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Isotope dilution high-performance liquid chromatography–electrospray tandem mass spectrometry assay for the measurement of 8-oxo-7,8-dihydro-2′-deoxyguanosine in biological samples

J.-L. Ravanat[a], B. Duretz[b], A. Guiller[b], T. Douki[a], J. Cadet[a,*]

[a]*Département de Recherche Fondamentale sur la Matière Condensée /SCIB/Laboratoire 'Lésions des Acides Nucléiques', CEA-Grenoble, 17, Avenue des Martyrs, F-38054 Grenoble Cedex 9, France*

[b]*Perkin-Elmer Applied Biosystems, 25 Av de la Baltique BP 96, 91943 Courtaboeuf Cedex, France*

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Abstract

A sensitive and specific assay aimed at measuring 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) has been developed by associating a reversed-phase liquid chromatographic separation with an electrospray tandem mass spectrometric detection. The HPLC–MS approach in the single ion monitoring (SIM) mode and the HPLC–MS/MS assay in the multiple reaction monitoring (MRM) mode have been compared, using isotopically labeled [M+4] 8-oxodGuo as the internal standard. The limit of detection of 8-oxodGuo was found to be around 5 pmol and 20 fmol for the HPLC–MS and HPLC–MS/MS methods, respectively. The HPLC–MS/MS assay is sensitive enough to allow the determination of the level of 8-oxodGuo in cellular liver DNA and in urine samples. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: DNA biomarkers; Oxidative stress; 8-Oxo-7,8-dihydro-2′-deoxyguanosine

1. Introduction

The measurement of oxidized bases in cellular DNA is a challenging analytical problem since the limit of the required sensitivity should approach one modification per 10^6 DNA bases in a few micrograms of DNA (for a review see [1]). Thus different analytical methods have been developed for such a purpose, mainly focused on the detection of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), one of the main oxidation products of DNA [2], use as a biomarker of in vivo oxidative stress (for a review see [3]). An HPLC separation associated with electrochemical detection (HPLC–EC) has been reported [4], allowing a sensitive detection of 8-oxodGuo. The latter assay has received numerous applications, and 8-oxodGuo has been shown to be produced in DNA under various conditions of oxidative stress (for a review see [3]). Simultaneously, a gas chromatographic method associated with a mass spectrometry detection technique (GC–MS) has been set up [5] for the measurement of 8-oxo-7,8-dihydroguanine (8-oxoGua), the corresponding free base, and also other DNA modifications [6]. The latter method is more versatile than the HPLC–EC assay, since it allows the detection of a wide array of lesions. In addition, the mass spectrometric detection makes possible the use of isotopically labeled deriva-

*Corresponding author.

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tive of the molecule of interest as the internal standards, increasing the accuracy of the determination [7]. In this respect, a number of oxidized DNA bases [8,9], including 8-oxo-7,8-dihydroguanine [10], labeled with stable isotopes (^{13}C, ^{15}N, ^{2}H) became recently available. For the GC–MS approach, a derivatization is requested since the DNA bases are not sufficiently volatile to be analyzed by gas chromatography. However, it was recently shown that the derivatization reaction induces significant oxidation of the overwhelming normal DNA constituents, including guanine [11,12], and also adenine and pyrimidine bases [13]. This artifactual oxidation reaction explains, at least partly, the higher levels of 8-oxoGua provided by the former GC–MS assay as compared with HPLC–EC [14]. To prevent such an artifactual oxidation to occur, a prepurification of the modified base to be measured is required prior to the derivatization step [11,13]. Interestingly, the levels of oxidized bases determined by the improved GC–MS assay are similar to those obtained by the HPLC–EC detection. However, the necessary prepurification of the targeted lesion prior to the GC–MS analysis constitutes a limitation to the application of the latter assay for routine analysis.

In the present study, we report the development of an HPLC–MS/MS method for the measurement of 8-oxodGuo. The assay combines the advantages of the two above mentioned methods: possibility of calibration with internal standards and versatility of the technique of detection on one hand, and straightforward analysis on the other hand. The application of HPLC–MS/MS with electrospray ionization, using isotopically labeled [M+4] 8-oxodGuo as the internal standard, allows the quantitation of 8-oxodGuo in cellular DNA samples as well as in urine.

2. Experimental

2.1. Chemicals

2′-Deoxyguanosine (dGuo), and 8-oxodGuo were purchased from Sigma, (St. Louis, MO, USA). [$^{15}N_3$,$^{13}C_1$] 8-OxodGuo was synthesized according to Stadler et al. [10]. HPLC grade acetonitrile, ammonium acetate and formic acid were from Prolabo (Fontenay-sous-Bois, France). Distilled deionized water was obtained from a Milli-Q system (Millipore, Molsheim, France).

2.2. Apparatus

On-line HPLC–MS measurements were carried out on a Perkin–Elmer 200 binary pumping system (Perkin–Elmer, Foster City, CA, USA). Loop injections were performed with a Perkin–Elmer 200 autosampler (Perkin–Elmer) using a Rheodyne 7125 injector with a 20 μl loop (Touzart et Matignon, Les Ulis, France). The 785 UV–vis detector (Perkin–Elmer) was set to 260 nm.

The RP-HPLC separations were achieved on a H5C18#15M octadecylsilyl silicagel Hypersyl (particle size: 5 μm, 150×2.0 mm I.D.) column obtained from Interchim (Montluçon, France). The mobile phase consisted of 10 mM ammonium acetate, adjusted to pH 4.8 with formic acid, and 5% acetonitrile. The eluent was degassed with helium prior to use and the flow-rate was 0.2 ml min^{-1}. The injection volume was typically 5 μl.

2.3. Electrospray ionization mass spectrometry

Positive ion mass spectra were acquired using a API 365 triple quadrupole mass spectrometer equipped with a turboionspray source (SCIEX, Thornill, Canada). The instrument response for 8-oxodGuo was optimized by infusion experiment of the pure compound dissolved in the mobile phase at a flow-rate of 5 μl min^{-1} with a Harvard Model 11 syringe pump (Harvard Apparatus, South Natick, MA, USA).

Electrospray ionization was performed with nitrogen as the nebulizing (1.23 l min^{-1}) and curtain (1.25 l min^{-1}) gas. An auxiliary gas (nitrogen) heated to 450°C was also used at a flow-rate of 8 l min^{-1} to improve the sensitivity. The total eluent was directed to the turboionspray source. The spray needle potential was set at 5500 V. Low energy CID mass spectra were obtained using nitrogen and a collision energy of 17 eV. Instrumental responses for the ions were optimized by ramping the ionspray voltage, the orifice voltage, the ring voltage, the collision energy (in the second quadrupole) and the

collision gas pressure. The size of the window that was used for parent selection was set to 1 during the optimization process and then to 0 during the acquisition.

2.4. Sample preparation

Standard samples: 8-OxodGuo and [$^{15}N_3$,^{13}C]-8-oxodGuo were dissolved in deionized distilled water at given concentrations which were determined by UV spectroscopy [15]. Appropriate dilutions of the solutions into the HPLC buffer were performed in order to obtain μ*M* (or less concentrated) solutions for the HPLC–MS analysis.

DNA samples: About 200 mg of liver tissue was dispersed in 1 ml of cold homogenization buffer (0.1 *M* NaCl, 30 m*M* Tris, 10 m*M* EDTA, 5 m*M* desferroxamine mesylate, 0.5% Triton X-100 and 10 m*M* ß-mercaptoethanol, pH 8.0) by using a 2 ml potter glass homogenizer. After homogenization, the sample was centrifuged at 1300×*g* for 15 min at 4°C. The pellet was washed with 0.5 ml of homogenization buffer and recovered by centrifugation (1300×*g*, 15 min, 4°C). Thereafter, the nuclear pellet was suspended into 3 ml of the extraction buffer (20 m*M* NaCl, 20 m*M* Tris, 20 m*M* EDTA, 5 m*M* desferroxamine mesylate, pH 8.0) and 200 μl of 10% SDS was added. The resulting suspension was homogenized in a 5 ml potter glass apparatus. Then, 100 μL of a 1 $mg\,ml^{-1}$ solution of proteinase (Qiagen, Hilden, Germany) was added and the sample was incubated for 1 h at 37°C. Subsequently, chloroform (1 ml) was added and the resulting solution was vigorously agitated for 30 s. After centrifugation at 3000×*g* for 5 min, the aqueous phase was collected, and the DNA was precipitated by addition of 300 μl of 4 *M* NaCl and 7.5 ml of cold 70% ethanol. The DNA pellet obtained after centrifugation (3000×*g*, 10 min) was rinsed using 500 μl of 70% ethanol, and then recovered by centrifugation.

The nucleic acid pellet was solubilized in 1 ml of RNase buffer (10 m*M* Tris, 1 m*M* EDTA, 2.5 m*M* desferroxamine mesylate, pH 7.4) to which 100 μl of RNase A (1 $mg\,ml^{-1}$, Sigma) and 10 μl of RNase T1 (100 $U\,ml^{-1}$, Sigma) were added. After incubation at 37°C for 1 h, 100 μl of a 4 *M* NaCl solution and 2.5 ml of ethanol (−20°C) were added. The sample was gently shaken until complete precipitation of DNA and then, centrifuged at 3000×*g* for 10 min at room temperature. The supernatant was discarded and the pellet gently rinsed with 500 μl of 70% ethanol. The liquid phase was discarded and the sample was left at room temperature for 5 min. The DNA pellet was then solubilized in 200 μl of deionized water, prior to DNA hydrolysis. DNA samples were digested, after addition of the internal standard, using nuclease P1 and alkaline phosphatase as previously described [16].

Urine samples: Urine was prepurified using a Maxi-Clean C18 (600 mg) cartridge obtained from Alltech (Deerfield, IL, USA) connected to a 5 ml syringe. Typically, 300 pmol of [M+4] 8-oxodGuo was added to 1 ml of urine. Then, the solution was filtered through a 0.22 μm filtration membrane and loaded on the C18 cartridges initially washed with 5 ml of methanol followed by 10 ml of water. Finally, the column was washed with 1 ml of water and 8-oxodGuo was eluted with 1 ml of 20% CH_3CN in water. Only 20 μl of the collected fraction was analyzed by HPLC–MS/MS.

3. Results

3.1. Mass spectrometry characterization of 8-oxodGuo

The oxidized nucleoside was analyzed in the positive electrospray ionization mode. Three main ions were observed in the full scan mass spectrum (Fig. 1). They correspond to the pseudo molecular ion $[M+H]^+$, the sodium adduct $[M+Na]^+$, and an intense fragment ion $[B+H_2]^+$, observed at m/z=284.0, m/z=306 and m/z=168.0, respectively.

The daughter mass spectrum (MS/MS spectrum) of the protonated molecular ion $[M+H]^+$ is shown in Fig. 2. The base peak at m/z=168.0 corresponds to the main fragment ion $[B+H_2]^+$ already observed in the full scan mass spectrum of 8-oxodGuo (*vide supra*), in agreement with previous observations [17]. Another fragment ion at m/z =117, corresponding to the protonated 2-deoxyribose was also observed. However it represented only about 3% of the base peak.

The [M+4] 8-oxodGuo is labeled on the base

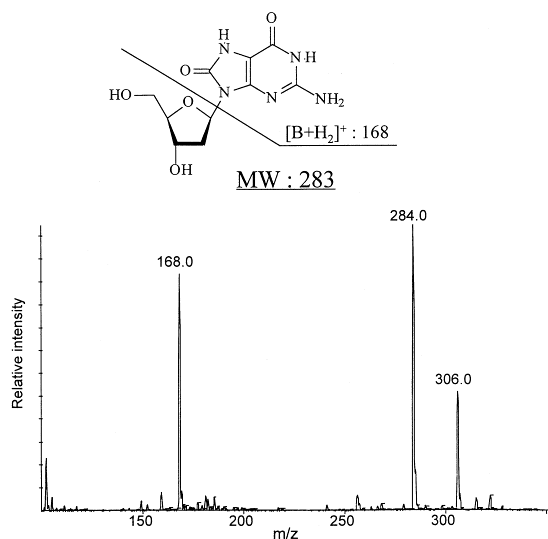

Fig. 1. Positive electrospray ionization mass spectrum of 8-oxodGuo.

moiety with three ^{15}N and one ^{13}C atoms [10], therefore the pseudo molecular ion $[MH]^+$ and the main fragment $[B+H_2]^+$ are expected to be 288.0 and 172.0 uma, respectively. Thus, for analytical purpose, in the SIM mode, the ions at m/z=284.0 and 288.0, for 8-oxodGuo and its isotopically labeled internal standard, respectively, were monitored. For HPLC–MS/MS analysis in the multiple reaction monitoring (MRM) mode, the transitions 284.0/168.0 and 288.0/172.0 were recorded.

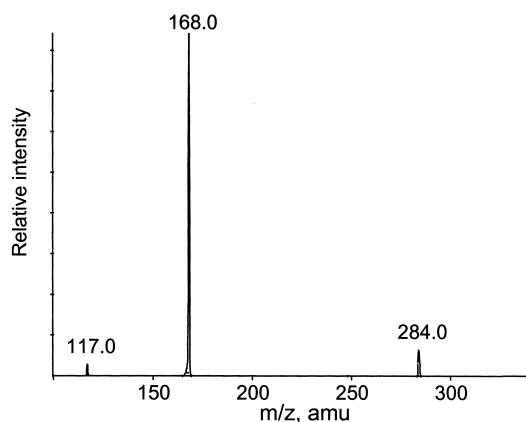

Fig. 2. Positive electrospray ionization mass spectrum (MS/MS) of 8-oxodGuo. The spectrum corresponds to the ion products of the pseudomolecular ion (m/z 284.0).

3.2. Sensitivity of the HPLC–MS and HPLC–MS/MS methods

The sensitivity of the detection of 8-oxodGuo provided by the HPLC–MS (SIM mode) and HPLC–MS/MS (MRM mode) assays was determined using the reference compounds. The ion current corresponding to the HPLC elution profile of the HPLC–MS analysis in the SIM mode of 5 pmol of both 8-oxodGuo and [M+4] 8-oxodGuo is shown on Fig. 3. Under the HPLC conditions used, both the normal and the labeled nucleosides elute at a retention time of 4.1 min. The injected amount (5 pmol) of 8-oxodGuo is close to the limit of detection provided by the HPLC–MS method. On the other hand, the limit of detection of 8-oxodGuo using the HPLC–MS/MS assay (MRM mode) is close to 20 fmol (Fig. 4).

A linear calibration curve was obtained using 5 pmol of [M+4] 8-oxodGuo as the internal standard and variable amounts of 8-oxodGuo ranging from 0.1 to 45 pmol (ten samples analyzed in triplicate). It is important to note that the slope of the curve (0.987) as well as the correlation coefficient (1.000) and the intercept (0.037) are close to 1 and 0, respectively.

3.3. Sample analysis

The HPLC–MS/MS method was used to measure the amount of 8-oxodGuo in DNA samples. The chromatograms corresponding to the HPLC–MS/MS analysis of enzymatic hydrolysates of extracted pig liver DNA and commercially available calf thymus

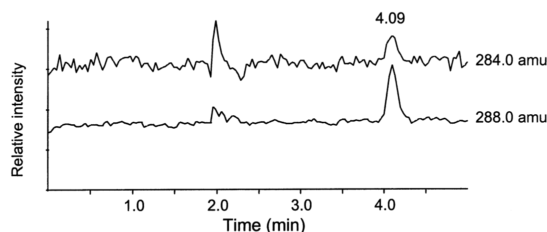

Fig. 3. HPLC–MS chromatograms obtained for the injection of 5 pmol of each 8-oxodGuo and [M+4] 8-oxodGuo in the SIM mode. The ions at m/z 284.0 (top chromatogram) and 288.0 (bottom chromatogram) for the detection of 8-oxodGuo and [M+4] 8-oxodGuo, respectively, were monitored.

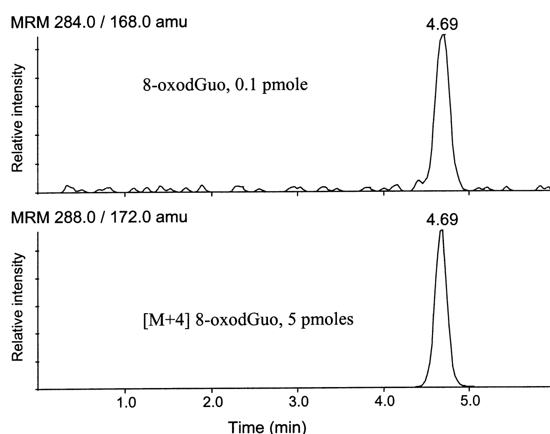

Fig. 4. HPLC–MS/MS chromatograms obtained for the injection of 0.1 pmol of 8-oxodGuo (top chromatogram) mixed with 5 pmol of [M+4] 8-oxodGuo (bottom chromatogram) in the MRM mode. The transitions 284.0/168.0 and 288.0/172.0 related to unlabeled and (M+4) labeled 8-oxodGuo, respectively, were monitored.

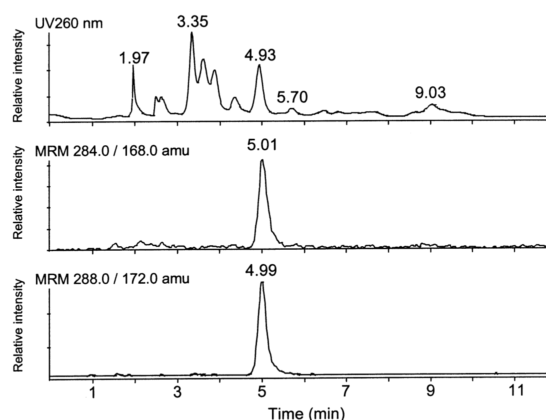

Fig. 6. HPLC–UV–MS/MS analysis of a human urine sample. UV (top chromatogram) and the MS/MS chromatograms (284.0/168.0 transition, middle chromatogram and 288.0/172.0 transition, bottom chromatogram) are represented. 8-OxodGuo elutes at 5 min under the HPLC conditions used.

DNA are reported in Fig. 5. Under the HPLC conditions used, 8-oxodGuo elutes at 4.7 min, whereas dGuo elutes at 3.8 min, as determined by UV absorbance detection set at 260 nm (data not shown). It should be pointed out that dGuo is partly oxidized at the output of the HPLC column, during the ionization process, since a m/z 284.0/168.0 transition is detected at the retention time corresponding to dGuo. The second peak observed at 4.7 min corresponds to 8-oxodGuo. The levels of 8-oxodGuo in liver and calf thymus DNA samples determined by the HPLC–MS/MS method were 0.7 and 2.8 per 10^5 dGuo, respectively.

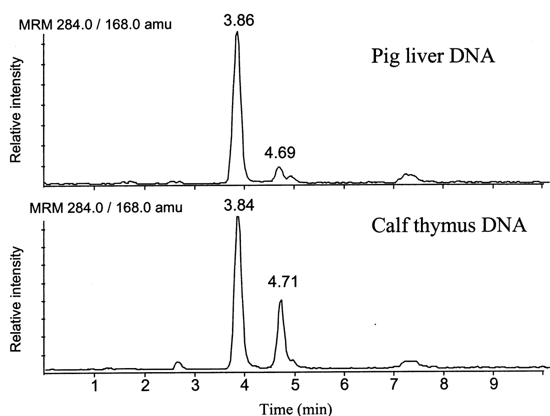

Fig. 5. HPLC–MS/MS chromatograms of the analysis of cellular pig liver (top chromatogram) and isolated calf thymus (bottom chromatogram) DNA samples. Only the 284.0/168.0 transition is represented. Under the HPLC conditions used, 8-oxodGuo and dGuo elute at around 4.7 and 3.85 min, respectively.

The possibility of applying the HPLC–MS/MS method to determine the amount of 8-oxodGuo in human urine was investigated. A quick prepurification of the urine sample, to which isotopically labeled 8-oxodGuo was added, was carried out. Then, the resulting solution was analyzed using the HPLC–MS/MS method. The products eluting at the output of the column were detected by both UV absorption at 260 nm and mass spectrometry under the above MRM conditions reported. As shown in Fig. 6, for both transitions (i.e. 284.0/168.0 and 288.0/172.0) a single peak, corresponding to the time of retention of 8-oxodGuo is observed. The level of 8-oxodGuo which was detected corresponds to 20 pmol ml^{-1} urine.

4. Discussion

The development of new methods for the measurement of oxidized bases and nucleosides in biological samples is still required. In this respect, the specificity and the sensitivity of the assays are the two major criteria to be taken into consideration for

further improvement of already existing analytical tools. For these purposes, it is assumed that the use of a mass spectrometry detection represents, at the moment, the best approach. The other main advantage of such a detector, is the possibility of using isotopically labeled internal standards, that could correct for eventual loss of the targeted product to be measured during the work-up. A mass spectrometry detection, associated with gas chromatography has been extensively used during the last decade for the detection of oxidized DNA bases [6]. However, such an approach requires the derivatization of the DNA samples that could generate artefactual oxidation of the overwhelming unmodified nucleobases if they are not removed (*vide supra*).

To our knowledge, only a few attempts have been made to monitor the formation of oxidized DNA bases in biological samples by HPLC coupled to mass spectrometry. This is mainly due to the poor sensitivity of the ionization methods available until recently, such as the thermospray technique [18]. More interestingly, the electrospray [19] and atmospheric pressure chemical ionization techniques represent better alternatives for the analysis of non volatile substances, mainly if they are associated with the high specific tandem mass spectrometry method. One example illustrating such an observation concerns the HPLC–MS/MS (electrospray ionization) measurement of 8-oxodGuo in DNA of rat after exposure to adriamycin [17]. The latter application showed already the suitability of the HPLC–MS/MS approach to monitor the formation of 8-oxodGuo in biological samples. However, the detection limit of the assay was not determined and accurate determination was not performed due mostly to the lack of an internal standard. Electrospray ionization mass spectrometry has been also applied successfully in the field of nucleic acid chemistry and biochemistry. This includes the characterization of synthetic [20] and modified [21] oligonucleotides, together with the detection of DNA adducts [22,23] and the analysis of modified 2′-deoxyribonucleosides [24]. Protonated molecular ion is generally the most abundant fragment observed for oxidized DNA bases together with the sodium and potassium adducts [24]. This is in agreement with the present observations. The mass spectrum of 8-oxodGuo (Fig. 1) exhibits a pseudomolecular ion at $[M+H]^+=284$ uma. The sodium adduct ion is also detected ($m/z=306$) together with the fragment corresponding to the loss of the 2-deoxyribose moiety. The latter ion $[B+H_2]^+$ results from the cleavage of the *N*-glycosidic bond with a transfer of an hydrogen atom from the sugar moiety, as already described for 2′-deoxy- and ribonucleosides [17,24]. The importance of the fragmentation (around 40% compared to the pseudomolecular ion) indicates that the *N*-glycosidic bound of the nucleoside is fragile. The MS/MS spectrum of 8-oxodGuo, obtained after collision-induced dissociation of the pseudomolecular ion at $m/z=284.0$ (Fig. 2) confirms that the major fragmentation involves the loss of the 2-deoxyribose moiety. A minor fragment, corresponding to the protonated 2-deoxyribose moiety is also observed at $m/z=117$.

The main advantage provided by electrospray ionization tandem mass spectrometry (MRM mode) for the detection of 8-oxodGuo is the significant increase in sensitivity with respect to the HPLC–MS (SIM mode) method. Using the SIM mode (HPLC–MS), the limit of detection is around 5 pmol of 8-oxodGuo injected (Fig. 3). The limitation of the sensitivity is mainly due to the occurrence of a high background due to solvent molecules aggregation. In this respect, it should be noticed that the background is higher for the profile recorded at $m/z=284$ compared to that at $m/z=288$. As expected, the use of the MRM mode (HPLC–MS/MS) leads to a considerable reduction of the background level. The quasi-absence of background (Fig. 4) leads to a significant increase in the sensitivity (about 20 fmol) by about two orders of magnitude with respect to the HPLC–MS approach. Interestingly, the observed sensitivity is close to that of the HPLC–EC detection method using coulometric detection [25]. Therefore, the HPLC–MS/MS assay is suitable for analysis of 8-oxodGuo within cellular DNA and also offers the possibility of using isotopically labeled internal standards. The calibration curve was found to be linear over a wide range of 8-oxodGuo, using 5 pmol of [M+4] 8-oxodGuo as the internal standard. The slope of the calibration curve and the intercept are close to 1 and 0, respectively. This indicates that 8-oxodGuo and related internal standard exhibit close similarity with respect to ionization and fragmentation. In addition, it may be inferred that no unlabeled 8-oxodGuo is contaminating [*M*+4] 8-oxodGuo.

Thus, the HPLC–MS/MS method has been used for the detection of 8-oxodGuo in commercially available calf thymus DNA and extracted pig liver DNA (Fig. 5). It is important to note that under the HPLC conditions used, dGuo and 8-oxodGuo elute at 3.85 and 4.7 min, respectively. Thus, the main peak detected at around 3.85 min for both samples represent the artifactual oxidation of dGuo that produce 8-oxodGuo, most probably during mass spectrometry analysis. Such an oxidation process stresses out the absolute necessity to separate 8-oxodGuo from dGuo in order to prevent any drawback to occur. Thus, direct injection of the sample into the mass spectrometer detector, without HPLC separation, could not be used to accurately determine the level of 8-oxodGuo in DNA samples. Interestingly, a separation was achieved using reversed-phase HPLC with solvents compatible with mass spectrometry detection. The levels of 8-oxodGuo detected under these conditions (Fig. 5), represent 0.7 and 2.8 8-oxodGuo per 10^5 dGuo for cellular pig liver DNA and isolated calf thymus DNA, respectively. These values are very similar to those determined in an independent way by HPLC coupled to electrochemical detection [26] which were 0.5 and 2.8 (data not shown) for pig liver DNA and calf thymus DNA, respectively.

The advantage of using the high specific isotope dilution tandem mass spectrometry detection method is illustrated by the determination of 8-oxodGuo in urine samples (Fig. 6). For such a detection, a three-dimensional HPLC method has been developed [27,28], in order to purify 8-oxodGuo prior to its quantification using an electrochemical detection method. However, the latter rather tedious assay is not suitable for routine analysis and the accuracy of the measurement may be questioned since no internal standard could be used. In contrast, application of the HPLC–MS/MS approach allows an accurate measurement of 8-oxodGuo in a relatively easiest way, requiring only 20 μl of urine. A very simple and fast prepurification was performed in order to remove the urine salts (that could decrease the sensitivity of the detection) and non polar constituents that could stick onto the HPLC column. Only one peak, corresponding to the retention time of 8-oxodGuo was observed on the HPLC–MS/MS chromatogram (Fig. 6). This indirectly demonstrates the specificity of the tandem mass spectrometric detection that allows accurate quantification of 8-oxodGuo in complex matrices. The level of 8-oxodGuo detected (20 pmol ml^{-1} urine) is in agreement with the values determined by the HPLC–EC approach [28].

5. Conclusion

We have demonstrated that the sensitivity of the HPLC–MS/MS method for the detection of 8-oxodGuo is compatible with the levels of the modified bases found in extracted DNA samples and in human urine. The specificity of the mass spectrometry detection together with the possibility of using isotopically labeled compounds as internal standards, represent a significant progress for the accurate determination of the level of 8-oxodGuo as compared with the traditionally widely used HPLC–EC method. This is particularly true for the measurement of oxidized DNA base in urine. Work is in progress in order to extent such a measurement to other oxidized bases and nucleosides, in both cellular DNA and biological fluids.

6. Abbreviations

dGuo	2′-deoxyguanosine
8-oxoGua	8-oxo-7,8-dihydroguanine
8-oxodGuo	8-oxo-7,8-dihydro-2′-deoxyguanosine
HPLC–EC	HPLC coupled to an electrochemical detector
HPLC–MS	HPLC coupled to mass spectrometry
HPLC–MS/MS	HPLC coupled to tandem mass spectrometry
GC–MS	GC coupled to mass spectrometry
SIM	Selected ion monitoring
MRM	Multiple reaction monitoring
CID	Collision-induced dissociation.

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