Toward consensus in the analysis of urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine as a noninvasive biomarker of oxidative stress

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ABSTRACT Of the DNA-derived biomarkers of oxidative stress, urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) is the most frequently measured. However, there is significant discrepancy between chromatographic and immunoassay approaches, and intratechnique agreement among all available chromatography-based assays and ELISAs is yet to be established. This is a significant obstacle to their use in large molecular epidemiological studies. To evaluate the accuracy of intra/intertechnique and interlaboratory measurements, samples of phosphate buffered saline and urine, spiked with different concentrations of 8-oxoG, together with a series of urine samples from healthy individuals were distributed to ESCULA members. All laboratories received identical samples, including 2 negative controls that contained no added 8-oxodG. Data were returned from 17 laboratories, representing 20 methods, broadly classified as mass spectrometric (MS), electrochemical detection (EC), or enzyme-linked immunoassorbant assay (ELISA). Overall, there was good within-technique agreement, with the majority of laboratories’ results lying within 1 SD of their consensus mean. However, ELISA showed more within-technique variation than did the chromatographic techniques and, for the urine samples, reported higher values. Bland-Altman plots revealed good agreement between MS and EC methods but concentration-dependent deviation for ELISA. All methods ranked urine samples according to concentration similarly. Creatinine levels are routinely used as a correction factor for urine concentration, and therefore we also conducted an interlaboratory comparison of methods for urinary creatinine determination, with the majority of laboratories’ results lying within 1 SD of the consensus value, irrespective of the analysis procedure. This study reveals greater consensus than previously expected, although concern remains over ELISA.—ESCUA [European Standards Committee on Urinary (DNA) Lesion Analysis], Evans, M. D., Olinski, R., Loft, S., Cooke, M. S. Toward consensus in the analysis of urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine as a noninvasive biomarker of oxidative stress. FASEB J. 24, 1249–1260 (2010). www.fasebj.org

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A significant component of endogenously and exogenously generated genotoxic insult involves oxidative processes, leading to the generation of nucleic acid oxidation products (1, 2). Furthermore, oxidative stress, including oxidatively generated modification of nucleic acids, is reported to be an important factor in many globally significant pathogenic conditions, including cancer, neurodegenerative diseases, diabetes, cardiovascular disease, chronic inflammatory diseases, and aging (3, 4). Noninvasive markers of oxidative stress-induced damage to the genome, such as those measurable in urine, have significant scope in terms of application to wide-scale, population-based studies, e.g., molecular epidemiological investigations (5). The sample throughput capacity of such studies would be significantly enhanced if multiple laboratories could be engaged in analysis of samples and the data combined. However, a prerequisite to such coordinated analysis is the establishment of robustly validated analytical procedures, where data can be reliably compared and amalgamated among laboratories. In addition, if such studies span multiple populations, reference ranges need to be established, in order to have application in a clinical context.

Measurement of urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) has received considerable attention as a biomarker of oxidatively generated damage to the genome (6). Not only is measurement of this lesion feasible via a noninvasive route, but it is also remarkably stable in this matrix...
(7), and multiple methodologies exist for its measurement (8). Although there have been some data to suggest that diet may contribute to urinary levels of thymine glycol and 8-oxo-7,8-dihydro-guanine (8-oxoGuA) (9–11), there has been no evidence to suggest that this applies to urinary levels of oxidatively modified 2’-deoxyribonucleosides (9, 12). Indeed, more recent data suggest that both urinary 8-oxodG and 8-oxoGuA levels are unaffected by diet (13, 14), removing this issue as a possible confounder. This matter is considered in more detail elsewhere (15).

However, discrepancies in the basal levels of urinary 8-oxodG have been noted when comparing chromatographic techniques (e.g., gas chromatography-mass spectrometry (GC-MS) following prior HPLC prepurification, or high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), or HPLC-electrochemical detection (HPLC-EC)) with ELISA, although all techniques have been shown to discriminate between healthy and nonhealthy subjects and possess good within-technique agreement [reviewed in Cooke et al. (6)]. Although ELISA has received widespread use, and is clearly accessible to the greatest number of laboratories, the discrepancy with chromatographic techniques continues to raise questions regarding its utility. Understanding the basis of this discrepancy will therefore aid our ability to address this, with a view to improving ELISA measurements. Furthermore, there is also growing clinical interest in the measurement of urinary 8-oxodG as a means to determine the role of oxidative stress in disease and evaluate intervention strategies (16–18); therefore the need for robust analytical procedures is paramount. In this context it is important to emphasize that the magnitude of change in urinary excretion may be rather small. For example, the effect of smoking is an increase of ∼15–50%, and the effect of cruciferous vegetable is a reduction of ∼30% (19–21). Therefore, the demand for specificity and agreement between laboratories and methods is high.

Discrepancies between different laboratories and techniques in assessment of the levels of 8-oxodG measured in intracellular DNA led to the formation of the European Standards Committee on Oxidative DNA Damage (ESCODD). Via large-scale interlaboratory validation exercises, ESCODD was able to identify and address several of the problems associated with the analysis of 8-oxodG in DNA (22, 23). To date, comparison of methods for the analysis of urinary 8-oxodG has been performed only in a limited number of small-scale intra- and interlaboratory studies, involving a few methods and laboratories (24–30). Following the precedent of ESCODD, and with financial support from Environmental Cancer Risk, Nutrition and Individual Susceptibility (ECNIS), a Network of Excellence operating within the European Union 6th Framework Program, the European Standards Committee for Urinary (DNA) Lesion Analysis (ESCUA; http://www.escula.org) was established to address issues associated with the analysis of urinary DNA biomarkers of oxidative stress. Because of its relatively widespread use, 8-oxodG was chosen as the first analytical target. Extension to a broader spectrum of nucleic acid derived-lesions, including nonoxidized lesions and other biomarkers derived from lipids and proteins, is planned. As a summary, the immediate and longer-term objectives of ESCULA include determining reference ranges, addressing intra- and interindividual variability, assessing sample collection procedures and correction factor issues, achieving a better understanding of the sources of DNA lesions in urine, and examining how levels of other urinary lesions compare to urinary 8-oxodG levels. Initially consisting of just 3 laboratories (30), ESCULA has expanded to include more than 26 participants, the majority based in Europe but also with participants from the United States and Asia. Most of the ESCULA laboratories contributed to the data in this article, which describes the first large-scale, multiassay, interlaboratory validation exercise examining the measurement of urinary 8-oxodG.

**MATERIALS AND METHODS**

**Test materials**

Samples were distributed to the participating laboratories by courier on dry ice from the University of Leicester, and acknowledgment of receipt was given. A concentrated stock of 8-oxodG (Sigma Chemical Co., Poole, UK) was prepared by dissolving the entire 5-mg content, as received, in 5 ml ultrapure water. Sequential 1:10 dilutions of this stock were used to make solutions to prepare the test materials described below. The test samples consisted of the following: 1) PBS samples, to simulate a physiological matrix, with 8-oxodG spiked to a final concentration of 0 (negative control), 0.5, 2, 8, 20, 80, and 200 ng/ml, labeled A to G; 2) urine samples (spot urine derived from one healthy individual) with 8-oxodG spiked to an added concentration of 0 (negative control), 0.5, 2, 8, 20, 80, and 200 ng/ml in addition to the baseline 8-oxodG level, labeled H to N; and 3) aliquots of 1 ml spot urine (first void, midstream) from 9 healthy adults, labeled O to W.

It was evident to the participating laboratories which samples were urine and which were PBS, but no indication was given as to the 8-oxodG concentration for those spiked with standard. The code for the samples was unknown to the participants until the analysis was complete; this included the sample distribution laboratory, in which sample preparation and analysis was performed by different people.

**Analytical procedures for 8-oxodG measurement**

Each laboratory used its own method for urinary 8-oxodG determinations, including sample preparation, even if the ultimate detection and/or separation technique was superficially similar. The salient features of the methodology
used by each laboratory are presented in Tables 1–3, along with references for additional information.

Creatinine determination

Many laboratories involved in the measurement of urinary 8-oxodG, including most of those involved in ESCULA, determine urinary creatinine concentration as a correction factor for urine concentration (28, 31). Of these, the majority use the Jaffe alkaline picrate method (32) or some variant thereof, either using an autoanalyzer or performing manual absorbance readings of the creatinine-picrate complex on a spectrophotometer. Three other approaches were used: direct detection of creatinine using HPLC-UV; Benedict/Behre chemistry (reaction of creatinine with 3,5-dinitrobenzene) on an autoanalyzer; and multistep enzymatic degradation of creatinine, liberating hydrogen peroxide, subsequently detected via a colored complex measured on an autoanalyzer (the exact identity of the detection reagent is dependent on the nature of the assay used).

Statistical analysis

All data were plotted and analyzed using Prism v5.0 (GraphPad Software Inc., La Jolla, CA, USA). Statistical comparisons were made using 1- and 2-way ANOVA with a Tukey post hoc test and a significance level of 0.05. Bland-Altman plots were also prepared to compare agreement between the technique groups for each sample type.

RESULTS

For this interlaboratory analytical exercise, 20 of the 25 ESCULA participating laboratories agreed to receive samples, and 17 laboratories returned results, consisting of 20 sets of data (3 laboratories providing data sets derived from 2 techniques).

Analysis of 8-oxodG standard added to PBS

Figure 1 (MS, HPLC-EC, and ELISA) shows the data for 8-oxodG standards dissolved in PBS (samples A–G) or urine (samples H–N), expressed as ng/ml 8-oxodG. Samples A and H contain no exogenously added 8-oxodG. Also shown on each plot, for samples B–G, are “target values” (known concentration of 8-oxodG in the sample). For samples A–N, we present an indication of the central tendency of the data, or “consensus value” (mean value for the level of 8-oxodG derived from individual laboratory measurements), which is included for completeness and used in statistical comparisons; the accompanying error bars indicate sd (where these values are >0).

Each technique group detected successive increases in 8-oxodG, albeit with a signal also detectable in PBS alone for some of the MS and ELISA methods (Fig. 1A, C). For the majority of the standards in PBS, most of the laboratories underestimated the level of 8-oxodG, compared to the calculated target value (Fig. 1). Statistically, the consensus mean values for the 80 ng/ml standard in PBS for the MS and EC methods (P<0.05) and 200 ng/ml in PBS for all 3 techniques (P<0.01) were significantly lower than the target value. Laboratory 7, however, very poorly differentiated the level of 8-oxodG between samples, and values from this laboratory were not included in calculating the consensus values shown in Fig. 1B.

Analysis of 8-oxodG standard added to urine

For the urine spiked with increasing concentrations of 8-oxodG standard (samples H–N) there was no significant difference among the techniques in terms of the consensus value obtained for each sample. However, it was noted that the dose-dependence trend was markedly suppressed for each technique, compared to standards in PBS. Here, 8 ng/ml 8-oxodG (sample K) and higher was reached for the chromatographic techniques. For ELISA, however, the dose response was not evident until 20 ng/ml 8-oxodG. The consensus value should be considered cautiously, particularly if reviewing the relative proximity of each laboratory’s determination to this value in the absence of a known target value. It is possible that a value from one center, considered an outlier relative to the consensus value, is actually closer to the real level of 8-oxodG in that sample than the consensus mean. However, it does illustrate the central tendency of the data. On the whole, the individual determinations of 8-oxodG, either in PBS or in spiked urine, for most of the laboratories were within 1 sd of the consensus mean, although the majority of values for laboratory 18 (samples H–N) tend to be toward to lower limit of, or below, 1 sd from the consensus mean.

Determination of urinary 8-oxodG

Figure 2 (MS, EC, and ELISA) shows the individual laboratory values and consensus mean values for 8-oxodG measured in each of the 9 urine samples from healthy subjects supplied to each laboratory. The data are all ranked in order of the mean consensus values for each technique. However, laboratory 14 (GC-MS) generated values above the upper limit of 1 sd above the consensus mean for 8 of 9 urine samples. Also of note is laboratory 10 (ELISA), which generated values outside the upper limit of 1 sd of the consensus mean for 5 of 9 urine samples. Values for laboratory 7 were included in this part of the analysis, as the problems evident in Fig. 1B no longer seemed to be apparent.

The consensus values for each urine sample were generally not statistically different among the techniques; however, this was not always the case. When excluding laboratory 14 (GC-MS) from the analysis, the ELISA consensus value was significantly higher (P<0.05) than the MS value for samples O, Q, R, and V. For sample U, the ELISA consensus value was significantly higher (P<0.01) than either EC or MS, irrespective of the inclusion of the data for laboratory...
14. Despite there being little difference in the consensus values between each technique, apart from those samples noted above, the sd values for the ELISA methods were, on the whole, notably greater than those for either of the chromatographic techniques. This was reiterated by the significant difference ($P < 0.05$) between the mean coefficient of variation for ELISA methods (62%), for the combined analysis of the 9 urine samples, compared to those for MS methods (minus GC-MS data; 29%), but not significantly different from that of EC methods (47%). Similarly, the residual variation was significantly lower for the EC methods than for ELISA methods, and significantly lower for the MS methods (excluding laboratory 14) than EC methods, when comparing results of ANOVA normalized values for each method, with inclusion of both sample alone and sample and laboratory as explanatory factors.

**Agreement of measurements between each technique**

The greatest agreement in terms of rank order was between MS and EC (Fig. 2A, B), with 5 of 9 samples agreeing. However, this was only when the GC-MS data were excluded (Fig. 2A). The rank of 3 samples agreed perfectly between EC and ELISA (Fig. 2B, C), but 5 agreed perfectly between MS and ELISA, although only when the GC-MS data were excluded (Fig. 2A, C). However, for all 3 methods, the consensus concentration ranks were within 1 or 2 rank places, except for sample V, for which the MS rank was 3 places from the EC and ELISA methods. This is attributed to the results from the GC-MS, as this problem is removed on exclusion of this laboratory’s data (Fig. 2A).

**TABLE 1. Methodology details for mass-spectrometry-based analysis**

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Sample vol.</th>
<th>Sample enrichment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (HPLC-MS/MS)</td>
<td>500 µl (0.5 µl injected)</td>
<td>SPE with Isolute® Env+ SPE (Kinesis, St. Neots, UK); eluate evaporated under vacuum and reconstituted in 50 µl mobile phase</td>
</tr>
<tr>
<td>4 (HPLC-MS/MS)</td>
<td>50 µl (5 µl injected)</td>
<td>None</td>
</tr>
<tr>
<td>4 (LC-GC/MS)</td>
<td>750 µl (500 µl injected)</td>
<td>LC-prepurification followed by freeze-drying of collected fractions, hydrolysis in 60% formic acid, and derivatization with BSTFA</td>
</tr>
<tr>
<td>9 (UPLC-MS/MS)</td>
<td>40 µl</td>
<td>Sample diluted with 0.1 M LiAc, pH 6.4 (40:60, v/v)</td>
</tr>
<tr>
<td>11 (HPLC-MS/MS)</td>
<td>120 µl (20 µl injected)</td>
<td>Online SPE with ODS-3 column, 5 µm, 4.6 × 33 mm (Inertsil; GL Sciences Inc., Tokyo, Japan)</td>
</tr>
<tr>
<td>14 (GC-MS)</td>
<td>800 µl</td>
<td>Sample diluted 1:10 with formic acid buffer (10% pH 2.75), SPE Oasis HLB columns (Waters); freeze-dried extract derivatized with BSTFA+1% TMCS</td>
</tr>
<tr>
<td>15 (HPLC-MS/MS)</td>
<td>20 µl</td>
<td>Online SPE with ODS-3 C18 column, 5 µm, 4.6 × 50 mm (Inertsil)</td>
</tr>
<tr>
<td>16 (HPLC-MS/MS)</td>
<td>100 µl (10 µl injected)</td>
<td>None</td>
</tr>
<tr>
<td>18 (HPLC-MS/MS)</td>
<td>50 µl (10 µl injected)</td>
<td>SPE (Oasis HLB)</td>
</tr>
</tbody>
</table>

All techniques LC-MS/MS, unless indicated otherwise. Literature references for methodology are provided, where available. NH₄Ac, ammonium acetate; SPE, solid phase extraction.

14. Bland-Altman plots are shown in Fig. 3 (comparisons between MS, EC, and ELISA for 8-oxodG standards in PBS), Fig. 4 (comparisons between MS, EC, and ELISA for 8-oxodG spiked into urine), and Fig. 5 (comparisons between MS, EC, and ELISA for healthy adult urine samples). Each figure consists of 3 panels related to the techniques being compared: A) MS vs. EC, B) MS vs. ELISA, and C) EC vs. ELISA. The plots represent the difference between paired measurements (consensus mean for each technique) plotted against the corresponding mean of the individual consensus means for each technique. The
comparisons between MS and EC show good agreement (with a narrow range for the 2 sd) for all standards, irrespective of whether added to PBS or urine (Figs. 3A and 4A). There is also reasonable agreement between MS and EC, when compared to ELISA, for standards spiked into PBS (Fig. 3B, C). However, this level of agreement worsens further for the standards spiked into urine, resulting in a wide range for the 2 sd (Fig. 4B, C), derived from the tendency for ELISA to report higher levels than chromatographic methods. This is demonstrated clearly in Fig. 4B, C, where the difference is close to or higher than the mean value for most samples. At the highest concentration of 8-oxodG spiked into urine, notable disagreement between the levels was detected by the MS/EC and ELISA methods (Fig. 4B, C).

Although based on a limited sample number of 9 urine samples, the agreement between the chromatographic and ELISA techniques becomes poorer as the mean level of urinary 8-oxodG becomes higher (Fig. 5B, C), again with the trend toward higher levels measured by ELISA.

### Creatinine analysis

Agreement between the ranked urinary creatinine determinations for each of those laboratories that measure this parameter is shown in Fig. 6. The majority of the values lie within 1 sd of the consensus mean, with no consistent exceptions, other than laboratory 4, with 5 of 9 samples outside 1 sd, but not consistently above or below.

### DISCUSSION

This is the first report of a major interlaboratory validation exercise aimed at assessing agreement between multiple different laboratories measuring urinary 8-oxodG using individual methods. In addition to assessing agreement between individual laboratories, it was hoped that some indication of possible reasons for any observed significant differences between laboratories and assay formats might be found. By using this information, it is hoped that steps toward harmonization between different laboratories, as well as between
techniques, may be achieved. Furthermore, it may be possible to make recommendations to some laboratories, and the research field, to address issues that are perceived as in need of modification. It should be noted that this is an initial step toward validation, with sample origin restricted to normal, healthy volunteers, and that further testing and comparison of situations with diseases and interventions will follow.

In terms of the mass spectrometric techniques, it is worth noting that, for most of the urine samples, GC-MS gave relatively high levels of 8-oxodG, distinct from LC-GC-MS. The GC-MS technique has been the subject of much discussion in ESCODD, with the conclusions that GC-MS alone, without prior sample cleanup, was not as favorable for the analysis of 8-oxodG in DNA. The issues may originate from the derivatization, at high temperature, of DNA hydrolysates, leading to artifactual oxidation of guanine. However, experiments performed by laboratory 14 exclude the possibility of artifactual formation of 8-oxodG during derivatization of urine samples (note added in proof; ref. 33 and unpublished results). It seems that isolation of 8-oxodG from urine by HPLC prior to derivatization (13) as carried out by laboratory 4 eliminated the oxidation of 2'-deoxyguanosine (dG); therefore, the results generated by GC/MS are comparable to the results of LC-MS/MS. In general, LC-MS/MS methods generate consistent results among the laboratories. However, laboratory 18 generated 8-oxodG values at the lower end of the range of values for LC-MS/MS analysis, for both the spiked urine samples and the 9 urine samples. The reasons for this are not clear, particularly as this technique uses solid-phase clean-up prior to the chromatography, which, with the use of an internal standard, should compensate for sample losses.

The much lower variation between different versions of the 2 types of chromatographic techniques, irrespective of whether MS or EC is used for detection, compared to ELISA, most likely arises from the use of 1) robust internal standardization in the MS procedures using mass labeled standards with negligible differences in chemical and physical properties compared to the target compound and 2) chromatographic separation and greater assurance of the identity of the compound being analyzed. The ELISA procedures would appear inherently more subject to variability in sample composition and thus potential interference, with an added component of interlaboratory variation possibly arising from the use of different primary antibodies, although only one laboratory used an antibody other than N45.1 (Table 3). Another significant limitation is the lack of linear response in the ELISA calibration curves with increasing concentration of 8-oxodG. Furthermore, internal standardization is not possible for ELISA. Interlaboratory within-technique agreement may be improved with the use of standards obtained from a common supplier, prepared in an identical manner (e.g., preparation and distribution from one center), and robustly quantified, for example, using a molar absorption coefficient.

There have been several reports in the litera-

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Sample vol.</th>
<th>Sample enrichment</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>I) Bondelut CH column; 2) HPLC system 1; fraction containing 8-oxodG collected</td>
</tr>
<tr>
<td>5</td>
<td>50 µl</td>
<td>Urine sample diluted 1:1 with 2× MP1; HPLC system 1: fraction containing 8-oxodG collected (C1, MP1); HPLC system 2: fraction containing 8-oxodG collected (C2, MP2 and MP3)</td>
</tr>
<tr>
<td>7</td>
<td>500 µl (50 µl injected)</td>
<td>SPE: bond elute C18(OH) (3 ml), bond elute strong cation exchange column (3 ml)</td>
</tr>
<tr>
<td>13</td>
<td>50 µl (10 µl injected)</td>
<td>SPE: anion exchange column</td>
</tr>
</tbody>
</table>

Literature references for methodology are provided, where available. MP, mobile phase; C1, column 1; C2, column 2; C3, column 3.
ture highlighting the differences between chromatographic and immunochemical determinations of urinary 8-oxodG, with the latter yielding values 4–10 times the level measured by chromatographic procedures (25, 26). The reasons for this discrepancy have largely been attributed to a lack of specificity of the primary antibody (6). In this study, the Bland-Altman plots, along with ranked data, suggest good agreement between MS and EC, based on consensus mean values. The agreement between these assays and ELISA is notably weaker. At the highest level of exogenous 8-oxodG added to urine, the methods disagree significantly with each other. However, this level of urinary 8-oxodG is not biologically relevant and may be related to variability at the high end of the ELISA standard curve.

For the unadulterated urine samples, ELISA and the chromatographic techniques show less agreement when the mean level of 8-oxodG in urine is relatively high ($\geq 10$ ng/ml). The apparent agreement between the chromatographic and ELISA methods is offset by the greater variability of the latter, which would also increase the number of persons recruited to a study, as a result of how $sd$ is involved in power calculations. This could have implications for the sensitivity of ELISA and

<table>
<thead>
<tr>
<th>Laboratory</th>
<th>Sample vol.</th>
<th>Calibration (range)</th>
<th>Primary Ab and origin</th>
<th>Primary Ab incubation conditions</th>
<th>Assay source</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>95 $\mu$l</td>
<td>0.1–10 ng/ml</td>
<td>Clone N45.1 ([JaICA])</td>
<td>Overnight, 4°C</td>
<td>In house, sample enrichment included</td>
<td>44</td>
</tr>
<tr>
<td>8</td>
<td>50 $\mu$l</td>
<td>1.25–40 ng/ml</td>
<td>Clone N45.1</td>
<td>1.5 h, room temperature</td>
<td>In house</td>
<td>45</td>
</tr>
<tr>
<td>10</td>
<td>25 $\mu$l</td>
<td>2.5–80 ng/ml</td>
<td>Clone 1F7</td>
<td>1.5 h, 37°C</td>
<td>In house</td>
<td>45</td>
</tr>
<tr>
<td>12 (ELISA 1)</td>
<td>50 $\mu$l</td>
<td>0.5–200 ng/ml</td>
<td>Clone N45.1</td>
<td>1 h, 37°C</td>
<td>New 8-OHDg Check ELISA ([JaICA])</td>
<td>46</td>
</tr>
<tr>
<td>12 (ELISA 2)</td>
<td>50 $\mu$l</td>
<td>0.125–10 ng/ml</td>
<td>Clone N45.1</td>
<td>Overnight, 4°C</td>
<td>Highly sensitive 8-OHDg Check ELISA ([JaICA])</td>
<td>47</td>
</tr>
<tr>
<td>19</td>
<td>50 $\mu$l</td>
<td>0.5–200 ng/ml</td>
<td>Clone N45.1</td>
<td>1 h, 37°C</td>
<td>Bioxtech 8-OHDg-EIA kit (Oxis Health Products Inc., Portland, OR, USA)</td>
<td>46, 47</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analytical conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration</td>
</tr>
<tr>
<td>External standard; 8-oxodG (1–100 ng/ml); no correction for loss of material during sample preparation</td>
</tr>
<tr>
<td>External standard; 8-oxodG (1–40 ng/ml); good correlation up to 200 ng/ml</td>
</tr>
<tr>
<td>External standard; 0.5–8 ng/ml, 4-point calibration curve; 2 ng/ml, 1-point calibration</td>
</tr>
<tr>
<td>5 ng/ml, 1-point calibration</td>
</tr>
</tbody>
</table>

TABLE 3. Methodology details for immunoassay-based analysis

Interlaboratory analysis of urinary 8-oxodG

Literature references for methodology are provided, where available. JaICA, Japan Institute for the Control of Aging (Shizuoka, Japan).
affect the ability to discriminate between two groups of individuals (33). It is reasonable to suggest that because of variable urine composition, immunoassays could be more adversely affected, simply because these techniques do not fractionate and uniquely identify the species being analyzed. Furthermore, the present investigations are restricted to urine from healthy volunteers. It is possible that when analyzing urine from individuals with pathological conditions, the issue of antibody specificity in the ELISA could be more problematic. Indeed, agreement between HPLC-EC and ELISA has been demonstrated to improve greatly fol-

Figure 1. Analysis of PBS and urine with added exogenous 8-oxodG by mass spectrometric, electrochemical, and immunoassay procedures. A) MS detection. B) HPLC-EC detection. C) ELISA immunoassay. Symbols correspond to laboratory and technique as indicated. ELISA 1, New 8-OHdG Check ELISA [Japan Institute for the Control of Aging (JaICA), Shizuoka, Japan]; ELISA 2, Highly Sensitive 8-OHdG Check ELISA (JaICA). Samples A–G are 8-oxodG standards (0, 0.5, 2, 8, 20, 80, and 200 ng/ml in PBS); samples H–N are 8-oxodG standards (final concentrations of 0, 0.5, 2, 8, 20, 80, and 200 ng/ml in a single urine sample derived from a healthy subject). For samples A–H, solid purple squares indicate target values (known amounts of exogenously added 8-oxodG). Solid red stars and error bars indicate consensus means ± sd.

Figure 2. Ranked mean level of urinary 8-oxodG in samples from 9 healthy individuals. A) MS detection. B) HPLC-EC detection. C) ELISA immunoassay. Symbols correspond to laboratory and technique as indicated. Solid red stars and error bars indicate consensus means ± sd.
lowing HPLC or SPE prepurification of samples prior to immunoassay (25, 34), although HPLC prepurification, at least, detracts from two of the major benefits of ELISA: speed and simplicity.

Large sd values indicated that certain urine samples presented a greater problem for analysis than others (e.g., samples V and R for MS; S and W for EC; and P, R, and U for ELISA). Interestingly, this issue appears to be detection method specific. Contaminants have been reported to affect some mass spectrometric techniques, making it impossible to quantify 8-oxodG in 10–20% of samples (35), and endogenous constituents of urine, such as urea, have been reported to markedly influence quantification by ELISA (36). In many cases, the precise basis for these problems is not clear and warrants further investigation.

The creatinine determinations reported by returning
laboratories generally agreed well, which is reassuring to those laboratories that perform this assay in their research laboratory, rather than via an accredited chemical pathology service.

This is the first interlaboratory study of urinary 8-oxodG determination conducted on a significant scale involving the comparison of multiple methods and analysis of identical samples. As a consequence, we have been able to make robust conclusions concerning the detection methods used (mass spectrometric, electrochemical, and immunoassay). These findings will have far-reaching implications, not least because noninvasive biomarkers of oxidative stress are increasingly being called for in large-scale molecular epidemiology studies of human disease, and therefore robust, accurate, reproducible, and high-throughput (37) assays are essential (38). To address this need, future studies from ESCULA will investigate further inter- and intra-assay variability, and aim to establish an external quality assurance scheme for participating laboratories.

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