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8-Oxo-7,8-dihydroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine concentrations in various human body fluids – implications for their measurement and interpretation

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Abstract

8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) is the most investigated oxidatively damaged DNA lesion product that has been associated with the development of aging, cancer, and some degenerative diseases. Here, we present the first LC-MS/MS method that enables the simultaneous measurement of its repair products in plasma and saliva, namely 8-oxo-7,8-dihydroguanine (8-oxoGua) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo). Using this method, we investigated the underlying transport mechanism of oxidatively damaged DNA between cellular compartments and biological matrices. Plasma, saliva and urine samples were collected concurrently from 57 healthy subjects. Various deproteinization methods were evaluated and the precipitants acetonitrile and sodium hydroxide-methanol were respectively selected for plasma and saliva samples due to their effect on recovery efficiencies and chromatography. The mean baseline concentrations of 8-oxoGua and 8-oxodGuo in plasma were demonstrated to be 0.21 and 0.016 ng/mL, while in saliva they were 0.85 and 0.010 ng/mL, respectively. A relatively high concentration of 8-oxoGua was found in saliva with a concentration factor (CF, concentration ratio of saliva to plasma) of 4 as compared to that of 8-oxodGuo (CF: 0.6), implying that 8-oxoGua in plasma may be actively transported to saliva whereas 8-oxodGuo was most dependent on a passive diffusion. Good correlations between urine and plasma concentrations were observed for 8-oxoGua and 8-oxodGuo, suggesting that blood was a suitable matrix in addition to urine. Significant correlation between 8-oxoGua and 8-oxodGuo in urine was only observed when the concentrations were not corrected for urinary creatinine, raising the issue

of applicability of urinary creatinine to adjust 8-oxoGua concentrations.

Key words: online SPE LC-MS/MS, oxidatively damaged DNA, plasma, saliva, deproteinization, transport mechanism

Abbreviations: 8-oxoGua, 8-oxo-7,8-dihydroguanine; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; LC-MS/MS, liquid chromatography-tandem mass spectrometry; CF, concentration factor; BER, base excision repair; ELISA, enzyme-linked immunosorbent assay; SPE, solid-phase extraction; ESI, electrospray ionization; LOQ, limit of quantification.

Introduction

Oxidatively damaged DNA is understood to be involved in the development of aging, cancer, and some degenerative diseases (Cooke et al. 2006; Tudek et al. 2010). 8-Oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) is an important DNA lesion product that can be generated in cellular DNA by hydroxyl radicals, singlet oxygen, and one-electron oxidants (Cadet et al. 2012). The detection of this DNA lesion product is considered important because of its abundance and mutagenic potential through G-to-T transversions during DNA replication (Cheng et al. 1992). It is generally accepted that oxidatively damaged DNA can be repaired, and the repair products are released into the bloodstream and consequently appear in the urine without further metabolism (Fraga et al. 1990). The modified nucleobase 8-oxo-7,8-dihydroguanine (8-oxoGua) and modified 2′-deoxyribonucleoside (8-oxodGuo) in urine represent the major repair products of oxidatively damaged DNA *in vivo*, presumably through the base excision repair (BER) and sanitization of the 2′-deoxyribonucleotide pool (Evans et al. 2010) although, to date, the precise source of urinary 8-oxodGuo remains unclear. Urinary 8-oxodGuo has been widely studied and used as a biomarker of oxidative stress (Analysis et al. 2010), whereas limited information is available concerning urinary 8-oxoGua. The reasons for this include previous studies which show that diet could contribute significantly to its urinary levels (Fraga et al. 1990) and the poor stability and solubility of 8-oxoGua which make it difficult to measure accurately (Helbock et al. 1998). However, two robust studies in humans have shown that urinary levels of 8-oxodGuo and 8-oxoGua are not influenced by diet (Cooke et al. 2005; Gackowski et al. 2001) and the stability of

8-oxoGua has also been recently examined and found to be fairly stable (~87 days in water with pH of ~7 or ~112 days in 5% methanol with pH of 11, at -20 °C) (Hu et al. 2010a). Furthermore, several reliable chromatographic-based methods have been developed for its detection in urine (Hu et al. 2010a; Roszkowski and Olinski 2012; Svoboda et al. 2006; Weimann et al. 2002). We believe that the detection of both 8-oxodGuo and 8-oxoGua in urine may improve the assessment of the whole-body burden of oxidative stress.

In addition to urinary analysis, several studies reported that the measurement of 8-oxodGuo in blood or saliva could also be useful; 8-oxodGuo concentrations in blood (e.g., serum) could directly reflect exposure to oxidative stress and subsequent DNA repair in the whole organism (Bloomer and Fisher-Wellman 2008; Sova et al. 2010), while salivary 8-oxodGuo could be specific for oxidative stress in the buccal cavity (Agha-Hosseini et al. 2012; Sezer et al. 2012) or whole organism (Komatsu et al. 2013; Su et al. 2012). However, most 8-oxodGuo measurements in blood and saliva have been performed by enzyme-linked immunosorbent assay (ELISA) that is known to be limited by a lack of specificity and sensitivity (Barregard et al. 2013). There is only one chromatographic-based approach that has successfully quantified 8-oxodGuo in blood and saliva (Bogdanov et al. 1999). To the best of our knowledge, there are no literature reports of the modified nucleobase 8-oxoGua being quantified in plasma or saliva. A strength of the measurement of 8-oxoGua in human body fluids is a much clearer understanding of its source, compared to 8-oxodGuo, namely the BER pathways (e.g. via human 8-oxoguanine DNA glycosylase 1).

Previously, we developed an isotope-dilution LC-MS/MS method coupled with online solid-phase extraction (SPE) for simultaneous determination of 8-oxoGua and 8-oxodGuo in urine (Hu et al. 2010a). In the present study, we attempted to extend this to the quantification of both 8-oxoGua and 8-oxodGuo in plasma and saliva. By doing so, we tried (i) to evaluate the feasibility of using other biomatrices (i.e., blood or saliva) other than urine, (ii) to investigate the correlation between the modified nucleobase 8-oxoGua and the most frequently measured biomarker of oxidative stress, 8-oxodGuo, and (iii) to explore the underlying transport mechanism of oxidatively damaged DNA in human body fluids.

Experimental

Chemicals

Solvents and salts were of analytical grade. Unlabeled 8-oxoGua and 8-oxodGuo were purchased from Sigma-Aldrich and the stable isotope internal standard, [$^{15}N_5$]-8-oxo-7,8-dihydro-2′-deoxyguanosine ([$^{15}N_5$]-8-oxodGuo) was from Cambridge Isotope Laboratories. The [$^{15}N_5$]-8-oxo-7,8-dihydroguanine ([$^{15}N_5$]-8-oxoGua) was synthesized as described previously (Hu et al. 2010a).

Subjects and sample collection

This study was approved by the Institutional Review Board of Chung Shan Medical University. Spot urine samples, and corresponding blood and saliva samples were concurrently collected from

57 apparently healthy individuals. A questionnaire was used to obtain data on age and body mass index (BMI). Blood samples were collected in EDTA vacutainer tubes and centrifuged at 3000 × g for 15 min to separate plasma. For saliva collection, all the subjects were asked to rinse their mouths with distilled water three times. Saliva was then collected by rolls of cotton wool, followed by centrifugation at 3000 × g for 10 min. All samples were kept at 4 °C during sampling and then stored at -20 °C prior to analysis.

Simultaneous determination of 8-oxoGua and 8-oxodGuo in urine

8-OxoGua and 8-oxodGuo concentrations in urine were measured using a validated method of LC-MS/MS with online SPE as previously reported by Hu et al. (2010a). Briefly, a 20 μL of urine was diluted 10 times with a solution containing 4 ng of [$^{15}N_5$]-8-oxoGua and 0.8 ng of [$^{15}N_5$]-8-oxodGuo as internal standards in 5% (v/v) methanol (MeOH)/1 mM ammonium acetate (AA). A 50 μL of prepared urine sample was directly injected into the online SPE LC-MS/MS. After automated sample cleanup, LC-MS/MS analysis was performed using an Agilent 1100 series HPLC system (Agilent Technology) interfaced with an API 3000 triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) with electrospray ion source (ESI). The samples were analyzed in the positive ion multiple reaction monitoring mode, and the transitions of the precursors to the product ions were as follows: *m/z* 168→140 (quantifier ion) and *m/z* 168→112 (qualifier ion) for 8-oxoGua, *m/z* 173→145 for [$^{15}N_5$]-8-oxoGua, *m/z* 284→168 (quantifier ion) and 284 → 140 (qualifier ion) for 8-oxodGuo, and *m/z* 289 → 173 (quantifier ion) for

[$^{15}N_5$]-8-oxodGuo. Urinary creatinine was determined using a HPLC-UV method (Yang 1998).

Simultaneous determination of 8-oxoGua and 8-oxodGuo in plasma and saliva

For plasma and saliva samples, four different deproteinization methods were evaluated including precipitation with organic solvents (MeOH or acetonitrile), zinc sulfate-sodium hydroxide ($ZnSO_4$-NaOH) (Polson et al. 2003) and sodium hydroxide-methanol (NaOH-MeOH) (Bogdanov et al. 1999). The detailed procedures are given in Supplementary Data I. Among these four deproteinization methods, deproteinization by acetonitrile (ACN) and NaOH-MeOH generally gave higher peak areas of both 8-oxoGua and 8-oxodGuo in plasma and saliva in the chromatograms (as shown in Supplementary Data I, Fig. S1 for plasma and Fig. S2 for saliva). The overall process efficiency of each deproteinization method was provided in Supplementary Data I (Table S1); the overall process efficiency (combination of the matrix effect and the pretreatment process recovery) was calculated from the ratio of the peak area of internal standard ([$^{15}N_5$]-8-oxoGua or [$^{15}N_5$]-8-oxodGuo) spiked before pretreatments to the peak area of internal standard in neat solution multiplied by 100 (Matuszewski et al. 2003). Although deproteinization by ACN and NaOH-MeOH gave higher process efficiencies, it was also noted that the retention time during chromatography was not stable (it should be the same as that in the neat solution) (see Fig. S1 and S2). A manual SPE using C18 cartridge was therefore applied after protein precipitation by ACN or NaOH-MeOH to further purify the plasma and saliva samples. Supplementary Data I (Table S2) showed the overall process efficiency of [$^{15}N_5$]-8-oxoGua and

[$^{15}N_5$]-8-oxodGuo in plasma and saliva after combined pretreatment with protein precipitation and manual SPE. Regarding a satisfactory process efficiency and chromatography for both 8-oxoGua and 8-oxodGuo, the protein precipitation by ACN and NaOH-MeOH were finally selected and applied for plasma and saliva samples, respectively, followed by the manual SPE, as described below:

For plasma analysis, 1 mL of plasma was added with 40 μL of internal standard solution containing 4 ng of [$^{15}N_5$]-8-oxoGua and 0.8 ng of [$^{15}N_5$]-8-oxodGuo. After addition of 3 mL of ACN, the samples were mixed and centrifuged at 3000 ×g for 10 min. The supernatant was dried and resuspended in 1 mL of deionized water. After addition of 0.3 mL of 1 M AA buffer (pH 5.25) and vigorous vortexing, the sample was loaded onto a Sep-Pak C18 cartridge (100 mg/1 mL; Waters, Milford, MA, USA) preconditioned with 1 mL of MeOH and 1 mL of deionized water. The cartridge was then eluted with 1 mL of 40% MeOH. The eluate was dried under vacuum and redissolved in 150 μL of 5% (v/v) MeOH containing 1 mM AA.

For saliva analysis, after addition of 40 μL of internal standard solution, aliquots of 2 mL of saliva samples were precipitated with 2 mL of 1 mM NaOH in MeOH and centrifuged for 10 min at 3000 ×g. The pellets were resuspended in 2 mL of MeOH and centrifuged for 10 min at 3000 ×g. The supernatants were combined, dried and resuspended in 1 mL of deionized water, adjusted to pH 7 with HCl. The resulting solutions were then processed for manual SPE as described earlier for plasma.

Pretreated plasma and saliva samples were analyzed using the same validated method of online

SPE LC-MS/MS as previously reported by Hu et al. (2010a) with a minor modification in chromatography gradient. An injection volume of 70 μL was used for plasma and saliva analysis.

Statistical methods

Mean and SD were used to describe the distributions of 8-oxoGua and 8-oxodGuo in urine, plasma and saliva as well as the demographic data for study subjects. The data were analyzed using the SAS statistical package (SAS, ver. 9.1). Pearson correlation coefficients were used to study the relationships between 8-oxoGua or 8-oxodGuo concentrations in urine, plasma and saliva. The concentration factor (CF) was used to investigate the possible transport mechanism for 8-oxoGua and 8-oxodGuo, by dividing the mean 8-oxoGua (or 8-oxodGuo) concentration in urine (or saliva) by its mean concentration in plasma (Haeckel and Hanecke 1996).

Results

Simultaneous determination of 8-oxoGua and 8-oxodGuo in plasma and saliva using online SPE LC-MS/MS

A representative online SPE LC-MS/MS chromatogram for 8-oxoGua and 8-oxodGuo in saliva of a healthy subject is shown in Fig. 1, while a representative online SPE LC-MS/MS chromatogram for plasma analysis is in Supplementary Data II Fig. S3. The positive electrospray ionization mass spectrum of 8-oxoGua contained a $[M+H]^+$ precursor ion at *m/z* 168 and product ions at *m/z* 140 (quantifier ion, Fig. 1A) and *m/z* 112 (qualifier ion, Fig. 1B) due to losses of one or two CO groups

(28 u or 56 u); a precursor ion at *m/z* 173 and product ion at *m/z* 145 characterized the [$^{15}N_5$]-8-oxoGua (Fig. 1C). Meanwhile, the $[M+H]^+$ precursor ion of 8-oxodGuo was *m/z* 284 and product ions appeared at *m/z* 168 (quantifier ion, Fig. 1D) and *m/z* 140 (qualifier ion, Fig. 1E), resulting from loss of the neutral 2′-deoxyribose moiety (116 u) or its combination with CO (28 u); a precursor ion at *m/z* 289 and product ion at *m/z* 173 characterized the [$^{15}N_5$]-8-oxodGuo (Fig. 1F). The retention times were 9 min and 13 min for 8-oxoGua and 8-oxodGuo, respectively.

The limit of quantification (LOQ) was estimated for a signal-to-noise (S/N) ratio of 10 from the sample chromatograms at the lowest validation level tested, using the quantification transition. Because 8-oxoGua and 8-oxodGuo are usually present in body fluid, there was no blank matrix available. In the present study, LOQ values were estimated from quantified levels present in non-spiked blank samples (Gracia-Lor et al. 2011). Using the present method, the LOQs in plasma were determined to be 0.04 and 0.008 ng/mL for 8-oxoGua and 8-oxodGuo, respectively, while the LOQs in saliva were 0.02 ng/mL for 8-oxoGua and 0.003 ng/mL for 8-oxodGuo. Overall process efficiencies (combination of the matrix effect and the pretreatment process recovery, Matuszewski et al. 2003) of 8-oxoGua and 8-oxodGuo in plasma were estimated to be 26-35 % and 74-88%, respectively, while they were 24-36 % for 8-oxoGua and 61-78% for 8-oxodGuo in saliva. It has to be mentioned that any variation in overall process efficiency is well compensated by the use of stable isotope internal standards added and therefore will not influence the accuracy of measurement.

8-OxoGua and 8-oxodGuo concentrations in plasma, saliva and urine

A total of 57 healthy subjects were recruited into the study. As shown in Table 1, the mean age and BMI of the subjects were 30.2 ± 6.9 years and 23.7 ± 5.4 kg/m^2, respectively. The overall mean concentrations of 8-oxoGua and 8-oxodGuo in plasma were 0.21 ± 0.09 and 0.016 ± 0.005 ng/mL, respectively. Salivary concentrations of 8-oxoGua and 8-oxodGuo were 0.85 ± 0.76 and 0.010 ± 0.007 ng/mL, respectively, while urinary 8-oxoGua and 8-oxodGuo were 11.9 ± 4.1 and 3.6 ± 1.3 ng/mg creatinine. Concentration distributions of 8-oxoGua and 8-oxodGuo in three matrices are plotted in Fig. 2.

The correlations between different matrices for 8-oxoGua (or 8-oxodGuo) concentrations were analyzed using Pearson correlation coefficients. It was found that the urinary 8-oxoGua concentrations were significantly correlated with the 8-oxoGua concentrations in plasma ($r = 0.291$, $P = 0.028$, see Fig. 3A). Urinary 8-oxodGuo concentrations were also correlated with 8-oxodGuo in plasma ($r = 0.737$, $P < 0.001$, Fig. 3B). No significant correlations were found between saliva and urine or saliva and plasma for both 8-oxoGua as well as 8-oxodGuo. Furthermore, the correlation between 8-oxoGua and 8-oxodGuo in each matrix was also investigated. As shown in Fig. 4, the best correlation between 8-oxoGua concentrations and 8-oxodGuo concentrations was obtained in urine ($r = 0.67$, $P < 0.001$), followed by plasma ($r = 0.425$, $P = 0.001$) and then saliva ($r = 0.377$, $P = 0.004$).

Discussion

We have successfully applied isotope-dilution LC-MS/MS coupled online SPE to quantify both 8-oxoGua and 8-oxodGuo in three human body fluids. To the best of our knowledge, this is the first report describing an assay to simultaneously determine levels of both 8-oxoGua and 8-oxodGuo in plasma and saliva.

Previous analytical methods were focused mostly on the quantification of 8-oxodGuo in urine, with some attempts to measure the 8-oxodGuo concentrations in blood or in saliva. Breton et al. (2003) used HPLC-ECD to measure 8-oxodGuo in human serum but failed to provide a satisfactory result due to the lack of validated pretreatment of serum. Lam et al. (2012) used UPLC-MS/MS with prior manual SPE purification to determine the 8-oxodGuo concentrations in plasma and saliva. The authors found that mean 8-oxodGuo in plasma was 0.04 ng/mL whereas the 8-oxodGuo in saliva was not detectable. In the present study, we have simultaneously quantified the 8-oxodGuo and 8-oxoGua both in saliva and plasma. This success is partly attributed to the use of our online SPE system, but also effective sample pretreatment which removed interfering compounds and decreased possible matrix effects.

The mean baseline plasma concentrations of 8-oxodGuo detected in this study (~0.016 ng/mL) for healthy adults were similar to the findings obtained by HPLC-ECD and LC-MS/MS (~ 0.013-0.040 ng/mL) (see Table 2), whereas they were considerably lower than those obtained by ELISA kits (up to ~5.9 ng/mL) (Pan et al. 2008; Sato et al. 2010). A similar discrepancy was also noted for salivary 8-oxodGuo analysis (~0.016 ng/mL in this study vs. up to 42.7 ng/mL by ELISA kit) (Su et al. 2009). The latest report by the European Standards Committee on Urinary (DNA)

Lesion Analysis (ESCULA) clearly demonstrates that ELISA kits are not specific for 8-oxodGuo in urine, giving urinary 8-oxodGuo values 2–6 times higher than those measured by chromatographic methods (Barregard et al. 2013). This study further suggested that the overestimation of 8-oxodGuo by ELISA assay may be more severe in plasma or saliva samples by over hundred times higher than those measured by chromatographic methods. Such phenomena could be due to extremely low concentrations of 8-oxodGuo, coupled with the presence of high molecular weight compounds, such as carbohydrates or proteins, which may interfere with the ELISA, as suggested elsewhere (Evans et al. 2008).

Despite the clear provenance of 8-oxoGua in body fluids, from the BER of DNA, its presence in body fluids has been much less studied, compared to 8-oxodGuo. Possible contributions from diet or RNA notwithstanding, this could be partly related to analysis of 8-oxoGua being more technically challenging than 8-oxodGuo. Furthermore, no ELISA kit exist for 8-oxoGua, whereas a number of commercial kits are available for 8-oxodGuo, which are relatively cheap and do not require specific technical skills or equipment. Nevertheless, recent studies have suggested that urinary excretion of 8-oxoGua is a promising biomarker of oxidatively damaged DNA. For instance, Loft et al. (2012) showed an increased risk of developing lung cancer among non-smokers with high excretion of 8-oxoGua. Increased 8-oxoGua excretion has been associated with exposure to air pollution, toxic metals, tobacco smoke and low plasma antioxidant levels (Suzuki et al. 1995; Yoshioka et al. 2008; Foksinski et al. 2007). Svoboda et al. (2006) showed that urinary excretion of 8-oxoGua was associated with life span and further suggested that urinary

8-oxoGua may be a better marker of oxidative stress than urinary 8-oxodGuo. In the present study, in addition to urinary 8-oxoGua, we also reported mean baseline concentrations of 8-oxoGua in plasma and saliva (Table 2), with this being the first report of salivary 8-oxoGua levels in the literature.

It was noted that the mole ratios of 8-oxoGua to 8-oxodGuo varied greatly among the three body fluids (see Table 1). For instance, the mole ratio of 8-oxoGua to 8-oxodGuo was 23.5:1 in plasma whereas it was lower (5.8:1) in urine. This suggested that 8-oxodGuo was excreted more efficiently from blood by the kidney than 8-oxoGua; the CF of 58 and 238 can be obtained for 8-oxoGua and 8-oxodGuo, respectively, by dividing the mean 8-oxoGua (or 8-oxodGuo) concentration in urine by its mean concentration in plasma (Supplementary Data III, Fig. S4).

Interestingly, the mole ratio of 8-oxoGua to 8-oxodGuo was much higher in saliva (217:1) than that of in plasma (23.5:1). Because the 8-oxodGuo concentrations in saliva were similar to those of in plasma (CF: 0.6), we propose that passive diffusion could be the major transport route for 8-oxodGuo passing between plasma and saliva. Although a recent review has suggested that endogenous and exogenous compounds transferred into saliva by passive diffusion show the strongest correlation between plasma and saliva concentrations (Michalke et al. 2014), such correlation between plasma and saliva concentrations for 8-oxodGuo was not observed in our study. One of reasons could be possibly due to that 8-oxodGuo is weakly acidic with pKa of 8.6 (Culp et al. 1989), and small changes of salivary pH during sampling will affect ionization and the distribution of such weak acid across the epithelial membrane (Haeckel 1993), influencing the

correlation between plasma and saliva concentrations. In contrast to 8-oxodGuo in saliva, much higher concentrations of 8-oxoGua were found in saliva with a CF of 4 (Supplementary Data III, Fig. S4), implying a different transport mechanism for 8-oxoGua. There may be three possible explanations for this; (i) a proportion of 8-oxoGua in saliva could derive from BER in cells of the oral mucosa, (ii) part of 8-oxoGua in saliva might also result from the degradation of oral mucosa cells during cell turnover since turnover rate of oral mucosa is approximately 2-3 times higher than that of epidermis of the skin (Winning and Townsend 2000), and (iii) 8-oxoGua is actively transported from plasma to saliva. It is difficult to prove that the BER reaction of oral mucosa could contribute to the 8-oxoGua in saliva. To test the other two hypotheses, two experiments were conducted (Supplementary Data IV and V). As DNA, released during cell turnover, might be further degraded to nucleobases by processes present in saliva, we therefore incubated calf thymus DNA (or 8-oxodGuo standard) with saliva at 37 °C for 4 h to investigate whether the enzymes in saliva are able to degrade the calf thymus DNA (or 8-oxodGuo standard) into nucleobases and consequently release 8-oxoGua. The results showed that after a 4 h incubation at 37 °C 8-oxoGua concentrations in saliva spiked with calf thymus DNA (or 8-oxodGuo standard) were similar to those of saliva without spiking (Supplementary Data IV, Table S3). This result demonstrated that if oral mucosal cells were degraded during cell turnover, the enzymes in saliva are not able to release 8-oxoGua from DNA (or 8-oxodGuo) and therefore could not contribute the 8-oxoGua in saliva. To explore the final hypothesis, we measured the structural analogs of 8-oxoGua and 8-oxodGuo, respectively, namely guanine (Gua, the native nucleobase) and 2′-deoxyguanosine (dGuo, the

native 2′-deoxyribonucleoside) in plasma, saliva and urine, to investigate whether the structural analogs of 8-oxoGua or 8-oxodGuo were higher in saliva than that of in plasma, implying an active transport mechanism. Interestingly, the result (Supplementary Data V, Table S4) showed that the mean concentration of Gua in saliva (mean: 59 ng/mL) was also higher than that of in plasma (mean: 16 ng/mL) with a CF of 3.7 (Supplementary Data V, Fig. S5), which was similar to that observed for 8-oxoGua (CF: 4, Supplementary Data III, Fig. S4). These results implied that both native and oxidized Gua are actively transported from plasma to saliva. Meanwhile, we found that the mean concentration of Gua (59 ng/mL, Supplementary Data V, Table S4) is only 69-times higher than 8-oxoGua (0.85 ng/mL in Table 1) in saliva. This result also supports our above finding that the degradation of oral mucosa cells cannot be the primary source of salivary 8-oxoGua; otherwise the ratio between native and oxidized bases would be similar to that seen in cells, reportedly anywhere between 0.3-6 8-oxoGua/10^6 Gua (Gedik et al. 2005; Dziaman et al. 2009). Taken together, the most likely explanation for the high concentration of 8-oxoGua measured in saliva could be due to the active transportation for 8-oxoGua (and Gua) from plasma to saliva, but the possibility cannot be ruled out that 8-oxoGua in saliva could originate from BER in the cells of the buccal cavity.

As shown in Fig. 2, we noted that the distribution range of 8-oxoGua concentration in plasma was narrow but became relatively wide in urine when adjusted for urinary creatinine. However, this phenomenon was not observed for 8-oxodGuo as the concentration distributions in plasma and urine displayed a comparable distribution range**.** Moreover, Fig. 3A showed only a modest

correlation between 8-oxoGua concentration in plasma and in urine adjusted with urinary creatinine ($r = 0.291$; $P = 0.028$), whereas a strong correlation was observed for 8-oxodGuo concentration in plasma and in urine adjusted with urinary creatinine, as shown in Fig. 3B ($r = 0.737$; $P < 0.001$). The above findings indicated that the use of urinary creatinine to adjust urinary flow for urinary 8-oxoGua concentration could be questionable, but it is appropriate for urinary 8-oxodGuo concentration. This result, showing that the suitability of using creatinine concentration for urinary 8-oxodGuo, is consistent with a previous finding reported by Barregard et al. (2013). The inapplicability of creatinine for urinary 8-oxoGua correction may also explain why a satisfactory correlation between 8-oxoGua and 8-oxodGuo in urine was only observed without adjustment for urinary creatinine (Fig. 4C). There was no correlation between 8-oxoGua and 8-oxodGuo after adjustment for urinary creatinine (data not shown, $P = 0.09$).

Conclusion

We have successfully quantified both 8-oxoGua and 8-oxodGuo in plasma, saliva and urine samples using online SPE LC-MS/MS. Among these three biomatrices, urine, being non-invasive, easy to collect and handle (less biohazard than blood sample), together with its direct measurement should be considered the best specimen, especially in large-scale studies. Alternatively, plasma samples may also be considered applicable as the concentrations of 8-oxoGua (or 8-oxodGuo) in plasma were significantly correlated with those in urine (see Fig. 3) although additional sample pretreatment is required. However, a previous report has suggested that plasma levels of

8-oxodGuo are not valid measures of oxidative stress (Poulsen et al. 2014). The authors state that because the level in plasma is determined mainly by the kidney function and not by the level of oxidative stress. Therefore when comparing different individuals the measure will not provide information about oxidative stress, but kidney function. We suggest that because molecules are excreted into the urine mainly by an active transport mechanism, impaired kidney function will not only influence the plasma level but also the urinary level, even a 24 h urine sample is collected. Therefore, if the kidney argument holds true for plasma, then it also applies to urine. This reiterates the importance of kidney function and determining urinary creatinine values as a surrogate for function. Saliva samples are not recommended for oxidative stress assessment in this study, because the source of oxidative DNA lesion products in saliva is not fully understood so far, especially for 8-oxoGua. As the concentrations of 8-oxoGua are highly correlated with 8-oxodGuo either in plasma or in urine (see Fig. 4), both lesion products and matrices should be considered to be good biomarkers of oxidative stress in humans. The greater 8-oxoGua concentration in plasma and urine (compared to 8-oxodGuo) is an additional advantage to its accurate measurement, but concerns remain over its correction by creatinine in urine. In addition to providing the analytical techniques in plasma and saliva analysis, the findings obtained in this study may help to add new insights into the transport of oxidatively DNA lesions between cellular compartments and biological matrices.

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Conflicts of Interest

None of the authors have any conflict of interest in relation to publication of this paper

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Table 1 Demographic characteristics and mean 8-oxoGua/8-oxodGuo concentrations of study subjects

Variable	Subjects (n = 57)
Age (y)	30.2 ± 6.9
BMI (kg/m^2)	23.7 ± 5.4
In plasma (ng/mL),	
8-oxoGua	0.21 ± 0.09
8-oxodGuo	0.016 ± 0.005
8-oxoGua/8-oxodGuo[a]	23.5 ± 10.4
In saliva (ng/mL),	
8-oxoGua	0.85 ± 0.76
8-oxodGuo	0.010 ± 0.007
8-oxoGua/8-oxodGuo	217 ± 127
In urine (ng/mg creatinine),	
8-oxoGua	11.9 ± 4.1 (12.2 ± 6.5)[b]
8-oxodGuo	3.6 ± 1.3 (3.8 ± 2.5)
8-oxoGua/8-oxodGuo	5.8 ± 1.9

[a] The ratio expressed in mol/mol

[b] as expressed in ng/mL

Table 2 Concentrations of 8-oxoGua and 8-oxodGuo in various biomatrices for healthy subjects in the literature, as measured by chromatographic based methods.

Matrix	8-oxoGua	8-oxodGuo	Methods	References
Plasma/serum	-[a]	0.0134 ± 0.002	HPLC-ECD	Bogdanov et al. (1999)
(ng/mL)	0.160	-	HPLC-ECD	Shin et al. (2001)
	-	0.022 ± 0.009	Online SPE LC-MS/MS	Hu et al. (2010b)
	-	0.040 ± 0.014	UPLC-MS/MS	Lam et al. (2012)
	0.21 ± 0.09	0.016 ± 0.005	Online SPE LC-MS/MS	This study
Saliva	-	0.0153 ± 0.003	HPLC-ECD	Bogdanov et al. (1999)
(ng/mL)	-	0.005 ± 0.003	Online SPE LC-MS/MS	Hu et al. (2010b)
	0.85 ± 0.76	0.010 ± 0.007	Online SPE LC-MS/MS	This study
Urine	11 ± 2.4	3.9 ± 2.0	HPLC-ECD	Svoboda et al. (2006)
(ng/mg creatinine)	11.4	3.5	HPLC-GC/MS	Obtulowicz et al. (2010)
	11.9 ± 4.7	4.4 ± 2.1	Online SPE LC-MS/MS	Hu et al. (2010a)
	11.9 ± 4.1	3.6 ± 1.3	Online SPE LC-MS/MS	This study

[a] Not measured

Figure legends

Fig. 1. LC-MS/MS coupled with online SPE chromatograms for 8-oxoGua (A-C) and 8-oxodGuo (D-F) in human saliva. Selected reaction-monitoring transitions of (A) *m/z* 168→140 and (B) *m/z* 168 →112 for 8-oxoGua, (C) *m/z* 173→145 for [$^{15}N_5$]-8-oxoGua, (D) *m/z* 284→168 and (E) *m/z* 284→ 140 for 8-oxodGuo, and (F) *m/z* 289→173 for [$^{15}N_5$]-8-oxodGuo. cps, counts per second.

Fig. 2. Distribution of 8-oxoGua and 8-oxodGuo concentrations in urine, plasma and saliva.

Fig. 3. Correlations between plasma and urine concentrations for (A) 8-oxoGua and (B) 8-oxodGuo.

Fig. 4. Correlations between 8-oxoGua and 8-oxodGuo in (A) plasma, (B) saliva and (C) urine.