Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Pharmaceutical and Biomedical Analysis 36 (2004) 101–104
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Study of urinary 8-hydroxydeoxyguanosine as a biomarker of oxidative DNA damage in diabetic nephropathy patients

G.W. Xu [a,*], Q.H. Yao [a], Q.F. Weng [a], B.L. Su [b], X. Zhang [b], J.H. Xiong [a]

[a] *National Chromatographic R&A Center, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116011, China*
[b] *Second Affiliated Hospitals of Dalian Medical University, Dalian 116023, China*

Received 15 January 2004; received in revised form 19 April 2004; accepted 24 April 2004

Available online 15 June 2004

Abstract

Increased oxidative stress induced by hyperglycemia may contribute to the pathogenesis of diabetic complications. Urinary 8-hydroxydeoxyguanosine (8-OHdG) has been reported to serve as a sensitive biomarker of oxidative DNA damage and also of oxidative stress. This article studied oxidative DNA damage in patients with diabetic nephropathy and in healthy control subjects by urinary 8-OHdG evaluations. Contents of 8-OHdG in urine were analyzed by capillary electrophoresis with end-column amperometric detection (CE-AD) after a single-step solid-phase extraction (SPE). Levels of urinary 8-OHdG in diabetic nephropathy patients with macroalbuminuria was significant higher than in control subjects (5.72 ± 6.89 μmol/mol creatinine versus 2.33 ± 2.83 μmol/mol creatinine, $P = 0.018$). A significant difference of 24 h urinary 8-OHdG excretions exists between the patients with macroalbuminuria and the patients with normoalbuminuria (19.2 ± 16.8 μg/24 h versus 8.1 ± 1.7 μg/24 h, $P = 0.015$). There was a positive correlation between urinary excretion of 8-OHdG and glycosylated hemoglobin (HbA_1c) ($r = 0.287$, $P = 0.022$). A weak correlation exists between the levels of 8-OHdG and triglyceride ($r = 0.230$, $P = 0.074$). However, the urinary 8-OHdG contents are not correlated with blood pressure and total cholesterol. The increased excretion of urinary 8-OHdG is seen as indicating an increased systemic level of oxidative DNA damage in diabetic nephropathy patients.

Keywords: Oxidative DNA damage; 8-Hydroxydeoxyguanosine; Diabetic nephropathy

1. Introduction

Reactive oxygen species (ROS) are involved in a diversity of biological phenomena such as inflammation, carcinogenesis, aging, and arteriosclerosis. It has been reported that ROS and increased oxidative stress might play an important role in the development of diabetic complications including nephropathy [1–3]. Hyperglycemia, a well recognized pathogenetic factor of long-term complications in diabetes mellitus, not only generates more ROS but also attenuates antioxidative mechanisms through glycation of the scavenging enzymes. Therefore, oxidative stress has been considered to be a common pathogenic factor of diabetic complications [4–6] including nephropathy [7].

8-Hydroxydeoxyguanosine (8-OHdG) is a product of oxidative DNA damage following specific enzymatic cleavage after ROS induced 8-hydroxylation of the guanine base in mitochondria and nuclear DNA [8]. When damaged DNA is repaired, 8-OHdG produced is excreted in urine without further metabolism [9,10]. Urinary 8-OHdG has been widely used as a sensitive marker of oxidative DNA damage and of the total systemic oxidative stress in vivo [11,12]. Recent reports [13–15] found an increased 8-OHdG level in mononuclear cells and urine in patients with insulin-dependent diabetes mellitus (IDDM) and non-insulin-dependent diabetes mellitus (NIDDM). Giugliano et al. [16] reported that the leukocyte or urinary 8-OHdG was related significantly with the severity of diabetic nephropathy and retinopathy. The study of Hinokio et al. [17] provided direct evidence that increased urinary 8-OHdG at entry was associated with the development of diabetic nephropathy after 5 years. But there are overall very few data on oxidative DNA damage in patients with diabetic complications. Thus, in this paper we

* Corresponding author. Tel.: +86 411 83693403; fax: +86 411 83693403.
E-mail address: dicp402@mail.dlptt.ln.cn (G.W. Xu).

0731-7085/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2004.04.016

investigated 8-OHdG in urine from 69 diabetic nephropathy patients and 30 healthy control subjects by using capillary electrophoresis with end-column amperometric detection (CE-AD) after a single-step solid-phase extraction (SPE) [24].

2. Experimental

2.1. Apparatus

An electrochemical analyzer (model CHI 800, CH Instruments, Austin, TX, USA) was used for end-column amperometric detection. In connection with a cell, the potentiostatic control of the electrode potential was used by means of a three-electrode system which consists of a carbon fiber microdisk electrode (about 30 carbon fibers with 6 μm diameter) as the working electrode, a Pt wire as the auxiliary electrode and a saturated calomel electrode (SCE) as the reference electrode. The reference electrode was connected to the electrolytic cell containing the analyte via a salt bridge filled with the same supporting electrolyte as in the cell. Carbon fiber microdisk electrode was constructed as described in reference [18].

Electrophoresis in the capillary was driven by a high voltage dc (0–30 kV) power supply (model NT-9123, Beijing Institute of New Technology, Beijing, China). Uncoated fused-silica capillaries (25 μm i.d.) were obtained from Yongnian Optical Conductive Fiber Plant (Yongnian, China) and flushed with 0.1 M sodium hydroxide solution overnight before use. The high voltage was applied at the injection end of the capillary, while the outlet end also as electrochemical cell was held at ground potential. The electrochemical cell was shielded in a copper box.

2.2. Chemicals and reagents

8-Hydroxydeoxyguanosine of analytical grade was obtained from Sigma (St. Louis, MO, USA) and used as received. Stock solutions of 8-OHdG were stored at 4 °C in refrigerator when not in use. KH_2PO_4, Na_2HPO_4, $Na_2B_4O_7{\cdot}10H_2O$ were all purchased from Shenyang Federal Reagent Corp. (Shenyang, China) and were analytical grade. Methanol (CH_3OH) was HPLC grade obtained from Tedia Company (USA). Bond Elut LCR SPE columns (C_{18}/OH, 500 mg, 6 ml) were purchased from Varian (Harbor City, CA, USA). A 12-port vacuum manifold was obtained from J&W Scientific (USA). All solutions were prepared with ultra-pure water from a Milli-Q water system (Millipore Corp., Bedford, MA, USA).

2.3. Study of the subjects

Twenty-four hours urine samples were collected from 69 type II diabetic patients (age range, 40–90 years, 62.9±12.1) from the Second Affiliated Hospitals of Dalian Medical University, China. Random urine samples were collected from 30 healthy donors from author's institute (age range, 40–70 years, 53.1 ± 7.2).

Urinary albumin excretion rate (UAER) is an important clinic index for the diagnosis of diabetic nephropathy. A patient is diagnosed as diabetic nephropathy when the UAER exceeds 200 μg/min constantly. In this study, the type II diabetic patients fulfilled the WHO diagnostic criteria for NIDDM. Based on Mogensen's estimate method for stages of diabetic nephropathy [19], the patients were classified into three groups according to the degree of UAER: the groups of normoalbuminuria (L group: below 20 μg/min, $n = 18$), microalbuminuria (M group: 20–200 μg/min, $n = 28$), and macroalbuminuria (H group: above 200 μg/min, $n = 23$). Some biochemical and demographic characteristics of the study groups of patients are presented in Table 1.

2.4. SPE pretreatment procedure of urine samples

Urine samples collected were titrated with 1 M HCl to the pH 4–5, then were frozen at −20 °C in refrigerator. Before analysis, urine was thawed at room temperature and was centrifuged at 1000 rpm for 5 min in order to remove precipitates, then was filtered through a 0.2 μm micropore filter membrane. A 12-port vacuum manifold supplied the vacuum of the SPE column. The Bond Elut C_{18}/OH cartridge was preconditioned with 10 ml of methanol and 10 ml of water in turn, and then 2 ml of urine was applied. The column was washed with 5 ml of water. 8-OHdG was eluted with 2 ml of 30% methanol. The eluate containing 8-OHdG was placed in a water bath at a temperature of 39 °C and evaporated into dryness. The concentrated sample was dissolved in 100 μl 30 mM phosphate solution (pH 6.5), thus a 20-fold concentration of the sample was obtained for analysis.

2.5. Analysis of urinary 8-OHdG by CE-AD method

A 85 cm capillary was used for electrophoresis. Electromigration injection was performed under 20 kV for 10 s. Electrophoresis was carried out in 30 mM Na–borate buffer solution (pH 9.10) at 22 kV. Furthermore, a novel sample focusing method named as dynamic pH junction reported by Chen and co-workers [26,27] was employed in this study to improve analytical sensitivity. And this focusing method was proved valid in increasing the concentration sensitivity for analyzing urinary 8-OHdG in the previous report of our group [24]. Optimal focusing was achieved with a high column efficiency ($N > 2.9 \times 10^5$) when the pH of the sample solution was 6.5 and the pH of BGE was 9.10. Therefore, 30 mM phosphate solution (pH 6.5) was used as sample matrix. The amperometric detection mode at a constant potential 0.45 V was performed by end column approach with the three-electrode system mentioned above.

Table 1
Clinical and demographic characteristics of the patients

Clinical parameters	UAER (μg/min)		
	L group	M group	H group
	<20	20–200	>200
Gender (male/female, *n*)	6/12	12/16	9/14
Age (years)[a]	58.8 ± 13.3	62.7 ± 12.3	66.3 ± 10.1
Triglyceride (mmol/l)[a]	1.3 ± 1.0	1.3 ± 0.6	2.4 ± 1.9
Total cholesterol (mmol/l)[a]	4.7 ± 1.4	5.4 ± 1.6	5.7 ± 1.4
HbA_1c (%)[a]	8.2 ± 1.8	9.6 ± 1.9	8.9 ± 1.6
BP(mmHg, syst/diast)[a]	137 ± 20/79 ± 7	141 ± 17/80 ± 11	148 ± 23/84 ± 12

[a] Data in the table are mean ± S.D.

2.6. Statistical analysis

One-way analysis of variance (one-way ANOVA) was used to compare 8-OHdG levels between groups. Correlation was tested by regression analysis on MS Excel software. Data in the text were given as mean ± S.D. Differences of $P < 0.05$ were considered statistically significant.

3. Results and discussion

The single-step SPE was efficient for separating 8-OHdG from interfering urinary matrix components. The recovery of 8-OHdG in the extraction procedure from 2 ml of urine was found to be 82.7 ± 6.9% ($N = 6$) and was constant over the concentration range from 20 to 100 nM. In contrast, the recovery of aqueous standard after the SPE procedure was found to be 87.5 ± 5.0% ($N = 4$).

The reproducibility of the peak current and the migration time in this experiment was determined by repeatedly ($n = 8$) injecting 1 μM 8-OHdG standard into the system under the above optimized conditions. The relative standard deviation (R.S.D.) was found to be 2.3% for peak current, and 1.1% for migration time. The calibration curve exhibits excellent linear behaviour over the concentration range from 10nM to 50 μM. The correlation coefficient was 0.9996. The average inter- and intra-day precision for quantitation was 1.26 and 3.97%, respectively.

The limit of detection for an aqueous standard of 8-OHdG was 4.3 nM (S/N = 3), which was 10-fold lower than that of the off-column CE-AD method reported by Weiss and Lunte [25] (50 nM) and almost five-fold lower than that of our previous study [24] (20 nM) for analyzing 8-OHdG. Since the concentration of a urine sample was increased 20-fold after the SPE step, the limit of detection for a urine sample was 0.22 nM. And the lower limit of the linear range also can be reduced to 1 nM that is enough to determine the low concentration of urinary 8-OHdG.

The greater improvement of detection limit in this method contributed mainly to two reasons. One is the employment of dynamic pH junction which increased concentration sensitivity. The other is the working electrode was a carbon fibre microdisk electrode and was laid at the end of the separation capillary instead of using a carbon fibre microcolumn electrode inserted into the capillary. Such a placement not only avoided interference of electric field brought from the high pressure applied on the capillary and greatly decreased the noise on the electrode.

An example of CE-AD analysis of 8-OHdG in urine extraction from a healthy volunteer was shown in Fig. 1. The peak of 8-OHdG was identified by migration time and/or spiking with 8-OHdG standard. The concentrations of urinary 8-OHdG were quantified by the five-point calibration.

The mean level of urinary 8-OHdG excretions was 4.15 ± 4.85 μmol/mol creatinine in diabetic nephropathy patients and 2.33 ± 2.83 μmol/mol creatinine in control subjects ($P = 0.058$). Results of the control subjects agreed well with those reported previously by HPLC-ECD [20]. The levels of 8-OHdG in urine of the three groups divided according to the UAER were shown in Table 2. No difference was observed between the healthy group and L group. Although the M group had higher averaged 8-OHdG contents in urine than the healthy group, there was no significant difference between the two groups ($P = 0.069$). A significant difference was found between H group and the healthy group ($P = 0.018$).

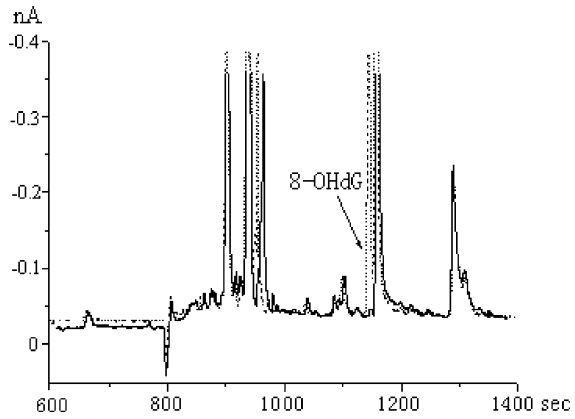

Fig. 1. Electropherogram of a spiked (dashed trace) and unspiked (solid trace) urine extract. Electrophoretic conditions: 25 μm × 85 cm capillary; BGE, 30 mM Na–borate, pH 9.10; injection, 20 kV, 10 s; separation voltage, 22 kV; detection potential, 0.45 V vs. SCE.

Table 2
The levels of 8-OHdG in urine of the patients compared to the healthy group

Subject	Group	Subject number	8-OHdG/creatinine (μmol/mol)	8-OHdG (μg/24 h)
Healthy group		30	2.33 ± 2.83	–
Diabetic	L	18	2.63 ± 2.94	8.1 ± 1.7
neuphropathy	M	28	3.84 ± 3.37	12.5 ± 10.3
patients	H	23	5.72 ± 6.89	19.2 ± 16.8

Data in the table are mean ± S.D.

The differences of 24 h urinary excretions of 8-OHdG were compared among the three patient groups. It was found that a noticeable difference existed between the H and L groups ($P = 0.015$), no significant difference was found between the M and L groups ($P = 0.15$).

By regression analysis, the urinary 8-OHdG excretions were found to be correlated positively with glycosylated hemoglobin (HbA_1c)($r = 0.287$, $P = 0.022$) showed in Fig. 2. The result is in agreement with the literature [14]. A weak correlation exists between the levels of 8-OHdG and triglyceride ($r = 0.230$, $P = 0.074$). However, the urinary 8-OHdG contents were not correlated with blood pressure and total cholesterol.

8-OHdG is known as a sensitive biomarker of oxidative DNA damage also of oxidative stress. The high urinary 8-OHdG levels in the patients with high albuminuria investigated in this study suggested that the increased oxidative stress has a primary role in the pathogenesis of diabetic nephropathy. Recent reports found an increased systemic oxidative stress in diabetic patients and in diabetic animal models [13,21–23]. Our study showed that the patients with normoalbuminuria had a lower urinary 8-OHdG level than the patients with high albuminuria. We speculated that the increased urinary 8-OHdG in the diabetic nephropathy patients might have resulted not only from the increased systemic oxidative stress, but also from the kidney in which oxidative stress increased caused by hyperglycemia.

In summary, the present study found high urinary excretion of 8-OHdG in diabetic nephropathy patients, especially in patients with macroalbuminuria. Further investigations are required to know the full meaning of the increased oxidative DNA damage in diabetic complications.

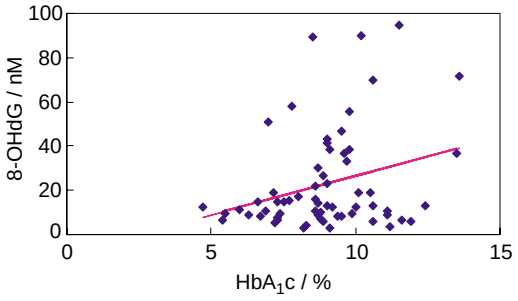

Fig. 2. Correlation between HbA_1c and 8-OHdG levels in urine.

Acknowledgements

We are thankful to the National Natural Science Foundation of China for financial support (No. 29975029), the high-technology development plan “863 project” (2003AA223061) of State Ministry of Science and Technology of China and the Knowledge Innovation Program of the Chinese Academy of Sciences.

References

[1] J.W. Baynes, Diabetes 40 (1991) 405–412.
[2] J.W. Baynes, S.R. Thorpe, Diabetes 48 (1999) 1–9.
[3] T. Nishikawa, D. Edelstein, X.L. Du, et al., Nature 404 (2000) 787–790.
[4] S. Suzuki, Y. Hinokio, K. Komatu, M. Ohtomo, M. Onoda, S. Hirai, M. Hirai, A. Hirai, M. Chiba, S. Kasuga, H. Akai, T. Toyota, Diabetes Res. Clin. Pract. 45 (1999) 161–168.
[5] S.P. Wolff, Z.Y. Jiang, J.V. Hunt, Free Radic. Biol. Med. 10 (1991) 339–352.
[6] S.P. Wolff, Br. Med. Bull. 49 (1993) 642–652.
[7] R.G. Larkins, M.E. Dunlop, Diabetologia 35 (1992) 499–504.
[8] M.S. Cooke, M.D. Evans, K.E. Herbert, Free Radic. Res. 32 (2000) 381–397.
[9] M.K. Shigenaga, B.N. Ames, Free Radic. Biol. Med. 10 (1991) 211–216.
[10] S. Loft, K. Vistisen, M. Ewertz, A. Tjnne´øland, H.E. Poulsen, Carcinogenesis 13 (1992) 2241–2247.
[11] M. Shigenaga, C. Gimeno, B.N. Ames, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 9697–9701.
[12] B. Halliwell, Free Radic. Res. 25 (1996) 57–74.
[13] P. Dandona, K. Thusu, S. Cooke, Lancet 347 (1996) 444–445.
[14] J. Leinonen, T. Lehtimaki, S. Toyokuni, et al., FEBS Lett. 417 (1997) 150–152.
[15] Y. Hinokio, S. Suzuki, M. Hirai, M. Chiba, A. Hirai, T. Toyota, Diabetologia 42 (1999) 995–998.
[16] D. Giugliano, A. Ceriello, G. Paolisso, Diabetes Care 19 (1996) 257–267.
[17] Y. Hinokio, S. Suzuki, M. Hirai, C. Suzuki, M. Suzuki, T. Toyota, Diabetologia 45 (2002) 877–882.
[18] W. Jin, Q. Weng, J. Wu, Anal. Chim. Acta 342 (1997) 67–74.
[19] C.E. Mogensen, C.K. Christensen, E. Ritlinghus, Diabetes 26 (1983) 64–78.
[20] D. Germadnik, A. Pilger, H.W. Rüdiger, J. Chromatogr. B 689 (1997) 399–403.
[21] N. Yuji, H. Goji, N. Naoto, I. Eiichiro, O. Hirotomo, Y. Toshikazu, New Technol. Med. 3 (2002) 92–96.
[22] M. Riikka, R. Vappu, X. Huang, et al., Hum. Genet. 105 (1999) 179–180.
[23] Y. Ihara, S. Toyokuni, K. Uchida, H. Odaka, T. Tanaka, H. Ikeda, H. Hiai, Y. Seino, Y. Yamada, Diabetes 48 (1999) 927–932.
[24] S.R. Mei, Q.H. Yao, L.S. Cai, J. Xing, G.W. Xu, C.Y. Wu, Electrophoresis 24 (2003) 1411–1415.
[25] D. Weiss, C. Lunte, Electrophoresis 21 (2000) 2080–2085.
[26] P. Britz-Mckibbin, D.D.Y. Chen, Anal. Chem. 72 (2000) 1242.
[27] P. Britz-Mckibbin, G. Bebault, D.D.Y. Chen, Anal. Chem. 72 (2000) 1729.