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Quantitative determination of urinary 8-oxo-7, 8-dihydro-2′-deoxyguanosine, 8-oxo-7,8-dihydroguanine, 8-oxo-7,8-dihydroguanosine, and their non-oxidized forms: daily concentration profile in healthy volunteers

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Abstract

We developed a new method for the simultaneous quantitative determination of 8-oxo-7,8-hydro-2′-deoxyguanosine (8-oxodGuo), 8-oxo-7,8-dihydroguanine (8-oxoGua), 8-oxo-7,8-dihydroguanosine (8-oxoGuo), and the corresponding non-oxidized forms, 2′-deoxyguanosine (dGuo), guanine (Gua) and guanosine (Guo), in human urine samples by liquid chromatography–tandem mass spectrometry. Differences in the ionization of analytes in different urine batches with variable matrix effects were effectively compensated for by internal standardization with stable isotope-labelled analytes. The method was sensitive enough to allow the determination of background levels of these biomarkers and was applied to characterize the inter- and intraindividual variability of biomarkers in the diurnal profile of concentrations in 24 healthy volunteers. When normalized for creatinine, none of the biomarkers was affected by sampling time, thus ruling out any circadian rhythm for nucleic acid oxidation in urine.

Keywords: *Nucleic acid oxidation; 8-oxo-7,8-dihydro-2′-deoxyguanosine; 8-oxo-7,8-dihydroguanine; 8-oxo-7,8-dihydroguanosine; LC-MS/MS; matrix effects; creatinine*

Introduction

Under conditions of oxidative stress, nucleic acids and the pool of nucleotides are vulnerable to oxidative attack by reactive oxygen species (ROS). An imbalance between the production and detoxification of ROS may occur during normal cellular metabolism and after exposure to exogenous agents, including ionizing radiation, tobacco smoke and other oxidizing chemicals (Cooke et al. 2003). 8-Oxo-7,8-dihydroguanine (8-oxoGua) is the most abundant oxidation product (Sekiguchi & Tsuzuki 2002), due to the low oxidation potential of guanine at the C-8 position (Steenken & Jovanovic 1997). Its mutagenic potential (Cheng et al. 1992) and the availability of suitable analytical methods boosted the determination of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) in biological matrices, such as blood cells, urine and cultured cells, with high sensitivity (Kasai 1997) in most of the studies dealing with the genotoxic consequences of oxidative stress. Although oxidized guanine in DNA from blood and tissues is a well-recognized biomarker of oxidative damage, the invasiveness of sampling procedures

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and the risk of artefactual oxidation during sample storage and DNA extraction (ESCODD 2000, Collins et al. 2004) have limited its application in large-scale human studies. More recently, several studies highlighted the role of different repair pathways leading to the formation of extracellular oxidized guanine derivatives, including 8-oxodGuo, 8-oxoGua and 8-oxo-7,8-dihydroguanosine (8-oxoGuo) (Lunec et al. 2002, Cooke et al. 2005). All these products are detectable in urine – with reduced sample manipulation and reduced risk of pre-analytical artefacts compared with blood and tissues – and are candidate biomarkers of nucleic acid oxidation and repair. Although the debate about the measurement and the sources of urinary oxidized guanine species in urine is still open (Cooke et al. 2008), it is known that urinary 8-oxoGua originates, at least in part, from the glycosylase activity of the base excision repair (BER) system on oxidized guanine residues of DNA. On the other hand, urinary concentrations of 8-oxodGuo may reflect either the repair of oxidized 2′-deoxyguanosine triphosphate in the cellular 2′-deoxyribonucleotide pool by the Nudix hydrolase MTH1 along with others with similar activities (Tsuzuki et al., 2001), or the repair of 8-oxodGuo from DNA by an endonuclease/nucleotidase-based DNA repair system (Bessho et al. 1993) or even repair by the nucleotide excision repair (NER) system (Patel et al. 2007, Reardon et al. 1997). Finally, 8-oxoGuo may originate from oxidized guanine in RNA, probably as a result of its turnover, rather than as a product of RNA repair mechanisms, that have not yet been well characterized (Nunomura et al. 2006). The turnover or repair of RNA may also be responsible for the generation of extracellular 8-oxoGua (Evans & Cooke 2004).

8-OxodGuo, and more recently also 8-oxoGua and 8-oxoGuo, have been considered as biomarkers of oxidative stress and have been associated with age-related diseases (Olinski et al. 2007) and occupational exposure to chemicals (Pilger & Rudiger 2006). Nevertheless, their application in epidemiological studies requires the characterization of both inter- and intraday variability, the background levels and the kinetics of excretion. The native non-oxidized species, i.e. guanine, guanosine and 2′-deoxyguanosine (Gua, Guo, dGuo) are excreted in urine together with the oxidized moieties. Although the biological meaning of these urinary markers is still unclear, their quantification could provide useful data to understand more clearly the role and the influence of some pathways, like cell death and cell turnover, as suggested by the European Standards Committee on Urinary (DNA) Lesion Analysis (ESCULA) (Cooke et al. 2008).

Several methods have been described for the determination of urinary 8-oxodGuo, including high-performance liquid chromatography (HPLC) with electrochemical detection, gas chromatography–mass spectrometry (GC-MS), liquid chromatography–(tandem) mass spectrometry LC-MS(/MS), and immunoassay (reviewed in Peoples & Karnes 2005). In the past decade, methods based on the use of MS have been extensively used to quantify 8-oxodGuo in biological samples to increase both sensitivity and selectivity of analytical determinations (Ravanat et al. 1998, Renner et al. 2000, Weimann et al. 2001, 2002, Lin et al. 2002, Sabatini et al. 2005, Harri et al. 2007, Malayappan et al. 2007) and as reference methods to assess the performance of simpler analytical approaches such as enzyme-linked immunosorbent assay (ELISA) (Cooke et al. 2006, 2009, Evans et al. 2008). LC-MS/MS has been demonstrated to be a powerful technique for the quantitative determination of biomarkers in complex biological fluids, like blood and urine, without requiring derivatization and extensive sample manipulation. Nevertheless, it is well know that the accuracy of LC-MS/MS could be affected by matrix effects (Manini et al. 2004, Van Eeckhuat et al. 2009). Two different types of matrix effect have been described, an 'absolute matrix effect' (defined as the ratio of the response of a standard present in a sample extract from one single matrix batch to the response of a standard in a neat solution), and 'a relative matrix effect' (defined as the comparison of matrix effect values between different batches of biofluids). In quantitative bioanalysis, where (pooled) blank matrix is applied in the production of calibration standards, the relative matrix effects are of primary concern and should be investigated as part of the development and validation of a bioanalytical method (Van Eeckhuat et al. 2009). Therefore, during the development of an analytical method for quantitative LC-MS(/MS) bioanalysis, it is necessary to generate data that allow the detection and quantification – or the exclusion – of a relative matrix effect ('batch-to-batch') (Matuszewski et al. 2003, 2006), and to ensure that, if existing, it does not affect assay precision, selectivity and sensitivity (Food & Drug Administration 2001).

The aims of the present study were: (1) to develop and validate a novel LC-MS/MS method for the simultaneous determination of 8-hydroxylated guanine derivatives (8-oxoGua, 8-oxoGuo, 8-oxodGuo) excreted in human urine together with the corresponding non-oxidized species (Gua, Guo, dGuo) taking into account matrix effects during method validation; (2) to validate non-invasive biomarkers of oxidative stress for application to molecular epidemiology studies by characterizing their inter- and intraindividual variability in the daily urinary excretion; (3) to evaluate whether these biomarkers of nucleic acid oxidation in spot urine samples are influenced by sampling time; and (4) whether analytical results should be normalized expressing their urinary excretion as a function of urinary creatinine.

Materials and methods

Chemicals

Guanine (Gua, purity ≥98%), guanosine (Guo, ≥98%), 2′-deoxyguanosine (dGuo, 99–100%), 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo, ≥98%), ascorbic acid, sodium hydroxide (NaOH), potassium phosphate monobasic, lithium acetate, ammonium hydroxide, formic acid, hydrogen peroxide (H_2O_2), dimethyl sulfoxide (DMSO), HPLC-grade water and methanol were purchased from Sigma-Aldrich (Milan, Italy). 8-Oxo-7,8-dihydroguanine (8-oxoGua, ≥90%) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo, ≥98%) were from Cayman Chemicals (Ann Arbor, MI, USA). Stable isotope-labelled compounds used as internal standards (ISs), i.e. [^{13}C]Gua (8-^{13}C, 98%), [$^{15}N_5$]Guo (U-$^{15}N_5$, 96-98%, used also as IS for 8-oxoGuo), [$^{15}N_5$]dGuo (U-$^{15}N_5$, 96–98%), and [$^{13}C_1$,$^{15}N_2$]8-oxoGua (8-$^{13}C_1$ 98%,7,9-$^{15}N_2$ 98%) were obtained from Cambridge Isotope Laboratories, Inc. (Cambridge, MA, USA). All the standards were used without further purification.

Synthesis of [15N5]8-oxo-7,8-dihydro-2′-deoxyguanosine standard

[$^{15}N_5$]8-oxodGuo was synthesized from [$^{15}N_5$]dGuo according to Hu et al. (2004), with some modifications. Briefly, 50 µl of [$^{15}N_5$]dGuo (3.75 mmol l^{-1}) were diluted in 1 ml of KH_2PO_4 (0.1 M, pH 4.4), 10 µl of ascorbic acid (0.1 M) and 10 µl of H_2O_2 were added, and the mixture was incubated at 37°C for 4 h. During incubation, the addition of ascorbic acid and H_2O_2 was repeated every 30 min. The solution was eluted on a 1-ml Bakerbond C_{18} SPE cartridge (J.T. Baker Chemical Co., Phillipsburg, NJ, USA) to remove part of the unreacted [$^{15}N_5$]dGuo, ascorbic acid and H_2O_2. The column was preconditioned with 2 ml of methanol, water and KH_2PO_4 (0.1 M). After the sample had been loaded, the column was washed with 1 ml of a KH_2PO_4 (0.1 M)/methanol solution (98/2, v/v) and eluted with 1 ml of a KH_2PO_4 (0.1 M)/methanol solution (85/15, v/v). The yield of the synthetic product was up to 30% for [$^{15}N_5$]8-oxodGuo.

Standard preparation

Standard stock solutions (about 5–10 mM) of Gua, 8-oxoGua and Guo were prepared in NaOH (126 mM), those of 8-oxoGuo and 8-oxodGuo in DMSO, and that of dGuo in water. The same solvents were used for the preparation of the solutions of the isotope-labelled ISs. All these solutions, except that of 8-oxoGua, were stable for up to 6 months. These stock solutions were divided in several aliquots and stored at –20°C. Due to its instability, 8-oxoGua stock solution was prepared weekly, stored at +4°C and its concentration (0.5–5 µM in 10 mM NaOH) was accurately determined by UV spectrometry (λ_{max} = 283 nm, ε = 8200) immediately before use. A fresh working solution containing Gua (75 µM), 8-oxoGua (45 µM), Guo (5 µM), 8-oxoGuo (2.5 µM), dGuo (0.8 µM) and 8-oxodGuo (0.8 µM) was prepared daily in water. This solution was further diluted to prepare the calibration standards. The working solutions containing stable isotope-labelled ISs were prepared daily.

Sample preparation and standard curves

In order to cover two orders of magnitude near the expected biological values reported in the literature (Cooke et al. 2008), calibration standard curves were obtained by spiking a pooled urine sample with standard mixtures at five concentration levels in the ranges reported in Table 1. Urine was thawed and added with an equal volume of an aqueous solution containing different concentrations of ISs (quoted in Table 1). To redissolve a possible precipitate containing the analytes, the sample was kept at 37°C for 10 min, then vortexed and centrifuged at 10 000*g* for 10 min. This procedure is known to release 8-oxoGua and 8-oxodGuo from the precipitate (Weimann et al. 2001). Each calibration level was injected in triplicate. Calibration curves were constructed by linear regression analysis of the area ratios analyte/IS versus the concentration of analytes injected. [$^{15}N_5$]Guo was also used as IS for 8-oxoGuo. As a 'blank' urine pool sample is not available, the detection limits (LODs) and the limits of quantification (LOQs) of all analytes except 8-oxoGuo were determined in the matrix by adding the isotope-labelled standards, and were calculated by using

Table 1. Calibration curve ranges, limits of detection (LODs), limits of quantification (LOQs), intraday and interday precision in urine of the liquid chromatography–tandem mass spectrometry method for the determination of oxidized and non-oxidized guanine derivatives.

	Range	IS	LODs[a]	LOQs[b]	%CV[c]	
Compound	(nM)	(nM)	(nM)	(nM)	Intraday	Interday
8-oxoGua	45–4500	2000	3	5	5.9	6.8
8-oxoGuo	2.5–250	n.a.	0.3	0.75	2.5	4.2
8-oxodGuo	0.8–80	80	0.1	0.75	3.4	5.3
Gua	75–7500	4000	10	25	2.2	4.0
Guo	15–1500	250	1	5	2.8	4.5
dGuo	0.8–80	20	0.1	0.75	3.2	5.1

8-oxoGua, 8-oxo-7,8-dihydroguanine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; Gua, guanine; Guo, guanosine; dGuo, 2′-deoxyguanosine; IS, internal standard.

[a]LOD (signal to noise (S/N) ratio = 3) calculated in urine under selected-reaction monitoring (SRM) conditions; [b]LOQs (S/N ratio = 10) calculated in urine under SRM conditions; [d]coefficient of variation calculated on a spiked urine sample injected six times on the same day for the intraday precision (n = 6) and three times on five different days for the interday precision (n = 15) at three concentration levels corresponding to the lower, the upper and 10-fold the lower value of the calibration range.

a criterion of a signal to noise ratio (S/N) = 3 for LODs and S/N = 10 for LOQs. Due to the lack of the corresponding isotope-labelled standard, the LOD and LOQ of 8-oxoGuo were determined in water. The intra- and interday precision were calculated at three concentration levels (corresponding to the lower, the upper and 10-fold the lower value of the calibration ranges, see Table 1) by determining the concentrations of the analytes in a spiked urine sample injected 6-fold on the same day and 3-fold on five different days, respectively.

The concentration of the guanine derivatives in urine samples were expressed as a function of creatinine concentration, measured by the method of Jaffe (Kroll et al. 1986).

Matrix effects and lithium acetate effect

To evaluate the relative matrix effect of different urine samples on the instrumental response, five urine samples from different subjects were selected on the basis of their creatinine values. Selected creatinine concentrations, spanning the physiologial creatinine range (2.65–26.6 mM), were 3.81, 7.70, 13.63, 18.85 and 26.73 mM. Each urinary sample was added with the standard mixture levels used for the calibration standard curve, diluted with an equal volume of the aqueous solution of the ISs, and injected in triplicate. The results were used to establish the performance of the analytical method, following the protocol proposed by Matuszewski (2006) for exogenous compounds, with some modifications due to the fact analytes are endogenous compounds and blank urine samples are not available. In particular, the absence or presence of a relative matrix effect on the quantification of analytes was assessed by comparing the slopes of the standard lines obtained for the five different samples. To this purpose, the following three parameters were calculated: 'slope CV (%)', 'slope difference (%)', and 'assay CV range (%)'. Slope CV (%) is the precision of standard line slopes constructed in five different batches of a biofluid, expressed as a coefficient of variation. A cut-off value of <3–4% has been suggested to establish if the method is practically free from a significant relative matrix effect (Matuszewski 2006). Slope difference (%) is the maximum percentage difference between the highest and the lowest slope values (divided by the lowest one and multiplied by 100) for standard curves prepared in different batches of a biofluid and corresponds to the maximum difference in the calculated concentration of an analyte in five batches studied that originates from the relative matrix effect. In practice, it represents the difference between the concentration obtained in an assay when an analyte present in one urine sample is analysed and its concentration calculated using a standard line prepared in a different urine batch (control, pooled urines). The larger the values in 'slope difference (%)' are, the more pronounced the relative matrix effect becomes. Finally, 'assay CV range (%)' is the range of coefficient of variation values determined at all concentrations used for constructing standard lines. It represents the overall method precision and should not exceed 8.7% in the absence of relative matrix effects (Matuszewski 2006). As the five urine samples had different contents of analyte(s), the resulting concentrations in spiked samples were not same, thus making impossible the calculation of analyte(s) response %CVs. Therefore, 'assay CV range (%)' was calculated on the response ISs at all concentrations used for constructing standard lines. Slopes of standard lines were determined from the linear regression analysis of the peak area ratio of analyte/IS versus analyte concentrations.

The same set of experiments on five urine batches was repeated by adding the IS mixture dissolved in a 100 mM lithium acetate solution, as described by Bogdanov et al. (1999).

LC-MS/MS conditions

Liquid chromatography was carried out with an Agilent HP 1100 Series HPLC apparatus consisting of a binary pump, a thermostated autosampler and a vacuum degasser. An additional PE Series 200 LC pump (Perkin Elmer) was used for the post-column addition of 0.07 ml min^{-1} of methanol to the chromatographic flow in order to improve the ionization efficiency. The LC system was coupled with a PE-Sciex API 365 triple-quadrupole mass spectrometer (Sciex, Concord, Canada) equipped with a TurboIonSpray™ interface (TISP). A Power Macintosh G3 computer was used for instrument control, data acquisition and processing. Chromatography was performed on an Atlantis® dC_{18} column (100 × 2.0 mm i.d., 3 µm; Waters, Milford, MA, USA) using variable proportions of 10 mM aqueous formic acid (pH 3.75) and methanol mixture as the mobile phase. Elution programme: 0% methanol, hold for 2.5 min; from 0% to 10% methanol in 7.5 min (linear gradient); 10% methanol, hold for 1 min; from 10% to 80% methanol in 2 min (linear gradient); 80% methanol, hold for 1 min; then back to the starting condition in 1 min and re-equilibration for 10 min. The flow-rate was 0.2 ml min^{-1}. The injection volume was 30 µl and each analysis required 24 min, including the re-equilibration time. The first (0–3 min) and the last (22–24 min) parts of the chromatographic run were diverted to waste using a 10-port valve (Valco Systems, Houston, Texas, USA). The temperature of the sample cooler in the autosampler was set at 10°C. Both analytes and ISs were ionized in positive ion mode and the detection was obtained in selected-reaction monitoring mode (SRM) after optimization of TISP-MS/MS parameters by infusing a 18 µM solution of each analyte in 80/20 (v/v) aqueous formic acid (10 mM, pH 3.75)/methanol. In the case of Gua and 8-oxoGua, a 10-fold more concentrated solution (180 µM) was used

for parameter optimization. For all analytes, $[M+H]^+$ was selected by first mass filter. After collision activation, the ions corresponding to the protonated nucleobase $[B+H_2]^+$ were selected by the last mass filter. Retention times, SRM transitions and collision energies are summarized in Table 2 for all analytes and ISs.

Sample collection

To evaluate the chronobiology of urinary excretion and both inter- and intraday variability of oxidized guanine derivatives, we recruited 24 healthy non-smoking volunteers (11 male, mean age 34.8 ± 5.4 years). They were asked to collect spot urinary samples at six different times in one single day (at 07.00 and 11.00 a.m., at 3.00, 7.00, 11.00 p.m. and at 07.00 a.m. of the day after) without changing their habits. Urine samples were divided into different fractions and immediately stored at –20°C until analysis, which was carried out within 30 days from collection. All the participating subjects provided their written informed consent and the sampling of biological material was carried out according to the Helsinki Declaration (World Medical Association 1964).

Urinary proteins

In order to account for possible interference of physiological variations in the kidney function, in the same urinary samples we determined the concentrations of two proteins for which the existence of a circadian variation has been demonstrated, e.g. retinol-binding protein (RBP) and albumin. The protein contents were measured by original and validated ELISAa, e.g. RBP by a sandwich ELISA (Lucertini et al. 1984) and albumin by a competitive ELISA (Alinovi et al. 1988). All markers were expressed as a function of urinary creatinine.

Table 2. Time window settings (retention time (RT), selected-reaction monitoring (SRM) transitions and collision energy (eV)) for liquid chromatography–tandem mass spectrometry analysis of guanine derivatives and internal standards.

Time window	Time frame (min)	Compound	RT (min)	SRM transition	eV
1	0–9	Gua	5.34	152 > 135	28
		[^{13}C]Gua		153 > 136	
		8-oxoGua	6.88	168 > 140	21
		[^{13}C, $^{15}N_2$]8-oxoGua		171 > 142	
2	9–22	Guo	13.35	284 > 152	16
		[$^{15}N_5$]Guo		289 > 157	
		8-oxoGuo	14.25	300 > 168	22
		dGuo	14.66	268 > 152	18
		[$^{15}N_5$]dGuo		273 > 157	
		8-oxodGuo	15.35	284 > 168	18
		[$^{15}N_5$]8-oxodGuo		289 > 173	

Gua, guanine; 8-oxoGua, 8-oxo-7,8-dihydroguanine; Guo, guanosine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; dGuo, 2′-deoxyguanosine; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine.

Statistical analysis

Statistical analysis was carried out with the SPSS software (version 15.0 for Windows®, Chicago, IL, USA). Linear regression analysis (Pearson's correlation) was used to construct calibration curves for each analyte. Normal distribution was assessed by the Kolmogorov–Smirnov test for all guanine derivatives, except for 8-oxoGua which followed a log-normal distribution. Data were expressed as mean ± SD for all biomarkers except 8-oxoGua, whose concentrations were expressed as geometric mean (GM) and geometric standard deviation (GSD). Analysis of variance (one-way ANOVA for repeated measures followed by *post-hoc* Tukey test) was performed using the GraphPad Prism 4 software (GraphPad, San Diego, CA, USA); for 8-oxoGua the Friedman test was applied. The reliability of measurements, the homogeneity of the scale and the homoscedasticity of variance were assessed, respectively, by the Cronbach's α (α >0.7), the interclass correlation coefficient (ICC >0.45) and the Mauchly test (p >0.05).

Results and Discussion

Liquid chromatography–mass spectrometry

The LC-MS/MS method allows the determination of the six analytes in authentic human urine samples in a single chromatographic run, as shown in Figure 1. The chromatogram has been divided into two acquisition periods in order to reduce the number of SRM transitions monitored at any given time, and to increase the S/N ratio. The settings for each acquisition period are summarized in Table 2. Compared with the method of Weimann et al. (2002), the only one to propose a method for the separation of both oxidized and non-oxidized guanine derivatives in urine, the chromatographic time was considerably reduced (24 min instead of 50 min) owing to the use of the Atlantis dC18 column, which has a higher retention of polar compounds in reversed-phase chromatography and can also operate in 100% aqueous mobile phases. The post-column addition of methanol was useful to improve the ionization efficiency of the early eluting compounds, Gua and 8-oxoGua, which elute with an almost completely aqueous mobile phase. The peaks of six analytes were baseline separated to avoid overestimation of the oxidized form. As already reported by others (Ravanat et al. 1998, Renner et al. 2000), we observed that about 1% of dGuo was oxidized into 8-oxodGuo in the ion source, but the chromatographic separation and the low

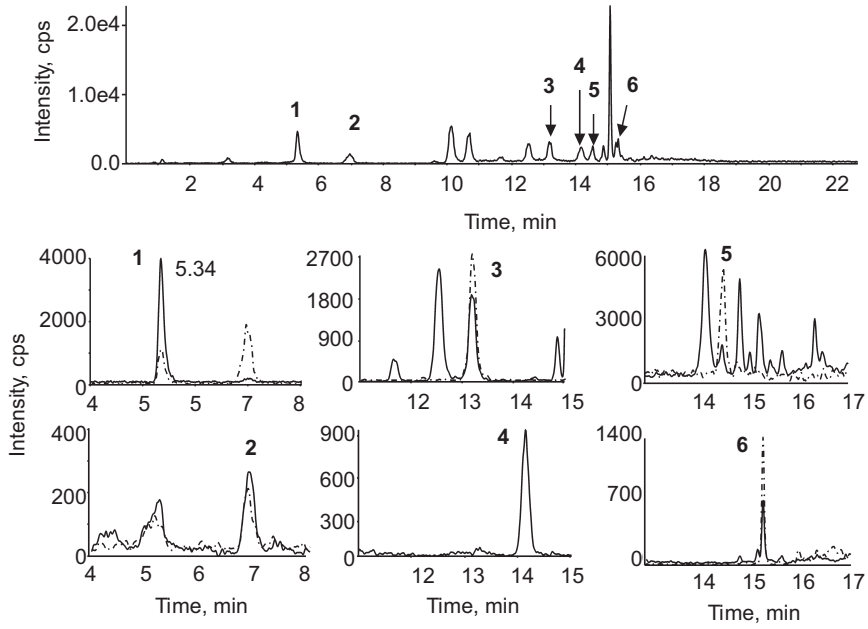

Figure 1. Chromatogram of a urine sample from a healthy subject. The upper part shows the total ion current (TIC), the lower part shows the extracted ion chromatograms of selected-reaction monitoring (SRM) transitions of analytes (continuous line) and the corresponding internal standard (IS) (dotted line). Compound identification and SRM transitions (in parentheses). 1 Gua, guanine (*m/z* 152→135, IS *m/z* 153→136); 2 8-oxoGua, 8-oxo-7,8-dihydroguanine (*m/z* 168→140, IS *m/z* 171→142); 3 Guo, guanosine (*m/z* 284→152, IS *m/z* 289→157); 4 8-oxoGuo, 8-oxo-7,8-dihydroguanosine (*m/z* 300→168); 5 dGuo, 2′deoxyguanosine (*m/z* 268→152, IS *m/z* 273→157); 6 8-oxodGuo, 8-oxo-7,8-dihydro-2′deoxyguanosine (*m/z* 284→168, IS *m/z* 289→173).

dGuo content in urine samples enabled us to avoid the risk of 8-oxodGuo overestimation.

The product–ion mass spectra of oxidized and non-oxidized guanine derivatives were similar to those reported by Weimann et al. (2002) using a API3000 triple quadrupole mass spectrometer, and have been already discussed elsewhere (Ravanat et al. 1998, Sabatini et al. 2005).

Method validation

The LODs and the LOQs determined in urine samples for each analyte are reported in Table 1. Although the LODs were higher than those reported by Weimann et al. (2002) who used a better performing API3000 triple quadrupole mass spectrometer, the sensitivity of the method allowed the quantification of all analytes in all urine samples analysed at the required levels. Despite the post-column addition of methanol, the sensitivity for the nucleobase Gua and its oxidation product 8-oxoGua was 10- to 100-fold lower compared with that of the corresponding nucleoside, the deoxynucleoside or their oxidation derivatives. Gua and 8-oxoGua are relatively more abundant in urine samples, and thus their lower sensitivity was not a limiting factor for the quantitative determination of these compounds.

As a stable isotope-labelled IS is not commercially available for 8-oxoGuo and the synthetic procedure described by others (Malayappan et al. 2007) was not successful, [$^{15}N_5$]Guo was used as IS for both the nucleoside Guo and its oxidized derivative. The retention time difference between Guo and 8-oxoGuo is less than 1 min and the two analytes elute with a very similar mobile phase composition in the middle of the chromatogram, where matrix effects are supposed to be less relevant. The results of the experimental set for the assessment of relative matrix effects showed that [$^{15}N_5$]Guo could be effectively used as IS for 8-oxoGuo (see below).

Due to the fact that a ‘blank’ urine sample is not available, both LODs and LOQs were estimated in the matrix from the response of the corresponding isotopically labelled ISs. The intra- and interday precision calculated by determining the concentrations of the analytes in a spiked urine sample injected 6-fold on the same day and 3-fold on five different days, and expressed as %CV, ranged between 2.2 and 5.9% and between 4.0 and 6.8%, respectively. In both cases, CVs were lower than 15%, which is in accordance with the recommendations by the Food and Drug Administration in the Guidelines for bioanalytical method validation (Food & Drug Administration 2001).

The concentrations of oxidized guanine derivatives determined in this study were of the same order of magnitude of those previously reported by other Authors using methods based on mass spectrometry (reviewed in Cooke et al. 2008). In particular, a good agreement with the results of Olinski and co-workers (Olinski et al. 2007, Foksinski et al. 2007) was found for 8-oxodGuo,

whereas the concentrations of 8-oxoGua reported in this study were about 1.5-fold higher than those obtained by the same authors using a HPLC-GC-MS method. To date, few data have been reported for 8-oxoGuo with some differences between them (Glintborg et al. 2006, Malayappan et al. 2007). Finally, no data exist for non-oxidized guanine derivatives expressed as a function of creatinine concentration and the comparison with the results published by Weimann et al. (2002) expressed as nmol per 24 h could only indicate some consistency in the order of magnitude of biomarker levels.

Matrix effect

The phenomenon known as the matrix effect is due to interference of co-eluting components from the sample matrix in the ionization process of the compound(s) of interest (ionization suppression or enhancement) (Tang & Kebarle 1993). As recommended by FDA guidelines, the evaluation of matrix effects should be part of the development and validation of a bioanalytical method (FDA 2001). To assess quantitatively the relative matrix effect, Matuszewski et al. (2003 and 2006) proposed determination of the slopes of standard curves constructed in five different batches of a biofluid during method validation. This experimental approach is based on the calculation of three parameters, namely 'slope CV (%)', 'slope difference (%)' and 'assay CV range (%)', which are indicative of the absence or presence of a relative matrix effect.

To our knowledge, this is the first method for the LC-MS/MS determination of biomarkers of nucleic acid oxidation in urine where the guidelines suggested by Matuszewski (2006) were applied for the validation of a bioanalytical method. The values of the three parameters calculated for each analyte of interest are summarized in Table 3. After the results published by Bogdanov et al. (1999), sample dilution with a lithium acetate neutral buffer has been widely applied to recover 8-oxodGuo from precipitate (Weimann et al. 2001, Malayappan et al. 2007). On the other hand, we noticed that the lithium acetate buffer generated a high concentration of ions in the TISP ion source, leading to some additional matrix effects, which jeopardized the reproducibility of ionization. For this reason, we performed the set of experiments for matrix effect assessment by diluting urine samples with both the IS mixture dissolved in water ('without lithium acetate') and the IS mixture prepared in a 10 mM lithium acetate buffer ('with lithium acetate'), as proposed by Bogdanov et al. (1999). The results in Table 3 shows that only in the case of samples treated 'without lithium acetate', the precision (slope CV%) of the standard line slopes not exceed 4%, which is the limit value for the method to be considered practically free from the relative matrix effects. Conversely, when samples were diluted 'with lithium acetate', the slope CV% values were significantly higher than 4% (up to 12.3%) for all analytes except 8-oxodGuo. The presence of lithium acetate buffer also led to higher values of the slope difference (ranging from 2.1 to 36.6%) compared with the 'without lithium acetate' treatment (1.1–7.3%), and also decreased the precision of the method, as assessed by an increase in the assay CV range (%). This was particularly true for the early eluting compounds, which generally are most susceptible to matrix effects due to the presence of salts, either naturally occurring in urine or added during sample pretreatment. These results prompted us to avoid the use of lithium acetate buffer for sample dilution, at least with this LC-MS/MS equipment. Moreover, both dilution methods ('without lithium acetate' and 'with lithium acetate') gave identical results in the determination of the endogenous concentration of all six analytes in the five urine batches used for method validation (data not shown), suggesting that any eventual precipitate could be effectively redissolved by warming the sample diluted with water.

The assay CV range (%) values – calculated on the IS responses for the five different urines and at all concentrations used for constructing calibration standard

Table 3. Summary of the bioanalytical method validation data obtained on five different urine batches, according to the protocol proposed by Matuszewski (2006).

	Slope CV (%)[a]		Slope difference (%)[b]		Assay CV range (%)[c]	
Compound	Without LiAc	With LiAc	Without LiAc	With LiAc	Without LiAc	With LiAc
8-oxoGua	2.9	6.9	7.3	20.4	1.3–7.6	1.8–5.2
8-oxoGuo	2.2	4.1	5.0	8.9		
8-oxo-dGuo	0.5	0.9	1.1	2.1	3.9–5.4	1.1–6.1
Gua	2.5	12.3	5.6	36.6	3.1–7.8	2.5–9.0
Guo	3.8	10.7	7.1	28.7	1.4–5.0	1.6–2.9
dGuo	2.7	5.1	6.8	13.5	2.2–3.8	1.0–6.1

8-oxoGua, 8-oxo-7,8-dihydroguanine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; Gua, guanine, Guo, guanosine; dGuo, 2′-deoxyguanosine. LiAc, lithium acetate.

[a]Precision value (coefficient of variation, CV%) of slopes of standard lines constructed in five different urine with creatinine concentrations (3.81–26.73 mM) in the normality range; [b]maximum difference between the highest and the lowest slope values divided by the lowest slope value and multiplied by 100; [c]range of coefficient of variation values (method precision) determined on the internal standards at all concentrations used for constructing standard lines.

lines (Table 3) – were comparable with the CV values calculated at three concentrations but in a single urine sample (reported in Table 1), indicating that the use of stable isotope-labelled compounds as IS effectively eliminates relative matrix effects. In fact, any eventual difference between the two CV values may be indicative of the contribution of relative matrix effect to the CV values obtained in different batches.

Daily profile of concentrations in healthy volunteers

To the best of our knowledge, this is the first study reporting the characterization of the concentration profile of oxidized and non-oxidized guanine nucleobases. From the point of view of occupational toxicology, the interest for oxidized guanine derivatives relies on their potential usefulness as biomarkers of exposure to oxidizing agents (Pilger & Rudiger 2006). On the other hand, urinary lesion levels provide an integrated measure of exposure intensity, endogenous antioxidant defence and inter-individual differences in DNA repair capability. In the validation of a new biomarker, the characterization of its background levels in the general population and the study of the inter- and intraindividual variability should be considered as a prerequisite. In the case of urinary biomarkers, the knowledge of the excretion kinetics in relation with the excretion profile of urinary creatinine (Viau et al. 2004) may be useful for the definition of the right sampling time (WHO 2001) and for a correct data interpretation and expression.

In a recent study on laminators (Manini et al. 2009), styrene-exposed workers showed lower levels of 8-oxodGuo/10^5 dGuo in white blood cell (WBC)-DNA but higher concentrations of U-oxoGuo compared with unexposed workers ($p=0.002$ and $p=0.008$, respectively, *t*-test for independent samples). Moreover, in a subgroup of subjects bearing the *hOGG1*Ser/Ser genotype, laminators showed lower levels of WBC 8-oxodGuo/10^5 dGuo and significantly higher concentrations of U-8-oxoGua than controls ($p=0.07$ and $p=0.01$, respectively). Interestingly, workers showed higher levels of *hOGG1* expression compared with controls ($p<0.0005$). Thus, styrene exposure seems to be associated with oxidation damage to nucleic acids, particularly to RNA and with an induction of the BER system. A possible influence of sampling time on observed differences between exposed subjects and controls seems to be ruled out by the present study, which does not support the existence of a circadian rhythm for guanine derivatives.

Figure 2 shows the diurnal variation of concentrations of six guanine derivatives (expressed as nM) and that of creatinine (expressed in mM). The lower part of each panel shows the mean ± SEM of the concentrations of the selected biomarker determined in spot urine samples collected at each sampling time from all 24 volunteers. In the upper part of each panel, the concentration of each biomarker is expressed as a function of creatinine concentration. As the main limitation of this study, we recognize that the collection of spot urine samples at six fixed times over 24 h without measuring urinary volumes did not allow us to calculate the excretion rates in the various time periods for these biomarkers and to evaluate the possible influence of the urinary flow on excretion rates (Greenberg & Levine 1989). Nevertheless, a striking parallel was apparent between the concentration profile of creatinine and that of all analytes. Although at visual inspection Gua at times T_2 and T_3 seems to be an exception, no significant difference was observed. Such an overlapping trend may be due both to a parallel production during muscular activity (accounting for most of aerobic metabolism in mammals and the main source of creatine catabolism) and to similar renal handling, thus making creatinine normalization appropriate for spot urine sample collection in routine biomonitoring. Moreover, expression of results as a function of creatinine (μmol mol Cr^{-1}) resulted in a reduction in the interindividual variability of biomarkers. In fact, the %CV of oxidized guanine derivatives calculated as expressing concentrations either in nM or as a function of creatinine concentration changed from 35% to 17% for 8-oxoGua, from 25% to 10% for 8-oxoGuo and from 27% to 12% for 8-oxodGuo, respectively. A similar change was observed for the non-oxidized forms, except for Gua for which the two %CV values were 21% and 27%, respectively. Taken together, these results suggest that creatinine normalization of biomarkers in spot urine samples could compensate for the potentially large intra- and interindividual differences in diuresis, as well as for differences in the lean body mass and physical workload.

The concentrations of urinary guanine derivatives in urine from 24 volunteers determined at each sampling time (T_1–T_6) are summarized in Table 4, together with the concentrations of two proteins measured in the same samples, i.e. albumin and RBP, which are known to be excreted with a circadian rhythm (Buzio et al. 1989). Results are expressed as mean ± SD for all biomarkers except 8-oxoGua, which followed a log-normal distribution and is expressed as geometric mean and geometric SD. All the values, expressed as a function of creatinine, were within the reference interval for healthy subjects. The goodness of sampling was confirmed by the existence of a circadian rhythm in the concentration profile of urinary proteins, with the peak (zenith) at two different times, at 7.00 p.m. for RBP (concomitant to switching from light to dark and subsequent crepuscular vision) and at 3.00 p.m. for albumin (concomitant to a renal hyperfiltration period). Conversely, one-way ANOVA for repeated measures (followed by *post-hoc* Tukey test) revealed that none of the guanine derivatives was

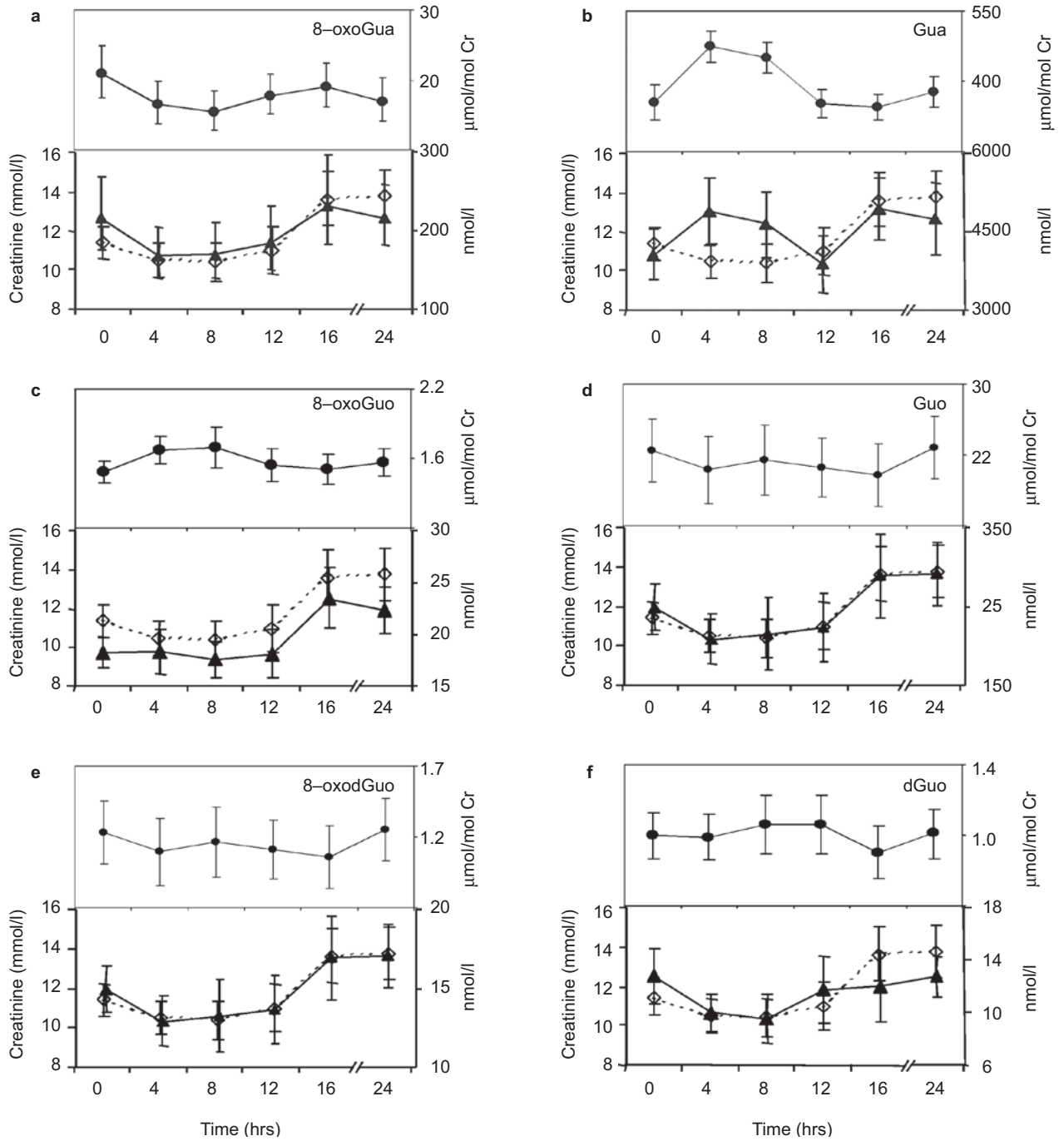

Figure 2. (a–f, lower part) Concentration profiles of creatinine (open diamonds, dotted line, left scales, as mM) and guanine derivatives (closed triangles, continuous line, right scales, as nM) in spot urine samples collected at six fixed times (at 07.00 and 11.00 a.m., at 3.00, 7.00, 11.00 p.m. and at 07.00 a.m. of the day after) from 24 healthy subjects. In the upper graph of each panel (a–f), the concentration profiles of the compound expressed as a function of creatinine is shown (closed circles, continuous line, right scales). Values are reported as mean ± SEM. 8-oxoGua, 8-oxo-7,8-dihydroguanine; Gua, guanine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; Guo, guanosine; 8-oxodGuo, 8-oxo-7,8-dihydro-2′deoxyguanosine; dGuo, 2′deoxyguanosine.

affected by significant variation during the day. This result has an important implication for the biomonitoring practice, as the sampling time does not seem to be critical for the assessment of nucleic acid oxidation in urinary samples.

Finally, the reliability of measurements, the homogeneity of the scale and the homoscedasticity of variance were tested by calculating the Cronbach's α, the ICC and the Mauchly test, respectively. In our set of data expressed as a function of urinary creatinine, the

Table 4. Urinary concentrations of guanine derivatives and proteins in spot samples collected at six different times in one single day from 24 volunteers. Values are reported as the mean ± SD for all biomarkers except 8-oxoGua (reported as geometric mean and geometric SD). Guanine derivatives are expressed as μmol mol creatinine^{-1}, creatinine as mmol l^{-1}, retinol-binding protein (RBP) as μg g creatinine^{-1} and albumin as mg g creatinine^{-1}.

Compound	T_1	T_2	T_3	T_4	T_5	T_6	*p*-Value
8-oxoGua	20.98 (2.24)	16.60 (2.26)	15.49 (2.28)	17.78 (2.11)	19.05 (2.08)	16.98 (2.30)	ns
8-oxoGuo	1.58 ± 0.36	1.83 ± 0.49	1.79 ± 0.71	1.66 ± 0.58	1.61 ± 0.56	1.75 ± 0.74	ns
8-oxodGuo	1.30 ± 0.44	1.43 ± 0.67	1.39 ± 0.53	1.18 ± 0.34	1.23 ± 0.44	1.26 ± 0.63	ns
Gua	355 ± 185	474 ± 159	451 ± 151	353 ± 136	345 ± 123	378 ± 155	ns
Guo	23.4 ± 14.7	21.5 ± 15.5	22.5 ± 15.9	21.7 ± 13.6	21.1 ± 14.1	23.7 ± 15.4	ns
dGuo	1.22 ± 0.61	0.92 ± 0.45	1.05 ± 0.71	1.18 ± 0.86	0.86 ± 0.61	1.02 ± 0.62	ns
Creatinine	11.41 ± 3.75	10.46 ± 3.80	10.37 ± 4.49	10.98 ± 5.59	13.61 ± 6.35	13.73 ± 6.27	ns
Albumin	3.74 ± 3.36$^{\wedge}$	5.12 ± 3.28	7.65 ± 7.49$^{\wedge}$	7.20 ± 5.81	5.14 ± 3.56	3.95 ± 3.46	<0.0002
RBP	33.9 ± 20.0$^{\#}$	37.4 ± 17.6$^{\#,*}$	43.0 ± 17.6$^{\#,*}$	63.2 ± 19.0$^{\#}$	72.7 ± 34.1$^{\#}$	32.49 ± 13.1	<0.0001

8-oxo-Gua, 8-oxo-7,8-dihydroguanine; 8-oxoGuo, 8-oxo-7,8-dihydroguanosine; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; Gua, Guanine; Guo, guanosine; dGuo, 2′deoxyguanosine; RBP, retinol-binding protein.

$^{\#}p<0.001$ T_1 vs T_4, T_1 vs T_5, T_2 vs T_5, T_3 vs T_5, T_4 vs T_6, T_5 vs T_6 for RBP; $^{*}p<0.01$ T_2 vs T_4, T_3 vs T_4 for RBP, $^{\wedge}p<0.05$ T_1 vs T_3, T_3 vs T_5, T_3 vs T_6 for albumin.

values of the Cronbach's α ranged from 0.857 for Gua to 0.973 for Guo, the ICC value was higher than 0.499 for all biomarkers and the Mauchly test showed as not significant for all the guanine derivatives, indicating that our results are reliable and have an internal coherency.

In conclusion, the LC-MS/MS method presented has been developed and validated taking into account the relative matrix effects, which were effectively compensated by internal standardization with isotope-labelled internal compounds. Moreover, the method showed adequate sensitivity and selectivity for quantitative determination of oxidized and non-oxidized guanine derivatives in human urine samples. Biomarkers of nucleic acid oxidation, determined in spot urine samples collected from 24 volunteers at 4-h time intervals in one single day, showed a high inter- and intraindividual variability, although none of the biomarkers was affected by significant variation during the day, as assessed by one-way ANOVA. Variability of biomarkers was significantly reduced by expressing their concentration as a function of the creatinine concentration (μmol mol Cr^{-1}). On the other hand, the parallel between the daily profile of concentrations of biomarkers and that of urinary creatinine suggests the applicability of normalizing biomarkers for creatinine concentration. Although the calculation of the 24-h excretion rate would be necessary to rule out a circadian rhythm in the excretion of biomarkers, our data seem to indicate that the sampling time is not very critical for the assessment of nucleic acid oxidation in urine.

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Declaration of interest

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