

ORIGINAL ARTICLE

Plasma and Urinary HPLC-ED Determination of the Ratio of 8-OHdG/2-dG in Parkinson's Disease

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SUMMARY

Background: Oxidative stress may be directly or indirectly involved in the pathogenesis of Parkinson's disease (PD). 8-hydroxy-2'-deoxyguanosine (8-OHdG) is the major product of DNA oxidative damage but its determination in plasma or urine may have controversial significance. The concentration of 8-OHdG not only depends on its oxidation rate but also on the efficacy of the DNA repairing systems.

Methods: We studied the ratio between 8-OHdG and 2-dG (the corresponding not hydroxylated base 2'-deoxyguanosine) in plasma and urine as a marker of oxidative stress in PD. This enabled the determination of the real DNA damage in terms of oxidation rate regardless of the efficacy of the DNA repairing mechanisms.

Results: We optimized two different analytical methods: one for 8-OHdG and the other for 2-dG, both based on a common preliminary solid-phase extraction step (SPE) followed by two different HPLC analytical separations with electrochemical detection (HPLC-ED).

The reliability of these methods was confirmed by analysing plasma and urine samples collected in parkinsonian patients and in age-matched healthy control subjects.

Conclusions: In urine samples, the measurement of 8-OHdG alone as well as the ratio 8-OHdG/2-dG were significantly different in healthy controls and PD patients. In plasma samples, only the ratio 8-OHdG/2-dG was significantly higher in PD compared to healthy controls showing that the ratio 8-OHdG/2-dG is a reliable diagnostic tool in studies on DNA oxidative damage.

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KEY WORDS

Oxidative stress, 8-hydroxy-2'-deoxyguanosine, Parkinson's disease, HPLC-ED

INTRODUCTION

Oxidative stress is widely recognized as a factor in many diseases, as either a cause or effect.

Chemicals, drugs, ionising radiation, solar light, cigarette smoking, and air pollution can increase the production of reactive oxygen species (ROS) that cause damage to DNA and to other macromolecules such as lipids and proteins [1,2].

The oxidative modification caused by ROS to biomolecules is a process held in check only by the existence of multiple antioxidant and repair systems as well as the replacement of damaged nucleic acids, proteins and lip-

ids. DNA is probably the most biologically significant target of oxidative attack, and it is widely thought that continuous oxidative damage to DNA is a significant contributor to the age-related development of the major cancer, aging, and degenerative diseases [3]. Although more than 20 different oxidative modifications of DNA bases have been identified, the major product of DNA impairment is 8-hydroxyguanine (8-OHG) and its nucleoside 8-hydroxy-2'-deoxyguanosine (8-OHdG).

As a consequence of the repair of DNA in vivo by exonucleases, the resulting 8-OHdG is excreted without further metabolism from cells and blood into urine. Thus, the plasma concentration and the urinary excretion of 8-OHdG reflect oxidative DNA damage and the "whole body" repair of DNA.

The concept of oxidative stress may be directly or indirectly involved in the pathogenesis of Parkinson's dis-

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ease (PD) [4]. Recent studies showed that oxidative damage to neurons by free radicals plays an important role in substantia nigra degeneration. Unfortunately, the data reported in the literature on the role of oxidative stress in PD are confusing. In addition, the determination of urinary 8-OHdG may only have controversial significance. Since measures of urinary levels of 8-OHdG mark the total extent of oxidative damage of DNA, a reduction in its excretion rate may be caused by a dietary assumption of free radical scavenger such as vitamin C, E, carotenoids, and polyphenols in sufficient amount [5, 6]. However, this reduction could result not only from a decreased oxidation of DNA bases (likely to be beneficial) but even from a diminished cellular turnover and repair of DNA (likely to be detrimental), as already reported in elderly patients with neurodegenerative pathologies.

To overcome the questions coming from the efficiency of repairing systems, it has been proposed that the determination of 8-OHdG directly in DNA extracted from peripheral blood leukocytes and the expression of the oxidative damage as a function of the corresponding not hydroxylated free base 2'-deoxyguanosine (2-dG) [7,8]. These methods, although more informative than the single assay of free 8-OH-dG in plasma and urine, are scarcely feasible and difficult to apply in routine analysis due to the complexity of pre-analytical procedures (i.e. double-strand DNA extraction and its enzymatic digestion before HPLC analysis).

Here we show that the ratio between 8-OHdG and 2-dG can be used to study DNA oxidative stress by analyzing plasma and urine and expressing the ratio for these matrices.

Indeed, using this method, we were able to highlight the real DNA damage in terms of oxidation rate independent from the efficacy of the DNA repairing mechanisms, which may be different between healthy controls and PD patients.

Therefore, we have optimized two different analytical methods, one for 8-OHdG and the other for 2-dG based on a common preliminary solid-phase extraction step (SPE) followed by two specific HPLC analytical separations with electrochemical detection (HPLC-ED) [9,10].

The reliability of these methods was shown by analyzing plasma and urine samples collected from two subgroups of parkinsonian patients, namely early stages (de novo) and advanced stages (PD) receiving levodopa therapy less than one year and over five years, respectively. The 8-OHdG/2-dG ratios determined in these patients were compared to age-matched healthy controls.

MATERIALS AND METHODS

Chemicals and solution preparation

All chemicals used, ammonium hydroxyde, sodium dihydrogen-orthophosphate, sulphosalicylic acid (SSA), hydrochloric acid, formic acid, citric acid, EDTA, so-

dium azide, and diethylamine were of analytical grade and purchased from Farmitalia (Milan, Italy). Water, acetonitrile, and methanol used for mobile phase and solution preparations were HPLC grade.

8-OHdG and 2-dG standards were purchased from Sigma-Aldrich (Milan, Italy), and cartridges for the solid phase extraction (MF C18 Isolute 50 mg, cod. 240-0005-A) from StepBio (Bologna Italy). Stock solutions of 8-OHdG (35 mmol/L) were prepared in water and kept at -80 °C until analysis. One aliquot of 8-OHdG stock solution was adequately diluted first in water and finally in plasma or urine matrix pooled from donor samples to obtain working spiked solutions ranging from 35 to 3500 pmol/L for plasma and from 35 to 3500 nmol/L for urine.

In the same way, stock solutions of 2-dG (37 mmol/L) were prepared in 0.5 mol/L ammonium hydroxyde and kept at -80 °C. At the time of analysis one aliquot was diluted first in water and finally in plasma or urine matrix to obtain working spiked solutions ranging from 0.37 to 3.7 µmol/L for plasma and from 370 to 3700 µmol/L for urine.

Collection of samples

Plasma samples were obtained from apparently healthy individuals (n = 13 controls, 6 males and 7 females), patients with idiopathic Parkinson's disease receiving levodopa therapy for over five years (n = 13 PD, 6 males and 7 females), and early stages PD subjects (n = 8 de novo, 4 males and 4 females) receiving therapy for less than 1 year.

The mean age of the subjects was 68 ± 12 years, all were non-smokers.

Blood samples were collected in vacuum sealed tubes containing EDTA. After sampling, the tubes were immediately centrifuged at 3500 rpm for 10 minutes at 4 °C and the plasma frozed at -80 °C.

First morning spot urine was also collected from healthy and PD subjects (4 males and 4 females for each group) and frozen until analysis.

Samples preparation

Both the standards and the unknown samples were processed according to the extractive procedure summarised below.

1 mL of EDTA plasma was deproteinized with 250 µL of SSA 15 % (w/v); after vortex mixing and centrifugation (10 minutes, 3500 rpm), 1 mL of supernatant was added with 100 µL NaH₂PO₄ 1.5 mol/L pH 4.5 and applied onto the SPE cartridge.

The urinary pH must also be modified before SPE extraction by the addition of 100 µL NaH₂PO₄ 1.5 mol/L pH 4.5 to 1 mL of centrifuged urine.

SPE cartridges were used according to the procedure of De Martinis-Bianchi with some modifications [10]. The columns were preconditioned with 1 mL acetonitrile, followed by 1 mL water and 1 mL NaH₂PO₄ 100 mmol/L pH 6.0.

1 mL of deproteinized plasma or buffered urine, spiked with 8-OHdG and 2-dG (working standard solutions) and not spiked (unknown samples), were applied to SPE cartridges and eluted without vacuum. Cartridges were then washed with 1 mL of water (washing step 1, W1) followed by 1 mL of NaH₂PO₄ 100 mmol/L, pH 6.0 (washing step 2, W2); both these washing solutions were collected in the same tube labeled W.

8-OHdG was finally eluted with 1 mL of NaH₂PO₄ 100 mmol/L, pH 6.0 acetonitrile 90:10, (v/v) (elution step, E).

For 8-OHdG analysis, one aliquot of the final SPE effluent E was injected into the chromatograph; for 2-dG analysis one volume of E solution was mixed with two volumes of washing solutions W before HPLC injection. In both cases, aliquots of 100 µL and 50 µL were injected for plasma and urinary assays, respectively.

8-OHdG and 2-dG were quantified by measuring peak areas via 712 Gilson software on the linear regression curve calculated for standard spiked solutions after subtraction of the nucleotide concentrations in the correspondent unspiked sample (standard 0).

Apparatus

The HPLC system consisted of a 307 pump model and a 234 autosampler model with a 100 µL loop, both from Gilson (Villiers-le-Bel, France). The separation was performed on a RP18 stainless-steel column (XBridge Shield 250 mm x 4.6 mm i.d.) packed with 5 µm particles (Waters, Milford MA, USA), with a 10 mm x 4.6 mm i.d. precolumn, packed with the same material.

Chromatographic conditions

For 8-OHdG analysis, the mobile phase was a mixture composed of formic acid 0.1 mol/L, citric acid 1.0 mmol/L, EDTA 0.5 mmol/L, sodium azide 7.7 mmol/L, diethylamine 24.0 mmol/L, and acetonitrile 4 % (v/v), pH 3 (not modified).

For 2-dG analysis, the mobile phase was a mixture composed of NaH₂PO₄ 50.0 mmol/L (pH 4.5) and acetonitrile 4 % (v/v).

A flow rate of 1.0 mL/min was used at room temperature, giving a pressure of about 120 kg/cm². In the optimized conditions, 8-OHdG elute with capacity factor (*k'*) of 4.1 and 2-dG of 2.8.

Electrochemical conditions

An electrochemical "coulometric" detector 5100 A (ESA, Bedford, MA, USA) fitted with a model 5011 high-sensitivity cell was used. For 8-OHdG, the applied potentials at the first and second electrode were 0.00 and + 0.50 volts, respectively, and for 2-dG analysis +0.40 and + 0.80 volts.

The current response was recorded at the second electrode only (2s time constant). Under the described conditions, the background current in 8-OHdG conditions was about 60 nA with a full scale deflection corresponding to 20 nA for plasma and to 200 nA for urine assay; in 2-dG conditions the background current was

about 150 nA with a full scale deflection of 50 nA and 5000 nA for plasma and urine assay respectively.

Statistical analysis

Statistical analysis was carried out with Stata 9.0 software [11]: for each series of data, Pearson correlation coefficients and Lin's concordance coefficient were used [12,13] as recommended in the most recent documents. The concordance between the series of data was estimated using Deming's regression model [14]. The level of significance was set at *p* < 0.05.

RESULTS

SPE setup: in order to optimize the sample pre-treatment step, different SPE procedures previously reported were tested [9,10]. In particular, we studied and optimized one washing step to reduce and minimize an important interfering compound that elutes with a retention time closely similar to 8-OHdG (*k'* = 3.8) in HPLC analysis.

After SPE purification, while 8-OHdG was recovered at 90 - 95 % in E solution, 2-dG was not retained in the cartridge and 70 - 75 % already eluted during washing steps W1 and W2 (tube W). Nevertheless, at the end of the purification and before HPLC analysis, two volumes of W solution were mixed with one volume of E. By injection of the obtained mixture, it was possible to analyze 2-dG accurately with good and nearly complete recovery (95 - 100 %).

Liquid chromatography optimisation: most authors have reported that the pH of the mobile phase in the range 3.5 - 7.0 was not relevant for the retention time of 8-OHdG. On the contrary, in our experience, a more acidic mobile phase (pH 3.0) permits a better resolution of the 8-OHdG peak from a major interferent that has a slightly greater *k'*, particularly for urine extracts. We have even observed that the percentage of acetonitrile in the mobile phase was very influential for the complete resolution of the 8-OHdG peak from that interfering component.

Because numerous analytical series may have a progressive "fusion" of the 8-OHdG peak with that close interfering component, a slight reduction in acetonitrile amount may overcome the problem.

In case of repeated injections of samples, we have repeatedly observed a progressive increment of baseline current. This is likely caused by a slow elution of high molecular weight molecules retained on the SPE cartridge during washing steps and co-eluted with 8-OHdG in the E solution.

The wash of the analytical column every 10 - 15 analytical runs with 20 - 30 mL of a mainly organic mobile phase composed of NaH₂PO₄ 50.0 mmol/L (pH 4.5) and acetonitrile 80 % (v/v) regenerate the column to the starting background values. When a HPLC system with dual pump is available, this step may be automatically

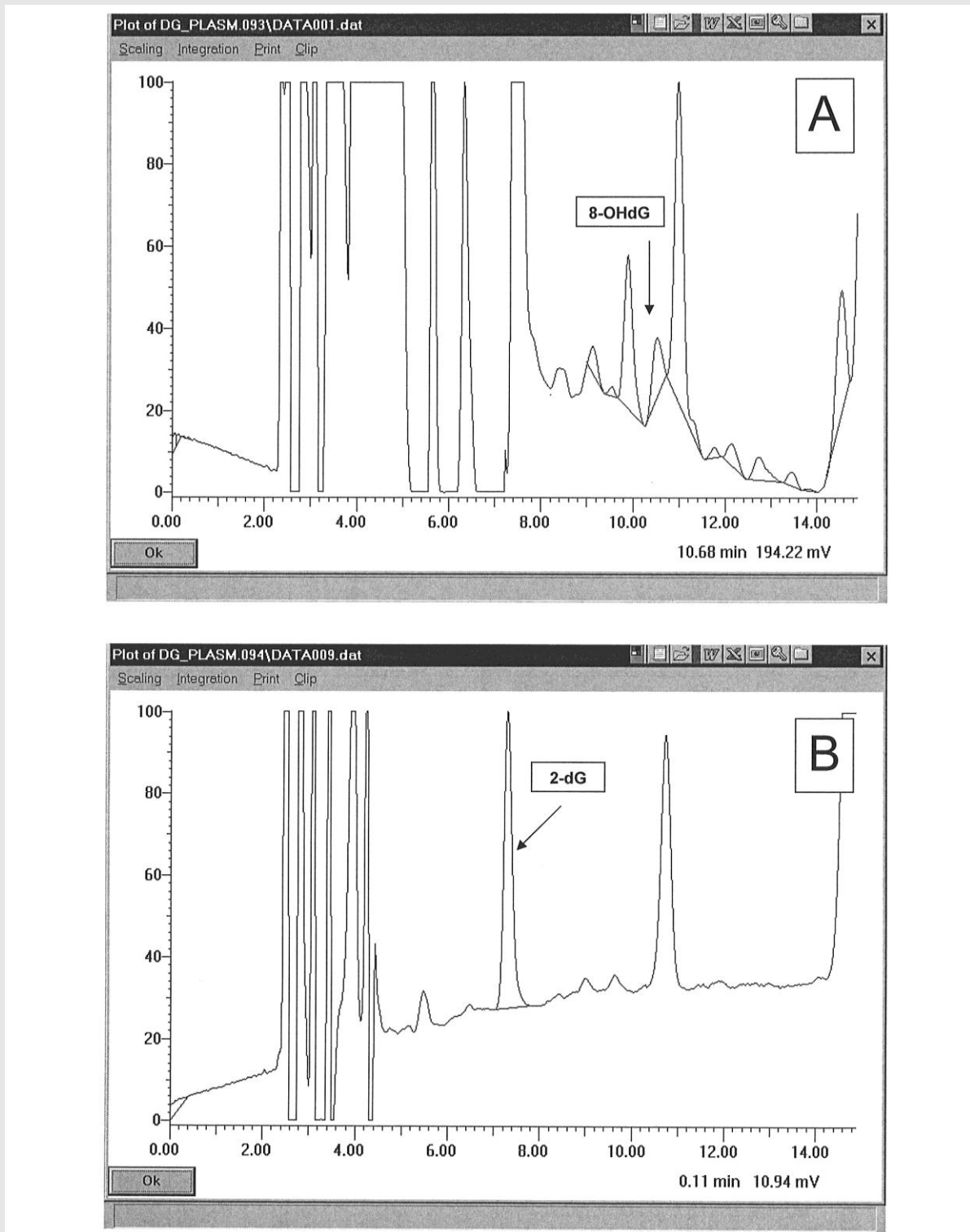


Figure 1. Typical chromatograms of a plasma extract in HPLC conditions for 8-OHdG (A) and 2-dG (B).

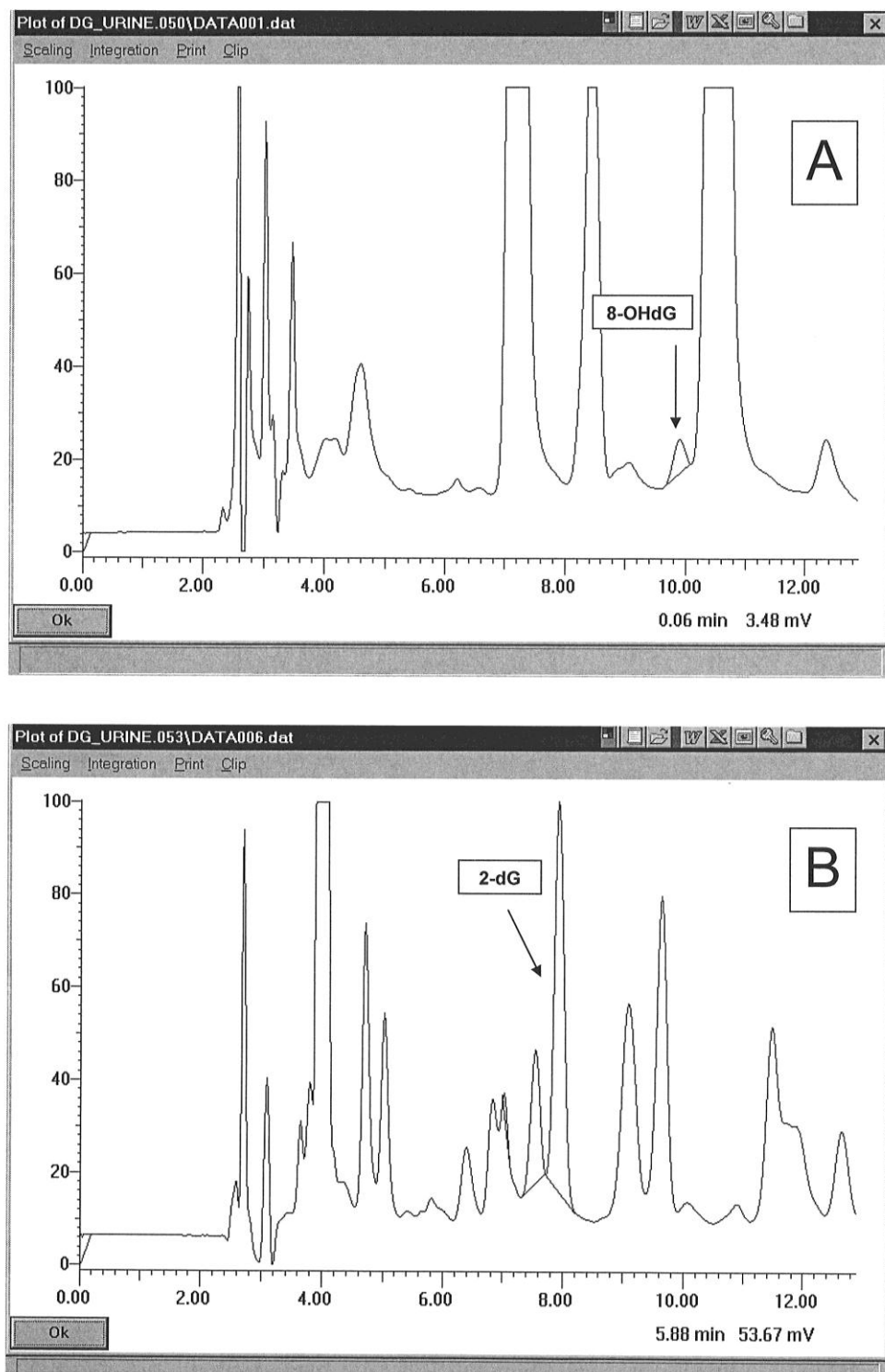
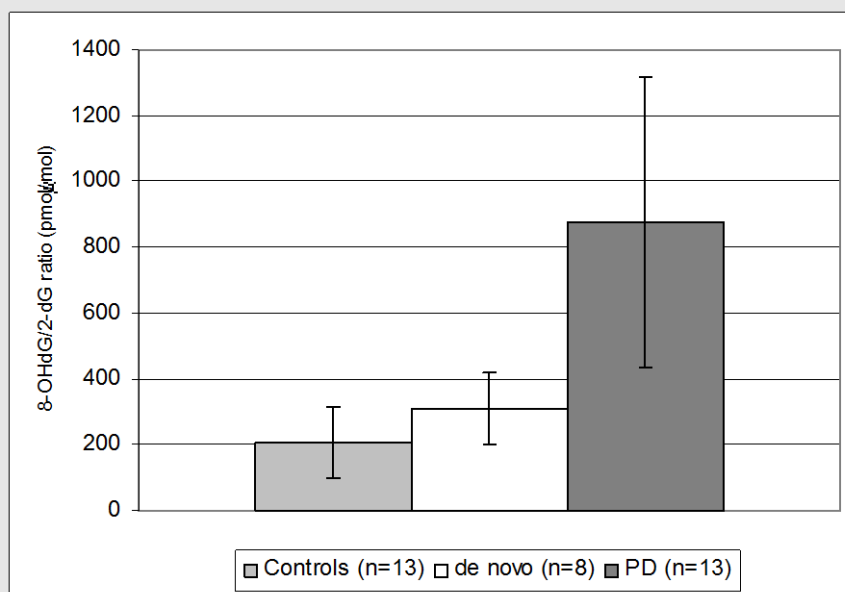


Figure 2. Typical chromatograms of a urine extract in HPLC conditions for 8-OHdG (A) and 2-dG (B).

Table 1. Plasma levels of 2-dG, 8-OHdG, and the 8-OHdG/2-dG ratio in the three groups studied.

	2-dG $\mu\text{mol/L}$		8-OHdG pmol/L		ratio 8-OHdG/2-dG $\text{pmol}/\mu\text{mol}$	
	mean	SD	mean	SD	mean	SD
Controls (n = 13)	1.48	0.36	308	172	208	109
de novo (n = 8)	1.60	0.44	499	251	311	111
PD (n = 13)	1.03	0.54	899	451	876	441

**Figure 3. Graphic representation (mean \pm SD) of the plasma 8-OHdG/2-dG ratio in the three groups studied.**

performed at the end of each analytical run and before the next injection.

Figures 1 and 2 shows typical chromatograms obtained from plasma and urine extracts, respectively, in the optimized conditions described above.

The intra- and inter-assay precision were calculated for both 8-OHdG and 2-dG by analysis in triplicate of plasma and urine spiked samples (n = 5). The mean results obtained showed a relative standard deviation less than 4 % for the intra-assay precision and less than 5 % for that of inter-assay. The accuracy calculated during the tests were within the range of ± 5 % compared to the expected values and sensitivity as signal to noise ratio (S/N = 2) was equal to 6 and 24 pmol/L for 8-OHdG and 2-dG, respectively.

Application in PD: in order to validate the proposed method, 2-dG and 8-OHdG were analysed in urine and EDTA plasma samples collected from PD patients subjected to levodopa therapy for a minimum of 5 years and in patients with the same pathology but in therapy for less than one year (de novo PD). These data were compared with those of healthy controls. Free 8-OHdG levels in plasma and urine were comparable with those previously reported in the literature [9,15,16]. The 8-OHdG/2-dG ratios obtained, expressed in pmol/ μmol (mean \pm SD), are reported in Table 1 and Figure 3 for plasma and in Table 2 and Figure 4 for urine.

Table 2. Urinary levels of 2-dG, 8-OHdG, and the 8-OHdG/2-dG ratio in controls versus PD subjects.

	2-dG μmol/L		8-OHdG nmol/L		ratio 8-OHdG/2-dG pmol/μmol	
	mean	SD	mean	SD	mean	SD
Controls (n = 8)	1299	721	20	17	15	14
PD (n = 8)	1037	444	319	210	308	173

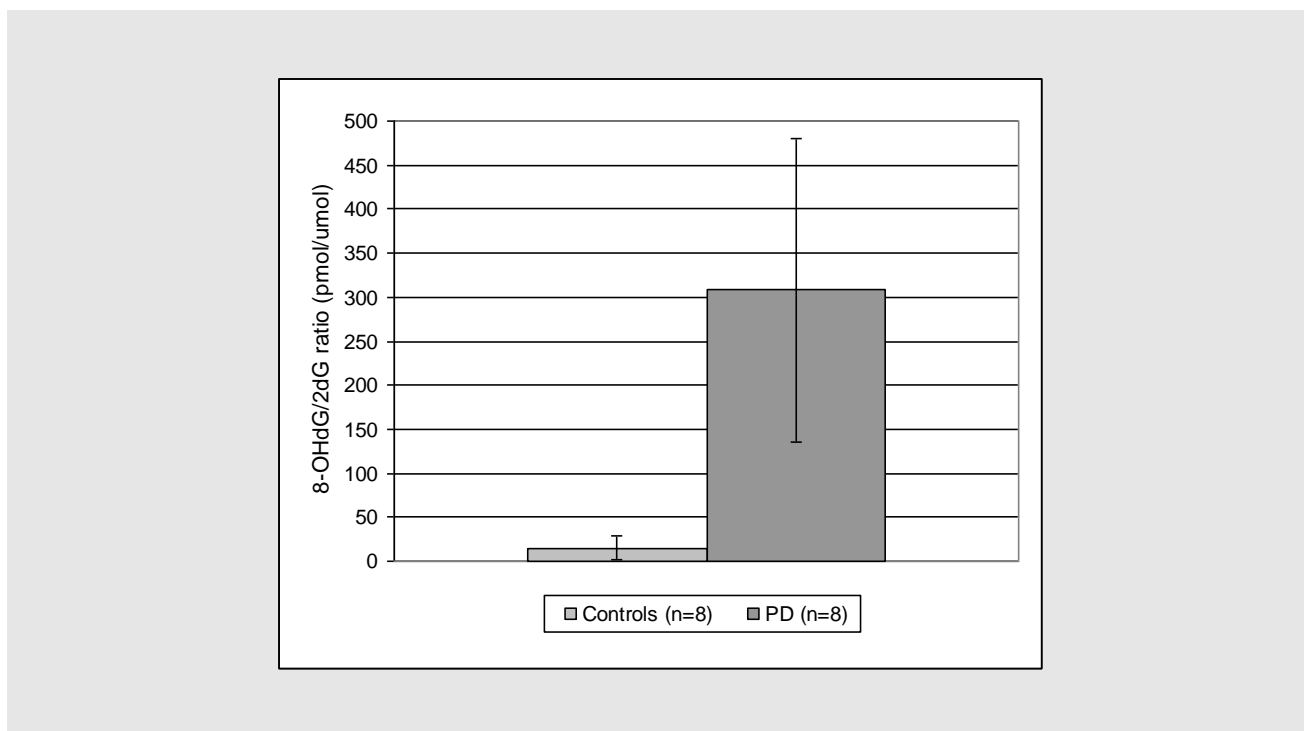


Figure 4. Graphic representation (mean ± SD) of the urinary 8-OHdG/2-dG ratio in controls versus PD subjects.

DISCUSSION

Recent advances in neurochemistry have shown the involvement of oxidative stress to the cascade leading to dopamine cell degeneration in PD. 8-OHdG has been widely accepted as a biomarker of DNA damage caused by reactive oxygen species. To study the relationship between endogenous oxidative damage and degenerative diseases associated with aging, several methods have been proposed (GC/MS, ELISA, LC/MS, capillary electrophoresis) in order to determine the 8-OHdG concentration in biological fluids.

As 8-OHdG oxidises readily, HPLC coupled with electrochemical detection (HPLC-ED) can detect it simply and at very low levels. Thanks to the optimisation of the SPE step and analytical HPLC separation, our method

combines easiness and reliability with a very high sensitivity; infact, the lower limit of detection was 10 pmol/L corresponding to one femtomol injected into the HPLC (signal to noise ratio of 2).

High sensitivity in the detection is very important particularly in plasma samples where 8-OHdG is detectable in very low amounts in healthy controls as well as in PD patients. The low concentration of 8-OHdG in plasma and/or urine is probably due to two different mechanisms in healthy controls and PD patients. In the former, there may be lower oxidative stress, in the latter a reduced DNA turnover, particularly in advanced stages. Our preliminary data in plasma samples (Table 1 and Figure 3) shows a marked but not statistically significant difference in 8-OHdG concentration between controls, de novo, and PD patients. In particular, 8-OHdG

levels in PD patients are from two (in de novo) to about three times (in PD) greater than controls. Further studies are ongoing in order to increase the number of observations and to strengthen its statistical significance.

An increase in diagnostic sensitivity may be still obtained with the complementary assay of the not-hydroxylated nucleoside 2-dG because the calculation of the ratio 8-OHdG/2-dG may reflect the real oxidative damage of DNA independently of its turnover.

In our data, 2-dG plasma levels in healthy controls and in de novo patients were comparable, while in PD these levels were slightly decreased, although they were not significant. On the contrary, when considering the ratio of 8-OHdG/2-dG in plasma, a greater and significant difference ($p < 0.01$) was found in both PD patient groups versus controls, in spite of the low number of observations compared.

In urine (Table 2 and Figure 4), however, the differences between controls and PD were significant for 8-OHdG ($p < 0.05$) as well as for 8-OHdG/2-dG ratio ($p < 0.01$).

Our future studies will clarify whether the plasma or urinary assay will be the preferable tool to better describe oxidative stress in PD.

CONCLUSION

Among the numerous types of oxidative damage, 8-OHdG is the most relevant marker for oxidative stress in DNA. Its determination together with the corresponding not hydroxylated nucleotide 2-dG and the consequent expression of the ratio 8-OHdG/2-dG may be a specific and sensitive marker in neurodegenerative diseases like PD.

The proposed analytical method is based on a SPE extraction of 8-OHdG and 2-dG from plasma and urine coupled with HPLC analysis with electrochemical detection. The excellent sensitivity and reliability of this method makes it a useful tool to evaluate DNA oxidative damage.

Our further studies aim to increment these preliminary observations in order to better and more accurately describe the oxidative phenomenon in neurodegeneration, with the objective to evaluate the antioxidant activity of dietary components and the effects of different therapeutic strategies to prevent or slow down neuron degeneration in PD.

Declaration of Interest:

The Authors declare that they have no conflict of interest with the topics treated in it, that the study was completely self-financed without intervention of pharmaceutical companies or any other sponsor.

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