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Journal of Pharmaceutical and Biomedical Analysis
32 (2003) 657–661
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

LC-APCI-MS/MS analysis of urinary 8-hydroxy-2′-deoxyguanosine

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Received 24 April 2002; received in revised form 7 June 2002; accepted 19 June 2002

Abstract

8-Hydroxy-2′-deoxyguanosine (8OHdG) is regarded as an important biomarker of oxidative DNA damage and it may be estimated by using different techniques in various biological matrices, most notably DNA and urine. In the case of DNA, artifactual oxidation may take place during the isolation of DNA, its hydrolysis and possible derivatization (as for GC-MS), invalidating the measurement of 8OHdG. Therefore, the direct analysis of 8OHdG excreted into urine was preferred. Interferences from the urine matrix were excluded by applying LC-APCI-MS/MS in the multiple reaction monitoring (MRM) mode. The abundant fragment ion at *m/z* 168 arising from 8OHdG was monitored in the urine sample of volunteers supplemented with tomato concentrate for different times. The procedure allowed the detection of levels of 8OHdG as low as 1 ng/ml in urine sample.

Keywords: 8OHdG; Urine; MRM; APCI; LC-MS

1. Introduction

Reactive oxygen species (ROS) are constantly generated in vivo from incomplete reduction of oxygen in the respiratory chain (energy metabolism) or produced consequently to exogenous exposures, such as ionizing radiation, cigarette smoking, pollutants, food contaminants, excess of food and alcohol and strong exercise [1,2]. ROS and other free radicals can cause oxidative damage to cellular biomolecules, with DNA being a significant target. Oxidative attack to DNA results in different base modification products, including 8-hydroxy-2′-deoxyguanosine (8OHdG, Fig. 1), or the corresponding base 8-hydroxyguanine (8OHG) [3]. These products represent important biomarkers of cellular oxidative stress and are parameters of interest for evaluating the possible influence of dietary antioxidants in limiting DNA damage. To measure 8OHdG in human DNA, several analytical methods have been suggested, such as HPLC-ECD, GC-MS, LC-MS and immunochemical procedures [4–6]. All these meth-

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0731-7085/03/$ - see front matter
doi:10.1016/S0731-7085(03)00172-9

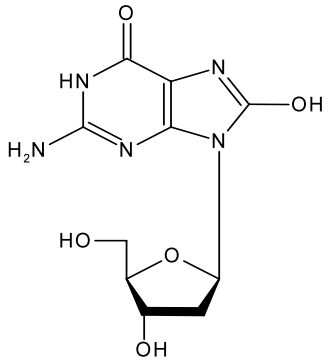

Fig. 1. Molecular structure of 8-hydroxy-2′-deoxyguanosine (8OHdG).

ods suffer from some limitations [7]. First of all, isolation of DNA and the subsequent acidic hydrolysis may cause artifactual oxidation of DNA bases, raising the apparent level of guanine oxidation products and invalidating the measurements.

In addition, sample preparation of DNA bases for GC-MS analysis requires derivatization, which may cause further artifactual oxidation. Consequently, the apparent level of guanine oxidation products may be overestimated. On the other side, HPLC analysis of 8OHdG after enzymatic or formic hydrolysis of DNA may yield underestimated data. For all these reasons, the quantitation of 8OHdG from plasma samples remains problematic [8].

The direct analysis of free 8OHdG in urine could be an alternative. In this case, there is no need to isolate and hydrolyze DNA, thereby excluding possible artifacts.

Indeed, the urine sample can be analyzed directly without any purification, except for the HPLC-ECD approach that requires a troublesome work-up of the urine to exclude interferences in the electrochemical detection.

LC-MS of urinary 8OHdG allows to overcome these drawbacks, as described by Weimann et al. [9]. In particular, LC-APCI-MS/MS in multiple reaction monitoring (MRM) mode has the potential to detect a low level of analytes through the monitoring of their characteristic fragment ions. This procedure has been applied to measure basal levels of urinary 8OHdG in healthy volunteers and the results are described in this paper.

2. Experimental

2.1. Chemicals

8-Hydroxy-2′-deoxyguanosine was purchased from Sigma-Aldrich (Milano, Italy). TFA was purchased from J.T. Baker (Milano, Italy). Methanol was acquired from BDH Laboratory Supplies (Poole, UK). All other reagents were HPLC grade (J.T. Baker, Deventer, Holland).

2.2. Samples urine preparation

The 24 h-urine sample of healthy subjects ($n = 12$) were collected and aliquots of 15 ml were acidified with 0.3 ml of 2 M HCl. The resulting solutions were centrifuged at $3000 \times g$ for 10 min and 100 μl were injected into the LC-APCI-MS system.

2.3. Calibration curve

A calibration curve was obtained from standard solutions of 8OHdG using samples concentrations in the range from 1 to 50 ng/ml and injecting 100 μl for each calibration point.

2.4. HPLC

A spectra series HPLC (Thermoquest, Milan, Italy) equipped with autosampler was used. Separations were performed using a Symmetry C_{18} column (250×4.6 mm) from Waters (Milford, MA). The eluent was methanol:water:trifluoroacetic acid (10:90:0.025, v/v/v). The flow-rate was 0.8 ml/min and the volumes injected were 100 μl.

A divert valve was inserted to discard the first 8-min eluate and after each run the column was rinsed with 90% methanol to wash impurities.

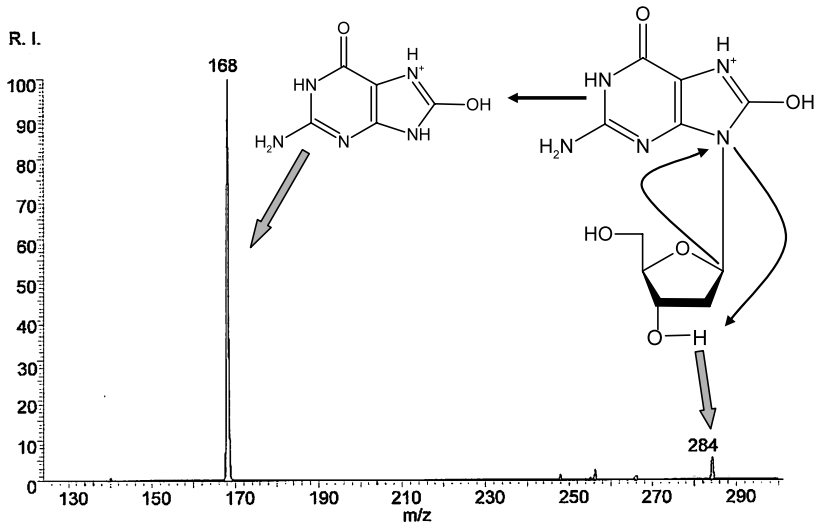

Fig. 2. MS/MS spectrum of protonated 8OHdG.

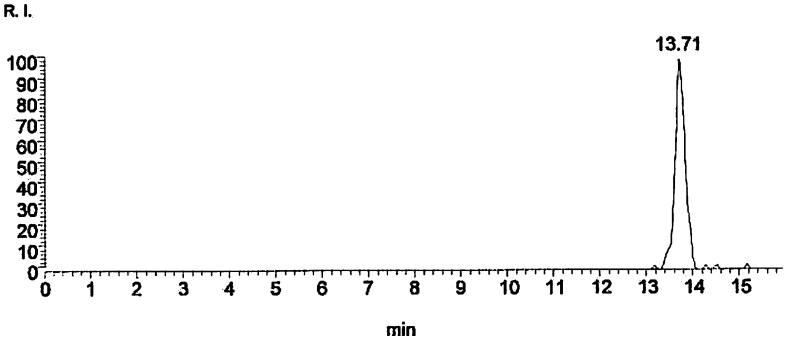

Fig. 3. Typical mass chromatogram of standard 8OHdG acquired in the MRM mode monitoring the daughter at *m/z* 168.

2.5. Mass spectrometry

The APCI mass spectra were obtained using a LCQ^{DECA} (Thermo Finnigan, Palo Alto, CA). The source temperature was 180 °C. The corona discharge voltage was 6 kV. MS/MS spectra of selected ions were obtained by resonance excitation, which was performed by applying a supplementary voltage corresponding to 30% of the maximum value (5 V peak-to-peak). The He pressure inside the trap was kept constant; the pressure directly read by ion gauge (in the absence of N_2 stream) was $2.8*10^{-5}$ Torr. LC-APCI-MS and MS/MS chromatograms were acquired in single ion monitoring (SIM) multiple reaction monitor (MRM) modes.

3. Results and discussion

In a first approach, 8OHdG was detected by LC-APCI-MS in the SIM mode. The mass chromatogram of standard 8OHdG (5 ng injected) was characterized by a peak (*m/z* 284) at 13.6 min.

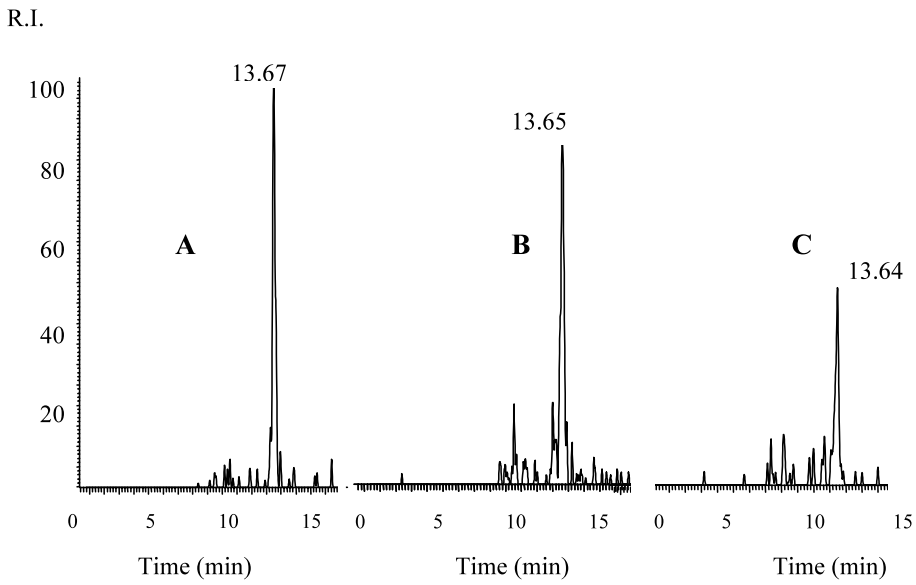

Fig. 4. Mass chromatograms, acquired in the MRM mode monitoring the daughter ion at *m/z* 168, of 24 h-urine collected at time 0 (A), 7 (B) and 21 (C) days from the same volunteers.

When this technique was applied to blank urine samples, very complex chromatograms were obtained. Indeed, several chemical species present in the urine matrix and different from 8OHdG yielded to the ion at *m/z* 284 and the detection of 8OHdG was unfeasible. Attempts to reduce this background through urine sample purification were unsuccessful. Besides this severe inconvenience, the limit of detection (LOD) in the SIM mode was $\approx$10 ng/ml and this value exceeds those expected in urine. For these reasons, LC-APCI-MS in the SIM mode was abandoned. By contrast, LC-APCI-MS/MS in the MRM mode was allowed to monitor an abundant fragment ion (*m/z* 168). This ion is produced from 8OHdG likely through an intramolecular hydrogen rearrangement (Fig. 2). A typical mass chromatogram of standard 8OHdG acquired in the MRM mode is given in Fig. 3. The linearity of the response was established up to 50 ng/ml, which is the range of interest in the analysis of urinary 8OHdG [11,13] and the correlation coefficient was higher than 0.996. The LOD was $\approx$1 ng/ml and also this value is consistent with those attainable in urine. The signal to noise ratio in the MRM mode was found to be about ten times higher than that achieved in the SIM mode. The overall reproducibility of quantitative analysis of 8OHdG was 3.4 and 5.1% for intra- ($n=4$) and inter- ($n=4$) day analysis, respectively. Most notably, the urine matrix did not interfere with the detection of the fragment ion at *m/z* 168.

The method was applied to evaluate the concentration of urinary 8OHdG in healthy subjects ($n=12$) given tomato concentrate (25 g) according to a protocol previously described [10,12]. Before supplementation, the mean content of urinary 8OHdG in the examined subjects was 9.4 ± 0.5 ng/ml, which is in agreement with literature data [11,13]. Typical mass chromatograms from 24 h-urine collected at time 0, 7 and 21 days from the same subjects are shown in Fig. 4. The total content of 8OHdG decreased to a final value of 3.8 ± 0.3 ng/ml. This trend was ascertained in ten subjects, thereby indicating that supplementation with tomato concentrate protects DNA against oxidative damage, as previously described by others authors [12].

From these results, it may be concluded that LC-APCI-MS/MS in the MRM mode allows the detection of urinary 8OHdG at levels suitable to evaluate the effect of dietary and herbal antioxidants on the reduction of DNA damage.

Acknowledgements

Professor Marisa Porrini is gratefully acknowledged for providing biological samples.

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