Assessment of measurement of salivary urea by ATR-FTIR spectroscopy to screen for chronic kidney disease

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Key Points

Salivary urea can be quantitated rapidly and accurately by infrared spectroscopy in the range relevant to that found in CKD patients.

The measured urea concentrations allowed discrimination of patients with stages 3, 4 or 5 CKD from each other and from healthy controls.

This provides a proof-of-concept that the technique could be developed as a novel, sensitive and cost-effective screening method for CKD.

Abstract

Stages of chronic kidney disease (CKD) are currently defined by estimated glomerular filtration rates (eGFR) and require measurement of serum creatinine concentrations. Previous studies have shown a good correlation between salivary and serum urea levels and the stage of CKD. However, quantitative salivary urea assays in current clinical use require costly and labour-intensive commercial kits which restricts the advantage of using saliva and limits wider applicability as a quick and easy means of assessing renal function. Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy has been shown to provide a potentially straightforward, reagent-free method for the identification of a range of disease-related biomarkers and is in current clinical use for analyses of the chemical composition of kidney stones. We assessed the feasibility of ATR-FTIR spectroscopy as an alternative method to measure salivary urea in patients with different stages of CKD. The ATR-FTIR spectra of dried saliva samples from 6 healthy controls and 20 CKD patients (stages 1-5) were analysed to provide their urea concentrations. The lower limit of detection of salivary urea by the ATR-FTIR spectroscopy method was 1-2 mM, at the lower end of the clinically-relevant range. Statistically significant differences in salivary urea concentrations were demonstrated between healthy subjects (4.1±0.5 mM) and patients with CKD stages 3-5 (CKD stage 3: 6.8±0.7 mM; CKD stage 4: 9.1±1 mM; CKD stage 5: 14.8±1.6 mM). These salivary urea concentrations correlated well with serum urea levels in the same patients measured by an automated analyser (Spearman's rank correlation coefficient of 0.71; $p<0.001$). The ability of the method to detect and stage CKD was assessed from the sensitivity and specificity parameters of a receiver operating characteristics (ROC) curve analysis. This proof-of-concept study demonstrates that quantitation of salivary urea by ATR-FTIR spectroscopy could provide a viable tool for rapid and cost-effective diagnosis of stages 3-5 CKD.

Introduction

Chronic Kidney Disease (CKD) has an estimated prevalence of 8-16% worldwide and has been reported to contribute increasingly to mortality globally[1] and to be a significant economic burden. Occurrence of CKD is predicted to rise sharply and it is estimated that 5.4 million people worldwide will be receiving renal replacement therapy (RRT) by 2030[2]. CKD causes significant morbidity and mortality, in part due to a lack of unique markers for diagnosis and delay in initiation of treatment. Many deaths caused by CKD may be preventable through more effective screening of high-risk populations such as patients with diabetes, hypertension, cardiovascular disease and HIV, or with limited access to healthcare.

Determination of renal function in current clinical practice requires measurement of serum creatinine, or other biomarkers of kidney function in the blood, to estimate Glomerular Filtration Rate (eGFR) using validated serum creatinine-based formulae such as the Modification of Diet in Renal Disease (MDRD) equation[3]. eGFR and albuminuria are the principal parameters used to define and stage CKD, according to Kidney Disease Improving Global Outcomes (KDIGO) criteria[4]. Calculation of eGFR is based on serum creatinine measurements and requires venepuncture expertise and facilities for blood sample collection and transfer to a laboratory for later analysis. Venepuncture can be difficult in some patients, especially children, and is associated with a small risk of infection. The process becomes more challenging outside a hospital or clinical setting, particularly in resource-poor countries where there may be a lack of trained personnel and equipment. Hence, recent efforts have focused on the potential use of venepuncture-free biofluids, such as saliva, for more rapid 'near-patient' testing and detection of kidney disease.

A number of studies have shown that various salivary components including urea, cortisol, creatinine, uric acid and phosphate correlate positively with the severity of CKD and are therefore potential indicators of renal function[5-8]. Of these, salivary urea has been particularly addressed because it has been shown to correlate positively with elevated serum urea levels found in patients with CKD[9,10]. Hence, salivary urea is a potential non-invasive alternative biomarker for detecting CKD. Nevertheless, current methods for detection and quantitation of salivary urea have some limitations. One recent simple method utilises dipsticks to measure salivary urea nitrogen (SUN) colorimetrically. However, this method is semi-quantitative and was shown to be less sensitive in patients with milder kidney disease when compared with serum analysis[11]. In contrast, the quantitative kits for detection of salivary urea are much more expensive in reagents, which need to be refrigerated, and also require substantially more time to perform the assay. The development of a more rapid and cost-effective method for

analysing salivary urea can improve CKD diagnosis, either by providing an additional parameter or as a potential alternative where blood testing is less convenient or unavailable.

Attenuated total reflection Fourier-transform infrared (ATR-FTIR) spectroscopy has been assessed as a diagnostic method for a wide range of diseases, including cancer and kidney disease[12-16]. It is a non-destructive analytical technique that can be used with complex biological materials, including cells, tissues or biofluids, often without requiring chemical or physical pre-treatment. Analyses of the resulting IR spectra can reveal signatures of disease biomarkers[17]. For example, ATR-FTIR spectroscopy can be used to quantitate creatinine and urea in both blood and urine, with an accuracy equivalent to that of assays in routine clinical use[18-21].

The aims of this study were (i) to determine whether salivary urea can be measured in the clinically-relevant range by ATR-FTIR spectroscopy; and (ii) to test the performance of this method to diagnose and stage CKD.

Materials and Methods

Saliva sample collection. Unstimulated saliva samples were collected from 6 healthy adults and 52 adult CKD patients attending the Nephrology Outpatient Department of the Royal Free Hospital, London, UK between March and August 2018. Subjects were asked to rinse their mouth with water and wait for 15 minutes prior to depositing 1-3 mL saliva into plastic collection tubes (in practice, the smallest volume of saliva practicable for centrifugation and collection of a 5 µL test aliquot was 100 µL). For convenience, saliva samples were stored at -20℃ after collection until salivary urea was measured, though preliminary tests showed no difference in using fresh samples stored at RT for at least 4 hours before analysis. All participants provided written informed consent at enrolment (Ethics number 7727 of the Royal Free London NHS Foundation Trust Research and Development Committee).

ATR-FTIR Spectroscopy. ATR-FTIR spectra were recorded with a Bruker IFS 66/S FTIR spectrometer equipped with a KBr beam splitter, a liquid-nitrogen-cooled mercury-cadmium telluride (MCT) detector and an ATR microprism (SensIR, 3 reflection silicon microprism with ZnSe optics). Spectra were recorded at room temperature versus a clean prism background in the 4000-600 cm^{-1} range. Power spectra were computed by Fourier transformation of 1000 (background; clean crystal surface) or 500 (saliva sample) averaged interferograms at 4 cm^{-1} resolution and were converted to absorbance spectra by calculation as $\log_{10} (I_{background}/I_{sample})$ at each wavenumber.

Generation of salivary urea calibration curve and estimation of detection limit. Saliva was provided by a healthy volunteer with normal urinalysis and no personal or family history of kidney disease. A 100 mM urea (purity $\geqq$ 99.5, Sigma-Aldrich) stock solution was prepared in ultra-pure water and was diluted into the saliva to obtain 500 µL saliva samples with 0-20 mM added urea. Samples were centrifuged at 10,000 x g_{av} for 10 minutes at 4 ℃ to remove any particulate material. Aliquots (5 µL) of the resulting supernatants were dried on the ATR-prism with a gentle stream of N_2 gas (200 mL/min) and sample spectra were recorded (Figure 1A). The areas of the 1449 cm^{-1} urea peaks and were integrated between limits of 1481 cm^{-1} and 1435 cm^{-1} and plotted versus added urea concentration to generate an initial calibration curve. The Y axis intercept (peak integral of healthy saliva with no added urea) was then used to determine the amount of endogenous urea present, which in this case was 3.5 mM. Hence, this endogenous urea was factored into the calculated total final concentrations of samples with added urea (0-20 mM) and were 3.5-22.8 mM. These concentrations provided the plot of peak area *versus* total urea concentration (Figure 1B) which was used for all subsequent measurements. The accuracy and reproducibility of the plot indicated that concentrations as low as 1-2 mM urea should be detectable. It should be noted that a new calibration curve would be required for other spectrometer configurations, though this has to be done only once for each new device.

Quantitation of salivary urea in controls and CKD patients. After thawing, 500 µL aliquots of normal and patient saliva samples were transferred to 1.5 mL Eppendorf tubes and centrifuged at 4 ℃ for 10 min at 10,000 x g_{av} (Eppendorf 5415R bench microcentrifuge). An aliquot (5 µL) of the supernatant was placed on the ATR prism, dried with a gentle stream of N_2 gas (200 mL/min) and spectra were recorded after stabilisation of the dried state. Each sample was analysed in triplicate and salivary urea was quantitated using the calibration plot of peak area versus total urea in Figure 1B.

Serum urea and creatinine measurement and CKD staging. Serum urea and creatinine were measured by an automated analyser (Cobas® 8000 modular analyzer series, Roche), calibrated according to the manufacturer's instructions. Serum creatinine, alongside other variables including age, gender, and race were used to estimate GFR using the Modification of Diet in Renal Disease (MDRD) equation and, hence, define stages of CKD[3, 4].

Statistical analyses. Salivary urea concentrations were plotted according to CKD stage and differences between CKD stages were assessed with a non-parametric one-way analysis of

variance (ANOVA) Kruskal-Wallis with Dunn's multiple comparison test. Correlation between serum and salivary urea was assessed by calculation of Spearman's rank correlation coefficient. Diagnostic performance of salivary urea to detect stages of CKD was assessed using receiver operating characteristic (ROC) analysis. The optimal threshold concentration of salivary urea to diagnose CKD was determined according to Youden's Index[23] and sensitivity and specificity at this threshold are reported. Analyses were performed with Graphpad Prism version 7 (www.graphpad.com). A *p*-value of <0.05 was considered statistically significant.

Results

ATR-FTIR spectra of dried saliva and quantitation of salivary urea

Figure 1A shows the ATR-FTIR spectra of a dried saliva sample from a healthy individual with different concentrations of added urea (0, 1, 2 and 3 mM), together with that of a dried sample of pure urea. Pure urea displays a prominent band at 1464 cm^{-1} (attributable to a v_{as}(C-N) mode) when dried in water, but this can be shifted to a lower wavenumber when urea is dried in complex mixtures, probably due primarily to retention of water molecules[22]. This is the case for urea in dried saliva where this band is centred around 1449 cm^{-1} (Figure 1A). An increase in intensity of this 1449 cm^{-1} urea band is seen in the dried saliva spectra with increasing additions of urea above 1 mM. This urea peak was chosen for quantitation as it is intense and has little interference from other materials in the saliva. A calibration curve was constructed using a healthy control saliva sample with added urea in the physiologically-relevant 1-20 mM range, with urea quantitated from the area of the 1449 cm^{-1} peaks integrated between 1481 cm^{-1} and 1435 cm^{-1} (Figure 1B). The peak area of the control saliva with no added urea is 0.8 (Y axis intercept in Figure 1B). Assuming that this arises solely from endogenous urea, it corresponds to 3.5 mM urea, a concentration within the expected range for healthy saliva samples[9, 25-27]. Hence total urea is calculated as endogenous + added urea and the plot with these total urea concentrations (lower X axis) was used to determine the total urea concentrations in control and CKD patient saliva samples described below.

Patient cohort

Of the 52 CKD patient saliva samples collected, 32 had either an insufficient saliva sample (n=6) for analysis or were contaminated with blood (n=7), sputum (n=7), or foreign material (n=12), and were excluded before ATR-FTIR analysis. The remaining 20 patient samples were included in this pilot study, alongside saliva samples from 6 healthy controls. Demographic and clinical data of the CKD patients are outlined in Table 1. Causes of CKD were: IgA nephropathy (n=3), diabetic nephropathy (n=5), lithium toxicity (n=2), hypertension (n=2),

systemic lupus erythematosus (n=1), minimal change disease (n=1), membranous nephropathy (n=1), membranoproliferative glomerulonephritis (n=1), previous nephrectomy (n=1), reflux nephropathy (n=1), obstructive uropathy (n=1), and unknown (n=1).

Quantitation of salivary urea from normal subjects and patients in different CKD stages by ATR-FTIR spectroscopy

Salivary urea concentrations (Supplementary Table 1) were estimated by ATR-FTIR spectroscopy from areas of the 1449 cm^{-1} urea peak referenced to the calibration plot of Figure 1B. The salivary urea concentrations in controls and in patients with different stages of CKD are shown in Figure 2. There were consistent differences in urea concentration ranges between the control (C) and CKD stage 3-5 groups. The mean values of salivary urea were: Control: 4.1±0.5 mM; CKD stage 3: 6.6±0.3 mM; CKD stage 4: 8.9±0.4 mM; CKD 5:14.3±0.6 mM, with significance of differences between groups shown in Figure 2. For CKD stages 1/2 (where eGFR was either normal or very slightly lower than normal), there was only a small increase in average salivary urea levels compared to the healthy control group; a greater sample size would be required to determine whether this difference is statistically significant.

The correlation between salivary and serum urea levels in CKD patients

Serum urea of CKD patients was measured by an automated analyser at the same time as the saliva samples were taken. Serum and salivary urea concentrations were positively correlated (Spearman's rank correlation coefficient of 0.71; p<0.001; Figure 3). The automated analyser also provided serum creatinine concentrations and these also showed the expected positive increase with advancing stages of CKD (Table 1).

Diagnostic performance of salivary urea

The performance of salivary urea measurement by ATR-FTIR spectroscopy for distinguishing different stages of CKD was assessed by Receiver Operating Characteristic (ROC) curve analyses, which provide estimates of the ability to resolve groups as the threshold value of the distinguishing parameter is varied[23]. The areas under such curves range from 1 for perfect resolution to 0.5 for zero resolution. Firstly, a ROC plot was constructed to test the resolution of a combined group of stages 3-5 CKD patients (i.e. eGFR < 60 mL/min) from a control group of healthy + stage 1+2 CKD patients (i.e. eGFR> 60 mL/min) (Table 2). At an optimal threshold concentration of 6.5 mM urea, this indicated a sensitivity (i.e. %true positive) of 87% and a specificity (i.e. 100 − %false positives) of 100%. Distinction of stages 4+5 CKD patients from the control group gave 100% sensitivity and 100% specificity at an optimal threshold of 8.1

mM urea whereas the stage 5 CKD patient group alone gave 100% sensitivity and 94% specificity at 11.2 mM urea.

Discussion

Although measurement of blood urea nitrogen is still often used together with serum or plasma creatinine to assess kidney excretory function, it is generally regarded as a less reliable measure than creatinine, which is used to estimate GFR. An increase in blood urea concentration is not so directly related to changes in GFR because it can be affected by additional factors, including dietary protein intake, gastrointestinal bleeding, dehydration, use of glucocorticoids or liver dysfunction. That said, measurement of urea levels in saliva is a potentially useful, rapid, convenient and cost-effective initial screening measure of kidney function in a busy clinical setting, and especially in more remote environments, as well as being with or near the patient. Our data illustrate its potential for this purpose and could guide the selection of patients for follow-up serum or plasma creatinine estimates and further investigation of kidney function.

We show here that urea at physiologically-relevant concentrations can be detected in the spectra of saliva samples. Urea has a prominent and characteristic v_{as}(C-N) urea band at 1464 cm^{-1} when dried in its pure state. However, this band is usually downshifted when urea is dried in complex mixtures, as is the case here where the band appears at 1449 cm^{-1} in the dried saliva samples in Figure 1A. This is probably due to the retention of water in the complex mixtures and the resulting H-bonding interaction differences in urea-urea and/or urea/water interactions in comparison to those of pure dry urea, previously discussed in relation to urea spectra in dried urine specimens[22, 24].

A urea calibration plot was established in the clinically-relevant concentration range by using the peak at 1449 cm^{-1}, integrated between 1481 and 1435 cm^{-1}, for a series of saliva samples to which urea was added, and taking into account the 3.5 mM urea present in the original healthy saliva sample. Quantitation of urea from this band allows salivary urea levels above 1-2 mM to be determined accurately, hence enabling quantitation in the clinically-relevant range of 3-40 mM[9, 25-27].

The concentrations of salivary urea measured by this method were slightly lower than the corresponding serum urea concentrations (Table 1). This is consistent with several published reports in the literature[9, 26, 27] and, as saliva is an ultrafiltrate of blood, the same components measured in saliva might be expected to be lower than in blood[28]. However, a few studies

have reported levels of salivary urea that are similar to, or even higher than, serum urea in some CKD patients[27, 29].

Importantly, the urea concentrations measured by the ATR-FTIR protocol are sufficiently accurate to provide a definitive diagnostic method of distinction between healthy subjects and those with stages 3-5 CKD (Figure 2) as defined by their eGFR values. In addition, the urea levels clearly increase with advancing CKD severity and allow each of stages 3-5 to be distinguished from each other. In this very small sample size, there was no statistically significant difference in concentrations of salivary urea between the healthy control group and stage 1-2 CKD patients, though the mean salivary urea concentration in the stage 1-2 CKD group was slightly higher. This may warrant further investigation with much larger sample sizes and a more standardised saliva collection protocol in order to determine statistical significance. However, patients classified with CKD stages 1-2 purely on the basis of eGFR anyway need other markers of kidney disease (e.g. abnormal urine or abnormal imaging) to be classified as having CKD. As such, a patient with CKD stage 2 may actually have a lower urea (and higher eGFR) than one that has 'normal kidney function'.

Despite this proof-of-concept study of its performance quality, to provide a fast, cost-effective method that could replace the more conventional clinical screening methods that require blood samples or other time- and cost-intensive methods, several issues of sample preparation, and in particular technology development, need to be resolved. Firstly, a much larger number of patients at well-defined CKD stages should be included, with both salivary and serum urea levels quantitated also by standard current methods. This would be aided by a more rigorous and consistent method of saliva collection – with the present protocol more than half of the samples were insufficient in terms of volume or unsuitable because of contaminants that made sample handling difficult. An improved collection protocol might include using agents such as chewing gum to stimulate saliva production[30] or providing subjects more time for appropriate oral hygiene prior to sample collection. Finally, although no chemical additives or reactions are required, and data acquisition is straightforward, our present protocol has involved centrifugation of samples before drying 5 µL aliquots onto the ATR prism surface. An improved protocol of saliva collection could obviate the need for centrifugation.

Most important is the need for simplification of the technology for clinical application. This requires development of simplified non-specialist spectrometers, and/or the development of multi-well plate readers for the preparation and analysis of many samples simultaneously. Automated data analysis methods that can provide urea concentrations are easily added,

hence allowing operation by staff with minimal training. Further enhancement of sensitivity and specificity of urea estimation is also possible with more global spectral analysis methods. The reader is referred to an excellent review by Finlayson et al[31] on problems and progress in these areas. To date, a number of other techniques have been used to measure salivary urea and the characteristics of these tests are compared to the method used in this study in Supplementary Table 2[10, 11, 32-36].

In summary, our results demonstrate that ATR-FTIR spectroscopy can be used to detect urea in saliva with an accuracy that is sufficient to identify and categorise patients as having stages 3, 4 or 5 CKD. Hence, the measurement method has the potential as a rapid, preliminary screening tool for stages 3-5 CKD, particularly for populations at high risk (e.g. obesity and diabetes) of developing CKD, or in countries with limited resources. It might also be used more widely in conjunction with the current clinical parameters of albuminuria and eGFR, which alone may not always provide sufficient accuracy[37-38].

Author Contributions

P. Rich and R. Unwin conceptualized the project. T.-L. Lin designed and performed the experimental work. R. Evans and J. Norman enabled patient saliva sample collection. P. Rich and T.-L. Lin analysed the data and wrote the initial draft. All authors reviewed and edited the manuscript.

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Tables

Table 1. Patient demographic and clinical data at the time of saliva collection.

	Number of patients (M/F)	Age (year)	Serum urea (mM)	Serum creatinine (μM)
CKD 1 & 2	2/3	42±17.1	7.1±1.2	78.2±14.9
CKD 3	5/0	65.2±8.4	12.9±2.9	159±12.5
CKD 4	5/0	57.6±19.7	15.8±1.6	298±76.3
CKD 5	1/4	71±5.3	23.1±2.1	434.2±330

Table 2. Diagnostic performance of salivary urea measurements.

Groupings *versus* control group eGFR (CKD Stages)	Sensitivity (%)	Specificity (%)	Area under ROC curve	Threshold of salivary urea concentration (mM)*
eGFR < 60 mL/min (Stage 3-5 CKD)	87	100	0.97	6.5
eGFR < 30 mL/min (Stage 4-5 CKD)	100	100	1	8.1
eGFR < 15 mL/min (Stage 5 CKD)	100	94	0.95	11.2

Diagnostic performance was assessed from ROC plots of true positives (sensitivity) versus false positives (100 - specificity) as urea concentration is varied. Areas under these curves (maximum 1.0) are a measure of diagnostic performance. *Threshold salivary urea concentrations to distinguish the CKD stages listed are those with the optimal Youden index, determined as the urea concentration at which the sum of sensitivity and specificity is maximal.

Figure Legends

Figure 1. ATR-FTIR spectroscopic detection of salivary urea and generation of a urea calibration curve.

(A) A saliva sample from a healthy individual was dried onto the prism after addition of 0 mM (blue), 1 mM (orange), 2 mM (black), or 3 mM (red) urea and spectra were then recorded. The spectrum of a dried sample of pure 3 mM urea (green) is shown for comparison. An increasing band at 1449 cm^{-1} with increasing added urea concentrations is clearly evident. (B) Urea calibration curve for saliva analysis. Saliva samples with 0-20 mM added urea were dried onto the ATR prism. The areas of the 1449 cm^{-1} peaks were integrated between 1481 cm^{-1} and 1435 cm^{-1} and plotted versus added urea concentrations (upper X axis). The r^2 value for the linear regression line of best fit is 0.99. Samples were measured in triplicate and error bars represent standard error of mean. The peak area of the control saliva with no added urea is 0.8 (Y axis intercept in 1B), corresponding to an endogenous concentration of 3.5 mM urea, a concentration within the expected range for healthy saliva samples. Hence total urea is added urea + 3.5 mM and the plot with these total urea concentrations (lower X axis) was used to determine the total urea concentrations in the patient samples.

Figure 2. Concentration of salivary urea in control subjects (C) and patients with different CKD stages.

Horizontal lines are mean values ± standard error of mean. Significance of differences between pairs of groups according to Dunn's multiple comparisons test are shown as * (p <0.05), **(p <0.01), ***(p <0.001).

Figure 3. Correlation between salivary and serum urea levels in CKD patients.

The serum and salivary urea concentrations are positively correlated with a Spearman's rank correlation coefficient of 0.71, p<0.001. The slope of the line of best fit was 1.5.