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# Gender-related reference intervals of urinary 8-oxo-7,8-dihydro-2′-deoxyguanosine determined by liquid chromatography-tandem mass spectrometry in Serbian population

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ABSTRACT

**Objectives:** Although there are many nucleobase modifications, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) is one of the dominant form of oxidative modifications of DNA. Urinary 8-oxodG is potentially the best non-invasive biomarker of oxidative stress. Defining reference interval for urinary 8-oxodG is a prerequisite for its clinical use as biomarker.

**Design and methods:** Reference population included 229 healthy Serbian adults (130 males and 99 females). The spot urinary 8-oxodG was determined using high performance liquid chromatography and tandem mass spectrometry (HPLC-MS/MS). Urinary creatinine was measured by the kinetic Jaffé method.

**Results:** Analytical performances of the HPLC-MS/MS: CVs within and between-run variations were 5.6% and 2.6%; LOD and LOQ were 1.65 nmol/L and 3.30 nmol/L; mean recovery and relative accuracy were 96% and 97%. Creatinine level was higher in males than in females, but no gender difference in 8-oxodG level was observed. Upon the adjustment of 8-oxodG to creatinine (8-oxodG/creatinine), higher values were obtained in females ($1.38 \pm 0.65$ nmol/mmol) than in males ($1.05 \pm 0.48$ nmol/mmol). Distribution of 8-oxodG/creatinine in spot urine sample was log-normal and gender-related reference intervals (estimated as the 2.5th–97.5th percentiles) were 0.45–2.22 nmol/mmol for males, and 0.54–3.11 nmol/mmol for females. Body mass index (BMI) affects excretion of the 8-oxodG in males, independently of urinary creatinine, while in females it does not. Therefore, BMI might contribute to the gender-related differences of 8-oxodG/creatinine in spot urine samples.

**Conclusions:** This is the first established gender-related reference intervals of spot urinary 8-oxodG/creatinine. Our results contribute to the full validation of 8-oxodG as biomarker of oxidative stress.



## Introduction

Reactive oxygen species (ROS) are formed in living cells, as part of normal physiological processes, as well as after exposure to UV radiation, tobacco smoke, and other carcinogenic substances [1]. Under normal physiological conditions there is a balance maintained between oxidants and antioxidant defenses. Disturbance of this balance in favor of oxidants, known as oxidative stress, leads to oxidative damaged of cellular components and may be an important factor in pathogenesis of many severe diseases (cancer, cardiovascular diseases, neurodegenerative diseases, chronic inflammatory diseases and aging) [2,3]. Mutagenic oxidative damage of the DNA, that may cause heritable alterations, includes base oxidation and fragmentation, inter/intra-strand cross-links, DNA–protein cross-links and sugar fragmentation. Due to their roles in the pathological processes, DNA lesions are recognized as a potential biomarker for the assessment of disease development risk and for the monitoring of therapy outcomes [4].

Although a broad range of nucleobase modifications are produced during oxidative damage of DNA, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) is the most investigated biomarker of nucleic acid oxidation. 8-oxodG is promutagenic DNA lesion, as it is able to mispair with adenine, leading to G:C→T:A transverse mutation, if it is not repaired prior to DNA replication [5]. Under certain conditions 8-oxodG may contribute to the development of human disease. Urinary 8-oxodG represents oxidation of breakdown products from the DNA and therefore

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determination of urinary 8-oxodG is potentially the best biomarker that reflect the effects of oxidative stress. Measurement of urinary levels of 8-oxodG is frequently used in epidemiological studies, due to the many advantages such as non-invasive and easily collected sample, its stability at −80 °C more than 10 years, with no pretreatments or preservatives [6]. However, many open questions regarding the analytical and clinical validation of this biomarker of oxidative stress still remains. A contribution to the analytical validation has been recently reported by European Standards Committee on Urinary (DNA) Lesion Analysis [7]. Their results reveal good agreement within different chromatographic techniques with mass spectrometric and electrochemical detection. New direct, isocratic HPLC/MS/MS method for the urinary 8-oxodG was recently evaluated [8]. This method employs the sensitivity of the HPLC-MS/MS with simple sample preparation and in the same time achieves good sensitivity and repeatability of measurement without the costly isotopically labeled internal standards.

Prerequisite for the clinical use of urinary 8-oxodG as a biomarker of oxidative stress is the establishment of reference intervals in different populations. Intention of this study was to establish a reference interval of 8-oxodG in spot urine in the well characterized Serbian healthy population, using highly sensitive liquid chromatography–tandem mass spectrometry (HPLC-MS/MS). We also evaluated the influence of gender, age, body mass index and tobacco smoking on the urinary 8-oxodG values.

## Materials and methods

### *Reference population*

Reference population consisted of 229 healthy middle age subjects (130 males and 99 females), from Belgrade and Pancevo (Republic of Serbia). The mean age of subjects was 45.3 ± 9.52 (range 21–70) years, and they were not professionally exposed to the severe oxidants. Individuals were recruited during the regular annual health control and all individuals have undergone regular physical examinations and laboratory testing. Selection of individuals was based on the normal laboratory test results and physical examination. From the initially recruited 255 subjects, participants with any chronic disease and medical therapy were excluded.

All study participants filled out a questionnaire with the basic information (date of birth, anthropometric data, use of medications) and smoking habits (65% were smokers). The influence of aging on the level of urinary parameters was tested between two groups, younger (≤50 years) and older (≥51 years) ones. The study has been approved by the institutions' ethical committees and was carried out according to the principles of the Declaration of Helsinki. Also, all participants have given informed consent to participate in the study.

### *Sample collection and analysis*

For the determination of the laboratory parameters phlebotomy was performed in the morning, after an overnight fast (≥10 h) using the vacutainer system (Becton Dickinson), and the samples analyzed on the same day. Biochemical and hematological parameters were measured using chemistry (Instrumentation Laboratory® ILab 650 and Beckman Synchron CX9 Pro) and hematology (Coulter® HmX) analyzers. Spot urinary creatinine was measured by the kinetic Jaffé method (Beckman Synchron CX9 Pro analyze).

The same morning when the phlebotomy was performed, the participants have collected a mid-stream spot urine sample. All urine samples were centrifuged at 3500 ×*g* for 15 min, and clear urine was frozen at −80 °C until analysis. The urinary 8-oxodG was determined using HPLC-MS/MS. Before analysis urine samples, calibrators and controls were vigorously mixed, sonicated for 1 min, and then centrifuged at 10,000 ×*g* for 5 min. A 20 μl sample of clear supernatant of each calibrator, control or test sample was injected into the HPLC-MS/MS system.

### *HPLC-MS/MS*

#### *Chemicals*

The reference chemical 8-oxodG, ammonium acetate (Sigma Ultra) and glacial acetic acid (ACS grade) were from Sigma (Sigma-Aldrich Co.). Acetonitrile (LC-MS Chromosolve) was from Fluka (Sigma-Aldrich Co.) and Milli-Q quality (18.2 MΩ cm) water was used. Calibrators and standard samples were prepared from the stock standard solution of 8-oxodG which was prepared in a water. From this stock solution (approximately 7 mmol/L) a diluted solution in water was prepared and the exact concentration was determined by an Ultraspec 3300pro UV/Vis Scanning Spectrophotometer (Amersham Biosciences) (ε 12,300 at 245 nm). The 8-oxodG stock and diluted solution were stored at −80 °C. A solution of 1200 nmol/L in water was prepared weekly from the diluted solution and stored at 4 °C for routine use and used daily to prepare working calibrators (0.0, 7.5, 15.0, 30.0, 60.0, 120.0 nmol/L) in thawed, pooled urine (stored at −80 °C) from healthy subjects. Control samples, prepared by spiking the pooled urine with appropriate amounts of standard, were stored at −80 °C.

#### *HPLC-MS/MS analysis*

Analyses were carried out on Thermo ACCELA™ (Thermo Scientific, Waltham, Massachusetts, USA) coupled to a triple quad Mass Spectrometer Thermo TSQ Quantum Access Max (Thermo Scientific, Waltham, Massachusetts, USA) with a heated electrospray ionization (HESI) interface. Samples were placed in a thermostated autosampler at 4 °C. Samples of 20 μL were injected onto a Thermo Scientific Hypersil GOLD aQ column (3 μm, 100 L×4.6 mm ID) secured by a guard cartridge (Thermo Scientific Hypersil GOLD aQ, 10 mm L×4 mm ID) and eluted at a temperature of 25 °C and a flow rate of 500 μL/min for 10 min with a freshly prepared isocratic mobile phase — ammonium acetate (10 mmol/L adjusted pH 4.3 with acetic acid) and acetonitrile (96.4:3.6 vol/vol). The solution was filtered through a 0.22 μm nylon filter before use. With an integrated diverting valve installed in the spectrometer, only the eluate fraction of 8-oxodG was delivered into the spectrometer, the rest of LC eluate was diverted to waste (0–5.5 min eluate going to waste, 5.5–7 min eluate going to mass spectrometer, 7–10 min eluate going to waste). MS/MS analysis was performed in a positive-ion mod. The spectrophotometer was first optimized by infusion of an 8-oxodG standard in water (7.0 μmol/L), using the integrated syringe pump (flow rate 10 μL/min). Both calibration and sample data were obtained by multiple reaction monitoring (MRM) acquisition: precursor ion ([M + H] + at m/z 284, Q1, product ion at m/z 168, Q3). The product ion was quantified using dwell time 500 ms/channel with unit mass resolution for Q1 and Q3. The spray voltage was 5000 V. The temperature in the capillary was adjusted to 280 °C, while the vaporizer temperature was set to 350 °C. Sheet gas pressure was set to 35 arbitrary units, while the auxiliary valve flow was set to 10 arbitrary units. MS resolution values were defined to correspond to a mass resolution of 0.7 Da. Collision energy for optimized compound was 12.0 eV. Other parameters were optimized to produce a maximum analyte signal (Collision cell pressure: 1.5 mTorr of Argon). All data were acquired and processed by Xcalibur software (Thermo Fisher, San Jose, CA, USA).

#### *Limit of detection, lower limit of quantification, precision, recovery and accuracy*

Detection limits of the instrument were determined as three times the signal to noise ratio of the 8-oxodG standard solution in water. As normal urine always contains 8-oxodG, the "true LOD" was calculated from the linearly fitted calibration curves as twice the SD of the intercept of the six calibration curves divided by the average slope. The lower limit of quantification (LOQ) for 8-oxodG in urine is twice the "true LOD" in