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BRIEF REPORT

WILEY

Is pre-heat necessary for the measurement of 8-oxo-7,8-dihydroguanosine and 8-oxo-7,8-dihydro-2′-deoxyguanosine in urine samples

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Abstract

Background: It is currently unclear for the necessary of pre-heating urine samples for the accurate determination of 8-oxo-7,8-dihydroguanosine (8-oxoG) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG). Thus, we conducted this study to evaluate the effect of pre-heat (i.e., to 37°C) on the accurate measurement of 8-oxoG and 8-oxodG in frozen urine samples.

Methods: Random urine samples from six healthy volunteers, six patients with renal dysfunction, and six patients with systematic diseases such as diabetes were collected, split, and stored at −80°C for up to 1 month. The frozen samples were thawed at room temperature (RT) or 37°C for different time, 10-fold diluted with ddH2O containing 1% formic acid, and determined by self-established LC–MS/MS method coupled with an ACQUITY™ Primer HSS T3 column.

Results: Thawing the samples at RT for 30 or 120 min, or at 37°C for 15 or 90 min did not affect the determination of 8-oxoG and 8-oxodG in urine samples. Moreover, no significant difference between thawing the urine samples at RT and 37°C was found after storing at −80°C for 1–3 months.

Conclusion: It is not always necessary to pre-heat the frozen urine samples to release 8-oxoG and 8-oxodG from precipitates, which is associated with different pre-treatment and determination methods.

KEYWORDS
8-oxo-7,8-dihydro-2′-deoxyguanosine, 8-oxo-7,8-dihydroguanosine, LC–MS/MS, pre-heat

1 | INTRODUCTION

As sensitive and crucial biomarkers for oxidative damage, 8-oxo-7,8-dihydroguanosine (8-oxoG) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) are extensively studied in recent years. The development of liquid chromatography–tandem mass spectrometry (LC–MS/MS) enabled the accurate determination of 8-oxoG and 8-oxodG in urine, with reduced sample manipulation and risk of pre-analytical artifacts than ELISA method.[1] In 2000, Allan et al.[2] reported a LC–MS/MS method for the determination of 8-oxoG and 8-oxodG in urine, and emphasized the necessary to redissolve the precipitates in urine samples by heating to 37°C for 10 min to release the measurements. Many following studies also chosen to thaw the frozen urine samples at 37°C, making the pre-treatment process more complex.[2–8] Moreover, the specific time of pre-heat and/or process of pre-treat were inconsistent, and was not effectively evaluated and reported.

A recent study published specific data to support the opinion that frozen urine samples must be warmed (37°C for 15 min) to redissolve any precipitates prior to analysis.[4] It was estimated that the release efficiency at room temperature (RT) is 14%–71% for 8-oxoG, and 14–66% for 8-oxodG in frozen urine samples, respectively.[4] However, only two situations were evaluated, some details such as sample blending were not mentioned in this study, and only supernatant after certification was further treated for analysis. There were also many studies thawing the frozen urine samples without warming.[9–13] Thus, it is still needed to evaluate whether pre-heat is necessary for the accurate measurement of 8-oxoG and 8-oxodG in frozen urine samples.

In this study, we more meticulously evaluated the necessary of pre-heat for the measurement of 8-oxoG and 8-oxodG in urine samples based on our laboratory self-established LC–MS/MS method.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Six apparently healthy volunteers were recruited from the Department of Laboratory Medicine of Peking Union Medical College Hospital (PUMCH), and their fresh urine samples were obtained. Six clinical patients with renal insufficiency and six patients with other systematic diseases such as diabetes and cancers were randomly recognized, and their clinical residual urine samples were obtained. All samples were mixed up, split, stored at −80°C, and measured before being frozen as baseline levels. After 1 week, 42 urine samples from six apparently healthy volunteers (seven same split samples for everyone) were simultaneously taken out, and separately pre-treated at seven different conditions including RT (22–24°C) for 30, 60 and 120 min, and at 37°C for 15, 30, 60 and 90 min. Moreover, these samples from all 18 individuals were also taken out after storing at −80°C for up to 1 month, and pre-treated at RT for 30 and 60 min, and at 37°C for 15 and 30 min. Then the levels of 8-oxoG and 8-oxodG in urine samples were determined based on a robustly self-established LC–MS/MS method. Moreover, during from October to November in 2021, urine samples of 18 apparently healthy check-ups were derived, mixed up, split, and stored at −80°C. These samples were separately measured at December, 2021 with thawing at RT for 60 min, and at February, 2022 with thawing at 37°C for 30 min.

This study was approved by the Ethics Committee of Peking Union Medical College and Chinese Academy of Medical Sciences at Peking Union Medical College Hospital (ZS-2486), and all apparently healthy participants provided informed consent for participation in the study.

2.2 | Preparation of calibration standards and urine samples

Powdered 8-oxoG (batch No., H942770; purity, 97%), 8-oxoG-*d3* (batch No., H942772; purity, 98%), 8-oxodG (batch No., O850250; purity, 96%), and 8-oxodG-*d3* (batch No., O850252; purity, 98%) were purchased from Toronto Research Chemicals. High-performance liquid chromatography grade methanol (batch No., A452-4; purity, 99.9%) and formic acid (batch No., L1670; purity, 100%) were purchased from Thermo Fisher Scientific. The stock solutions of both calibration standards and internal standard (IS) were dissolved, and further diluted with ddH2O containing 1% formic acid. An aqueous solution of 8-oxoG-d3 (5 ng/ml) and 8-oxodG-d3 (5 ng/ml) containing 1% formic acid was used as the IS working solution. After full mix up, 10 μl of urine sample was diluted with 90 μl ddH2O containing 1% formic acid, mixed with 10 μl of IS solution, transferred into a 2-ml 96-well collection plate, certificated at 2163 *g* for 2 min, and directly injected 10 μl into the system.

2.3 | Measurement method

A Waters TQ-XS triple quadrupole MS/MS system coupled with an ACQUITY™ Primer HSS T3 column (2.1 × 100 mm, 1.8 μm VanGuard™ FIT, Part No. 186009471) were used for the measurement of 8-oxoG and 8-oxodG. Both 8-oxoG and 8-oxodG were measured in positive electrospray ion mode, and the optimized MS/MS settings are summarized in Table S1. Eluent A consisted of ddH2O with 0.1% formic acid, and Eluent B was pure methanol. At a flow rate of 0.3 ml/min, gradient elution was performed by changing Eluent A: Eluent B (V: V) as follows: 0.0–0.5 min, 99: 1; 0.5–1.5 min, 99: 1–90: 10; 1.5–2.3 min, 90: 10; 2.3–3.3 min, 90: 10–80: 20; 3.3–3.4 min: 80: 20–10: 90; 3.4–3.9 min: 10: 90–5: 95; 3.9–4.5 min, 5: 95–99: 1; 4.5–5.0 min: 99: 1.

2.4 | Statistical analysis

Statistical analysis was performed using SPSS version 22.0 (IBM) and GraphPad Prism 7.0 (GraphPad Software). Two-sided *p*-values < 0.05 were considered statistically significant.

3 | RESULTS

The conditions of mass spectrographic and chromatographic were optimized to achieve adequate retention and separation of analytes onto the column (Figure S1). Of six apparently healthy volunteers, two had significant precipitates in their urine samples. No significant difference between thawing the samples at RT and 37°C was found (Figure 1). Moreover, the time putting the samples at RT or 37°C did not affect the measurement of 8-oxoG and 8-oxodG in urine samples, which meant setting at RT for 30 min was enough for complete dissolution of 8-oxoG and 8-oxodG from precipitates.

To exclude the effect of storing time and individual differences, the urine samples of all 18 volunteers including six apparently healthy volunteers and 12 clinical patients with renal insufficiency or other systematic diseases kept frozen at −80°C, and were further evaluated after storing for 1 month. More than half of these urinary samples had apparent precipitates after this frozen process. Still, no significant difference was found between pre-treatment at 37°C and RT situations for all 18 individuals, and thawing these samples at RT for 30 min was enough for complete dissolution of 8-oxoG and 8-oxodG from precipitates (Figure 2).

As shown in Figure S2, there was no significant difference (8-oxoG, $p = 0.692$; 8-oxodG, $p = 0.065$) for the results of urinary 8-oxoG and 8-oxodG in 18 apparently healthy check-ups between thawing at RT for 60 min (previous measurement after storing at −80°C for more than 1 month) and 37°C for 30 min (new measurement after storing at −80°C for more than 3 months). The measurement standard deviations between two determinations were less than 10%.

4 | DISCUSSION

In this study, we found it not always necessary to thaw the frozen urine samples at 37°C, and setting at RT for 30 min with ample blending and dilution were enough for the release of 8-oxoG and 8-oxodG from precipitates in urine samples. The levels of urinary 8-oxoG and 8-oxodG in healthy individuals measured with thawing

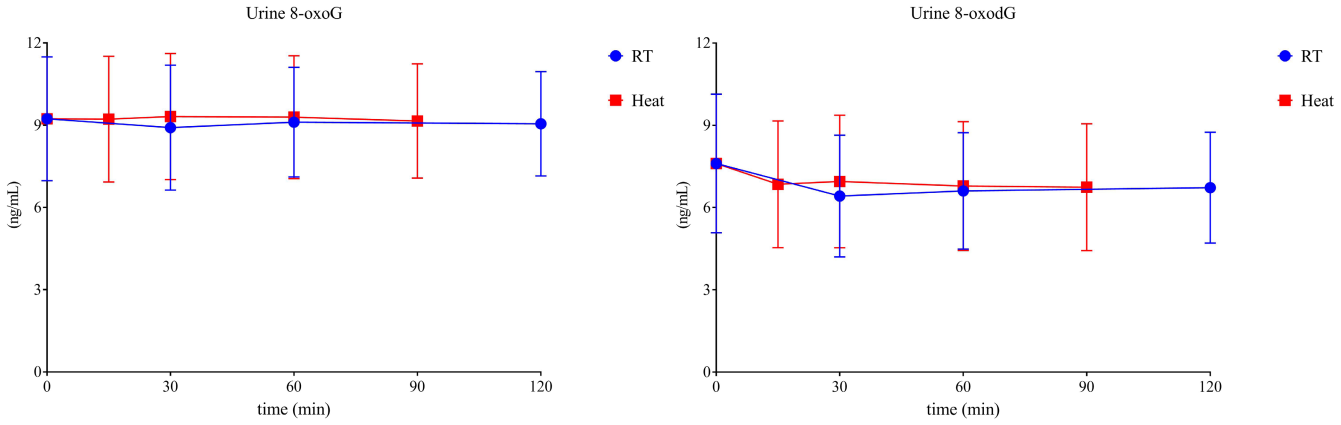

FIGURE 1 The effect of pre-heat after storing at −80°C for 1 week in healthy individuals 8-oxoG, 8-oxo-7,8-dihydroguanosine; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine

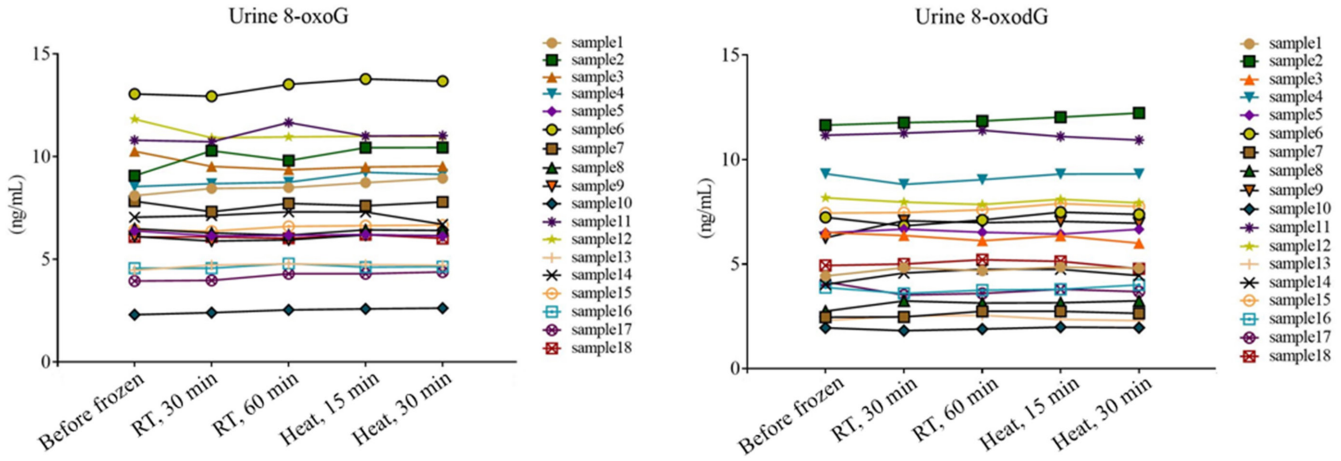

FIGURE 2 The effect of pre-heat after storing at −80°C for 1 month in 18 individuals 8-oxoG, 8-oxo-7,8-dihydroguanosine; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanosine

at RT for 30 min in this study was close to and even higher than that measured with thawing at 37°C in the previous study [8-oxoG, 6.18 (3.06, 10.58) ng/ml; 8-oxodG, 3.63 (1.47, 6.51) ng/ml].[4] Except for healthy individuals, we also enrolled the patients with renal dysfunction and other systematic diseases to large the application of this conclusion. Of these 18 individuals, half of them had participations, and three of them had large participations in their urine samples. Still, no significant difference between thawing at RT and 37°C was found.

In the process, we mixed the urine samples up well after thawing without discarding precipitates, diluted with ddH2O containing 1% formic acid, which was benefit for the complete dissolution of 8-oxoG and 8-oxodG. Previous studies[4,12] found that diluting the urine with five volumes of ddH2O or two volumes of 8% MeOH, or 1:1 with 0.1 M lithium acetate (pH 6.4) at RT could encourage oxidized guanine lesions from the precipitates into solution. Other studies without warming process chose to dilute the urine samples 10-fold with 5% (v/v) methanol/1 mM ammonium acetate, 10- or 20-fold with 5% MeOH (v/v) solvent with 0.1% formic acid, and so on.[9,11,13] Since we also used ddH2O to dilute the urine samples and found no significant difference with ddH2O containing 1% formic acid, we supposed that the dilution process contributed to the release of 8-oxoG and 8-oxodG from precipitates. However, nearly all studies did not point out whether they evaluated the release efficiency of 8-oxoG and 8-oxodG from urinary precipitates under different pre-treatment and determination method. Without efficient evaluation, these different pre-treatment steps across previous studies may lead to some of the apparent assay variability between laboratories.[14,15]

To our knowledge, this was the first study to evaluate the effect of heating process (37°C) for different time on the release of 8-oxoG and 8-oxodG from urinary precipitates in both healthy individuals and patients with different diseases. Since the dilution process could contribute to the release of 8-oxoG and 8-oxodG from precipitates, thus, whether it is necessary to pre-heat the urine samples may depend on different pre-treatment methods. To make the pre-treatment process easy, we suggest to evaluate whether it is needed to heat the urine samples under different pre-treatment and measurement methods. Furthermore, the robustly self-established LC–MS/MS method used in this study could effectively distinguish the interferes in serum and urine samples with the limit of quantification of 5 pg/ml (unpublished data), making sure the accuracy of determination.

In conclusion, whether the process of heating urine samples for the release of 8-oxoG and 8-oxodG is necessary and the specific details needs to be evaluated under different pre-treatment and determination methods to make sure the accuracy of measurements with simpler pre-treatment methods.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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