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8-Oxo-2'-deoxyguanosine as a biomarker of tobacco-smoking-induced oxidative stress

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ABSTRACT

7,8-Dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dGuo) is a useful biomarker of oxidative stress. However, its analysis can be challenging because 8-oxo-dGuo must be quantified in the presence of dGuo, without artifactual conversion to 8-oxo-dGuo. Urine is the ideal biological fluid for population studies, because it can be obtained noninvasively and it is less likely that artifactual oxidation of dGuo can occur because of the relatively low amounts that are present compared with hydrolyzed DNA. Stable isotope dilution liquid chromatography–selected reaction monitoring/mass spectrometry (LC-SRM/MS) with 8-oxo-[¹⁵N₅]dGuo as internal standard provided the highest possible specificity for 8-oxo-dGuo analysis. Furthermore, artifact formation was determined by addition of [¹³C₁₀N₅]dGuo and monitoring of its conversion to 8-oxo-[¹³C₁₀N₅]dGuo during the analytical procedure. 8-Oxo-dGuo concentrations were normalized for interindividual differences in urine flow by analysis of creatinine using stable isotope dilution LC-SRM/MS. A significant increase in urinary 8-oxo-dGuo was observed in tobacco smokers compared with nonsmokers either using simple urinary concentrations or after normalization for creatinine excretion. The mean levels of 8-oxo-dGuo were 1.65 ng/ml and the levels normalized to creatinine were 1.72 μg/g creatinine. Therefore, stable isotope dilution LC-SRM/MS analysis of urinary 8-oxo-dGuo complements urinary isoprostane (isoP) analysis for assessing tobacco-smoking-induced oxidative stress. This method will be particularly useful for studies that employ polyunsaturated fatty acids, in which a reduction in arachidonic acid precursor could confound isoP measurements.

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Reactive oxygen species (ROS) generated during normal cellular metabolism are detoxified by a suite of antioxidant enzymes including superoxide dismutases, catalases, glutathione peroxidases, and thioredoxins, as well as by dietary antioxidants [1–5]. Oxidative stress occurs when ROS overwhelm the endogenous detoxification pathways such as during inflammation [6], viral and bacterial infections [6], metabolism of endogenous molecules such as estrogens [7], metabolism of drugs such as etoposide [8],

metabolism of environmental chemicals such as benzo[a]pyrene [9], or tobacco smoking [10]. During oxidative stress, ROS can cause oxidative damage to cellular DNA [1,11] as well as to the trinucleotide precursors of DNA [12]. 8-Oxo-dGuo is by far the most studied of the DNA adducts that arise through ROS-mediated oxidative damage to DNA [13,14].

Previous studies have revealed that significant amounts of dGuo are excreted in the urine [15–20]. This raised the possibility that adventitious oxidation of dGuo to 8-oxo-dGuo could occur during the urine extraction and analysis, as we have previously shown for cellular DNA [11,21]. It is noteworthy that rigorous feeding studies have shown that dietary 8-oxo-dGuo is not excreted in the urine [22,23] and a number of studies have demonstrated that urinary 8-oxo-dGuo does not arise from cell death [24–26]. However, it is of significant concern that urinary 8-oxo-dGuo measurements could not be validated in the carbon tetrachloride rat model, one of the most widely accepted animal models of oxidative stress [27]. Despite this potential problem, urinary 8-oxo-dGuo has become widely accepted as a measure of oxidative DNA-base damage [14]. This is because urinary 8-oxo-dGuo is quite stable [19] and urine can be readily acquired through a noninvasive procedure. Furthermore, there are multiple methods available for the analysis of urinary

Abbreviations: 8-oxo-dGuo, 7,8-dihydro-8-oxo-2'-deoxyguanosine; dGuo, 2'-deoxyguanosine; dGMP, 2'-deoxyguanosine monophosphate; ECD, electrochemical detection; ELISA, enzyme-linked immunosorbent assay; GC-MS, gas chromatography–mass spectrometry; hOGG, human 8-oxo-guanine glycosylase; HPLC, high-performance LC; HQC, high quality control; LC-SRM/MS, liquid chromatography–selected reaction monitoring/mass spectrometry; LQC, low quality control; MQC, middle quality control; MTH, mammalian homolog of *Escherichia coli* MutT; Q, quadrupole; ROS, reactive oxygen species; SPE, solid-phase extraction; TQ, triple quadrupole

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8-oxo-dGuo, including enzyme-linked immunosorbent assay (ELISA) [18,24,28,29], stable isotope dilution gas chromatography–mass spectrometry (GC–MS) [22,25,26,30], and high-performance LC coupled with electrochemical detection (ECD) [15,18,19,31–33]. LC–MS-based methodology has proved to be particularly useful for urinary 8-oxo-dGuo analysis and so the approach described in this critical methods paper is based upon concepts described in these previous studies [16,17,20,28,34–58].

The cleanup methods employed for the urine before injection into the mass spectrometer have included offline SPE and immunoaffinity column purification [33], two-steps of offline cleanup followed by HPLC/ECD [32], or offline HPLC prepurification followed by GC–MS analysis [30]. Newer methods have used an SPE cleanup step, coupled with LC–SRM/MS analysis [16,53,58]. Concentrations determined by LC–MS were correlated with those obtained by ELISA measurements using an assay in which the primary antibody incubation was conducted at 4 °C [44]. Interestingly, although the mean amounts determined by LC–MS and ELISA were similar (Table 1), there were substantial inter-individual differences [44]. In a similar study conducted by Garratt et al. [28], there was a much greater difference between the LC–MS and the ELISA values at both 4 and 37 °C (Table 1). The differences that were observed between LC–MS- and ELISA-based assays can be explained in part by the effect of urea on the antibody–antigen interaction that occurs in the ELISA [29]. As a result, the reported urinary 8-oxo-dGuo concentrations obtained by ELISA-based methodology have questionable validity [14]. This was particularly evident when urine samples were analyzed from individuals with a pathological condition such as cystic fibrosis [28].

Principles

Three base excision repair enzymes, human MutY homolog [59], hOGG1 [60], and hOGG2 [61], are involved in the repair of 8-oxo-dGuo-derived lesions in DNA, whereas the hydrolase enzyme mammalian homolog of *Escherichia coli* MutT (MTH) 1 removes 8-oxo-dGuo from the trinucleotide pool [40,62]. It is this pathway that is considered to be the major source of urinary 8-oxo-dGuo (Fig. 1) [12]. Stable isotope dilution LC–SRM/MS methods are potentially more specific than ELISA-based methodology for the analysis of 8-oxo-dGuo because they can separate the individual oxidized DNA- and RNA-derived base adducts. In general, a triple-quadrupole (TQ) mass spectrometer operated in the SRM mode is employed for the analysis of urinary 8-oxo-dGuo. In this mode of operation, a precursor ion is preselected and resolved in quadrupole (Q) 1 of the TQ and fragmented by collision-induced dissociation in Q2, and the resultant product ion is analyzed in Q3. Under optimal operating conditions, the precursor to product ion “reaction” is monitored many times per second, resulting in extremely reproducible chromatographic peak shape and intensity. In this way, a stable isotope-labeled analog internal standard is used to establish the presence of an endogenous analyte using both the LC retention time and MS/MS mass selection of the TQ platform. This level of specificity cannot be attained with any other bioanalytical technique employed for biomarker analysis.

An authentic stable isotope-labeled analog of an analyte has physicochemical properties identical to those of the endogenous analyte except for its mass. The term stable isotope dilution refers to the use of a stable isotope-labeled internal standard spiked into a sample at a known concentration. The response ratio between

Table 1

Reported values for urinary 8-oxo-dGuo normalized to creatinine concentrations in nonsmoking subjects.

Technique	Nonsmoking subjects (n)	Mean (nmol/mmol creatinine)	SD (nmol/mmol creatinine)	Mean (µg/g creatinine)	SD (µg/g creatinine)	Mean (ng/ml)	SD (ng/ml)	Ref.
HPLC–ECD	60	2.70	1.88			8.26	6.11	[32] ^a
LC–MS	35			4.69	1.70	5.87	2.61	[39] ^b
LC–MS	20	4.65	2.09					[44] ^c
ELISA (4 °C)	20	3.44	1.62					[44] ^c
ELISA (37 °C)	20	7.86	3.92					[44] ^c
HPLC–GC–MS	115			3.86	NP	6.01	5.22	[46]
HPLC–ECD	115			4.20	NP	6.52	4.59	[46]
ELISA (37 °C)	115			18.7	NP	29.8	31.3	[46]
LC–MS	6	2.42	NP					[16]
LC–MS	33	1.27	0.93					[28]
ELISA (4 °C)	33	6.88	2.33					[28]
ELISA (37 °C)	33	5.92	1.95					[28]
LC–MS	50			3.70	2.00	6.20	4.80	[51]
LC–MS	48	0.72	0.45	1.72	1.07	1.65	1.68	This study

NP, not provided.

^a Converted from µmol/mol creatinine to nmol/mmol creatinine and nM to ng/ml.

^b Converted from µg/g creatinine to ng/mg creatinine and µg/L to ng/ml.

^c Converted from pmol/µmol creatinine to nmol/mmol creatinine.

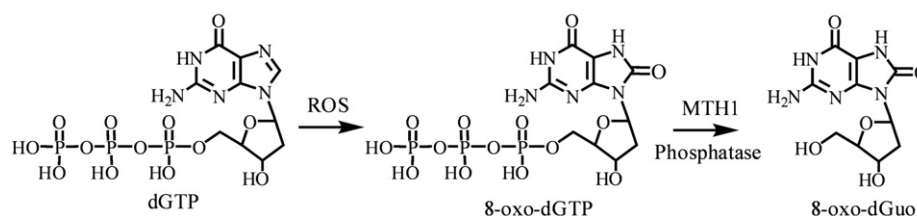


Fig. 1. Scheme for the formation of urinary 8-oxo-dGuo.

the analyte and the labeled compound can then be interpolated onto a standard curve to calculate the absolute amount of analyte in the unknown sample. Therefore, the stable isotope internal standard offers a means to verify the presence of the analyte and normalize experimental variables such as sample storage and matrix suppression. The use of structural analogs as internal standards, rather than authentic isotope-labeled analogs, is undesirable because they will have different retention times and ionization properties compared with the analyte of interest. Therefore, differential ionization can occur between an analyte and a structural analog in the source of the mass spectrometer. This difference arises in part from suppression of ionization by constituents present in the biofluid that is being analyzed and can lead to significant imprecision during quantitative analyses [63]. Unfortunately, suppression effects vary with chromatographic retention time and with biofluid samples from different individuals [64]. It is therefore extremely difficult to standardize the amount of suppression occurring within any particular sample [65].

The ideal control offered by an authentic isotope-labeled internal standard is not always possible because for many biomarkers only deuterated and structural analogs are available. Deuterated forms of a compound are not perfect internal standards, because there is a small but significant separation of the deuterium analog internal standards and their corresponding endogenous protium forms during LC analysis [66]. This slight difference in chromatography can result in differential suppression or enhancement of ionization and affect the quality of the analytical data. Fortunately, [$^{15}\text{N}_5$]dGuo and [$^{13}\text{C}_{10}^{15}\text{N}_5$]dGuo analogs are available so the corresponding labeled 8-oxo-dGuo internal standards can be readily synthesized [11,34,53,67]. Previous reports have described the use of both in-house synthesized stable isotope-labeled internal standards and commercially available 8-oxo-[$^{15}\text{N}_5$]dGuo [14,58] for the quantification of 8-oxo-dGuo in urine. Typically, ^{15}N - and ^{13}C -labeled internal standards have LC retention times identical to those of the corresponding protium forms [68]. Structural analogs are even less representative of the endogenous compound, because in addition to differences in LC retention time, the structural analog can show different absorptive losses. Selective binding to active sites on glassware or other surfaces can occur during extraction and LC analysis, leading to significant analyte loss. Whereas a structural analog might not account for this loss, an isotope-labeled internal standard has identical physicochemical properties and is therefore lost at the exact same rate as the endogenous analyte. Because of this feature of stable isotope analogs, they may act as carriers, preventing the loss of trace amounts of analyte during extraction and analyses [69]. Finally, variability introduced during compound isolation can be fully controlled by an authentic isotope-labeled standard [68].

As noted in previous studies (including our own) the specificity of LC-SRM/MS analysis of 8-oxo-dGuo arises from the use of a unique transition from the protonated molecule (MH^+) at m/z 284 to a product ion derived from the loss of the protonated ribose moiety (m/z 116) at m/z 168 [11,34,53,58]. Similar specific transitions 5 Da higher in mass were employed for the internal standard 8-oxo-[$^{15}\text{N}_5$]dGuo from m/z 289 to m/z 173, and for the marker of artifactual oxidation (8-oxo-[$^{13}\text{C}_{10}^{15}\text{N}_5$]dGuo) transitions 15 (MH^+) and 5 Da (product ion) higher in mass were used from m/z 299 to m/z 178. Thus, three parameters have to be correct to satisfy the analytical constraints required for identification of urinary 8-oxo-dGuo. The analyte must have the correct MH^+ at m/z 284, the correct product ion at m/z 168, and a retention time identical to that of the internal standard (Fig. 2). This potentially provides higher specificity than can be obtained with HPLC-ECD because an internal standard with identical physicochemical properties cannot be used with this methodology. Interfering substances present in the urine were removed using SPE columns. A parallel standard curve was obtained in urine compared with a standard curve constructed in

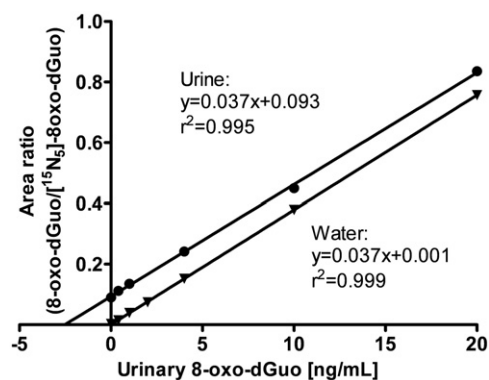


Fig. 2. Calibration curve constructed with authentic standards, performed in water and urine.

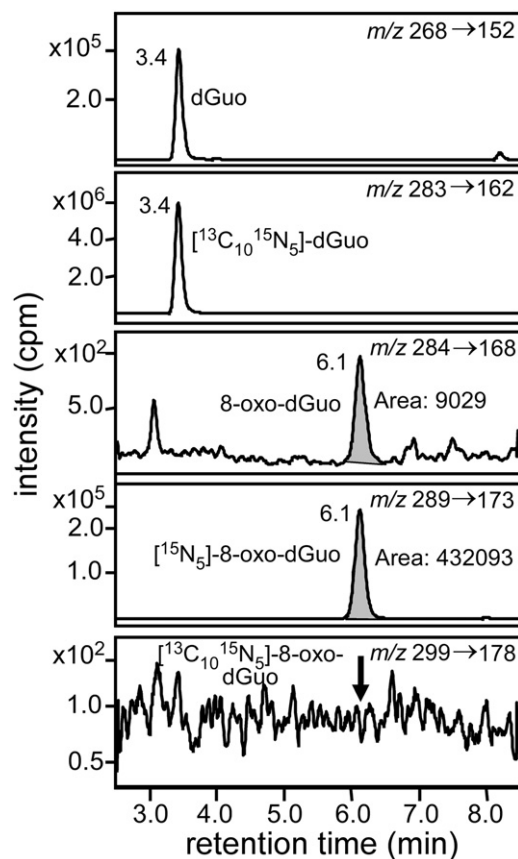


Fig. 3. LC-SRM/MS chromatograms from a nonsmoker's urine sample.

water (Fig. 3). Parallelism of the urine and water standard curves, which is important when analyzing endogenous analytes such as 8-oxo-dGuo, provided further validation of the assay specificity [68].

Validation of the critical methods assay was conducted on 5 separate days with five replicates at the lower limit of quantitation (0.2 ng/ml), as well as with low quality control (LQC; 0.4 ng/ml), middle quality control (MQC; 4 ng/ml), and high quality control (HQC; 20 ng/ml) samples. Precision and accuracy were within the range of $\pm 15\%$ and between 85 and 115%, respectively. Analysis of study samples was conducted using standard curves covering the range of concentrations found in the urine (Fig. 2) together with two LQC samples, two MQC samples, and two HQC samples. Assays were repeated if the QC values were outside the range of 15% for precision or 85 to 115% for accuracy. Artifact formation was determined by addition of [$^{13}\text{C}_{10}^{15}\text{N}_5$]dGuo and monitoring its conversion to

8-oxo-[¹³C₁₀¹⁵N₅]dGuo during the analytical procedure (Fig. 2). 8-Oxo-dGuo concentrations were normalized for interindividual differences in urine flow by analysis of creatinine using a stable isotope dilution LC–SRM/MS assay that was based upon a previously reported procedure [16]. These methods can then be employed to determine whether there is a relationship between urinary 8-oxo-dGuo and tobacco smoking as a biomarker of tobacco-smoke-induced oxidative stress.

Materials and methods

Chemicals and supplies

1. 7,8-Dihydro-8-oxo-2'-[¹⁵N₅]deoxyguanosine (8-oxo-[¹⁵N₅]dGuo) (Cambridge Isotope Laboratories, Cat. No. NLM-6715).
2. [¹³C₁₀¹⁵N₅]-dGuo (Cambridge Isotope Laboratories, Cat. No. CNLM-3900).
3. 8-Oxo-dGuo (Sigma–Aldrich, Cat. No. H5653).
4. Desferal (Sigma–Aldrich, Cat. No. D9533).
5. Formic acid (Sigma–Aldrich, Cat. No. 56302).
6. Sodium chloride (Sigma–Aldrich, Cat. No. S7653).
7. Chelex 100 resin (Bio–Rad, Cat. No. 143-2832).
8. Methanol, acetonitrile, and water (all Optima grades) were from Fisher Scientific.
9. Oasis HLB (30 mg, 1 ml) (Waters, Cat. No. 94225).
10. Conical glass tubes, 10 ml (Kimble, Cat. No. 73790-10).

Study participants and urine samples

Urine samples were obtained from nonsmokers ($n=48$) and from cigarette smokers ($n=85$) who had smoked for a minimum of 6 years and a maximum of 60 years (mean 34 years). Samples, which were provided during a clinic visit, were not collected at predetermined times after the last cigarette had been smoked. Subjects were healthy individuals participating in an ongoing study approved by the University of Pennsylvania Institutional Review Board (Protocol 800924). Smoking status was assigned based on questionnaires, which requested information on smoking history, packs/day, and use of other tobacco products. All of the smoking subjects were cigarette smokers except for one individual who also smoked one cigar/day. Urine samples were collected in 20-ml polypropylene tubes fitted with a screw cap. The tubes were capped and labeled and urine samples were stored at -80°C until analysis.

Sample preparation

1. Positive displacement automated 1-ml pipette (Mettler Toledo, Cat. No. MR-1000).
2. Hamilton gas-tight glass syringe (Fisher Scientific, Cat. No. 13-684-81).
3. 24-port SPE vacuum manifold (Fisher Scientific, Cat. No. 03-251-253).
4. Centrifuge (Sorvall, Cat. No. 75004377).
5. Vortex (Fisher Scientific, Cat. No. 02-215-360).
6. Analytical nitrogen evaporator, 24 sample positions (Fisher Scientific, Cat. No. NC9892499).

Liquid chromatography

1. Phenomenex Kinetex C18 column (100 × 2.1-mm i.d., 2.6 μm) (Phenomenex, Cat. No. 00D-4462-AN).
2. Guard column C18 cartridge (0.5 μm × 0.004 in.) (Phenomenex, Cat. No. AF0-8497).

3. HPLC: an Agilent 1200 series HPLC pump (Agilent Technologies, Santa Clara, CA, USA) was used. It was equipped with an autosampler and thermo controller (set at 4°C). The column heater was set at 30°C .
4. The mobile phase A was water with 0.02% formic acid and mobile phase B was acetonitrile. The linear gradient was as follows: 3% B at 0 min, 3% B at 2 min, 20% B at 8 min, 80% B at 8.1 min, 80% B at 11 min, 3% B at 11.1 min, and 3% B at 15 min with a flow rate of 0.2 ml/min. Injections of 10 μl were made.

Mass spectrometry

An Agilent Technologies 6460 triple-quadrupole mass spectrometer equipped with a JetStream source was operated in positive mode, but any triple-quadrupole instrument could be used. The column effluent was diverted to waste for the first 3 min and the last 5 min of the analysis to prevent extraneous material from entering the mass spectrometer. The Agilent 6460 operating conditions were as follows: gas temperature was set at 275°C and the gas flow was set to 8 L/min. Sheath gas temperature was 400°C and the sheath gas flow was set to 10 L/min. The capillary voltage was set to 3500 V. The nozzle voltage was set to 1000 V. The following transitions were monitored: m/z 284 (MH^+) → m/z 168 [MH^+ -2'-deoxyribose+H] transition for 8-oxo-dGuo and m/z 289 (MH^+) → m/z 173 [MH^+ -2'-deoxyribose+H] transition for 8-oxo-[¹⁵N₅]dGuo. For dGuo and m/z 268 (MH^+) → m/z 152 was monitored and for the labeled [¹³C₁₀¹⁵N₅]dGuo m/z 283 (MH^+) → m/z 162. Any labeled 8-oxo-[¹³C₁₀¹⁵N₅]dGuo that was formed during sample preparation from the added [¹³C₁₀¹⁵N₅]dGuo was monitored by the transition m/z 299 (MH^+) → m/z 178.

Protocol

Preparation of standards and calibration curve solutions

Individual primary stock solutions of 8-oxo-dGuo and 8-oxo-[¹⁵N₅]dGuo (1 μg/ml) were prepared in methanol and stored at -80°C . For [¹³C₁₀¹⁵N₅]-dGuo a stock of 10 μg/ml was prepared in methanol as well. Working solutions were prepared by serial dilutions with methanol. One large urine sample (500 ml) was obtained from a never smoker and used for the preparation of QC samples. Calibration curves were prepared by spiking 8-oxo-dGuo in 250 μl of urine from a never smoker who had not been exposed to second-hand smoke with 250 μl of 1 M NaCl with 100 μM desferal in Chelex-treated water, followed by the addition of 20 μl of internal standard solution (500 ng/ml). 8-Oxo-dGuo was analyzed in the range 0.4–20 ng/ml. Daily 8-point calibration samples (0, 0.2, 0.4, 1, 2, 4, 10, and 20 ng/ml) were prepared and analyzed together with two each of low, medium, and high QC samples (LQC 1, MQC 4, and HQC 20 ng/ml). Concentrations are expressed as means ± standard deviation.

Sample preparation

The urine samples were stored at -80°C until the night before analysis. The samples were thawed at 4°C overnight and a 250-μl aliquot was taken from each tube after being centrifuged for 3 min (10,000g) to remove any precipitates. With a setup containing two vacuum manifolds it is best to do at one time 34 urine samples, 8 calibration point samples, and 6 QC samples.

1. Label one set of conical glass tubes with calibration, QC, and urine sample numbers.

2. Add using the glass syringe 20 μ l of internal standard solution (500 ng/ml) and 20 μ l of [$^{13}\text{C}_{10}$ $^{15}\text{N}_5$]-dGuo 10 μ g/ml to all of the tubes.
3. Add using the glass syringe 10 μ l of corresponding standard solution to the calibration- and QC-labeled tubes.
4. Add 250 μ l of 1 M NaCl with 100 μ M desferal in Chelex-treated water to all tubes with the automated 1-ml pipette.
5. Add 250 μ l of water with 100 μ M desferal in Chelex-treated water to all tubes that were used for calibration and QC samples.
6. Add 250 μ l from each thawed urine sample to the tube labeled with the corresponding number using the automated pipette.
7. Vortex each tube for 5 s.

SPE preparation

1. Label Oasis HLB cartridges exactly as the labeled tubes for samples.
2. Insert them in the vacuum manifold.
3. Precondition with 1 ml of acetonitrile added using the automated 1-ml pipette without vacuum.
4. Precondition with 1 ml of water added using the automated 1-ml pipette without vacuum.
5. Load the samples without vacuum. Change the pipette tip for every sample!
6. Wash with 1 ml Chelex-treated water added using the automated 1-ml pipette without vacuum.
7. Wash with 1 ml 5% methanol in Chelex-treated water added using the automated 1-ml pipette without vacuum.
8. With the vacuum attached, dry the cartridges under vacuum for 5 min.
9. Insert a labeled set of clean glass tubes to collect the samples.
10. Add to the SPE tubes 0.7 ml of 50% acetonitrile using the automated pipette to elute the analytes. It might be necessary to apply the vacuum for a few seconds to get the cartridges wet, but the elution should be done without vacuum.
11. Remove the tubes from the manifold and dry the samples with the nitrogen evaporator.

HPLC sample preparation

1. Add 100 μ l of water/acetonitrile (97/3) to the tubes containing the dried-down samples using an automated pipette.
2. Vortex for 10 s.
3. Label the HPLC vials.
4. Move the resuspended samples into the labeled HPLC with the pipette. Change the tip for every sample!

Calculations and expected results

Usually each analytical instrument has software that would do the calibration and QC samples automatically, after which the amount of 8-oxo-dGuo in all the analyzed samples would be calculated. The instrument software package is used to calculate the peak areas based on the correct retention time. The peak areas for 8-oxo-dGuo and [$^{15}\text{N}_5$]-8-oxo-dGuo are shaded in Fig. 3. To get the calibration curve, one would calculate the area ratio for each of the calibration points, and those ratios were plotted against known concentrations of 8-oxo-dGuo (Fig. 3). From the calibration point one would find the equation of the line in the form

$$y = ax + b,$$

where y represents the area ratio and x the concentration.

For an unknown sample, one could find the y value by calculating the area ratio of the analyte (8-oxo-dGuo) area over the internal standard ([$^{15}\text{N}_5$]-8-oxo-dGuo) area. With the calculated y , one could back-calculate the concentration:

$$x = (y - b)/y$$

Using this method, urine samples from apparently healthy smokers (85) and nonsmokers (48) were analyzed (Fig. 4). The concentration of 8-oxo-dGuo was found to vary widely, between 0.6 and 15.7 ng/ml for the smokers (Fig. 4A). The concentrations in the nonsmoker subjects were closer in range, varying between 0.2 and 4.1 ng/ml (Fig. 4A). The mean urinary 8-oxo-dGuo concentration for 48 nonsmokers was 1.65 ng/ml with a standard deviation (SD) of 1.68 ng/ml and the mean concentration for 85 smokers was 2.83 ng/ml with an SD of 2.67 ng/ml (Fig. 4A). When the values were normalized for creatinine concentrations, there was little effect on the range of values. The mean of the 8-oxo-dGuo concentrations in nonsmokers' urine was 0.72 nmol/mmol creatinine (SD=0.45 nmol/mmol creatinine) and the mean concentration in the smokers' urine was significantly higher at 1.07 nmol/mmol creatinine (SD=1.50 nmol/mmol creatinine) (Fig. 4B). These values

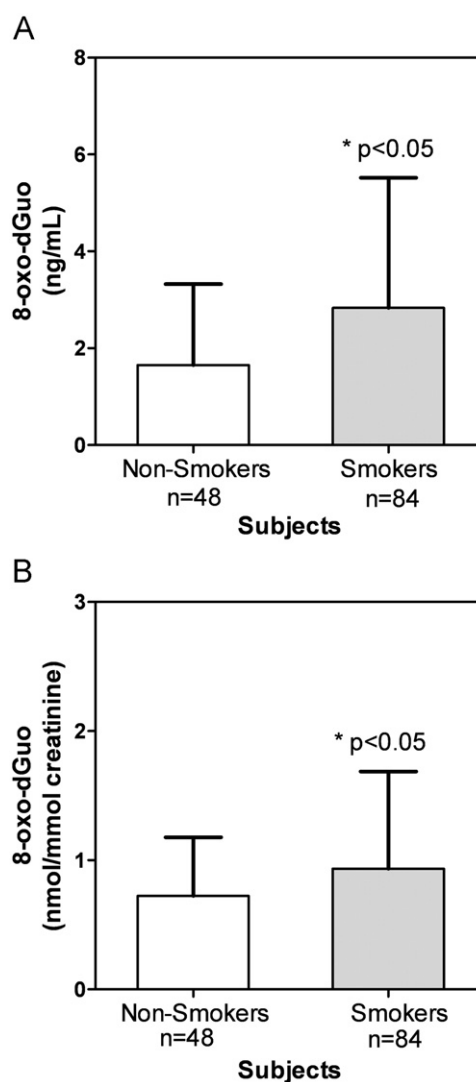


Fig. 4. 8-Oxo-dGuo concentrations in urine from apparently healthy nonsmokers and smokers. (A) Concentrations in ng/ml urine. (B) Concentrations normalized to creatinine (nmol/mmol creatinine). A two-tailed, unpaired t test with Welch's correction for unequal variances and a confidence interval of 95% were used to determine statistical significance.

correspond to a mean of 1.72 $\mu\text{g}/\text{mg}$ creatinine (SD=1.10 $\mu\text{g}/\text{mg}$ creatinine) for the nonsmokers and a mean of 2.21 $\mu\text{g}/\text{mg}$ creatinine (SD=1.79 $\mu\text{g}/\text{mg}$ creatinine) for the smokers. There was no significant difference in the urinary creatinine concentrations between nonsmokers and smokers. The mean values for nonsmokers ($n=48$) were 1.22 mg/ml (SD=1.16 ng/ml) or 10.77 mM (SD=10.25 mM) and for smokers ($n=84$) were 1.42 mg/ml (SD=1.10 ng/ml) or 12.59 mM (SD=9.81 mM).

Caveats

DNA damage, which occurs during oxidative stress, results in the formation of 8-oxo-dGuo [9,11,70]. The 8-oxo-dGuo is excised from DNA by glycosylase-mediated repair, which results in the release of 8-oxo-guanine rather than 8-oxo-dGuo [60,61]. Therefore, analyses of urinary 8-oxo-guanine cannot distinguish between RNA and DNA damage. In contrast, oxidative damage to the trinucleotide pool results in the formation of 8-oxo-2'-deoxyguosine triphosphate, which is hydrolyzed by MTH1 to release 8-oxo-2'-deoxyguosine monophosphate (8-oxo-dGMP) rather than 8-oxo-guanine [62]. The 8-oxo-dGMP is then converted to 8-oxo-dGuo by cellular phosphatases [71]. Therefore, urinary 8-oxo-dGuo concentrations are thought to reflect oxidative damage to the trinucleotide pool rather than to DNA [12]. Ideally, it would be best to analyze urinary 8-oxo-dGuo in 24-h urine samples so that the possible changes in the glomerular filtration rate (GFR) during that period would have a minimal effect on the concentration of 8-oxo-dGuo. Unfortunately, this is often not possible in biomarker studies as it is difficult to collect urine for an entire 24-h period. Spot urine samples are frequently used as an alternative because they are simple to collect and pose minimal subject inconvenience. However, spot urinary 8-oxo-dGuo concentrations may fluctuate because of many factors (such hydration status) that are unrelated to its rate of formation. This means that changes in urinary 8-oxo-dGuo concentrations from shorter collection times might simply reflect modulation in GFR during a particular collection period.

The concept of creatinine adjustment to normalize for changes in GFR, which was originally proposed by Vought et al. [72], depends upon daily urinary creatinine excretion by a healthy individual being constant [73]. Creatinine is formed nonenzymatically from creatine (primarily in the muscle) at an almost steady-state rate of approximately 2% of the creatine pool per day [74]. Creatine itself can be formed endogenously from glycine and arginine through the transamidinase-mediated intermediate formation of guanidinoacetate, which is then converted into creatine by *N*-guanidinoacetate methyltransferase-mediated methylation by *S*-adenosylmethionine [75]. The rate of creatine synthesis is closely regulated by feedback inhibition of transamidinase. Thus, on a creatine-free vegetarian diet, this pathway is fully activated, and adequate guanidinoacetate is synthesized from its amino acid precursors [76]. Conversely, creatinine that is ingested from meat partially or totally represses transamidinase to modulate its endogenous production. Creatinine is formed nonenzymatically from creatine through cyclization and dehydration or by the intermediate formation of phosphocreatine. The resulting creatinine then diffuses into the circulation and appears in the urine after glomerular filtration.

Daily urinary excretion of creatinine derived from muscles occurs at a rate of approximately 1 g/day (1 g/20 kg of muscle mass) [77]. The normal daily urinary excretion of creatinine is relatively stable for an individual, with a daily variation of between 4 and 8%; however, there are substantial interindividual differences, which are dependent upon sex, height, weight, race, age, and other factors [78]. This means that considerable

uncertainty could be introduced when using creatinine excretion as a normalization factor. Nevertheless, adjustment for creatinine concentration is commonly used for ELISA-, GC-MS-, HPLC-ECD-, and LC-MS-based assays of urinary 8-oxo-dGuo (Table 1) [16,28,32,39,44,46,52]. Conversely, total urinary nicotine concentrations, which provide an index of smoking topography, are rarely normalized for creatinine [79]. Another possible confounding factor is the general use of colorimetric assays for the analysis of urinary creatinine. We have found that this underestimates creatinine concentrations by 20% (data not shown) compared with LC-MS-based methodology similar to that described by Teichert et al. [16]. Therefore, it is conceivable that additional uncertainties exist in much of the 8-oxo-dGuo data that have been published when the simple colorimetric assay was employed to analyze urinary creatinine.

Alternative approaches have been advocated such as using timed urine collections and then normalizing to the urinary creatinine excretion rate rather than its concentrations. However, there could still be uncertainty in the actual timing of the urine collection unless it is conducted under carefully controlled conditions. A more innovative approach has been proposed by Warrack et al. [80] for use in metabonomic analyses of urinary metabolites. This involves normalization to urine osmolality, which is a direct measure of total endogenous metabolic output. Using this normalization method, it was possible to reduce variation among biological replicates, which was not corrected by the use of creatinine concentrations [80]. There are as yet no reports on the use of either of these approaches for the analysis of urinary 8-oxo-dGuo. Therefore, in future studies, it will be necessary to evaluate the utility of these methods for normalizing urinary 8-oxo-dGuo concentrations in spot urine samples to take account of potential intra- and interindividual differences in GFR.

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