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Short communication

Assay for the determination of urinary 8-hydroxy-2′-deoxyguanosine by high-performance liquid chromatography with electrochemical detection

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Abstract

A high-performance liquid chromatographic procedure with electrochemical detection is described for the determination of urinary 8-hydroxy-2′-deoxyguanosine, a major oxidative DNA lesion induced by radical forming agents. A two-step solid-phase extraction procedure was followed for extracting 8-hydroxy-2′-deoxyguanosine from human urine and the analysis was performed on a RP-18 analytical column under isocratic conditions. The limit of detection of 8-hydroxy-2′-deoxyguanosine in urine was found to be 0.9 n*M*. The non-invasive assay provides an indirect measurement of oxidative DNA damage.

Keywords: 8-Hydroxy-2′-deoxyguanosine; DNA

1. Introduction

The so-called reactive oxygen species (ROS) are generated as byproducts of normal cellular metabolism [1] in addition, ionizing radiation, metals or tumor promoting agents can lead to a formation of these reactive species, which can modify DNA [2–4]. DNA is a permanent target to ROS and 8-hydroxy-2′-deoxyguanosine (8OHdG), in particular, is one of the major oxidative adducts formed by radical-induced damage to DNA. ROS have also been suggested to contribute to physiological changes associated with aging, cancer and degenerative diseases [5–9]. Environmental agents such as benzene [10], benzo[*a*]pyrene [11] or mineral dusts [12] have been reported to induce 8OHdG formation in humans.

Oxidative DNA damage may result directly from the interaction of free oxygen radicals with various groups in the DNA structure or indirectly from the activation of endonucleases [13]. Of about 20 known oxidative adducts in DNA, 8OHdG has earned much interest due to its mutagenic potential [8,14–18]. Since the formation of ROS is a continous process, antioxidant defenses and intracellular repair mechanisms have evolved to avoid the rapid and lethal accumulation of oxidative DNA damages. The repair of DNA containing 8OHdG involves a N-glycosylase, which releases the free base 8-oxoguanine and non-specific enzymes, which excise DNA adducts to release deoxynucleotides, which are subsequently hydrolyzed to deoxynucleosides [19–

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21]. These water soluble repair products are excreted into the urine without further metabolism. Since 8OHdG is not absorbed through the digestive system its level is not influenced by diet. Thus 8OHdG has been proposed as urinary biomarker for in vivo oxidative DNA lesions [22].

In order to study conditions of "oxidative stress" and possible correlations between diseases and the exposure of individuals to ROS inducing agents, fast, sensitive and simple-to-use methods for the analysis of oxidative damage are needed. Several methodologies for the measurement of oxidated bases in the urine have been proposed, originally by Ames and co-workers [20,22,23] which are largely based on high-performance liquid chromatography with electrochemical detection (HPLC–EC). The use of polyclonal antibodies against 8OHdG has been described to improve the analyte purification prior to analysis [24,25]. Loft et al. developed an automated triple column switching HPLC technique with isocratic separation and electrochemical detection for the determination of 8OHdG in urine [26]. An alternative analytical strategy for quantitation of 8OHdG in human urine used a single extraction step for the sample preparation and a coupled-column HPLC–EC method for analysis [27,28].

In this paper, we describe a rapid and sensitive assay for determination of urinary 8OHdG based on reversed-phase HPLC–EC following a two-step solid-phase extraction.

2. Experimental

2.1. Chemicals and reagents

All chemicals were of analytical reagent grade quality. Water, acetonitrile and methanol of chromatographic grade were obtained from Merck (Darmstadt, Germany). Bond Elut LCR solid-phase extraction columns (C_{18}/OH, 500 mg, 2.8 ml) were obtained from Varian (Harbor City, CA, USA), 8OHdG from WAKO Chemicals GmbH (Neuss, Germany). Five milligrams of 8OHdG were dissolved in 100 ml of water to give a 176-μM stock solution. The concentration of the solution was estimated by spectrophotometry using the spectral data ϵ_{245} (12300) and ϵ_{293} (10300) [29]. The 1/10 diluted stock solution was aliquoted and stored at −20°C. Working standards (55–880 n*M*) were prepared weekly and kept at 4°C.

2.2. Apparatus

A Hewlett-Packard (Waldbronn, Germany) Series 1050 pump system (with pulse damper and autosampler) was used, connected to a Hewlett-Packard 1049A amperometric detector. The electrochemical cell was equipped with a glassy carbon working electrode operated at +0.6 V versus a Ag/AgCl reference electrode. The system was operated at 50 nA full range deflection. Data acquisition was performed by Hewlett-Packard HPLC 2D ChemStation software.

2.3. Columns

The separation of 8OHdG was carried out on two EcoCART (125×3 mm I.D.) analytical columns attached in series, packed with 4-μm Supersphere 100 RP-18 [endcapped] (Merck). A LiChroCART (4 mm x 4 mm I.D.) packed with 5-μm LiChrospher 100 RP-18 [endcapped] (Merck) was used as a guard column.

2.4. Chromatographic analysis

The mobile phase used for isocratic elution of 8OHdG was composed of 50 m*M* KH_2PO_4 (pH 3.5), 2.5% acetonitrile, 1% methanol (solvent A); the solvent mixture for the wash step contained 50 m*M* KH_2PO_4, 25% acetonitrile, 25% methanol (solvent B). Both solutions were filtered by vacuum through a 0.22-μm cellulose acetate filter and used within 5 days. The flow-rate was 0.5 ml/min and the temperature of analysis was 40°C.

2.5. Urine collection and clean-up procedure

Urine specimens from ten healthy persons (four men, six women), pooled together and with 8OHdG standard added, were used for developing the sample preparation steps. The pH of urine was adjusted to

4–5 by the addition of 2 *M* HCl and 5-ml aliquots of urine were kept frozen at −20°C. Each sample passed at least one freeze–thaw step and was centrifuged at 1500 *g* for 5 min before processing to remove precipitates. The supernatant underwent the cleanup procedure by solid-phase extraction. The Bond Elut C_{18}/OH cartridges were preconditioned with 10 ml of methanol, 5 ml of water and 10 ml of 50 m*M* KH_2PO_4, pH 7.5 (buffer A). A 2-ml volume of urine premixed with 0.5 ml of 220 n*M* 8OHdG standard was applied to the first column. The column was washed with 3 ml of buffer A and 3 ml of 5% methanol in buffer A. 8OHdG was eluted with 3 ml of 15% methanol in buffer A. The eluate then was diluted with 5 ml of water, mixed and applied to another conditioned C_{18}/OH column. The column was dried under vacuum and the absorbed material was eluted with 1.5 ml of 20% methanol in buffer A. To remove methanol, the samples were evaporated for 1.5 h in a SpeedVac Concentrator SVC200H (Savant, Holbrook, NY, USA) and subsequently filled up with buffer A to give a final volume of 1.5 ml: 50–80 μl of the prepared eluate was injected into the HPLC system.

2.6. Analysis of 8OHdG

Early morning urine was used for analysis and the 8OHdG content was related to the amount of creatinine. A standard at a concentration of 88 n*M* was injected at least twice before the first analysis to check the retention time and signal-to-noise ratio level. Control standard was also injected at the end of a series. Prior to each subsequent urine sample, a 47-min wash run was performed as follows: 0–17 min, 100% solvent A; 18–27 min, 0–50% solvent B; 28–32 min, 50% solvent B; 33–42 min, 50–0% solvent B; 43–47 min solvent A. The wash step was followed by an electric treatment for preparing the surface of the glassy carbon electrode, pulsing the potential between +0.6, +1.5 and −1.0 V, 60 cycles within 1 min. After an equilibration time of 30 min the baseline was stabilized and the system ready for the next injection. Quantification of 8OHdG in urine was done by the method of peak-height measurement using the linear regression curve for aqueous standard solutions (55, 110, 220, 440 n*M*).

3. Results and discussion

The two-step solid-phase extraction was efficient for separating 8OHdG from interfering urinary matrix components. The recovery of 8OHdG in the extraction procedure from 2 ml of urine was found to be 45.8±4.5% ($n=5$) and was constant over the concentration range from 55 to 440 n*M*. In contrast the recovery of aqueous standard after the column extraction steps was found to be 92.8±5.0% ($n=12$). The accuracy and precision of the procedure were ascertained by adding standards of 8OHdG to urine with known concentration and analysing the samples of each concentration (Table 1). The columns for the clean-up procedure were equilibrated at pH 7.5 heeding that the recovery was dependent on the pH conditions. At pH 9, 8OHdG did not bind to the column. An example of HPLC–EC analysis of 8OHdG in human urine is shown in Fig. 1. The identity of 8OHdG present in urine was demonstrated by determination of the retention time of authentic 8OHdG and of urine with 8OHdG standard added. Under the described conditions the retention time for 8OHdG was 13.8 min and the coeluted urinary components produced no interference peaks. The limit of detection defined as the peak height equal to three times the signal-to-noise ratio of the detector was 0.9 n*M* 8OHdG when 2 ml of urine was extracted and 50 μl was injected into the HPLC system. To estimate the coefficients of variation within- and between-series, two extracted samples from a spiked urine pool (88 n*M*) have been measured on ten different days at the beginning and at the end of a series of determinations. The coefficient of variation between mean values determined from different series of analysis was 14% and the coefficient of variation within series was 3%. The coefficient of variation between series of measurement of the 88 n*M* 8OHdG standard, stored for two

Table 1
Precision and accuracy of the determination of 8OHdG in urine

Concentration added (n*M*)	Concentration measured (mean ± S.D., $n=5$) (n*M*)	C.V. (%)
55	53.7±6.6	12.3
110	103.8±8.1	7.8
220	227.5±10.0	4.4
440	436.3±16.3	3.7

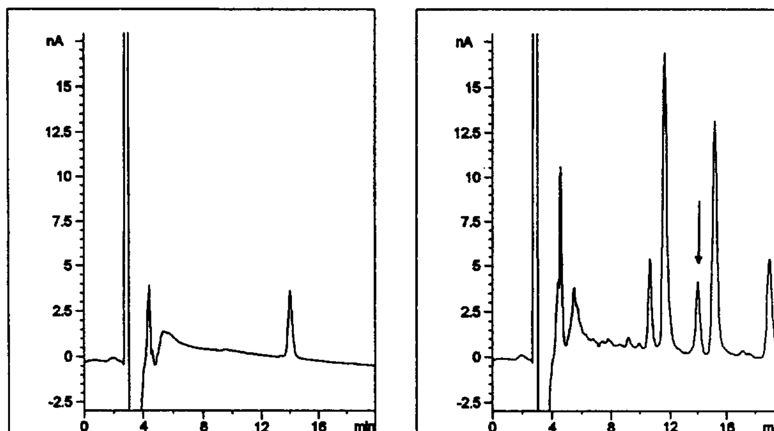

Fig. 1. HPLC–EC analysis of 8OHdG. Left: aqueous standard of 60 n*M* 8OHdG; Right: urine after solid-phase extraction procedure containing 60 n*M* of added standard.

weeks at 4°C, was 9% without a detectable influence of the storage conditions on the 8OHdG standard concentration.

The calibration with aqueous standard solutions, as well as the method of sample addition, require evaporation of methanol from the eluate of the solid-phase extraction in order to give comparable peaks. The calibration curves derived from peak heights were linear over the investigated range. The regression equation for aqueous standard solutions was $y=0.064x-0.021$ with a correlation coefficient greater than 0.999. Urine from 60 healthy non-smokers (29 men aged 28–52 years, 31 women aged 25–45 years) yielded an 8OHdG concentration between 2.4 and 93.3 n*M* and between 0.16 and 8.23 μmol/mol creatinine respectively. The mean normal concentration was 29.2±21.6 n*M* 8OHdG and 2.70±1.88 μmol 8OHdG/mol creatinine.

Since there is reason to believe that the excision of 8OHdG in DNA is a fast process, the urinary excretion of 8OHdG is considered to be approximately equivalent to its formation in DNA. The average amount of 8OHdG excreted by non-smoking healthy individuals has been estimated to be 213±84 pmol/kg/24 h [25], which corresponds to an excretion of 110–250 8OHdG residues per cell per day, assuming 5×10^{13} nucleated cells per adults and ignoring a tissue specifity of 8OHdG formation. The mean level of 8OHdG excretion found in our study and calculated per 0.5 l urine and 73 kg body weight corresponds to 200 pmol 8OHdG/kg. This is in accordance with the 130–300 pmol 8OHdG/kg per day for healthy humans, published by Shigenaga and Ames [20].

The advantage of the proposed method is that it is simple to use and faster than previously published methods. The detection limit of 0.9 n*M* of urinary 8OHdG is well below the concentrations of 8OHdG found in normal human urine and the method offers an approach for estimating oxidative DNA damage.

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