

**Urine 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), a specific marker of
oxidative stress, using direct, isocratic LCMSMS:
method evaluation and application in study of biological variation in healthy adults**

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Abstract

Background: Urine 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) is a specific biomarker of oxidative stress. We evaluated a modified LC-MS/MS assay for urine 8-oxodG and determined biological variation in healthy adults.

Method: Untreated urine was injected into an isocratic LC-MS/MS system (positive-ion MRM mode). Urine 8-oxodG in 51 healthy volunteers was measured; within- and between-day variations in 23 healthy volunteers were investigated.

Results: Dose-response was linear to 452nmol/L; limit of detection=2.3nmol/L; within-run and between-run CVs were <3.0% and <4.7%, respectively; recovery=97%-101%; accuracy=97.7-103.5%. Urine 8-oxodG (median, mean [SD]): 1.70, 1.70[0.60]nmol/mmol creatinine (n=51). Men had higher (p=0.027) levels than women matched for age and body mass index: mean (SD), 1.90[1.60]; n=26 vs. 1.50[0.55];n=25. Within- and between-day variations were wide but random. No significant differences were seen overall across four time-points within one day (p=0.407) or at the same time-point across five consecutive days (p=0.381).

Conclusions: The method has advantages of speed and relative simplicity as it does not require sample pre-treatment for 8-oxodG extraction, the use of internal standard or gradient LC elution and has high linearity, specificity, precision and recovery. Biological variation in urine 8-oxodG is wide, but no within- or between-day differences at the group level were seen in healthy adults.

1. Introduction

Guanine is the most easily oxidized nucleotide base, and its oxidation forms 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG, also known as 8-OHdG) [1,2]. Measurement of 8-oxodG in DNA is a marker of oxidation-induced damage and, possibly, cancer risk, but 8-oxodG measurement in cells is problematical due to post-sampling changes and artifact [2-4]. Measurement of 8-oxodG in urine is more straightforward. Extracellular 8-oxodG is excreted in urine without further metabolism. It is stable in urine, and levels are not affected directly by diet or cell death [2-4]. The origin of 8-oxodG in urine is not clear, but it is believed to be from sanitation of the nucleotide pool [2]. This makes urine 8-oxodG a potentially specific and robust biomarker of 'whole body' oxidative stress [4-8].

Concentrations of 8-oxodG in human urine can cover a wide range due to the high variation in water content of urine, but are generally in the low nanomolar range [9]. At such low concentrations it is necessary to use an analytical technique that has a low limit of detection (LOD) and wide measuring range, as well as high specificity and precision. High-performance liquid chromatography with tandem mass spectrometry (LC-MS/MS) offers a

sensitive and specific tool for 8-oxodG measurement. Published LC-MS/MS methods for urine 8oxo-dG generally employ a sample pre-treatment step, often solid phase extraction, and require the use of an isotopic internal standard to correct for loss during this step [10,11]. In addition, the HPLC separation preceding entry to the spectrometer is usually by gradient elution [10,11]. The sample workup, internal standard and gradient requirements add to cost and time of analysis. Moreover, and importantly, published data on normal biological variation of urine 8-oxodG are lacking, making it difficult to interpret urine 8-oxodG data from clinical studies and intervention trials.

In this study, a relatively simple, isocratic elution, direct injection HPLC-ESI/MS/MS method for urine 8-oxodG was evaluated in relation to analytical performance characteristics (linearity, precision, recovery, limit of detection). In addition, urine 8-oxodG in a sample of healthy men and women in our local population was determined, and biological variation (within- and between- day) in urine 8-oxodG in healthy subjects was investigated. We also measured, in pilot study, urine 8-oxodG in a small number of newly diagnosed cancer patients to explore disease related effects on 8-oxodG.

2. Materials and methods

2.1. Chemicals

8-oxodG (listed in product catalogue as 8-hydroxy-2'-deoxyguanosine) and ammonium acetate (Sigma Ultra) were purchased from Sigma (Sigma-Aldrich Inc., St. Louis, MO). Glacial acetic acid (AR grade) and acetonitrile (Hipersolv for HPLC) were from BDH (VWR International Ltd., Poole, UK). Water was of Milli-Q quality (18.2M Ω .cm).

Standard samples or calibrators and controls: a stock standard solution of 8-oxodG was prepared in a small quantity of water. From this stock solution, the exact concentration of an aqueous diluted solution was determined by a Beckman Coulter DU Series 700 UV/Vis Scanning Spectrophotometer (Beckman Coulter, Inc., Fullerton, CA) (ϵ 12,300 at 245nm) [12]. The stock and the diluted solutions were stored at -80°C. A solution of 1,127nM in water was prepared weekly from the diluted solution and stored at 4°C for routine use and used daily to prepare working calibrators (0.0, 7.1, 14.1, 28.2, 56.5, 113.0nM) in thawed, pooled urine (stored at -80°C) from apparently healthy subjects. Three control samples,

prepared by spiking the pooled urine with appropriate amounts of standard, were made, stored at -80°C , and thawed for use as needed.

2.2. Urine samples, calibrators and controls

Mid-stream urine samples were collected and stored at -80°C . Calibrators were prepared freshly each time by spiking known amounts of 8-oxodG standard into freshly thawed pooled urine. All test samples, calibrators and controls were vigorously mixed, sonicated for one min, and then centrifuged at 10,000g for 5min. A 25- μl sample of clear supernatant of each calibrator, control or test sample was directly injected into the HPLC-ESI/MS/MS system.

2.3. HPLC-ESI/MS/MS analysis

HPLC separation was achieved using a Chromolith Performance RP-18e column (4.6mm ID x 100mm L, particle size: 2 μm x 13nm pore) (Merck KGaA, Darmstadt, Germany) secured by a guard cartridge (C₁₈, 3mm ID x 4mm L) (Phenomenex, Torrance, CA). The freshly prepared isocratic mobile phase was a solution of ammonium acetate (10mM, adjusted to pH 4.3 with acetic acid) and acetonitrile (96.4:3.6 in volume). The

solution was filtered through a 0.22- μm Millipore filter (Millipore Corporation, Bedford, MA) before use. With an integrated diverting valve installed in the spectrometer, only the eluate fraction of 8-oxodG was delivered into the spectrometer, the rest of the LC eluate was diverted to waste. Peak separations were done at a flow rate of 0.5ml/min for 10.0min (0–7.5min eluate going to waste, 7.5–10.0min eluate going to the mass spectrometer), followed by a wash period of 6.0min at 2ml/min, washing with the same mobile phase. The HPLC system (Agilent 1100 Series, quaternary pump, G1311A and micro vacuum degasser, G1379A, Agilent Technologies Inc., Waldbronn, Germany), equipped with a non-thermostatted well-plate autosampler (G1313A), was linked to a 3200 QTRAP mass spectrometer from Applied Biosystems/MDS Sciex (MDS Inc., Concord, ON, Canada). MS/MS analysis was performed in positive ion mode with a TurboIonSpray[®] source. The spectrometer was first optimized by infusion of an 8-oxodG standard in water (7.0 μM), using the integrated syringe pump (flow rate at 5 $\mu\text{l}/\text{min}$). Both calibration and sample data were obtained by multiple reaction monitoring (MRM) acquisition: precursor ion ($[\text{M}+\text{H}]^+$ at m/z 284, Q1), product ion (at m/z 168, Q3). The product ion was quantified using dwell time

500ms/channel with unit mass resolution for Q1 and Q3. The source parameters were set as follows: curtain gas (nitrogen), 12.0psi; collision gas (nitrogen), medium, ionspray voltage, 5500V; temperature, 375°C; ion source gas (air) 1, 11.0psi; ion source gas (air) 2, 32.0psi; and interface heater, on. The parameters for the optimized compound were: declustering potential, 26.0V; entrance potential 3.5V; collision energy, 19.0eV; and, collision cell exit potential, 3.9V. All data were acquired and processed by Analyst[®] Software 1.4.2 (MDS Inc., Concord, ON, Canada).

2.4. Limit of detection, lower limit of quantification and ion suppression effects

The absolute limit of detection (LOD) for the MS detector was defined as three times the signal-to-noise ratio of the 8-oxodG standard solution in water. In the absence of 8-oxodG-free urine samples, LOD in urine matrix, the 'true LOD' of the assay, was calculated as follows: six calibration curves ($y = mx + b$) were constructed after subtracting the 8-oxodG urinary basal peak areas from the areas determined for each calibrator. LOD in urine matrix was calculated as twice the SD of the intercept, b , of the six calibration curve divided by the average slope, m . The lower limit of quantification (LOQ) for 8-oxodG in urine was assessed

by calculating 4 times SD of b, divided by m, from the calibration curves. The system was also tested for ion suppression effects at the critical elution time of 7-10min during method development [13].

2.5. Precision, recovery and accuracy

Precision was checked with three 8-oxodG-spiked pooled urine controls (spiked levels at 7.1, 14.1 and 56.5nM) which were stored at -80°C along with subject samples until analysis. Within-day precision was performed by 6 replicate samples tested within one day. Between-day precision was tested over 6 different days. Recovery of 8-oxodG was assessed in urine by addition, to normal pooled urine, of 8-oxodG in three different concentrations (7.1, 28.3, 56.5nM), with each tested 6 times and in parallel with the same amounts added to water. Mean recovery (%) was estimated from the 8-oxodG concentration measured in urine divided by those measured in water, while accuracy was assessed from the concentration found in urine divided by the nominal (expected) concentration. The detection reproducibility in a urine sample under measuring conditions was also tested using an 8-oxodG control

containing a spike of 14.1nM 8-oxodG. This was left at room temperature and measured every 20 samples over the course of almost 24 hours.

2.6. Subjects and experimental design

Fifty one apparently healthy, non-obese Chinese adults, all non-smokers, were recruited with their informed consent. There were 26 men [mean (SD), age (y): 39.5(6.7); BMI (kg/m²): 22.6(2.4)] and 25 women [age: 38.0(8.3); BMI: 20.5(2.1)]. All procedures involving subjects complied with the Declaration of Helsinki (revised 2008).

For the investigation of the biological variation of urine 8-oxodG, the work was divided into 3 parts: (1) male to female differences study (n=51); (2) within-day variation study for each individual and overall for the group (n=23); and (3) day-to-day variation study for each individual and overall for the group (the same 23 subjects). These 23 subjects comprised 11 males [mean (SD), age (y): 38.1(5.1); BMI (kg/m²): 22.8(2.2)] and 12 females [age: 37.3(7.7); BMI: 20.7(2.2)]. From each subject repeated samples of mid-stream urine were collected into plain bottles as follows:

- i. For male/female differences study: samples of first voided urine in the morning were

collected, with samples collected on the same day for all 51 subjects.

- ii. Within-day variation: four urine samples from each of the 23 subjects were collected over the course of a single day; the times of collection were: morning (first-voided urine); 1:00-2:00 pm; 6:00-7:00 pm; the last-voided urine before sleep.
- iii. Between-day variation: samples of first-voided urine were collected from each of the 23 subjects on five consecutive days.

All urine samples were stored at 4°C at the point of collection. On arrival in the laboratory, urine was centrifuged at 3,500g for 15min, and clear urine was aliquoted and stored at -80°C until needed. On the day of analysis, thawed urine was vortex mixed, sonicated for one minute and centrifuged at 10,000g for 5min, then 25µl of urine was injected directly into the LC-MS/MS system without further pre-treatment.

To explore disease-related effects on urine 8-oxodG, we also performed a very preliminary pilot study in which 8-oxodG in urine collected from 11 newly diagnosed, consenting, cancer patients immediately prior to their beginning treatment was measured.

2.7. Creatinine determination

Urine creatinine concentration was measured on the day of collection using a Dade Dimension RxL analyzer (Dade Behring Inc., Newark, NJ) and a kinetic Jaffe method for creatinine kit (Dade Behring, Milton Keynes, UK).

2.8. Data analysis

Data were performed using Graphpad Prism, version 3.0 (San Diego, CA).

Distribution of data was tested for normality before analysis. The unpaired t-test was used to investigate male/female differences in urine 8-oxodG, and to compare results in healthy subjects and cancer patients. Pearson's correlation was used to investigate the relationship of urine 8-oxodG with age and BMI. Biological variation was investigated using repeated measures analyses of variance and Bonferroni multiple comparison post-test. Male-female difference in biological variation was investigated by the Mann-Whitney test. The total analytical variation (CV_A) was calculated from the individual CVs of 8-oxodG and creatinine assays, using the formula: $[(CV_{8\text{-oxodG}})^2 + (CV_{\text{creatinine}})^2]^{1/2}$. The mean \pm 2SD (n=51) was set as a preliminary reference range for urine 8-oxodG. The total between-individual coefficient of variation (CV_{TB}) of urine 8-oxodG was obtained by first calculating average values per

individual and then calculating standard deviation and mean of these individual averages.

CV_{TB} is the sum of the overall biological between-individual CV (CV_{BB}) and the CV_A . So,

CV_{BB} was calculated as follows: $CV_{BB} = [(CV_{TB})^2 - (CV_A)^2]^{1/2}$. The total within-individual

CV (CV_{TW}) of urine 8-oxodG was obtained by calculating the average of individual CV,

which was obtained from standard deviation and mean per individual. CV_{TW} is the sum of the

overall biological within-individual CV (CV_{BW}) and the CV_A . Hence, CV_{BW} was calculated

as follows: $CV_{BW} = [(CV_{TW})^2 - (CV_A)^2]^{1/2}$. For biological variation information to be of any

real value, the variance of a constituent must be homogeneous. Index of heterogeneity was

used to reach a quick decision on whether the within-individual variances showed

heterogeneity. The index of heterogeneity is the ratio of observed CV_{TW} to theoretical CV,

which is $[2/(n-1)]^{1/2}$ where n is the average number of specimens collected per individual.

The standard deviation of the difference between this ratio and its expected value of unity

under the hypothesis of no heterogeneity of the true with subject variances is $1/(2n)^{1/2}$. It may

be concluded that significant heterogeneity is present if the ratio differs from unity by at least

twice this standard deviation [14]. The desirable analytical goal for imprecision was set at 0.5

CV_{BW} [14]. The index of individuality was calculated as CV_{BW}/CV_{BB} and was used to evaluate the utility of population based reference values. When the index of individuality is less than 0.6, conventional population based reference values are of very limited value in the detection of unusual (or changed) results for a particular individual. Repeated sampling with comparison of the measured difference with reference change value may be superior to reference intervals for clinical interpretation at the individual level. In contrast, when index of individuality is more than 1.4, observed values can be compared usefully with reference values [14]. The reference change value (RCV), i.e., the change in a result that makes it significantly different from a previous result with $p < 0.05$, was calculated as follows: $RCV = 2.8 \times [(CV_{BW})^2 + (CV_A)^2]^{1/2}$ [14].

3. Results

Typical chromatograms of 8-oxodG, including one investigating possible ion suppression effect, are shown in Fig. 1. The single, near symmetrical peak obtained indicates good LC resolution of 8-oxodG. No evidence of interaction or competition with other ions was seen, as evidenced by absence of ion suppression around the 8-oxodG peak eluate. A

linear dose response was obtained over a wide range (7-452nM). A typical calibration line is shown in Fig. 2.

Figures 1 and 2 near here

The absolute LOD, the 'true' LOD and LOQ (in urine) were 1.8, 2.3 and 4.6nM, respectively. Water was used as the blank sample since normal urine contains 8-oxodG. To determine the absolute LOD, various amounts of 8-oxodG prepared in water were injected to obtain a peak with signal to noise ratio >3 [Fig. 3]. Within-day and between-day CVs (n=6 for each of three 8-oxodG concentrations, 7.1, 14.1 and 56.5nM) were, respectively, 0.4-3.0% and 2.5-4.7% [Table 1]. Mean recovery and relative accuracy of 8-oxodG in the assay were, respectively, 97.1-101.0% and 97.7-103.5% across three different 8-oxodG concentrations (7.1, 28.3 and 56.5nM, n=6 for each) [Table 1]. The reproducibility (CV) of 8-oxodG measurement in a urine sample kept at room temperature and measured repeatedly over a 24 hour period was 6.5% (n=5). The within-run and between-run CVs of urinary creatinine assay were 1.7-1.9% and 1.6-2.2%, respectively (n=6 for each). The desirable analytical goal for imprecision ($0.5 \times CV_{BW}$) for creatinine-standardized urinary 8-oxodG analysis was

estimated to be 9.1% or better. Using the largest CVs of 8-oxodG and creatinine controls, the total CV_A was found to be 5.6%. These figures for linearity, precision, recovery and LOD meet analytical goals and compare well with published methods (see discussion section).

Figure 3 near here

Table 1 near here

Urine 8-oxodG results of our 51 subjects are presented in Table 2. Urine 8-oxodG (nmol/mmol creatinine) in men was slightly but significantly ($p=0.027$) higher than in women. No significant correlation was seen between urine 8-oxodG and age ($r^2=0.006$; $p=0.592$) or between urine 8-oxodG and BMI ($r^2=0.002$; $p=0.783$) (data not shown).

Table 2 near here

Individual variations, both within- and between-day, were wide [Fig. 4], however no statistically significant differences in urine 8-oxodG were seen in the group overall in samples collected at four different time-points within the same day. Urine 8-oxodG results (mean (SD), $n=23$ for each) for first voided morning urine and for urine collected at midday, evening and before sleep were, respectively, 1.7(0.52), 1.6(0.61), 1.7(0.65) and 1.6(0.61)

nmol/mmol creatinine ($P>0.05$). In samples collected at the same time-point over 5 consecutive days biological variation was also wide, but no statistically significant between-day differences were seen in the group overall. Mean (SD) urine 8-oxodG results were: Monday 1.6(0.57); Tuesday 1.5(0.50); Wednesday 1.6(0.52); Thursday 1.6(0.51); Friday 1.5(0.54) nmol/mmol creatinine ($P>0.05$).

Figure 4 near here

The total within-individual CV (CV_{TW}) of all subjects was 19.1%. The CV_{BW} (overall biological within-individual CV) was 18.3%. When results were compared for gender, no statistically significant difference in CV_{BW} between males and females was detected ($p=0.131$) although the CV_{BW} in males (15.1%) was smaller than females (20.7%). The total between-individual CV (CV_{TB}) of all subjects was 31.3%. The CV_{BB} (overall biological between-individual CV) was 30.8%. The CV_{BB} in males (24.8%) was smaller than in females (36.7%) but this difference was not statistically significant.

The index of heterogeneity of all subjects, males and females was, respectively, 0.36, 0.30 and 0.40. In each case this is less than twice of the corresponding standard deviations of

the difference between the index of heterogeneity and their expected value of unity under the hypothesis of no heterogeneity of the true with subject variances (0.50). So, homogeneous within-individual variances were found in this current study. The index of individuality of all subjects was found to be 0.59. There was marked individuality with low index of individuality. Stratification by sex is considered when reference values are being considered. The index of individuality for males and females, respectively, was 0.61 and 0.56. The reference change values (RCVs) of urinary 8-oxodG for all subjects, males and females were, respectively, 53.5, 45.1 and 59.9%.

Mean (SD) urine 8-oxodG concentration in 11 cancer patients was 2.71 (0.78) nmol/mmol creatinine, which was significantly ($P<0.0001$) higher than in the healthy subjects.

4. Discussion

Urine 8-oxodG has been represented and used as a biomarker of oxidation-induced DNA damage [2,3,5-9,15-18]. However, 8-oxodG in urine originates not from repair of oxidized DNA, but most probably from oxidation-induced modifications to the nucleotide pool [15]. As such, urine 8-oxodG represents a sensitive, stable and specific marker of

oxidative stress at the whole body level [2-9,19]. Using urine as a sample eliminates the requirement for DNA extraction and 8-oxodG release from DNA, with its associated problems and errors [2,8,20], and testing urine simplifies the assay in other ways also. To elaborate briefly, 8-oxodG molecules in urine are freely dispersed in the liquid matrix.

Therefore, it is not necessary to perform digestion or solid phase extraction before introducing the sample into the HPLC unit. The near 100% recovery of 8-oxodG in the method described reflects this, and means that an isotopic internal standard is not needed, as it is when there is a likelihood of loss during extensive sample workup and extraction.

Furthermore, although other oxidized bases may be present in urine (the main one being deoxyguanine (dG)), good HPLC separation prevents their interference. Under the HPLC conditions used, dG elutes at ~4min (data not shown), far away from 8-oxodG, which elutes at 8.5min. Therefore, in the eluate of interest (~8.5min) no dG is present to be oxidized to 8-oxodG during electrospray ionization. If dG is of interest to measure, it is a simple matter to allow the 4min eluate to enter the spectrometer for measurement, but this still will not cause

interference with 8-oxodG measurement (or vice versa) because of their distinctly different elution times.

In the method described, the column used is designed to elute with high flow rate while avoiding increased back-pressure. Therefore, after elution of the peak of interest, the flow rate can be increased to clean the column, making isocratic elution feasible, and a very reproducible chromatogram is obtained. By programming only the LC column eluate containing the 8-oxodG to enter the mass spectrometer, contamination is minimized and the life of the detector is not compromised. In this way urine clean-up can be omitted, saving both sample handling time and cost. The run time for one sample in the current assay was 16min, including the column washout time. The effectiveness of peak separation and on-line cleaning was checked by neat urine injection (without using the spectrometer) with simultaneous monitoring of signals from electro-chemical and photo-diode array detectors (results not shown). No marked increase of back-pressure or peak distortion was observed even after performing >300 sample injections.

Several methods for urine 8-oxodG measurement exist (immunoassays, HPLC with electrochemical detection, as well as LC-MS/MS methods), and results have been reported in various units, including ng/dl, nmol/l, nmol/24h, ng/mg creatinine and nmol/mmol creatinine [see Table 3 and references 19-33]. This makes it difficult to compare results of different studies. To compensate for the highly variable water content of urine, urine 8-oxodG results should be concentration standardized, i.e. expressed relative to urine creatinine. Methods that employ LC-MS/MS are preferred due to the high sensitivity and specificity of this technique [8,15,21-30,33]. Immunoassay gives markedly higher results due to lack of specificity of the antibodies, and immunodetection methods are not recommended [15,18,30,31]. Furthermore, urea, abundant in urine, has recently been found to cross-react in immunoassay methods [32].

In published LC-MS/MS methods for 8-oxodG, sample pre-treatment requires the use of an internal standard to maintain satisfactory recovery and low LOD. In the method presented here, recovery was excellent, though it is noted that the true LOD (of 2.3 nM) was higher than in some published methods [Table 3]. However, absolute 8-oxodG concentrations

in normal urine are in the range of 3.0-40 nM, indicating that the true LOD of 2.3 nM of this method is acceptable [24,30,34,35].

Table 3 near here

When the urinary 8-oxodG results (as nmol/mmol creatinine) of this current investigation of 51 healthy Chinese adults were analyzed by gender, males had slightly but significantly higher levels than females. The reasons are not clear, but it is noted that most of female subjects were pre-menopausal. Pre-menopausal females have lower iron status than men, and women also tend to have higher plasma ascorbic acid concentrations and lower metabolic rate than men [36,37]. Together, these factors are likely to lower oxidative stress in women. We saw no age-related increases in urine 8-oxodG in this study, but this may have been because subjects were all apparently healthy and no subjects of advanced age were recruited. Further study is needed to see if this biomarker is affected by age *per se*, or if healthy elderly subjects retain similar values to healthy younger subjects.

The issue of what magnitude of change can be expected to occur normally in urine 8-oxodG has not been well studied. Data presented indicate that a change of ~50% in urine 8-

oxodG can be expected to occur normally in an individual. The high and random variation observed in the level of urinary 8-oxodG within and between individuals may be explained by genetic polymorphisms of the genes such as CYP2E1 and GSTT1 genotypes involved in metabolism and detoxification [37,38], to differences in antioxidant status as well as the lifestyle of the individuals, and even to psychological and occupational stresses such as physical labour and day-night shift work [39-42].

Regardless of the cause, the wide intra-individual variation means that, in clinical study of individuals, a series of measurements is needed to track pathological or treatment-related effects on oxidative stress. However, there was no evidence of a diurnal or any common pattern of change in urine 8-oxodG within a day or between days in healthy adults. This indicates that time of sampling is not critical. Furthermore, data indicate that a sustained trend of change in urine 8-oxodG concentration in an individual or group is likely to be caused by a change in oxidative stress, and very unlikely to be caused by normal biological variation. To fully validate a biomarker for clinical use, it must be shown to be different in healthy vs. diseased subjects, and to relate to stage of disease. Such evaluation is outside the

scope of this methodological and biological variation study, but our very preliminary data on cancer patients (who could be expected to be in a state of increased oxidative stress) showed they had significantly higher urine 8-oxodG than the healthy subjects studied. However, it must be noted that these subjects were not age or sex matched to the healthy group, and the number was very small. Results need to be confirmed in larger study.

In conclusion, the urine 8-oxodG LC-MS/MS method presented does not require sample pre-treatment for 8-oxodG extraction, or the use of an internal standard, or gradient LC elution. It has the advantages of speed, simplicity and lower cost when compared to LC-MS/MS methods that do require these steps, and it demonstrates high specificity, precision and recovery. Within-day and between-day variations in urine 8-oxodG in healthy adults are wide but random, and no differences at the group level were seen over the course of a single day or over five consecutive days.

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List of abbreviations

8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; HPLC, high performance liquid chromatography; LC, liquid chromatography, LC-MS/MS, liquid chromatography-tandem mass spectrometry; HPLC-ESI/MS/MS, high performance liquid chromatography-electrospray ionization-tandem mass spectrometry; MRM, multiple reaction monitoring; LOD, limit of detection; CV, coefficient of variation; CV_A , total analytical coefficient of variation; CV_{BB} , overall biological between-individual coefficient of variation; CV_{BW} , overall biological within-individual coefficient of variation; CV_{TB} , total between-individual coefficient of variation; CV_{TW} , total within-individual coefficient of variation

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Figure Legends

Fig. 1. Test on ion suppression effect: under multiple reaction monitoring mode (m/z 284 \rightarrow 168 amu): (a) mobile phase (20 μ l) was injected during infusion of aqueous 8-oxodG (3.2 μ M) and no ion suppression occurred; (b) neat urine (20 μ l) was injected with infusion of 8-oxodG and strong ion suppression, due to compounds present in urine matrix, occurred at 2.7-3.7min; (c) neat urine (25 μ l) spiked with 8-oxodG (14.1nM in urine) in a normal chromatographic run, 8-oxodG elution peak at 8.0-8.8min.

Fig. 2. Typical calibration curve of the assay over 8-oxodG concentration 7 to 113nM.

Fig. 3. Test on absolute limit of detection of 8-oxodG with chromatograms showing: (a) water (25 μ l) injected to observe the baseline; (b) absolute LOD of 8-oxodG in water (45fmol injected), peak at 8.7min with S/N ratio >3 .

Fig. 4. Biological variation in creatinine-adjusted urine 8-oxodG concentrations (medians and error bars of ranges) in samples from 23 healthy Chinese adults: (a) within-day variation in individual subjects, samples collected at 4 time-points over a single day; (b) day-to-day

variation in individual subjects, samples collected at the same time of day for 5 consecutive

days.

Table 1. Precision, recovery and accuracy data. (a) Within-day (n=6 at each of three

concentrations) and between-day (n=6 at each of three concentrations) precision;

(b) Recovery and accuracy were assessed at three levels (n=6 for each): recovery was

performed in pooled urine spiked with 8-oxodG compared with same spike into water.

Accuracy was calculated from nominal (calculated) values.

(a)

Nominal conc.(nM)	Peak area (counts)						CV (%)
	1	2	3	4	5	6	
Within-day precision							
7.1	562	522	572	552	552	552	3.0
14.1	1142	1162	1182	1132	1162	1182	1.8
56.5	4432	4452	4442	4462	4412	4432	0.4
Between-day precision							
7.1	535	536	536	501	544	540	2.9
14.1	1155	1176	1106	1191	1170	1150	2.5
56.5	4415	4506	4106	3981	4300	4400	4.7

(b)

Nominal conc. (nM)	Recovery (%)						Mean (%)	Mean accuracy	
	Peak area (counts)							Absolute (nM)	Relative (%)
	1	2	3	4	5	6			
Urine									
7.1	560	580	610	630	520	610	99.8	7.35	103.5
28.3	2280	2140	2140	2240	2200	2210	97.1	27.66	97.7
56.5	4490	4300	4520	4470	4480	4600	101.0	56.24	99.5
Water									
7.1	573	607	605	588	617	611			
28.3	2265	2225	2305	2235	2195	2275			
56.5	4605	4595	4645	4645	4565	4555			

Table 2. Urine 8-oxodG excretion in 51 healthy Chinese adults

	8-oxodG (nmol/mmol creatinine)		
	n	Mean (SD)	Median(Range)
All Subjects	51	1.7 (0.6)	1.7 (0.6-3.3)
Males	26	1.9 (0.6)*	1.9 (0.7-3.3)
Females	25	1.5 (0.6)	1.4 (0.6-2.5)

*significantly higher than in females ($p=0.027$).

Table 3. Comparison of characteristics and analytical performance of 8-oxodG assays.

Method	Urine	Sample Treatment	Run Time Condition Cleaning	Results in Human Urine Mean(SD) n =	LOD	Reference
HPLC-ESI/MS/MS	25µl	Sonication, centrifugation	16min isocratic on-line	1.7 (0.6) ^a n=51	1.8nM or 45fmol	This paper
HPLC-ESI/MS/MS	65µl	Dilution, precipitation, redissolved	10min isocratic *	~0.26(0.04) ^a n=4	2nM or 40fmol	Malayappan <i>et al.</i> , 2007 [21]
HPLC-ESI/MS/MS	500µl	SPE	25 min gradient	1.8–4.2 ^b n=3	0.35fmol	Sabatini <i>et al.</i> , 2005 [22]
HPLC-ESI/MS/MS	500µl	SPE	10min isocratic -	5.59(3.34) ^c n=49	0.024 ng/ml or 1.7fmol	Hu <i>et al.</i> , 2004 [23]
HPLC-APCI/MS/MS	15ml, inject 100µl	Acidification, centrifugation	15min isocratic **	9.4(0.5) ^c n=12	1ng/ml	Pietta <i>et al.</i> , 2003 [24]
HPLC-ESI/MS/MS	unclear	Dilution, precipitation, redissolved	40min isocratic cleaning unclear	-	0.3nM or 7.5fmol	Weimann <i>et al.</i> , 2001 [25]
HPLC-ESI/MS/MS	3ml	SPE	20min gradient	2.1 ^c n=9	0.2ng/mL or 7fmol	Renner <i>et al.</i> , 2000 [26]
HPLC-ESI/MS/MS	1ml	SPE	>11min isocratic	20pmol/ml	20fmol	Ravanat <i>et al.</i> , 1998 [27]
HPLC-ESI/MS/MS	25µl	Dilution, centrifugation	51min gradient equilibrate 25min	28(2)nmol /24h n=20	0.5nM or 12.5fmol	Weimann <i>et al.</i> , 2002 [33]
HPLC-EC	2mL	SPE	20min isocratic	2.08(1.23) ^b n=15		Chen <i>et al.</i> , 2005 [25]
HPLC-EC	2ml	SPE	20min isocratic	1.61(0.95) ^a n=44		Pilger <i>et al.</i> , 2002 [28]
HPLC-EC	100 µl	2 Switching columns	>1h isocratic	5.65(2.77) ^c n=72		Yoshida <i>et al.</i> , 2002 [30]
ELISA	50 µl	Antibody incubation	>3h (batch assay)	11.13(5.77) ^c n=72		Yoshida <i>et al.</i> , 2002 [30]

^a, µmol/mol (or nmol/mmol) creatinine; ^b, ng/mg creatinine; ^c, ng/ml (or µg/l) urine; * guard column renewed

every 100 injections and back-flush cleaning every 50 urine samples; ** columns cleaned (time not stated) with

90% methanol after each run; ELISA, enzyme-linked immunosorbent assay; EC, electro-chemical detection;

SPE, solid phase extraction; APCI, atmospheric pressure chemical ionization.

Fig. 1 (a)

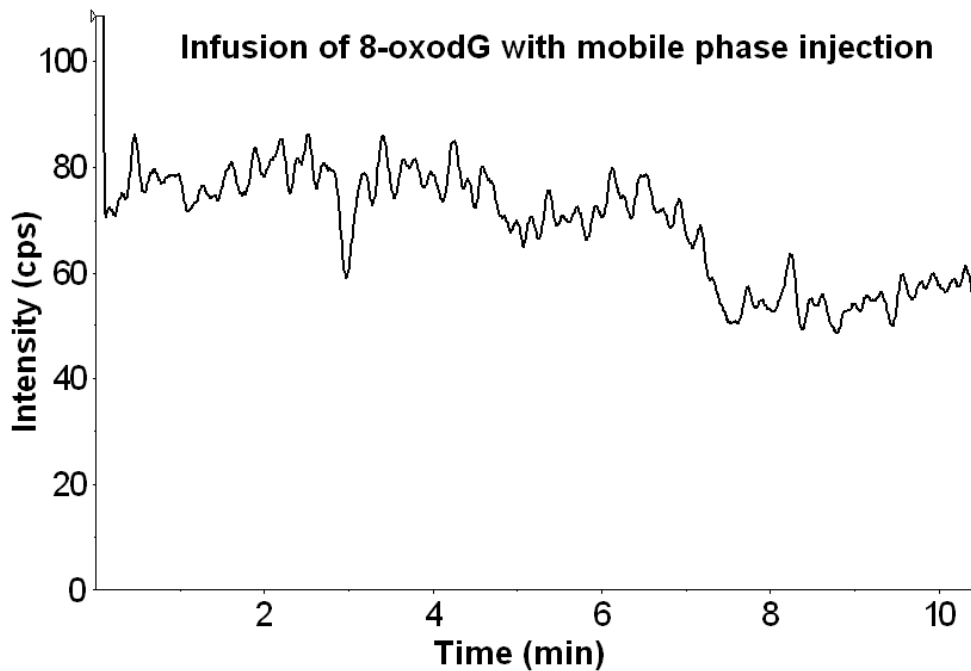


Fig. 1 (b)

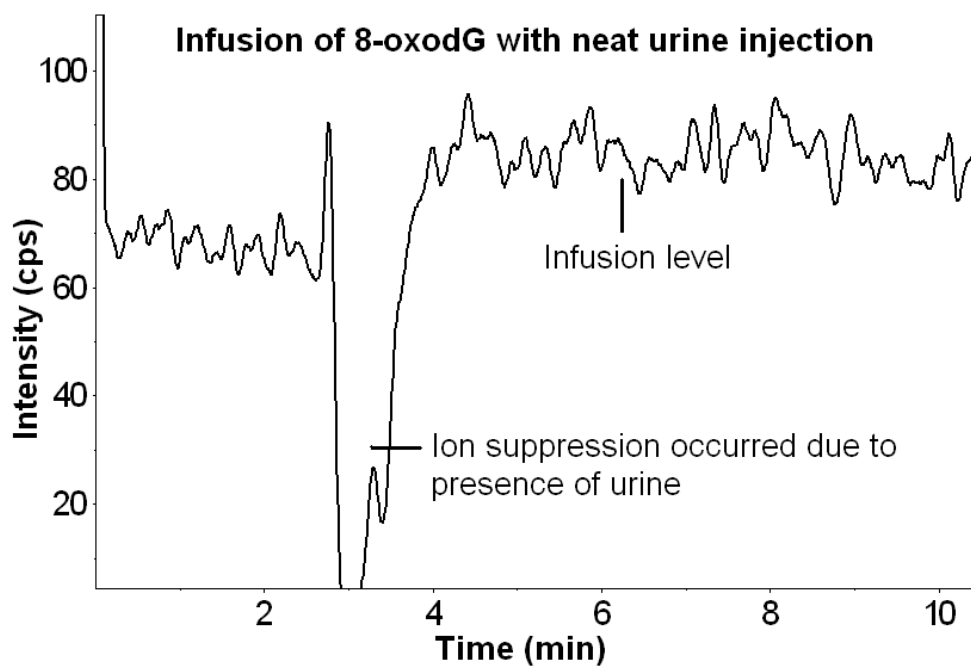


Fig. 1 (c)

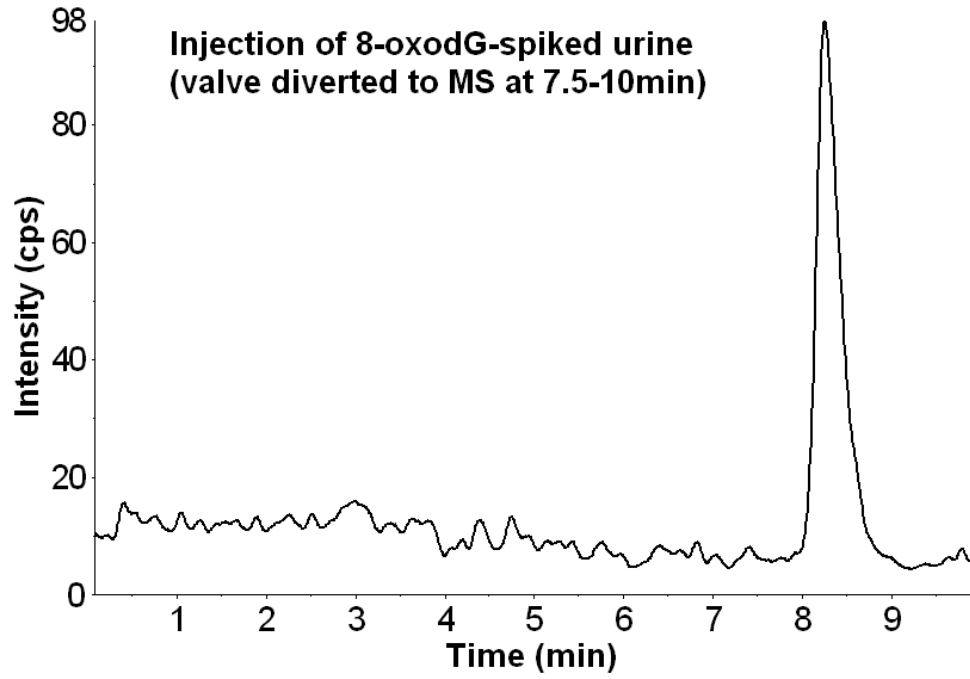


Fig. 2

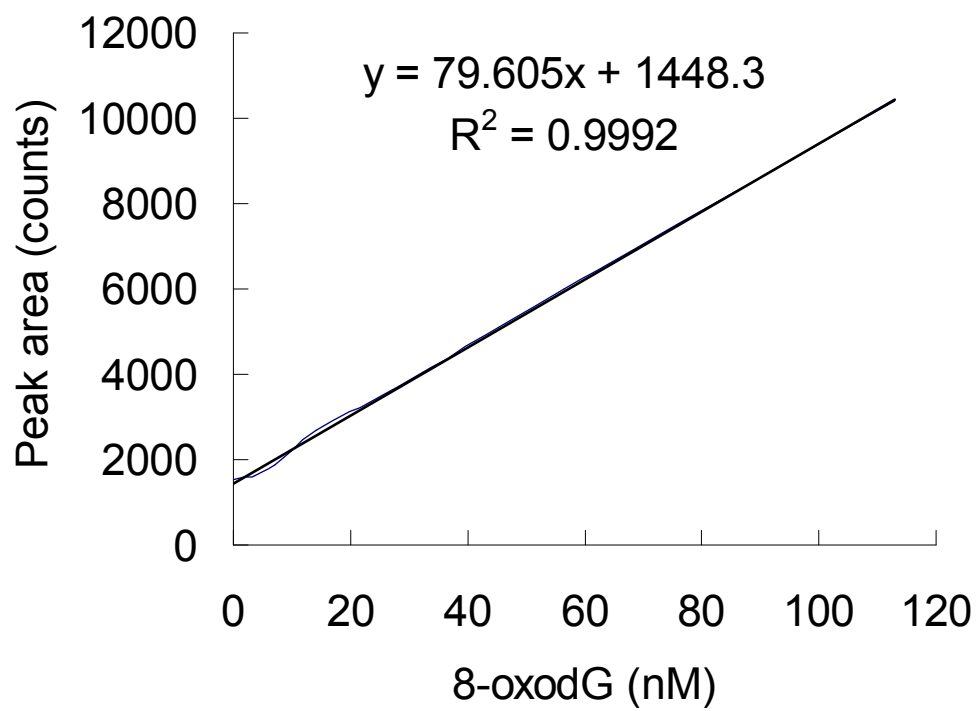


Fig. 3 (a)

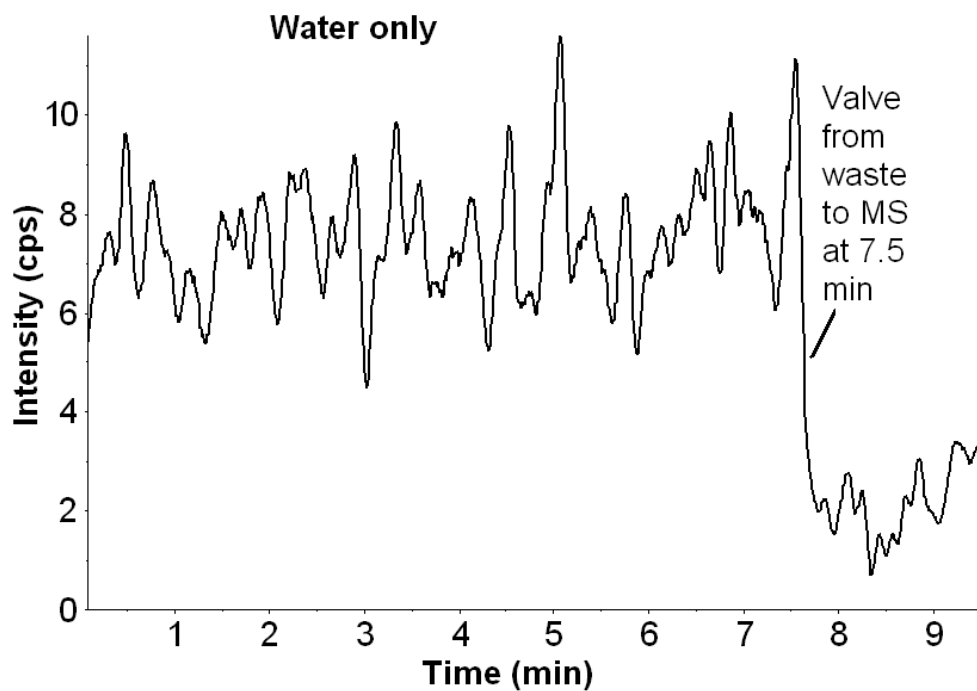


Fig. 3 (b)

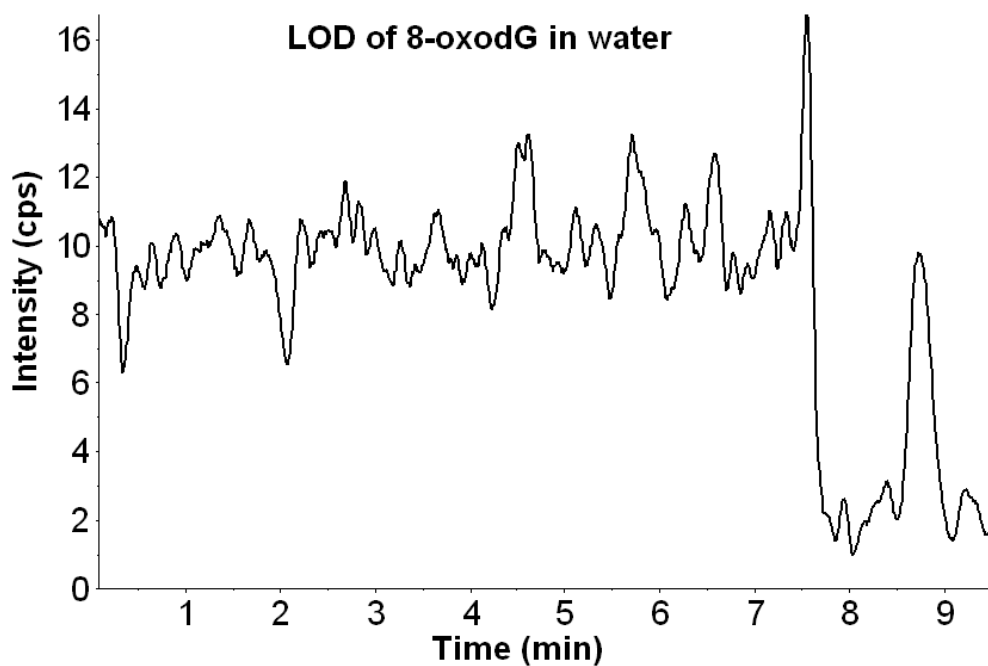


Fig. 4 (a)

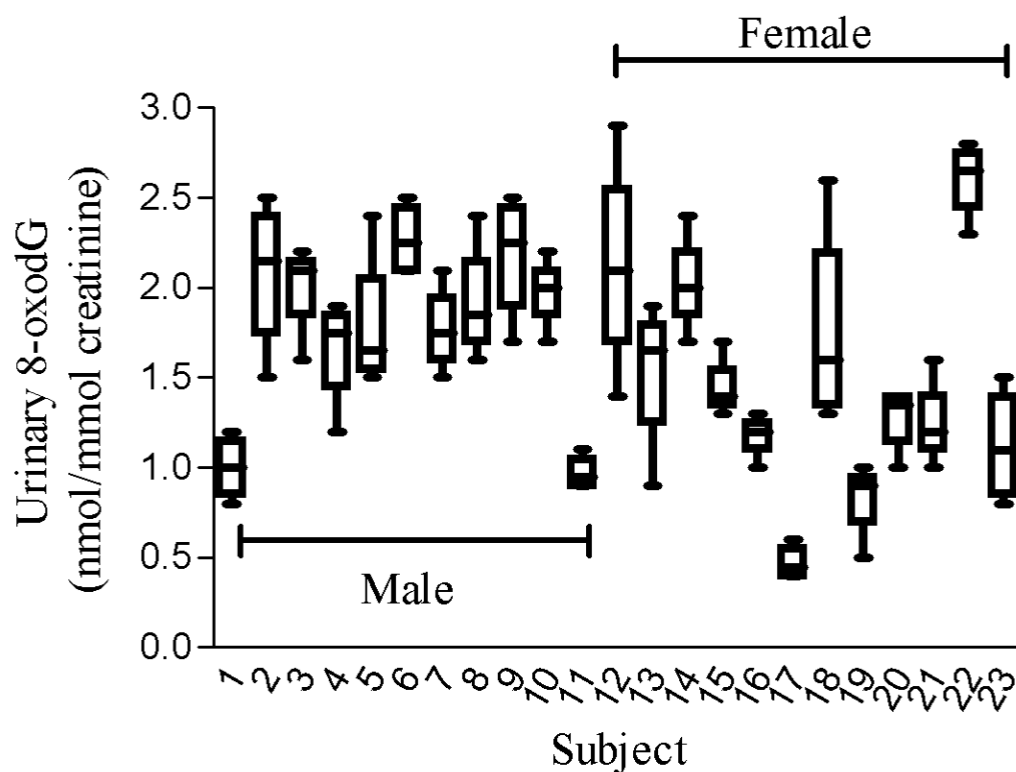


Fig. 4 (b)

