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Measurement of 8-oxo-7,8-dihydro-2′-deoxyguanosine and 8-oxo-7,8-dihydro-guanosine in cerebrospinal fluid by ultra performance liquid chromatography–tandem mass spectrometry

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ABSTRACT

Increased levels of nucleosides modified by oxidation in human cerebrospinal fluid (CSF) have several times been reported in Alzheimer patients and patients suffering from Parkinson's disease. The focus has especially been on nucleosides containing the 8-hydroxylation of guanine. Only few reports on quantification of the ribonucleoside 8-oxo-7,8-dihydro-guanosine (8oxoGuo) in CSF have been published, whereas more have been published on the quantification of the deoxy-ribonucleoside 8-oxo-7,8-dihydro-2′-deoxyguanosine (8oxodGuo). The reports on the quantification of 8oxodGuo concentrations in CSF report absolute concentrations varying by a factor $> 10^5$ in healthy humans. This could indicate that there is a serious specificity problem in some of the methods. In this paper an isotope-dilution UPLC–MS/MS method with high specificity and sensitivity for the quantification of 8oxoGuo and 8oxodGuo in CSF is presented. LLOQ for the two analytes is determined to 4pM and 2pM, respectively. The calibration curves has been tested to be linear in the range from 4 to 3,000pM for 8oxoGuo and between 2 and 3,000pM for 8oxodGuo. Using a weighting factor of 1/x the correlation coefficient "r" for both analytes is > 0.999.

1. Introduction

Measurement of modifications in nucleic acids by oxidation has been challenging as it requires highly specific and sensitive methodology. Moreover, pre-analytical challenges have been identified because of spurious/artificial oxidation during the preparative procedures of tissues leading to reporting levels varying with 5 orders of magnitude [1,2]. The ESCODD (European Standards Committee on Oxidative DNA damage) collaboration produced protocols to remedy the pre-analytical problems [3,4]. In the ESCODD program focus was only on the oxidation of guanine in DNA, although more than 100 different modifications have been described [5]. Measurement of this lesion as breakdown/repair product has been established for matrices like urine and cerebrospinal fluid (CSF) [6–8], and has been expanded to the corresponding RNA modification of guanine, 8-oxo-7,8-dihydro-guanosine (8oxoGuo) in urine [8,9], CSF [10–12] and tissue [13,14].

The brain is the most energy consuming organ in the body and is considered to be highly exposed to oxidative stress, however, the result of the high oxygen concentration has not been examined in details. Recently, an increased focus on oxidation of RNA has emerged, and it seems that this nucleic acid is more prone to oxidation than DNA, so far demonstrated particularly for degenerative brain diseases, for review compilation see [15]. The biological consequences have so far mainly been theoretical [16], but recently it has been shown that oxidized RNAs accumulates and associates with polyribosomes and may lead to ribosomal stalling [17], and that oxidation of micro RNA fundamentally can change the regulatory property [18]. The RNA machinery is very complex and surprisingly little investigated, although it could be a prime target for disease mechanisms.

Both direct estimation in brain tissue and estimation of breakdown products represent analytical challenges because the levels are low [7]. In this paper we describe a methodology developed to estimate low levels in cerebrospinal fluid that requires small sample volumes, thereby making it possible to do investigations in experimental animals

Abbreviations: 8oxodGuo, 8oxo-7,8-dihydro-2′-deoxyguanosine; 8oxoGuo, 8-oxo-7,8-dihydro-guanosine; CSF, cerebrospinal fluid; dGuo, 2′-deoxyguanosine; EC, electrochemical; ESCODD, European Standards Committee on Oxidative DNA damage

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as well as in humans and a high specificity from the use of quantitative isotope dilution mass spectrometry.

2. Material and methods

2.1. Chemicals

8-oxo-7,8-dihydro-2′-deoxyguanosine (8oxodGuo), was from Berry & Associates (Dexter, MI), 8-oxo-7,8-dihydro-guanosine (8oxoGuo) was from BioLog (Bremen, Germany), [^{13}C,^{15}N$_2$] 8-oxo-7,8-dihydro-guanosine ([^{13}C,^{15}N$_2$] 8oxoGuo) (Toronto Research Chemicals, North York, ON, Canada).

[^{15}N$_5$] 8-oxo-7,8-dihydro-2′-deoxyguanosine ([^{15}N$_5$] 8oxodGuo) and [^{15}N$_5$] 8-oxo-7,8-dihydro-guanosine ([^{15}N$_5$] 8oxoGuo) were synthesized in house from [^{15}N$_5$] 2′-deoxyguanosine and [^{15}N$_5$] guanosine (Cambridge Isotope Laboratories, Inc., Tewksbury, MA) by electrochemical oxidation and the products were purified by preparative HPLC. 8-^{18}O-oxo-7,8-dihydro-2′-deoxyguanosine (8-^{18}O-oxodGuo) was a gift from Dr. Miral Dizdaroglu, National Institute of Standards and Technology (Gaithersburg, MD).

Acetic acid was of LC–MS quality from Merck and acetonitrile was of LC–MS quality from Honeywell/Riedel-de Haën (Sigma-Aldrich Co. Ltd. (Steinheim, Germany)). Water was obtained from a Milli-Q Gradient A10 system (Millipore, Bedford, MA).

2.2. Samples

The human CSF samples that were used to validate the method were anonymized excess fluid from the Clinical Biochemistry Department Rigshospitalet, Denmark. According to Danish law it is allowed to use excess fluid from diagnostic procedures for E.G. method development and testing as long as the samples are anonymized.

Samples from ten healthy controls from the Danish Dementia Research Centre, Rigshospitalet, Copenhagen, were analyzed. The project was approved by the ethical committee of the Copenhagen Capital Region and all subjects gave informed consent.

CSF samples (10–12 mL) were obtained by lumbar puncture, collected in polypropylene tubes and gently mixed. The samples were centrifuged at 2000 × *g* for 10 min at 4 °C to remove cells and other insoluble materials and stored in polypropylene tubes at −80 °C pending analysis.

Table 1
UPLC Gradient Program.

Time (Minutes)	Flow (μL/min)	%A	%B
0.0	200	100	0
1.5	200	100	0
17.5	200	96.1	3.9
24.0	200	86.4	13.6
24.1	200	0	100
26.0	200	0	100
26.1	250	100	0
35.9	300	100	0
36.0	200	100	0

Eluent A: 0.5% acetic acid. Eluent B: Acetonitrile. Column temperature was 1 °C.

2.3. Apparatus

2.3.1. UPLC

A Waters Acquity UPLC system consisting of an Acquity Sample manager and an Acquity Binary pump was used. The column used was an Acquity UPLC™ HSS T3 column (2.1 × 100 mm, 1.8 μ) protected by an Acquity UPLC™ HSS T3 VanGuard pre-column (2.1 × 5 mm, 1.8 μ) both obtained from Waters (Wexford, Ireland). The mobile phase was: eluent A, 0.5% acetic acid; Eluent B, Acetonitrile. The loop size was 250 μL and the injection volume 150 μL.

The linear UPLC gradient is shown in Table 1.

The column was immersed in a cooling bath with a temperature maintained at 1 °C. This temperature is chosen because the nucleosides retains better at low temperature, making it possible to better focus the nucleosides on top of the column before the elution starts and to use large injection volumes without getting very broad peaks [8].

2.3.2. Mass spectrometry

The UPLC was connected to a Waters XEVO TQ-s triple quadrupole mass spectrometer with electrospray ion source and controlled by MassLynx software version 4.1 (Waters, Wexford, Ireland). The software controlled valve that is part of the mass spectrometer was used to divert the eluent fractions that contained the analytes into the mass spectrometer. The early (0–15.6 min) and late eluting (24.9–36.0 min) fractions were diverted to waste, thereby reducing contamination of the ion source.

Electrospray ionization was performed in the negative ion mode for both 8oxodGuo and 8oxoGuo. For the two analytes the $[M-H]^-$ ions were selected by the first mass filter. Following collision activation the most intense fragment ions were selected by the last mass filter for quantification. With the current sensitivity 8oxoGuo have sufficiently high concentration in CSF to include a qualifier ion. The second most intense product ion was used as qualifier ion. The mass spectrometric recording parameters are presented in Table 2.

Argon was used as collision gas. Nitrogen was used as nebulizer, cone and, desolvation gas. The electrospray probe temperature was 650 °C. Individual tuning files were used for both analytes to achieve maximum sensitivities.

For accurate quantification using LC–MS stable isotopic labeled internal standards were used.

2.4. Sample preparation

2.4.1. CSF

The frozen CSF was thawed on ice and the samples were kept on ice whenever possible. 100 μL CSF was added 20 μL internal standard mixture. The samples were then filtered through Amicon® Ultra 0.5 mL centrifugal filters (10 K MWCO)(Merck Millipore Ltd. Cork, Ireland). The samples were centrifuged at 20,000 rpm for 10 min at 0 °C on an Eppendorf 5417R centrifuge (Eppendorf AG, Germany). The filters were added 50 μL eluent A and were again centrifuged for 10 min. This washing was repeated so washing was done twice. The combined filtrates were transferred to a sample manager vial and injected. It takes approximately 1.5 h to prepare 30 samples after the samples were thawed (The sample capacity of the centrifuge used). The bottleneck here is the sample capacity of the centrifuge. If using a centrifuge with a higher sample capacity or using more than one centrifuge more samples

Table 2
Mass Spectrometric (Multiple Reaction Monitoring) Parameters.

	Precursor ion [*m/z*]	Quantifier ion [*m/z*]	Coll. Energy [eV]	Qualifier ion [*m/z*]	Coll. Energy [eV]	Ratio	Time Window [Min]
8oxoGuo	298	208	18	165	24	3.9	21.0–23.5
[^{13}C,^{15}N$_2$] 8oxoGuo	301	211	18	–	–	–	21.0–23.5
8oxodGuo	282	192	18	–	–	–	23.5–25.0
[^{15}N$_5$] 8oxodGuo	287	197	18	–	–	–	23.5–25.0

can be prepared without using much more time.

3. Results

The developed method has been used for the measurement of ten samples from healthy controls from the Danish Dementia Research Centre, Rigshospitalet, Copenhagen. The mean results have been included in Table 3 for a comparison with results obtained using other presented methods for the quantification of 8oxoGuo, 8oxodGuo and 8oxoGua in CSF from healthy volunteers.

3.1. Validation

The presented method has been validated for the quantification of 8oxodGuo, and 8oxoGuo in CSF.

3.1.1. Limit of quantification

The lower limit of quantification (LLOQ) was determined as being the lowest concentration with CV < 20%. Based on the standards it was determined to be approximately 1 pM for 8oxodGuo (CV 9.4%). A chromatogram of a standard containing 1pM 8oxodGuo and 10pM 8oxoGuo is shown in Fig. 1. In real samples, however, it is difficult to determine the LLOQ precisely since it is impossible to obtain CSF samples without the analytes and impossible to get certified reference material. In real samples we have had problems finding a sufficient high number of samples with a low content of 8oxodGuo to be able to measure the CV close to the LLOQ. Instead we have defined an S/N ratio limit of 10 to be the LLOQ. The sample shown in Fig. 2 has been analyzed 9 times. The average S/N ratio for 8oxodGuo in the nine analyses is 35 and the average concentration is measured to be 6.4pM. Thus it can be calculated that a sample having an 8oxodGuo concentration of 6.4pM divided by 3.5 can be expected to have an S/N ratio of 10 and thus the LLOQ is determined to be 1.8pM or rounded 2pM.

The concentrations of 8oxoGuo found in CSF samples were never close to the LLOQ. Measurement of a 10pM 8oxoGuo standard gave a CV of 3.2% (see chromatogram Fig. 1). The same pooled CSF as above showed on average an 8oxoGuo concentration of 69.9pM (see chromatogram Fig. 2). At this level the 8oxoGuo within-day reproducibility was 5.5% and the between-day reproducibility was 6.2%. (See Table 4). The average S/N ratio for the nine times analysis of the sample is 190. By dividing 69.9pM by 19 it can be calculated that a sample having an 8oxoGuo concentration of 3.7pM can be expected to have an S/N ratio of 10 and thus the rounded LLOQ for 8oxoGuo is 4pM.

3.1.2. Linearity, precision, and accuracy

The linearity and range of the calibration curves were evaluated for

Table 3
Reported concentrations of 8oxoGuo, 8oxodGuo and 8oxoGua measured in CSF.

Analyte	Concentration [nM]	Technique	Reference
8oxoGuo	0.1	LC-EC	[10]
8oxoGuo	0.1	LC-EC	[11]
8oxoGuo	0.097	LC-EC	[12]
8oxoGuo	0.089[c]	UPLC–MS/MS	This paper
8oxodGuo	0.0035	LC-EC	[7]
8oxodGuo	0.0053	LC-EC	[19]
8oxodGuo	0.0060[f]	SPE, LC-EC	[20]
8oxodGuo	0.0064[f]	SPE, LC-EC	[21]
8oxodGuo	0.0146[b]	LC-EC	[22]
8oxodGuo	< 0.22[e]	ELISA	[23]
8oxodGuo	0.26	HPLC, GC–MS	[24]
8oxodGuo	0.7[a]	HPLC, GC–MS	[25]
8oxodGuo	5.2[d]	ELISA	[26]
8oxodGuo	680	SPE, GC–MS	[27]
8oxodGuo	0.0108[c]	UPLC–MS/MS	This paper
8oxoGua	0.31	HPLC, GC–MS	[24]
8oxoGua	1	HPLC, GC–MS	[25]

[a] Measured in cancer patients, since it was impossible to obtain CSF from healthy volunteers.
[b] Measured in children.
[c] Measured in healthy controls aged 61.4 ± 7.0 years.
[d] A cross-reaction with 8oxoGuo is reported, but 2 orders of magnitude lower than that of 8oxodGuo.
[e] Almost all values reported at their limit of detection.
[f] The authors report a detection limit of 20 pmol. To be able to measure the levels reported here will then require in excess of 3 L of CSF, so the reported levels are uncertain.

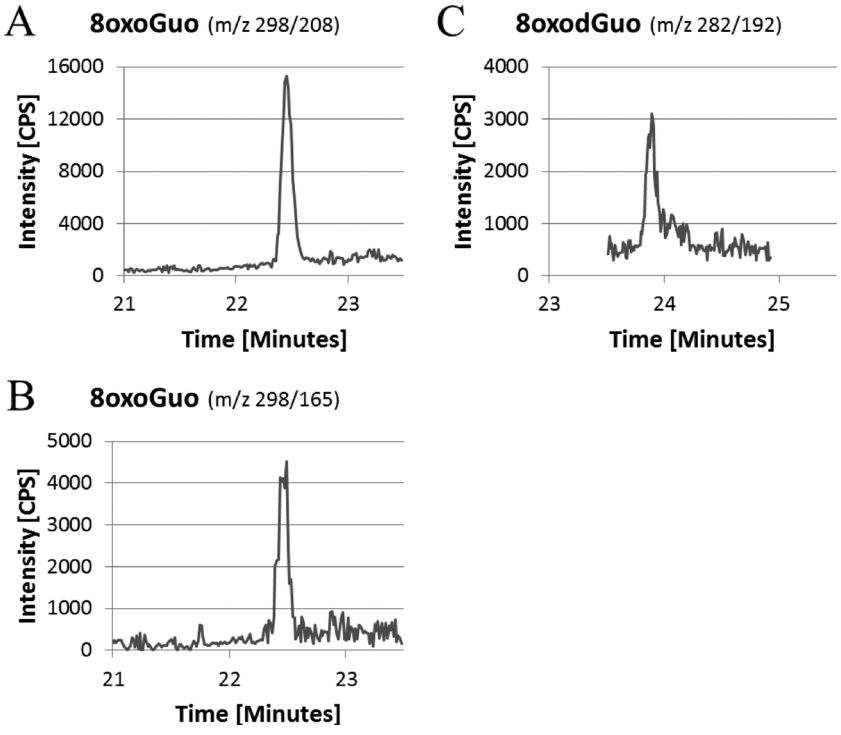

Fig. 1. UPLC-ESI–MS/MS chromatograms of a watery standard containing 1pM 8oxodGuo and 10pM 8oxoGuo. Panel A shows the mass transition corresponding to the 8oxoGuo quantifier ion. Panel B shows the mass transition corresponding to the 8oxoGuo qualifier ion. Panel C shows the mass transition corresponding to the 8oxodGuo quantifier ion.

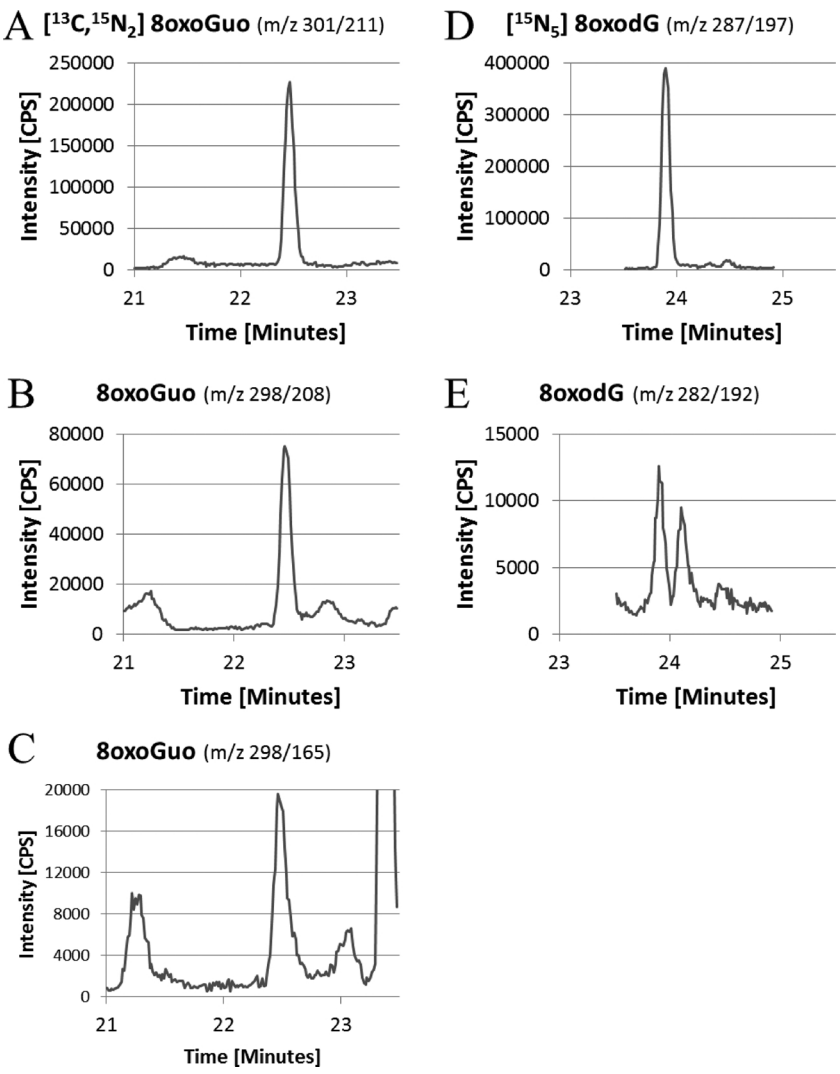

Fig. 2. UPLC-ESI–MS/MS chromatograms of a cerebrospinal fluid sample containing low concentrations of the two analytes (6.4pM 8oxodG and 69.9pM 8oxoGuo). Panel A shows the mass transition corresponding to the 8oxoGuo stable isotopic labeled internal standard. Panel B shows the mass transition corresponding to the 8oxoGuo quantifier ion. Panel C shows the mass transition corresponding to the 8oxoGuo qualifier ion. Panel D shows the mass transition corresponding to the 8oxodGuo stable isotopic labeled internal standard. Panel E shows the mass transition corresponding to the 8oxodGuo quantifier ion.

8oxodGuo and 8oxoGuo with 11 levels of standards over the working concentration range (0; 1; 3; 5; 10; 30; 50; 100; 300; 500; 1000 and 3,000pM). The calibration standards were prepared by serial dilution with water from 1 mM stock solutions of the two analytes. 8oxodGuo was tested to be linear in the range 2–3000 pM and 8oxoGuo was tested to be linear in the range 4–3000 pM according to FDA guidelines. The linearity was not tested above these ranges. Linear relationship was obtained for both analytes in the full concentration range, using a weighting factor of 1/x. The correlation coefficient "r" for both analytes is > 0.999.

Since it is not possible to get CSF samples without the two analytes it was necessary to test the accuracy of the method by spiking CSF that already contained the analytes. In order to obtain the most precise and realistic results a CSF sample that contained low concentrations of the analytes was needed and thus the same pool of CSF that was used for the determination of the LLOQs was used. This pooled CSF was spiked with A: 5pM 8oxodGuo and 50pM 8oxoGuo; B: 15pM 8oxodGuo and 150pM 8oxoGuo, and C: 30pM 8oxodGuo and 300pM 8oxoGuo (In each case 1.7 mL CSF was added 100 µL spike solution). These measurements were only performed in triplicate on each of the three days as it was very difficult to find more CSF with such a low content of the analytes among the excess CSF from diagnostic procedures. To measure the precision and accuracy in the medium to high concentration range pooled CSF having a higher background concentration of the two analytes could be used and it was much easier to obtain CSF samples in this concentration range. Thus it was possible to measure the precision and accuracy in five replicates on each day at each spike level. This pooled CSF was spiked with D: 100pM 8oxodGuo; E: 750pM 8oxodGuo and 8oxoGuo, and F: 2000pM 8oxodGuo and 8oxoGuo (In each case 3.1 mL CSF was added 100 µL spike solution). All the results obtained on spiked samples were corrected by the dilution factor.

The accuracy of the method was calculated by subtracting the initial values from the final (spiked samples) values and the result compared with the spiked amount. The accuracy was expressed as the average percentage recovery in the spiked CSF samples. For 8oxodGuo it was 94.9% and for 8oxoGuo it was 97.0%. The results are shown in Table 4.

The average within-day precision was 4.4% for 8oxodGuo and 3.8% for 8oxoGuo. The average between-day precision was 4.0% and 4.6%, respectively. The overall precision of the method was 6.3% for 8oxodGuo and 6.1% for 8oxoGuo. Results are shown in Table 4.

3.1.3. Ion suppression

It has turned out, that individual series often shows different levels of ion suppression apparently varying with the concentrations of the analytes. We have had series with low ion suppression, series with about 50% ion suppression and a post mortem series that sometimes showed up to around 90% ion suppression. Because of this the ion suppressions for 8oxoGuo and 8oxodGuo were measured in 6 CSF samples of which two had low concentrations, two had medium concentrations and the last two had high concentrations of the two analytes.

The ion suppression was measured by filtering the 6 CSF samples and washing the filters from the samples before the internal standards

Table 4
Precision and Accuracy.

8oxodGuo						
		Concentration	Accuracy	Precision		
	N	Mean (pM)	Recovery[a] (%)	RSD_W (%)	RSD_B (%)	RSD_T (%)
A	9	6.4	–	6.6	6.2	9.0
A + 5pM	9	11.2	102.2	2.9	7.0	7.6
A + 15pM	9	20.3	94.8	3.4	2.6	4.3
A + 30pM	9	35.3	97.5	1.7	4.0	4.4
B	15	19.3	–	5.7	5.8	8.2
B + 100pM	15	113.8	94.5	9.9	2.6	10.3
B + 750pM	15	687.5	89.1	1.9	2.6	3.3
B + 2000pM	15	1921.9	95.1	1.7	2.8	3.3
Mean	96 (72)	–	94.9	4.4	4.0	6.3
8oxoGuo						
		Concentration	Accuracy	Precision		
	N	Mean (pM)	Recovery[a] (%)	RSD_W (%)	RSD_B (%)	RSD_T (%)
A	9	69.9	–	5.5	6.2	8.3
A + 50pM	9	115.9	99.8	2.0	4.4	4.9
A + 150pM	9	214.3	98.9	2.8	2.1	3.6
A + 300pM	9	368.2	100.8	1.8	3.4	3.9
B	15	166.9	–	4.5	5.8	7.4
B + 750pM	15	852.4	91.4	4.6	4.5	6.4
B + 2000pM	15	2114.4	97.4	4.4	5.0	6.6
Mean	81 (57)	–	97.0	3.8	4.6	6.1

The table shows the results for the 8 CSF samples used in the validation. A pooled CSF sample (Sample A) containing low initial concentrations of 8oxodGuo and 8oxoGuo was spiked with low concentrations of the two analytes. The measurements on these samples were only performed in triplicate on each of the three days as it was very difficult to find more CSF with such a low initial content of the analytes. A pooled CSF sample (Sample B) containing slightly higher initial concentrations of 8oxodGuo and 8oxoGuo was spiked with medium to high concentrations of the two analytes. The measurements on these samples were performed five times on each of the three days. The recovery was calculated by subtracting the initial concentrations from the final concentrations and comparing with the spiked levels.
RSD_W; Within-day reproducibility; RSD_B: Between-day reproducibility, and RSD_T: Overall precision for each analyte.
[a] Calculated as: $[(C_{spikedsample} - C_{naturalsample})/(X\ pM)] \times 100$, where X is the respective spike level.

Table 5
Ion Suppression.

	8oxodGuo		8oxoGuo	
Sample	Concentration	Ion Suppression	Concentration	Ion Suppression
CSF low concentration 1	15.1 pM	2%	136.0 pM	−2%
CSF low concentration 2	10.0 pM	4%	161.3 pM	1%
CSF medium concentration 1	59,1 pM	−4%	299,8 pM	7%
CSF medium concentration 2	257,9 pM	−5%	706,8 pM	9%
CSF high concentration 1	571,7 pM	57%	2475,6 pM	60%
CSF high concentration 2	382,0 pM	9%	2416,6 pM	18%

The table shows the ion suppression measured in 6 different CSF samples. Two of the samples have low concentrations of 8oxodGuo and 8oxoGuo, two samples have medium concentrations, and two samples have high concentrations of the two analytes. As can be seen from the table CSF samples with low concentrations of the two analytes show less ion suppression than CSF samples with higher concentrations of the two analytes.

were added. After the filtering and washing 130 μL of the filtrate were added internal standards and analyzed as the standards. In this way a loss of ISTD during sample preparation is not included in the calculation of the ion suppression and identical concentrations of ISTD were present in both samples and standards before analysis by LC–MS/MS. 135 μL was injected for both the standards and the samples. Ion suppression due to matrix effects was then estimated from the ratio of the average peak area of the internal standards in the aqueous calibration standards compared to the same areas in the CSF samples. The results showed that the areas of the internal standards were virtually the same in the low concentration samples and the standards. The middle concentration samples showed little ion suppression and the high concentration samples showed ion suppression up to 60%. See Table 5.

It has been tested, if dilution of the post mortem CSF samples mentioned above with water (up to 10 times) was possible. The quantitative results were independent of whether the samples were diluted or not. On average the results before and after dilution only deviated by less than 2% for both analytes. Fortunately, as it seems like the CSF samples with high ion suppression also contains high concentrations of the two 8-oxo analytes the effect of the ion suppression on the detection limits has not caused any problems.

3.1.4. 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 CSF can be found without detectable endogenous levels of the analytes, this requirement could only be used with respect to the ISTDs. No significant peaks appeared in the 6 CSF samples that could interfere with the internal standards.

Table 6
Recovery.

	8oxodGuo		8oxoGuo	
Concentration	Mean Recovery	RSD (n = 3)	Mean Recovery	RSD (n = 3)
10pM	94.1%	18.4%	100.4%	14.0%
100pM	80.7%	1.1%	80.7%	0.3%
1000pM	79.8%	0.4%	80.7%	4.2%

The table shows the recovery through the filter at 3 different concentrations of the two analytes. The experiments were done in triplicate. The recovery was measured by adding the internal standards to the standards after filtration. Two alternative stable isotopic labeled internal standards were used as carriers and added before the standards were filtered. In this way the normal situation in a sample was mimicked.

Regarding 8oxoGuo an alternative selectivity requirement was defined as follows: The peak area ratio of the quantifier and the qualifier ions should not deviate from the average ratio in the standards by more than ± 25%. This is the requirement for analysis of banned substances within the EU [28]. The requirement was met for 8oxoGuo in all of the 10 CSF test-samples. For 8oxodGuo the concentration in CSF was usually very low and thus it was not possible to use a qualifier ion. In positive ion mode it is possible to see a qualifier ion in the samples. Where the ratio could reliably be determined (Where the S/N ratio was sufficiently high) the ratio was well inside the limits, confirming, that the peak corresponds to 8oxodGuo.

3.1.5. Recovery

The recovery during sample preparation was measured in triplicate at each level by filtering 100 µL of a standard containing 10pM 8oxodGuo and 8oxoGuo; 100pM 8oxodGuo and 8oxoGuo or 1000pM 8oxodGuo and 8oxoGuo. The standards were also added 10 µL of alternative internal standard solution. The alternative internal standards were; 8-^{18}O-oxodGuo and [$^{15}N_5$] 8oxoGuo. The alternative internal standards were added at a similar concentration to the concentrations of the usual internal standards. After the initial filtration the filter was washed two times with 50 µL eluent A and the combined filtrates were added 10 µL internal standard solution (twice the usual concentration) before the standard was analyzed by LC–MS/MS.

The recovery for 8oxodGuo and 8oxoGuo is presented in Table 6.

3.1.6. Carry over

To test if the method suffered from carry-over a blank sample was injected immediately after a standard containing 1000 (3000) pM of the two analytes. The blank sample showed no trace of any of the two analytes or their internal standards showing that carry-over was not a problem.

4. Discussion

The purpose of this study was to provide a method for the quantification of 8oxodGuo, and 8oxoGuo in CSF that required only 100 µL CSF or less.

By coincidence it was observed, that by using a time window with recording in positive ion mode (between 16.0 and 20.5 min) just before recording the two analytes in negative ion mode a signal increase of approximately 50% could be obtained as measured on the internal standards of the analytes. It is assumed that this is caused by eliminating a charging effect in the ion source.

During sample preparation the CSF samples are filtered. After filtering a sample the filter is washed twice with 50 µL eluent A to wash out the analytes from the liquid that is retained in the filter. As the analytes are focused at the front of the UPLC column a large volume can be injected without compromising the chromatography. Thus to diminish the loss of analytes as much as possible the filter is washed twice. After filtration 150 µL filtrate is injected on the UPLC column.

Initially when measuring the recovery it turned out that the recovery at the 10pM level was often low especially for 8oxoGuo (down to 60%). At the higher concentrations (100 and 1,000pM) it was around 80%. In a normal sample internal standards are added before the sample is filtered and thus it was investigated if the recovery for the analytes could be improved especially at the low concentrations by using a carrier for each analyte. Two alternative stable isotopic labeled internal standards were used as carriers and added before the standards were filtered. In this way the normal procedure for a sample is mimicked. The usual internal standards were first added after passage of the filter. Now the recoveries at all levels were around 80% or higher. For 8oxodGuo at 10pM it was around 200%. It turned out that the alternative internal standard 8-^{18}O-oxodGuo had become polluted by some unlabeled 8-oxodGuo. Thus we had to run three zero samples and subtract the average 8oxodGuo value from all the 8oxodGuo values. This is probably the reason why the RSD for 8oxodGuo at the 10pM level is quite high since two values of similar magnitude had to be subtracted from each other. Without the addition of the alternative ISTD the recovery for 8oxodGuo was 84%, RSD 9.8% at 10pM (n = 7).

The recoveries were now calculated to be between 78.9% and 100.4% for the two analytes (see Table 6).

Concentrations of 8oxodGuo in CSF reported in the litterature vary considerably as depicted in Table 3. The reported typical average levels of 8oxodGuo from healthy persons vary by a factor > 10^5. Such a high variation can stem from biological variation, pre-analytical and analytical variation. The variation between laboratories is much higher than the variation within laboratories, which points at analytical including pre-analytical variation as the major source.

The analytical variation could E.G. be caused by lack of specificity of some of the methods, artificial oxidation during sample handling, contamination and calibration errors.

Thus in order to obtain high specificity UPLC–MS/MS and use of both quantifier and qualifier multiple reaction monitoring pairs have been used in the presented method. In order to be able to measure the two analytes in the low pM range in a 100 µL CSF sample the method need to be able to quantify a few hundred amol of analyte. It will, however, likely be possible to improve the sensitivity even further than in this method by using capillary columns or by using positive ionization mode. Initially we used positive ion mode, but often encountered problems with overlapping peaks. A Chromatogram showing the problem with overlapping peaks for 8oxodGuo is shown in Fig. 3. This problem was solved by using negative ion mode where the S/N ratio is almost as good as in positive ion mode for 8oxodGuo, but the signals (and the noise) are much weaker in negative ion mode (See Table 7). A comparison between the fragmentation of 8oxodGuo in positive and negative ion mode has been described by Hua et al. [29]. For 8oxoGuo the loss in S/N ratio is higher by switching to negative ion mode, but as the concentration of 8oxoGuo in CSF is much higher than the LLOQ this causes no problems.

The huge inter-laboratory variation in the reported levels of especially 8oxodGuo (see Table 3) cannot easily be explained by true differences due to different selection of the persons donating the samples. The levels that have been measured in healthy humans in this experiment correspond best with the lowest levels previously reported for 8oxodGuo in CSF.

The most obvious reason for the huge inter-laboratory variation would be lack of specificity of the methods giving the highest 8oxodGuo concentrations, similarly to what has been reported for methods based on ELISA [30,31]. As the other methods measuring high 8oxodGuo levels, however, are based on electrochemical detection or mass spectrometric detection, the specificity ought to be fairly high to high. A reason here could be artificial oxidation of 2′-deoxyguanosine (dGuo) during sample preparation as have previously been observed in GC–MS measurements of 8oxodGuo in tissue samples [32]. When analyzing 8oxodGuo by GC–MS then first the nucleosides have to be acid hydrolyzed at elevated temperatures to liberate the free nucleobases and

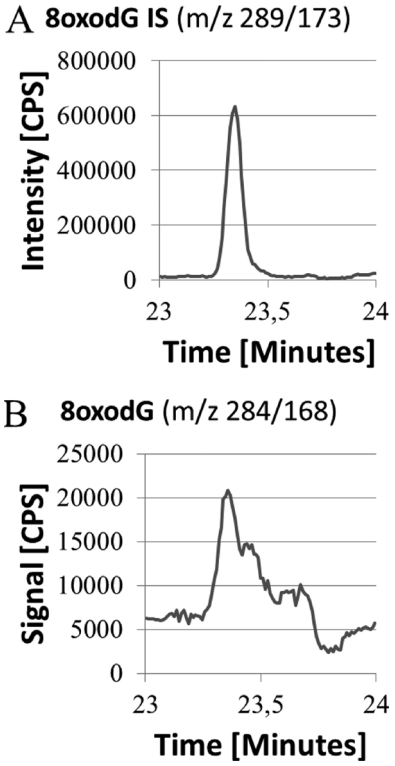

Fig. 3. The figure shows the problem with overlapping peaks observed in positive ion mode. The problem seems to occur in less than 10% of the samples.

Table 7
Sensitivity in positive contra negative ion mode.

	Positive Ions		Negative Ions		Ratio Positive/Negative Ions	
Analyte	Peak Height	S/N	Peak Height	S/N	Peak Height	S/N
8oxodGuo	66,671 cps	270	13,275 cps	140	5.0	1.9
8oxoGuo	80,660 cps	440	8,674 cps	60	9.3	7.3

The table compares the signals and the S/N ratios for a 10pM standard recorded in both positive and negative ion mode. The S/N ratio for 8oxodGuo is almost as good in negative ion mode as in positive ion mode even though the signal is a factor of 5 higher in positive ion mode. For 8oxoGuo both the S/N ratio and the signal are much better in positive than in negative ion mode.

then the free nucleobases have to be derivatized also at elevated temperatures to make them amenable to GC–MS. During these harsh conditions artificial oxidation of the non-oxidized dGuo can occur. Often the non-oxidized dGuo and guanine are removed by some kind of pre-purification to eliminate the risk of artificial oxidation of these compounds during hydrolysis and derivatization. When quantifying 8oxodGuo in tissue, however, the ratio between 8oxodGuo and dGuo is usually around $1:10^6$ and thus only a minute spurious oxidation could distort the measurements. In CSF, on the other hand, we have measured the concentration of dGuo to be approximately 2 nM. If it is assumed, that this is a typical dGuo concentration in CSF it is thus difficult to imagine, that artificial oxidation could explain an oxidation ratio in the range of 1:10 or even higher in the GC–MS based experiments. A more likely explanation could be calibration or contamination errors. The many steps in the sample preparation for the GC–MS analysis may increase the risk for contamination errors.

To the best of our knowledge the presented method is the first LC–MS based method for quantification of 8oxodGuo and/or 8oxoGuo in CSF. Contrary to GC–MS the free nucleosides can be measured directly by LC–MS without need for hydrolysis and derivatization before analysis. Several LC–MS based methods have been published for the quantification of 8oxodGuo and/or 8oxoGuo in other biological fluids like urine, plasma and saliva [33–35], but apparently not in CSF. The measured values in these fluids are higher than the levels we have detected for 8oxodGuo in CSF. Thus a reason for the lack of other LC–MS based methods for the quantification of 8oxodGuo in CSF could likely be lack of sufficient sensitivity. In the development of this method it has turned out, that it is very important that the LC–MS system is very clean. Thus if the mass spectrometer has just had a PM or if it had been used E.G. for measuring 8oxodGuo or 8oxoGuo in tissue samples it was necessary to flush the system for several days prior the CSF experiments before the sensitivity was back to normal.

Basically two different methods based on HPLC with electrochemical (EC) detection has been published for the detection of 8oxodGuo in CSF that apparently shows the ability to measure levels in the low pM range. One of the methods is based on pre-concentration of the samples on a carbon column that is very efficient in retaining the analyte [7,19,22] (They used 2–3 mL CSF). ESA is, however, no longer able to produce and supply these carbon columns. The other LC-EC method [20,21] is based on a solid phase extraction and up-concentration before LC-EC analysis. The authors, however, report a detection limit of 20 pmol so in this way the method would require in excess of 3 L of CSF to be able to measure the reported levels of 8oxodGuo in CSF (It is reported to require 1 mL), so obviously something is wrong. It may thus be that the method published in this paper is presently the only method capable of measuring the low levels of 8oxodGuo present in CSF. At least to the best of our knowledge it is the only method requiring only 100 µL CSF to obtain sensitivity to get detection limits in the low pM range.

5. Conclusion

A very sensitive and specific method based on UPLC–MS/MS for the quantification of 8oxodGuo, and 8oxoGuo in CSF requiring a sample size of only 100 µL has been developed.

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