6-2016

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Abstract

ELISA is commonly used for the detection of urinary 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG), a marker of whole body oxidative stress. However, the method has been criticized for high inter-laboratory variability and poor agreement with chromatographic techniques. We performed an inter-laboratory comparison of 8-oxodG assessed in 30 urine samples and a urine spiked with four different concentrations of 8-oxodG by ELISA using standardized experimental conditions, including: sample pre-treatment with solid-phase extraction (SPE), performing analysis using a commercial kit from a single manufacturer and strict temperature control during the assay. We further compared the ELISA results with high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) and performed tentative identification of compounds that may contribute to the discrepancy between both methods. For all but one participating laboratory (Data 1) we observed consistent ELISA results lying mostly within 1 SD of the mean 8-oxodG concentration. Mean 8-oxodG levels assessed by ELISA correlated with the data obtained by HPLC-MS/MS ($R=0.679$, $p<0.001$). The correlation improved when Data 1 were excluded from the analysis ($R=0.749$, $p<0.001$). In the correlation plot we identified three outlying urine samples; one with an ELISA 8-oxodG concentration lower, and two with 8-oxodG levels higher, than those measured by HPLC-MS/MS. Omitting these samples further improved inter-methodology agreement ($R=0.869$, $p<0.001$). In the outliers with high 8-oxodG estimates various aromatic and heterocyclic compounds were tentatively identified using gas chromatography-mass spectrometry (GC/MS); application of authentic standards revealed the presence of saccharides, including D-glucose and D-galactose as putative interfering substances. In summary, assay standardization improved ELISA inter-laboratory agreement, although some variability is still observed. While the assay shows reasonable correlation with HPLC-MS/MS, there are still compounds in urine

that affect the anti-8-oxodG antibody binding, contributing to overestimation of 8-oxodG by ELISA in some samples. Thus, despite significant improvement, ELISA still cannot be considered a robust alternative to the chromatographic techniques.

Keywords

8-oxodG, ELISA, HPLC-MS/MS, inter-laboratory agreement, oxidative stress, standardization, urine

Abbreviations

8-oxodG, 8-oxo-7,8-dihydro-2'-deoxyguanosine; CV, coefficient of variation; GC-MS, gas chromatography-mass spectrometry; HPLC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; SPE, solid-phase extraction

Introduction

Oxidative stress, resulting from the imbalance between levels of antioxidants and oxidants in the organism, in favor of the latter, represents a major source of damage to macromolecules [1]. Although DNA oxidation causes the formation of a number of oxidized products [2], 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) is the most abundant and most commonly studied oxidized deoxynucleotide [3]. This compound may be measured in blood, saliva, urine or other biological matrices [4]. However, due to the availability of urine and high stability of 8-oxodG in this matrix [5], urinary levels of 8-oxodG have become a popular biomarker of whole body oxidative stress [6]. Although several methods of 8-oxodG detection have been developed, they may be generally categorized into chromatographic and immunochemical approaches [4,7]. Chromatographic techniques, particularly high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS), are considered the gold standard methods of urinary 8-oxodG detection. However, for many laboratories obtaining and running expensive chromatographic instrumentation is not feasible. Therefore, antibody-based techniques (mostly competitive ELISA) are widely used despite their potential shortcomings stemming from non-specific interactions of antibodies with compounds in the urine [3].

The complex nature of urine, which consists of many chemicals including inorganic salts, urea, various organic compounds and organic ammonium salts [8], is main challenge to urinary analyzes of 8-oxodG. These compounds may interfere with analytical procedures, particularly with antibody-based methods. To overcome this potential source of analytical bias, solid-phase extraction (SPE)-based protocols are sometimes used for sample pre-treatment [9–11]. It has been shown that the SPE pre-treament significantly improves the quality of ELISA data [9,11]. Over the years, several comparisons of 8-oxodG levels analyzed by chromatography and ELISA have been made [7,11–20]. The

agreement between the methods ranged from no correlation (r=0.14, p=0.95) [15] to almost perfect agreement (r=0.98, p<0.001) [17]. However, despite reasonable correlations observed in some studies, most of the authors reported several-fold higher 8-oxodG concentrations estimated by ELISA than those measured by chromatography.

A recent inter-laboratory and between-technique comparison of urinary 8-oxodG analyzes organized by the European Standards Committee on Urinary (DNA) Lesion Analysis (ESCULA) revealed that not only there is poor agreement between the data obtained by ELISA and chromatography, but also high inter-laboratory variability for those study participants that used ELISA [7,20]. The studies concluded that ELISA variability is greater than the variability of chromatographic methods and that ELISA cannot be used to determine absolute levels of 8-oxodG. Moreover, high inter-laboratory ELISA variability currently prevents the method from being used for pool data from multiple labs for subsequent analysis.

In the present study we took advantage of ELISA improvements described previously [11,16] to conduct the inter-laboratory comparison of 8-oxodG analysis in 30 urine samples and a urine spiked with four concentrations of 8-oxodG. The main aim of our work was to verify whether application of a common ELISA protocol would help to decrease the inter-laboratory variability so that it is comparable with that observed for chromatographic techniques. We obtained data from five participating laboratories. All participants used a commercial ELISA kit from a single manufacturer and adhered strictly to the common protocol that included the pre-treatment of urine using SPE and incubation of the purified samples with the primary antibody at 4 °C. We further compared the obtained data with HPLC-MS/MS analysis and identified samples for which there was a greater disagreement between the methods. In these samples we aimed to identify compounds that might contribute to the discrepancy.

Materials and Methods

Samples collection

Spot urine samples were obtained from 30 non-smoking pregnant women (aged 21-38 y) in Hospital Ceske Budejovice, Czech Republic. These subjects participated in another study focused on the effect of environmental pollution on the health of mothers and their newborns. All subjects gave signed informed consent and could cancel their participation at anytime. The study was approved by the Ethical Committee of the Institute of Experimental Medicine in Prague.

Urine samples were frozen at -20 °C immediately after collection and stored at -80 °C after delivery to the Institute of Experiment Medicine. Before analysis in individual laboratories, the samples were thawed and aliquoted. A urine sample obtained from a healthy male individual (age 43) was spiked with 8-oxodG to reach the final concentration of the compound of 0.25; 0.5; 1.0 and 2.5 ng/mL. The samples were coded; the code was not known to persons performing the assay. The samples were then shipped on dry ice to the study participants.

Pre-treatment of the samples and ELISA

Before analysis, the samples were thawed and centrifuged for 10 min at 13,000 g. The supernatants were used for further analysis. Solid-phase extraction was performed as previously described [10] with modifications introduced in [11]. For SPE, 250 µL of urine was diluted to 1 mL with HPLC water. After purification and drying under a stream of nitrogen, the samples were reconstituted in 250 µL PBS, prior to addittion to the ELISA plate.

For ELISA, the Highly Sensitive 8-OHdG Check kit (JaICA, Shizuoka, Japan) was used. The samples were analyzed in triplicate, 50 µL sample/well, according to the

manufacturer's instructions. The instructions included omitting the outer plate wells and incubation of the samples with the primary antibody at 4 °C overnight. The results were expressed in ng/mL, but for further analyzes they were normalized per creatinine content (nmol 8-oxodG/mmol creatinine).

HPLC-MS/MS analysis

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Creatinine assay

The analysis of creatinine was performed by the Jaffe picric acid method [21] individually in each participating laboratory. The inter-laboratory variability was very low; coefficient of variation (CV) was around 5%. The samples were analyzed in duplicate; creatinine concentrations were expressed in mmol/L.

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Profiling organic compounds in selected urine samples using GC/MS and HPLC-MS/MS

The preparation of samples was identical as for ELISA, with the exception of the solvent used to dissolve the SPE eluate (methanol instead of PBS). For the analysis, 1 µL of the eluate was used; for the identification of saccharides, 50 µL of the eluate was transformed to trimethylsilyl derivatives before the analysis. GC separation was performed on a fused silica SLB-5ms capillary column (30 m x 0.20 mm x 0.20 µm; Sigma-Aldrich, St. Louis, MO, USA) with helium as the carrier gas. A Saturn 2100T ion trap mass spectrometer (Varian, Walnut Creek, CA, USA), which operated in electron ionization and full scan mode at an electron ionization energy of 70 eV, was used for the detection of the unknown substances in urine samples. Tentative identification of unknown selected compounds was performed by comparing of their mass spectra with the spectra of the NIST (The National Institute of Standards and Technology) library.

The same samples (1 µL of the eluate dissolved in methanol) were also analyzed by HPLC-MS/MS a TripleQuad 6410 triple quadrupole mass spectrometer (Agilent, Santa Clara, CA, USA) equipped with an electrospray ion source (ESI), an Agilent 1200 Binary Pump System with an autosampler and a MassHunter software system. The ionization of the analytes was performed in the positive ion mode and full scan detection. The separation of compounds was achieved on a reverse-phase mode using a Supelcosil LC-PAH HPLC column (150 mm x 3 mm, 5 µm; Supelco, Bellefonte, PA, USA).

Statistical analysis

First, inter-laboratory variability of 8-oxodG concentrations determined by ELISA was tested using ANOVA; the differences between individual laboratories were analyzed using Tukey *post-hoc* test. Then, mean ELISA 8-oxodG values and SD were calculated and compared with 8-oxodG concentrations from the HPLC-MS/MS analysis by paired *t*-tests. Agreement between the participating laboratories and between techniques was assessed by Pearson correlations and Bland-Altman plots. All calculations were performed using SPSS IBM 20 (Chicago, IL, USA).

Results

The samples were analyzed in five laboratories (Lab 1-Lab 5) from which six sets of ELISA data (Data 1-Data 6) and one set of HPLC-MS/MS results were obtained. In one laboratory (Lab 5), the ELISA of the urine samples, but not the samples spiked with 8-oxodG, was performed by two persons independent of each other thus generating to sets of data (Data 5 and Data 6). The laboratory responsible for HPLC-MS/MS also conducted ELISA (Lab 4, Data 4). The ELISA results were pooled and analyzed separately for the urine sample spiked with 8-oxodG and for unspiked urine.

Analysis of 8-oxodG in the urine sample spiked with 8-oxodG

We first analyzed the ability of ELISA to distinguish between urine samples spiked with increasing concentrations of 8-oxodG. The results showed consistent data for all participating laboratories with the exception of Lab 1 (Data 1; Fig. 1A, Table 1). This laboratory detected higher 8-oxodG levels in all tested samples, the estimated concentrations laying outside the SD error bars. This resulted in CV ranging from 23.8% to 47.2% and significant differences of 8-oxodG concentrations between participants detected by ANOVA ($p=0.013$). Excluding Data 1 from the analyzes significantly decreased the inter-laboratory variability, although we still observed some samples (those analyzed by Lab 4, Data 4) exceeding the SD interval (Fig. 1B). Also the CV decreased to the range of 13.7% - 19.6% and no significant difference was found between the laboratories ($p=0.675$) (Table 1). The mean ELISA values showed the concentration trend of 8-oxodG, although it was not detected by all laboratories particularly for samples with low 8-oxodG levels. Comparison of ELISA results with HPLC-MS/MS showed that, unexpectedly, 8-oxodG concentrations measured by chromatography were about 60% higher than those detected by ELISA (Table 1). The difference between the methods was statistically significant ($p<0.001$), although there was a good correlation between both data sets ($R=0.982$, $p=0.003$ and $R=0.990$, $p=0.001$ for ELISA data both including and excluding Data 1, respectively; Fig. 2A and 2B). The level of agreement between ELISA and HPLC-MS/MS results was assessed using Bland-Altman plots (Fig. 2C and 2D). The plots show the mean value of the two measurements plotted against the difference between these values. They confirmed lower 8-oxodG estimates by ELISA but otherwise reasonable agreement between the methods. The agreement further improved after Data 1 were excluded from the analysis.

Analysis of 8-oxodG in unspiked urine samples

We further analyzed the inter-laboratory variability of 8-oxodG levels in selected 30 urine samples. The results demonstrate mostly consistent data across all samples, although we observed some outliers (Fig. 3). The greatest departure from the mean values was found for the results obtained by Lab 1, for which 50% samples lay outside the SD interval. The outliers were also found among data from other participants, particularly Lab 4, although the deviation from the mean value was less pronounced. The CV of 8-oxodG concentrations calculated for individual samples ranged from 10.6% to 53.8%, with the mean CV value of 26.6%. Excluding Data 1 from the analysis significantly decreased the variability, resulting in the CV ranging between 8.6% and 29.0% (the mean CV value of 17.6%). This approach, however, emphasized the outliers among Data 4 and changed the rank order (Fig. 4). The ELISA data obtained in individual laboratories correlated mostly well, with the R value exceeding 0.8 in all but one case ($p<0.001$; Table 2). The exception was, however, Data 1, for which the R value ranged from 0.337 to 0.752 with one non-significant result (Table 2). The ELISA results from individual laboratories significantly correlated with the HPLC-MS/MS data (R ranging from 0.606 to 0.781, $p<0.001$), again with the exception of Data 1 for which the correlation was poor and non-significant (Table 2).

The between-technique comparison identified a significant correlation between the mean ELISA and HPLC-MS/MS data ($R=0.679$, $p<0.001$; Fig. 5A). Although the correlation was significant, we observed three notable outliers: one sample with 8-oxodG concentration identified by the ELISA as being lower than that by the chromatography (sample 18) and two samples with ELISA 8-oxodG estimates higher than by HPLC-MS/MS (samples 20, 21; the three outliers circled in red in Fig. 5B). Excluding Data 1 improved the correlation ($R=0.749$, $p<0.001$; Fig. 5B), but the outliers were still present. Again, the

ELISA underestimated 8-oxodG concentrations, although the disagreement was less obvious as can be seen in Bland-Altman plots (Fig. 5B and 5D). The plots also showed the three outliers mentioned before. For all of them, the disagreement exceeded the confidence limits for bias of 2SD.

To see how the results would change if the three outliers were not present, we calculated correlations and prepared Bland-Altman plot after excluding these samples from the analysis. The correlation between the ELISA and the HPLC-MS/MS data further improved ($R=0.869$, $p<0.001$; Fig. 6A), as did the between-technique agreement. The ELISA still underestimated 8-oxodG levels (the difference analyzed by the paired t-test being significant, $p<0.001$), although the interval for confidence limits for bias was narrower (Fig. 6B).

Profiling organic compounds in the ELISA outliers

The discrepancy between the methods observed for the three outlying samples did not seem to be primarily driven by non-consistent data generated by the ELISA in individual laboratories. Although some samples lay outside the SD interval in the inter-laboratory comparison (Fig. 4), the CV was acceptable (12.9; 15.8 and 24.2%, for sample 20, 18 and 21, respectively) and the ELISA data from all laboratories were consistently higher (for samples 20, 21) or lower (for sample 18) than the chromatography results. Thus, we assumed that the lack of agreement is method-specific, probably associated with specific compounds causing cross-reactivity of the primary antibody used in ELISA. To elucidate potential differences in chemical composition of the urine, we performed GC-MS and HPLC-MS/MS analyzes of the outlying samples and compared them with the sample for which 8-oxodG concentration was comparable using both methods (sample 13, denoted further as the control sample).

The GC-MS allows detection of volatile non-polar and moderately polar compounds as well as their quantification and tentative identification. The chromatograms of the samples 20 and 21 (higher 8-oxodG concentrations by ELISA) were characterized by a number of peaks that were not present in the chromatogram of the control sample 13 (Fig. 7A, 7B, 7D). On the other hand, the number of peaks in sample 18 was lower than in the control sample 13 (Fig. 7C and 7D). Most of the compounds tentatively identified based on the chromatograms contained aromatic or heterocyclic rings (Table 3). The sum of their concentrations was greatest for sample 21 for which the highest disagreement between the methods was observed (2.33-fold difference in 8-oxodG concentration between ELISA and HPLC-MS/MS).

Detection of saccharides by the GC-MS based on the application of authentic standards revealed a similar trend with the highest concentration sum observed for sample 21. In this sample we identified almost 26-fold higher concentration of beta-D-galactose and almost 30-fold higher concentration of beta-D-glucose than in the control sample 13 (Table 4).

For profiling the organic compounds not detectable by the GC-MS we used the HPLC-MS/MS. Although this method did not allow tentative identification of the compounds in the eluates, we found different peak profiles in the individual samples. This was particularly true for sample 20 and 21 when compared with the control sample 13 In these two samples we observed extra peaks not detected in samples 13 and 18 suggesting the presence of compounds possibly interfering with 8-oxodG detection by ELISA (Fig. 8).

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Discussion

Due to the relative simplicity, speed and low cost of the laboratory equipment and chemicals, ELISA is considered an attractive alternative to the chromatographic methods

for detection of 8-oxodG in urine samples. However, there is a concern regarding the specificity of the anti-8-oxodG antibodies presently available and thus the agreement between the ELISA and chromatography data. Moreover, ELISA seems to be prone to higher inter-laboratory variability than chromatography [7,20] which currently disqualifies the method from application in large, multicenter studies and/or meta-analyzes. Several attempts have been made to improve ELISA-chromatography agreement while analyzing urinary 8-oxodG levels. They included HPLC pre-purification of urine before ELISA [13], incubation of the primary antibody with the samples at lower temperature (4 °C) [16] and application of urease to remove urea that has been shown to cross-react with the primary antibody [17]. However, to the best of our knowledge, our study is the first one to address the issue of substantial inter-laboratory variability previously observed for ELISA. Also, for the first time, the SPE pre-treament together with the standardized protocol was tested on a larger sample set with the aim to improve ELISA-chromatography agreement.

As the clone of anti-8-oxodG antibody and the ELISA format (commercial kit vs. in-house assay) have been shown to significantly impact the results [7,11], we opted for the commercial ELISA kit provided by a single manufacturer (Highly Sensitive 8-OHdG Check kit; JaICA, Shizuoka, Japan) to be used by all participating laboratories. Moreover, the urine samples were processed and the ELISA performed using a standardized protocol that has been shown to give best results when compared with the HPLC-MS/MS [11]. This included samples pre-purification by SPE previously advocated for HPLC-MS/MS application [10] and incubation of the samples with the primary antibody at 4 °C overnight, an approach tested by Evans et al. [16] and now recommended by the kit manufacturer. The results were corrected to creatinine levels as we have shown that creatinine cross-reacts with the anti-8-oxodG antibody and creatinine normalization improves between-technique correlation [11]. One of the participating laboratories further analyzed 8-oxodG

levels by HPLC-MS/MS. The data were then tested for inter-laboratory agreement and the mean ELISA 8-oxodG levels were compared with the HPLC-MS/MS data to check for between-technique variability.

Even though the standardized ELISA protocol was used, one of the participating laboratories delivered results that in many cases were not in agreement with other ELISA data and that exhibited poor correlation with both other ELISA results and HPLC-MS/MS data. Despite our efforts we were not able to identify the source of disagreement for these data. To exclude the impact of potential (although not proven) experimental issues, we presented our data both with and without the outlying ELISA results. Excluding these results helped to improve both inter-laboratory and between-technique correlation and agreement, particularly for the urine sample spiked with different 8-oxodG concentrations. For this sample we observed concentration-dependent increase of 8-oxodG and good correlation and agreement with the HPLC-MS/MS data, although the chromatography detected lower 8-oxodG levels than the ELISA. This result was unexpected as most of the previous studies comparing these techniques proved that the ELISA overestimated 8-oxodG concentrations, probably due to the non-specific interaction of the primary antibody with the urine compounds [7,12–15,17–22]. However, in another study, the ELISA underestimated 8-oxodG levels after incubation of the samples with the primary antibody at 4 °C, although this difference was not statistically significant [16].

Excluding the outlying Data 1 from the analysis of unspiked urine samples improved inter-laboratory variability, although we still observed the samples exceeding the SD interval. Overall, the correlation between the ELISA data from individual laboratories was statistically significant with correlation coefficient mostly above 0.8. The correlation between ELISA and HPLC-MS/MS was also significant, but correlation coefficients were lower. In the correlation and Bland-Altman plots we detected three notable outliers which

significantly contributed to the discrepancy between both sets of data. Omitting them further improved the data agreement bringing the correlation coefficient to R=0.869 and narrowing confidence limits for bias in Bland-Altman plot. While two of these outliers exhibited higher 8-oxodG estimates by the ELISA, the third sample had more than 40% higher 8-oxodG concentration detected by the HPLC-MS/MS.

As the three outliers were clearly the samples causing most of the between-technique disagreement (after omitting Data 1), we attempted to characterize the profile of organic compounds by GC-MS and HPLC-MS/MS. This combined approach allowed us to detect compounds with various chemical properties, taking advantage of public databases to tentatively identify GC-MS detected peaks and authentic standards to identify selected mono- and disaccharides. The two samples with higher ELISA 8-oxodG estimates exhibited a number of compounds either not present in the control sample 13, or present at very low concentrations. This suggests that 8-oxodG overestimation is caused by the cross-reactivity of the antibody with these (and possibly other) compounds that remained in the urine despite the SPE pre-treatment. Although the identification is tentative, we can broadly classify them as aromatic and heterocyclic compounds, e.g. compounds containing structures similar to 8-oxoguanine in 8-oxodG. Furthermore, authentic standards allowed identification of several monosaccharides, from which concentrations of beta-D-galactose and alpha- and beta-D-glucose in two ELISA outliers (samples 20 and 21) several fold exceeded those in the control sample 13. The structures of these compounds are partially similar to 2'-deoxy-D-ribose in 8-oxodG. Moreover, it has been reported that the epitope of the primary antibody spans from the hydroxyl group on C8 of guanine to the 2'-deoxyribose backbone [23], making the cross-reactivity of the antibody with the above-mentioned monosaccharides likely. It should be mentioned that although the concentrations of these monosaccharides were relatively high compared to the control

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sample 13, they were within a lower interval of the physiological range for healthy subjects reported in the literature [24]. This implies that provided that the two monosaccharides contribute to the cross-reactivity of the primary antibody, urine samples from any person may yield false-positive 8-oxodG concentrations. However, we should point out that our experiments did not conclusively prove the cross-reactivity of the primary antibody with the compounds detected in the chromatograms. To do that, we would have to use the compounds suspected from the cross-reactivity as competitors in the ELISA and check the levels of antibody binding inhibition. This was, however, out of the scope of our present study but points to the direction of future work. Some such tests have been done previously for several nucleosides, urea, creatine and creatinine with no cross-reactivity detected [23], although later studies found cross-reactivity with urea [17] and creatinine [11]. While the effect of these compounds can be eliminated by SPE or normalization per creatinine levels, there are probably many other urine components that affect the ELISA 8-oxodG assessment and the effect of which cannot be removed by the above-mentioned approaches.

The third outlier contained lower 8-oxodG concentration than the control sample 13. This result is difficult to explain by the simple cross-reactivity of the primary antibody with urine components. Evans et al. speculated that other high molecular weight compounds in urine, dissimilar to 8-oxodG (proteins, saccharides), may also contribute to ELISA vs. HPLC-MS/MS disagreement [16]. As the 8-oxodG ELISA has a competitive format, we suggest that such compounds could possibly physically block the binding of the primary antibody to the antigen in the sample thus facilitating the interaction of the antibody with the antigen immobilized to the bottom of the wells thus decreasing the estimate of 8-oxodG in the urine. However, if true, these compounds are hypothetical as they were not detected in any of the chromatograms.

The data presented in our manuscript indicate that: (1) Inter-laboratory comparison of the ELISA results is possible, although with some reservations. The fact that we were not able to identify the reason for discrepancy observed particularly for Data 1 is worrying and suggests that under certain circumstances the method may not generate reproducible results. For inter-laboratory analyzes this may be relatively easily overcome by administration of a urine sample to all study participants that would serve as a common standard. Subsequently, the data from the laboratories that showed discrepancies in the concentrations of these standards would be excluded. (2) The agreement between 8-oxodG ELISA and HPLC-MS/MS can be significantly improved by approaches described previously, specifically urine pre-treatment by SPE [11] and incubation of the primary antibody with the samples at 4 °C [16]. However, even these steps do not guarantee a perfect agreement between the techniques, as (yet to be identified) compound(s) in some urine samples may interfere with the antibody binding and cause falsely high or low 8-oxodG estimates. This fact particularly complicates a routine application of ELISA for 8-oxodG analysis, as currently there is no way to identify the outliers without running HPLC-MS/MS analysis alongside ELISA. However, this would eliminate any advantage of ELISA as a fast, easy and relatively inexpensive method.

Commented [5]: It is true that the kit is standardized and the 8-oxodG standards provided with the it could be used to revealed variability between laboratories. However, this approach would eliminate urine processing (centrifugation, dilution) and SPE where errors may also arise. That's why I thought urine samples might be better as common standards.

Although it is a known fact that there exists a discrepancy between ELISA and chromatography and the latest study describing the steps that would improve between-technique agreement was published two years ago [11], new papers still appear that do not take the necessary improvement steps into account. Out of 17 human studies published between 2013 and 2015 that we found in the PubMed database using keywords “8-oxodG” or “8-OHdG” “urine” and “ELISA” and for which we were able to retrieve full-text, only one acknowledged improvement of ELISA by the incubation of primary antibody at 4 °C [25] and none of them used the SPE pre-treatment step. Furthermore, we found two

studies that described development of new methods for 8-oxodG detection in urine [26,27]. The authors of both studies validated the new methods by comparing the results with 8-oxodG concentrations measured by ELISA kits rather than by the gold standard chromatographic methods. Moreover, the urine samples were not pre-treated, except for removal of precipitates by centrifugation and filtration.

In light of the results of our present study, it should be advisable to always combine the 8-oxodG ELISA analysis with the SPE pre-treatement keeping in mind that even these steps will not guarantee reliable 8-oxodG detection for some samples.

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Summary

In the present study we showed that using a standardized protocol and a commercial kit from a single manufacturer led to a good inter-laboratory agreement of 8-oxodG analysis by ELISA for most of the participating laboratories. However, to eliminate a laboratory/laboratories generating possibly outlying data, common urine standards should be distributed along the analyzed samples.

Commented [8]: As I mentioned before I think that extra steps needed for urine processing may also result in errors, therefore urine as a common standard might be better…

Creatinine determination might introduce some errors, but in our study the results were very consistent between labs: the CV was only around 5%. I mentioned that in the Methods.

The modified ELISA reported here substantially improved agreement of the method with HPLC-MS/MS analysis. However, we still detected some samples with 8-oxodG values that differed from the HPLC-MS/MS data. Because it is impossible to predict such samples and thus to exclude them from the analysis, we conclude that currently the ELISA is not suitable as a replacement for chromatographic methods. To achieve that, interfering substances would have to be identified and pre-purification steps would have to be further optimized.

It is essential for the scientific community to be aware of advantages of the modified ELISA and apply the recommended steps that are currently known to improve inter-laboratory and between-technique agreement. However, at the same time, the limitations

of the assay should be considered when planning the experiments to avoid generation of misleading data. More work is needed to to further improve the quality of the ELISA data.

Acknowledgments

We are grateful to Japan Institute for the Control of Aging (JaICA) for providing us with the 8-oxodG ELISA kits.

P.R., A.A., A.R. and R.J.S. were supported by GA CR 301-13-13458S.

Figure Legends

Figure 1. 8-OxodG concentrations in unspiked urine (sample 1) and the urine sample spiked with known concentrations of 8-oxodG (sample 2-5) analyzed by ELISA in all participating laboratories (Fig. 1A) and in four laboratories after Data 1 have been excluded (Fig. 1B). Mean 8-oxodG values (red horizontal sign) and SD (red vertical bars) are shown.

Figure 2. Correlation and between-technique agreement for 8-oxodG concentrations in unspiked urine and the urine sample spiked with known concentration of 8-oxodG measured by ELISA and HPLC-MS/MS. The ELISA data from all participating laboratories correlated well with chromatography (R=0.982, p=0.003; Fig. 2A); the correlation improved slightly after Data 1 have been excluded from the analysis (R=0.990, p=0.001; Fig. 2B). However, Bland-Altman plot showed underestimation of 8-oxodG levels by ELISA (Fig. 2C, 2D). The between-technique agreement improved after Data 1 have been removed from the analysis (Fig. 2D).

Figure 3. Ranked mean 8-oxodG concentrations and SD in urine samples analyzed by ELISA in all participating laboratories. Mean 8-oxodG values (red horizontal sign) and SD (red vertical bars) are shown.

Figure 4. Ranked mean 8-oxodG concentrations and SD in urine samples analyzed by ELISA in the participating laboratories after excluding Data 1. Mean 8-oxodG values (red horizontal sign) and SD (red vertical bars) are shown.

Figure 5. Correlation and between-technique agreement for 8-oxodG levels in urine samples analyzed by ELISA and HPLC-MS/MS. There was a significant correlation between ELISA data from all participating laboratories and HPLC-MS/MS (R=0.679, p<0.001; Fig. 5A), that further improved after Data 1 have been excluded from the analysis (R=0.749, p<0.001; Fig. 5B). Bland-Altman plots showed underestimation of 8-oxodG

Commented [9]: Regarding the target 8-oxodG values in the figure – I was considering it but then I realized that I was not sure how to do that. The 8-oxodG standard of a known amount was added to a urine sample with unknown 8-oxodG concentration. Probably the most precise value we have is 2.58 nmol/mmol (the result of HPLC-MS/MS) but this is about 50% higher than the mean ELISA results. So, we would have to use some ELISA data. But which would be appropriate? The mean value, or the mean value minus Data 1? Well, at this moment I decided against showing the target value :-)

But if my thoughts are wrong, please correct me...

levels by ELISA and improved agreement after removal of Data 1 from the analysis (Fig. 5C, 5D). There were three notable outliers detected in both correlation and Bland-Altman plots (samples 18, 20, 21; circled in red in Fig. 5B). Sample 13 (the control sample): near perfect between-technique agreement has been obtained; the sample was used as a control in identification of compounds in the outliers (more details in the text and Fig. 7).

Figure 6. Correlation and between-technique agreement for 8-oxodG levels in urine samples analyzed by ELISA and HPLC-MS/MS after the three outlying samples have been excluded. The correlation between the data improved ($R=0.869$, $p<0.001$; Fig. 6A), although the difference between the methods was still statistically significant ($p<0.001$). The ELISA underestimated 8-oxodG levels, although the between-technique agreement improved resulting in narrower interval for confidence limits for bias (Fig. 6B).

Figure 7. GC-MS chromatograms and tentative identification of organic compounds in the urine samples for which the ELISA 8-oxodG levels were substantially higher (sample 20, Fig. 7A; sample 21, Fig. 7B) and lower (sample 18, Fig. 7C) than those assessed by the HPLC-MS/MS. In sample 13 comparable 8-oxodG concentrations were obtained by both methods (Fig. 7D).

Figure 8. HPLC-MS/MS chromatograms of organic compounds in the urine samples for which the ELISA 8-oxodG levels were substantially higher (sample 20, Fig. 8A; sample 21, Fig. 8B) and lower (sample 18, Fig. 8C) than those assessed by the chromatography. In sample 13 comparable 8-oxodG concentrations were obtained by both methods (Fig. 8D).

Table 1. Creatinine-normalized 8-oxodG levels in unspiked urine and in the urine sample spiked with known concentration of 8-oxodG: pooled ELISA data from all participating laboratories; the data after excluding the outlying results; the results obtained by HPLC-MS/MS.

Sample (ID)	8-oxodG (ELISA, all data)		8-oxodG (ELISA, excl. Data 1)		HPLC-MS/MS
	Mean±SD (nmol/mmol)[a]	CV (%)	Mean±SD (nmol/mmol)[b]	CV (%)	8-oxodG (nmol/mmol)
Urine (1)	1.73±0.49	28.5	1.53±0.21	14.0	2.58
Urine + 8-oxodG 0.25 ng/mL (2)	2.16±0.85	39.2	1.75±0.24	14.0	3.08
Urine + 8-oxodG 0.5 ng/mL (3)	2.17±0.73	33.5	1.86±0.26	14.2	3.11
Urine + 8-oxodG 1 ng/mL (4)	2.76±1.31	47.2	2.19±0.30	13.7	3.47
Urine + 8-oxodG 2.5 ng/m (5)	3.65±0.87	23.8	3.36±0.66	19.6	4.67

[a] p=0.013, results of ANOVA, a comparison of all laboratories running ELISA

[b] p=0.675, results of ANOVA, a comparison of laboratories running ELISA after excluding Data 1

Table 2. Pearson correlation between 8-oxodG levels in 30 urine samples measured by ELISA in individual laboratories and by HPLC-MS/MS.

	ELISA Data 2	ELISA Data 3	ELISA Data 4	ELISA Data 5	ELISA Data 6	HPLC-MS/MS
ELISA Data 1	R=0.543; p=0.002	R=0.752; p<0.001	R=0.462; p=0.013	R=0.514; p=0.004	R=0.337; p=0.068	R=0.267; p=0.153
ELISA Data 2		R=0.908; p<0.001	R=0.916; p<0.001	R=0.917; p<0.001	R=0.900; p<0.001	R=0.751; p<0.001
ELISA Data 3			R=0.888; p<0.001	R=0.845; p<0.001	R=0.730; p<0.001	R=0.606; p<0.001
ELISA Data 4				R=0.846; p<0.001	R=0.837; p<0.001	R=0.636; p<0.001
ELISA Data 5					R=0.887; p<0.001	R=0.735; p<0.001
ELISA Data 6						R=0.781; p<0.001

Table 3. Tentative identification of abundant compounds in selected urine samples by GC-MS.

	Sample ID (concentration, ng/ml)			
Compound	18	20	21	13
Benzeneacetic acid, methyl ester	463	24716	30532	1034
2-Methyl-4-propyl-thiazole	6.2	10031	8852	0
Azidotrimethylsilane	0	30251	48094	118
Benzamide	0	560	478	0
Benzeneacetamide	0	10702	14361	0
Benzeneacetic acid	0.5	286	386	0.5
5-Amine_N(Phenylmethyl)-1H-Tetrazole	0	22879	16382	5.3
3,4-Dibenzyloxyphenol	0	1808	2118	65.8
1,3-Diphenyl-2-propanone	0.6	2493	2073	45.8
N-(phenylacetyl)-glycine	0.3	9741	12793	20

4-OH-2-Propenoic acid	1.5	449	891	1.4
Pregnane-3,20-diol diacetate (peak 1)	592	1746	2472	1163
Pregnane-3,20-diol diacetate (peak 2)	22.6	86.8	290	102

Table 4. Identification and quantification of saccharides in selected urine samples by GC-MS based on authentic standards. Fold difference is related to comparison of saccharide concentrations between samples 18, 20, 21 and the control sample 13.

	Sample ID (concentration, ng/ml) (fold difference)			
Compound	18	20	21	13
alfa-D-Galactose	4.9 (0.02)	11.9 (0.05)	15.5 (0.06)	239
D-Mannose	550 (1.48)	745 (2.00)	444 (1.19)	372
beta-D-Galactose	52.3 (1.29)	75.2 (1.86)	1031 (25.5)	40.5

alfa-D-Glucose	56.7 (1.31)	119 (2.75)	1276 (29.5)	43.2
beta-D-Glucose	119 (0.64)	628 (3.36)	1431 (7.67)	187
alfa-Lactose	162 (0.40)	161 (0.40)	306 (0.76)	404
Trehalose	6.5 (1.63)	0.0 (0)	0.0 (0)	4.0
beta-Lactose	112 (0.28)	154 (0.39)	342 (0.86)	396

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