Use of Colorimetric Test Strips for Monitoring the Effect of Hemodialysis on Salivary Nitrite and Uric Acid in Patients with End-Stage Renal Disease: A Proof of Principle

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BACKGROUND: Initial screening of potential biomarkers for monitoring dialysis was performed with saliva samples collected from patients with end-stage renal disease (ESRD). A more thorough analysis of the most promising markers identified in the initial screening was conducted with saliva samples acquired at hourly intervals throughout dialysis to monitor analyte concentrations as dialysis progressed. We observed that salivary nitrite (NO2−/H11002) and uric acid (UA) concentrations consistently decreased as dialysis progressed. Test strip measurements showed that mean salivary concentrations of NO2− and UA were decreased in ESRD patients by 86% and 39%, respectively, compared with 15% and 9% for time-matched controls. Comparison of test strip results with calibrated solution-based assays suggests that the test strips can semiquantitatively measure salivary concentrations of NO2− and UA.

CONCLUSIONS: The colorimetric test strips monitored changes in salivary NO2− and UA concentrations that occurred in ESRD patients during dialysis. The test strips may prove useful for noninvasively evaluating dialysis progress and may also be useful for monitoring renal disease status.

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Whole saliva is a mixed oral fluid derived from the major and minor salivary glands. In addition, saliva contains constituents of nonsalivary origin, including a variety of microorganisms and their products, blood cells, desquamated epithelial cells, and food debris. Saliva also contains serum-derived components resulting from passive diffusion via gingival crevices (1); therefore, saliva has been proposed to be a good surrogate of blood for diagnostic purposes. Furthermore, saliva can be collected noninvasively and more easily by minimally trained personnel (2-5).

With the goal of developing a diagnostic test for the simultaneous detection of multiple markers in saliva, we selected end-stage renal disease (ESRD)5 as a suitable target disease state, because the disorder is a well-defined phenotype and its effect on blood composition is well known. Owing to the contribution of serum-derived components to whole saliva, we hypothesized that changes in serum composition caused by hemodialysis would be reflected in saliva. Several studies have discovered that salivary concentrations of biomarkers are decreased during dialysis, suggesting...
that saliva tests could be used to evaluate dialysis efficacy (6–9).

We describe the ability to monitor salivary nitrite (NO$_2^-$) and uric acid (UA) concentrations as a possible method for the surveillance of dialysis treatment efficacy. Once the trends associated with these analytes were confirmed, a simple method of multiplexed detection was required. The development of simple and robust testing methods for point-of-care (POC) use is of great interest, and the ideal method should be inexpensive and easy to interpret by both healthcare workers and patients, particularly in the home-testing setting. Colorimetric test strips provide an ideal format to fulfill the requirements of POC diagnostic tests (10, 11). We converted NO$_2^-$ and UA-detection chemistries into a test strip format, which we used to rapidly measure concentrations of these analytes in saliva samples collected from ESRD patients in a dialysis clinic.

Materials and Methods

Materials
Tetra(tetramethylammonium) salt (Sodium Green indicator), 6-methoxy-N-(3-sulfopropyl)quinolinium (SPQ), and an Amplex Red Uric Acid/Uricase Assay Kit were obtained from Invitrogen. Reagent-grade DMSO, 0.1-mol/L NO$_2^-$ calibrator solution, solid NaCl, 2,3-diaminonaphthalene (DAN), citric acid, N-(1-naphthyl)ethylenediamine, sulfanilamide, UA, copper(II) sulfate, sodium citrate, and 2,2'-biquinoline-4,4'-dicarboxylic acid disodium salt hydrate (sodium bicinchoninate) were obtained from Sigma–Aldrich. A roll of vinyl 20 mil thick (1 mil = 1/1000 in) was obtained courtesy of Hi-Tech Products (Buena Park, CA). Acid-free double-sided tape was obtained from Scotch 3M. Whatman chromatography paper (Grade 3MM CHR) was obtained from Fisher Scientific. Test strips were imaged with either an Epson Perfection 3490 flatbed scanner or a Canon Powershot A530 digital camera. Histogram analysis of test strip images was completed with Adobe Photoshop.

Patients and Sample Collection
We recruited 43 ESRD patients undergoing regular hemodialysis for the study. Twelve patients participated in the initial phase to screen potentially informative analytes in saliva samples collected before and after dialysis, 12 participated in the second phase in which saliva samples were collected at hourly intervals throughout dialysis, and 19 patients participated in the POC test strip study. For the POC study, we also recruited 10 healthy control volunteers in the Clinical Research Center at Boston University School of Dental Medicine. Patient volunteers were recruited at a chronic dialysis center affiliated with Boston University Medical Center (BUMC). All study participants signed consent forms according to BUMC-approved institutional review board regulations. Participants were asked to fast for 2 h before providing saliva samples. For the 3 stages of the study, samples were collected in the following 3 manners: (a) once before and once after dialysis on a weekly basis for a 2-month period in the initial analyte-screening phase; (b) at hourly intervals throughout dialysis, with the final sample collected just before the conclusion of dialysis in the confirmatory analyte-screening phase; or (c) once before and once immediately after dialysis (approximately 4 h later) in the test strip study. Salivary flow was stimulated by mastication of a 1.5-g wax bolus (Parafilm) at a mastication rate of 30 strokes per minute. Participants were asked to expectorate every 30 s into a Falcon tube kept on ice until the collection volume totaled 5.0 mL. Samples were immediately processed by centrifugation at 27 000g for 20 min at 4 °C. Aliquots (1.0 mL) of saliva supernatant were placed in 1.5-mL polyethylene tubes and stored at −80 °C until use. For the test strip study, we collected approximately 1.0 mL of whole saliva for an initial test strip measurement in the clinic (see the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol54/issue9), collected an additional volume of approximately 3.0 mL, and processed the samples with the centrifugation and fractionation procedures described above.

Analysis of Saliva Composition
A SpectraMax Gemini microplate spectrofluorometer (Molecular Devices) and untreated, flat-bottom, 96-well black microtiter plates (Costar; Corning) were used for solution-based fluorescence assays. SoftMax Pro software (Molecular Devices) was used for data acquisition and analysis.

Uric Acid
Salivary UA concentrations were measured with an Amplex Red Uric Acid/Uricase Assay Kit according to the manufacturer’s instructions. In brief, 5 μL of saliva was diluted with 45 μL of deionized water and combined with a solution of uricase, horseradish peroxidase, and Amplex Red indicator in each well of the microtiter plate. Fluorescence was measured with excitation and emission wavelengths of 550 nm and 590 nm, respectively.

Sodium
Sodium Green indicator was used to measure the Na$^+$ concentration. Six aqueous Na$^+$ calibrators (0.2–6.25 mmol/L) were prepared from a 1-mol/L NaCl stock solution. We pipetted 50 μL of either undiluted saliva or Na$^+$ calibrators into microtiter plate wells to
react with 50 µL of 10 µmol/L Sodium Green in DMSO. The excitation and emission wavelengths used for fluorescence measurements were 507 nm and 532 nm, respectively.

**CHLORIDE**

Cl\(^{-}\) concentrations were measured with the fluorescent indicator SPQ. Seven aqueous Cl\(^{-}\) calibrators (1.56–100 mmol/L) were prepared from a 1-mol/L NaCl stock solution. We allowed 100 µL of either undiluted saliva or Cl\(^{-}\) calibrators to react with 50 µL of 2 mmol/L SPQ in deionized water. Fluorescence was measured with excitation and emission wavelengths of 344 nm and 443 nm, respectively.

**NITRITE**

NO\(_2^-\) concentrations were measured fluorometrically with DAN (12, 13). Eight aqueous NO\(_2^-\) calibrators (0.39–50 µmol/L) were prepared from a 0.1-mol/L NO\(_2^-\) stock solution. Saliva samples were diluted with 3 volumes of deionized water. We then pipetted 100 µL of diluted saliva or the NO\(_2^-\) calibrator into microtiter plate wells to react for 15 min with 10 µL of 0.1 g/L DAN in 0.6 mol/L HCl. Before measuring the fluorescence, we added 10 µL of 2.8 mol/L NaOH to each well. Fluorescence was measured with excitation and emission wavelengths of 365 nm and 450 nm, respectively.

**TEST STRIP STUDY AND CONFIRMATORY ANALYSIS**

Chromatography paper was impregnated with solution-based NO\(_2^-\) and UA-detection chemistries (14–17). The 2 test papers were then immobilized onto vinyl backing material, which was then hand-cut into strips. The colorimetric test paper for salivary NO\(_2^-\) measurement uses the principle of the Griess reaction, a common method for NO\(_2^-\) quantification (17). When a sample containing NO\(_2^-\) comes into contact with the paper, the citric acid impregnated in the paper converts NO\(_2^-\) to nitrous acid, which then diazotizes sulfanilamide. The diazotized sulfanilamide then couples with N-(1-napthyl)-ethylenediamine to produce a red-violet azo compound. The intensity of the resulting color is proportional to the amount of NO\(_2^-\) present. The colorimetric test paper for UA is based on a bicinchoninate chelate method (14). When the UA test paper is exposed to a sample containing UA, Cu(II) is reduced to Cu(I), which then forms a chelate with sodium bicinchoninate. The intensity of the resulting deep-violet precipitate is proportional to the amount of UA present in the sample. Detailed information on test strip fabrication and characterization is available in the online Data Supplement.

Saliva samples were collected from 19 ESRD patients in the BUMC dialysis clinic immediately before and immediately after treatment. Ten control individuals also donated 2 samples each. In parallel with the approximate time required for ESRD patients to undergo a complete dialysis treatment, the first sample was collected at time zero, and the second sample was collected 4 h later. Samples were processed as described above, frozen, and sent to Tufts University for storage at ~80 °C. Aliquots of saliva supernatant were thawed and analyzed by immersing test strips in the samples and imaging the strips on a desktop digital scanner. Test strip color intensities were measured by means of the histogram function in Adobe Photoshop and converted to concentrations by comparing the intensities with a calibration curve of digitally analyzed test strip color intensities produced by calibrator solutions of known concentration (see the online Data Supplement). We also tested saliva samples with solution-based quantitative assays for NO\(_2^-\) and UA, as described above (the DAN method and the Amplex Red Uric Acid/Uricase Assay Kit, respectively).

We directly compared the test strip assays and the solution-based assays by analyzing the same set of saliva samples with both methods. The net changes in NO\(_2^-\) and UA concentrations (initial at t = 0 minus final at t = 4 h) measured with the test strips were compared with those obtained with the microtiter plate methods for the same set of patients and control individuals. The degree of agreement of the net differences for the 2 test methods was examined with Bland–Altman plots. For each participant, the difference between the net change in concentration measured with the test strips and that obtained with the microtiter plate–based assay was plotted against the mean concentration change obtained by both methods.

**Results**

**INITIAL SCREENING**

We initially measured several analytes in patient saliva samples collected on a weekly basis, with the aim of identifying analytes that demonstrated a distinguishable and consistent change from before to after dialysis (see the online Data Supplement) (18). To further refine our choices, we then investigated the most promising markers by testing saliva samples collected at 1 h intervals throughout dialysis. We compiled the results of the secondary screening to obtain the changes in concentration that occurred throughout the dialysis procedure for each patient. As shown in Fig. 1, the UA and NO\(_2^-\) results correlated best with dialysis progression, because a net decrease in their concentrations was observed in all 12 patients (Fig. 1, A and D). Na\(^+\) and Cl\(^{-}\) did not consistently show the same trends (Fig. 1, B and C). Owing to sample-volume limitations for several time points, we could not acquire complete time traces for every patient. Despite this lack of informa-
tion for several patients, we collected pre- and postdialysis samples for every patient and measured all variables in these samples. Thus, we were able to evaluate the overall effect of dialysis on analyte concentrations. Although several patients exhibited fluctuations in UA and NO$_2$ concentrations throughout dialysis before the final measurement, we consistently observed net decreases in the concentrations of these analytes from predialysis samples to postdialysis samples. Normalization and averaging of all patient data provide a simplified representation of the trends we observed for both UA and NO$_2$ across all 12 patients (Fig. 2). Furthermore, the normalized predialysis and postdialysis values were significantly different with no overlapping SDs at the 95% confidence interval ($P < 0.0001$ for both UA and NO$_2$). The net decreases in concentration observed for both analytes from predialysis samples to postdialysis samples support their potential utility as markers of dialysis efficacy.

**TEST STRIP STUDY**

We observed a wide range of initial/predialysis NO$_2$ concentrations among ESRD patient samples with the test strips (Fig. 3). Salivary NO$_2$ concentrations measured with the test strips decreased after dialysis in all but 1 ESRD patient. Compared with the ESRD patients, the control samples showed no consistent trend in salivary NO$_2$ concentration between the time zero and the 4-h time points. The concentrations for 3 control individuals increased over the 4-h time period, and the range of initial concentrations was less variable. Salivary UA concentrations measured with the test strips decreased in all but 2 ESRD patients, whereas, again, we observed no consistent trend in salivary UA concentrations in the time-matched control samples. The UA concentration increased from the zero time point to the 4-h time point in 5 of the 10 control volunteers. Although the mean initial NO$_2$ and UA values for the ESRD patients (161 μmol/L and 202 μmol/L, respectively) and the healthy controls (75.2 μmol/L and 160 μmol/L) were not significantly different ($P = 0.15$ and 0.12, respectively), the mean net changes in concentration observed for the 2 populations were significantly different ($P = 0.018$ and 0.0077 for NO$_2$ and UA, respectively).

**Discussion**

We hypothesize that salivary UA is a suitable marker for qualitatively evaluating dialysis efficacy, given that patients experiencing renal failure have high plasma and salivary UA concentrations (19) that are significantly decreased by dialysis (8, 9, 20). Goll and Mookerjee also observed that UA and creatinine concentra-
tions in whole saliva correlated significantly with those in serum and speculated that a saliva diagnostic test would be useful for reducing the number of blood tests for anemic and pediatric patients with renal disease (6). The results we have presented support the notion that changes in UA concentrations occurring during dialysis can be monitored in saliva; we therefore believe that salivary UA warrants further examination for its clinical utility.

Nitric oxide (NO), a strong vasorelaxant, is continually produced by the kidneys via the metabolism of L-arginine (21). Owing to the complicated functions of NO in the human body, its role in ESRD has been debated (21–23). Rapid oxidation of NO to nitrate (NO$_3^-$) and NO$_2^-$ occurs in biological fluids; therefore, NO$_3^-$ and NO$_2^-$ have been examined as qualitative markers of NO production (22). Of note, however, is that NO constitutes only a small fraction of the source of NO$_3^-$ and NO$_2^-$ in serum and saliva. A major determinant of serum NO$_3^-$ and NO$_2^-$ is the catabolism of proteins and amino acids, and dietary sources are another (24). Nonetheless, research has suggested a possible association of increased NO metabolite concentrations in plasma with enhanced NO production and decreased renal function (25). The concentration of NO$_2^-$ plus NO$_3^-$ in serum has been observed to decrease more than either blood urea nitrogen (BUN) or creatinine following dialysis, and the concentrations of these 2 analytes have been suggested as a potential indicator of renal impairment (26). Similar associations of NO$_3^-$ concentrations have been observed in pre- and postdialysis plasma (27). Because salivary NO$_3^-$ is reduced to NO$_2^-$ by bacteria in the oral cavity (28) and because both NO$_3^-$ and NO$_2^-$ are removed from the blood by dialysis (26), salivary NO$_2^-$ has also been selected as a suitable candidate marker for monitoring dialysis efficacy.

The gold standard test for evaluating dialysis efficacy is to measure pre- and posttreatment BUN concentrations. The reduction ratios for BUN, as well as for salivary NO$_2^-$ and UA, were calculated as: [(predialysis concentration) – (postdialysis concentration)]/ (predialysis concentration). Comparison of the salivary UA, NO$_3^-$, and BUN reduction ratios revealed some correlation ($r = 0.24$ and $0.43$ for UA and NO$_2^-$, respectively; Table 1). At least one of the 2 salivary analytes yielded similar reduction ratios in 9 of the 12 patients. For patients 2, 7, and 12, although the UA and NO$_2^-$ reduction ratios did not correlate with the BUN reduction ratio ($r = -0.81$ and $-0.55$ for UA and NO$_2^-$, respectively), salivary UA and NO$_2^-$ concentrations were both substantially decreased after dialysis. Of note, however, is that there is no a priori reason why saliva measurements for these analytes should

![Fig. 2. UA and NO$_2^-$ data normalized to the mean predialysis values.](image-url)

Presented are box-and-whisker plots and group means with 95% confidence intervals for UA (A and B, respectively) and NO$_2^-$ (C and D, respectively). All of the time point results were significantly different ($P < 0.05$) from mean baseline (predialysis) values except for the 1-h NO$_2^-$ time point. • (A and C), outliers.
necessarily correlate with BUN concentration. Given the conflicting observations regarding the correlation of salivary urea with BUN (6, 29), we have sought to offer evidence for alternative salivary markers that may be useful for noninvasively monitoring dialysis efficacy and renal function. The screening of a larger patient cohort—including comparisons of salivary \( \text{NO}_2^- \) and UA concentrations with salivary carbonyl, serum \( \text{NO}_2^- \), and serum UA concentrations, as well as with other common serum (creatinine and/or BUN) or urine tests—could encourage the acceptance of saliva testing for evaluating dialysis efficacy. The results of the preliminary screening tests conducted in microtiter plates suggest that 2 salivary analytes, UA and \( \text{NO}_2^- \), exhibit net decreases in concentrations in all patients throughout the dialysis process. Thus, we developed colorimetric test strips to rapidly and easily measure the concentrations of these 2 analytes in saliva.

Although the mean predialysis \( \text{NO}_2^- \) and UA concentrations obtained with test strips (Fig. 3) do not agree with those obtained with the microtiter plate assays in preliminary screening experiments (Fig. 1), it is important to point out that these 2 studies were performed with different individuals. Although the results of the 2 methods do not agree precisely, they give the same qualitative result for the 2 different patient groups when the net predialysis to postdialysis change in concentration is taken into account. When we used the 2 methods to test the same population, an examination of the Bland–Altman plot shows that the microtiter plate and test strip data were in good agreement for \( \text{NO}_2^- \) (Fig. 4). The one exception was when the net concentration change was >700 \( \mu \text{mol/L} \). In this instance, the results obtained with the 2 methods differed by nearly 300 \( \mu \text{mol/L} \), but such a change is extreme, and both methods readily identified it as a large reduction. In this case, the test strip assay underestimated the net concentration change, compared with the solution-based assay. For UA, the 2 methods were in good agreement for nearly 75% of the samples. Although the UA test provides a lower agreement rate than the \( \text{NO}_2^- \) test, the combination of

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<th>4</th>
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<td>0.67</td>
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<tr>
<td>( \text{NO}_2^- ) RR</td>
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<td>0.62</td>
<td>0.82</td>
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*UA and \( \text{NO}_2^- \) RRs for patients 2, 7, and 12 (boldfaced values) did not correlate with the BUN RR (\( r = -0.81 \) and \( -0.55 \) for UA and \( \text{NO}_2^- \), respectively), but salivary UA and \( \text{NO}_2^- \) concentrations were both substantially decreased after dialysis.
the 2 tests may provide useful information regarding the effectiveness of a dialysis treatment. These results demonstrate that the net changes in salivary NO$_2^-$ and UA concentrations obtained with the 2 methods were in agreement and suggest that the test strips can be used for semiquantitative measurements.

In comparison, the test strip method yields instantaneous results for NO$_2^-$ and UA simultaneously, whereas the solution-based assays require longer assay times and expensive analytical instrumentation. Although the biocinchoninate-chelate UA-detection method is not as selective as UA detection based on uricase (30), the presence of interfering substances in saliva is believed to be minimal; therefore, changes in UA levels should not be masked in the test strip assay (16). A comparison of test strip results with those obtained with the uricase-based detection method showed that the qualitative changes in UA concentrations obtained with the 2 testing methods were similar (Fig. 4). Furthermore, nonenzymatic detection methods are preferable to enzymatic detection methods for test strip preparation because the shelf lives are longer and the reagents do not require any additional special storage conditions. After several months of storage in an airtight container with desiccant, the NO$_2^-$/UA test strips produced measurements comparable to those obtained with a freshly prepared batch (data not shown).

Several studies have examined the compositional changes in saliva produced by dialysis (8, 9), but the present investigation is the first to offer a method for simultaneously monitoring 2 salivary analytes for qualitatively assessing dialysis efficacy. It is important to note that in some cases a single analyte does not change while the second analyte shows a decrease consistent with the clinical status. Such a possibility underscores the potential value of performing multianalyte tests when making clinical decisions. Clearly, these analytes and this method require further study with saliva samples to conclusively evaluate their clinical utility. With the acceptance of salivary NO$_2^-$ and UA as qualitative biomarkers, the test strip method we have presented may also become useful for patients with progressive renal failure or who are undergoing peritoneal dialysis, because baseline analyte concentrations can be established for individual patients and the concentrations can be monitored routinely by the patients in the convenience of their homes. Furthermore, for ESRD patients undergoing dialysis, technicians can quickly monitor salivary analyte concentrations throughout treatment to determine if a patient has achieved a successful dialysis before the standard completion time. We hypothesize that salivary NO$_2^-$ and UA could be used in addition to the current gold standard blood tests (BUN and creatinine) used to evaluate renal function.

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