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Fast quantification of the urinary marker of oxidative stress 8-hydroxy-2′-deoxyguanosine using solid-phase extraction and high-performance liquid chromatography with triple-stage quadrupole mass detection

Thomas Renner[a], Thomas Fechner[b], Gerhard Scherer[a,*]

[a]*ABF, Analytisch-biologisches Forschungslabor, Goethestrasse 20, D-80336 Munich, Germany*
[b]*PE Biosystems, Paul-Ehrlich-Strasse 17, D-63225 Langen, Germany*

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Abstract

Exogenous and endogenous oxidants constantly cause oxidative damage to DNA. Since the reactive oxidants itself are not suitable for analysis, oxidized bases like 8-hydroxy-2′-deoxyguanosine (8OHdG) are used as biomarkers for oxidative stress, either in cellular DNA or as elimination product in urine. A simple, fast and robust analytical procedure is described for urinary 8OHdG as an indicator of oxidative damage in humans. The adduct was purified from human urine by applying a single solid-phase extraction step on LiChrolut EN®. After evaporation of the eluate, the residue was resolved and an aliquote was injected into a HPLC system with a triple quadrupole mass spectrometer. The limit of detection was 0.2 ng ml^{-1} (7 fmol absolute) when using one product ion as quantifier and two further product ions as qualifier. The coefficient of variation was 10.1% ($n=5$ at 2.8 ng ml^{-1} urine). The sample throughput was about 50 samples a day. Thus, this method is more sensitive and much faster than the common method using HPLC with electrochemical detection. The results of a study with nine volunteers investigated at six time-points each over 5 days are presented. The mean excretion of 8OHdG was 2.1 ng mg^{-1} creatinine (range 0.17–5.9 ng mg^{-1} creatinine; 4 of 53 samples were below the LOD). A relatively large intra- (relative SD 66%) and inter-individual (relative SD 71%) variation in urinary 8OHdG excretion rates was found.

Keywords: Oxidative stress; 8-Hydroxy-2′-deoxyguanosine

1. Introduction

Although oxygen is essential for aerobic organisms, a variety of reactive oxygen species (ROS) are formed during metabolic pathways. Cells have developed several mechanisms to neutralize ROS. However, if the capacity of their natural defence is exceeded, a variety of damages can occur [1]. Oxidative stress is defined as an increase of ROS production [2] leading to oxidative damage of cellular proteins, lipids and DNA [2,3].

A number of specific lesions caused by ROS, including 8-hydroxy-adenine (8-OH-ADE), 8-hydroxy-guanine (8-OH-GUA) or thymine glycol, have been identified by several analytical approaches [4].

*Corresponding author. Tel.: +49-89-535-395; fax: +49-89-532-8039.

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For the quantification of the very low levels of oxidative DNA damage generated by endogenous metabolic processes, the determination of urinary thymine glycol and thymidine glycol by HPLC with UV detection was suggested [5]. With the identification of 8-hydroxy-2′-deoxyguanosine (8OHdG) as an urinary marker for oxidative DNA damage, the determination of oxidatively damaged DNA bases was simplified [6]. The determination 8OHdG can be sensitively and selectively performed using HPLC with electrochemical detection (HPLC–ED) [7]. The urinary excretion of 8OHdG caused by excision repair and enzymatic hydrolysis has prompted the development of a noninvasive assay of urinary 8OHdG using HPLC–ED [8]. Although the oxidation of guanine in DNA is certainly not the only source of the urinary 8OHdG, its quantification has become a common marker for oxidative stress. Several analytical procedures have been described during the last decade using solid-phase extraction (SPE) [9–13] and HPLC [14,15] with column switching technique [10,12,13,16–19]. Recently, a method was described using for the first time a LC–MS–MS system for quantification of 8OHdG [20].

We developed a fast, easy and robust method for the accurate determination of urinary 8OHdG using SPE and HPLC–MS–MS in order to facilitate the sample preparation (Table 1). This rapid and accurate method should increase the range of possible applications in biomonitoring studies.

Table 1
Characteristics of the described method for the determination of 8-hydroxy-2′-deoxyguanosine using SPE and LC–MS–MS

Linearity (ng ml^{-1})	0.2–100
Equation of calibration	Area=m×conc.+b Slope: m=1688 Intercept: b=−84 Correlation coeff.: r=0.998
LOD[a] (ng ml^{-1})	0.2
Recovery[b] (%)	98.6±2.8
Accuracy[c] (%)	98
RSD[d] LC–MS–MS (%)	2.5
RSD[e] of total procedure (%)	10.1

[a] LOD: limit of detection.
[b] Mean±standard deviation; 5 samples at 1 ng ml^{-1}.
[c] Stocking experiment; 3 samples at 5 ng ml^{-1}.
[d] RSD: relative standard deviation; 7 samples at 5 ng ml^{-1}.
[e] RSD: relative standard deviation; 5 samples at 2.8 ng ml^{-1}.

2. Material and methods

2.1. Chemicals

8-Hydroxy-2′-deoxyguanosine ($C_{10}H_{13}N_5O_5$, MW=283.2; CAS 88847-89-6) of analytical grade was obtained from Sigma (Deisenhofen, Germany). LiChrolut EN® extraction cartridges (200 mg, 3-ml-PE cartridge), potassium dihydrogen phosphate, ammonium acetate and acetic acid (all of analytical grade), acetonitrile, methanol and water (all of gradient grade) were obtained from Merck (Darmstadt, Germany).

2.2. Urine samples

Urine samples of nine volunteers (two women and seven men) were collected during a 5-day controlled study. On days 1 and 4 urine was sampled for 24 h (two samples). On days 2 to 3, 12 h urine fractions ware collected (four samples). The mean age of the subjects was 32.3±9.4 years (range 22–49). Four of the volunteers were smokers (mean daily cigarette consumption: 7.2±5.7, range 4–16). During the study, the diet was not controlled except on day 2 when all subjects consumed a lunch rich in smoked ham and cheese.

2.3. Sample preparation and analysis by LC–MS–MS

Urine samples were kept at about 4°C during sampling and stored frozen at −20°C until analysis after 10 days. The freshly thawed urines were homogenized by vigorous shaking for 1 min. A volume of 3 ml of the urine samples was applied on a LiChrolut EN® cartridge previously conditioned with 3×3 ml of acetonitrile, methanol and 25 mM potassium dihydrogen phosphate buffer (pH 5.5), each. After washing with 3 ml of 25 mM potassium dihydrogen phosphate (pH 5.5) and 2 ml of water the

cartridge was dried in a gentle stream of nitrogen. Before elution of the 8OHdG with 2×1 ml of methanol, the cartridge was washed with 2 ml of acetonitrile to remove further components of the urinary matrix. The methanolic eluates were reduced to dryness by applying a stream of nitrogen and resolved in 300 μl of 10 m*M* ammonium acetate containing 2% methanol (pH 4.3).

Ten microliters of the extract were injected onto a Supersphere 100 RP18 endcapped HPLC column (125×2 mm, 4 μm) with an identical guard column (10×2 mm; all Merck) maintained at 25°C using a HPLC system consisting of a PE 200 autosampler and two PE 200 micro pumps (all Perkin Elmer, Überlingen, Germany). A gradient was formed with 10 m*M* ammonium acetate, adjusted to pH 4.3 with acetic acid, and methanol starting with 1% methanol raised to 80% within 15 min. The flow-rate was 0.2 ml min^{-1}. The HPLC was directly coupled to a triple stage quadrupole mass spectrometer (API 3000, PE Biosystems, Langen, Germany) equipped with a TurboIonSpray™ source. The product ion 168 *m*/*z* resulting from the precursor ion $[M+H]^+$ *m*/*z* 284 in the multiple reaction monitoring (MRM) mode with positive ionisation was used as quantifier and two further product ions (*m*/*z* 112 and 140) as qualifiers. The retention time of 8OHdG was 7.2 min. A six-point linear calibration curve was established for external standard solutions over a range of 0.2–10 ng ml^{-1}. The overall recovery was determined by the use of five aqueous standard solutions processed in the same way as the samples. All results were corrected for the recovery rate. A six-point calibration was run before and after the series of 53 samples. After every tenth sample a standard (5 ng ml^{-1}) was injected to monitor the accuracy.

3. Results

A typical chromatogram of a standard solution (0.5 ng ml^{-1}) and a real sample (0.44 ng ml^{-1}) is shown Fig. 1. The most intensive product ion of 8OHdG in the multiple reaction monitoring (MRM) mode using electrospray in the positive polarity was *m*/*z* 167.8 (Fig. 2). Although acetonitrile has a

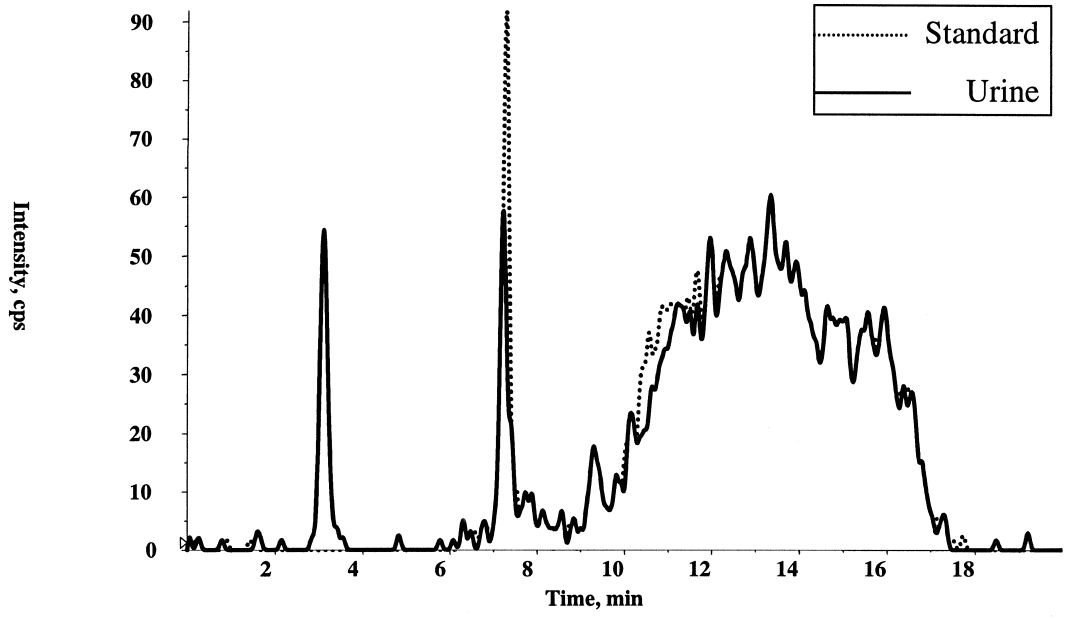

Fig. 1. Chromatogram in the MRM mode (*m*/*z* 284.1 to *m*/*z* 167.8) using the LC–MS–MS system (HPLC PE 200, API 3000) and a Superphere 100 RP18 endcapped (125× 2 mm, 4 μm) with gradient formed of 10 m*M* ammonium acetate and methanol as described in the text. Retention times: 3.1 min (2dG), 7.2 min (8OHdG). Dotted line: aqueous standard solution (0.5 ng ml^{-1}). Solid line: urine sample after SPE (0.44 ng ml^{-1}).

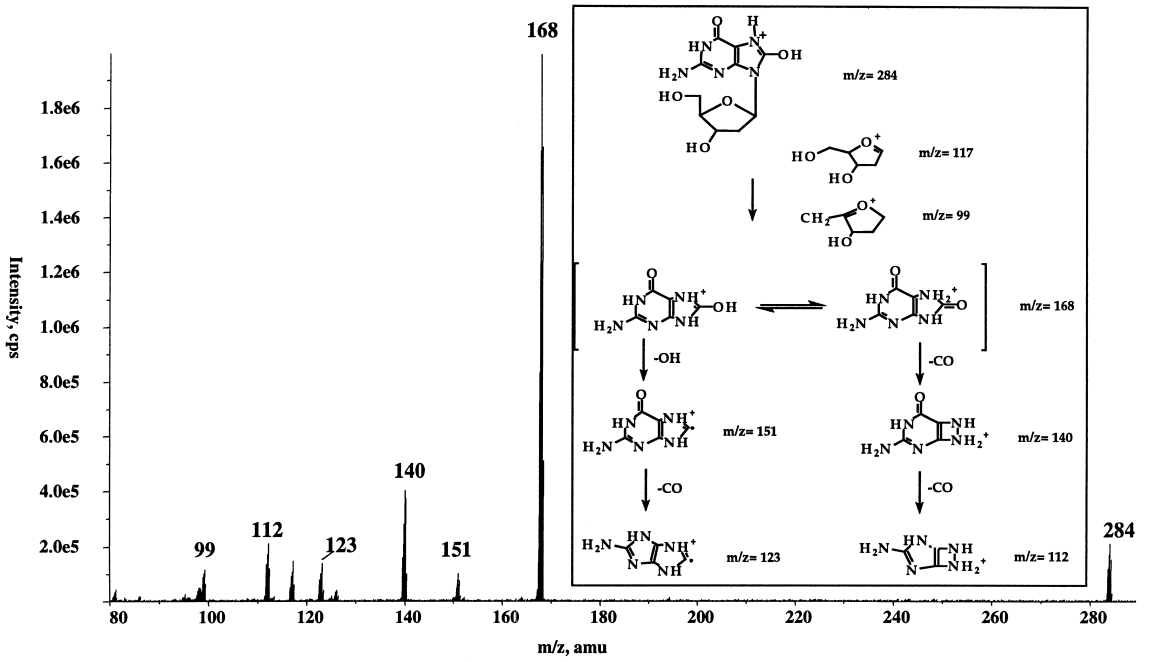

Fig. 2. Product ion spectrum and scheme of fragmentation of the 8OHdG molecule ion $[M+H]^+$ (m/z 284.1) in the positive electrospray mode using the API 3000.

higher elution strength than methanol, 8OHdG enriched on LiChrolut EN® was not eluted by applying 4 ml acetonitrile on the enrichment cartridge. Therefore, acetonitrile could be used as a solvent in the washing step of the SPE procedure. Nether the less, the solid-phase extraction of 8OHdG does not remove the matrix entirely. But no disturbance in the chromatogram can be seen. The peak eluting at 3.1 min which can be seen in the urine sample (Fig. 1) belongs to 8OHdG formed from 2-deoxyguanosine (2dG) due to oxidation in the ion source. Fig. 3 shows a chromatogram of an aqueous standard of 2dG (MW=267.2). The MRM chromatogram of the corresponding precursor ion m/z 268.1 $[M+H]^+$ to the product ion m/z 152.1 shows a peak at 3.1 min. With the same retention time a peak is found in the MRM m/z 284.1 to m/z 167.8 which is representative for 8OHdG. Therefore, 2dG is oxidized to 8OHdG during the ionization process in the Turbo-Ion source.

The limit of detection (LOD) of 0.2 ng ml^{-1} was determined as the three-times signal-to-noise ratio of a standard solution. The recovery of the total procedure was 98.6±2.8% in five independent extractions of standard solutions. Since the chromatogram of aqueous standard solutions did not show any differences to the urine samples, the recovery rate was used for the real samples. The instrumental precision, determined as the intra-assay variation of repeated injections of standards (5 ng ml^{-1}, $n=7$), was 2.5%. The intra-assay precision for the total procedure of an urine sample (2.8 ng ml^{-1}) was found to be 10.1% ($n=5$). The accuracy due to stocking experiments at a level of 5 ng ml^{-1} was 98% ($n=3$).

The urinary excretion of 8OHdG of nine healthy volunteers over a period of 5 days is summarised in Fig. 4. The mean excretion of the smokers (1.7±0.7 ng mg^{-1} creatinine) was not significantly different from that of the non-smokers (2.4±0.7 ng mg^{-1} creatinine). The excretion of 8OHdG ranged from <0.2 ($n=4$) to 5.9 ng mg^{-1} creatinine. The intra-

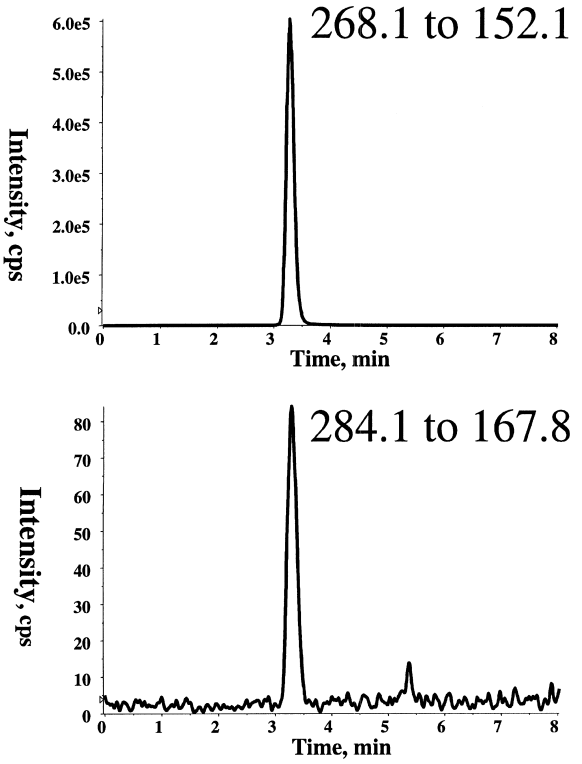

Fig. 3. Chromatograms of an aqueous standard solution of 2dG (1 μg ml^{-1}) in the MRM mode (*m*/*z* 268.1 to *m*/*z* 152.1; *m*/*z* 284.1 to *m*/*z* 167.8) using the LC–MS–MS system (HPLC PE 200, API 3000) and a Superphere 100 RP18 endcapped (125×2 mm, 4 μm) with gradient formed of 10 m*M* ammonium acetate and methanol as described in the text. Retention times: 3.1 min (2dG). MRM (268.1 to 152.1): 2dG. MRM (284.1 to 167.8): oxidized 2dG (=8OHdG).

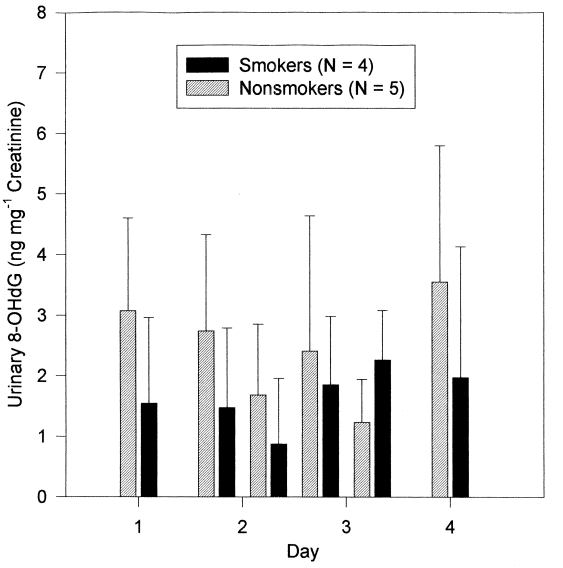

Fig. 4. Urinary excretion of 8OHdG of four smokers and five non-smokers at six time-points over a period of 5 days (means and standard deviations).

and inter-individual variations of the 8OHdG excretion rates were about similar (~70%).

4. Discussion

The described method is a sensitive and reliable tool for the determination of the urinary excretion of 8OHdG even at the low levels of non-occupationally exposed subjects. We also observed the formation of 8OHdG form 2dG in the electrospray ion source during the ionization process [20]. Therefore, the chromatographic separation of 2dG from 8OHdG using HPLC is essential. Using the described method 2dG (R_t=3.1 min) was well separated from 8OHdG (R_t=7.2 min; Fig. 1).

Previously published urinary excretion rates of 8OHdG are summarized in Table 2. The mean 8OHdG levels in urine from healthy subjects ranged from 2.3 to 7.8 ng mg^{-1} creatinine. The mean 8OHdG excretion in our study was 2.1 ng mg^{-1} creatinine and thus slightly lower than previously reported. In contrast to earlier studies [10,11,17,18], there was no significant difference between the 8OHdG excretion of smokers and non-smokers presumably due to the small number of subjects which were investigated and to the fact that the smokers in this study were light smokers.

Elevated urinary levels of 8OHdG were found for occupationally exposed persons. Tagesson et al. [18,19] reported 8OHdG excretion rates of 3.5±1.4, 3.7±1.4, 4.8±2.1 and 4.3±1.9 ng mg^{-1} creatinine for employees in the asbestos, rubber, azo-dye and glass industry, respectively. Also chemotherapeutically treated cancer patients showed significantly higher levels of 8OHdG in their urine (6.1±5.7 ng

Table 2
Published data for urinary excretion rates of 8-hydroxy-2′-deoxyguanosine of healthy subjects

Author	Method	8-Hydroxy-2′-deoxyguanosine in urine (ng mg^{-1} creatinine)
K.C. Cundy et al. [9]	SPE+HPLC–ED	7.8±4.6 (n=4)
D.S. Bergtold et al. [14]	SPE+GC–MS	4.0±1.3 (n=23; 300±100 pmol kg^{-1} d^{-1})
K. Vistisen et al. [10]	SPE+LC–LC–ED	2.7±1.1 (n=53; 213±84 pmol kg^{-1} d^{-1}; non-smoker) 4.4±0.1 (n=30; 328±9 pmol kg^{-1} d^{-1}; smoker)
E.-M. Park et al. [15]	SPE+HPLC–ED	2.3±1.1 (n=63; 172±79 pmol kg^{-1} d^{-1})
S. Loft et al. [17]	SPE+LC–LC–ED	2.7 – 4.1 (n=168; 250±50 pmol kg^{-1} d^{-1})
C. Tagesson et al. [19]	LC–HPLC–ED	2.7±1.0 (n=41)
J. Suzuki et al. [11]	SCE–SPE+CPC+HPLC–ED	3.8±2.0 (n=6; non-smoker) 7.4±1.5 (n=7; smoker)
C. Tagesson et al. [21]	LC–HPLC–ED	2.8±1.2 (n=71; men) 3.2±1.2 (n=58; women)
H. Verhagen et al. [12]	SPE+LC–LC–ED	4.0 – 5.8 (n=10; 350 – 500 pmol kg^{-1} 24 h^{-1}; women)
C. Tagesson et al. [18]	SPE+LC–LC–ED	3.6±1.8 (n=37; 11.1±5.6 nmol l^{-1}, men, non-smoker) 3.1±1.4 (n=63; 9.6±4.4 nmol l^{-1}, women, non-smoker) 4.5±2.0 (n=12; 13.7±6.1 nmol l^{-1}, men, smoker) 3.6±1.6 (n=31; 11.0±4.9 nmol l^{-1}, women, smoker)
D. Germadnik et al. [13]	SPE+HPLC–ED	6.8±4.7 (n=60)
H.W. Rüdiger (in preparation)	SPE+HPLC–ED	6.2±3.8 (n=69)

mg^{-1} creatinine) than untreated controls (71 men: 2.8±1.2 ng mg^{-1} creatinine; 58 women: 3.2±1.2 ng mg^{-1} creatinine) [21]. The about similar and high intra-individual and inter-individual variation in the urinary excretion of 8OHdG (~70%) indicates that additional, as yet unknown factors may influence the formation and excretion of 8OHdG. This should be investigated by also including other oxidized DNA bases (e.g., 8OHdA, thymine glycol, thymidine glycol) in addition to 8OHdG using LC–MS–MS.

We suggest the described fast method due to its very high sensitivity (LOD 7 fmol absolute) and selectivity as a suitable tool for further research in this field. The high sample throughput makes this method for the determination of urinary 8OHdG suitable for the monitoring of a large number of subjects.

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