROC analyses. The first form is the intentional incorporation of riboflavin into components of treatment pills, a unique feature of our study design. This allowed us to use active treatment ingredients, daidzein and genistein, as an internal, gold standard reference of truth of yes/no ingestion of study pills also containing riboflavin. The second form, and the most commonly used form of a gold standard of truth of adherence, is supervised ingestion. We investigated the ROCs of riboflavin, daidzein, and genistein using the 3-components-in-1 study pill under both supervised ingestion in a previous study (Ramanujam et al., 2011) and unsupervised ingestion in the current study.

We showed previously that the pharmacokinetics of these two isoflavones in humans (Lu et al., 1996) are similar to that of riboflavin when studied under supervised ingestion conditions (Ramanujam et al., 2011). The excretion of all three analytes peaked at 6–8 hrs and was near completion 24 hrs after ingestion. When the ROC of riboflavin was measured in this previous study with supervised pill ingestions, it had AUCs of 0.99 and 0.94 for urine collected between 0–8 and 8–24 hrs, respectively (Ramanujam et al., 2011). In the present study in which study pill ingestion occurred at home and was unsupervised, we found the area of the riboflavin ROC to be 0.91 (Fig. 2). Thus, time of urine sampling relative to last oral dosing affects areas of ROC. An area of 1 would represent a perfect binary adherence classification tool. An AUC of 0.91 for riboflavin indicates that there is a 91% likelihood that any randomly selected individual (from either the placebo or treatment groups) who is adherent will have higher urinary riboflavin than any randomly selected individual who is not adherent. Similarly in the active treatment group, the isoflavone ROCs indicate that any randomly selected subject who is adherent will have 92% and 87% likelihood of higher urinary daidzein and genistein, respectively, than any randomly selected non-adherent subject. Thus, riboflavin performs well as a binary classification tool for adherence to a daily dosing regimen.

Usefulness

Four examples are shown below.

A marker of ingestion

Adherence assessment has traditionally relied upon convenient and inexpensive approaches such as residual pill counting with or without electronic surveillance methods. But these approaches do not detect whether pills are taken or discarded. Use of riboflavin as marker that is an ingredient of study pills can assure that ingestion has taken place. Riboflavin taken in amounts provided in this study has a bright yellow color when excreted in urine. Subjects in placebo and active treatment groups may detect this coloration after pill ingestion. In spite of a possible association of adherence of pill ingestion with bright yellow urine color, subjects from placebo and isoflavone groups both brought back urine that showed no

riboflavin (see examples in Fig. 1) indicating no ingestion within the last 24 hrs. In this RCT, subjects were asked to take study pills daily for 5 consecutive days per week with two study pill holidays. It is possible that urine samples negative for pill ingestion could be from study visits that might occur less than one to up to 3 days of last pill dosing, and this may partly explain the variable adherence shown in Figure 1, e.g., 1 of 8 (Fig. 1A) and 8 of 8 (Fig. 1C and 1F) as positive adherence. A combination of electronic methods that document accessing pill containers and tracer methodology that documents pill ingestion may be optimal for assessing medication adherence in clinical studies.

An independent marker of adherence when active treatments such as daidzein and genistein exhibit large inter-individual differences in metabolism (Lu et al., 1996, Ren et al., 2001). In this study, the AUC of the riboflavin ROC was not significantly different from daidzein, whereas that for genistein was significantly different from riboflavin and daidzein (Table 1). This is consistent with the well-known larger inter-individual differences in metabolism of genistein, which has a lower urinary recovery of 0 to 30% compared to the recovery of 50 to 80% for daidzein (Lu et al., 1996, Ren et al., 2001). Our data support the need and the usefulness of an independent adherence marker, such as riboflavin, when the metabolism of an active ingredient is highly variable.

Useful for comparing adherence between RCT groups

In adherence assessment, a false positive (1-specificity) is a non-adherent subject wrongly classified as adherent, while a false negative (1-sensitivity) is an adherent subject wrongly classified as non-adherent. A Youden index derived from ROC data is a commonly used cut-off point where specificity and sensitivity are both maximized from ROC data. The Youden index for riboflavin was 1.42 $\mu\text{g/mL}$ (Table 1). A Youden index is equivalent to assuming pre-test probability of 0.5 and economic cost ratio of 1, i.e., the cost ratio of false positive to false negative being the same (Table 1). However, assuming pre-test probability of 0.5, for a cost ratio decreasing to 0.8 or increasing to 1.2, the cut-off for riboflavin remains 1.42 $\mu\text{g/mL}$. This concentration is, therefore, an optimal threshold for adherence decision for riboflavin. Using this cut-off point, we found that the rates of medication adherence decreased with duration of intervention but did not differ between placebo group and isoflavone active treatment group, as would be expected in a well-randomized study. The temporal change in adherence rate is consistent with previous reports, even in the absence of severe side effects (Blaschke, 2012).

Quality control of study samples

We also found, unexpectedly, that riboflavin inclusion in study pills was useful as a quality measure for correct provision of the study drug (Figure 3). We suspect that instances of providing the wrong pills to some subjects might result from errors during manufacturing, given that these error rates were not balanced by randomization, in contrast to all baseline characteristics that were balanced between treatment groups (Lu et al. manuscript submitted). Moreover, detection of these errors allowed us to model statistical analyses accordingly when studying biological effects (Lu et al., manuscript submitted).

Limitation

Many strengths have been discussed above. The tracer technology in this proof-of-concept project may be inconvenient to study subjects and can be costly to operate if a large number of urine samples are needed. However, the tracer technology has the potential of being developed into a home-based assay, should the concept become popular in clinical trial communities. The possibility that study subjects ingested study pills only on the date of urine collection could compromise the reliability of the tracer technology for measuring true rate of adherence. However, this cheating behavior should be balanced out between RCT groups and should not affect its usefulness as an adherence covariate. We did not test the shelf-life of riboflavin in tablets, which may not be a problem as we were able to measure its urinary concentrations in subjects who participated for 2 years (Figs. 1C & 1D), although rigorous testing is required. In a prior study, we tested the stability of riboflavin in urine stored at -20°C and found it to be stable for analyses after repeated defrosting.

Conclusions

We have shown that riboflavin is an accurate and useful marker for adherence to daily oral dosing in a RCT. Its accuracy is 0.91 as a binary classifier. It is useful because it assesses ingestion rather than the accessing of study pills, can be used as a marker that is independent of active ingredients and can be incorporated into both placebo and active treatment pills, and can serve as a quality control measure for the provision of study pills. We suggest that use of riboflavin as an adherence tracer can be effectively combined with residual pill counting in future clinical trials.

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Abbreviations used

HPLC	high performance liquid chromatography
GC-FID	gas chromatography-flame ionization detection
AUC	area under the curve
ROC	receiver operating curve
RCT	randomized clinical trial
GEE	generalized estimating equation

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Figure 1.

Examples of female subjects with low (A and D) medium (B and E) and high (C and F) degrees of adherence to medication in the placebo (A to C) and isoflavone-treatment (D to F) groups in this double blind randomized placebo-controlled study. Riboflavin (x), daidzein (o) and genistein (Δ) concentrations were measured in a 12-hour overnight urine sample collected just prior to each study visit (0 indicates start of treatment, 0 baseline visits and >0 visits during treatment).

Figure 2.

Receiver operating curves (ROC) and corresponding areas under the curves (AUC) comparing the sensitivity and specificity of riboflavin as a tracer for adherence to two active treatment components daidzein and genistein. Urinary concentrations of riboflavin, daidzein, and genistein in subjects assigned to the isoflavone study group were used for pair-wise analyses. Baseline data represent no exposure to all three components (truth of negative adherence) and data from follow-up visits during the intervention phase of the study represented exposure to study pills (truth of positive adherence).

Figure 3.

Examples of including riboflavin as a tracer for adherence to the medication in identifying study samples from subjects that were provided the wrong study pills. Arrows indicate times of errors in pill dispensing. Panels A–C: subjects assigned to placebo but with isoflavone exposure (indicated by arrows); Panel D: a subject in the isoflavone group who ingested one batch of placebo pills (indicated by an arrow).

Receiver operating curve analyses of riboflavin daidzein and genistein as adherence markers for medication ingestion^a

Table 1

	Area Under the Curve(95% CI)	Method	Cut-off Point (µg/ml)	Sensitivity	Specificity
Riboflavin	0.91 (0.89 0.93) P<0.00001	Pre-test p=0.5 Cost ratio=1	142 ^b	0.77	0.91
		Close to '0, 1'	1.16 ^c	0.80	0.87
		Youden index	1.42 ^d	0.77	0.91
Daidzein	0.92 (0.90, 0.94) P<0.00001	Pre-test p=0.5, Cost ratio=1	2.06 ^b	0.80	0.96
		Close to '0, 1'	1.83 ^c	0.82	0.92
		Youden index	2.06 ^d	0.80	0.96
Genistein	0.87 (0.85, 0.90) P<0.00001	Pre-test p=0.5, Cost ratio=1	2.20 ^b	0.72	0.95
		Close to '0, 1'	1.90 ^c	0.78	0.89
		Youden index	2.20 ^d	0.72	0.95

^aSee Fig. 1 for receiver operating curves (ROCs)

^bData point on a ROC assuming pre-test probability of 0.5 and cost ratio of false positive to false negative being 1

^cData point on a ROC with the shortest distance to false positive being 0 and sensitivity being 1

^dData point on a ROC being farthest to the 45 degree diagonal line