The L-arginine/NO biosynthetic pathway is involved in many physiologic and pathologic processes, including vasodilation and inhibition of platelet aggregation and adhesion. Nitrate (NO$_3^-$) and nitrite (NO$_2^-$) are the most abundant circulating and excretory NO metabolites in humans. In humans on a standardized low nitrate/nitrite diet, circulating nitrite reflects constitutive NO synthase activity (1), whereas measurement of nitrate in urine is a reliable noninvasive method to assess whole-body NO synthesis in humans (2).

Current analytical methods for the measurement of nitrate and nitrite include spectrophotometry, chemiluminescence, capillary electrophoresis, assays based on the Griess reaction, HPLC, and gas chromatography–mass spectrometry (GC-MS) (3). Among these assays, GC-MS methods form the basis for reference methods (4). Numerous HPLC methods using ultraviolet, electrochemical, or fluorescence detection have been reported for the analysis of nitrate and nitrite (3). The majority of them, however, have not been validated against MS, and they are mostly limited to human plasma (5) or rat urine (6). Recently, a commercially available HPLC-Griess system has been used for the analysis of urinary nitrite/nitrate in humans (7), but it has not been externally validated, and analysis of nitrate requires its reduction to nitrite.

Here I report on a simple, rapid, GC-MS-validated anion-pairing HPLC method with ultraviolet absorbance detection at 205 nm for the accurate measurement of nitrate in human urine without sample pretreatment other than dilution of samples with the mobile phase. This HPLC method is not suitable to measure nitrate in human plasma.

HPLC analyses were performed with a Pharmacia LKB pump Model 2248 and an analytical column [250 × 4 mm (i.d.) packed with Nucleosil 100–5C$_{18}$ AB (Macherey-Nagel)]. The mobile phase consisted of water–acetonitrile (90:10 by volume) contained 10 mmol/L tetrabutylammoniumhydroxide sulfate (Merck), which serves as the anion-pairing agent. The pH of the mobile phase was adjusted to 8.4 by addition of sodium dihydrogen phosphate and 10 mol/L NaOH, and the flow rate was 1 mL/min. The variable ultraviolet–visible detector (Model Spectroflow 783; Kratos Analytical) was set at 205 nm. Analyses were performed at ambient temperature (22–26 °C). Sodium nitrate (purity >99.9%) and sodium nitrite (purity >99%) were purchased from Riedel-de Haën. Stock solutions of nitrate (80 mmol/L) and nitrite (10 mmol/L) were prepared in distilled water and diluted as appropriate with the mobile phase.

Mixtures of synthetic nitrate and nitrite containing these compounds at a constant molar ratio of 8:1 in the ranges 0–8000 μmol/L for nitrate and 0–1000 μmol/L for nitrite were used to prepare calibration curves. The reten-
The measurements were linear up to a concentration of 1000 μmol/L for both anions. A polynomial fit within the whole concentration range of nitrate yielded the regression equation: \( y = -0.46x^2 + 9665x - 58920 \) \((r = 0.999)\).

Quantitative determination of urinary nitrate was performed by diluting centrifuged (800 g for 5 min) native urine (250-μL aliquots) with 750 μL of the mobile phase and injecting a 20-μL aliquot. Total analysis time was 15 min. Overnight, a mobile phase consisting of water-acetonitrile (10:90 by volume) was pumped at a flow rate of 0.1 mL/min.

We added nitrate to urine samples at relevant concentration ranges. The samples were diluted with the mobile phase (1 part urine to 3 parts mobile phase by volume), and 20 μL of the resulting solutions was injected. Linear regression analysis of peak area \((y)\) and concentration \((x)\) of nitrate added \((0–1600 \text{ μmol/L})\) yielded a mean (SD) slope of 8654 (63) and intercept of 2408000 (13016) arbitrary units \((r = 0.999)\) for a urine sample with a basal nitrate concentration of 1093 μmol/L.

We validated the HPLC method for the quantitative determination of nitrate in urine by adding nitrate in triplicate to a fresh urine sample (Table 1). Linear regression analysis between measured \((y)\) and added \((x)\) nitrate concentrations yielded a slope (SD) of 0.99 (0.01) and intercept of 1099 (6) μmol/L \((r = 0.999)\). Imprecision \((CV)\) for analysis of a urine sample was 2.9% at a mean (SD) nitrate concentration of 1112 (32) μmol/L \((n = 5)\). A 1-mL aliquot of the same urine sample was diluted with 3 mL of the mobile phase and analyzed by HPLC on 5 consecutive days. Overnight, the diluted urine sample was stored at 4°C in a refrigerator. The CV was 4.3% at a mean (SD) concentration of 1108 (48) μmol/L.

We measured nitrate in 24 urine samples from patients suffering from various diseases by the present HPLC method and by a previously described GC-MS method \((4)\). Urine samples were aliquoted (1 mL) and frozen at
-20 °C until being measured in parallel by the present HPLC method (sample dilution, 1:4 by volume; 20-μL aliquots injected; flow rate, 1 mL/min; detection at 205 nm) and by the GC-MS method (derivatization of 100-μL aliquots). GC-MS analyses were carried out on a Hewlett-Packard MS Engine 5989A. Sodium [15N]nitrate (declared aliquots injected; flow rate, 1 mL/min; detection at 205 °C) was obtained from Sigma and used as internal standard. In all urine samples, including six quality-control samples (4). Selected-ion monitoring of m/z 62 for unlabeled nitrate and m/z 63 for [15N]nitrate was carried out in the negative-ion chemical ionization mode as described previously (4). The recovery (SD) and imprecision (SD) of the GC-MS method were 95 (3)% and 1.3 (0.7)%, respectively.

Representative HPLC chromatograms from the analysis of synthetic and urinary nitrate are shown in Fig. 1A. HPLC analysis of urine samples showed a peak eluting with the retention time of synthetic nitrate. GC-MS analyses of the 24 urine samples, however, revealed urinary nitrate concentrations in the range 4.3–8.4 μmol/L, which cannot account for the HPLC peak with the retention time of nitrite.

N^C-Nitro-L-arginine is a frequently used exogenous inhibitor of NO synthase. In methods requiring reduction of nitrate to nitrite, N^C-nitro-L-arginine may erroneously contribute to nitrite (8). The present HPLC method is not affected by N^C-nitro-L-arginine (Sigma-Aldrich) because of its shorter retention time of 2.7 min.

Linear regression analysis between the nitrate measured by the HPLC method (y) and those measured by the GC-MS method (x) yielded a mean (SD) slope of 1.16 (0.06) and y-intercept of -121 (61) μmol/L (r = 0.973). The mean (SD) ratio of the nitrate values measured by HPLC to those measured by GC-MS was 0.98 (0.23). The results were also compared by plotting the difference for nitrate measurements (HPLC – GC-MS) vs the mean concentration (Fig. 1B) according to the method of Bland and Altman (9). Urinary creatinine was determined by a recently described HPLC method (10).

To ensure that the limit of quantification was above the concentrations seen in urine, we injected 20-μL aliquots of a 2 μmol/L solution in the mobile phase, which was equivalent to 40 pmol of nitrate, and measured the absorbance at 205 nm; the experiment was performed in quadruplicate. The HPLC peak observed at the retention time of nitrate was measured with a signal-to-noise ratio of 67:1 and an imprecision (CV) of 3.9%, suggesting that the limit of quantification is below 8 μmol/L.

The proposed MS-validated, isocratic, anion-pairing, reversed-phase HPLC method is simple, rapid, specific, and suitable for the accurate measurement of nitrate in human urine. The most important advantages of the present HPLC method over other reported HPLC methods for urinary nitrate are its accuracy, simplicity, speed, and conformity. All of these advantages result from the ability of the method to analyze urine directly without pretreatment. In particular, the omission of the reduction step overcomes serious analytical problems that may occur in other methods that require reduction of nitrate to nitrite (4, 9). The simplicity and streamlined sample treatment of the proposed method make it suitable for automated analysis of nitrate in human urine. Nevertheless, we consider the GC-MS method reported previously by our group (4) to be superior to the HPLC method described here, and it should be used preferably when possible.

References


Enzyme Immunoassay for Serum Autoantibody to Survivin and Its Findings in Head-and-Neck Cancer Patients, Joseph T. Chang,¹ Fen-Hua Wong,² Chun-Ta Liao,³ I-Hou Chen,³ Hung-Ming Wang,⁴ and Ann-Joy Cheng⁵

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Several proteins associated with malignant transformation of cells can induce autoantibodies (1–4). These autoantibodies, such as those to p53, are detectable in serum and may serve to monitor tumor progression (4, 5). Survivin, a recently cloned 16.5-kDa apoptosis inhibitor be-