Synthesis of 8-oxo-7,8-dihydro-2'-deoxyguanosine from 2'-deoxyguanosine using Cu(II)/H₂O₂/ascorbate: A new strategy for an improved yield

Patrícia S. Peres, Andressa Valerio, and Glaucia R. Martinez

Laboratório de Oxidações Biológicas, Departamento de Bioquímica e Biologia Molecular, Setor de Ciências Biológicas, Universidade Federal do Paraná (UFPR), Curitiba, Brazil.

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The free nucleoside 2'-deoxyguanosine (dGuo) is the most susceptible to oxidation by reactive oxygen species (ROS) compared to the other free nucleosides, and its oxidation product 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) has been used as a biomarker to quantify oxidative stress damage. We investigated different reactions using Fe²⁺ or Cu²⁺ and H₂O₂ in order to identify the reaction with the best yield. HPLC coupled with a UV detector and microTOF mass spectrometry were used to detect and confirm the identity of 8-oxodGuo. The optimized reaction synthesized 8-oxodGuo with a yield of 72.0%, much higher than that previously described in the literature. Our improved method for 8-oxodGuo synthesis could be extremely useful for assays that require the synthesis of internal standards labeled with stable isotopes.

Several diseases, such as metabolic syndrome, hypertension, cataracts, diabetes, and cancer, are related to oxidative stress (1), which consists of an imbalance between oxidant and antioxidant species in favor of the oxidants. Reactive oxygen species (ROS), such as hydrogen peroxide (H₂O₂), superoxide anion (O₂^{•-}), hydroxyl radical (•OH), and singlet oxygen (¹O₂), can react with biomolecules, causing damage and dysfunction in living organisms (2). ROS are able to damage proteins, lipids, and nucleic acids, and the damage to DNA is removed not through degradation but only by repair. An important repair system is the base excision repair (BER) pathway, which has been conserved from bacteria to mammals (3). Nevertheless, not all DNA lesions are properly removed, and those that remain constitute a great threat because if they are not repaired, they may lead to mutation and contribute to aging or carcinogenesis (4–5).

As the half-life of ROS inside cells is very short, it becomes difficult to monitor the amount of oxidant present. Therefore, researchers usually monitor the levels of oxidative damage biomarkers, mainly for the oxidation of lipids, proteins, and DNA. 2'-deoxyguanosine (dGuo) is a purine nucleoside that is the target of several types of ROS, resulting in various oxidation products such as 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo), which is used to monitor damage in DNA. dGuo can be generated by the direct oxidation of guanine by addition of a hydroxyl radical to C-8 of the imidazole ring of guanine (6) or by reaction with singlet oxygen (7).

Many publications describe the detection of 8-oxodGuo in DNA (and also urine), with the majority of methods relying on HPLC-electrochemical detection or LC-MS/MS. However, due to difficulties such as the lack of sensitivity resulting from low yields of the oxidation products, instability of some products, and optimi-

zation of assays that may require the synthesis of internal standards labeled with stable isotopes (8), it is very important to develop new strategies of 8-oxodGuo synthesis. As 8-oxodGuo has been used as a biomarker of DNA oxidation caused by ROS, labeled 8-oxodGuo synthesis plays an indispensable role in the study of guanine oxidation products since stable isotope-labeled internal standards are required for LC-MS/MS-based detection of 8-oxodGuo, which has become the gold standard for accurate and reproducible results.

In order to obtain labeled 8-oxodGuo, one can oxidize labeled dGuo. Several studies have reported the synthesis of 8-oxodGuo (9–19), and a summary of the main reactants is provided in Table 1. For instance, Kasai and Nishimura observed that the C8 of dGuo was hydroxylated by ascorbic acid in the presence of O₂ in 0.1 mM phosphate buffer (pH 6.8) at 37°C and that adding H₂O₂ reinforced the reaction

METHOD SUMMARY

Here we present an improved method for synthesizing 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodGuo) from 2'-deoxyguanosine (dGuo) dGuo, Cu²⁺, H₂O₂ and ascorbate. Our new approach adds a pre-purification step that guarantees 8-oxodGuo stability for at least 3 months and produces a 72.0% yield.

Table 1. Review of 8-oxodGuo synthesis reactions.

Authors	Substrate	Ascorbic acid	EDTA	Copper source	Iron source	H ₂ O ₂	pH	Temperature	Time
Brodie et al., 1953 (9) *	6 mM (diverse)	14 mM	6.5 mM	-	1.3 mM	-	6.7	37°C	2 h
Kasai and Nishimura, 1984 (10)	dGuo, 2.5 mM	14 mM	6.5 mM	-	1.3 mM	50 mM	6.8	37°C	3 h
Aruoma et al., 1991 (11)	DNA, 0.5 mg/mL	100 μ M	100 μ M	25 μ M	25 μ M	2.8 mM	7.4	37°C	1 h
Lloyd and Philips, 1999 (12)	DNA, 0.5 mg/mL	-	0 to 5 mM	250 μ M	-	50 mM	-	37°C	1 h
Yoshino et al., 1999 (13)	DNA, 100 μ g	0.2 mM	-	0.2 mM	-	-	7.4	37°C	1 h
Singh et al., 2003 (14)	dG, 1 mg/mL	10 mM	-	1.2 mM	-	5%	-	Room	15 min
Mucha et al., 2005 (15)	DNA, 25 μ g/mL	-	-	50 μ M or 5 mM	-	500 μ M or 50 mM	7.4	37°C	1 h or 90 min
Cerchiaro et al., 2009 (16)	GTP, 1 mM	1 mM	-	10 μ M	-	-	7.4	37°C	4 h
Fleming et al., 2011 (17)	dGuo, 2 mM	2 mM	10 mM at end of reaction	1 mM	-	10 mM	7.0	22 °C	1 h
Spencer et al., 2011 (18)	DNA ,300 μ g/mL	150 μ M	-	30 μ M	-	-	7.4	37°C	4 h
Bergstron et al., 2012 (19)	dGuo, 400 μ M	-	-	10 μ M	-	200 μ M	5.3	37°C	1 h

Only the main reactants are presented. For more details, please check the corresponding references. (*): synthesis of hydroxylated products other than 8-oxodGuo.

(10). Singh et al. synthesized 8-oxodGuo from [¹⁵N₅]-dGuo, 170 mM ascorbic acid, 350 μ L of 20 mM CuSO₄, and 250 μ L of 30% H₂O₂ (14).

Here we tested reactions reported by others (9–10,14), improving and characterizing them in order to obtain a better yield. Reactions from References 10 and 14 were used as standards for comparison.

Materials and methods

Reagents and equipment

Experimental procedures were performed using 2'-deoxyguanosine (Sigma-Aldrich, St. Louis, MO), ascorbic acid (Merck, Rio de Janeiro, Brazil), EDTA (Sigma-Aldrich), Fe₂SO₄·7H₂O (Synth, Diadema, Brazil), CuSO₄ (Merck, Darmstadt, Germany), H₂O₂ (Merck), and oxygen gas (Air Liquide, Curitiba, Brazil). PBS was prepared as follows: 68 mM NaCl, 13.4 mM KCl, 40.5 mM Na₂HPO₄, and 7.35 mM KH₂PO₄ at pH 6.8. A C18 Bond Elut 500 mg column with a capacity of 3 mL (Agilent Technologies, Santa Clara, CA) was used for pre-purification. A 500 W Hg light bulb (Phillips, Mauá, Brazil) was used for irradiation of Reactions III and V. Separations and identi-

fication of products were performed using the HPLC SIL 20AC (Shimadzu, Tokyo, Japan) and a microTOF Q-II (Bruker, Billerica, MA).

Oxidation of dGuo

Reaction I was performed as proposed by Kasai and Nishimura (10), and Reaction II was performed as described by Singh et al. (14). These reactions were chosen as the Standard Reactions to be improved upon. Variations of Reactions I and II were prepared, which were referred to as Reactions III, IV, V, VI, VII, VIII, IX, and X and are described in Table 2.

Pre-purification of 8-oxodGuo

In order to avoid degradation of 8-oxodGuo after collecting and lyophilizing it, pre-purification by solid-phase extraction (SPE) right after the reaction and before its collection was routinely carried out. Pre-purification was performed using a C18 Bond Elut 500 mg column with a 3 mL capacity (Agilent Technologies). Columns were prepared with 3 mL ultrapure water, followed by 1 mL methanol and another 1 mL ultrapure water. Then, 500 μ L reaction mixture was applied to the column, followed by 1 mL 5%

methanol, 1 mL 7% methanol, and 1 mL 10% methanol. The last 1 mL of eluate was collected and used for downstream analysis, such as checking for degradation after months of storage or even its use in other reactions. Pre-purification must be done immediately after the synthesis reaction.

Detection and quantification of dGuo and 8-oxodGuo

The free nucleoside dGuo and its oxidation product 8-oxodGuo were detected by HPLC using a Thermo Scientific Hypersil BDS C18 (250 × 2.1 mm, 5 μ m) (Thermo Fisher Scientific, Waltham, MA) column under 0.2 mL/min flow and a UV diode array detector set at 254 nm. The mobile phase was under 1 of 2 different conditions: Condition 1 was an isocratic flow composed of 15% methanol in 0.1% formic acid, and Condition 2 was a gradient of 0.1% formic acid (A) and methanol (B) as follows: 0–20 min, 5% B; 20 min, 15% B; 25 min, 20% B; and 30 min, 5% B. After identification by UV detection, the flow was directed to a microTOF Q-II set at 180°C and -4.5 kV. The 8-OxodGuo concentration was determined using its molar extinction coefficient (ϵ_{293} 8-oxodGuo = 9,700 cm⁻¹·mol⁻¹·L) (20).

Table 2. Reactions for dGuo oxidation.

Reaction	Composition	Condition
I (Fe ²⁺ /ASC/H ₂ O ₂)	2.5 mM dGuo + 14 mM ascorbic acid + 6.5 mM EDTA + 1.3 mM FeSO ₄ + 50 mM H ₂ O ₂ + PBS	Heat in water at 37°C under agitation for 3 h.
II (Cu ²⁺ /ASC/H ₂ O ₂)	3.7 mM dGuo + 10 mM ascorbic acid + 1.2 mM CuSO ₄ + 5% H ₂ O ₂	Room temperature for 15 min.
III (Fe ²⁺ /ASC/O ₂)	2.5 mM dGuo + 70 mM ascorbic acid + 6.5 mM EDTA + 1.3 mM FeSO ₄ + O ₂	Heat in water at 37°C for 3 h under O ₂ bubbling and irradiation.
IV (Fe ²⁺ /ASC/H ₂ O ₂)	2.5 mM dGuo + 70 mM ascorbic acid + 6.5 mM EDTA + 1.3 mM FeSO ₄ + 50 mM H ₂ O ₂	Heat in water at 37°C under agitation for 3 h.
V (Cu ²⁺ /ASC/O ₂)	2.5 mM dGuo + 70 mM ascorbic acid + 6.5 mM EDTA + 1.3 mM CuSO ₄ + O ₂	Heat in water at 37°C for 3 h under O ₂ bubbling and irradiation.
VI (Cu ²⁺ /ASC/H ₂ O ₂)	2.5 mM dGuo + 70 mM ascorbic acid + 6.5 mM EDTA + 1.3 mM CuSO ₄ + 50 mM H ₂ O ₂	Heat in water at 37°C under agitation for 3 h.
VII (Cu ²⁺ /ASC/H ₂ O ₂)	2.5 mM dGuo + 140 mM ascorbic acid + 1.3 mM CuSO ₄ + 5% H ₂ O ₂	Heat in water at 37°C under agitation for 15 min, 1 h, or 3 h.
VIII (Cu ²⁺ /ASC/H ₂ O ₂)	3.7 mM dGuo + 140 mM ascorbic acid + 1.3 mM CuSO ₄ + 5% H ₂ O ₂	Heat in water at 37°C under agitation for 1 h.
IX (Cu ²⁺ /ASC/H ₂ O ₂)	3.7 mM dGuo + 140 mM ascorbic acid + 10 mM CuSO ₄ + 5% H ₂ O ₂	Heat in water at 37°C under agitation for 15 min, 1 h, or 3 h.
X (Cu ²⁺ /ASC/H ₂ O ₂)	3.7 mM dGuo + 140 mM ascorbic acid + 1.3 mM CuSO ₄ + 10% H ₂ O ₂	Heat in water at 37°C under agitation for 1 h.

ASC stands for ascorbic acid, PBS is phosphate buffer saline (68 mM NaCl, 13.4 mM KCl, 40.5 mM Na₂HPO₄, and 7.35 mM KH₂PO₄ at pH 6.8).

Figure 1. Reaction VII performed for different times compared with Standard Reactions I and II. Incubation and detection were performed as described in the “Materials and methods.” Reaction I was composed of 2.5 mM dGuo, 14 mM ascorbic acid, 6.5 mM EDTA, 1.3 mM FeSO_4 , and 50 mM H_2O_2 in PBS. Reaction II was composed of 3.7 mM dGuo, 10 mM ascorbic acid, 1.2 mM CuSO_4 , and 5% v/v H_2O_2 . Reaction VII was composed of 2.5 mM dGuo, 140 mM ascorbic acid, 1.3 mM CuSO_4 , and 5% v/v H_2O_2 . Results are expressed as mean $\pm$ SD of two independent experiments in duplicate.

Statistical analysis

Reported values, presented as the mean $\pm$ SD difference between groups, were analyzed by ANOVA followed by Tukey’s post-hoc test using GraphPad Prism 6 software. The significance level was set as $P < 0.05$.

Results and discussion

A summary of all reactions performed in this study is provided in Table 2. Reactions I and II were performed as described originally, since they were chosen as the Standard Reactions (10,14). Reactions III, IV, V, and VI were adaptations of reactions presented in Table 1 that were performed as described in Table 2 and then injected into the HPLC instrument using a 0.2 mL/min isocratic flow composed of 15% methanol (condition 1). In this first trial, 8-oxodGuo was detected only in the reactions that contained CuSO_4 (Reactions V and VI). In fact, it has been shown previously that reactions containing Cu^{2+} are more efficient at damaging DNA than reactions with equimolar Fe^{3+} (11,21). Since Reaction VI was easier to perform as it does not include O_2 bubbling, it was chosen as the reaction to be carried out in the next step of this study.

In order to obtain a better resolution for the chromatography of the remaining dGuo from ascorbic acid and to better collect 8-oxodGuo, the isocratic flow of

15% methanol (Condition 1) was changed to a gradient composed of 0.1% formic acid (A) and methanol (B) as follows: 0–20 min, 5% B; 20 min, 15% B; 25 min, 20% B; and 30 min, 5% B (Condition 2). As expected, this change better separated dGuo from 8-oxodGuo, as dGuo retention time was 8.35 min, and 8-oxodGuo retention time was 12.40 min. The identity of 8-oxodGuo was confirmed by diode array detection and MS analysis (data shown in the Supplementary Material). Reactions were re-analyzed using this new mobile phase gradient (Condition 2).

However, degradation of 8-oxodGuo was observed since a sample stored 1 week in the freezer after synthesis failed to be detected using HPLC. This could possibly be due to the presence of Cu^{2+} in the solution, which could oxidize 8-oxodGuo into other products. In fact, oxidation of dGuo may yield a plethora of products in addition to 8-oxodGuo (22–24). Interestingly, the balance between the products due to different reaction conditions has been recently published (24). In this study, our main goal was to provide an efficient method for maximizing the production of 8-oxodGuo, but our approach probably decreases the formation of the other oxidation products. Thus, aiming to remove as much Cu^{2+} from the solution after the reaction as possible, a pre-purification SPE step was added to the process, using a C18 Bond Elut 500 mg column with 3 mL capacity as described above. From this point on, reactions were prepared, pre-purified on the C18 Bond Elut column, and then purified by HPLC under Condition 2. Samples were then lyophilized and stored

Figure 2. Reaction VIII compared with Standard Reaction II performed at different temperatures. Standard Reaction II was performed under different temperatures for 15 min, and Reaction VIII was performed at 37°C for 1 h. Incubation and detection were performed as described in the “Materials and methods.” Reaction II was composed of 3.7 mM dGuo, 10 mM ascorbic acid, 1.2 mM CuSO_4 , and 5% v/v H_2O_2 . Reaction VIII was composed of 3.7 mM dGuo, 140 mM ascorbic acid, 1.3 mM CuSO_4 , and 5% v/v H_2O_2 . Results are expressed as mean $\pm$ SD of two independent experiments in duplicate.

at -20°C in a freezer for future use. All data presented here were obtained after this pre-purification by SPE. This step reduces the final product yield compared with the yield obtained immediately after synthesis, probably due to the loss of 8-oxodGuo in the column. However, it is important to guarantee that the 8-oxodGuo will not be degraded. This pre-purification also protects HPLC columns from deterioration. Indeed, the 8-oxodGuo formed by Reaction VIII was kept in aqueous solution at -20°C in a freezer and analyzed 3 months after its synthesis, and it retained its original concentration.

Figure 3. Kinetic study of Reaction II at 37°C and 45°C. Reactions were incubated and 8-oxodGuo was detected as described in the “Materials and methods.” Reaction II was composed of 3.7 mM dGuo, 10 mM ascorbic acid, 1.2 mM CuSO_4 , and 5% v/v H_2O_2 . Results are expressed as mean $\pm$ SD of two independent experiments in duplicate.

Figure 4. Comparison among Reactions VIII, IX, and X. Reactions were incubated, and 8-oxodGuo was detected as described in the “Material and methods.” Reaction VIII was composed of 3.7 mM dGuo, 140 mM ascorbic acid, 1.3 mM CuSO_4 , and 5% v/v H_2O_2 . Reaction IX was composed of 3.7 mM dGuo, 140 mM ascorbic acid, 10 mM CuSO_4 , and 5% v/v H_2O_2 . Reaction X was composed of 3.7 mM dGuo, 140 mM ascorbic acid, 1.3 mM CuSO_4 , and 10% v/v H_2O_2 .

In order to determine whether the absence of EDTA would increase the final amount of 8-oxodGuo, Reaction VI was also performed without EDTA, and as expected, the yield was greater. Indeed, EDTA was found to decrease DNA base damage by Cu^{2+} (11) and to increase DNA base damage induced by ROS in the presence of Fe^{3+} and H_2O_2 (25). Reaction VI was then modified to be done without EDTA but with higher concentrations of ascorbic acid (140 mM) and H_2O_2 (5% v/v); this is referred to as Reaction VII. This modification increased the final product yield (Figure 1). Reaction VII was then performed at 37°C under agitation for 15 min, 1 h, and 3 h in order to choose the best reaction time. The 1 h reaction was then chosen for the next steps as it resulted in the best yield (Figure 1).

As shown in Figure 1, Reaction VII is undoubtedly more effective than the Standard Reaction I. This comparison is easy to make because both reactions started with 2.5 mM dGuo. In order to provide a better comparison between Standard Reaction II and Reaction VII, the latter was modified to start from 3.7 mM dGuo—just as with Reaction II—and, under these conditions, the reaction is referred to as Reaction VIII (Figure 2). Reaction I was then performed not only at room temperature (21°C), as originally described, but also at 24°C, 33°C, 37°C, and 45°C. 8-OxodGuo synthesis by Reaction II proved to be temperature-dependent up to 45°C with 15 min of reaction time (Figure 2).

Nevertheless, 8-oxodGuo formation could also be time-dependent, so Reaction II was performed at 37°C (Figure 3A) and 45°C (Figure 3B), not only for 15 min but also for 30 min and 1 h. Although slight variations were observed among the groups, Reaction II carried out for its original time of 15 min and at 45°C was more effective at producing 8-oxodGuo, resulting in a yield of 64.5%. Even so, Reaction VIII was the most efficient with a yield of 72.0% (Figure 3).

The Cu^{2+} ion is necessary for dGuo oxidation, as well as H_2O_2 . Therefore, it was essential to evaluate whether a change in the amount of these components would affect 8-oxodGuo generation. To that end, Reaction VIII was first performed with much more CuSO_4 [i.e., 10 mM (Reaction IX)], which led to lower 8-oxodGuo production (Figure 4). The next step was to test the role of H_2O_2 in the reaction. That was done by doubling the amount from the original Reaction VIII, which we refer to as Reaction X. As this modification had no effect (Figure 4), it is better to use less H_2O_2 , (i.e., 5% v/v H_2O_2), as in the original Reaction VIII.

The reaction proposed by this study (Reaction VIII; composed of 3.7 mM dGuo, 140 mM ascorbic acid, 1.3 mM CuSO_4 , and 5% v/v H_2O_2 with the pre-purification step) provided a 72.0% yield of 8-oxodGuo. That represents a much higher yield than the 2.4% and 21.4% previously observed in Reactions I and II when also performed with the pre-purification step. Thus, we have developed a superior method for synthesizing 8-oxodGuo from dGuo that can be used in situations that require a large amount of 8-oxodGuo or labeled 8-oxodGuo generated from isotopically labeled dGuo.

Author contributions

P.S.P., A.V., and G.R.M. conceptualized the study and outlined the protocol. P.S.P. and A.V. carried out the experiments. P.S.P. performed data analysis and wrote the manuscript. G.R.M. supervised the whole study and edited the manuscript.

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Competing interests

The authors declare no competing interests.

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Address correspondence to Glauca R. Martinez, Laboratório de Oxidações Biológicas, Departamento de Bioquímica e Biologia Molecular, Setor de Ciências Biológicas, Universidade Federal do Paraná (UFPR), 81531-980, Curitiba, PR, Brazil. E-mail: grmartinez@ufpr.br

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