Urine Nitrogen as a Biomarker for the Validation of Dietary Protein Intake[1]

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ABSTRACT When validated for completeness, 24-h urine nitrogen obtained from repeated 24-h urine collections has provided useful insights into the validity of dietary assessments, underreporting behaviors and the structure of measurement errors that are associated with different methods. This is particularly so when nitrogen is combined with another marker in 24-h urine samples, potassium. Although the collection of 24-h urine is a tedious procedure, the method is readily accessible and comparatively inexpensive. Other markers of dietary intake and intermediate risk markers may also be measured in the 24-h urine that is obtained. J. Nutr. 133: 921S–924S, 2003.

KEY WORDS: • *urine nitrogen* • *dietary assessments* • *biomarkers* • *protein* • *epidemiology*

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Used primarily as a biomarker of the validity of dietary assessments, 24-h urine nitrogen is the most well-known biological marker. Individual results from published metabolic studies where dietary intake is kept constant over prolonged periods of time show a fair correlation between daily nitrogen intake and daily urine-nitrogen excretion. The use of 24-h urine nitrogen depends on the assumption that subjects are in nitrogen balance and there is no accumulation due to growth or repair of lost muscle tissue or loss due to starvation, dieting or injury. This was appreciated as early as 1924, when it was suggested that actual protein intake as assessed from 24-h urine excretion was far lower than the recommended level (1).

The apparent accuracy of 24-h urine nitrogen as a biological marker led to the suggestion that it be used to validate estimates of protein intake from various dietary survey methods (2). In 1980 Isaksson summarized a number of studies carried out by his group and showed that estimates of protein intake obtained from 24-h recalls of food intake were low when compared with the urine nitrogen, but those estimated from diet histories and records were in good agreement with the urine values (2). Van Staveren also found good agreement between 24-h urine and diet-history estimates of protein intake (3). Reported protein intake in obese subjects (from a diet history) was only 46 g, but on the basis of 24-h urine collections, it was 87 g. In another study, subjects who were overweight or diabetic seemed to report their prescribed diet rather than what they were actually eating as judged by the urine nitrogen excretion (4,5).

24-h urine nitrogen for validation of individual estimates of protein intake

These comparisons could only be made on a group basis, because each individual contributed only one or two 24-h urine collections. Other early comparisons between average urine nitrogen and dietary intake have been summarized (6).

To investigate the applicability of using 24-h urine nitrogen to validate estimates of protein intake on an individual basis, four men and four women were given their usual varying diet over a 28-d period while they lived in a metabolic suite (7). Duplicates of diets were made up each day for each individual, and 24-h urine and fecal collections were also made during this period. Diet, urine, feces and skin losses were measured for their nitrogen contents (7). As judged by the correlation coefficient of 0.99, there was almost complete agreement between the 28-d diet and the urine estimation. Urine nitrogen underestimated intake at higher levels of protein intake and overestimated at lower levels, but a constant factor for fecal and skin losses can be used to counteract this, and output from urine can be expressed as a ratio of intake, 0.81 (7). Although this study was based on results from a comparatively small group, a later meta-analysis of a large set of data has confirmed that urine nitrogen should be ~80% of dietary intake on average (8). However agreement between individual estimates of usual protein intake and the 24-h urine-nitrogen output are not as good if fewer observations on each individual are made and if the collections are not verified for their completeness.

Number of days of recording intake or excretion required for characterization of an individual

Daily variation is such that on any one day an individual is not likely to be in balance; therefore measurements of intake and output are required over several days to characterize the relationship within an individual with reasonable reliability. The expected correlations between daily intake and output are in the region of 0.5 when a single day's data are used, and a low estimate of precision yields a coefficient of variation of 24%. When 8 d of urine collections and 18 d of dietary observation

[1] Published as part of *The Journal of Nutrition* supplement publication "Biomarkers of Nutritional Exposure and Nutritional Status." This series of articles was commissioned and financially supported by International Life Sciences Institute, North America's Technical Committee on Food Components for Health Promotion. For more information about the committee or ILSI N.A., call 202-659-0074 or E-mail ilsina@ilsi.org. The opinions expressed herein are those of the authors and do not necessarily represent the views of ILSI N.A. The guest editor for this supplement publication was Jo Freudenheim, University at Buffalo, State University of New York, Buffalo, NY 14214.

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0022-3166/03 $3.00 © 2003 American Society for Nutritional Sciences.

are available, the correlation improves to 0.95, and the coefficient of variation falls to 5%. Several 24-h collections that have been validated for completeness are therefore required to make accurate comparisons with individual dietary intake data; the exact number depends on the reliability that is required. With an average coefficient of variation of 13% in urine-nitrogen excretion, 8 d of collections will estimate nitrogen output to within 5%. In this case, the expected ratio of urine nitrogen to dietary nitrogen is 0.81 ± 0.05 for valid estimates of dietary intake (7).

Verification of the completeness of 24-h urine collections

Earlier studies used creatinine to check on the completeness of urinary collection. Creatinine excretion is dependent upon both creatinine intake (primarily from meat in the diet) and creatinine production (which is proportional to fat-free mass). When diets vary considerably within individuals especially in their meat content, creatinine excretion is not a reliable marker of completeness. When collections are known to be complete, within-subject variability of urinary creatinine has been shown to be on the order of 10%, which is similar to that for urinary nitrogen.(9,10). Between-subject variation in creatinine excretion is ~23% in a mixed population, which is again similar to total nitrogen (9).

Two external makers are in use to verify the completeness of 24-h urine collections. Lithium is completely absorbed and excreted and has been used, for example, to assess sodium consumption when sodium is added as cooking or table salt (11). Lithium can also be used to assess the completeness of 24-h urine collections, although the salt does have to be given to subjects every day some days before the intended 24-h urine collection so that equilibrium can be achieved.

Para-aminobenzoic acid (PABA)[3] is actively absorbed and excreted, so it can be used to check on the day of the 24-h collection to verify completeness (10). The method consists of three 80-mg tablets of PABA that are taken with meals. This is quantitatively excreted within 24 h, so that single 24-h collections that contain < 85% of the PABA marker can be classified as unsatisfactory either because the tablets have not been taken or because one or more specimens were omitted from the collection. There is a systematic difference, particularly in urea and total nitrogen, between collections that are deemed complete by the PABA method and ones that contain < 85% of the PABA marker (9,10). Omission of such a marker therefore causes underestimation of 24-h urine nitrogen or urea output and contributes to the poor agreement between estimates of usual intake, diet and estimates of 24-h urine output. PABA has been used extensively in methodological studies carried out in the U.K. (12–16), Italy (17), France (18), the U.S.A. (19 and unpublished data, A.T. Subar et al. 2002), Denmark (20) and Germany (21).

Partial 24-h urine collections

Owing to the difficulty of obtaining complete 24-h collections, replacement with partial collections is sometimes suggested. Correlations between nitrogen in partial collections with full 24-h collections on the order of 0.50–0.81 have been found (22,23). However the extent of agreement probably depends on the timing of diet and main meal consumption, which varies among different populations, so that it is not possible to make general statements of the utility of partial collections other than to say they are less accurate than full 24-h collections for estimating protein intake.

To investigate the possibility that repeat overnight collections would improve comparisons between dietary nitrogen intake and nitrogen from partial urine collections, 39 men were asked to keep an overnight urine collection immediately before making a 24-h collection on eight occasions. The correlation between the mean nitrogen excretion from eight collections and the excretion from a single 24-h collection was 0.692, whereas the correlation between eight 24-h collections and a single overnight collection was 0.285 and with repeat overnight collections was 0.297 (unpublished data, C. Kehoe, University of Ulster at Coleraine, 1993). Even repeat overnight collections cannot replace the necessity for full 24-h urine collections, and a single collection is better than none if several cannot be obtained from each individual in validation studies.

Use of 24-h urine nitrogen to assess underreporting in individual dietary assessments

Providing sufficient complete 24-h urine collections are obtained and verified for completeness and depending on the assumption that subjects are in nitrogen balance (with no gain and no loss due to starvation or injury), dietary estimates can be compared with urine excretion on an individual basis. When these criteria are met, individuals who are judged to underreport by the 24-h urine-nitrogen method also tend to be classified as underreporters by the doubly labeled water method (12,24).

In a study of the validity of different methods of dietary assessment, 160 women were studied at home on four occasions (seasons) over the course of 1 y. At each season, the participants were asked to complete 4 d of weighed-food records. The volunteers were also asked to provide two 24-h urine collections on each occasion so that over the year each individual provided 16 d of weighed dietary records and eight 24-h urine collections (15). The completeness of the urine collections was assessed using the PABAcheck method, and only those that were complete were used to validate the dietary assessments. Average nitrogen intake from the 16-d weighed records was 11.2 ± 2.3 g nitrogen/d and that from nitrogen excretion in the complete 24-h urine was 9.84 ± 1.78 g nitrogen/d, so that the average ratio of urine nitrogen to dietary nitrogen was 0.91 ± 0.09. This was greater than the ratio of 0.81 ± 0.05 that would be expected if the average results from all individuals were valid (15).

To determine which if any of the individual results were valid, the ratio of urine nitrogen to dietary nitrogen was sorted, and data were examined as quintiles of the distribution of the urine nitrogen/diet nitrogen ratio. Means of this ratio ranged from 0.76 in the lower quintile of the distribution to 1.13 in the upper quintile. Examination of correlations between urine and dietary nitrogen, ratios of energy intake to basal metabolic rate (BMR), correlations of the energy intake/BMR ratio with the urine nitrogen/diet nitrogen ratio, body mass index (BMI) and body weight indicated that mean values from the 20% of the individuals assigned to the top quintile were different from data from the 80% of the individuals assigned to the other four quintiles (15).

All data were therefore considered separately for individuals in the top quintile and for individuals in the other four quintiles of the distribution in the urine nitrogen/diet nitrogen ratio. Not only were individuals in the top quintile heavier with a lower energy intake/BMR ratio than the others, but their intakes

[3] Abbreviations used: BMI, body mass index; BMR, basal metabolic rate; EPIC, European Prospective Investigation into Cancer; FFQ, food frequency questionnaire; PABA, *para*-aminobenzoic acid; PABAcheck, test for completeness of urine collection (Laboratory for Applied Biology, London).

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of energy and all energy-yielding nutrients calculated from weighed records were significantly lower than those from individuals in the other quintiles. On average there was an 18-g difference in reported fat consumption and a 27-g difference in reported sugar consumption between the average values reported in the top and the other four quintiles according to the urine nitrogen/diet nitrogen ratio. Mean consumption of cakes, breakfast cereals, milk, eggs, fats and sugars was also significantly lower in those individuals classified in the top quintile of the distribution. However there was no difference in reported consumption of meat, fruits, vegetables and potatoes between these underreporters and the other 80% of the population who gave valid records, nor were there differences in vitamin C or carotene. Underreporting did not seem to be limited to weighed records, because reported intakes of energy and energy-yielding nutrients by these individuals were as likely when they used another dietary assessment method, a food-frequency questionnaire (FFQ) (15).

Correlations between urine nitrogen and dietary nitrogen from different methods of dietary assessments

The extent to which methods are able to place individuals in the correct part of the distribution of intake can be examined using correlation coefficients between individual estimates of nitrogen intake from different methods and estimates of output from repeat 24-h urine collections. **Table 1** summarizes results from studies that have used two or more methods to assess intake and compared results with 24-h urine-nitrogen excretion. Correlations are greater between the biomarker and estimates of intake from records than from estimates of intake using FFQ. Energy adjustment may increase the magnitude of the correlation coefficients between results from methods and 24-h urine nitrogen. Using residuals to energy-adjust the data, estimates for nitrogen intake from an FFQ showed the most improvement in comparison with urinary nitrogen, from 0.24 to $r = 0.48$. However the correlations between 24-h urine nitrogen and protein intake assessed from weighed and estimated records remained high and did not change with energy adjustment (25).

Analysis of measurement error in individual estimates

For large epidemiologic studies, it is now common practice to correct for measurement error in the assessment of relative risk using regression calibration, and the correction factors are derived by comparison of the method in use (such as an FFQ) with a reference method (such as a record). However, this practice relies on the assumptions that errors in the reference instrument are uncorrelated with both true intake and with errors in the method in use. These assumptions can be examined if repeat estimates of intake and repeat biomarker comparisons are available. Kipnis et al. (8,30) have reexamined the work comparing repeat measures of intake of nitrogen from different methods of dietary assessment and with repeat 24-h urine-excretion values. Using a new measurement-error model that allows for individual bias (for example, in the tendency to underreport food intake), they showed that neither of these assumptions were true, leading to greatly underestimated attenuation factors and consequently underpowered studies.

In a recent study to assess the accuracy of methods in the European Prospective Investigation into Cancer (EPIC) U.K. cohorts, repeat biomarker estimates were also obtained from EPIC participants over a 9-mo period. Urinary nitrogen was estimated from 2–6 complete 24-h urine collections in 134 subjects. PABA was used to verify the completeness of the 24-h urine collections. Subjects completed two FFQ and two 7-d food diaries, and the second diary and FFQ were sent at varying times over the course of the study. The 24-h urine samples were not collected during the time that subjects were recording their dietary intake, which made it more likely that any errors between the dietary method and biomarker were completely independent of one another. In both men and women, results calculated from the 7-d food diary were much closer to estimates of output from urinary nitrogen than those calculated from the FFQ; see Table 1 (29).

The design of this study also allowed error-variance analysis to be conducted from the repeated dietary intake measures and the repeated urine collections. Marked differences in error variances associated with the different dietary assessments were shown. The most accurate method, the 7-d food diary, had substantially less error variance than the FFQ. Using the urine nitrogen as an index of true intake, the correction factors for measurement error of relative risk estimates from the dietary assessment methods could be estimated. Correction factors for regression dilution from the food diary were only 2.0, whereas those for the FFQ were too large to use with confidence: 9.0 for nitrogen. Furthermore the confidence limits around these estimates for the FFQ became impossibly wide: 1.7–16.2 (31).

Use of 24-h urine nitrogen for calibration of dietary assessments

To meet the sample-size requirements for studies in gene-nutrient interactions in chronic disease, pooling of data from multiple cohorts is becoming common practice. This also increases the heterogeneity of dietary habits, which is useful for overcoming measurement error in individual dietary assessments. However because each participating center may have used different methods of dietary assessment, all of which can have different measurement errors, calibration is then

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TABLE 1

Correlations between estimates of intake using different methods and 24-h urine nitrogen output in various studies

Number of urine collections	Number of individuals	Correlation between 24-h urine nitrogen and nitrogen obtained using dietary methods				Reference
		Weighed record	Records	FFQ[1]	24-h recall	
8	153	0.69	0.65	0.24	0.10	(15)
4	52	0.41	—	0.11	—	(16)
3	38	0.77	—	0.45	—	(26)
4	134	—	—	0.45	0.51	(27)
4	76	—	0.54	0.27	—	(28)
6	146	—	0.57–0.67	0.21–0.29	—	(29)

[1] FFQ, food-frequency questionnaire.

necessary to correct for any bias in mean intake that can be associated with these methods (32). Within the main EPIC study, a standardized computerized 24-h recall method, EPICSOFT, has been developed and administered to representative subsamples within each cohort (33). Within each subsample, 100–350 participants also kept single 24-h urine collections that were verified for completeness using PABA to assess the validity of the EPICSOFT method. Initial results suggest a high correlation between mean nitrogen intake and nitrogen excretion levels across the populations studied, which allows confidence to be placed in the validity of the calibration method (Slimani et al., unpublished data).

Other biomarkers for protein intake and future research

Although robust and reliable, the Kjehldahl technique for measuring total nitrogen requires specialized apparatus including acid-proof fume cabinets. With the advent of new technology for measuring total nitrogen such as those based on the Dumas technique, measurement is becoming less of a problem. Urea, however, is routinely measured in clinical laboratories, and comparisons of total nitrogen and urea nitrogen show good agreement at least for individuals who consume more than adequate protein. Under these conditions, urine urea nitrogen is 85 ± 2% of total urine-nitrogen excretion. At lower protein intakes however, the contribution of other sources of nitrogen to the total, especially creatinine, are greater (9).

Future research for the purpose of validating dietary assessments requires the development of a greater variety of nutritional biomarkers of diet that reflect a wide selection of food items for which extensive food-composition data should exist and that include markers for fat and carbohydrate. It is unlikely that other biomarkers will replace nitrogen for the purpose of validation of dietary intake of protein. Excretion of 3-methylhistidine in 24-h urine has been suggested as a marker of meat consumption, because this amino acid is released and excreted in urine after muscle-protein breakdown. However 3-methylhistidine is insufficiently accurate to use for validation purposes due to the high and variable baseline excretion, which depends on body muscle mass. Correlations between intake and excretion were poor in one comparison (34). Excretion of 1-methylhistidine varies according to the type of meat eaten (beef, pork or chicken), and there is a dose response over a range of 100–300-g meats (35).

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