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Urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine values determined by a modified ELISA improves agreement with HPLC–MS/MS

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ABSTRACT

ELISA is widely used for urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) analysis. It is the method of choice for laboratories that lack specialized chromatographic instrumentation. It allows fast, high-throughput sample analysis without a need for extensive samples processing. However, a lack of agreement between ELISA and chromatographic methods confines its application to the assessment of relative urinary 8-oxodG levels. We investigated various ELISA modifications, seeking optimal conditions that would yield a good agreement between ELISA and high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS). Purification of urine by solid phase extraction (SPE), then incubation with the anti-8-oxodG antibody at 4 °C overnight and subsequent normalization of 8-oxodG levels per urinary creatinine resulted in a near-perfect correlation and agreement in mean levels between ELISA and HPLC–MS/MS ($r = 0.917$, $p < 0.001$; and paired t-test $p = 0.803$, respectively). Our data show that, after introduction of a simple modification, ELISA quantification urinary 8-oxodG substantially improves. Although more sample manipulation is required, the method retains its key advantages over chromatography (high-throughput analysis that does not require expensive instrumentation). This represents a significant advance for the ELISA, and encouraging its use in more studies adding to our knowledge of the role of this biomarker of oxidative stress in health and disease.

1. Introduction

Urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) is a popular biomarker of whole body oxidative stress [1]. Urine has many advantages over blood or other biological matrices. Samples can be collected non-invasively, and can be obtained from vulnerable subjects, including young children and the elderly. Stability of 8-oxodG is another strength, not being affected by 15 y storage at −20 °C [2], nor is prone to artifactual formation from dG oxidation [3]. For urinary 8-oxodG analysis two types of methods predominate: chromatographic techniques (e.g., high-performance liquid chromatography coupled with mass spectrometric or electrochemical detection), and immunochemical methods (e.g., competitive ELISA) [4,5]. Although high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) is considered the gold standard method of 8-oxodG analysis, in some laboratories its use is restricted due to high cost of the equipment and the need for expert personnel operating the system. In contrast, ELISA represents a method that does not require specialized equipment; the analysis is fast and high-throughput and may be performed by individuals with little training. For these reasons standardization and improvement of ELISA so that its results are comparable with chromatographic techniques would be beneficial for many laboratories worldwide.

Several authors have attempted to increase ELISA specificity by sample pre-treatment and/or changing the incubation temperature of the primary antibody with the sample [4,6–8]. Unfortunately, ELISA remains unable to provide absolute values of 8-oxodG which are comparable with chromatographic methods, and has attracted a lot of criticism, and the conclusion that ELISA, in its present form, cannot be recommended for accurate quantification of 8-oxodG in human urine [4,9].

In the present study we aimed to develop an ELISA that would improve 8-oxodG quantification in urine compared to HPLC–MS/MS. For this we tested various combinations of urine pre-treatment including incubation with urease [8] and purification by solid-phase extraction (SPE) [10], in conjunction with both a commercial kit (Highly Sensitive 8-OHdG Check, JaICA, Shizuoka, Japan) and a previously described in-house assay [11].

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2. Material and methods

Healthy males (n = 5; aged 23–50), recruited from staff and students of the University of Leicester, provided 10 spot urine samples over a period of two weeks. All volunteers gave informed, written consent to participate in the study. Approval had been granted by the University of Leicester, College of Medicine, Biological Sciences and Psychology Non-Clinical Ethics Committee.

Urine samples (20–30 mL) were collected into Universal containers and stored at −20 °C until analysis. Before analysis, 1–2 mL aliquots of urine were centrifuged at 14,000×g for 5 min and supernatant transferred to 1.5 mL tubes.

2.1. Urease treatment

Urine samples (1 mL) were treated with 16.25 U urease (Jack bean urease, type C-3, Sigma–Aldrich, St. Louis, MO, USA) for 2 h at 37 °C. The reaction was stopped by adding N-ethylmaleimide (Sigma–Aldrich, St. Louis, MO, USA) to a final concentration of 4 mM and incubation for 5 min at room temperature. The precipitate was removed by centrifugation at 14,000×g for 5 min. For urines not processed by SPE, the pH of the samples was checked and adjusted to approx. 7.0 with 6 M HCl. After treatment the samples were either used directly for downstream application, or stored at −20 °C.

2.2. SPE purification

SPE was performed as described by Lam et al. [10] with modified sample volumes: for HPLC–MS/MS, 150 μL of urine was spiked with 3 pmol of [$^{15}N_5$]-8-oxodG internal standard and diluted to 1 mL with HPLC grade water; for ELISA, 200–300 μL of urine was diluted to 1 mL with HPLC water. After SPE dried samples for HPLC–MS/MS were reconstituted in 50 μL mobile phase, incubated at −20 °C for 30 min and centrifuged at 14,000×g for 10 min at 4 °C. Supernatants were then transferred to HPLC vials. For ELISA, dried samples were reconstituted in 200–300 μL PBS (depending on the volume used for SPE), prior to adding to the plate.

2.3. HPLC–MS/MS analysis

HPLC–MS/MS analysis was performed as described previously [12].

2.4. ELISA

Samples were analyzed in triplicate, 50 μL sample/well. 8-OxodG levels were expressed either in ng/mL, or in nmol/mmol creatinine. Two ELISA approaches we evaluated:

1. Highly Sensitive 8-OHdG Check kit (JaICA, Shizuoka, Japan): conducted according to the manufacturer's instructions. Samples were incubated with the primary antibody at 4 °C overnight.
2. In-house ELISA: The assay was performed as previously described [11]. Incubation of urine samples with primary antibody was performed either 1.5 h at room temperature or at 4 °C overnight.

2.5. Creatinine assay

To normalize 8-oxodG levels per creatinine content in the samples, creatinine levels were analyzed using a commercial kit (Arbor Assays, Ann Arbor, MI, USA) according to manufacturer's recommendations. The samples were analyzed in duplicate; creatinine concentrations were expressed in mmol/L.

2.6. Statistical analysis

Statistical analyses were performed by SPSS IBM version 20 (Chicago, IL, USA). After checking normality of the data distribution by Kolmogorov–Smirnov test, paired t-tests were used to compare 8-oxodG levels obtained by individual methods. Correlations between the data were calculated by Pearson correlation analyses.

3. Results

To test various ELISA modifications and to identify conditions that gave the best agreement between ELISA and HPLC–MS/MS, we collected 10 urines and for each of them tested combinations of urease treatment with SPE purification and, in the case of the in-house assay, with two primary antibody incubation temperatures (Fig. 1). From each urine we obtained 8 samples for the in-house assay and 4 samples for the JaICA kit. The total number of samples analyzed by ELISA was 120.

Table 1 shows 8-oxodG results expressed in ng/mL. We identified two main ELISA modifications which gave reasonable correlation with HPLC–MS/MS and/or mean 8-oxodG levels close to those measured by chromatography:

(i) Samples pre-treated with urease and SPE followed by the analysis either by the JaICA kit, or the in-house assay at 4 °C.
(ii) Samples purified with SPE analyzed by the JaICA kit.

The in-house assay yielded best correlation with HPLC–MS/MS (r = 0.748), but absolute 8-oxodG levels differed significantly from those measured by chromatography (p = 0.029). For the JaICA kit the correlation coefficients were lower (r = 0.668 and 0.617 for urease + SPE and SPE only pre-treatment, respectively), but 8-oxodG levels did not differ significantly from the HPLC–MS/MS data (p = 0.590 and 0.261 for urease + SPE and SPE only pre-treatment, respectively). The results indicated that urease treatment only, particularly when accompanied by 8-oxodG analysis by the in-house assay, gave the highest 8-oxodG levels. Urease catalyses urea decomposition to carbon dioxide and ammonium which further forms ammonium hydroxide. Because urea concentrations in urine are high (up to 30 mg/ml, or 0.5 M) [8], the amount of ammonium hydroxide formed may reach up to 1 M. We analyzed the effect of NH_4OH (1 mM to 1 M) on N45.1 binding and found that the presence of 0.5 M and 1 M NH_4OH causes false detection of 8-oxodG (corresponding to concentration about 0.21–0.25 ng/mL 8-oxodG, respectively).

Normalization per creatinine levels resulted in a significant improvement of correlations of ELISA data with the HPLC–MS/MS results (Table 2). Correlation coefficients increased to 0.914, 0.930 and 0.917 for the samples pretreated with urease + SPE analyzed by the JaICA kit, pretreated with urease + SPE analyzed by the in-house assay at 4 °C and SPE-purified only analyzed by the JaICA kit, respectively. More importantly, the absolute 8-oxodG values in samples analyzed by the kit still did not differ from those analyzed by HPLC–MS/MS (results of paired t-test: p = 0.566 and 0.803, for samples pretreated with urease + SPE, and SPE only, respectively). A comparison of correlations between 8-oxodG levels measured by the JaICA ELISA in samples without any pre-treatment or creatinine normalization and HPLC–MS/MS, and between 8-oxodG levels measured by the JaICA kit in samples purified by SPE and HPLC–MS/MS before and after creatinine normalization is shown in Fig. 2. In this figure we also report a comparison between 8-oxodG levels analyzed by both methods ranked according to increasing concentrations of 8-oxodG assessed by HPLC–MS/MS. Although modified ELISA clearly improved agreement between both methods, there is still some discrepancy, particularly for samples with lowest 8-oxodG levels.

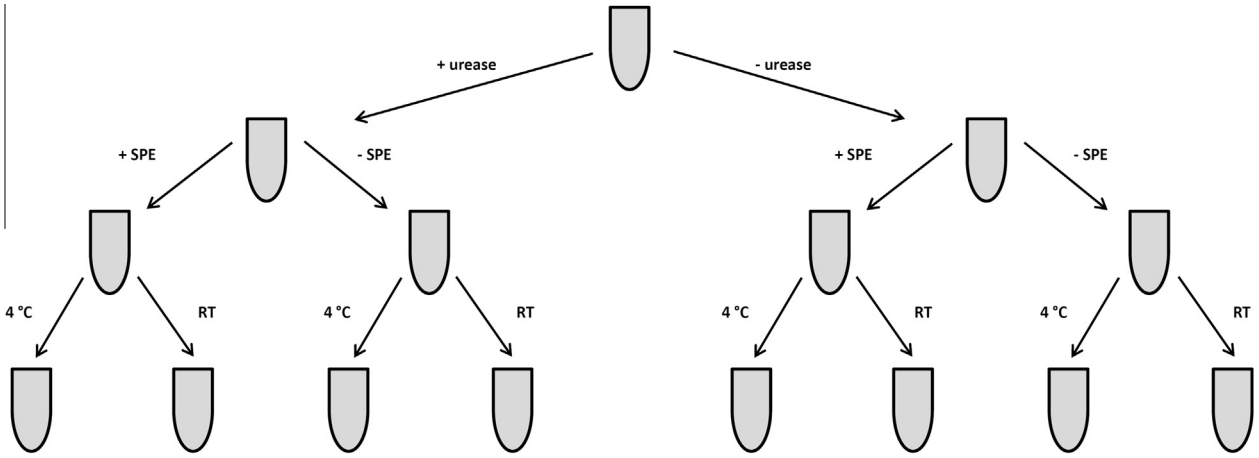

Fig. 1. Overview of ELISA optimization steps. The chart illustrates steps tested to improve ELISA. Ten urine samples were obtained and each of them treated as depicted in the figure. Samples analyzed by the JaICA kit were incubated with the primary antibody at 4 °C overnight, while for the in-house assay a 1.5 h incubation at room temperature (RT) and overnight at 4 °C were tested.

Table 1
Comparison of 8-oxodG levels (ng/mL) in urine samples ($n = 10$) analyzed by modified ELISAs and HPLC–MS/MS.

Detection method	8-oxodG (ng/mL)		Comparison with HPLC–MS/MS	
	Mean	SD	Correlation (r; p)	Paired t-test (p)
JaICA: urease, SPE, 4 °C	4.64	2.55	0.668; 0.035	0.590
In house: urease, SPE, RT	8.46	3.17	0.516; 0.127	0.001
In house: urease, SPE, 4 °C	6.06	3.28	0.748; 0.013	0.029
JaICA: urease, 4 °C	7.09	4.25	0.643; 0.045	0.024
In house: urease, RT	28.5	13.9	0.499; 0.142	<0.001
In house: urease, 4 °C	18.6	10.1	0.513; 0.129	0.001
JaICA: SPE, 4 °C	5.23	3.08	0.617; 0.057	0.261
In house: SPE, RT	9.60	4.68	0.292; 0.413	0.005
In house: SPE, 4 °C	7.81	4.94	0.314; 0.377	0.045
JaICA, 4 °C	6.52	4.31	0.716; 0.020	0.046
In house: RT	18.8	13.3	0.477; 0.163	0.005
In house: 4 °C	13.0	9.36	0.537; 0.109	0.009
HPLC–MS/MS	4.28	2.50		

Table 2
Comparison of 8-oxodG levels (nmol/mmol creatinine) in urine samples ($n = 10$) analyzed by modified ELISAs and HPLC–MS/MS.

Detection method	8-oxodG (nmol/mmol)		Comparison with HPLC–MS/MS	
	Mean	SD	Correlation (r; p)	Paired t-test (p)
JaICA: urease, SPE, 4 °C	1.32	0.43	0.914; <0.001	0.566
In house: urease, SPE, RT	2.84	1.48	0.780; 0.008	0.001
In house: urease, SPE, 4 °C	1.80	0.79	0.930; <0.001	0.002
JaICA: urease, 4 °C	1.88	0.58	0.788; 0.007	0.010
In house: urease, RT	8.31	2.57	0.863; 0.001	<0.001
In house: urease, 4 °C	5.26	1.72	0.803; 0.005	<0.001
JaICA: SPE, 4 °C	1.43	0.40	0.917; <0.001	0.803
In house: SPE, RT	3.00	1.27	0.750; 0.012	<0.001
In house: SPE, 4 °C	2.19	0.63	0.682; 0.030	0.002
JaICA, 4 °C	1.67	0.72	0.672; 0.033	0.180
In house: RT	4.92	1.79	0.735; 0.015	<0.001
In house: 4 °C	3.15	0.88	0.516; 0.127	<0.001
HPLC–MS/MS	1.40	0.76		

The improved agreement between ELISA and HPLC–MS/MS after creatinine normalization suggests an association between 8-oxodG measured by ELISA and urinary creatinine levels. We therefore calculated correlations between both parameters for ELISA variants and HPLC–MS/MS. This showed a significant correlation between 8-oxodG and creatinine, measured by ELISA, but not by HPLC–MS/MS (Table 3). Further experiments revealed a weak cross-reactivity of the primary antibody with creatinine in the urine, corresponding to ~10% inhibition of antibody binding in the competitive ELISA or the equivalent of 0.13 ng/mL 8-oxodG, for a physiological creatinine concentration of 20 mmol/L [13]. Therefore it is essential that 8-oxodG concentrations measured by ELISA should always be normalized per creatinine to obtain data comparable with HPLC–MS/MS analysis.

4. Discussion

Herein, we report a modification of ELISA that improves the quantification of urinary 8-oxodG yielding results that are in agreement with the gold standard HPLC–MS/MS technique. This was

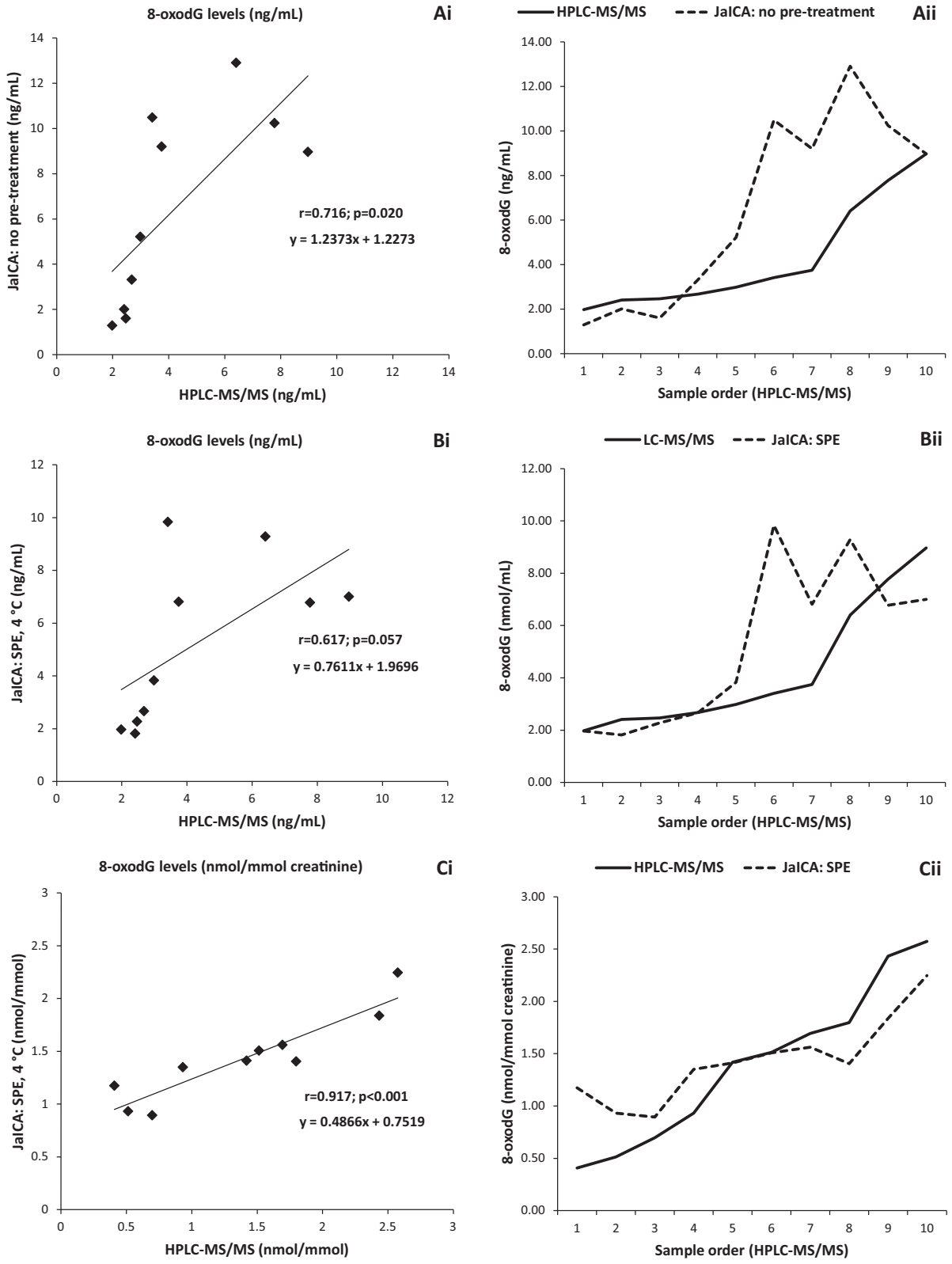

Fig. 2. Correlation (Ai–Ci) and rank order agreement (Aii–Cii) between urinary 8-oxodG levels measured by the JaICA ELISA and HPLC–MS/MS. 8-oxodG levels were measured by ELISA in samples (A) without any pre-treatment, results expressed in ng/mL urine; (B) following SPE with results expressed in ng/mL urine; (C) following SPE, with results normalized per creatinine.

achieved by a combination of relatively simple pre-treatment steps and incubation of samples with the primary antibody at 4 °C.

The primary antibody N45.1 used for 8-oxodG detection in most ELISA studies [5] was characterized by Toyokuni et al. [14]. The antibody is relatively specific: it recognizes both the 8-oxo-7,8-dihydroguanine and 2′-deoxydeoxyribose structures in 8-oxodG and also cross-reacts with 8-oxo-7,8-dihydroguanosine (8-oxo-Guo), but at concentrations two orders higher than 8-oxodG. The

Table 3
Correlations between 8-oxodG levels, as measured by modified ELISAs and HPLC–MS/MS, and urinary creatinine levels.

Detection method	Correlation (r; p)
JaICA: urease, SPE, 4 °C	0.872; 0.001
In house: urease, SPE, 4 °C	0.761; 0.011
JaICA: SPE, 4 °C	0.907; <0.001
HPLC–MS/MS	0.344; 0.330

authors reported no cross-reactivity with either unmodified 2′-deoxyribonucleosides, or other components of urine, including urea, creatine, and creatinine [14]. However, a lack of agreement between absolute urinary 8-oxodG levels measured by ELISA and chromatographic techniques has been widely reported [4,7,8,15–21] indicating some cross-reactivity.

There have been three notable attempts to increase the specificity of urinary 8-oxodG measurement by ELISA. (i) HPLC prepurification of urine prior to ELISA improved the correlation between ELISA and HPLC with electrochemical detection from ($r = 0.550$, $p < 0.01$) to ($r = 0.833$, $p < 0.001$) [16]. However, this modification is not practical due to the requirement for HPLC, which effectively eliminates all advantages of ELISA, particularly speed and simplicity. (ii) Evans et al. [7] showed that decreasing the temperature of the primary antibody incubation step significantly ($p < 0.0001$) decreased the mean 8-oxodG values, compared to values determined at 37 °C, resulting in no significant difference between mean levels determined by ELISA and HPLC–MS/MS, although the correlation was not high ($r = 0.65$, $p = 0.005$). This was subsequently incorporated into the JaICA ELISA protocol. The authors further speculated that urine components structurally dissimilar to 8-oxodG, including proteins and carbohydrates, might interfere with N45.1 yielding false-positive signals and suggested urine pre-treatment using SPE as a method to remove such components [7]. (iii) By fractionating urine samples and performing ELISA on the fractions, Song et al. [8] identified urea to be a compound cross-reacting with N45.1, seemingly contradicting Toyokuni et al. [14]. This contradiction is explained by the concentrations in Toyokuni et al. study [14] being too low, with a maximum of 100 μM, as urea concentrations may reach up to 0.5 M, at which concentration cross-reactivity with the primary antibody occurs [8]. Song et al. demonstrated that agreement between ELISA and HPLC improved when 8-oxodG concentrations were corrected per urea content, or when urine samples were pre-treated with urease [8]. However, the authors also noted that urea explained only part of the discrepancy and showed that 8-oxoGuo cross-reacts with N45.1 at concentrations 7-fold higher than 8-oxodG. However, this observation contradicts others [7,14], and levels of urinary 8-oxoGuo are reported to be closer to 2-fold greater than 8-oxodG [22], making cross-reactivity with urinary 8-oxoGuo unlikely.

In the present study, we used a popular commercial ELISA and an in-house assay, developed in our laboratory [11], to identify methodological modifications that would result in best agreement with HPLC–MS/MS. Although the in-house assay and the JaICA kit share the same primary antibody (N45.1), 8-oxodG levels detected by both ELISA variants differ. Moreover, there were substantial differences between 8-oxodG levels for each pre-treatment option.

In general, highest 8-oxodG/mL levels were generated by the in-house assay when the primary antibody was incubated at room temperature. This was expected and confirms earlier reports. Moreover, there was no correlation between the ELISA and HPLC–MS/MS. When this was repeated at 4 °C, 8-oxodG levels decreased, but were still higher than those obtained by the JaICA ELISA. This can be explained partly by differences in real concentrations of 8-oxodG used to construct standard curve. 8-OxodG used in the in-house assay was diluted to an expected concentration of 1 mg/mL, but not confirmed analytically. Thus, weighing errors and/or impurities in 8-oxodG may cause variation in urinary levels of 8-oxodG when compared with the JaICA ELISA. The 8-oxodG solution used to coat ELISA plates, may also affect the accuracy. While the in-house assay uses a concentration of 5 ng 8-oxoGuo/bovine serum albumin conjugate/50 μL/well, there are no corresponding details available for the kit.

Interestingly, pre-treatment of the samples with urease, without subsequent SPE purification, gave increased 8-oxodG estimates measured by both the in-house assay and the kit.[1]

The increase was about 2-fold and 3-fold, for the JaICA ELISA and in-house assay respectively. This result was unexpected and contradicts the data published by Song et al. [8]. While we have no explanation for this discrepancy, we hypothesized that compound(s) produced by the urease reaction (e.g., NH_4OH) might affect binding of the primary antibody. This is supported by the fact that 8-oxodG levels, measured by the JaICA kit before creatinine normalization, in samples treated with urease followed by SPE were lowest of all tested pre-treatment options and closest to HPLC–MS/MS. In our experiments, we observed the effect of high NH_4OH concentrations, theoretically present in the urine after urease treatment, on N45.1 binding. Since samples containing NH_4OH were neutralized before ELISA, it is unlikely that alkaline pH would be responsible for high 8-oxodG estimates, suggesting that changes in ion concentrations may affect antibody binding. Ammonium hydroxide is removed from the samples during SPE, eliminating the interference with antibody and decreasing the over-estimation of 8-oxodG levels. Support for this effect being antibody-related is derived from the fact that urease treatment had no effect on 8-oxodG levels measured by HPLC–MS/MS (data not shown).

While SPE purification is one of the key steps for improvement of 8-oxodG ELISA, it may potentially cause a partial loss of the sample during workup. However, recovery of 8-oxodG for the SPE method we used in our study was reported to be 88% with intra- and inter-day variability of 4.0 and 10.2%, respectively [10]. This variability is low and is comparable with inter-assay variability of ELISA and should not have any significant impact on the results.

Although 8-oxodG levels (ng/mL) detected by the JaICA kit in samples pre-treated with urease + SPE or urease only did not differ significantly from the levels detected by HPLC–MS/MS, correlations were not perfect. For the in-house assay, the correlation with chromatography was better ($r = 0.748$) but the mean ELISA results were significantly higher than those obtained by HPLC–MS/MS. Normalization of 8-oxodG levels per urinary creatinine significantly improved correlations between ELISA and chromatography and, for samples treated with SPE only, also improved the agreement in absolute 8-oxodG levels. Further investigation showed a strong correlation ($r \geqslant 0.761$) between 8-oxodG levels (ng/mL) measured by ELISA and urinary creatinine. No such correlation was observed for 8-oxodG analyzed by HPLC–MS/MS, suggesting that the result is antibody-related. As creatinine shares a common CO–NH structure with urea and 8-oxodG (Fig. 3), we speculated that N45.1 might also bind to it. Although Toyokuni et al. [14] did not observe any cross-reactivity of N45.1 with creatinine, their maximal creatinine concentration was 100 μM, whereas in our study creatinine levels reached up to 33 mM (average value 16.6 mM), similar to those reported by Barr et al. [13]. We therefore examined the effect of physiological concentrations of creatinine (20 mM) and found it recognized by N45.1. Thus, creatinine also contributes to the ELISA over-estimation of urinary 8-oxodG. This underlines the need for normalization of urinary 8-oxodG levels for creatinine.

Taken together, our results demonstrate improved ELISA determination of urinary 8-oxodG levels. For the JaICA kit, urease treatment before SPE is not required, making sample preparation

[1] A result observed also by others – Prof. Regina Santella, Columbia University, NY, USA (personal communication).

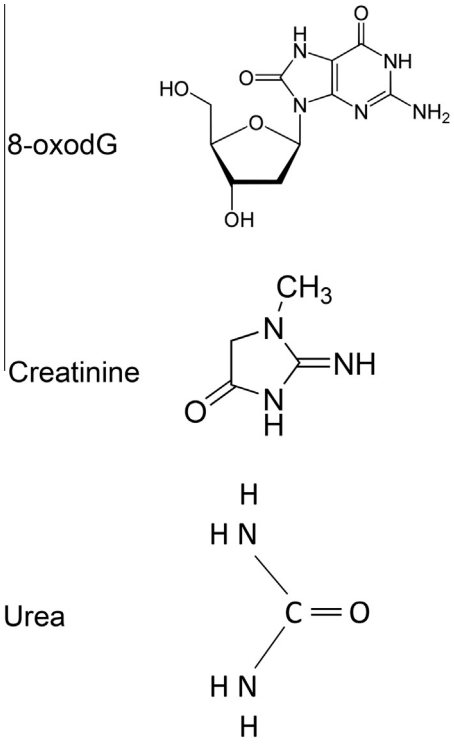

Fig. 3. Molecular structure of 8-oxodG, creatinine and urea, demonstrating a common CO–NH structure.

faster; whereas the in-house assay benefits from urease treatment to improve the correlation with the HPLC–MS/MS. This suggests that combinations of our improvements may be required for different variants of the 8-oxodG ELISA. Finally, the primary antibody must be incubated with the samples at 4 °C and 8-oxodG levels must be normalized per urinary creatinine concentration. Although some of the ELISA advantages, namely the speed and easiness of sample processing and analysis, may be compromised, this may be mitigated by using 96-well format SPE columns, conceivably even eluting directly into the 8-oxodG ELISA plate. Such modified ELISAs might equally be applied to the assessment of 8-oxodG in other biological matrices (e.g., plasma, saliva and cell culture medium) to remove potentially interfering substances. This remains a method that can be performed without the need for expensive instrumentation, which is an insurmountable obstacle for many laboratories, and presents all laboratories the potential for improved measurement of this valuable biomarker of oxidative stress.

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