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Original Contribution

Automated method for the direct analysis of 8-oxo-guanosine and 8-oxo-2′-deoxyguanosine in human urine using ultraperformance liquid chromatography and tandem mass spectrometry

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ABSTRACT

The potential use of oxidative stress-induced DNA and RNA damage products as biomarkers is an important aspect of biomedical research. There is a need for assays with high specificity and sensitivity that also can be used in molecular epidemiology studies with a large number of subjects. In addition there is a need for assays that can measure more than one product from DNA oxidation. We present a sensitive, precise, and accurate method for quantitative analysis of the oxidized nucleosides 8-oxoGuo and 8-oxodG in human urine. The assay is based on automated sample handling using a BIOMEK 3000 Workstation, and UPLC-ESI(+)-MS/MS analysis. High specificity is evidenced by the use of qualifier ions for both analytes. The quantification limit in urine samples is 1 nM for both analytes. Accuracy and precision were documented, showing average recoveries of 106.2% (8-oxoGuo) and 106.9% (8-oxodG), and overall precision (within-day and between-days) of 6.1 and 4.4%, respectively.

Introduction

Free oxygen radicals and other reactive oxygen species (ROS) are a major source of damage to cellular components. Oxidative damage to macromolecules, such as proteins, lipids, and DNA, can result in cytotoxic effects and has been associated with development of several common age-related diseases, e.g., cancer and diabetes, representing important health issues in the Western world [1]. RNA damage has not received similar attention [2], but has recently been demonstrated to occur at higher rates [3]. Identification of proper biomarkers of oxidative damage to macromolecules would afford information on the predisposition and prognosis of certain pathologies. Specific and sensitive tools that can analyze a large number of samples are essential for evaluating the predictive value for developing diseases as well as the effect of various interventions in diseases associated with oxidative stress.

As a result of repair and degradation to individual nucleosides 8-oxodG and 8-oxoGuo are excreted into the urine and can be used to investigate the body burden of DNA and RNA damage, respectively [3], and has as such been used to investigate the effects of, e.g., brussels sprout, exercise, olive oil, smoking, and smoking cessation in a number of different intervention trials.

The urinary excretion of, e.g., 8-oxodG is dependent on a functional DNA repair mechanism. It can be calculated that if no DNA repair functions were functional, 1–5% of human DNA would be oxidized within a short period of years, and this is not compatible with living. In previous in vivo experiments, we showed that the carcinogen nitropropane induced high tissue levels of oxidized DNA that were repaired within 24 h, and that the increase in the number of oxidized 8-oxodG moieties in tissue corresponds very closely to the amount excreted into urine [4]. This indicates that the origin of urinary 8-oxodG is from tissue DNA. In most situations concerning humans, the tissue levels are stable within the period of investigation, e.g., a disease like hemochromatosis or diabetes [5]. The excretion into urine of oxidized species will consequently balance the number formed per time unit, e.g., 24 h. Changes in repair will lead to changes in tissue levels, but as soon as a new steady-state situation has been established, the number of oxidized species formed or excreted will again be balanced. Urinary excretion is therefore a measure of "oxidative stress" to DNA, and is independent of changes in DNA repair.

Abbreviations: 8-oxodG, 8-oxo-2′-deoxyguanosine; 8-oxoGuo, 8-oxo-guanosine; ISTD, internal standard.

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It should be recognized that this is very different from the measurement of tissue levels, which are influenced by formation rate as well as repair rate and only can be interpreted as "oxidative stress" provided that the repair rate is unchanged.

Various analytical techniques have been used for 8-oxodG quantification, including HPLC with electrochemical detection (HPLC-ECD), gas chromatography–mass spectrometry (GC-MS), and ELISA [6]. The analytical procedures for urinary adducts are challenging indeed. Complex sample cleanup is often required due to interferences inherent to the urine matrix, where the presence of many potentially interfering substances in concentrations far greater than those of the adducts set demands for high selectivity and low limit of quantification for unequivocal identification and quantification.

Liquid chromatography–tandem mass spectrometry (LC-MS/MS) is a powerful technology that can overcome the sensitivity and selectivity issues in analysis of DNA and RNA adducts. Substituting HPLC with UPLC provides more narrow and higher peaks and thus improved sensitivity. This opens up the possibility of including a qualifier ion for ultimate specificity, and for automated integration and quantification, even at very low concentration levels.

There is a need for assays with high specificity and sensitivity that also can be used in molecular epidemiology studies with a large number of subjects. In addition there is a need for assays that can measure more than one product from oxidation of nucleic acids.

The present method based on UPLC-ESI(+)-MS/MS fulfills these demands. Moreover, the method is fast and automated to a degree that makes is suitable for use in molecular epidemiology, i.e., can be applied to a large number of samples.

Materials and methods

Chemicals

Glacial acetic acid and acetonitrile were from Merck KgaA (Darmstadt, Germany) and 25% aqueous ammonia was supplied from J.T. Baker (Deventer, the Netherlands). Purified water was obtained from a Milli-Q Gradient A10 system (Millipore, Bedford, MA). All other solvents were of analytical grade. Lithium acetate dihydrate was from Sigma-Aldrich Co. Ltd. (Steinheim, Germany).

8-oxoGuo was purchased from BioLog (Bremen, Germany), 8-oxodG was purchased from Berry & Associates (Dexter, MI), and their respective internal standards $^{15}N_5$-8-oxodG and $^{15}N_5$-8-oxoGuo were synthesized as previously reported [3]. Concentrations of the stock solutions were determined by measuring the UV absorbance in water (8-oxoGuo; $\varepsilon_{293\ nm} = 10{,}300\ M^{-1}cm^{-1}$ [7], and 8-oxodG; $\varepsilon_{293\ nm} = 10{,}300\ M^{-1}cm^{-1}$ [8]). Stock solutions of each analyte were prepared in water and stored at −80°C. A 1 μM aqueous working solution of the two of the analytes was prepared frequently and stored at −20°C in Eppendorf tubes at 500 μL. At the day of analysis, a tube of 1 μM standard was thawed and used for preparation of calibration standards in the concentrations 1.0, 3.0, 10.0, 20.0, 40.0, and 60.0 nM. Working solution containing the two internal standards (50 nM) was prepared in 100 mM lithium acetate buffer, pH 6.4, and stored at −20°C. QC samples were prepared from a pool of urine samples, which was thoroughly mixed and stored at −20°C in portions of 500 μL. The analyte concentrations in the QC sample were19.5 nM of 8-oxoGuo and 15.9 nM of 8-oxodG.

Table 1
UPLC gradient program

Time (min)	Flow (mL/min)	%A	%B
0.0	0.20	100	0
0.5	0.20	100	0
15.0	0.20	93	7
16.0	0.20	76	24
17.0	0.20	10	90
17.6	0.30	10	90
19.0	0.30	10	90
20.0	0.25	100	0
20.5	0.20	100	0
23.0	0.20	100	0

Eluent A: 2.5 mM ammonium acetate, pH 5. Eluent B: acetonitrile.
Column temperature was 1°C.

Table 2
Setting of the mass spectrometer

Parameters	8-oxoGuo	8-oxodG
Ionspray voltage (V)	4500	4500
Declustering potential (V)	37	25
Focusing potential (V)	320	230
Entrance potential (V)	6.3	8.1
Focusing lens 1 (V)	-7,7	-7.3
Prefilter (V)	-14.6	-18.1
Focusing lens 2 (V)	-27.0	-20.0
Collision energy (eV)	20 (m/z 168)	19 (m/z 168)
	45 (m/z 140)	41 (m/z 140)
Collision cell exit potential (V)	8.2	8.4

Instrumentation

The automated sample handling was performed on a Biomek 3000 robot (Beckman Coulter, CA, USA) equipped with a 200 μL single channel tool (P200) and an eight-channel 200 μL tool (MP200).

The chromatographic separation was performed on an Acquity UPLC system (Waters, Milford, MA, USA), which was equipped with a binary solvent delivery manager, and a sample manager. The used column was an Acquity UPLC BEH Shield RP18 column (1.7 μm, 2.1 × 100 mm) protected with in-line filter (4 × 2 mm, 0.2 μm) both obtained from Waters. The mobile phase was (A) 2.5 mM ammonium acetate, adjusted to pH 5.0 with glacial acetic acid, and (B) acetonitrile. The UPLC gradient program is given in Table 1. Constant column temperature (1°C) was achieved using a Comfort Heto Chill Master (Holm & Halby, Brøndby, Denmark). A sample volume of 50 μL was injected on the column.

The MS detection was performed on an API 3000 triple quadrupole mass spectrometer (Sciex, Toronto, Canada) equipped with an ESI ion source (Turbospray) operated in the positive mode. The UPLC/MS system was controlled by Analyst ver. 1.4.2, with the additional program ICON, Acquity 1.30 from Waters. The vaporizer temperature was 450°C. Nitrogen was used as nebulizer, auxiliary, and collision gas. A second pump, PE200 micro (Perkin Elmer, Norwalk, CT, USA), delivered a postcolumn flow of acetonitrile (150 μL/min) to enhance the electrospray ionization (subsequently, acetonitrile was substituted with methanol, due to the global temporary shortage of acetonitrile). Interface settings were manually optimized at the LC conditions prevailing when the analyte in question elutes into the MS; see Table 2 for details. Detections were performed in MRM mode; "high resolution mode" corresponding to a peak width of 0.5 amu at half the maximum peak height (0.5 FWHM) was used in the first quadrupole (Q1) and a peak width of 0.4 amu in the second quadrupole (Q3). The MS/MS transitions selected for 8-oxoGuo were m/z 300→168 (250 ms) and m/z 300→140 (250 ms); the corresponding transition for $^{15}N_5$-8-oxoGuo was m/z 305→173 (100 ms) and m/z 305→145 (200 ms). The MS dwell time is shown in parentheses. The MS/MS transition selected for 8-oxodG was m/z 284→168 (250 ms) and 284→140 (250 ms); the corresponding transition for $^{15}N_5$-8-oxodG was m/z 289→173 (100 ms) and m/z 289→145 (200 ms). Product ion mass spectra of the two analytes are shown in Fig. 1. To reduce contamination of the ion source, an automated switching valve (6-port 2-position) from VICI (Schenkon, Switzerland) was used to divert the eluent fraction that contained the analytes into the mass spectrometer, and everything else to waste.

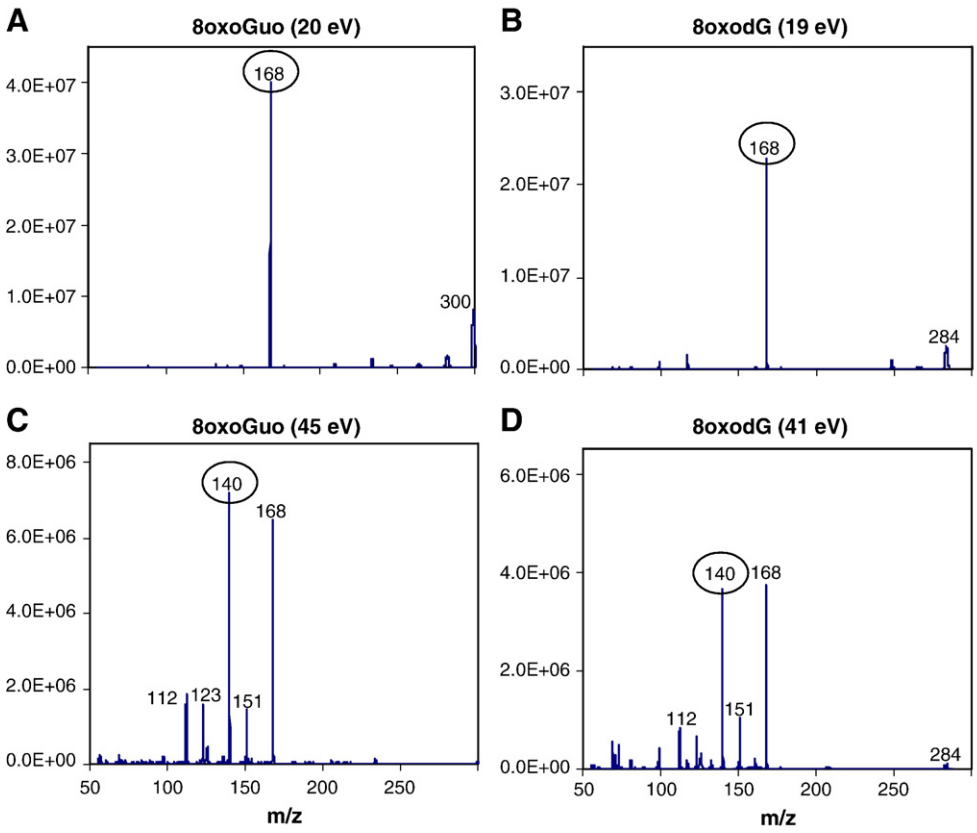

Fig. 1. Product ion spectra of 8-oxoGuo and 8-oxodG at collision energies selected to obtain the optimal signal for the product ions m/z 168 and m/z 140, respectively. (A) 8-oxoGuo, 20 eV, (B) 8-oxodG, 19 eV, (C) 8-oxoGuo, 45 eV, (D) 8-oxodG, 41 eV. Even at the optimized collision energies, the intensity of the ion m/z 168 is about five times the intensity of the ion m/z 140. The latter is thus used for confirmation.

Sample handling

Urine samples were stored at −20°C prior to analysis. The frozen urine was thawed, mixed, and heated to 37°C for 5 min to redissolve possible precipitate and thus release eventual trapped analytes from the precipitate [9]. The samples were then centrifuged at 10,000 rpm for 5 min, and placed on the Biomek 3000 (Beckman Coulter, CA, USA) deck. The Biomek Workstation performed all further tasks fully automated.

The sample preparations were conducted in 350 μL 96-well plates and were carried out in three steps: (1) 90 μL 100 mM lithium acetate buffer was added to each well by the MP200 tool. (2) A 110-μL aliquot of urine, calibration standard, or QC was consecutively added to each sample well by the P200 tool. (3) The amount of 90 μL of internal standard solution of stable isotope-labeled 8-oxoGuo and 8-oxodG, prepared in a 100 mM lithium acetate buffer, pH 6.4, was added to each sample well by the MP200 tool and mixed. The prepared 96-well plate was sealed with aluminum foil seals (Beckman Coulter) and placed in the UPLC sample manager. An aliquot (50 μL) of the solution was injected into the UPLC system.

Validation procedures

A validation program was executed according to the FDA guidelines [10], including selectivity, accuracy, precision, linearity, ion suppression, and LLOQ. Validation with respect to stability of samples and standards was performed in a previous study [11]. The challenge in designing the validation protocol relates to the fact that no human urine can be found without detectable endogenous levels of the studied adducts. The usual requirement for determination of selectivity, measuring a minimum of six blank matrix samples, was therefore only possible with respect to the two internal standards, but not for the two analytes. Also, no accredited reference material is available. Thus, the analytical accuracy could be determined only from the recovery in fortified urine samples.

Quantification

Quantification was based on the signal peak area from the transitions 300/168 (8-oxoGuo) and 284/168 (8-oxodG) relative to the signal peak area of the respective internal standards. The transitions 300/140 (8-oxoGuo) and 284/140 (8-oxodG) were applied as qualifier ions to confirm the presence of the analyte and the absence of false contributions from coelution of similar components in the urine sample. The signal ratio of the quantifier/qualifier ions was calculated for each sample, and the mean ratio was calculated for the standards. A ratio in the urine sample diverging >25% from that of the calculated mean of the standards indicates interference from a coeluting compound. In such instances, the result was discarded or, if possible, calculated from the signal ratio of the qualifier ion and the internal standard.

The calibration curves are expressed as the ratio between the analyte peak area and the ISTD peak area, as a function of the analyte concentration. As ISTD peak area are used the ion traces m/z 305/173 and m/z 289/173.

The ion traces of the internal standard qualifier ions are recorded as well, but these ions are applied for quantification only in the case of interference in the primary ion trace of the internal standard (m/z 305/173 and m/z 289/273). In such rare cases, an alternative calibration curve was prepared using the peak area of the analyte relative to the peak area of the ISTD qualifier instead of the usual ISTD peak. The particular interfered sample was then quantified using the alternative calibration curve.

Results

Sample processing

The proposed method is based on minimal manual manipulation and transfer. Except from the initial sample heating and centrifugation, and preparation of standards for the calibration curve, all further steps were carried out automatically. The BIOMEK robot performed the pipetting steps required for transferring aliquots of the urine samples, controls, and calibration standards to the respective wells at the autosampler plate, and the addition and mixing with buffer and internal standard, according to a program specifically designed for this application. Standards used for the calibration curve were placed in the beginning and end of the plate.

In the programming several parameters can be adjusted to optimize the accuracy and precision of the preparation steps. The most critical issue in this respect was to prevent differences in the handling of urine and water samples (standards), and to avoid leftover droplets at the pipette tips. These problems were solved by reducing aspiration and dispensing speed (25% of default) and by reducing speed of the pipette arm when leaving the liquid (5% of default). The robot time for preparation of a 96-well plate was approximately 1 h.

Chromatography and mass spectrometry

Fig. 2 represents a typical UPLC-MS/MS chromatogram for 8-oxoGuo and 8-oxodG in a urine sample, showing the traces of the quantifier ions (m/z 300/168 and 284/168), qualifier ions (m/z 300/140 and 284/140), and the corresponding traces from the ^{15}N-labeled internal standards (m/z 305/173 and 289/173) and (m/z 305/145 and 289/145). The latter traces of the internal standards are included in the routine analysis only as a backup, to be applied for quantification if the primary peak from the internal standard is subjected to interference.

During the method development several interfering peaks were observed in the chromatograms from analysis of urine samples, despite the specific MS/MS detection. Three different Acquity UPLC columns were evaluated prior to the final choice of the BEH Shield RP18 column (1.7 μm, 2.1 × 100 mm). A very flat gradient was applied to achieve sufficient retention of the analytes and to separate them from interfering peaks, which sometimes exceeded the analyte peak height with more than one order of magnitude. Initially, the column temperature was kept at 5°C in order to reduce the analysis time. However, as retention and separation of the hydrophilic analytes were considerably improved by decreasing the column temperature to 1°C, this temperature was preferred. The final gradient program is listed in Table 1.

Due to the low content of acetonitrile in the mobile phase at the time of analyte elution, in particular for 8-oxoGuo, a postcolumn flow of acetonitrile was applied to assist the electrospray ionization and thus increase the sensitivity. The sensitivity and repeatability were further improved by increasing the injected sample volume from 15 to 50 μL. Due to the initial mobile phase conditions of 100% aqueous buffer combined with the low column temperature, the analytes were concentrated at the front of the column during the injection and not released to travel through the column until the gradient starts. Thus, despite this rather large injection volume, the narrow peak shapes were not compromised as illustrated in Fig. 2.

As the analytes are formed endogenously, related compounds with almost the same polarities, masses, and fragmentation patterns can be expected to be present in the urine and may potentially interfere with the analyte response. In fact, interfering peaks were a considerable concern during method development. To improve the specificity of the detection, mass resolution was increased to 0.5 amu at half the maximum peak height (0.5 FWHM). Furthermore, two specific fragment ions of each analyte were included in the analysis, and an ion ratio within a certain range was required for a positive identification. Both initiatives intensify the demands to the method sensitivity, because higher mass resolution decreases the signal, and because the specific detection of two fragment ions is limited by the ability to measure the peak from the ion with the lowest intensity. For the present analytes, the intensity of the qualifier ions (m/z 140) was only about 20% of the signal of the quantifier ion (m/z 168), even when the optimized collision energies of the respective ions were applied (see Fig. 1).

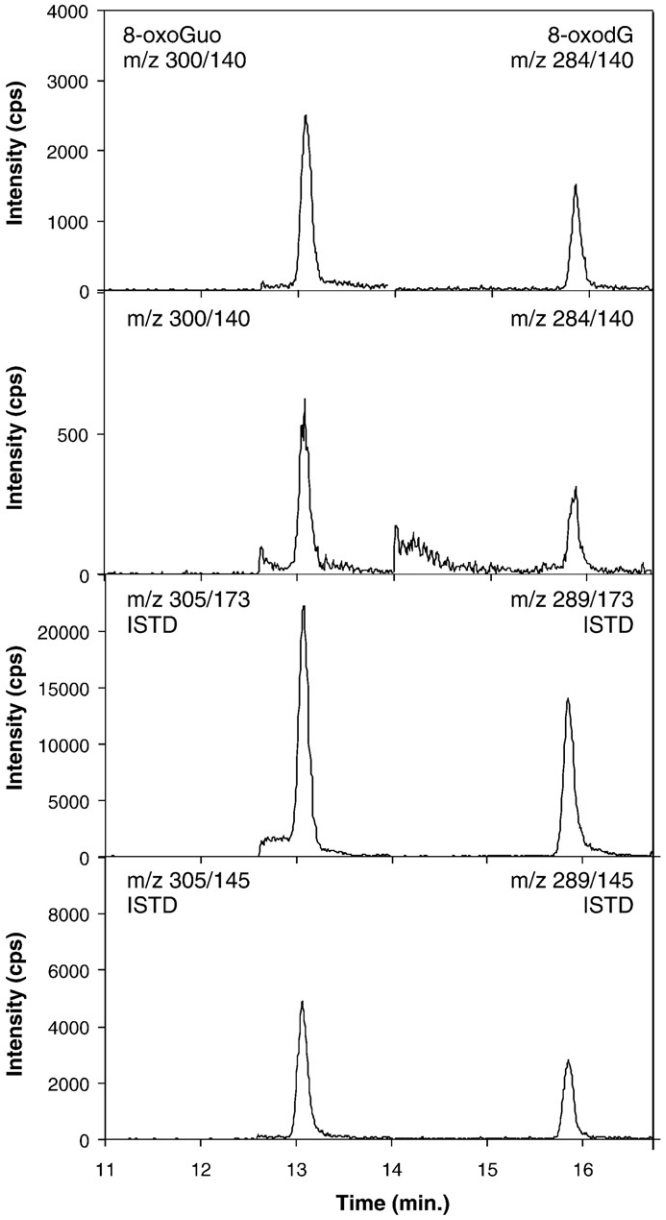

Fig. 2. UPLC/ESI-MS/MS chromatograms of a urine sample, showing the mass transitions corresponding to 8-oxoGuo, 8-oxodG, and the $^{15}N_5$-labeled internal standards. The urine sample contained 5.9 nM 8-oxoGuo, and 3.8 nM 8-oxodG. Only the fraction eluted from the column at the time 12.4–16.5 min was led to the mass spectrometer.

The importance of the qualifier ions are exemplified in Fig. 3, showing chromatograms of two urine samples with significant interference to the peak of 8-oxodG. In particular the interference in the sample shown in Fig. 3B might not have been identified without the qualifier ion. Hence, in that case the reported concentration of that sample might have been much too high.

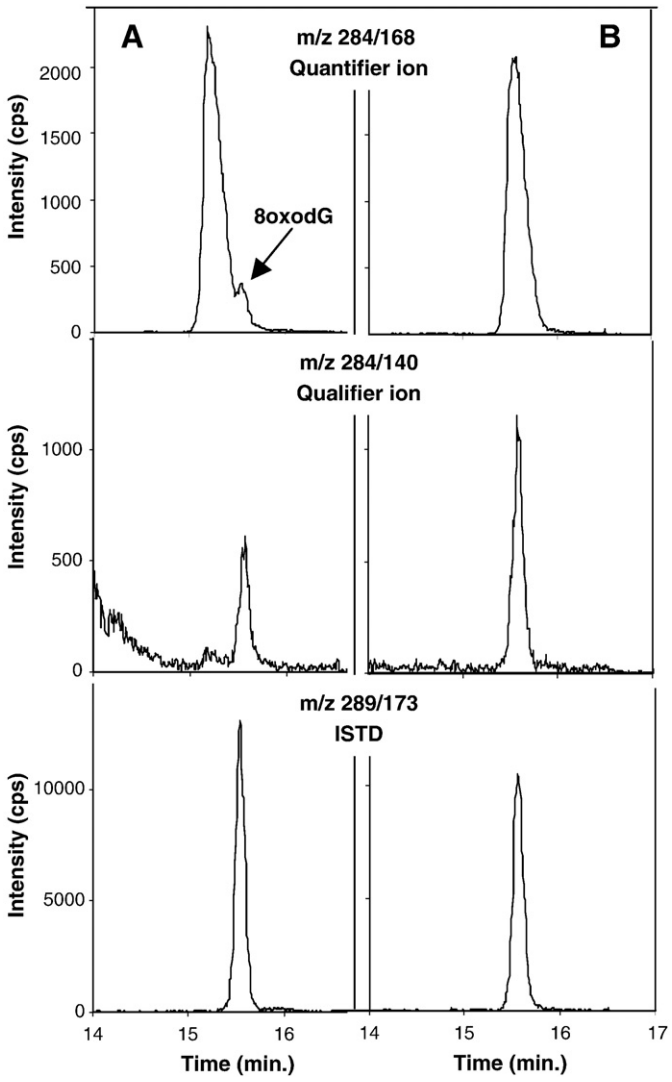

Fig. 3. Examples of interference in urine samples and the necessity of the qualifier ion. (A) The peak of the quantifier ion of 8-oxodG is only just visible at the backside of a huge interfering peak, and could not be properly integrated. The qualifier ion confirmed that 8-oxodG was present, and was used for quantification in this particular sample. (B) The analyte peak at m/z 168 is totally covered by an interfering peak. The peak is slightly broadened, but as there is only one peak and the retention time is correct, it might have been accepted for quantification if not a considerable deviation of the quantifier/qualifier ion ratio was observed. The analyte concentration was calculated using the qualifier ion, as the different shape of the quantifier peak clearly indicated the presence of an interfering peak, and thus the signal from the qualifier ion appeared to be reliable.

The automated integration procedure was controlled by visual inspection of the integration marks on each individual peak. The Analyst software performed acceptable autointegration in about 145 of 160 samples, this means that manual setting of integration start and stop was applied in about 10% of the samples, in most cases only for one of the six peaks.

Validation characteristics

Limit of quantification

The lower limit of quantification (LLOQ) was 0.4 nM for both 8-oxoGuo and 8-oxodG, based on the quality requirements of a S/N $\geq$5:1 and CV<20%. Due to dilution of the sample with buffer and internal standard as part of the sample preparation, this corresponds to a concentration of 1.0 nM in the original urine sample. To ensure the specificity throughout the measured concentration range, we additionally required a S/N$\geq$3:1 for the qualifier ion at the quantification limit. On average, the S/N ratios at 1.0 nM were 8–30 for the quantification ions and 4–14 for the qualifier ions.

Linearity, precision, and accuracy

The linearity and range of the calibration curves were evaluated with 7 levels of standards in duplicates over the working concentration range 1.0–100 nM. This corresponds to concentrations of 0.38–38 nM in the injected sample, after dilution with buffer and internal standard. Linear relationships were obtained for both analytes in the concentration range investigated, using a weighting factor of $1/x^2$. However, as the urine concentration of 8-oxoGuo and 8-oxodG rarely exceeds 60 nM, the validation was performed using a calibration curve in the range 1–60 nM.

Repeatability and accuracy studies were performed to compile method performance. The *within-day* and *between-day* variances were estimated from three series of eight human urine samples in triplicate. The urine samples were chosen to cover a broad concentration range. Precision is expressed as the percentage of relative standard deviation (RSD, %). The average within-day precision was 4.4 and 3.7% for 8-oxoGuo and 8-oxodG, respectively. The corresponding values for between-day precision was 4.0 and 2.3%. The overall precision of the method was 6.1 and 4.4%, respectively.

Accuracy was expressed as the percentage recovery in three urine samples, fortified with 10 or 40 nM of both analytes. The average recovery was 106.2% (8-oxoGuo) and 106.9% (8-oxodG).

These obtained accuracy and precision values are comparable to those previously reported from similar methods [3,12–14].

Detailed results from the validation regarding precision and accuracy are shown in Table 3.

Blank solvent samples analyzed within the batch verified that there was no detectable carryover from urine samples or the highest standard sample. The oxidative adducts were stable in human urine throughout at least three freeze–thaw cycles, determined by analysis on fresh aliquots compared to urine samples that were refrozen and reanalyzed twice over the next 3 weeks as part of the reproducibility study.

Ion suppression

Ion suppression due to matrix effects was estimated from the ratio of the average peak area of the internal standard in the urine samples compared to the aqueous calibration standards, as identical amounts of ISTD have been added to urine samples and standards. Considerable ion suppression was observed for 8-oxoGuo, on average 42%, whereas the corresponding value for 8-oxodG was only 17% (calculated as [1–(ISTD peak area$_{urine}$)/(ISTDpeak area$_{water}$)]$\times$100). Despite the ion suppression of 8-oxoGuo, the S/N-ratio of the 8-oxoGuo peaks exceeded 10 in urine samples with contents of the analyte close to the quantification limit.

Selectivity

The requirement for determination of selectivity includes the analysis of blank matrix samples from at least six sources, within which no traces of the respective analytes should be detectable. However, as no human urine can be found without detectable endogenous levels of the studied adducts, this requirement could only be fulfilled with respect to the internal standards.

Regarding the two analytes, alternative selectivity requirements were defined as follows: (1) Peaks from the quantifier ion as well as the qualifier ion should both be present in the chromatogram, and the retention time of the two peaks should not differ with more than 0.05 min, (2) The peak height ratio of the two peaks should not deviate from the average ratio in the standards with more than $\pm$25%. These requirements were fulfilled in all the urine samples involved in the validation.

Table 3
Validation results

Urine	N	8-oxoGuo (8-oxo-guanosine)						8-oxodG (8-oxo-deoxyguanosine)					
		Concentration level		Accuracy		Precision		Concentration level		Accuracy		Precision	
		Mean (nM)	CI 95[a] (nM)	Recovery[b] (%)	RSD_W (%)	RSD_B (%)	RSD_T (%)	Mean (nM)	CI 95%[a] (nM)	Recovery[b] (%)	RSD_W (%)	RSD_B (%)	RSD_T (%)
A	9	20.0	18.9–21.1		5.0	5.1	7.1	16.2	15.6–16.8		4.3	1.3	4.5
B	9	9.1	8.4–9.8		8.3	5.3	9.8	6.4	6.2–6.6		3.0	2.3	3.7
C	9	6.9	6.6–7.2		4.1	2.9	5.1	4.8	4.6–5.0		5.7	3.5	6.7
D	9	31.6	29.3–33.9		6.7	6.9	9.6	33.7	31.8–35.6		5.7	4.7	7.4
E	9	23.9	22.9–24.9		4.8	2.3	5.4	27.9	27.4–28.4		1.9	1.2	2.2
F (B + 10 nM)	9	19.4	18.8–20.0	103.6	2.7	3.1	4.1	16.7	16.1–17.3	103.0	3.7	2.8	4.7
G (B + 40 nM)	9	53.3	51.6–55.0	110.6	2.0	3.6	4.1	51.0	50.2–51.8	111.4	1.8	0.8	2.0
H (C + 10 nM)	9	17.4	17.0–17.8	104.3	1.4	2.9	3.3	15.4	14.9–15.9	106.3	3.5	2.1	4.1

Results for the eight urine samples involved in the validation: urine concentration (nM), 95% confidence intervals, recovery, within-day repeatability (RSD_W) and between-day reproducibility (RSD_B), and the overall precision (RSD_T) for each analyte.

[a] Calculated as: $[C_{mean} \pm t_{95\%,\ 8} \times SD]$. The standard deviation applied is calculated from RSD_T of the respective sample.

[b] Calculated as: $[(C_{spiked\ sample} - C_{natural\ sample})/(x\ nM)] \times 100$, where x is the respective spike level, 10 or 40 nM.

Using the qualifier ion for quantification

If the peak of the quantifier ion cannot be properly integrated due to severe interference, the qualifier ion may be used for quantification as an alternative. To test the validity of quantification using the qualifier ion, the two urine samples shown in Fig. 3 were fortified with 10 and 40 nM analyte standard, respectively, and analyzed together with the original urine samples. Accuracy was in the range 80.2–104.5% for 8-oxoGuo and 82.2–101.9% for 8-oxodG. Even if there was only interference for 8-oxodG in the urine samples, but not for 8-oxoGuo, the recovery was similar for both analytes.

The calibration curve based on the qualifier ion was linear in the measured range (1.0–60 nM).

Discussion

Measurement of oxidatively modified nucleic acid products is a major analytical challenge This is demonstrated by the fact that reported levels of oxidized nucleic acids in similar types of biological samples have differed by orders of magnitude, depending on the methodology applied for analysis, in particular the chromatographic based methods compared to ELISA. To identify and overcome the problems that cause these discrepancies, a number of research groups have joined in comparative interlaboratory studies, such as ESCULA (European Standards Committee for Urinary (DNA) Lesion Analysis) [15].

The primary challenge regarding urine samples is the specificity of the detection, as there are a huge number of molecules, some in very high concentrations, that can interfere or mimic, e.g., 8-oxodG and 8-oxoGuo. In the present paper we present the first method for analysis of 8-oxodG as well as 8-oxoGuo in urine samples that meets the recommended requirements for identification set by the Commission of the European communities, which implies the use of two fragment ions for the secure identification of the measured substance [16].

Due to the improved specificity, the present method may contribute to settle the discrepancies between hitherto published methodologies. The concentration range measured in urine samples by this method is comparable with the concentration range in earlier reports from our laboratory using LC-MS/MS [17–19], but very different from those measured by immunological assays [20,21].

In recent years several methods have been published on the measurement of 8-oxodG alone or together with other DNA and RNA oxidation products in urine [3,12–14,22–27]. It seems like the trend is toward shorter analysis times [13,14]. While short analysis time naturally is very desirable, it at the same time increases the risk of obtaining overlapping chromatographic peaks since the chromatogram will then be more compressed. Partly overlapping peaks can easily be detected by visual inspection of the chromatograms, but fully overlapping peaks are hard to detect without the use of a qualifier ion. When using qualifier ions it is important to make sure that the qualifier ion is characteristic and to check that the ratio between the quantifier ion and the qualifier ion is the same as the ratio in a pure standard of the same analyte. Obtaining a qualifier ion for measurement of nucleosides in positive-ion mode electrospray and at the same time keeping sufficient sensitivity is not as straightforward as it may seem. In the product ion spectra of protonated nucleosides usually only one intense product ion is observed corresponding to the protonated nucleobase. This may cause a marked loss in LLOQ if the qualifier ion peak has to have a reasonable size at this concentration level. Even without a qualifier ion it may be a major issue to obtain a sufficiently low LLOQ. Thus, an extra effort must be made in order to compensate for the loss in LLOQ when a qualifier ion is included. In this method the compensation consists of the use of postcolumn addition of acetonitrile, the use of UPLC with the concurrent improvement in sensitivity because of the narrower peaks and the reduced risk of ion suppression, and the on column up-concentration of the analytes, which enables the use of large injection volumes without suffering in peak width. To the best of our knowledge only one other method uses a qualifier ion for 8-oxodG [13] and we have been unable to find papers with use of a qualifier ion for both analytes. Whether this is because of lack of sufficient sensitivity by the use of a qualifier ion or the assumption that the quantifier transition (loss of mass 116 or 132) is sufficiently specific on its own to ensure that only the analyte and nothing else is being measured is the reason for the lack of use of qualifier ions is unknown. The losses of mass 116 or 132 may initially seem quite specific, but since the analytes are formed endogenously then often a lot of compounds with similar polarity (and thus similar retention on the UPLC column) and similar fragmentation pattern can be expected in the urine. It is thus highly recommended to use qualifier ions and the usefulness is shown in Fig. 3. In addition to the mentioned precautions then the use of high-resolution mode can often reduce or eliminate the possible interference from intense peaks at neighboring masses.

An extra bonus from using qualifier ions is achieved in the case where it is impossible to make a proper quantification using the quantifier ion, due to partly overlapping chromatographic peaks (as exemplified in Fig. 3A). It may, however, be possible from the peak height ratio of the qualifier/quantifier ions to determine if it is the correct analyte peak. If this is the case, then things can be inverted and thus the qualifier ions can be used for quantification. By using this strategy the number of samples that cannot be quantified directly can be reduces to close to zero. In the rare cases where the quantifier ion peak is totally covered by an interfering peak (as exemplified in Fig. 3B), it should be noted that quantification with the qualifier ion implies a loss of specificity though, as then only one specific ion is involved for identification.

Conclusions

Urinary excretions of 8-oxodG and 8-oxodGuo are measures of oxidative stress to nucleic acids.

A sensitive, robust, and highly specific method has been developed for the quantification of the urinary RNA and DNA adducts 8-oxoGuo and 8-oxodG. The method is validated with respect to linearity, precision, and accuracy, and the quality assurance results achieved are comparable to previously reported results from similar methods.

The analysis of real samples was demonstrated. The method was shown to be highly sensitive with a quantification limit (LLOQ) of 0.4 nM, corresponding to a concentration of 1.0 nM in the urine sample. As qualifier ions are included for both analytes, and detectable at the LLOQ, the method meets the requirements for specific detection by mass spectrometry as recommended within the European Union [16]. The presented method may thus be useful to settle discrepant results from other analytical methods.

Due to the automated sample preparation and ability to analyze more than 150 urine samples within one batch, the method is useful for large-scale studies. With a considerable part of the sample preparation performed automatically and automated integration of signal peaks the human involvement is minimal. The UPLC MS/MS analysis of two 96-well plates, containing 162 urine samples, is completed within about 75 h, which means that analysis of thousands of samples from, e.g., molecular epidemiological studies is feasible within a reasonable time and at reasonable cost.

This approach comprises the combination of automated sample handling with the high sensitivity and selectivity of liquid chromatography–mass spectrometry. Consequently, a significant throughput increase has been achieved along with an elimination of tedious labor and its consequential tendency to produce errors.

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