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RESEARCH ARTICLE

Interlaboratory comparison of methodologies for the measurement of urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine

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Abstract

Urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) is widely used as a marker of oxidative stress. Here we report the comparison of two, distinct chromatographic assays with an enzyme-linked immunosorbent assay (ELISA). The chromatographic assays displayed good agreement (r =:0.89, $p < 0.0001$), whereas there was markedly worse, albeit still significant, agreement with the ELISA (high-pressure liquid chromatography followed by gas chromatography (HPLC-GC/MS), $r = 0.43$; HPLC with electrochemical detection (HPLC-EC), $r = 0.56$; $p < 0.0001$). Mean values differed significantly between the chromatographic assays and the ELISA (HPLC-GC/MS 3.86, HPLC-EC 4.20, ELISA 18.70 ng mg^{-1} creatinine; $p < 0.0001$). While it is reassuring to note good agreement between chromatographic assays, this study reveals significant short-comings in the ELISA, which brings into question its continued use in its present form.

Keywords: *Oxidative stress; 8-oxodGuo; ELISA; HPLC; GC/MS; urine; biomarkers; molecular epidemiology*

Introduction

Oxidative stress is purported to have an important role in a variety of pathological conditions, including cancer, cardiovascular disease and neurodegenerative disease (Olinski et al. 2002, Cooke et al. 2003). Oxidative damage to DNA perhaps receives the most attention, for not only can this lead to mutation (Cheng et al. 1992), but it may have numerous other effects upon cell function, such as acceleration of telomere shortening or modification of gene expression and cell signalling (Evans & Cooke 2004). Overall, this demonstrates that oxidative modification of DNA possesses clear mechanistic relevance to the pathogenesis of both malignant and non-malignant disease (Evans & Cooke 2006).

Of all the identified oxidative modifications of DNA, the nucleobase 8-oxo-7,8-dihydro-guanine (8-oxoGua), or its 2′-deoxyribonucleoside equivalent 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), is the most studied, and is widely acknowledged as a biomarker of oxidative stress (Kasai 1997). Numerous approaches can determine cellular DNA levels of 8-oxodGuo (Guetens et al. 2002) but, in addition to being necessarily invasive, many of the procedures

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also risk the production of artefactual damage during sample work-up (Gedik & Collins 2005), which rather limits its utility in molecular epidemiology studies. However, measurement of 8-oxodGuo in urine, has excellent stability ($>$10 years) (Loft et al. 2006), and can circumvent issues of invasiveness and artefact (Lin et al. 2004). These properties are crucial to the development of assays which will improve our understanding of the role of oxidative stress in disease, and the development of biomarkers for this condition. Another prerequisite is that such assays should be well validated, in terms of being robust and reliable, with interlaboratory consensus. Furthermore, the origin of the lesion in urine should be understood. The presence of 8-oxodGuo in urine is thought to derive, in part, from the activity of nudix hydrolases (also known as pyrophosphohydrolases), which hydrolyse the modified 2′-deoxyribonucleotide triphosphate (8-oxodGTP) to the corresponding monophosphate, which cannot be rephosphorylated, removing 8-oxodGTP as a potential substrate for DNA polymerases (discussed in Cooke et al. 2008). Consequently, measurement of urinary 8-oxodGuo may provide information concerning the prevention of misincorporation (Cooke et al. 2005).

The principal chromatographic techniques applied to the analysis of 8-oxodGuo in urine are high-pressure liquid chromatography (HPLC) prepurification followed by gas chromatography (HPLC-GC/MS) (Gackowski et al. 2001), liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) (Weimann et al. 2002, Malayappan et al. 2007), and (column switching) liquid chromatography with electrochemical detection (HPLC-EC) (Loft et al. 1998, Kasai 2003), or immunoassay (enzyme-linked immunosorbent assay, ELISA) (Cooke et al. 1998, Rossner et al. 2007). The most frequently used ELISA applied to urinary analysis of 8-oxodGuo is that manufactured by the Japanese Institute for the Control of Aging (JaICA; '8-OH-dG Check'). However, there is significant precedent in the literature for this approach reporting greater levels of urinary 8-oxodGuo than chromatographic methods. Previous typical mean values for urinary 8-oxodGuo, in healthy volunteers, by the JaICA ELISA kit have ranged from 8.16 to 11.13 μg g^{-1} creatinine (Shimoi et al. 2002, Yoshida et al. 2002), compared with 0.79–2.13 μg g^{-1} creatinine, for example, by chromatographic techniques (discussed in Lin et al. 2004). It should also be noted that the reproducibility of 8-oxodGuo analysis by ELISA can also be very low. When the same urine samples were analysed repeatedly (30 times), the raw 8-oxodGuo values showed a very high coefficient of variation (CV, 28.4–47.3) (Yoshida et al. 2002).

A more recent report indicates that strict temperature control during the incubation steps of the JaICA ELISA can decrease mean values (e.g. 1.67 pmol μmol^{-1} creatinine for ELISA vs 0.41 pmol μmol^{-1} creatinine for LC-MS/MS (Cooke et al. 2006b), but this has still not resolved the problem. Further complicating this issue, there have been instances when the ELISA has been shown not to correlate with chromatographic techniques (Prieme et al. 1996, Cooke et al. 2006b), while in other reports they do correlate, using HPLC-EC (Shimoi et al. 2002, Yoshida et al. 2002) and LC-GC/MS (Cooke et al. 2006a). However, it appears that the good correlation noted by Yoshida et al. was obtained after certain erroneous data points were excluded. Specifically, data from an ELISA plate would be excluded if there was $>$ 25% variation in 8-oxodGuo values from any of the three different standard urine samples applied to the plate (Yoshida et al. 2002). This implies a problem with the uniformity of commercial ELISA plates.

To date, no study has made comparisons between more than one chromatographic assay and ELISA, and there is only a single report of a comparison between two chromatographic assays (Harri et al. 2007). Such experiments would be the first step towards establishing validated assays for application in future molecular epidemiology studies. As it was for the European Standards Committee on Oxidative DNA Damage previously, a goal of the European Standards Committee on Urinary (DNA) Lesion Analysis (ESCULA) (Cooke et al. 2008) is to achieve consensus between the various methods for measuring urinary 8-oxodGuo. Here we report the comparison of three distinct methods for the analysis of urinary 8-oxodGuo: HPLC-GC/MS, HPLC-EC and ELISA, in order to begin the process of validating assays, which can ultimately be used to establish reference ranges for healthy individuals and patient groups with specific disease, stratified for parameters known to affect oxidative stress (e.g. age and smoking).

Methods

Subjects and sampling

Urine samples in the present study were collected from Swedish men ($n = 140$), aged 20–60 years (mean 43 years), participating in the EU-funded, 'EMECAP' (European Mercury Emissions from Chloralkali Plants) project (Barregard et al. 2006, Jarosinska et al. 2006). While the primary purpose of that project was to quantify the internal dose of mercury in chloralkali workers and general populations living close to mercury cell chloralkali plants, this population presented the opportunity to compare multiple methods of analysing urinary 8-oxodGuo. Of the 140 subjects, 57 were chloralkali workers exposed to inorganic mercury and 83 were age-matched control subjects living in the same region. Because there were only small differences between exposed workers and controls, we pooled these 140 subjects into a single group for the method comparisons.

Each subject provided a first-void urine sample. The samples were subsequently aliquotted into multiple polyethylene tubes (cryotubes 4.5 ml, Nunc™, Roskilde, Denmark), and stored frozen at –25°C. The sample and background data collection was performed in 2002. Subsequently the samples were sent 'blindly' to the following laboratories for 8-oxodGuo analysis: HPLC-GC/MS (Olinski's laboratory, Bydgoszcz, Poland), HPLC-EC (Kasai's laboratory, Kitakyushu, Japan) and ELISA (Cooke's laboratory, Leicester, UK).

Urine sample preparation for HPLC purification with GC/MS analysis

To 2 ml of human urine were added 0.05 nmol of [$^{15}N_5$] 8-oxodGuo (Gackowski et al. 2001). After centrifugation (2000*g*, 10 min), the supernatant was filtered through a Millipore GV13 0.22 µm syringe filter and 500 µl of the filtrate was injected onto the HPLC system. HPLC purification of urinary 8-oxodGuo was performed according to the method described by Ravanat et al. (1999) with some modifications. Briefly, urine samples spiked with labelled compounds were injected onto a Supelcosil LC 18 column (250 × 10 mm) equipped with Supelguard LC18 guard column (20 × 4.6 mm), both from Supelco (Sigma-Aldrich, Poole, UK). A 30 min linear gradient elution was performed (0.5% acetic acid at 0 min to 0.5% acetic acid with an addition of 5% of acetonitrile 30 min), at a flow rate of 3 ml min^{-1}. After this time the column was washed with 70% of acetonitrile for 20 min and equilibrated with 0.5% acetic acid for 10 min prior to a further injection.

The collected fractions were dried by evaporation under reduced pressure in a Speed–Vac system. The 8-oxodGuo fraction was treated with 400 µl of 60% formic acid (Sigma-Aldrich, Poole, UK) for 30 min at 130°C. Subsequently, samples were prepared for GC/MS analysis which was performed according to the method described by Dizdaroglu (1994), adapted for additional [$^{15}N_5$] 8-oxoGua analyses (*m/z* 445 and 460 ions were monitored).

Urine sample preparation and HPLC-EC analysis

Urine samples were defrosted and mixed with an equal volume of 4% (v/v) acetonitrile in double-distilled water, containing the ribonucleoside marker 8-oxoguanosine (8-oxoGuo; 120 µg ml^{-1}), 130 mM NaOAc and 0.6 mM H_2SO_4. The treated urine samples were then stored at 5°C overnight, before being centrifuged at 15 700*g* for 5 min. Samples were transferred to plastic HPLC injector vials and 20 µl was injected, using an automated HPLC system similar to that described in detail previously (Kasai 2003). In essence, the system comprised a sampling injector (Gilson 231XL), a pump (Gilson 307) for the anion exchange guard (MCI GEL CA08F, 7 µm, 1.5 × 50 mm) and main column (MCI GEL CA08F, 7 µm, 1.5 × 150 mm) in HPLC-1 (flow rate was 50 µl min^{-1} and column oven was set at 65°C), a UV detector (Gilson UV/VIS-151 with micro cell), a second pump (Gilson 307) for the analysis of the 8-oxodGuo fraction with a reverse-phase column (Shiseido [Tokyo, Japan], Capcell Pak C18, 5 µm, 4.6 × 250 mm) in HPLC-2 (flow rate was 0.9 ml min^{-1} and column oven set at 40°C), connected with an EC detector (ESA Coulochem II) and two switch valves. A third pump (Gilson 307) was used to backwash the guard column (flow rate 70 µl min^{-1}) for 32 min after valve switching at around 13 min after each sample injection. For HPLC-1 the solvent used was 2% acetonitrile in 0.3 mM sulfuric acid; in HPLC-2 the solvent was composed of 10 mM phosphate buffer (pH 6.7), 5% methanol, plus an antiseptic, Reagent MB (KCG reagent; MC Medical Inc., Tokyo, Japan; 100 µl l^{-1}), and was recycled for a period of 1 week. The guard column was back washed with 0.5 M ammonium sulphate:acetonitrile (7:3 v/v). Both the 8-oxoGuo marker peak, used for automatic peak detection, and the 8-oxodGuo fraction, were detected at 305 nm (Svoboda & Kasai 2004). After automatic peak detection the 8-oxodGuo fraction was collected by valve switching and then injected on HPLC-2 to be detected by a Coulochem II EC detector (ESA) with a guard cell (5020) and an analytical cell (5011); applied potentials: guard cell = 400 mV, E1 = 150 mV and E2 = 350 mV. The total time between analysis of consecutive samples was 52 min. The automatic peak detection was controlled by software from Gilson and chromatograms were recorded and integrated with the computer software (Unipoint 3.30).

Urine sample preparation and ELISA

Following thawing and centrifugation (300*g* for 10 min) of the urine samples, the supernatants (50 µl per well) were applied to the competitive ELISA plate according to the protocol (JaICA, Fukuroi, Japan). On the basis of our previous observations (Cooke et al. 2006b) we adhered strictly to the incubation temperature of 37°C for the primary antibody, as described by the manufacturer.

Urinary creatinine

Urinary creatinine levels were determined by the photometric 'Jaffe' method at the University Hospital in Gothenburg, Sweden.

Statistics

Associations between assays were assessed by plots and the Pearson correlation coefficient (r_p), as well as the Spearman rank correlation coefficient (r_s). Differences between assays were tested with paired *t*-tests of untransformed and log-transformed results. Results were also expressed per mg creatinine in order to normalize for urine concentration, and allow comparison with previous literature values. The possible impact of smoking and age was tested in multiple linear regression models including these covariates and the creatinine concentration of the samples. Statistical analyses were performed using the SAS package, version 9.1. Values $p < 0.05$ were considered to be statistically significant.

Results

Descriptive statistics are shown in Table 1. The mean levels of urinary 8-oxodGuo were similar to those previously reported in the literature, for samples collected from the general population, when comparing like-for-like analytical methods: chromatography (Harri et al. 2007); ELISA (Leinonen et al. 1997, Thompson et al. 1999). As shown in the Table 1, the mean 8-oxodGuo levels, determined by the HPLC-EC assay, were on average ~10% higher than the HPLC-GC/MS method (paired analysis, $p < 0.01$). Mean levels by the ELISA assay were 5–7 times higher than those found for the other two methods ($p < 0.0001$).

Figure 1A shows the strong, linear association between the concentrations of urinary 8-oxodG, analysed by HPLC-GC/MS and HPLC-EC ($n = 115$, $r_p = 0.89$, $p < 0.0001$; Table 1). Expressed in another way, had the result been duplicates from the same assay, the coefficient of variation would have been only 25%. The strength of the association was identical based on all 140 samples ($r_p = 0.89$). The Bland–Altman plot (Figure 1B) shows that the association between results from the two assays was similar over the whole range of concentrations although, as would be expected, the absolute deviations between results increased with concentration.

In contrast, the linear associations between urinary 8-oxodGuo concentrations measured by ELISA and (1) HPLC-GC/MS ($r_p = 0.17$, $p = 0.08$), and (2) HPLC-EC methods ($r_p = 0.28$, $p = 0.003$), were much weaker. As evident from Table 1 and Figures 2A and 3A, the results using ELISA provide, on average, much higher concentrations of urinary 8-oxodG. Consequently the associations with the ELISA assay were stronger using the Spearman rank correlation coefficient, which puts less weight on the extremes, and reflects the rank order of the sample concentrations, irrespective of the fact that ELISA results in paired samples are higher than the results for the HPLC-based methods ($r_s = 0.43$, $p < 0.0001$ when comparing with HPLC-GC/MS and $r_s = 0.56$; $p < 0.0001$ when comparing with HPLC-EC; Figures 2A and 3A). Bland–Altman plots, of the data represented in Figures 2A and 3A, show that the association between results from the two assays was similar (within 95% limits of agreement) ata relatively low concentration of urinary 8-oxodG ($< 40\,\mathrm{ng\,ml^{-1}}$) although, as would be expected, the absolute deviations between results increased significantly with increasing concentration. The plots for ELISA vs HPLC-GC/MS and ELISA vs HPLC-EC were almost identical in terms of mean bias and 95% limits of agreement.

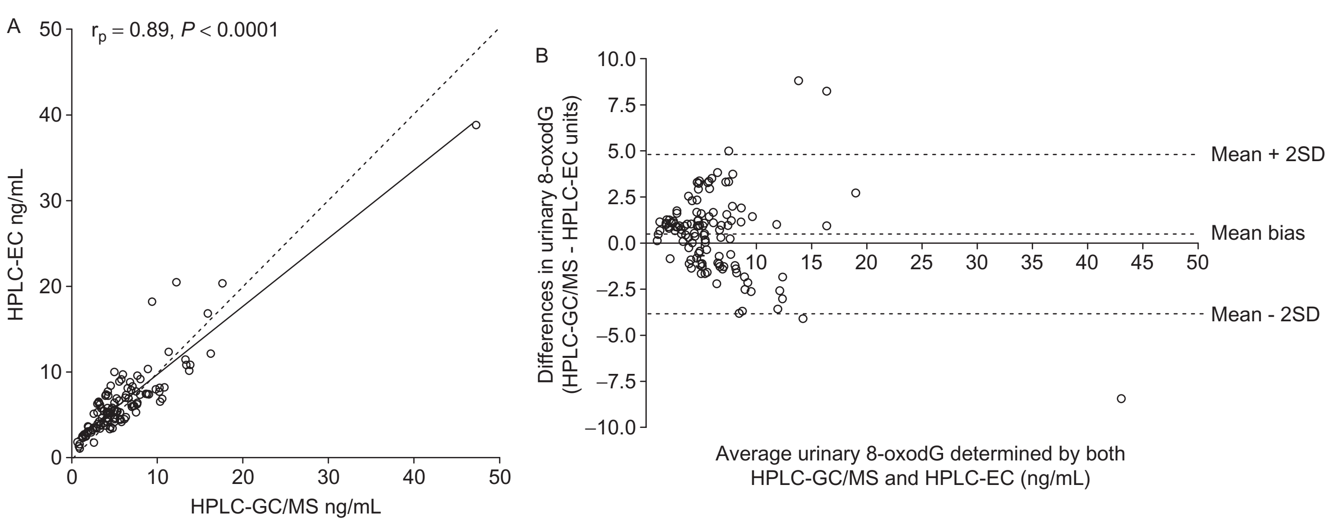

Figure 1. (A) Comparison of urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) concentrations as determined by high-performance liquid chromatography with electrochemical detection (HPLC-EC) and HPLC followed by gas chromatography (HPLC-GC/MS). Lines of linear regression (−) and perfect correlation (·····) are both shown. (B) Bland–Altman plot displaying the comparison of HPLC-EC and HPLC-GC/MS techniques. The plot shows the differences between paired measurements plotted against their respective means. The 95% limits of agreement (mean bias ± 2 SD) and mean bias are shown.

Table 1. Comparison of urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) by three different methods, in 115 Swedish men aged 20–60 years.

Method	Mean 8-oxodGuo (ng ml^{-1})[a]	Median[b]	SD	Min	Max
HPLC-GC/MS	6.01	4.99	5.22	0.66	47.3
HPLC-EC	6.52	5.62	4.59	1.07	38.8
ELISA	29.8	18.0	31.3	2.89	172

HPLC-GC/MS, high-performance liquid chromatography followed by gas chromatography; HPLC-EC, HPLC with electrochemical detection; ELISA enzyme-linked immunosorbent assay.

[a]Mean 8-oxodGuo in ng mg^{-1} creatinine was 3.86 for HPLC-GC/MS, 4.20 for HPLC-EC and 18.70 for ELISA; mean creatinine concentration 1.6 g l^{-1}.

[b]For HPLC-GC/MS and HPLC-EC results were available for 140 men. Mean 8-oxodGuo values were very similar to those shown above: 6.29 ng ml^{-1} (HPLC-GC/MS) and 6.74 ng ml^{-1} (HPLC-EC).

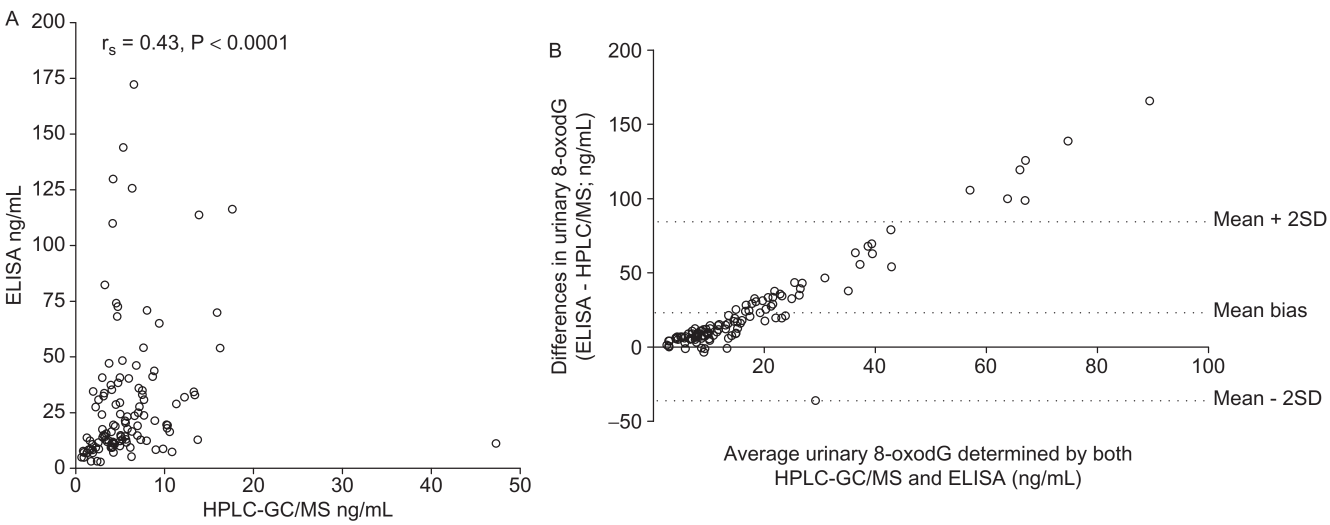

Figure 2. (A) Comparison of urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) concentrations as determined by enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography followed by gas chromatography (HPLC-GC/MS). (B) Bland–Altman plot displaying the comparison of ELISA and HPLC-GC/MS techniques. The plot shows the differences between paired measurements plotted against their respective means. The 95% limits of agreement (mean bias ± 2 SD) and mean bias are shown.

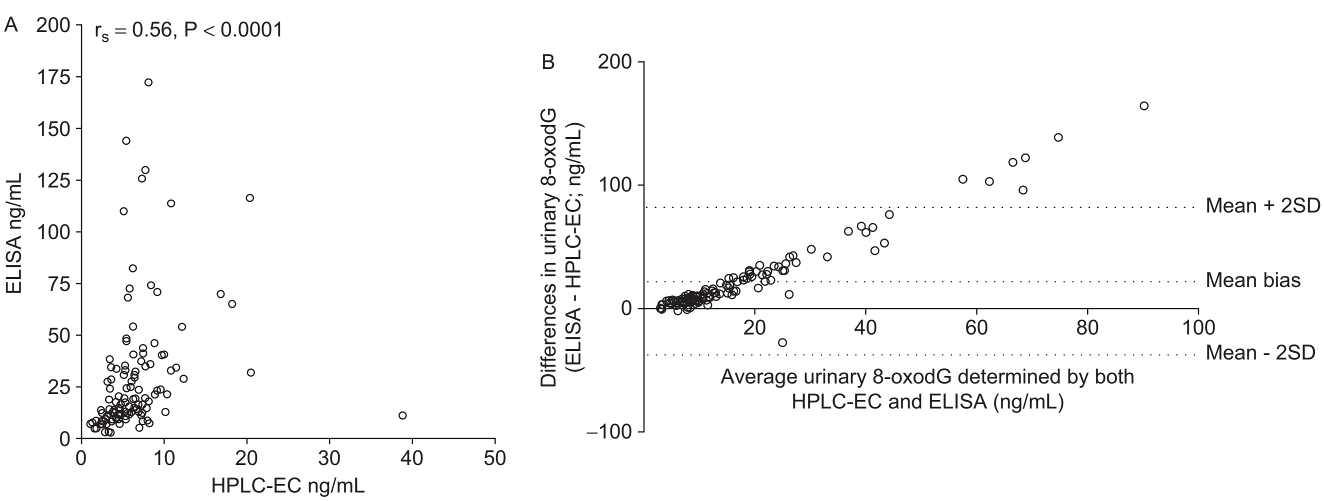

Figure 3. (A) Comparison of urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) concentrations as determined by enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography with electrochemical detection (HPLC-EC). (B) Bland–Altman plot displaying the comparison of ELISA and HPLC-EC techniques. The plot shows the differences between paired measurements plotted against their respective means. The 95% limits of agreement (mean bias ± 2 SD) and mean bias are shown.

The levels of urinary 8-oxodGuo in self-reported smokers were slightly higher, as measured by both HPLC-GC/MS and HPLC-EC (24% and 14% higher, respectively; smoking data were only available for $n = 110$ subjects), than in non-smokers. Surprisingly, the results using ELISA show, on average, 16% lower urinary 8-oxodGuo in smokers. In linear multiple regression models with self-reported smoking (yes/no), age and creatinine concentration as independent variables, the increase with creatinine concentration was by far the strongest effect, significant ($p < 0.0001$) for all assays. The effect of smoking on urinary 8-oxodGuo was statistically significant in the results from the HPLC-EC assay ($p = 0.03$) and borderline for the HPLC-GC/MS assay ($p = 0.05$). We also took the opportunity to examine the effect of age upon urinary 8-oxodGua; this was small and not statistically significant.

Discussion

There is a need for well-validated, preferably non-invasive, biomarkers of oxidative stress, that can be applied to the rapid analysis of samples from a large-scale study. Immunoassay approaches have the potential to fulfil this criterion, as well as being easily established in even the most poorly equipped of laboratories, and with little need for specialist experience or equipment. However, the on-going lack of agreement between immunoassay and chromatographic techniques, widely considered to be the 'gold standard', limits the usefulness of this approach.

Cursory examination of the literature would indicate that there appears to be reasonable within-technique agreement in urinary 8-oxodGuo levels, in healthy adults (discussed in Cooke et al. 2000). As mentioned above, a number of studies have compared chromatographic approaches with ELISA, with markedly different findings. However, there has been little formal comparison of chromatographic techniques, with only one report in the literature, which also showed a good correlation between HPLC-MS/MS and HPLC-EC ($r = 0.93$, $n = 246$ samples) (Harri et al. 2007). Herein, we demonstrate the strong agreement between HPLC-EC and HPLC-GC/MS, although mean 8-oxodGuo values by HPLC-EC were significantly higher than by HPLC-GC/MS. It is of interest that Harri et al. (2007) also noted that, on the whole, the HPLC-EC method gave higher levels, although this was not examined for statistical significance. A possible explanation for this finding might derive from the use of mass labelled, internal standards in the HPLC-MS/MS assay. Nevertheless, both chromatographic methods were sensitive enough to show the expected slight increase in urinary 8-oxodGuo among smokers (see below).

Consistent with previous studies, we demonstrated that ELISA generates significantly higher values for urinary 8-oxodGuo. Possible reasons for this overestimation are a source of much discussion within the literature; for example, some authors claim that the monoclonal antibody used in the JaICA ELISA kit, and indeed other commercially available kits, is not sufficiently specific for the detection of 8-oxodG. There is experimental evidence to show that the antibody used in the ELISA kit does not cross-react with 8-oxoGua or the unmodified nucleoside/base. However, it does cross-react with 8-oxoGuo and 8-oxodGMP (Evans et al. 2008), although the former is not in sufficient quantities in urine to interfere (Weimann et al. 2002), and the presence of the latter, in urine, has not been demonstrated (Weimann et al. 2004). Most recently, Evans et al. showed that performing the primary antibody incubation at 4°C overnight, increases the selectivity of the ELISA (Evans et al. 2008). This modification to the protocol brings mean urinary 8-oxodG levels into agreement with chromatographic methods.

We also showed a significant correlation between ELISA and two, distinct, chromatographic techniques. As there is a reasonable association between the ranks of the values for ELISA and chromatographic techniques, it is perhaps not surprising that the ELISA can identify associations with 8-oxodGuo in pathological conditions (Rossner et al. 2007, Chung et al. 2008), although this technique should be expected to be less precise than HPLC-based techniques. It is also of concern that the relationship between ELISA and chromatographic techniques can be so variable – sometimes correlating with chromatographic techniques, as reported here, and sometimes not (e.g. Prieme et al. 1996). It might be speculated that the basis for this variability derives from some intrinsic variability in the urine samples used in one study, compared with those used in another, e.g. a higher concentration of interfering compounds. Certainly, isolation of 8-oxodGuo from the urine, and hence removal of any interfering constituents, with subsequent analysis by ELISA, vastly improves the agreement between ELISA and HPLC-EC ($r = 0.550$ prior to purification, and $r = 0.833$, post-purification) (Shimoi et al. 2002). In itself, this implies that this is either an issue of specificity, or some other kind of assay interference. Of course, this detracts from any benefit offered by ELISA, and is not really a practical solution.

The source of the ELISA should also be considered. As we have highlighted previously, certain manufacturer's kits have severe shortcomings over and above those noted here (Cooke et al. 2006b). Taken together, and in particular given the ability for absolute identification of target compound by MS, it is reasonable to assume that the chromatographic techniques reflect the 'true' concentrations of the modified 2′-deoxyribonucleoside, 8-oxodGuo.

There are surprisingly few studies with a principal focus upon urinary 8-oxodGuo and smoking. Of these, the general finding is that levels of 8-oxodGuo are elevated in the urine of smokers, compared with non-smokers (Prieme et al. 1998, Hu et al. 2006, Loft et al. 2006); our findings, using chromatographic techniques, agree with this. However, the reverse was true based upon ELISA estimates, which is difficult to reconcile, although a previous study of smoking status and urinary 8-oxodGuo, determined by ELISA, failed to establish a significant difference between the two groups (Besaratinia et al. 2001).

This study is the first step towards obtaining validated methods for measuring urinary 8-oxodGuo, highlighting the limitations of immunoassay, and bringing into question its continued use in its present form. A larger study is needed, incorporating multiple methods, and analysing pure standards, as well as multiple urine samples, and this is a goal of ESCULA. Finally, it should be noted that, while presently the most studied product of oxidatively generated damage to DNA, 8-oxodGuo is not the sole product (Cooke et al. 2003). Other lesions exist which, under various cellular conditions, may be more abundant than 8-oxodGuo (Pang et al. 2007, Dedon 2008). This presents the possibility of other lesions being studied in urine, and other biological matrices, as potential biomarkers of oxidative stress and disease, another matter under consideration by ESCULA.

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References

Barregard L, Horvat M, Mazzolai B, Sällsten G, Gibicar D, Fajon V, Dibona S, Munthe J, Wängberg I, Haeger Eugensson M. 2006. Urinary mercury in people living near point sources of mercury emissions. *Sci Total Environ* 3681:326–334.

Besaratinia A, Van Schooten FJ, Schilderman PA, De Kok TM, Haenen GR, Van Herwijnen MH, Van Agen E, Pachen D, Kleinjans JC. 2001. A multi-biomarker approach to study the effects of smoking on oxidative DNA damage and repair and antioxidative defense mechanisms. *Carcinogenesis* 223:395–401.

Cheng KC, Cahill DS, Kasai H, Nishimura S, Loeb LA. 1992. 8-Hydroxyguanine, an abundant form of oxidative DNA damage, causes G----T and A----C substitutions. *J Biol Chem* 2671:166–172.

Chung CJ, Huang CJ, Pu YS, Su CT, Huang YK, Chen YT, Hsueh YM. 2008. Urinary 8-hydroxydeoxyguanosine and urothelial carcinoma risk in low arsenic exposure area. *Toxicol Appl Pharmacol* 2261:14–21.

Cooke MS, Evans MD, Dizdaroglu M, Lunec J. 2003. Oxidative DNA damage: mechanisms, mutation, and disease. *Faseb J* 1710:1195–1214.

Cooke MS, Evans MD, Dove R, Rozalski R, Gackowski D, Siomek A, Lunec J, Olinski R. 2005. DNA repair is responsible for the presence of oxidatively damaged DNA lesions in urine. *Mutat Res* 5741-2:58–66.

Cooke MS, Evans MD, Herbert KE, Lunec J. 2000. Urinary 8-oxo-2′-deoxyguanosine – source, significance and supplements. *Free Radic Res* 325:381–397.

Cooke MS, Evans MD, Podmore ID, Herbert KE, Mistry N, Mistry P, Hickenbotham PT, Hussieni A, Griffiths HR, Lunec J. 1998. Novel repair action of vitamin C upon in vivo oxidative DNA damage. *FEBS Lett* 4393: 363–367.

Cooke MS, Olinski R, Loft S; European Standards Committee on Urinary (DNA) Lesion Analysis. 2008. Measurement and meaning of oxidatively modified DNA lesions in urine. *Cancer Epidemiol Biomarkers Prev* 171:3–14.

Cooke MS, Rozalski R, Dove R, Gackowski D, Siomek A, Evans MD, Olinski R. 2006a. Evidence for attenuated cellular 8-oxo-7,8-dihydro-2′-deoxyguanosine removal in cancer patients. *Biol Chem* 387:393–400.

Cooke MS, Singh R, Hall GK, Mistry V, Duarte TL, Farmer PB, Evans MD. 2006. Evaluation of enzyme-linked immunosorbent assay and liquid chromatography-tandem mass spectrometry methodology for the analysis of 8-oxo-7,8-dihydro-2'-deoxyguanosine in saliva and urine. *Free Radic Biol Med* 4112:1829–1836.

Dedon PC. 2008. The chemical toxicology of 2-deoxyribose oxidation in DNA. *Chem Res Toxicol* 211:206–219.

Dizdaroglu M. 1994. Chemical determination of oxidative DNA damage by gas chromatography-mass spectrometry. *Methods Enzymol* 234:3–16.

Evans M, Cooke M. 2006. Oxidative damage to DNA in non-malignant disease: biomarker or biohazard? In: Volff J-N, editor. *Genome and disease*, vol. 1. Basel: Karger. p. 153–166.

Evans MD, Cooke MS. 2004. Factors contributing to the outcome of oxidative damage to nucleic acids. *Bioessays* 265:533–542.

Evans MD, Singh R, Mistry V, Sandhu K, Farmer PB, Cooke MS. 2008. Analysis of urinary 8-oxo-7,8-dihydro-purine-2′-deoxyribonucleosides by LC-MS/MS and improved ELISA. *Free Radic Res* 4210:831–840.

Gackowski D, Rozalski R, Roszkowski K, Jawien A, Foksinski M, 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 Radic Res* 356:825–832.

Gedik CM, Collins A. 2005. Establishing the background level of base oxidation in human lymphocyte DNA: results of an interlaboratory validation study. *Faseb J* 191:82–84.

Guetens G, De Boeck G, Highley M, van Oosterom AT, de Bruijn EA. 2002. Oxidative DNA damage: biological significance and methods of analysis. *Crit Rev Clin Lab Sci* 394-5:331–457.

Harri M, Kasai H, Mori T, Tornaeus J, Savela K, Peltonen K. 2007. Analysis of 8-hydroxy-2'-deoxyguanosine in urine using high-performance liquid chromatography-electrospray tandem mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci* 8531-2:242–246.

Hu CW, Wang CJ, Chang LW, Chao MR. 2006. Clinical-scale high-throughput analysis of urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine by isotope-dilution liquid chromatography–tandem mass spectrometry with on-line solid-phase extraction. *Clin Chem* 527:1381–1388.

Jarosinska D, Barregård L, Biesiada M, Muszynska-Graca M, Dabkowska B, Denby B, Pacyna J, Fudala J, Zielonka U. 2006. Urinary mercury in adults in Poland living near a chloralkali plant. *Sci Total Environ* 3681:335–343.

Kasai H. 1997. Analysis of a form of oxidative DNA damage, 8-hydroxy-2’-deoxyguanosine, as a marker of cellular oxidative stress during carcinogenesis. *Mutat Res* 3873:147–163.

Kasai H. 2003. A new automated method to analyze urinary 8-hydroxydeoxyguanosine by a high-performance liquid chromatography-electrochemical detector system. *J Radiat Res Tokyo* 442:185–189.

Leinonen J, Lehtimäki T, Toyokuni S, Okada K, Tanaka T, Hiai H, Ochi H, Laippala P, Rantalaiho V, Wirta O, Pasternack A, Alho H. 1997. New biomarker evidence of oxidative DNA damage in patients with non-insulin-dependent diabetes mellitus. *FEBS Lett* 4171:150–152.

Lin HS, Jenner AM, Ong CN, Huang SH, Whiteman M, Halliwell B. 2004. 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. *Biochem J* 380:541–548.

Loft S, Deng XS, Tuo J, Wellejus A, Sørensen M, Poulsen HE. 1998. Experimental study of oxidative DNA damage. *Free Radic Res* 296:525–539.

Loft S, Svoboda P, Kasai H, Tjønneland A, Vogel U, Møller P, Overvad K, Raaschou-Nielsen O. 2006. Prospective study of 8-oxo-7,8-dihydro-2’-deoxyguanosine excretion and the risk of lung cancer. *Carcinogenesis* 276:1245–1250.

Malayappan B, Garrett TJ, Segal M, Leeuwenburgh C. 2007. Urinary analysis of 8-oxoguanine, 8-oxoguanosine, fapy-guanine and 8-oxo-2’-deoxyguanosine by high-performance liquid chromatography-electrospray tandem mass spectrometry as a measure of oxidative stress. *J Chromatogr A* 11671:54–62.

Olinski R, Gackowski D, Foksinski M, Rozalski R, Roszkowski K, Jaruga P. 2002. Oxidative DNA damage: assessment of the role in carcinogenesis, atherosclerosis, and acquired immunodeficiency syndrome. *Free Radic Biol Med* 332:192–200.

Pang B, Zhou X, Yu H, Dong M, Taghizadeh K, Wishnok JS, Tannenbaum SR, Dedon PC. 2007. Lipid peroxidation dominates the chemistry of DNA adduct formation in a mouse model of inflammation. *Carcinogenesis 288*:1807–1813.

Prieme H, Loft S, Cutler RG, Poulsen HE. 1996. Measurement of oxidative DNA injury in humans: evaluation of a commercially available ELISA assay. In: Kumpulainen JT, Salonen JT, eds. *Natural antioxidants and food quality in atherosclerosis and cancer prevention.* London: The Royal Society of Chemistry. p. 78–82.

Priemé H, Loft S, Klarlund M, Grønbaek K, Tønnesen P, Poulsen HE. 1998. Effect of smoking cessation on oxidative DNA modification estimated by 8-oxo-7,8-dihydro-2’-deoxyguanosine excretion. *Carcinogenesis* 192:347–351.

Ravanat JL, Guicherd P, Tuce Z, Cadet J. 1999. Simultaneous determination of five oxidative DNA lesions in human urine. *Chem Res Toxicol* 129:802–808.

Rossner P Jr, Svecova V, Milcova A, Lnenickova Z, Solansky I, Santella RM, Sram RJ. 2007. Oxidative and nitrosative stress markers in bus drivers. *Mutat Res* 6171-2:23–32.

Shimoi K, Kasai H, Yokota N, Toyokuni S, 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* 118:767–770.

Svoboda P, Kasai H. 2004. Simultaneous HPLC analysis of 8-hydroxydeoxyguanosine and 7-methylguanine in urine from humans and rodents. *Anal Biochem* 3342:239–250.

Thompson HJ, Heimendinger J, Haegele A, Sedlacek SM, Gillette C, O’Neill C, Wolfe P, Conry C. 1999. Effect of increased vegetable and fruit consumption on markers of oxidative cellular damage. *Carcinogenesis* 2012:2261–2266.

Weimann A, Belling D, Poulsen HE. 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* 302:E7.

Weimann A, Riis B, Poulsen HE. 2004. Oligonucleotides in human urine do not contain 8-oxo-7,8-dihydrodeoxyguanosine. *Free Radic Biol Med* 3611:1378–1382.

Yoshida R, Ogawa Y, 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* 1110:1076–1081.