Free Radical Biology and Medicine 172 (2021) 350–357

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original article

Quantification of 8-oxo-7,8-dihydro-2′-deoxyguanosine and 8-oxo-7,8-dihydro-guanosine concentrations in urine and plasma for estimating 24-h urinary output

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ARTICLE INFO

Keywords:
Oxidative stress
RNA oxidation
DNA oxidation
Mass spectroscopy
Urine
Plasma

ABSTRACT

Among markers for oxidative stress urinary excretion 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) and 8-oxo-7,8-dihydro-guanosine (8-oxoGuo) have been widely used in controlled and epidemiological studies, and are considered to represent intracellular markers of oxidation of DNA and RNA in the entire organism, respectively. Although being non-invasive, urinary methods have shortcomings. There is no established method for analysis of 8-oxodGuo and 8-oxoGuo in plasma and the few plasma values presented in the literature vary greatly. We here present a liquid chromatography mass spectrometry method with full validation for analysis of 8-oxodGuo and 8-oxoGuo in plasma. Further, we investigated the basis for our previously physiological model and show that a single plasma sample can be used to estimate the 24-h production of 8-oxoGuo, whereas we challenge the use of urinary 8-oxodGuo/creatinine ratio or plasma 8-oxodGuo as measures of oxidative stress.

1. Introduction

Measurement of modifications of nucleic acids by oxidation has advanced as an important non-invasive biomarker of oxidative stress to be used in large epidemiological studies and clinical trials [1–3]. The oxidized nucleoside (8-oxodGuo) and the ribonucleoside (8-oxoGuo) measured in 24-h or spot urine samples [4,5] are examples of non-invasive biomarkers used in epidemiological or clinical controlled studies. Most often, only spot-urine samples are available and correction by urine creatinine concentrations have been used to correct urinary flow. Many biobanks or clinical studies do not store urine whereas plasma samples are often abundant. Plasma values represent the balance between release from the cells and excretion by the kidneys and; thus their plasma concentration cannot be interpreted as a measure of "oxidative stress" since the concentration does not discriminate between changes in these two processes [4,5]. With the argument that at steady state conditions, 24-h urine excretion represent the total formation in the cells in the collection period, we recently developed a physiologically based model where a single spot urine sample or plasma sample can be used to estimate the 24-h urine excretion of 8-oxodGuo and 8-oxoGuo [5]. While the methodology for quantification of 8-oxodGuo and 8-oxoGuo in urine is well established and validated, there is no validated method available for measurement of both these nucleosides and the nucleobase in plasma or serum by liquid-chromatography tandem mass spectrometry, and the values reported in the literature vary greatly (Table 1). The 2–300 times lower concentration of the analytes in plasma compared to urine [6,7] is a great analytical challenge, particularly also because removal of proteins is necessary and because high sensitivity increases the risk for interfering peaks in low concentrations. To overcome these challenges, we developed a column-switching MS/MS method. The presented chromatography-MS/MS method performs well with plasma samples and compares with our previous LC-MS/MS methods [8,9] for the measurement of 8-oxodGuo and 8-oxoGuo in urine; it requires less sample, gives cleaner chromatograms, has better sensitivity and shorter run times.

We tested the 10-year stability of 8-oxoGuo and 8-oxodG in urine, and their short-term stability in stored plasma samples. In addition we

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https://doi.org/10.1016/j.freeradbiomed.2021.06.014
Received 8 June 2021; Accepted 15 June 2021
Available online 22 June 2021

Table 1
Reported levels of 8-oxodGuo in plasma and serum from the literature.

8-oxodGuo [nmol/L]	Method	Sex	Matrix	Reference
0.015[a]	LC-MS/MS	Females	Plasma	[24]
≈0.016	LC-MS/MS		Plasma	[14]
0.027[a]	LC-MS/MS	Males	Plasma	[24]
0.046	LC/LC-EC		Plasma	[25]
0.047	LC/LC-EC		Plasma	[7]
0.051	LC-MS/MS		Plasma	[26]
0.053	LC-MS/MS		Plasma	This paper
0.057	LC/LC-MS/MS		Plasma	[6]
0.069	LC-EC		Plasma	[27]
≈0.075	LC-MS/MS		Plasma	[28]
0.077	LC-MS/MS		Plasma	[19]
0.08	LC-MS/MS		Serum	[29]
0.14	LC-MS/MS		Plasma	[29]
0.3	CIEIA		Serum	[30]
≈0.7	LC-EC		Serum	[31]
0.743	ELISA		Serum	[32]
0.883	ELISA	Males	Serum	[33]
0.905	?		Serum	[34]
0.919	ELISA	Females	Serum	[33]
1.77	ELISA	Females	Plasma	[35]
1.81	ELISA		Plasma	[36]
12.0	ELISA		Plasma	[37]
16.5	ELISA		Plasma	[38]
36.7	ELISA		Plasma	[39]
≈160	ELISA		Plasma	[40]
≈625	ELISA		Serum	[41]
8-oxoGuo [nmol/L]	**Method**		**Matrix**	**Reference**
0.110	LC-EC		Plasma	This paper
0.127	LC/LC-MS/MS		Plasma	[27]
8-oxoGua [nmol/L]	**Method**		**Matrix**	**Reference**
≈0.03	LC-MS/MS		Plasma	[14]
0.96	LC-EC		Serum	[42]
1.25	LC-MS/MS		Plasma	[6]
≈1	LC-MS/MS		Plasma	Unpublished data from our lab

[a] Apparently the reported levels are below their LLOQ.

investigated the assumptions that the nucleosides and creatine are treated identically by the kidneys, a prerequisite for our physiological model [5], by measuring the ratio of nucleosides and creatinine in plasma and urine obtained simultaneously.

2. Materials and methods

2.1. Chemicals

8-oxoGuo was purchased from BioLog (Bremen, Germany) and 8-oxodGuo from Berry & Associates (Dexter, MI). Internal standards, [^{13}C, $^{15}N_2$] 8-oxoGuo and [^{13}C, $^{15}N_2$] 8-oxodGuo, were purchased from Toronto Research Chemicals (North York, ON, Canada).

Methanol (HPLC-grade), lithium acetate dihydrate, and acetic acid were all from Sigma-Aldrich Co. Ltd. (Steinheim, Germany). Purified water was obtained from a Milli-Q Gradient A10 system (Millipore, Bedford, MA). QC samples were prepared in urine and stored at −20 °C.

2.2. Apparatus

The chromatographic separation was performed with a 3-column-setup on an Acquity UPLC I-class system (Waters, Milford, MA, USA), equipped with two binary pumps (denoted as primary and secondary pump) and a column manager with 2-position-6-port valves to control the column-switching -system. The system was supplied with an external column-oven (Mikrolab, Aarhus, Denmark). The MS/MS detection was performed by a Waters Xevo TQ-S triple quadrupole mass spectrometer set to positive ionization electrospray mode.

2.3. Column-switching LC-MS/MS configuration

Column 1: A cation-exchange precolumn, Nucleogel sugar 810 H 30 × 4 mm, from Macherey-Nagel. Column 2: A VanGuard precolumn (HSS T3, 1.8 µm, 2.1 × 5 mm) from Waters. Column 3: A Kinetex Polar C18, 30 × 2.1 mm, 2.6 µm, from Phenomenex. Column 1 was held at 60 °C. The second and third column were kept at 4 °C to retain the analytes on column 2 during elution of the analytes from column 1, and to enhance their separation on column 3. The primary pump delivered an isocratic flow of 0.1 mL/min of 100% 0.5 mM H_2SO_4. At the secondary pump 0.1% acetic acid was used as eluent A, and 50% methanol as eluent B. A 15 min regeneration- and cleaning-program was applied for every 25 samples, using 25 mM H_2SO_4 at the primary pump (column 1) and 50% methanol at the secondary pump (column 2 and 3), keeping the flow at 0.1 mL/min for 15 min. Column 2 and 3 are eluted with the same mobile phase from the same pump, no 2. Note that column 1 is a HPLC column, column 2 is just a small UPLC precolumn that works as a trap without much chromatography, and column 3 is a HPLC column.

For quantification the MS/MS-transitions *m/z* 300/168 (8-oxoGuo) and *m/z* 284/168 (8-oxodGuo) were used, relative to the corresponding signal of the internal standards (*m/z* 303/171 and *m/z* 287/171, respectively). The transitions *m/z* 300/140 (8-oxoGuo) and *m/z* 284/140 (8-oxodGuo) were applied for confirmation purposes. The 3-column-setup is shown in Fig. 1.

2.4. Sample preparation (urine)

The frozen urine (−20 °C) was thawed, mixed, and heated to 37 °C for 5 min followed by centrifugation at 10,000×*g* for 5 min. Internal standard solution and standards for calibration (1.0–60.0 nmol/L) were prepared in 0.5 mM H2SO4. The final sample preparation in 96-well plates was carried out by a Biomek 3000 Automated worksation (Beckman Coulter, CA, USA), at which 20 µL of urine/calibration

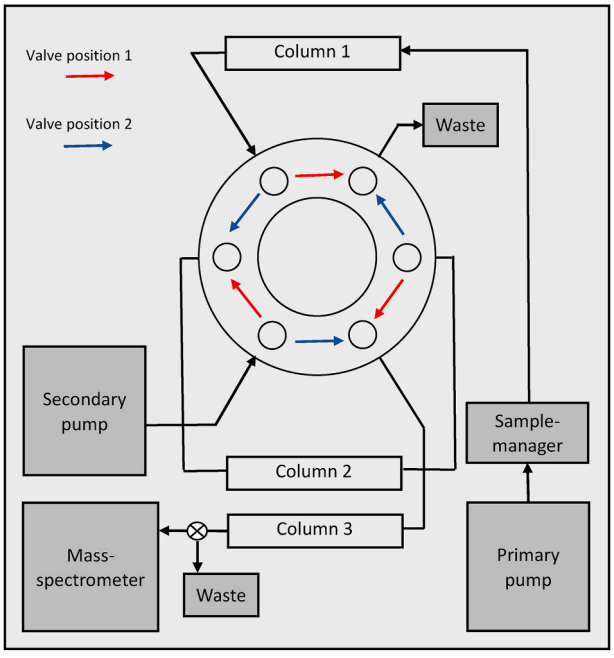

Fig. 1. Schedule of the 3-column-setup configuration. Column 1: Cation-exchange precolumn, Nucleogel sugar 810 H 30 × 4 mm (Macherey-Nagel). Column 2: VanGuard precolumn, HSS T3, 1.8 µm, 2.1 × 5 mm (Waters). Column 3: Kinetex Polar C18, 30 × 2.1 mm, 2.6 µm (Phenomenex). By the switch of valve position, the analytes were trapped at column 2 as they eluted from column 1, and finally eluted to column 3 for separation.

standard/QC sample was mixed with 50 μL of internal standard solution and 180 μL buffer 0.5 mM H2SO4. Heating to 37 °C [10] and dilution with buffer [7] ensure release of trapped analytes in precipitate in the samples [7]. A volume of 50 μL was injected onto the LC-system.

2.5. Sample preparation (plasma)

The frozen plasma samples (EDTA-plasma) were thawed and mixed. A volume of 300 μL was transferred to an Ultracel 3 K ultrafiltration tube (Merck Millipore, Carrigtwohill, Ireland), and 50 μL of internal standard solution was added. After centrifugation for 45 min at 20.000 rcf, the filtrate was transferred to a vial for analysis. Calibration standards in the range 0.01–1.0 nmol/L were prepared in 0.5 mM H_2SO_4. A volume of 100 μL was injected onto the LC-system.

2.6. Analysis of creatinine

Urine creatinine concentration was measured using the Jaffe technique, by measuring the UV-absorbance of the chemical complex formed by creatinine and picric acid. Plasma creatinine was measured by Department of Clinical biochemistry, Rigshospitalet by an accredited method (international standard DS/EN ISO 15189:2013).

2.7. Validation procedures

The analytical method was validated in accordance with the Commission of the European communities and FDA [11,12], including selectivity, accuracy, precision, linearity, LLOQ, ion suppression and stability of samples and standards and the use of both quantifier and qualifier ions.

2.8. Collection of plasma- and urine samples

Simultaneously obtained EDTA-plasma and urine samples, n = 15, originating from anonymized donors and collected for quality control purposes were used for comparing plasma and urine samples. The urine and plasma samples were stored at −20 °C until analysis. Excess plasma from 6 subjects with type 2 diabetes from previous trials, anonymized and stored for quality control purpose were also analyzed. As part of the anonymization process identity of the original trials and persons is not known. As part of the QC scheme of the laboratory we stored aliquots of analyzed urine samples from all projects since 2008, to enable stability testing of urine samples stored at −20 °C. To test the analyte stability in urine at −20 °C, a total of 160 urine samples were retrieved from various projects, which had previously been analyzed in our laboratory during the period 2008–2019 and afterwards stored at −20 °C. Use of anonymized samples for quality control do not require ethical approval according to Danish law.

3. Results

3.1. Optimization of the chromatographic conditions

Two cation-exchange precolumns, Nucleogel sugar 810 Ca and Nucleogel sugar 810 H, from Macherey-Nagel, were tested for the initial separation of the plasma ultrafiltrate. Besides the separation based on ion-exchange, their high affinity for sugar molecules, in particular ribose, were expected to be advantageous for separation of the nucleosides, 8-oxoGuo and 8-oxodGuo, from the complex matrix components in both plasma and urine. Acceptable retention was achieved at both columns. Thus, Nucleogel sugar 810 H, based on H^+-exchange, was preferred as an acidic mobile phase was most suitable for the combination with retention on column 2.

In heart-cutting LC-LC (Fig. 1) it is important to be able to trap and up-concentrate the analytes at the front of the second column in order to obtain sharp chromatographic peaks. It has previously been found that in order to trap the two analytes on a reversed phase column an almost 100% aqueous eluent and low temperature is necessary [13]. At such conditions, the analytes could be trapped for 5 min on the HSS T3 pre-column (column 2), which was sufficient to trap the analytes during elution from column 1.

3.2. Selection of mobile phases

It was initially attempted to use 0.1% acetic acid as aqueous mobile phase A for all three columns as this was found to provide the best MS-signal for the two analytes. The otherwise recommended use of dilute sulfuric acid on column 1 was avoided at first to prevent non-volatile sulfate ions from contaminating the mass spectrometer by following the analytes from column 1 and further through the system. However, a continuous decrease of the analyte retention on column 1 was observed, which might have been caused by a too low concentration of the counter ion (H^+) in the mobile phase.

Hence, the mobile phase at column 1 was changed to 0.5 mM sulfuric acid, whereas 0.1% acetic acid was maintained for column 3. The valve shifts between the columns and a diverter valve between column 3 and the mass spectrometer ensured that most of the remaining sulfate ions and matrix components were sent to waste.

3.3. Column temperature

According to the manufacturer, the separation efficiency on column 1 is best at high temperature (maximum at 90 °C), whereas it requires a very low temperature (4 °C which is equal to the lower limit for the column manager) to trap the polar analytes on column 2. A high temperature at column 1 thus narrows the analyte peaks and thereby the necessary time window for elution to column 2, whereas a too high temperature of the mobile phase from column 1 reduces the ability to trap the analytes at column 2. These opposite concerns were balanced by keeping column 1 at 60 °C.

3.4. Optimization of chromatographic stability

Initially a signal decline was observed during long runs (>50 samples). It turned out that due to retention time drift on column 1 an increasing part of the analyte peaks elute before the scheduled valve-shifts. Stable retention times on column 1 were achieved by two modifications: 1) inserting a 15-min regeneration-program which flushed the column with 25 mM H_2SO_4 for 15 min for every 25 analyses, and 2) the initial use of 100 mM lithium acetate for dilution of the urine samples was replaced by 0.5 mM sulfuric acid. After this, repeated analyses of the same urine samples proved that batches of 192 samples and standards could be analyzed without signal drift of the batch run (approximately 54 h).

4. Validation of the analytical method (urine)

4.1. Linearity and limit of quantification

Linear relationships were obtained for both analytes in the concentration range 0.5–200 nmol/L, using a weighting factor of $1/x^2$. As the concentration of 8-oxoGuo and 8-oxodGuo in urine rarely exceeds 60 nmol/L and rarely is below 1 nmol/L, the usual calibration range is reduced to 1–60 nmol/L.

The lower limit of quantification (LLOQ) is 0.5 nmol/L for both 8-oxoGuo and 8-oxodGuo, based on the quality requirements of CV<20%.

4.2. Accuracy and precision

The *within-day* and *between-day* variations were estimated from three series of seven human urine samples, all prepared in triplicate and measured at different days, covering the concentration range 1.5–65.5

nmol/L. Two of these samples were fortified with 10 nM and 25 nM, respectively, for estimation of accuracy. The average within-day precision was 1.8% for 8-oxoGuo and 3.0% for 8-oxodGuo, and the between-day precision was 4.4% and 4.9%, respectively. The average recovery in the fortified samples was to 97.3% for 8-oxoGuo and 92.8% for 8-oxodGuo.

In addition, satisfying agreement was achieved from analysis of 160 urine samples comparing the present method as well as the previous method [9]: On average 101% (8-oxoGuo) and 99% (8-oxodGuo), only 10 values (6%) deviated from average with more than 10%. Results are shown in Fig. 2.

4.3. Selectivity

Two fragment ions from each analyte were measured to confirm the presence of the analytes and the absence of interference from similar compounds in the urine samples: *m/z* 300/168 *m/z* and 300/140 for 8-oxoGuo and *m/z* 284/168 and 284/140 for 8-oxodGuo. The requirements for confirmation were as follows: 1) peaks from both transitions should be present in the chromatogram, and the retention time of the two peaks should not differ by more than 0.05 min, and 2) the ratio between an analytes peak areas should not deviate from the average ratio in the standards by more than ±30% [11].

4.4. Ion suppression

Ion suppression due to matrix effects was estimated to 16–17% based on the ratio of the average peak area of the internal standard in the urine samples compared to the aqueous calibration standards.

4.5. Stability of urine samples and calibration standards

The stability of 8-oxodGuo in urine samples have previously been tested and the 8-oxodGuo content was found to be stable for 15 years [15]. To the best of our knowledge, the long-term stability of 8-oxoGuo in urine has not been tested previously. For this purpose, we measured the recovery of 8-oxoGuo and 8-oxodGuo in 160 urine samples from various projects analyzed between 2008 and 2019 with our previous

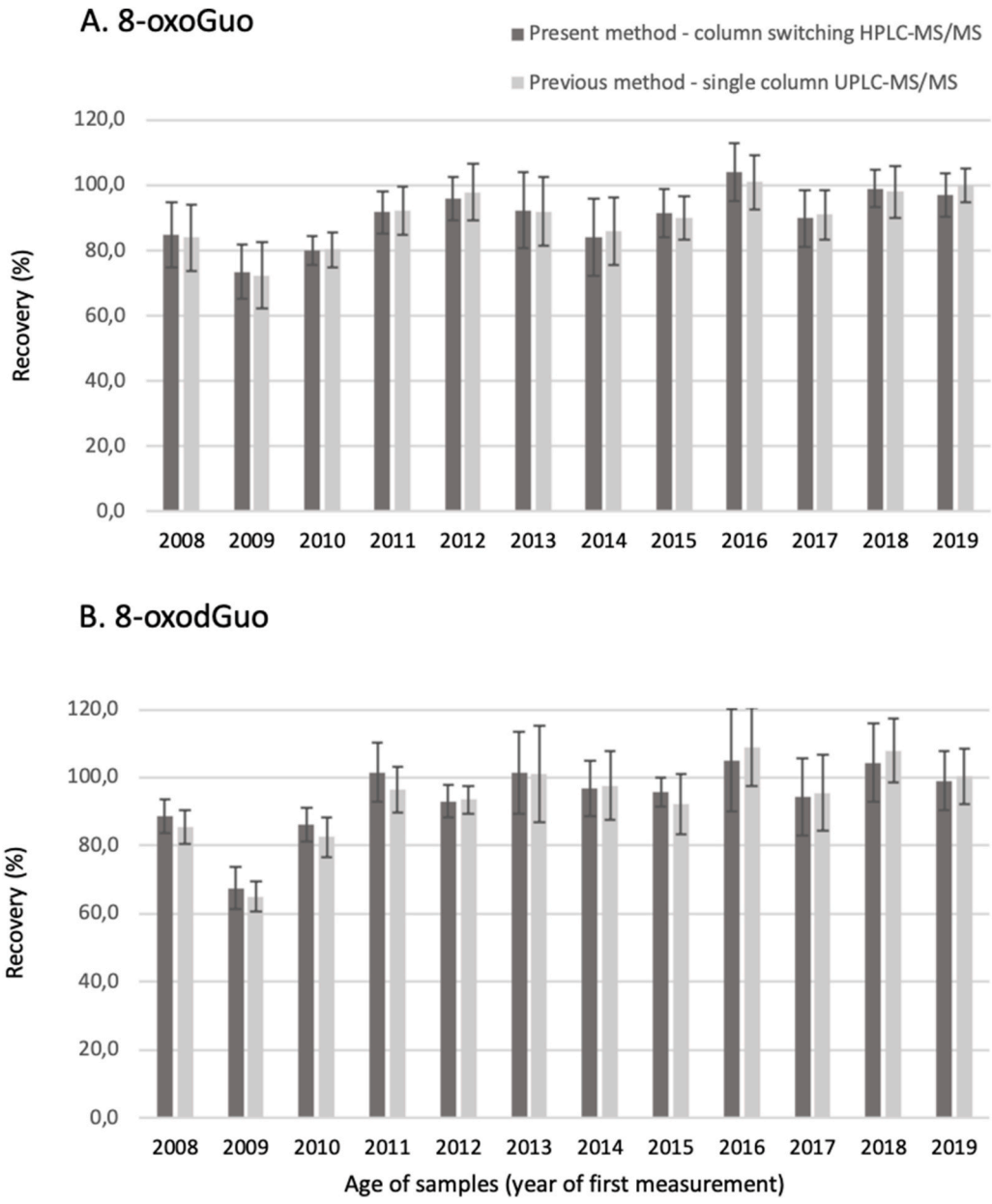

Fig. 2. Recovery of 8-oxo-7,8-dihydro-2'deoxyguanosine (8-oxodGuo) and 8-oxo-7,8-dihydroguanosine) 8-oxoGuo in urine samples, stored at −20 °C. The 160 urine samples were previously analyzed for the target compounds in the period 2008–19. The samples were measured by the new method as well as the previous method [8], in order to 1) ensure that differences between new and older results was not due to analytical bias, and 2) to confirm the quantitative compliance of the new method.

LC-MS/MS methods [8,9]. The samples have been stored at −20 °C for quality control purposes. Using the original results as reference value, the average recovery was 91% for 8-oxoGuo and 96% for 8-oxodGuo. There was, however, a tendency towards a slightly lower recovery in samples stored more than 10 years, as shown in Fig. 2. The 160 samples were measured by the method presented in this paper was used to examine the agreement between the method described here and previous method [9] and to ensure that differences between the method described here and the original results was not due to analytical bias. As shown in Fig. 3, the results confirmed good agreement. The advantages of the new method presented here is the improved sensitivity, which allows analysis of the 1000x lower concentrations in plasma compared with urine, and the shorter time of analysis, 14 min compared with the 23 min of the previous method.

Repeated analyses of prepared samples (calibration standards, QC and urine samples) after 1, 2 and 4 weeks documented that reproducible results can be obtained after storage at 4 °C for at least 4 weeks (data not shown).

5. Validation of the analytical method (plasma), linearity and limit of quantification

Linear relationships were obtained for both analytes in the concentration range 0.01–1.0 nmol/L, using a weighting factor of $1/x^2$. Fig. 3 presents a chromatogram from analysis of a plasma sample with a concentration of 8-oxoGuo at 0.077 nmol/L and 8-oxodGuo at 0.017 nmol/L. The latter is close to the limit of quantification.

5.1. Accuracy and precision

The *within-day* and *between-day* variations were estimated from three series of five human plasma samples, all prepared in triplicate and measured at three different days, covering the concentration range 0.078–0.244 nmol/L (8-oxoGuo) and 0.015–0.172 nmol/L (8-oxodGuo). Two of these samples were fortified with 0.100 nM and 0.200 nM, respectively, for estimation of accuracy. The average within-day precision was 4.9% for 8-oxoGuo and 4,6% for 8-oxodGuo, and the between-day precision was 3,0% and 6,7%, respectively. The average recovery was to 81.6% for 8-oxoGuo and 79,2% for 8-oxodGuo.

5.2. Selectivity

As for the specificity described for urine, similar requirements were fulfilled for all the plasma samples used in the validation.

5.3. Loss of sensitivity due to clean-up and ion suppression

The loss of analytes during sample clean-up by ultrafiltration was examined in plasma from two different persons. The isotope-labelled internal standards were added before and after ultrafiltration, respectively, and the concentrations were measured in all samples (triplicate of each type). The recovery after ultrafiltration was calculated to 36% for 8-oxoGuo and 26% for 8-oxodGuo. We hypothesize that the considerable loss from filtration might be due to binding to the proteins retained by the filter, since 75% of the sample volume passed to the collection tube. We tried to increase recovery by addition of 4% 2-propanol to the plasma before ultrafiltration, similarly to recommendations for on-line clean-up of plasma to release analytes from proteins. Though, this did not improve recovery either.

Ion suppression due to matrix effects was estimated to 42–46% based on the ratio of the average peak areas of the internal standards added to the plasma filtrate compared to the aqueous calibration standards. This is surprising as it was expected that the majority of salts and other matrix component would be diverted to waste.

Despite the low recovery and high ion-suppression, isotope labelled internal standards added to the plasma before ultrafiltration compensate correctly for the loss during clean-up and ionization, and the sensitivity of the method was sufficient to quantify the analytes in plasma, achieving the same concentration levels as reported by others.

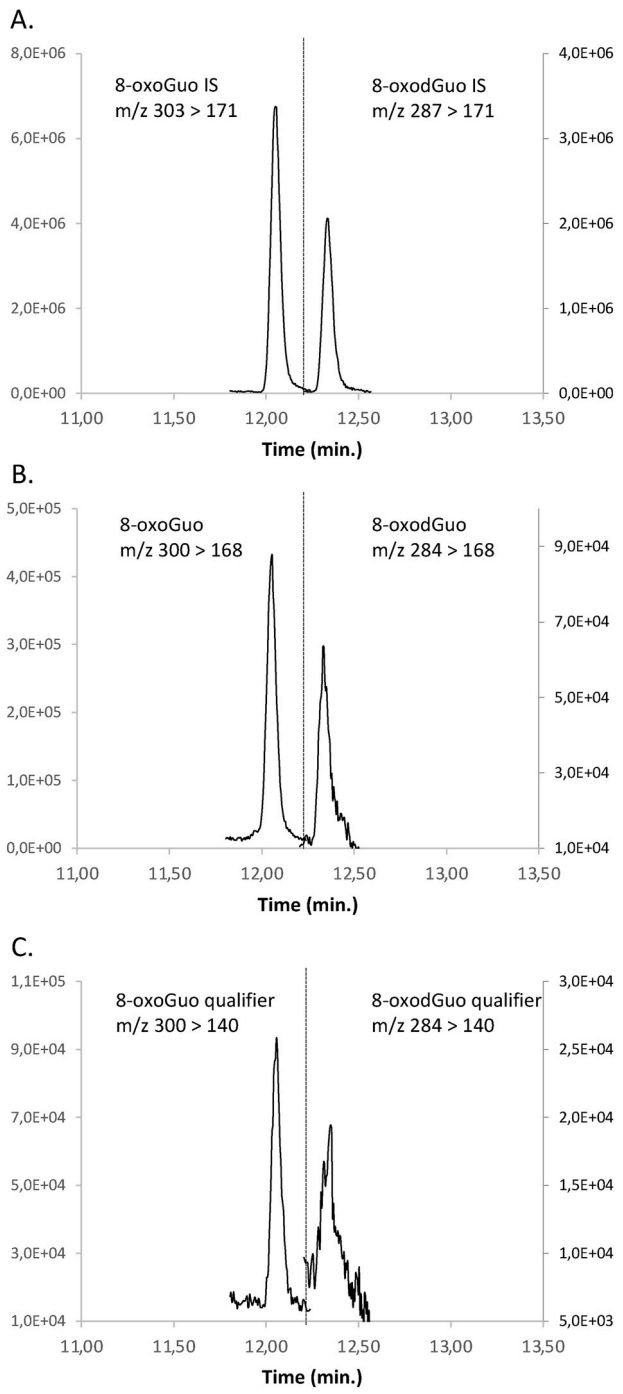

Fig. 3. Chromatogram from analysis of plasma. LC-LC/ESI-MS/MS chromatograms of a plasma sample, showing the mass transitions corresponding to 8-oxo-7,8-dihydro-2'deoxyguanosine (8-oxodGuo), 8-oxo-7,8-dihydroguanosine (8-oxoGuo), and the [13C, 15N2]-labelled internal standards. The sample contained 0.077 nmol/L of 8-oxoGuo, and 0.017 nmol/L of 8-oxodGuo. Note the different scales at the y-axis due to the lower response of 8-oxodGuo compared to 8-oxoGuo. Only the fraction eluted from the column at the time 11.8–12.6 min was transferred to the mass spectrometer.

5.4. Stability of samples

The analytes were stable in the raw plasma for at least one week after

storage at 20 °C, 4 °C and −20 °C, respectively. Prepared samples were stable for one week at 4 °C.

5.5. Blood samples

EDTA-plasma was used for method validation. Moreover, serum and citrate plasma were frequently used during the method development and we observed no differences using these types of plasma or serum (data not given).

5.6. Measured concentrations of 8-oxoGuo and 8-oxodGuo in plasma

The plasma concentrations of 8-oxoGuo and 8-oxodGuo in the control group (n = 15) were 0.11 nmol/L(sd = 0.05) and 0.05 nmol/L(sd = 0.02), respectively. The plasma concentrations of 8-oxoGuo was 1.6 times higher in patients with type 2 diabetes versus subjects without diabetes (p = 0.015, unpaired *t*-test), consistent with earlier reports on urinary excretion [16]. Plasma concentrations of 8-oxodGuo was 1.3 times higher in subjects with type 2 diabetes, which did not reach statistical significance (p = 0.11, unpaired *t*-test), Table 2.

5.7. Relation between 8-oxoGuo or 8-oxodGuo and creatinine in plasma and urine

The urinary 8-oxodGuo and 8-oxoGuo concentrations in controls as well as the plasma/urinary ratios for 8-oxodGuo, 8-oxoGuo and creatinine are given in Table 3. There was no correlation between plasma and urine 8-oxoGuo concentrations (r = 0.33,95% CI: 0.21 to 0.72, p = 0.22) whereas there was a significant correlation between plasma 8-oxodGuo and urine 8-oxodGuo concentrations (r = 0.7995% CI: 0.28 to 0.89, p = 0.003), plots not given. These two correlation coefficients are different, p = 0.07).

The ratio of 8-oxoGuo in plasma/urine and the ratio of creatinine in plasma/urine were similar whereas the ratio of 8-oxodGuo in plasma/urine was lower than that of creatinine plasma/urine concentration, Table 4.

Plots of the plasma/urine concentration ratios of 8-oxodGuo and 8-oxoGuo versus that of creatinine are given in Fig. 4. The slope linear regression of the 8-oxodGuo ratio versus the creatinine ratio was of 1.81 (sd = 0.06) and R-squared correlation was 0.98. Similar values for 8-oxoGuo ratio versus creatinine ratio were obtained (slope 1.18 (sd = 0.06) R-squared 0.98). As can been seen from Fig. 4 there is one outlier. We redid the regression analysis after removing this outlier, and found that this separated the 8-oxodGuo and the 8-oxoGuo versus creatinine somewhat more, and that the latter was closer to the line of unity. Therefore, removal of the outlier further strengthened the conclusions (data not shown).

Table 2
Plasma 8-oxo-7,8-dihydro-2'deoxyguanosine (8-oxodGuo), 8-oxo-7,8-dihydroguanosine,(8-oxoGuo) and creatinine concentrations in controls and type 2 diabetics.

	Variable		Controls (n = 15)	Type 2 diabetes (n = 6)	Total (n = 21)	p-value
1	Plasma 8oxoGuo (nmol/L)	mean (sd)	0.110 (0.047)	0.180 (0.060)	0.130 (0.060)	0.015
2	Plasma 8oxodG (nmol/L)	mean (sd)	0.052 (0.020)	0.068 (0.020)	0.057 (0.022)	0.14
3	Plasma creatinine μmol/L)	mean (sd)	81.2 (13.0)	72.1 (14.6)	78.6 (13.8)	0.16

Table 3
Urinary 8-oxo-7,8-dihydro-2'deoxyguanosine (8-oxodGuo), 8-oxo-7,8-dihydroguanosine (8-oxoGuo) and creatinine concentrations in controls.

	Variable		
1	Urine 8oxoGuo nmol/L, n = 15	mean (sd)	15.2 (8.7)
2	Urine 8oxodG nmol/L, n = 15	mean (sd)	14.4 (9.3)
3	Urine creatinine mmol/L, n = 15	mean (sd)	10.2 (5.7)

Table 4
Plasma/urine ratio for 8-oxo-7,8-dihydro-2'deoxyguanosine (8-oxodGuo), 8-oxo-7,8-dihydroguanosine (8-oxoGuo) and creatinine.

	Variable		
1	Ratio of 8-oxoGuo in plasma over urine, n = 15	mean (sd)	0.011 (0.010)
2	Ratio of 8-oxodGuo in plasma over urine, n = 15	mean (sd)	0.006 (0.006)[a]
3	Ratio of creatinine in plasma over urine, n = 15	mean (sd)	0.012 (0.011)

[a] Ratio significantly different from that of 8-oxoGuo and creatinine (p-value 0.02).

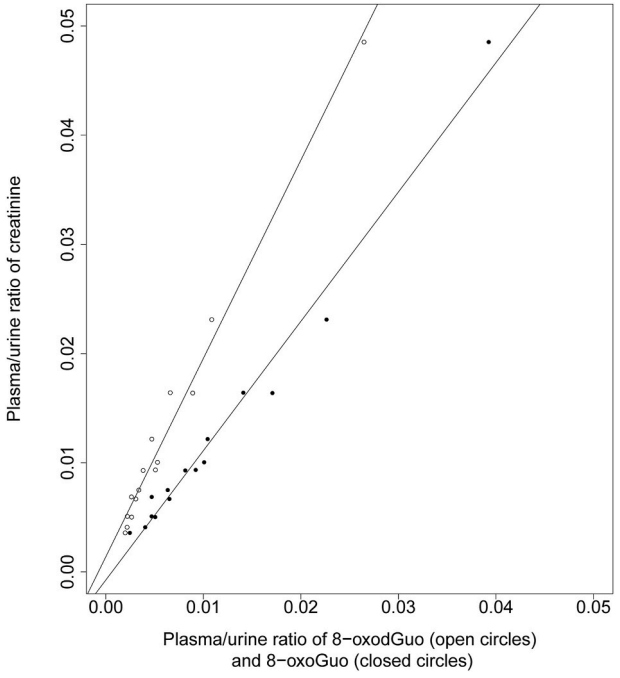

Fig. 4. Ratios of plasma/urine of 8-oxo-7,8-dihydro-2'deoxyguanosine (8-oxodGuo) and 8-oxo-7,8-dihydroguanosine (8-oxoGuo) versus the plasma/urine concentration ratio in 15 persons where plasma and urine samples are obtained at the same time. The 8oxodGuo slope is 0.46 (sd = 0.06) and the 8-oxoGuo slope is 1.18 (sd = 0.06).

6. Discussion

The current study presents and validates a method, based on HPLC heart-cut-column-switching tandem mass spectrometry, to measure plasma concentrations of the oxidized nucleoside, 8-oxodGuo, and the ribonucleoside, 8-oxoGuo, of guanine. This method is validated according to US and European standards and can be used in large epidemiological studies and in controlled clinical trials. In our lab it is semi-automated with an automatic pipetting robot. We also showed stability of plasma samples upon storage.

The reported concentrations in human plasma of 8-oxoGuo, and the nucleobase 8-oxodGuo vary considerable in the literature, the concentrations found with the current method are in agreement with the reported concentrations (Table 1) of 8-oxodGuo in plasma measured by

chromatographic methods, and we can with this validated method establish that the human plasma levels of 8-oxodGuo are approximately 0.05 nmol/L, and the levels of 8-oxoGuo are around 0.100 nmol/L. It should be noted that the previous methods for plasma measurements have not been validated according to present standards, and some rely on custom made chromatography columns that cannot be obtained commercially, e.g. antibody columns and carbon-based columns [7], and they could not easily be reproduced (personal experience and personal communication). Furthermore, with the present validated methodology that relies on commercially available commodities, storage of urine samples for 10+ years is possible without loss of 8-oxoGuo. The theoretical plasma values calculated with our physiological model is 0.200 nmol/L for 8-oxodGuo and 0.233 nmol/L for 8-oxoGuo [5]. Taken into consideration that the variation between individuals is large with values ranging up to more than 10 fold [17,18], the theoretical and found concentrations are in reasonable agreement. Because the plasma method is newly developed it is not possible to test the long-term stability of plasma samples, but 4 weeks storage at −20 °C is without loss, so we have no indications of 8-oxodGuo or 8-oxoGuo break-down during storage.

Some authors have suggested to use plasma values as a marker for oxidative stress, by measuring oxidized nucleosides in plasma [6,19,20], and also found a correlation between plasma values and creatinine corrected values in urine. The approach of these investigations is to compare the use of plasma concentrations of the oxidized nucleoside and the creatinine corrected nucleoside as two different biomarkers of oxidative stress. However, from a physiological point of view, plasma concentration of e.g., 8-oxoGuo in steady state conditions mainly will be determined by kidney function, i.e. glomerular filtrations rate, just as plasma creatinine in the clinic is used as a measure of kidney function. Kidney disease will result in higher plasma values of substances removed by filtration, such as creatinine or 8-oxoGuo, but the higher values cannot be interpreted as increased oxidative stress, it is just a physiological consequence of reduced glomerular filtration rate. We have considered this is details in a physiological (4,5) model where we assumed that creatinine and the oxidized nucleosides are treated identically, i.e., excretion without reabsorption. In the present study we examined this assumption by comparing the ratio of creatinine in plasma/urine with that of the nucleosides to investigate if the two molecules are treated identically in the kidney. For 8-oxoGuo and creatinine we found very similar ratios, as evaluated by the average values and the slope of their linear regression (Fig. 4). For 8-oxodGuo the ratio is about 50% of expected, i.e. the creatinine value, indicating an active process in the kidneys, presumably reabsorption. Surprisingly, this is independent of the concentration, i.e., the curve in Fig. 4 is linear in the entire concentration range. This indicates that the process does not have a threshold value, as is known e.g., for glucose. This will introduce an overestimation of about 50% of the calculated 24-h urinary excretion of 8-oxodGuo from the plasma concentration measurement, however only a slight overestimation of the 24-h hour urinary excretion of 8-oxoGuo. We therefore conclude that our physiological model is valid for 8-oxoGuo, but have to be reconsidered for 8-oxodGuo.

With this in mind, it is also necessary to reconsider the use of urinary 8-oxodGuo/creatinine concentrations in urine as a measure of oxidative stress and consider if the finding that 8-oxodGuo/creatinine is not prognostic for death in type 2 diabetes whereas 8-oxoGuo/creatinine [16,21] is due to the unexpected handling of 8-oxodGuo by the kidneys and possible invalidity of using creatinine correction for urinary 8-oxodGuo spot samples.

We only included analysis of the oxidized nucleoside and ribonucleoside of guanine, not the oxidized base itself (8-oxoGua). The reason for this is that the origin of the base and where it is oxidized is problematic. First it is not known if it originates from RNA or DNA, and second if it originates from the various precursor pools and/or from cell death or cell turnover. Further, although it does not seem to originate from the diet in humans as it does in the rat, this question needs systematic examination before it definitely can be ruled out.

Many authors have suggested the use of a panel of biomarkers to characterize oxidative stress in more details. However, the number of possible outcomes or patterns grow dramatically with the number of biomarkers and very large cohorts are needed to find such patterns and very large trials to test their relation to e.g. morbidity or mortality in groups where it has been stablished that oxidative stress plays a major role. Furthermore, it has been argued that the use of multiple biomarkers "is often limited, due to lacking analytical or clinical validation, or technical difficulties" [22].

ELISA kits specifications often claim they measure 8-oxodGuo, or more recently both 8-oxodGuo and 8-oxoGuo, e.g., the Cayman kit. From Table 1 it is clear that both urinary and plasma values measured by ELISA are much higher than measured by HPLC mass spectrometry, supporting our previous findings [23]. Taken together this further supports our notion that the ELISA kits available do not have the specificity of HPLC-MS/MS, they presumably not only have affinity for both 8-oxodGuo and 8-oxoGuo but also for other un-identified substances in both urine and plasma. We caution their usage and particularly their interpretation as a measure of oxidative nucleic acid modifications.

In summary, we present a validated method to measure 8-oxodGuo and 8-oxoGuo in human plasma and urine, we show that urine samples can be stored for 10+ years for analysis, and verify our proposal that 24-h urine excretion of 8-oxoGuo can be estimated from a single plasma and urine sample. We also raise concerns about the use of urinary 8-oxodGuo/creatinine ratio as a measure of oxidative stress, whereas the urinary 8-oxoGuo/creatinine ratio and calculation of 24-h 8-oxoGuo production from plasma 8-oxoGuo can be used interchangeably.

Declaration of competing interest

The authors declare no conflict of interest.

Acknowledgements

Katja Luntang Christensen is acknowledged for her help with preparation and analysis of samples.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.freeradbiomed.2021.06.014.

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