Assessment of Dietary Nitrate Intake by a Self-Administered Questionnaire and by Overnight Urinary Measurement

PIET A VAN DEN BRANDT,* WALTER C WILLET** AND STEVEN R TANNENBAUM†


The relationship between dietary intake and urinary excretion of nitrate was investigated among 35 male and 24 female graduate students in Boston. The dietary assessment method consisted of a self-administered semi-quantitative food frequency questionnaire currently used for large-scale epidemiological studies. Calculated mean daily nitrate intake was 1.83 mmol for men and 2.36 mmol for women; broccoli and green leafy vegetables accounted for 60% of the total. Urinary measurements involved two overnight specimens with a mean collection time of approximately 13 hours. The ratio of intra-to-inter individual variance in urinary nitrate excretion (lambda) was 1.87. The simple correlation coefficient between intake and excretion of nitrate was found to be 0.20; after correction for the within-person variation by using lambda, the correlation coefficient was 0.28. Adjustment for gender, age and Quetelet’s index in multiple regression analyses resulted in a partial correlation coefficient between nitrate intake and excretion of 0.37 (p = 0.008). Correction for within-person variation in urinary excretion increased this partial correlation coefficient between intake and excretion to 0.59 (95% CI = 0.03 to 0.87). These data suggest that a self-administered questionnaire may provide useful information on usual nitrate intake, and indicate the need to pursue this possibility further.

Increasing interest in relationships between long-term dietary intake and the occurrence of chronic disease has stimulated the development and evaluation of methods to measure dietary factors among large groups of individuals. Methods based on questionnaires or biochemical measurements may both be useful, depending on the parameter being assessed and on the practical constraints imposed by the particular study design. For any method it will be important to evaluate the reproducibility and validity of the measurement. Since long-term intake is important in most epidemiological hypotheses, interview or biochemical parameters that reflect intake over a short period (such as a single day) may be of limited use, even though highly accurate for that short interval.2

When possible, the use of biochemical measurements to validate dietary questionnaires is appealing since the sources of error should be largely independent. In a previous study3 we evaluated the capacity of our semi-quantitative food frequency questionnaire to measure vitamin E and carotenoid intake using plasma levels of these nutrients for comparison. However, the use of biochemical measures for this purpose is frequently limited because many are insensitive to dietary intake over much of the dose-response range, or are highly variable from day to day. Urinary nitrate has been proposed as an estimate of the dietary nitrate intake, after the observation that 65–70% of ingested nitrate is excreted in the urine during the following 24 hours and less than 1% in faeces.4 Nitrate has been hypothesized to play a role in the aetiology of certain gastrointestinal cancers, notably gastric cancer.5,6 Nitrate may be converted into nitrite in foods, in the stomach7 and in the oral cavity,8,9 nitrate and nitrite can react with secondary amines or amides to form N-nitroso compounds.10,11 The relation of nitrate intake with cancer risk has been investigated in various epidemiological studies.12–14 Attention has also been given to the cancer risk associated with nitrate in drink-
ing water because of the increasing use of nitrogenous fertilizers. In none of these studies was evidence found to support a positive association.

We therefore assessed the within-person variability of timed overnight urinary nitrate excretion measurements and the influence of demographic and other factors on these levels. We then used these urinary measures to evaluate questionnaire estimates of nitrate intake. Finally, we evaluated the effects of within-person variation and other variables on the association between questionnaire estimates of intake and urinary measurement.

MATERIALS AND METHODS

Subjects
In April 1984 we invited a random sample of the student population of the Harvard School of Public Health to participate in this study. The sample consisted mainly of North American and European students, but also included four Japanese students. Sixty-one students agreed to participate, but two failed to complete all procedures. Hence, our analyses are based on 59 subjects consisting of 35 males and 24 females, age 30.3 (1 SD ± 4.5) and 28.9 (± 6.5) years respectively. Subjects were unaware of the fact that the investigation concerned the consumption and excretion of nitrate, since that might have interfered with their intake. The procedures used in this study were approved by the Committee on the Use of Human Subjects at the Harvard School of Public Health, and all individuals signed an informed consent form.

Overall Design
Participants were asked to collect timed, overnight urine specimens on two occasions, separated by two weeks. To impose minimal inconvenience on the subjects, they were asked to start the urine collection after they had arrived at home in the evening and to continue until the next morning, recording the exact time of starting and stopping. We appreciated that the overnight specimens would be less optimal than full 24-hour collections; however, we wished to evaluate a method that might be feasible on a much larger scale. In this way urine samples covering a period of approximately 13 hours were obtained. The dietary questionnaire was completed at the time the first urine specimen was obtained.

Dietary Questionnaire
The semi-quantitative food frequency questionnaire we employed is a questionnaire that is being used in a variety of epidemiological studies. Earlier versions of this form were validated previously with respect to the intake of carotene, retinol, and vitamin E as well as other nutrients. The current self-administered questionnaire consists of 120 specified foods; the participant is asked how often, on average, a specified quantity of each food was consumed over the past year. Nine responses are possible, ranging from never to six or more times a day.

As described elsewhere, nutrient scores were computed by summing the products of the frequency and nutrient composition of the specified serving size for each food. Food composition values for nitrate were derived from a report of the National Research Council on the health effects of nitrate, and a compilation by White. For some food items the nitrate content was derived by extrapolation, since published data were not available. Nitrate from drinking water was not included in our calculation; however, the concentration of this ion in the Boston municipal water supply is less than five parts per million. Assuming an average intake of one litre of water per day, the intake from this source would be less than 0.05 mmol/24 hours (which is less than 2% of intake from food sources).

Urine Collection
Timed overnight urine specimens were collected in two litre plastic bottles containing 50 ml of 3% HCl solution as a bacteriostatic agent. On the morning that the collection was completed, four 10 ml aliquots of urine were taken and immediately frozen at −20°C for chemical analysis. At both collection periods subjects were asked about any infections they might have had at that time, since this might influence the nitrate content of the urine (D A Wagner, personal communication).

Chemical Analysis
Urinary nitrate concentrations were determined via reduction with a high-pressure cadmium column as described by Green et al. Creatinine concentration was measured by flame photometry.

Statistical Analyses
Statistical analyses were carried out using the BMDP statistical package. Urinary values were expressed as excretion rates per hour; in the analyses comparing dietary and urinary values, the mean of the excretion rates for the two collection periods was used as the dependent variable. Highly skewed variables were logarithmically transformed to meet normality assumptions. Dietary intake and excretion values were tested for gender-specific differences using Student's t-test.

Analysis of variance was performed on the two repeated urinary measurements per person to examine the components of variability in nitrate excretion as
described by Beaton. In this way estimates of the inter-individual \( (s_i^2) \) and intra-individual variance \( (s_e^2) \) were computed. The intra-individual variance is composed of the biological intra-individual variation (which may be largely due to daily variation in diet) as well as random measurement errors in the urinary values. The ratio of the intra-individual to the inter-individual variance components (lambda) provides an indication of the reproducibility of the urinary excretion values. Lambda can also be used to determine the degree of attenuation in the estimated correlation or regression coefficient describing the relationship between the dietary and urinary variables that is due to within-person variation. Conversely, these coefficients can be corrected for within-person variation by using lambda. In this application, the corrected correlation coefficient can be thought of as the correlation that would describe the relationship between the questionnaire measurement and an infinite number of urine specimens per subject. The relationship between the true and observed coefficient is as follows:\(^{22}\)

\[ r_t = r_o \sqrt{\frac{1}{1 + \frac{1}{k}}} \]

where \( r_o \) = observed correlation coefficient
\( r_t \) = true correlation coefficient
\( k \) = number of measurements per person
\( \lambda \) = ratio of within-person variance to between-person variance.

Confidence intervals for the corrected correlation coefficient were also computed.\(^{22}\)

We first computed simple correlation coefficients to compare nitrate intake and urinary output. In the final analyses we utilized multiple regression to assess the influence of several predictors of the urinary excretion rate simultaneously. Statistical significance is expressed as two-sided p-values throughout the text.

RESULTS
General Description
The daily intake of nitrate as estimated by the questionnaire was lower in males \( (1.83 \pm 0.79 \text{ mmol}) \) than in females \( (2.96 \pm 2.05 \text{ mmol}) \); however, this difference did not reach statistical significance. Broccoli, spinach, other greens and lettuce accounted for 60% of the calculated nitrate intake among the 59 subjects; each of these items was reported more frequently by women.

The excretion rate of nitrate was found to be higher among men than among women (see Table 1). Thus, men were estimated to have a lower intake of nitrate, although their excretion was higher, whether expressed as mmol/hr or \( \mu \text{mol/hr/kg} \). If we assume that overnight urine samples are representative of 24-hour excretion, then the proportion of ingested nitrate that would be excreted was 1.04 in males and only 0.37 in females.

Estimates of the within-person and between-person variances, as well as the ratio of these two variance components, are given in Table 2. The variance ratio for nitrate is 1.87, while that for creatinine is 0.36.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Men ((n = 35))</th>
<th>Women ((n = 24))</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time of collection (hrs)</td>
<td>13.0 ± 2.3</td>
<td>12.5 ± 1.8</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Nitrate (mmol/hr)</td>
<td>0.08 ± 0.04</td>
<td>0.05 ± 0.02</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(( \mu \text{mol/hr/kg} ))</td>
<td>1.15 ± 0.67</td>
<td>0.81 ± 0.42</td>
<td>0.011</td>
</tr>
<tr>
<td>Creatinine (mg/hr)</td>
<td>67.6 ± 9.4</td>
<td>42.5 ± 9.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>(mg/hr/kg)</td>
<td>0.98 ± 0.10</td>
<td>0.78 ± 0.17</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Table 1: Overnight urinary excretion of nitrate and creatinine among 59 men and women \((\text{mean} \pm 1 \text{ SD})\). Data were collected in Boston, USA during 1984.

Relation Between Intake and Excretion of Nitrate
The Pearson correlation coefficient between nitrate intake and excretion per hour was 0.20 \((p > 0.05)\).

Although this simple correlation was not statistically significant, it has not been corrected for variables that might influence (ie confound) the relationship between intake and excretion rate. In Table 3 Pearson moment correlation coefficients are reported between potential confounders and the excretion rate.

As noted above, men had a higher excretion rate than women. Quetelet’s Index \((\text{weight/height}^2)\) and age were positively correlated with nitrate excretion. Caloric intake, as well as the intake of most macronutrients (not presented), did not appear to be strongly related to the excretion rate. Subsequent regression analyses were carried out with and without adjustment for caloric intake. Since the results were not materially different and nitrate-rich foods in general contain few calories, caloric intake was left out of the regression model presented. We did not find an effect of reported infection on the measurement of nitrate excretion.

Adjusting the relationship between intake and excretion for these potentially confounding variables resulted in higher partial correlation coefficients in some instances (Table 4). Allowing for gender or Quetelet’s Index changed the partial correlation coefficient between intake and excretion of nitrate substantially.

Multiple regression analysis was performed to control for the effects of several predictors simultaneously. The results of both univariate regression and multiple regression analyses for nitrate are shown in Table 5. Only nitrate intake, gender and Quetelet’s Index were significantly associated with nitrate excretion in the
Table 2  Variation in urinary excretion levels of nitrate and creatinine in 59 men and women, Boston, 1984

<table>
<thead>
<tr>
<th>Variable</th>
<th>Within-person variance</th>
<th>Between-person variance</th>
<th>Variance ratio (lambda)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitrate (μmol/hr)</td>
<td>1604.0</td>
<td>859.6</td>
<td>1.87</td>
</tr>
<tr>
<td>Creatinine (mg/hr)</td>
<td>70.9</td>
<td>199.3</td>
<td>0.36</td>
</tr>
</tbody>
</table>

multiple regression model. Inclusion of age did not affect the estimated regression coefficients, nor R². The partial correlation coefficient between nitrate intake and excretion after controlling for Quetelet's Index, gender and age was 0.37. Since men reported a lower nitrate intake, but were observed to have a higher excretion of the ion, we also tested for possible interaction between gender and nitrate intake; no evidence was found for a statistically significant interaction.

As suggested by Beaton et al. and Liu et al., we used the ratio of within-to-between-person variance to adjust the correlation coefficient between nitrate intake and excretion. Utilizing the observed simple correlation coefficient from Table 4, and the values for lambda from Table 2, the corrected coefficient adjusted for within-person variation was 0.28 (95% confidence interval (CI) = -0.09, 0.58). This simple correlation, however, does not reflect the effects of variables that would normally be controlled in any epidemiological analysis, such as age and sex, or that influenced the relation between intake and excretion in this data set, such as Quetelet's Index. We therefore also calculated the value of lambda for nitrate excretion after adjusting each individual level for the variables in Table 5 (adjusted levels were computed as the residuals of the excretion values regressed on the predictor variables). As expected, the value of lambda increased from 1.87 to 3.13 since sources of inter-individual variation were removed while the intra-individual variation remained unchanged. This adjusted value of lambda was then used to correct the partial correlation from Table 5, which represents the correlation between the adjusted excretion and intake levels. Although the correlation coefficient increased substantially with this correction (0.59), the associated 95% confidence interval was wide (0.03, 0.87).

DISCUSSION

In this population of graduate students, we observed a moderately high within-person variability in the overnight urinary excretion of nitrate. The intra-to-inter individual variance ratio (lambda) for nitrate excretion was found to be 1.87. Although this is less than the lambda-value reported for sodium of 3.20, the intra-individual variation in nitrate excretion remains considerable. The use of a single overnight specimen will therefore be of limited utility for characterizing an individual's long-term intake or excretion of this ion, although it may be useful for comparing populations. In simple bivariate analyses, nitrate intake based on a self-administered dietary questionnaire was only weakly correlated with excretion measured by the average of two overnight urine samples. However, adjustment for additional variables in multiple regression analysis increased the association between intake and excretion, and further correction for within-person variability in urinary excretion suggested that the questionnaire may actually provide reasonable discrimination of individual intakes of nitrate.

The predictive value of overnight urine specimens was studied, among others, by Watson and Langford, who compared excretion rates in specimens collected overnight and during the full 24 hours. They reported correlations for sodium and potassium excretion between the overnight and 24-hour excretion rates of 0.76 and 0.73 respectively. While in their experiment the mean duration of overnight collection was only 7.9 hours, in our study this was almost 13 hours. Our specimens would therefore be expected to be more representative of the 24-hour period. Bartholomew and Hill showed that urinary nitrate reaches its maximum 4-6 hours after an oral nitrate load, and returns to the baseline value within 18 hours. This suggests that overnight specimens may be useful in the case of nitrate, also considering the usual consumption time of foods rich in nitrate. In our analysis, we computed the excretion rate per hour and used this as our criterion variable to compare with intake.

Few estimates have been made of the nitrate intake of individuals. The estimates so far have generally been based on population averages. White estimated daily nitrate intake as 1.19 mmol/day in the US; per capita intakes for European countries vary mostly between 1.11 and 2.51 mmol/day, while Japanese per capita estimates amount to 4.52 mmol/day. In two recent British studies individual nitrate intake was estimated

Table 3 Pearson correlation coefficients between urinary nitrate excretion levels and potential predictors in 59 men and women, Boston, 1984

<table>
<thead>
<tr>
<th>Nitrate excretion, mmol/hr (ln)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M = 1, F = 2)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age, yrs (ln)</td>
<td>0.013</td>
</tr>
<tr>
<td>Quetelet's Index, kg/m²</td>
<td>0.004</td>
</tr>
<tr>
<td>Calories, kcal (ln)</td>
<td>0.110</td>
</tr>
</tbody>
</table>
Table 4  Partial correlation coefficients between intake and excretion of nitrate after adjustment for different covariates among 59 men and women, Boston, 1984

<table>
<thead>
<tr>
<th>Adjusting for</th>
<th>Partial r (nitrate intake versus excretion)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nothing (simple r)</td>
<td>0.20</td>
<td>0.132</td>
</tr>
<tr>
<td>Gender</td>
<td>0.38</td>
<td>0.003</td>
</tr>
<tr>
<td>Age (1a)</td>
<td>0.16</td>
<td>0.232</td>
</tr>
<tr>
<td>Quetelet's Index</td>
<td>0.25</td>
<td>0.058</td>
</tr>
</tbody>
</table>

by a food frequency questionnaire and a diet record. Forman et al. estimated daily nitrate intake to be 1.89 mmol in areas at low risk for gastric cancer and 1.19 mmol in high risk areas. Using 48-hour dietary records, Chilvers et al. estimated this intake to be 1.78 ± 1.25 mmol among 404 adults (177 men, 227 women). No differences according to gender were reported. We noted lower intakes in males, although this difference was not significant. Chilvers et al. also measured urinary nitrate output and found mean 24-hour excretion levels of 1.94 ± 1.21 mmol, which was slightly higher than their intake (one urine specimen was collected per subject). In our study using two urine collections of approximately 13 hours we estimated excretion rates to be 0.08 ± 0.04 mmol/hr in men and 0.05 ± 0.02 mmol/hr in women, respectively. If our overnight excretion rates would be representative for the daily output, our corresponding 24-hour excretion levels would be 1.90 ± 1.06 mmol for men and 1.08 ± 0.59 mmol for women.

The percentage of ingested nitrate that was excreted into the urine estimated by our methods differed substantially between males and females. The reasons for this are not clear, but include the possibilities of differential reporting of intake by gender, and chance, particularly since the questionnaire asked about usual intake of foods over the past year and the urines represented only two days. While differences in metabolism of nitrate between men and women cannot be excluded, we have little reason to suspect they might exist. It is also possible, of course, that the questionnaire underestimates intake for males. However, in earlier validation studies on beta-carotene intake the same questionnaire was able to discriminate between men and women adequately. In that study women were shown to have both higher plasma levels and higher dietary intake of carotene than men.

Overall, assuming that our urine measurements reflected the entire 24-hour intake we found the proportion of ingested nitrate that was excreted to be 77% in men and women combined. Excreted nitrate represents the combination of exogenous intake and endogenous synthesis corrected for metabolic losses. The apparent recovery from urine of ingested nitrate will be influenced by the relative proportion of these two inputs. Assuming that endogenous synthesis is relatively constant for an individual, higher ingested amounts of nitrate will lead to lower apparent recovery in urine if the endogenous component is not accounted in this balance. The 77% recovery, however, is in agreement with the estimates made for recovery of deNO₃, where approximately half the metabolic losses appear to be due to the action of the gastrointestinal flora. Despite the substantial variation in nitrate excretion within a person, a reasonable correlation between intake and excretion was observed after adjustment for gender and Quetelet’s Index.

Caloric intake was not correlated with nitrate excretion rate. This might be expected since foods rich in nitrate generally contain few calories (e.g., vegetables). The absence of any age effect on excretion was expected since the population was relatively homogeneous with respect to age. This group of public health students was also unusual with regard to the Quetelet’s Index: males had a higher index than females, contrary to what is generally found. However, no one was grossly obese. This had no adverse effects on the study, since the effect

Table 5  Predictors of nitrate excretion (mmol/hr), logistically transformed, in 59 men and women living in Boston during 1984

<table>
<thead>
<tr>
<th></th>
<th>Univariate regression</th>
<th></th>
<th>Multiple regression</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>coef (SE)</td>
<td>p</td>
<td>coef (SE)</td>
<td>p</td>
</tr>
<tr>
<td>Intercept</td>
<td></td>
<td></td>
<td>-5.46</td>
<td></td>
</tr>
<tr>
<td>Nitrate intake (in mmol/day)</td>
<td>0.18 (0.13)</td>
<td>0.149</td>
<td>0.31 (0.11)</td>
<td>0.006</td>
</tr>
<tr>
<td>Gender (M = 1, F = 2)</td>
<td>-0.59 (0.15)</td>
<td>&lt;0.001</td>
<td>-0.58 (0.14)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Quetelet’s Index (kg/m²)</td>
<td>0.11 (0.04)</td>
<td>0.006</td>
<td>0.07 (0.03)</td>
<td>0.035</td>
</tr>
<tr>
<td>Age (1a years)</td>
<td>1.16 (0.47)</td>
<td>0.017</td>
<td>0.49 (0.41)</td>
<td>0.240</td>
</tr>
<tr>
<td>R²</td>
<td></td>
<td>0.42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Partial r (nitrate intake vs excretion)</td>
<td>0.37</td>
<td></td>
<td>0.005</td>
<td></td>
</tr>
</tbody>
</table>
of these variables could be controlled for in multiple regression analyses.

In conclusion, our data suggest that a simple self-administered questionnaire may provide useful information on usual nitrate intake. However, these findings should be replicated among larger and more diverse populations, since the performance of the questionnaire and the between-person variation in dietary sources of nitrate may be different in other demographic groups. The performance may also be different when subjects are living in areas where the nitrate content of drinking water is elevated; questions on water consumption will have to be added in that case.

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