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8-Oxo-7,8-dihydroguanine and 8-oxo-7,8-dihydro-2′-deoxyguanosine Levels in Human Urine do not Depend on Diet

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In the present study, we used the method involving HPLC pre-purification followed by gas chromatography with isotope dilution mass spectrometric detection for the determination of 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) and 8-oxo-7,8-dihydroguanine (8-oxoGua) in human urine. The mean levels of 8-oxoGua and 8-oxodGuo in the urine samples of the subjects on unrestricted diet were respectively 1.87 nmol/kg 24 h (±0.90) and 0.83 nmol/kg 24 h (±0.49), and in the case of the groups studied, they did not depend on the applied diet. The sum of the amounts of both compounds in urine can give information about the formation rate of 8-oxoGua in cellular DNA. It is also likely that the levels of modified nucleo-base/side in urine sample are reflective of the involvement of different repair pathways responsible for the removal of 8-oxodGuo from DNA, namely base excision repair (BER) and nucleotide excision repair (NER).

Keywords: 8-Oxo-7,8-dihydroguanine; 8-Oxo-7,8-dihydro-2′-deoxyguanosine; Human urine; DNA repair pathways

Abbreviations: 8-oxoGua, 8-oxo-7,8-dihydroguanine; 8-oxodGuo, 8-oxo-7,8-dihydro-2′-deoxyguanosine; BER, base excision repair; NER, nucleotide excision repair; ROS, reactive oxygen species

INTRODUCTION

Reactive oxygen species (ROS) are the products of partial reduction of oxygen. These species which include superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) are continuously produced in living cells as by-products of normal metabolism.

It was shown that $^{\cdot}OH$ radical attack upon DNA, generates a whole range of DNA damage, among the modified bases. Some of these

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modified DNA bases have considerable potential to damage the integrity of the genome.[1–3]

8-oxo-7,8-dihydroguanine (8-oxoGua) is one of the most critical lesions. The presence of 8-oxoGua residues in DNA leads to GC to TA transversion, unless repaired prior to DNA replication.[1] Therefore, the presence of 8-oxoGua may lead to mutagenesis. Furthermore, many observations indicate a direct correlation between 8-oxoGua formation and carcinogenesis *in vivo*.[2–4]

It is generally accepted that the products of repair of this kind of DNA damage are excreted into the urine without further metabolism.[5–7] There is a common belief that the presence of the 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) modified nucleoside in urine represents the primary repair product of the oxidative DNA damage *in vivo* and that this compound may be a mirror involvement of nucleotide excision repair pathway (NER).[8,9] However, oxidatively damaged DNA bases are repaired by the BER pathway and NER mechanisms.[10,11] Moreover several glycosylases, which specifically recognise and remove 8-oxoGua in human cells, have been recently described.[12,13] Therefore, the assays which are able to determine the level of 8-oxodGuo as well as the amount of 8-oxoGua in urine, may reflect oxidative damage of cellular DNA better.

The analysis of 8-oxoGua in urine presents particular difficulties,[9,14] and until now, there has been no reliable assay for its detection. Recently, a new technique was developed which allowed for simultaneous determination of 8-oxodGuo and 8-oxoGua in the same urine sample.[15] This method involved HPLC prepurification followed by gas chromatography with isotope dilution mass spectrometric detection (GC/MS). It is also noteworthy that recently developed LC/MS/MS technique is a method of choice for such a purpose.[16,17]

In the present study, we applied the technique described by Ravanat *et al.*,[15] for the detection of 8-oxodGuo and 8-oxoGua in the human urine. Since a previous study demonstrated that diet could influence the level of 8-oxoGua in rat urine,[18] we wanted to discover whether a similar phenomenon could be seen in humans.

MATERIALS AND METHODS

Subjects

To check dietary influence on the level of both products of oxidative DNA damage, we used a single person who acted as his/her own control and thus the difficulties presented by using a group of individuals drawn at random from the genetically heterogeneous human population were avoided. For these experiments, two groups of subjects were recruited; (i) a group of healthy non-smokers consisting of five males and seven females (average age 32 years) who were fed a nucleic acid-free diet (the diet consisted of glucose and starch); (ii) a group of non-smoking, surgically treated patients (eight males and four females, average age 55 years) who postoperatively were infused with 21/24 h of 10% glucose (the solution also contained 5.75 g/l of NaCl, 0.38 g/l of KCl, 0.38 g/l of $CaCl_2$, 0.2 g/l $MgCl_2$, 4.62 g/l of CH_3COONa and 0.9 g/l of citrate) for three consecutive days. The cause of operation on the studied patients was abdominal aortic aneurysm or intestinal ileus. Further characteristics of the studied groups are presented in Table I. Subjects were not entered into the trial for regular intake of antioxidants or other drug with antioxidant properties.

The study was approved by the medical ethics committee of The Ludwik Rydygier Medical University in Bydgoszcz, Poland, No. 48/99 and 03.02, 10/2000 (in accordance with Good Clinical

TABLE I General characteristics of the studied groups

Gender (male/female)	13/11
Body weight±SD (SEM) (kg)	69.6±15.7 (4.5)
Age±SD (SEM) (years)	43.5±11.1 (3.2)

Practice, Warsaw 1998) and all the patients gave informed consent.

Urine Sample Preparation

0.5 nmol of [$^{15}N_3$, ^{13}C] 8-oxoGua and 0.05 nmol of [^{18}O] 8-oxodGuo and 10 μl of acetic acid (Sigma, HPLC grade) were added to 2 ml of human urine. Isotopic purity of the applied standards were 97.65 and 85% respectively. After centrifugation, (2000 × *g*, 10 min) supernatant was filtered through a Millipore GV13 0.22 μm syringe filters and 500 μl of this solution was injected onto HPLC system. In the pilot study, isotopically labelled internal standards of unmodified compounds (1 nmol of [$^{13}C_3$] Gua and 1 nmol of [$^{15}N_5$] dGuo) were added to the urine samples to monitor fractions containing both these compounds and to avoid an overlapping of the peaks containing the modified and unmodified base/nucleoside. Isotopic purity of the applied standards were 96.4 and 98.0% respectively.

HPLC Purification and GC/MS Analysis

Urine HPLC purification of 8-oxoGua and 8-oxodGuo were performed according to the method described by Ravanat *et al.*[15] with some modifications. Briefly; the HPLC system consisted of GP 40 gradient pump, Gina 50 autosampler (both from Dionex), SPD M10 AVP diode array detector from Shimadzu and Foxy Jr. fraction collector from Isco Inc. Urine samples enriched in labelled compounds were injected onto Supelcosil LC 18 column (250 × 10 mm^2) equipped with Supelguard LC18 guard column (20 × 4.6 mm^2), both from Supleco. A 30 min linear gradient elution was performed (0.5% acetic acid at start to 0.5% acetic acid and 10% of acetonitrile), at a flow rate of 3 ml/min. After this time, the column was washed with 70% of acetonitrile for 10 min and equilibrated with 0.5% acetic acid for 20 min prior to a further injection. The effluent was monitored with UV detector at 220–360 nm.

Using isotopically labelled internal standards for 8-oxoGua, 8-oxodGuo, Gua and dGuo, we were able to avoid an overlapping of the peaks containing the modified and unmodified base/nucleoside (see also adequate statement under "urine sample preparation"). The collected fractions were dried by evaporation under reduced pressure in a Speed-Vac system and treated with 400 μl of 60% formic acid (Sigma) for 30 min at 130°C. Upon acid hydrolysis, 8-oxodGuo yields 8-oxoGua which was detected using MS analyses. Formic acid was also applied to fractions containing the base because the treatment improved the quality of GC/MS analyses (not effecting quantitative evaluation). Subsequently, samples were prepared for GC/MS analysis as described.[19]

GC/MS analysis was performed according to the method described by M. Dizdaroglu,[19] adapted for additional [^{18}O] 8-oxoGua analyses (*m*/*z* 442 and 457 ions were monitored).

RESULTS

For evaluation of the accuracy and reproducibility of the analysed compounds, the quantitative analyses of 8-oxoGua and 8-oxodGuo were repeated seven times on the same urine samples. To check accuracy and recovery, the spiked urine samples were analysed (10 pmol of 8-oxodGuo and 50 pmol of 8-oxoGua were added to 1 ml of urine sample). Quality control data for the analysis of 8-oxoGua and 8-oxodGuo are presented in Table II and standard curves for both compounds are reported in Fig. 1.

A previous study revealed that the amount of 8-oxoGua in urine reached the lowest value, 2–3 days after switching to a nucleic acid-free diet.[18] Therefore, in our study, the urine samples (24 h output) were collected after three days' nucleic acid-free diet was completed and from the same persons, 3–5 days after returning to the normal,

TABLE II Quality control data for the analysis of 8-oxoGua and 8-oxodGuo in urine

	8-OxoGua	8-OxodGuo
n	7	7
Average (pmol/ml)	102.78	33.15
Standard deviation (pmol/ml)	3.20	2.16
% of variation	3.11	6.54
Recovery (%)	97±4	94±6

unrestricted diet. Since there were no substantial differences between the groups studied, the data for both of them were combined (Figs. 2 and 3, Tables III and IV).

The mean levels of both analysed compounds were comparable in the studied groups regardless of the applied diet (Figs. 2 and 3, Tables III and IV). These results suggest that in the case of humans, diet has no influence on the level of either 8-oxoGua or 8-oxodGuo in their urine.

DISCUSSION

There have been only a small number of studies concerning 8-oxoGua analysis in human urine.[5,6,14,15,18,20] When the HPLC/EC technique was used, the values were lower than in our study.[6] This discrepancy may be at least partially explained by poor recovery of 8-oxoGua from urine and by lack of internal standard. When recovery of the modified base was improved[14] its level in the urine sample was very similar to the presently reported data (118.0 and 130.0 nmol/day respectively).

In the previous study, it was shown that diet can influence the level of 8-oxoGua in rat urine.[18] On the contrary, our experiments clearly show that diet has no influence on the level of both products of oxidative DNA damage in human urine. There may be several reasons for this inconsistency: (i) the methodological approach was different in both experiments. Park *et al.*[18] used immunoaffinity pre-purification (using antibody, which recognised 8-oxodGuo and related adducts) and HPLC with EC detection for the determination of modified base and the nucleoside. However, in their subsequent paper the authors stated that "8-oxoGua in urine presents particular difficulties, and a modified method for analysis of this species is

TABLE III Statistical analyses of the urinary excretion of 8-oxoGua (combined data for both groups of the subjects—see materials and methods)

	Nucleic acid free diet	*Normal diet*
Mean±SD (SEM) (nmol/24 h)	131.9±58.1 (11.9)	130.0±62.6 (12.8)
Mean±SD (SEM) (nmol/(kg 24 h)	1.90±0.83 (0.17)	1.87±0.90 (0.18)
Confidence interval (±95%) (nmol/24 h)	107.4–156.5	103.6–156.4
Confidence interval (±95%) (nmol/(kg 24 h)	1.54–2.25	1.48–2.25
Mean difference±SD (SEM) CI (nmol/24 h)	−1.9±69.3 (14.1) CI: −31.2–27.3	
Mean difference±SD (SEM) CI (nmol/(kg 24 h)	−0.03±0.99 (0.20) CI: −0.45–0.39	
P-value, that mean difference = 0	0.8913	

TABLE IV Statistical analyses of the urinary excretion of 8-oxodGuo (combined data for both groups of the subjects—see materials and methods)

	Nucleic acid free diet	*Normal diet*
Mean±SD (SEM) (nmol/24 h)	60.4±27.3 (5.6)	57.7±33.9 (6.9)
Mean±SD (SEM) (nmol/(kg 24 h)	0.87±0.39 (0.08)	0.83±0.49 (0.10)
Confidence interval (±95%) (nmol/24 h)	48.9–72.0	43.3–72.0
Confidence interval (±95%) (nmol/(kg 24 h)	0.70–1.03	0.62–1.03
Mean difference±SD (SEM) CI (nmol/24 h)	−2.8±35.4 (7.2) CI: −17.7–12.2	
Mean difference±SD (SEM) CI (nmol/(kg 24 h)	−0.04±0.51 (0.10) CI: −0.25–0.17	
P-value, that mean difference = 0	0.7057	

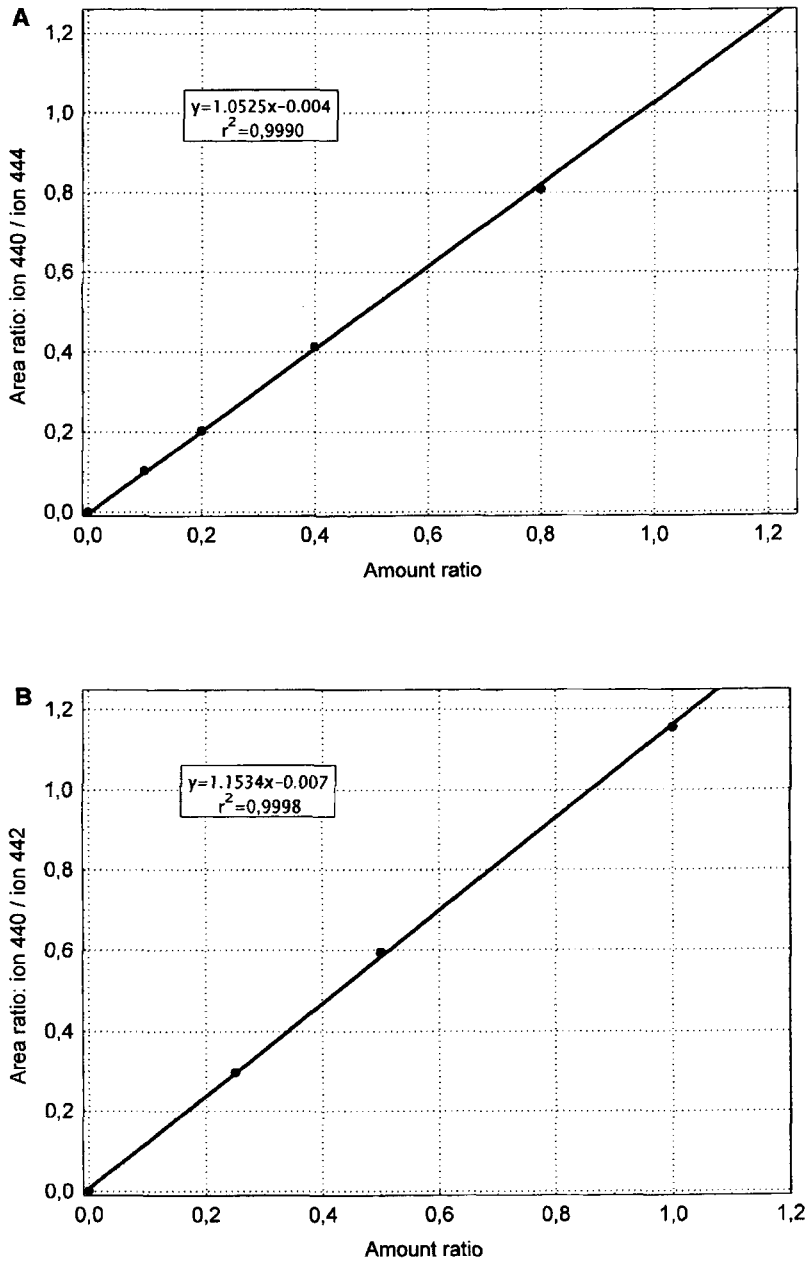

FIGURE 1 Calibration curves for [$^{15}N_3$, ^{13}C] 8-oxoGua (A) and [^{18}O] 8-oxodGuo (B) obtained by plotting the relative ratio of the target ion and of the isotopically labelled compound versus increasing relative amounts of both compounds. The ions used for detection of 8-oxodGuo are those of 8-oxoGua since the nucleoside is hydrolysed by formic acid treatment prior to GC/MS analysis.

required".[14] Therefore, in our experiments we used a recently developed technique, which involved HPLC pre-purification followed by gas chromatography with isotope dilution mass spectrometric detection.[15] In addition to unequivocal identification of the analysed compounds and high sensitivity, isotopically labelled internal standards used in this approach allowed

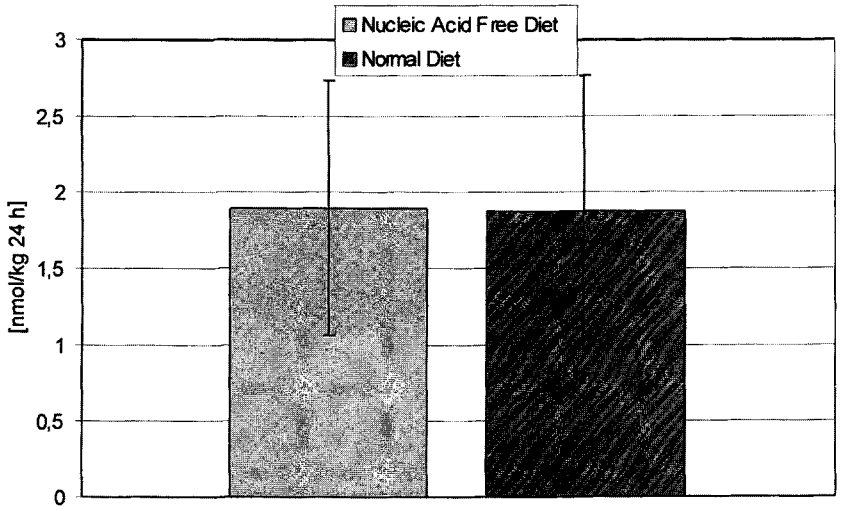

FIGURE 2 Urinary excretion of 8-oxoGua (combined data for both groups of the subjects—see materials and methods).

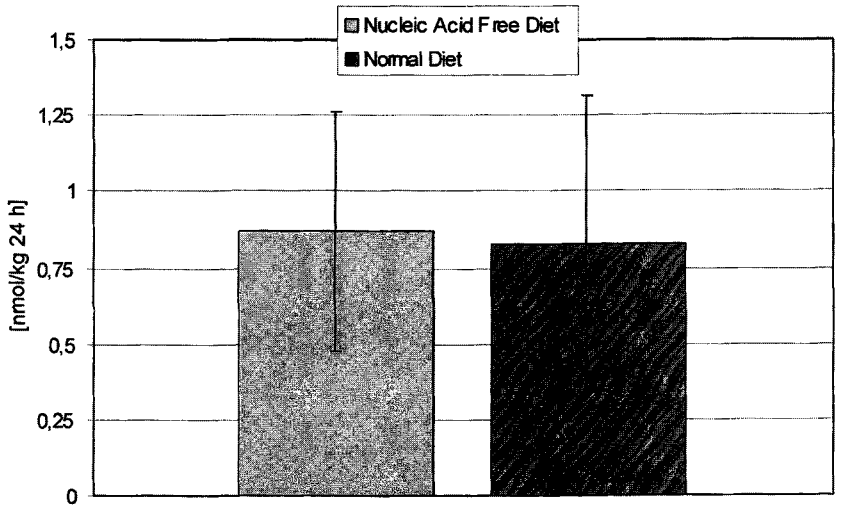

FIGURE 3 Urinary excretion of 8-oxodGuo (combined data for both groups of the subjects—see materials and methods).

compensation for eventual losses of the analysed products. However, it is rather unlikely that methodological differences alone may explain the above mentioned discrepancies. More likely, they may also be explained by (ii) different metabolism of purines in rats and humans.[21–23] This conclusion is supported by the fact that the level of 8-oxoGua in human urine of the subjects on normal, unrestricted diet, detected in our study (Fig. 2), and the level in urine of rats fed a diet without nucleic acids[18] are in the same range (1.87 and 3.975 nm/kg 24 h respectively) while there were substantial differences for these values when the comparison was made between humans on unrestricted diet and rats fed a normal diet (1.87 and 50.545 nmol/kg 24 h). Interestingly, the level of 8-oxoGua excised from DNA of human diploid fibroblast cells grown in cell culture (about 12 pmol per 10^9 cells per day[22] is also in the same range as found in our study (taking into consideration the fact that the number of DNA containing cells in a 70 kg

human has been estimated between 0.8×10^{13} and 7×10^{13}).[24,25] Moreover, inter-individual differences of 8-oxoGua and 8-oxodGuo excretion in the case of the subjects on unrestricted diet are the same (nine-fold range).

In good agreement with a previous study[18] the levels of 8-oxodGuo in the urine samples were also similar regardless of the diet. Therefore, the sum of amounts of both compounds in urine can give information about the formation rate of 8-oxoGuo in cellular DNA. It is also likely that the levels of the modified nucleo-base/side in urine sample are reflective of the involvement of different repair pathways responsible for the removal of 8-oxoGua from DNA, namely BER and NER.[8,11,26–28] Several glycosylases, which specifically recognise and remove 8-oxoGua have been identified in mammals including humans.[12,13] Therefore BER is supposed to play essential role in repair of this modification.[11,26,27] It has been suggested that NER acts simply as a "back up" system in the repair of oxidative DNA damage[11] although the existence of alternative repair pathway independent of OGG1 glycosylase has been found in an *ogg*1-/- cell line.[29]

However, we cannot exclude a possibility that other than repair processes can contribute to 8-oxoGua and 8-oxodGuo level in human urine. A further possible source of the modifications in urine may be that they derive from dead cells.[30] Urinary level of 8-oxoGua may also include a contribution from oxidized RNA, particularly if mechanisms exist to maintain the integrity of RNA molecule (but such a mechanism(s) has not yet been detected). The oxidation of the cellular nucleotide pool is a potential source of excreted adducts. All these possibilities remain to be tested.

In conclusion, we have found that urinary excretion of 8-oxoGua and 8-oxodGuo does not depend on diet in the case of humans. The level of both compounds may be a good indicator of the formation of oxidative damage in cellular DNA.

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