A high-throughput and sensitive methodology for the quantification of urinary 8-hydroxy-2'-deoxyguanosine: measurement with gas chromatography-mass spectrometry after single solid-phase extraction

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8-Hydroxy-2'-deoxyguanosine (8OHdG) is a widely used biomarker for the measurement of endogenous oxidative DNA damage. A sensitive method for the quantification of 8OHdG in urine by single solid-phase extraction and GC-MS (gas chromatography with MS detection) using selective ion monitoring is described in the present study. After solid-phase extraction, samples are freeze-dried, derivatized by trimethylsilylation and analysed by GC-MS. The urinary 8OHdG was quantified using heavy isotope dilution with [¹⁸O]8OHdG. The recovery of 8OHdG after the solid-phase extraction ranged from 70 to 80 % for a wide range of urinary 8OHdG levels. Using 1 ml of urine, the limit of quantification was > 2.5 nM (2.5 pmol/ml) and the calibration curve was linear in the range 2.5–200 nM. This method was applied to

measure 8OHdG in urine samples from 12 healthy subjects. The intra- and inter-day variations were < 9 %. Urinary 8OHdG levels in spot urine samples from four healthy subjects were also measured for 1 week and, again, the variation was small. The presence of H₂O₂ in urine did not cause artifactual formation of 8OHdG. Since this assay is simple, rapid, sensitive and reproducible, it seems suitable to be used as a routine methodology for the measurement of urinary excretion of 8OHdG in large population studies.

Key words: gas chromatography-mass spectrometry, 8-hydroxy-2'-deoxyguanosine, oxidative DNA damage, solid-phase extraction, urine.

INTRODUCTION

Free radicals and other reactive species are generated continuously *in vivo* during physiological and pathological processes, and many of them can cause oxidative damage to DNA [1]. Oxidative damage to DNA can contribute to mutation and cancer [2–4]. Although many different oxidative DNA damage products have been identified [4,5], the guanine-derived lesion 8OHGua (8-hydroxyguanine) and its corresponding deoxynucleoside 8OHdG (8-hydroxy-2'-deoxyguanosine) have been subjected to the most intensive study, and the urinary level of 8OHdG is regarded as an important biomarker of endogenous oxidative damage to DNA and its precursors [6].

The quantification of urinary 8OHdG has been extensively studied and several different methods, such as HPLC with ECD (electrochemical detection), HPLC with MS, GC-MS, CE (capillary electrophoresis) and immunoassay, have been established [7–14]. Since urine is a complex matrix with highly variable composition, extensive clean-up procedures are usually required before the assay of 8OHdG. Semi-preparative HPLC purification with or without prior SPE (solid-phase extraction) is often used to clean-up the urine samples [9,15,16]. These procedures are expensive and time-consuming. At present, urinary 8OHdG is most frequently measured by HPLC–ECD [6], but the method can be complex and extensive clean-up procedures are required [7]. The accuracy of many HPLC–ECD methods has been questioned [10]. An immunoassay for 8OHdG with a commercial monoclonal antibody (denoted N45.1) is becoming more popular and has many advantages, e.g. good sensitivity, simplicity, convenience and good correlation with the well-established HPLC–ECD method

[17–19]. However, this immunoassay might also detect the 8OHdG in oligomers or 8-OHdGMP [20]. Furthermore, HPLC prepurification might also be required [18]. HPLC with MS has also been reported previously [6,10]. However, owing to its high cost, its use is limited at present. Another potential problem is that urine contains H₂O₂, sometimes at high concentrations, especially after drinking coffee [21,22]. The potential effect of this on artifactual 8OHdG generation has not been considered in the past.

We describe in the present study a simple and rapid assay for the measurement of urinary 8OHdG. The urine samples were cleaned up through a straightforward single extraction with Waters Oasis HLB cartridges, and 8OHdG was measured with GC-MS using a stable isotope-labelled analogue of 8OHdG as the internal standard.

MATERIALS AND METHODS

Materials

8OHGua and 8OHG (8-hydroxyguanosine) were purchased from Aldrich and Cayman Chemical (Ann Arbor, MI, U.S.A.) respectively. 8OHdG, dG (2'-deoxyguanosine) and G (guanosine) were obtained from Sigma. [¹⁸O]8OHdG (internal standard, *N* + 2) was a gift from Dr Miral Dizdaroğlu (National Institute of Standards and Technology, Gaithersburg, MD, U.S.A.). The application of [¹⁸O]8OHdG as an internal standard for the measurement of 8OHdG in urine and other biological samples has been reported previously [9,23–25]. Heavy isotope-labelled [8-¹³C,7,9-¹⁵N₂]-8OHGua (*N* + 3) was obtained from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). Formic acid (98 %,

Abbreviations used: AUC, area under the curve; CE, capillary electrophoresis; CV, coefficient of variation; dG, 2'-deoxyguanosine; ECD, electrochemical detection; G, guanosine; GC-MS, gas chromatography with MS detection; 8OHdG, 8-hydroxy-2'-deoxyguanosine; 8OHG, 8-hydroxyguanosine; 8OHGua, 8-hydroxyguanine; SIM, selective ion monitoring; SPE, solid-phase extraction.

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v/v; Lancaster, Morecombe, U.K.), HPLC-grade methanol (EM Science, Gibbstown, NJ, U.S.A.) and Milli-Q water were used to prepare aqueous solutions in the experiments.

Subjects

Healthy non-smoking human volunteers from this research laboratory were used to provide spot urine samples. No food, drink or lifestyle restrictions were applied.

Creatinine assay

The concentration of creatinine in urine was measured using a creatinine kit (555-A) purchased from Sigma Diagnostics (St. Louis, MO, U.S.A.) by the method of Slot [26]. Briefly, the formation of acid-sensitive chromogen after reduction of the sample with picrate was measured at 500 nm. The urinary 8OHdG level was normalized with creatinine concentration and is presented as μmol of 8OHdG/mol of creatinine.

Urine clean-up procedure

Fresh urine was collected in 50 ml centrifuge tubes and samples were taken for creatinine measurement. Since the urine concentration is highly variable between different subjects and in the same subject at different times, the volume of urine used for the assay of 8OHdG was adjusted according to its creatinine level. For concentrated urine samples with creatinine concentrations higher than 10 mM, 800 μl of urine was used for the assay; for urine samples with creatinine concentrations from 5 to 10 mM, 1600 μl of urine was used; for dilute urine samples with creatinine concentrations less than 5 mM, 3200 μl of urine was used. [^{18}O]8OHdG (50 pmol) was added to the urine sample as the internal standard; 1 vol. of formic acid (10%) was mixed with 10 vol. of urine. After the acidified urine samples were incubated at 4 °C for 1 h, they were centrifuged at 10 000 *g* for 10 min at 4 °C. The supernatant was collected and Waters Oasis® HLB Vac cartridges (60 mg of packing material) were used for clean-up. HLB Vac cartridges were preconditioned first with 5 ml of methanol and then with 8 ml of 20 mM formic acid (pH $\approx$ 2.75). After centrifugation, the acidified urine sample was diluted with 20 mM formic acid to a final volume of approx. 5.8 ml, and the diluted urine was loaded on to the preconditioned HLB Vac cartridges. The diluted urine passed through the cartridges at approx. 2 ml/min under a vacuum of approx. 5 mmHg. After loading, 2 ml of 20 mM formic acid was added to flush the cartridges with a similar speed and pressure. Finally, 1.5 ml of 17.5% (v/v) methanol in 20 mM formic acid was used to elute 8OHdG from the cartridges. To avoid cross-contamination and maximize the recovery, the cartridges were totally dried under vacuum after each step of clean-up. Each cartridge was used to clean-up one urine sample only. The collected fraction that contains 8OHdG was transferred into an autosampler vial (La-Pha-Pack, Langerwehe, Germany), frozen at -80 °C and dried with a vacuum freeze-dryer (BioTran, Kangwon, South Korea). During our preliminary study, we found that 8OHdG was stable in 20 mM formic acid for at least 5 h at 4 °C.

Derivatization

The method for derivatization of 8OHdG and 8OHGua was modified from published methods [27–29]. The freeze-dried samples were purged with pure nitrogen and sealed with short thread caps with Teflon septa (La-Pha-Pack). Acetonitrile/

ethanethiol (15 μl ; 3:1, v/v) was added to the vial to solubilize the urinary residue and subsequently 60 μl of bis(trimethylsilyl)trifluoroacetamide (containing 1% trimethylchlorosilane) was added to the vial for derivatization at room temperature (23 °C).

GC-MS analysis

After derivatization, the samples were analysed on a Hewlett–Packard 5973 mass selective detector interfaced with a Hewlett–Packard 6890 gas chromatograph, which was equipped with an automatic sampler and a computer workstation. The injection port and the GC-MS interface were kept at 250 °C and 290 °C respectively. Separations were performed on a fused silica capillary column (length 12 m and internal diameter 0.2 mm) coated with cross-linked 5% phenylmethylsiloxane (film thickness, 0.33 μm). Helium was the carrier gas with a flow rate of 1 ml/min. The derivatized sample (1 μl) was injected into the GC injection port using a split ratio of 6:1. Column temperature was first held at 190 °C for 2 min, then increased from 190 to 290 °C at the rate of 20 °C/min and, finally, maintained at 290 °C for another 2 min. The running cycle was less than 10 min. Mass spectrum scan for each standard compound was performed at the beginning of the assay development. After observation of the major ions for each compound, SIM (selective ion monitoring; *m/z* 440 and 458 for 8OHGua; *m/z* 443 and 458 for stable isotope-labelled 8OHGua; *m/z* 383 and 643 for 8OHdG; *m/z* 385 and 645 for [^{18}O]8OHdG; *m/z* 555 for dG; *m/z* 643 for G; *m/z* 731 for 8OHG) was performed for quantification using the electron-ionization mode at 70 eV, with the ion source maintained at 230 °C.

Derivatization reaction time-course study

To optimize the derivatization time, a time-course study was performed. 8OHdG standard (200 pmol) was added to urine. After SPE, 0.5 nmol of heavy isotope-labelled 8OHGua (internal standard, *N* + 3) was added to the vials containing the fraction from the final step of the sample clean-up procedure. The samples were freeze-dried and derivatized as described above. These samples were injected for GC-MS analysis 2, 4, 6, 8, 10, 12 and 24 h after the start of derivatization. The derivatization reaction time-course study was performed in triplicate. 8OHGua was used as the internal standard, since 2 h are sufficient for the derivatization of 8OHGua [29]. The amount of 8OHdG derivative could be expressed as the AUC (area under the curve) of the ion at *m/z* 383 (8OHdG) divided by the AUC of the ion at *m/z* 443 (8OHGua).

Recovery study

The recovery study was performed with three different urine samples, namely highly concentrated urine (creatinine, 22.2 mM), moderately concentrated urine (creatinine, 5.5 mM) and dilute urine (creatinine, 3.4 mM). As mentioned above, 800, 1600 and 3200 μl of urine were used according to the creatinine level. We added 50, 100 and 200 pmol of 8OHdG before or after SPE. After extraction, 8OHGua was added to the vial as an internal standard. The recovery study was performed in triplicate. The recovery was calculated using the formula:

Recovery (%)

$$= \frac{\text{AUC}_{\text{ion383}} (8\text{OHdG added before SPE}) / \text{AUC}_{\text{ion443}}}{\text{AUC}_{\text{ion383}} (8\text{OHdG added after SPE}) / \text{AUC}_{\text{ion443}}} \times 100 \%$$

Linearity and sensitivity study

Samples of 2.5, 5.0, 10.0, 25.0, 50.0, 100.0 or 200.0 pmol of [^{18}O]8OHdG were added to 1 ml of urine and analysed as described above. Then, 0.2 nmol of stable isotope-labelled 8OHGua ($N+3$, internal standard to calibrate the variation between injections of GC) was spiked into each sample after solid-phase clean-up. These samples were freeze-dried and analysed by GC-MS after derivatization. The measured concentrations of [^{18}O]8OHdG were presented as the AUC of the ion at m/z 385 (8OHdG) divided by the AUC of the ion at m/z 443 (8OHGua). This linearity and sensitivity study was performed in triplicate. The quantification limit of [^{18}O]8OHdG was defined as a signal-to-noise ratio of 10:1.

Accuracy study

Samples of 0.0, 10.0, 25.0, 50.0, 75.0 and 100.0 pmol of unlabelled 8OHdG were added to 1 ml of urine. [^{18}O]8OHdG (50.0 pmol) was also added to each urine sample as an internal standard. These urine samples were cleaned up and analysed as described above. The amounts of externally added 8OHdG were calculated by the subtraction of the endogenous 8OHdG, calculated as described below, from the total amounts of detected 8OHdG. The accuracy study was performed in triplicate.

Intra- and inter-day measurement of urinary 8OHdG in 12 healthy subjects

Urine samples were collected from 14 healthy subjects (non-smokers; eight male and six female) on the same morning. Creatinine concentration was measured and different amounts of urine were transferred to small centrifuge tubes according to the creatinine level. [^{18}O]8OHdG (50 pmol) was added to the urine sample as an internal standard. The urine samples underwent sample clean-up, derivatization and GC-MS as described above. The concentration of 8OHdG was calculated using the formula:

$$8\text{OHdG (nM)} = ([\text{AUC}_{\text{ion}383} / \text{AUC}_{\text{ion}385}] \times 50) / \text{volume (ml)}$$

Both intra- and inter-day measurements were replicated five times. The samples for intra-day study were kept at -80°C before SPE clean-up.

Measurement of 8OHdG in spot urine samples from four healthy subjects during a 1 week period

Spot urine samples were collected from four healthy male subjects (non-smokers) each day for 1 week. The urine was collected between 9:00 and 17:00 h. No restrictions on food, drink or lifestyle were applied. The creatinine level was measured immediately. The urine samples were stored at -80°C before clean-up and GC-MS analysis.

The impact of H_2O_2 on the generation of 8OHdG in human urine

It has been reported that both dG and H_2O_2 are present in human urine, raising the possibility of artifactual generation of 8OHdG by reactive species from urinary H_2O_2 [21,22,29,30]. Therefore we performed a study to explore this issue. Urine samples (0.8 ml) from three healthy volunteers were subjected to four different treatments, namely incubation with 1 nmol of dG (final concentration, $1.25\ \mu\text{M}$), incubation with $50\ \mu\text{M}$ H_2O_2 , incubation with $50\ \mu\text{M}$ H_2O_2 and 1 nmol of dG (final concentration, $1.25\ \mu\text{M}$), incubation with $100\ \mu\text{M}$ H_2O_2 and 1 nmol of dG (final

concentration, $1.25\ \mu\text{M}$). The incubations were for 24 h at room temperature and [^{18}O]8OHdG was used as an internal standard. After incubation, the urine samples were analysed and compared with non-incubated control samples.

RESULTS

GC-MS behaviour of derivatized 8OHGua, 8OHdG, 8OHG, dG and G

8OHGua is routinely measured in this laboratory and the major ions of its trimethylsilyl derivative are of m/z 440 (molecular ion) and m/z 455 (characteristic ion, $M-\text{CH}_3$) [27,29]. For its stable isotope-labelled internal standard, m/z 443 (molecular ion) and m/z 458 (characteristic ion, $M-\text{CH}_3$) are also measured. 8OHGua eluted at 4.45 min under our chromatographic conditions.

The GC-MS behaviour of the trimethylsilyl derivative of 8OHdG has been well described [28]. To ensure that our results agree with the published results, pure standards of 8OHdG, 8OHG, dG and G were derivatized and injected into GC-MS and monitored using the scan mode. Similar to the published results [28], the $B+1$ ion was the most intense ion (m/z 383) in the spectrum of derivatized 8OHdG. Less intense ions were of m/z 368, 628 and 643 (results not shown). The ratios between these ions were all similar to those obtained by Dizdaroglu [28]. Our scan mode result was in good agreement with the well-described mass spectrum of 8OHdG. 8OHG, dG and G can also be present in urine [30]. Since the molecular structures of 8OHdG, 8OHG, dG and G are quite similar, they could generate some of the same ions after fragmentation, making the measurement of 8OHdG problematic. Indeed, we found that 8OHG, dG and G shared some ions with 8OHdG: 8OHG had the most intense ions of m/z 383 and 368; dG had a minor ion of m/z 368; and G had the moderate ions m/z 368 and 643. Fortunately, 8OHdG, 8OHG, dG and G had different retention times on our GC column. 8OHdG eluted at 6.89 min, 8OHG eluted at 6.75 min, dG eluted at 6.83 min and G eluted at 6.84 min.

Since m/z 383 is the most intense ion of 8OHdG after fragmentation and this channel had less interference from the urine according to our preliminary study, we adopted m/z 383 for SIM. Although m/z 368 is the second most intense ion for 8OHdG, this channel often had interference from other urinary constituents; m/z 628 is a weak ion. Thus we did not measure these two ions. However, we also included m/z 643 in the SIM and used it as a secondary reference standard for 8OHdG. For the measurement of [^{18}O]8OHdG (internal standard), SIM with ions of m/z 385 and 645 was applied. To assess 8OHG, dG and G in urine, the parent ions of 8OHG (m/z 731), dG (m/z 555) and G (m/z 643) were also included in SIM.

Derivatization reaction time-course study

The derivatization reaction time course for 8OHdG is shown in Figure 1. It was found that the derivatization reaction was fairly rapid. After 6 h, a slowing of the reaction was observed and the derivatized product was stable for at least 24 h. Thus all subsequent urine samples were incubated with derivatizing agents for more than 6 h before GC-MS analysis and, normally, all analyses were completed within 24 h after the samples were derivatized. A 2 h derivatization was sufficient for 8OHGua as reported previously [29].

Chromatogram of urinary 8OHdG

A representative chromatogram of urinary 8OHdG is shown in Figure 2. [^{18}O]8OHdG (50 pmol) was added to the urine sample

Figure 1 Time course for the derivatization reaction of 8OHdG

Results are the means $\pm$ S.D. for $n = 3$ independent experiments.

as an internal standard. The upper channel shows the ion of m/z 383 (endogenous 8OHdG), whereas the lower channel shows the ion of m/z 385 ($[^{18}\text{O}]8\text{OHdG}$ as the internal standard). Both endogenous 8OHdG and $[^{18}\text{O}]8\text{OHdG}$ had sharp peaks in the chromatogram, well separated from other urinary content peaks. Sharp and isolated peaks of 8OHdG and $[^{18}\text{O}]8\text{OHdG}$ could be obtained in most of the urine samples after single SPE. However, according to our experience with approx. 1000 urine samples during the assay development, 10–20% of the urine samples had contaminants that co-eluted with 8OHdG or $[^{18}\text{O}]8\text{OHdG}$ and made the quantification difficult. Fortunately, these contaminants in urine were non-subject-specific and usually disappeared on another sampling day.

During the preliminary study, it was found that 8OHGua did not bind well to HLB Vac cartridges and > 80% eluted during sample loading and washing with 20 mM formic acid. Thus we could not use HLB Vac cartridges for the measurement of 8OHGua. The ion of m/z 731 eluting at 6.75 min indicated that 8OHG was present in most of the urine samples as expected. dG (m/z 555) and G (m/z 643) were also present in some of the eluates at low levels.

Table 1 Recovery study of 8OHdG in urine samples from three different healthy subjects (A–C)

Values are means $\pm$ S.D. ($n = 3$ samples).

Amount of 8OHdG added (pmol)	[Creatinine] (mM) . . .	Recovery (%)		
		A	B	C
	22.2		5.5	3.4
50		76.9 $\pm$ 1.0	83.8 $\pm$ 1.4	83.0 $\pm$ 6.0
100		73.8 $\pm$ 4.6	79.2 $\pm$ 5.7	76.3 $\pm$ 5.5
200		76.2 $\pm$ 4.0	81.3 $\pm$ 4.6	82.8 $\pm$ 7.2

Recovery study

The recovery of 8OHdG after SPE ranged from 70 to 80% in each of the three different samples at various 8OHdG concentrations (Table 1). The recovery was consistently good over a wide range of urine samples (from very dilute to very concentrated) and was not affected by increasing the amount of 8OHdG from 50 to 200 pmol. During our preliminary study, we confirmed that $[^{18}\text{O}]8\text{OHdG}$ had the same recovery in the HLB cartridge as unlabelled 8OHdG. Although the use of an isotope-labelled standard compensates for recovery, the high recovery achieved in this study ensures a high sensitivity of the method.

Linearity study

Since the endogenous urinary 8OHdG made accurate determination of the quantification limit difficult, we used $[^{18}\text{O}]8\text{OHdG}$ instead of normal 8OHdG to spike the urine samples. The calibration curve was linear in the range 2.5–200.0 nM ($R^2 > 0.99$; Figure 3). Even 200 pmol of 8OHdG did not overload the SPE cartridges. The detection limit (signal-to-noise ratio, 10:1) of urinary $[^{18}\text{O}]8\text{OHdG}$ was at least as low as 2.5 nM 8OHdG when 1 ml of urine was assayed. The reported levels of 8OHdG and those measured by us are usually higher than 2.5 nM [10,11].

Figure 2 Chromatogram of 8OHdG and heavy isotope-labelled 8OHdG (internal standard) in urine samples

Upper channel: ions of m/z 382.9–383.9 for unlabelled 8OHdG present in urine. Lower channel: ions of m/z 384.9–385.9 for ^{18}O -labelled internal standard of 8OHdG.

Figure 3 Linearity study of [^{18}O]8OHdG in human urine

Results are the means $\pm$ S.D. for $n=3$ independent experiments.

Figure 4 Accuracy study

Results are the means $\pm$ S.D. for $n=3$ independent experiments.

Accuracy study

Results of the accuracy study are shown in Figure 4. The endogenous 8OHdG in this urine sample was found to be 10.56 ± 0.71 nM. The total amounts of 8OHdG were proportional to the externally spiked amounts of 8OHdG ($R^2 > 0.99$) and the 8OHdG added could be measured accurately at all the levels tested ($100 \pm 6.5\%$).

Intra- and inter-day measurement of urinary 8OHdG in 12 healthy subjects

We collected 14 spot urine samples from healthy subjects and 12 urine samples were successfully measured. Two urine samples (one from a male and one from a female subject) produced serious interference at the channel of m/z 383. Similar problems occurred in approx. 10–20% of all the urine samples we studied with the channels of m/z 383 or 385. However, according to our experience, the possibility of interference in the channel of m/z 383 was higher when compared with the channel of m/z 385.

Results of this study are presented in Tables 2 and 3. The CV (coefficient of variation) values of the levels of 8OHdG in both intra- and inter-day measurements ($n=5$) of these 12 samples were all $<9\%$. The urinary level of 8OHdG was in the range 3.58–115.24 nM, indicating a broad distribution of 8OHdG concentration. The concentration of creatinine was in

Table 2 Intra-day measurement of the 8OHdG levels in urine samples from 12 healthy subjects ($n=5$ samples for 1 day)

Values are means $\pm$ S.D.

Subject code	Gender	Creatinine concentration (mM)	8OHdG concentration (nM)	8OHdG/creatinine ($\mu\text{mol/mol}$)	CV (%)
1	M	3.79	4.31 ± 0.16	1.14 ± 0.04	3.8
2	M	26.01	115.24 ± 1.27	4.43 ± 0.05	1.1
3	M	21.71	42.39 ± 0.93	1.95 ± 0.04	2.2
4	F	3.42	3.58 ± 0.25	1.05 ± 0.07	6.9
5	M	14.58	27.37 ± 2.10	1.88 ± 0.14	7.7
6	F	2.58	4.40 ± 0.35	1.71 ± 0.14	8.0
7	M	20.68	34.45 ± 1.14	1.67 ± 0.06	3.3
8	M	1.16	3.99 ± 0.15	3.45 ± 0.13	3.8
9	F	2.15	4.20 ± 0.28	1.95 ± 0.13	6.7
10	F	8.31	11.28 ± 0.91	1.36 ± 0.11	8.0
11	F	1.38	3.91 ± 0.29	2.83 ± 0.21	7.5
12	M	22.71	47.69 ± 1.89	2.10 ± 0.08	4.0

Table 3 Inter-day measurement of the 8OHdG levels in urine samples from 12 healthy subjects ($n=5$ different days)

Values are means $\pm$ S.D.

Subject code	8OHdG/creatinine ($\mu\text{mol/mol}$)	CV (%)
1	1.12 ± 0.04	3.7
2	4.43 ± 0.06	1.3
3	1.96 ± 0.06	3.2
4	1.07 ± 0.04	4.2
5	1.78 ± 0.11	6.0
6	1.69 ± 0.13	7.5
7	1.72 ± 0.10	5.6
8	3.33 ± 0.30	8.9
9	1.82 ± 0.14	7.9
10	1.26 ± 0.11	8.6
11	3.10 ± 0.26	8.3
12	2.12 ± 0.10	4.5

the range 1.16–26.01 mM, indicating high variation in urine concentration. After normalizing the 8OHdG level with creatinine concentration, the urinary excretion of 8OHdG was in the range 1.14–4.13 μmol of 8OHdG/mol of creatinine, with a mean value of 2.13 ± 0.99 μmol of 8OHdG/mol of creatinine (mean $\pm$ S.D.). The mean values of 8OHdG were 2.37 ± 1.15 and 1.78 ± 0.68 μmol of 8OHdG/mol of creatinine in male and female subjects respectively. It seemed that men tended to excrete more 8OHdG compared with women, consistent with previous reports [31]. However, the difference was not statistically significant. Furthermore, we only had seven male and five female subjects, and such a limited sample size made accurate statistical analysis difficult. Similar results were reported previously [11]. The inter-individual variation of urinary 8OHdG observed in the present study was also within the reported range [11].

Measurement of 8OHdG in spot urine samples from four healthy subjects during a 1 week period

The urinary levels of 8OHdG in spot urine samples from four healthy male subjects are shown in Figure 5. The average values of 8OHdG were 1.84 ± 0.31 , 1.39 ± 0.13 , 1.96 ± 0.44 and 1.64 ± 0.22 μmol of 8OHdG/mol of creatinine in subjects D, E, F and G respectively. The CV ranged from 9.0 to 22.1%. Thus the urinary level of 8OHdG in spot urine was fairly consistent over a week.

Figure 5 Excretion level of 8OHdG in spot urine from four healthy subjects during a 1 week period

Table 4 No impact of H_2O_2 on the generation of 8OHdG in human urine

H, I and J represent the subject codes.

Conditions	8OHdG concentration (nM)		
	H	I	J
Control	14.28	15.36	10.24
Urine + 1 nmol of dG	13.92	15.64	9.85
Urine + 50 μ M H_2O_2	14.54	15.48	10.38
Urine + 50 μ M H_2O_2 + 1 nmol of dG	14.74	15.06	10.68
Urine + 100 μ M H_2O_2 + 1 nmol of dG	14.58	14.89	9.98

The impact of H_2O_2 on the generation of 8OHdG in human urine

Urine was spiked with dG and/or H_2O_2 to test the possibility of artifactual 8OHdG generation. Up to 100 μ M H_2O_2 was used, which is far greater than the levels present in most urine samples [21,22]. The levels of 8OHdG in the urine sample from the three subjects studied ranged from 10 to 15 nM. No significant difference was observed between different treatment groups (Table 4). The variations (CV) between different treatments were all < 5%, suggesting that H_2O_2 has negligible effect on the generation of 8OHdG in human urine under such circumstances. In any case, only trace levels of urinary dG were present in our eluates, making such an artifact unlikely. Even when we added 1 nmol of dG (final concentration, 1.25 μ M) with 100 μ M H_2O_2 to urine, no significant amount of extra 8OHdG was detected. Furthermore, this study also confirmed no significant artifactual 8OHdG generation during derivatization, since no significant change in urinary 8OHdG levels was observed in urine samples containing an additional 1 nmol of dG (final concentration, 1.25 μ M).

DISCUSSION

8OHdG required a longer derivatization time compared with 8OHGua, presumably because it has more hydroxy groups. Fortunately, a derivatization time of ≥ 6 h was still acceptable, since samples could be run in batches and were stable for at least 24 h.

The recovery rate of 8OHdG was high (70–80%) and consistent. This is an advantage, since urine samples with highly

variable contents would be obtained in population studies. The peak of 8OHG (m/z 731) was only at trace level, probably due to a low recovery of 8OHG in our clean-up procedure. According to our preliminary study, the recovery of 8OHG was only approx. $20.7 \pm 3.0\%$. Hence, our procedure is less suited to measure 8OHG compared with 8OHdG. Similarly, the recovery of dG was also low ($28.1 \pm 5.1\%$). Recovery of G was better ($60.0 \pm 7.2\%$); however, it was only detected at trace level in urine.

We found that this simple and rapid assay method for urinary 8OHdG was highly reproducible (CV < 9%), although according to our experiences with approx. 1000 urine samples, 10–20% of the samples had contaminants that made the quantification of 8OHdG unsuccessful. The non-permanent interference at channels of m/z 383 or 385 might be caused by dietary influence. Indeed, recent studies indicate that it is far less of a problem if subjects fast overnight (H.-S. Lin, A. M. Jenner, C. N. Ong, S. H. Huang, M. Whiteman and B. Halliwell, unpublished work). However, at the present state of development, we are unable to identify the interfering molecules. Generally, the values of 8OHdG in highly concentrated urine were less variable compared with the values of 8OHdG in diluted urine. Thus we adjusted the volume of urine for the assay according to the creatinine level. The urinary 8OHdG levels observed in the present study are within the ranges reported in various previous studies (approx. 1–3 μ mol of 8OHdG/mol of creatinine; Table 5). However, our clean-up procedure was simpler and less time-consuming. Simplicity is the major advantage of this assay method.

An average day-to-day variation of 15.4% in 8OHdG was observed in spot urine samples from four healthy volunteers over a period of 1 week (Figure 5), even though they made no attempt to 'standardize' their lifestyle or the time of urine collection. This suggests that the urinary excretion of 8OHdG might be a good biomarker of endogenous oxidative stress, since its level was not highly variable. Pilger et al. [11] performed a similar study on the measurement of 8OHdG level in 24 h urine from two subjects in 10 continuous days using HPLC–ECD with double SPE clean-up. The 8OHdG level in that study was quite variable (CV, 37 and 57%). However, without an internal standard, it is difficult to measure 8OHdG accurately with HPLC–ECD.

It is generally supposed that urinary 8OHdG derives from repair of DNA and its precursor pool, although the detailed processes are not fully clarified and diet appears to have no impact on its production [6,9,11]. Thus the urinary excretion of 8OHdG might be a reflection of the average total oxidative stress to DNA/DNA precursor pool of all body cells [10]. Since 8OHdG is regarded as an important biomarker for oxidative DNA damage, its assay in urine samples has been well reported. Currently, HPLC–ECD, HPLC plus MS, GC-MS and immunoassay are the most popular methods. Generally, extensive clean-up procedures with solid-phase extraction and/or semi-preparative HPLC are required. These are time-consuming and the handling of large numbers of samples is difficult. In the present study, we simplified the clean-up procedure through single SPE with HLB cartridges and this made a quick clean-up possible. The time for one round of SPE for 20 urine samples was approx. 20 min, and 500 urine samples could be cleaned up within one working day by one person using a 20-channel extraction manifold. HLB SPE cartridges have many advantages. During our assay development, we also tried C_{18} SPE cartridges. However, the clean-up effects, even with double extraction, using C_{18} cartridges were not as good as those with single extraction using HLB cartridges. After the urine samples were acidified with 10% formic acid, no other pH adjustment was required. This also saved time. Furthermore, the HLB contains water-wettable material and can be dried during extraction. For normal C_{18} cartridges, the cartridges must always be kept wet

Table 5 Comparison of urinary 8OHdG levelsResults are expressed as means $\pm$ S.D., where *n* represents the number of subjects.

Subjects	Clean-up procedure and assay method	Urinary 8OHdG level	Reference
Healthy subjects (non-smokers)	Single SPE, GC-MS	Male: 2.37 ± 1.15 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 7); female: 1.78 ± 0.68 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 5); total population mean: 2.13 ± 0.99 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 12); male: $3.99\text{--}115.24$ nM; female: $3.58\text{--}11.28$ nM	Present study
Healthy subjects	HPLC with MS	28 ± 2 nmol/24 h (<i>n</i> = 20)	[30]
Smokers and non-smokers	HPLC with MS	Smokers: 3.19 ± 1.22 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 80); ex-smokers: 3.06 ± 2.10 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 58); never-smokers: 2.92 ± 0.90 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 96)	[32]
	HPLC purification, formic acid hydrolysing 8OHdG into 8OHGua, GC-MS measurement of 8OHGua to quantify 8OHdG	30 ± 15 nM (ten urine samples)	[16]
Healthy men	Column switching, HPLC-ECD	1.91 ± 0.80 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 318)	[33]
Healthy subjects and patients after surgical treatment	HPLC purification, formic acid hydrolysing 8OHdG into 8OHGua, GC-MS measurement of 8OHGua to quantify 8OHdG	Nucleic acid-free diet: 60.4 ± 27.3 nmol/24 h (<i>n</i> = 24); normal diet: 57.7 ± 33.9 nmol/24 h (<i>n</i> = 24)	[9]
Healthy subjects	Double SPEs, HPLC-ECD	Non-smokers: 1.94 ± 1.21 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 70); smokers: 2.11 ± 1.22 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 78); total population mean: 2.03 ± 1.21 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 178)	[11]
Healthy subjects	Two-column-switching HPLC-ECD	Male: 2.05 ± 0.79 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 1254); female: 2.14 ± 0.93 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 1254); total population mean: 2.09 ± 0.86 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 2507)	[34]
Healthy subjects	Purine-selective porous carbon column, column-switching HPLC-ECD	Male: 1.70 ± 0.22 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 104); female: 1.83 ± 0.17 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 96); children (3–9 years): 2.14 ± 0.23 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 32); newborn: 6.20 ± 0.46 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 150)	[7]
Healthy subjects	Single SPE, HPLC with MS	Smokers: 0.79 ± 0.32 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 4); non-smokers: 1.11 ± 0.32 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 5)	[35]
Healthy subjects and haemochromatosis patients	Double SPEs, HPLC purification, formic acid hydrolysing 8OHdG into 8OHGua, GC-MS measurement of 8OHGua to quantify 8OHdG	Laboratory staff: 1.33 ± 0.29 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 11); haemochromatosis patients: 1.39 ± 0.40 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 12); medical students: 1.58 ± 0.84 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 15)	[31]
Healthy subjects	Purification with a cation-exchange column and a cellulose partition column, HPLC-ECD	Smokers: 3.43 ± 0.68 (<i>n</i> = 7) $\mu\text{mol/mol}$ of creatine; non-smokers: 1.76 ± 0.89 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 6)	[36]
Quartz-exposed workers and patients with silicosis	Double SPEs, HPLC-ECD	Quartz-exposed workers: 2.27 ± 0.91 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 63); patients with silicosis: 2.00 ± 0.92 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 42)	[37]
Healthy subjects	HPLC purification, HPLC-ECD	Smokers: 1.35 ± 0.50 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 30); non-smokers: 1.16 ± 0.35 $\mu\text{mol/mol}$ of creatine (<i>n</i> = 30)	[38]
Swimmers and distance runners	Double SPEs, HPLC-ECD	Swimmers: 2.27 ± 0.48 $\mu\text{mol/mol}$ of creatine (before swimming), 2.47 ± 0.44 $\mu\text{mol/mol}$ of creatine (after swimming) (<i>n</i> = 9); runners: 1.42 ± 0.47 $\mu\text{mol/mol}$ of creatine (before running), 1.56 ± 0.46 $\mu\text{mol/mol}$ of creatine (after running) (<i>n</i> = 9)	[39]

during the clean-up, except during the final elution step. These characteristics of HLB made the clean-up procedure easy and rapid. Although derivatization of 8OHdG took more than 6 h, the derivatized samples were stable for at least 24 h. Thus a large number of samples could be derivatized at the same time. Usually, we cleaned up the urine samples on the morning of the first working day and freeze-dried them overnight. On the next morning, the dried samples were derivatized and, in the late afternoon, these samples were placed on the autosampler and GC-MS analysis was started and kept running overnight. On the third morning, we performed data analysis of the GC-MS results. With this simple and rapid method, we could analyse approx. 600 urine samples within 1 week.

Sensitivity of this assay was not a problem for the measurement of urinary 8OHdG. Its quantification limit was at least as low as 2.5 nM, a concentration lower than most of the urine 8OHdG levels reported. Furthermore, since we could load more urine sample on to the HLB cartridges, a larger signal of 8OHdG could be easily obtained if required.

Since H_2O_2 and dG were observed in urine, part of the urinary 8OHdG might be generated by oxidizing dG into 8OHdG by Fenton chemistry or by derivatization artifacts [21,22,29,30,40]. However, we confirmed that H_2O_2 had negligible effects in oxidizing urinary dG into 8OHdG. Furthermore, no significant arti-

factual generation of 8OHdG during derivatization was observed. Indeed, this was as expected, since we derivatized at room temperature in the presence of an antioxidant, namely ethanethiol [29].

In conclusion, a simple, rapid, sensitive and reliable methodology for the analysis of urinary excretion of 8OHdG was developed. Since large numbers of urine samples can be analysed in a reasonable time, e.g. 600 samples/week, this assay is suitable for use in the routine measurement of urinary excretion of 8OHdG for large-scale epidemiological studies.

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REFERENCES

- Halliwell, B. and Gutteridge, J. M. C. (eds.) (1999) Oxidative stress: adaptation, damage, repair and death. In *Free Radicals in Biology and Medicine*, pp. 246–350, Oxford University Press, Oxford
- Beckman, K. B. and Ames, B. N. (1997) Oxidative decay of DNA. *J. Biol. Chem.* **272**, 19633–19636
- Halliwell, B. (2002) Effect of diet on cancer development: is oxidative DNA damage a biomarker? *Free Radical Biol. Med.* **32**, 968–974
- Cooke, M. S., Evans, M. D., Dizdaroglu, M. and Lunec, J. (2003) Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J.* **17**, 1195–1214
- Dizdaroglu, M., Jaruga, P., Birincioglu, M. and Rodriguez, H. (2002) Free radical-induced damage to DNA: mechanisms and measurement. *Free Radical Biol. Med.* **32**, 1102–1115

- 6 Cooke, M. S., Evans, M. D. and Lunec, J. (2002) DNA repair: insights from urinary lesion analysis. *Free Radical Res.* **36**, 929–932
- 7 Bogdanov, M. B., Beal, M. F., McCabe, D. R., Griffin, R. M. and Matson, W. R. (1999) A carbon column-based liquid chromatography electrochemical approach to routine 8-hydroxy-2'-deoxyguanosine measurements in urine and other biologic matrices: a one-year evaluation of methods. *Free Radical Biol. Med.* **27**, 647–666
- 8 Saito, S., Yamauchi, H., Hasui, Y., Kurashige, J., Ochi, H. and Yoshida, K. (2000) Quantitative determination of urinary 8-hydroxydeoxyguanosine (8-OH-dG) by using ELISA. *Res. Commun. Mol. Path. Pharmacol.* **107**, 39–44
- 9 Gackowski, D., Rozalski, R., Roszkowski, K., Jawien, A., Foksinski, M. and Olinski, R. (2001) 8-Oxo-7,8-dihydroguanine and 8-oxo-7,8-dihydro-2'-deoxyguanosine levels in human urine do not depend on diet. *Free Radical Res.* **35**, 825–832
- 10 Poulsen, H. E., Loft, S., Jensen, B. R., Sorenson, M., Hoberg, A.-M. and Weimann, A. (2001) HPLC-ECD, HP LC-MS/MS (urinary biomarkers). In *Critical Reviews of Oxidative Stress and Aging: Advances in Basic Science, Diagnostics, and Intervention* (Richard, G. C. and Henry, R., eds.), pp. 233–256. World Scientific, Singapore
- 11 Pilger, A., Ivancsits, S., Germadnik, D. and Rudiger, H. W. (2002) Urinary excretion of 8-hydroxy-2'-deoxyguanosine measured by high-performance liquid chromatography with electrochemical detection. *J. Chromatogr. B* **778**, 393–401
- 12 Mei, S. R., Yao, C. H., Cai, L. S., Xing, J., Xu, G. W. and Wu, C. Y. (2003) Capillary electrophoresis with end-column amperometric detection of urinary 8-hydroxy-2'-deoxyguanosine. *Electrophoresis* **24**, 1411–1415
- 13 Kvasnicova, V., Samcova, E., Jursova, A. and Jelinek, I. (2003) Determination of 8-hydroxy-2'-deoxyguanosine in untreated urine by capillary electrophoresis with UV detection. *J. Chromatogr. A* **985**, 513–517
- 14 Chiou, C. C., Chang, P. Y., Chan, E. C., Wu, T. L., Tsao, K. C. and Wu, J. T. (2003) Urinary 8-hydroxydeoxyguanosine and its analogs as DNA marker of oxidative stress: development of an ELISA and measurement in both bladder and prostate cancers. *Clin. Chim. Acta* **334**, 87–94
- 15 Holmberg, I., Stal, P. and Hamberg, M. (1999) Quantitative determination of 8-hydroxy-2'-deoxyguanosine in human urine by isotope dilution mass spectrometry: normal levels in hemochromatosis. *Free Radical Biol. Med.* **26**, 129–135
- 16 Ravanat, J. L., Guicherd, P., Tuce, Z. and Cadet, J. (1999) Simultaneous determination of five oxidative DNA lesions in human urine. *Chem. Res. Toxicol.* **12**, 802–808
- 17 Toyokuni, S., Tanaka, T., Hatton, Y., Nishiyama, Y., Yoshida, A., Uchida, K., Hiai, H., Ochi, H. and Osawa, T. (1997) Quantitative immunohistochemical determination of 8-hydroxy-2'-deoxyguanosine by a monoclonal antibody N45.1: its application to ferric nitrilotriacetate-induced renal carcinogenesis model. *Lab. Invest.* **76**, 365–374
- 18 Shimoi, K., Kasai, H., Yokota, N., Toyokuni, S. and Kinae, N. (2002) Comparison between high-performance liquid chromatography and enzyme-linked immunosorbent assay for the determination of 8-hydroxy-2'-deoxyguanosine in human urine. *Cancer Epidemiol. Biomarkers Prev.* **11**, 767–770
- 19 Yoshida, R., Ogawa, Y. and Kasai, H. (2002) Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine values measured by an ELISA correlated well with measurements by high-performance liquid chromatography with electrochemical detection. *Cancer Epidemiol. Biomarkers Prev.* **11**, 1076–1081
- 20 Cooke, M. S., Lunec, J. and Evans, M. D. (2002) Progress in the analysis of urinary oxidative DNA damage. *Free Radical Biol. Med.* **33**, 1601–1614
- 21 Long, L. H., Evans, P. J. and Halliwell, B. (1999) Hydrogen peroxide in human urine: implications for antioxidant defense and redox regulation. *Biochem. Biophys. Res. Commun.* **262**, 605–609
- 22 Long, L. H. and Halliwell, B. (2000) Coffee drinking increases levels of urinary hydrogen peroxide detected in healthy human volunteers. *Free Radical Res.* **32**, 463–467
- 23 Rozalski, R., Gackowski, D., Roszkowski, K., Foksinski, M. and Olinski, R. (2002) The level of 8-hydroxyguanine, a possible repair product of oxidative DNA damage, is higher in urine of cancer patients than in control subjects. *Cancer Epidemiol. Biomarkers Prev.* **11**, 1072–1075
- 24 Gackowski, D., Speina, E., Zielinska, M., Kowalewski, J., Rozalski, R., Siomek, A., Paciorek, T., Tudek, B. and Olinski, R. (2003) Products of oxidative DNA damage and repair as possible biomarkers of susceptibility to lung cancer. *Cancer Res.* **63**, 4899–4902
- 25 Rozalski, R., Winkler, P., Gackowski, D., Paciorek, T., Kasprzak, H. and Olinski, R. (2003) High concentrations of excised oxidative DNA lesions in human cerebrospinal fluid. *Clin. Chem.* **49**, 1218–1221
- 26 Slot, C. (1965) Plasma creatinine determination. A new and specific Jaffé reaction method. *Scand. J. Clin. Lab. Invest.* **17**, 381–387
- 27 Dizdaroglu, M. (1985) Application of capillary gas chromatography-mass spectrometry to chemical characterization of radiation-induced base damage of DNA: implications for assessing DNA repair processes. *Anal. Biochem.* **144**, 593–603
- 28 Dizdaroglu, M. (1986) Characterization of free radical-induced damage to DNA by the combined use of enzymatic hydrolysis and gas chromatography-mass spectrometry. *J. Chromatogr.* **367**, 357–366
- 29 Jenner, A., England, T. G., Aruoma, O. I. and Halliwell, B. (1998) Measurement of oxidative DNA damage by gas chromatography mass spectrometry: ethanethiol prevents artifactual generation of oxidized DNA bases. *Biochem. J.* **331**, 365–369
- 30 Weimann, A., Belling, D. and Poulsen, H. E. (2002) Quantification of 8-oxo-guanine and guanine as the nucleobase, nucleoside and deoxynucleoside forms in human urine by high-performance liquid chromatography-electrospray tandem mass spectrometry. *Nucleic Acids Res.* **30**, E7
- 31 Prottigente, A. R., England, T. G., Rehman, A., Rice-Evans, C. A. and Halliwell, B. (2002) Gender differences in steady-state levels of oxidative damage to DNA in healthy individuals. *Free Radical Res.* **36**, 157–162
- 32 Harman, S. M., Liang, L., Tsitouras, P. D., Gucciardo, F., Heward, C. B., Reaven, P. D., Ping, W., Ahmed, A. and Cutler, R. G. (2003) Urinary excretion of three nucleic acid oxidation adducts and isoprostane F_{2α} measured by liquid chromatography-mass spectrometry in smokers, ex-smokers, and nonsmokers. *Free Radical Biol. Med.* **35**, 1301–1309
- 33 Kasai, H., Iwamoto-Tanaka, N., Miyamoto, T., Kawanami, K., Kawanami, S., Kido, R. and Ikeda, M. (2001) Life style and urinary 8-hydroxydeoxyguanosine, a marker of oxidative DNA damage: effects of exercise, working conditions, meat intake, body mass index, and smoking. *Jpn. J. Cancer Res.* **92**, 9–15
- 34 Nakano, M., Kawanishi, Y., Kamohara, S., Uchida, Y., Shiota, M., Inatomi, Y., Komori, T., Miyazawa, K., Gondo, K. and Yamasawa, I. (2003) Oxidative DNA damage (8-hydroxydeoxyguanosine) and body iron status: a study on 2507 healthy people. *Free Radical Biol. Med.* **35**, 826–832
- 35 Renner, T., Fechner, T. and Scherer, G. (2000) Fast quantification of the urinary marker of oxidative stress 8-hydroxy-2'-deoxyguanosine using solid-phase extraction and high-performance liquid chromatography with triple-stage quadrupole mass detection. *J. Chromatogr. B* **738**, 311–317
- 36 Suzuki, J., Inoue, Y. and Suzuki, S. (1995) Changes in the urinary excretion level of 8-hydroxyguanine by exposure to reactive oxygen-generating substances. *Free Radical Biol. Med.* **18**, 431–436
- 37 Pilger, A., Germadnik, D., Schaffer, A., Theiler, A., Pils, P., Sluka, F., Winkler, N. and Rudiger, H. W. (2000) 8-Hydroxydeoxyguanosine in leukocyte DNA and urine of quartz-exposed workers and patients with silicosis. *Int. Arch. Occup. Environ. Health* **73**, 305–310
- 38 Pourcelot, S., Faure, H., Firooz, F., Ducros, V., Tripiet, M., Hee, J., Cadet, J. and Favier, A. (1999) Urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine and 5-(hydroxymethyl) uracil in smokers. *Free Radical Res.* **30**, 173–180
- 39 Inoue, T., Mu, Z. S., Sumikawa, K., Adachi, K. and Okochi, T. (1993) Effect of physical exercise on the content of 8-hydroxydeoxyguanosine in nuclear-DNA prepared from human-lymphocytes. *Jpn. J. Cancer Res.* **84**, 720–725
- 40 Halliwell, B., Long, L. H., Yee, T. P., Lim, S. and Kelly, R. (2004) Establishing biomarkers of oxidative stress, the measurement of H₂O₂ in human urine. *Curr. Med. Chem.*, in the press

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