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Development of quantitative analysis of 8-nitroguanine concomitant with 8-hydroxydeoxyguanosine formation by liquid chromatography with mass spectrometry and glyoxal derivatization

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Abstract

Under inflammatory conditions, both 8-nitroguanine (NO_2Gua) and 8-hydroxydeoxyguanosine (8-OHdG) are found in tissues. Measurements of the two types of damaged bases on nucleotides are expected to provide information pointing to the possible correlation between inflammation and carcinogenesis. For the establishment of an *in vivo* model, in this study, a sensitive and precise method for the determination of NO_2Gua, which uses liquid chromatography with mass spectrometry (LC–MS) and 6-methoxy-2-naphthyl glyoxal (MTNG) derivatization, was developed *in vitro*. The procedure for DNA digestion in this method is identical to that widely used for 8-OHdG measurement, which enables us to detect the two damaged bases in the same DNA sample. In order to validate our method, we measured NO_2Gua levels in DNA sample using LC–MS. A mass spectrometer equipped with an electrospray atmospheric pressure ionization source and operated in the negative ion mode (ESI^-) was set up with selective ion monitoring at *m/z* 391 and 394 for NO_2Gua-MTNG and [^{13}C, $^{15}N_2$]-NO_2Gua-MTNG as surrogate standard, respectively. The average recoveries from DNA samples spiked with 25, 50 and 250 nM NO_2Gua were 99.4, 99.8 and 99.1% with correction using the added surrogate standard, respectively. The limit of quantification was 3.0 nM for NO_2Gua. To ascertain the applicability of our method to DNA samples harboring the two damaged bases, we measured NO_2Gua and 8-OHdG levels in calf thymus DNA treated with $ONOO^-$. As a result, both NO_2Gua and 8-OHdG levels were clearly increased with $ONOO^-$ dose dependency, the amount of NO_2Gua at the high dose $ONOO^-$ being almost the same as those of 8-OHdG. LC–MS was able to determine NO_2Gua in a small amount of DNA sample, and is therefore expected to be a very powerful tool for the evaluation of DNA damage induced by reactive nitrogen species.

Keywords: Nitroguanine; Peroxynitrite; LC–ESI/MS; 8-OHdG; Reactive nitrogen species; Reactive oxygen species

1. Introduction

Reactive oxygen species produced by inflammatory cells are proposed to contribute to inflammation-mediated carcinogenesis by inducing oxidative DNA damage in both animals and humans [1–4]. 8-Hydroxydeoxyguanosine (8-OHdG), a form of guanine oxidized at the C-8 position, is considered to be fairly stable and to be the most abundant oxidative lesion among the many oxidized nucleosides [5]. The development of high-performance liquid chromatography with ultraviolet/electro chemical detection (HPLC–UV–ECD) for the sensitive and precise analysis of 8-OHdG has furthered our understanding of its biological function [6]. 8-OHdG can induce GC:TA transversion, which is frequently found in tumor genes in a variety of cancers [7,8].

In the case of inflammatory disease, induced NO synthetase generates a large amount of nitric oxide (NO) [9]. NO also may exert mutagenicity through the deamination of deoxyribonucleosides and/or nucleotides [10,11]. In addition, NO is responsible for the formation of genotoxic nitrosamines by reacting with secondary or tertiary amines [12,13]. Alternatively, NO also reacts with superoxide anion to produce peroxynitrite ($ONOO^-$), which can initiate reactions characteristic of hydroxyl radical, nitronium ion and nitrogen dioxide radical

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[14]. As $ONOO^-$ is relatively stable, NO-mediated DNA damage might involve not only deamination but also nitration of DNA [15,16]. In fact, it was found that $ONOO^-$ readily reacts with guanine to form 8-nitroguanine (NO_2Gua), which is then hydrolyzed to release free base NO_2Gua [17]. Subsequent formation of an apurinic site in DNA induces GC:TA transversion, as in the case of 8-OHdG formation. As $ONOO^-$ is an oxidizing agent as well as a nitrating one, the nitration of DNA might occur concomitantly with 8-OHdG formation [18–20]. Namely, the resultant 8-OHdG adducts and/or specific DNA base substitutions do not give any clues to determine their causes. Therefore, the development of a method that simultaneously analyzes 8-OHdG and NO_2Gua is requisite to investigating inflammation-mediated DNA damage.

The simultaneous analysis of 8-hydroxyguanine and NO_2Gua in DNA or RNA using LC–ECD has already been reported, in which the detection limit is comparatively high at 20 fmol/injection [20]. However, measurement by LC–ECD requires reduction of NO_2Gua by a reducing agent such as sodium hydrosulfite [20,21] which results in low reproducibility due to the reaction efficiency. In addition, pretreatment with an affinity column or solid phase extraction is needed to separate the two high-polarity substances from impurities [22], which lead to lowering of measurement precision.

In this study, we performed liquid chromatography with mass spectrometry (LC–MS) using a glyoxal derivatization reagent for guanine compounds to depress the polarity of NO_2Gua [23–28], and confirmed measurement precision by compensation with a stable isotope. The column-switching (CS) system was also adopted to purify and condense the sample online. The sample preparation procedures in our new method involved a DNA digestion method widely used for the measurement of 8-OHdG, which enabled us to measure NO_2Gua from one part of the obtained sample, and 8-OHdG from the other part. We were able to quantify NO_2Gua and 8-OHdG levels in calf thymus DNA treated with $ONOO^-$.

2. Materials and methods

2.1. Chemicals and reagents

Ammonium acetate, hydrochloric acid (HCl), phenyl glyoxal hydrate (PG), 2′-deoxyguanosine (dG) monohydrate, dimethylsulfoxide (DMSO) and HPLC-grade acetonitrile were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). NO_2Gua and 8-nitroguanine-8-^{13}C-7,9-$^{15}N_2$ ([^{13}C, $^{15}N_2$]-NO_2Gua) were purchased from Toronto Research Chemicals Inc. (North York, Canada). Diethylenetriaminepentaacetic acid (DTPA) as a chelating agent, deoxyribonucleic acid from calf thymus, 8-OHdG and alkaline phosphatase were purchased from Sigma (St. Louis, MO). 6-Methoxy-2-naphthyl glyoxal hydrate (MTNG) was acquired from Alfa Aesar (Ward Hill, MA). Glyoxal solution (G) and *p*-hydroxyphenyl glyoxal (HPG) were purchased from Fluka (Buchs, Switzerland). $ONOO^-$ was obtained from Dojindo Co. (Kumamoto, Japan). Nuclease P1 was obtained from Yamasa Shoyu Co. (Chiba, Japan). Purified water was obtained with Milli-Q gradient A 10 equipped with an EDS polisher (Millipore, Bedford, MA). All other chemicals used were of specific analytical or HPLC grade.

2.2. Standard solutions and derivatization solutions

Stock solutions (50 μM) of NO_2Gua were prepared in purified water. Working solutions for calibration (3–500 nM) were prepared by adding an adequate amount of [^{13}C, $^{15}N_2$]-NO_2Gua as surrogate standard (aqueous solution) and diluting with digestion buffer (20 mM sodium acetate buffer at pH 4.8/1.0 M Tris–HCl buffer at pH 8.0/3.0 M sodium acetate buffer at pH 5.1 = 10/1/1) at appropriate concentrations. MTNG solution (0.5%, w/v) was prepared by dissolving 5.0 mg of MTNG in 1.0 ml of DMSO, and was stored at −80 °C. Solvents used were HPLC grade.

2.3. MTNG derivatization

A 200 μl aliquot of a test solution in a test tube was spiked with 100 μl of NO_2Gua standard diluted with digestion buffer and 10 μl of 2.5 M HCl and 40 μl of MTNG dissolved in DMSO. After mixing, the solution was allowed to stand at room temperature for 1.5 h. A 100 μl portion of the final reaction mixture was used for CS-LC–MS.

2.4. DNA digestion and derivatization methods

In order to prevent the formation of oxidative by-product during DNA isolation, DNA was digested by slight modifying the method of Nakae et al. [6]. Treated calf thymus DNA was adjusted to 1.5 ml by adding 2-propanol and immediately centrifuged at 15,000 × *g* for 10 min at 4 °C. After washing with ethanol, the pellet was dissolved in 0.2 ml of 20 mM sodium acetate buffer, pH 4.8. DNA was enzymatically hydrolyzed by adding 4.0 μl Nuclease P1 to obtain a concentration of 40 units/ml. The mixture was incubated at 70 °C for 15 min. After addition of 20 μl of 1.0 M Tris–HCl buffer (pH 8.0), 4.0 μl of alkaline phosphatase was added to give a final concentration of 20 units/ml. The mixture was incubated at 37 °C for 1.0 h. Then, after addition of 20 μl of 3.0 M sodium acetate buffer (pH 5.1), the mixture was passed through 100,000 NMWL filter (Millipore, Bedford, MA). Then, a 50 μl portion of the digested solution was injected into the LC–UV–ECD for 8-OHdG and dG analysis. Forty microliters of MTNG solution and 10 μl of 2.5 M HCl were added to a 100 μl portion of the remaining digested solution and the mixture was allowed to stand at room temperature for 1.5 h. One hundred microliters aliquot of the derivatized sample solution was injected into the CS-LC–MS for NO_2Gua-MTNG analysis.

2.5. Instrumentation

In the NO_2Gua assay, the LC–MS system was an Agilent LC-MSD Superior Line (Agilent Technologies, Palo Alto, USA) equipped with an electrospray ionization (ESI) source. An Agilent pump was used to induce flow to elute the sample from the extraction column. A Shimadzu LC-10AS Pump (Shimadzu,

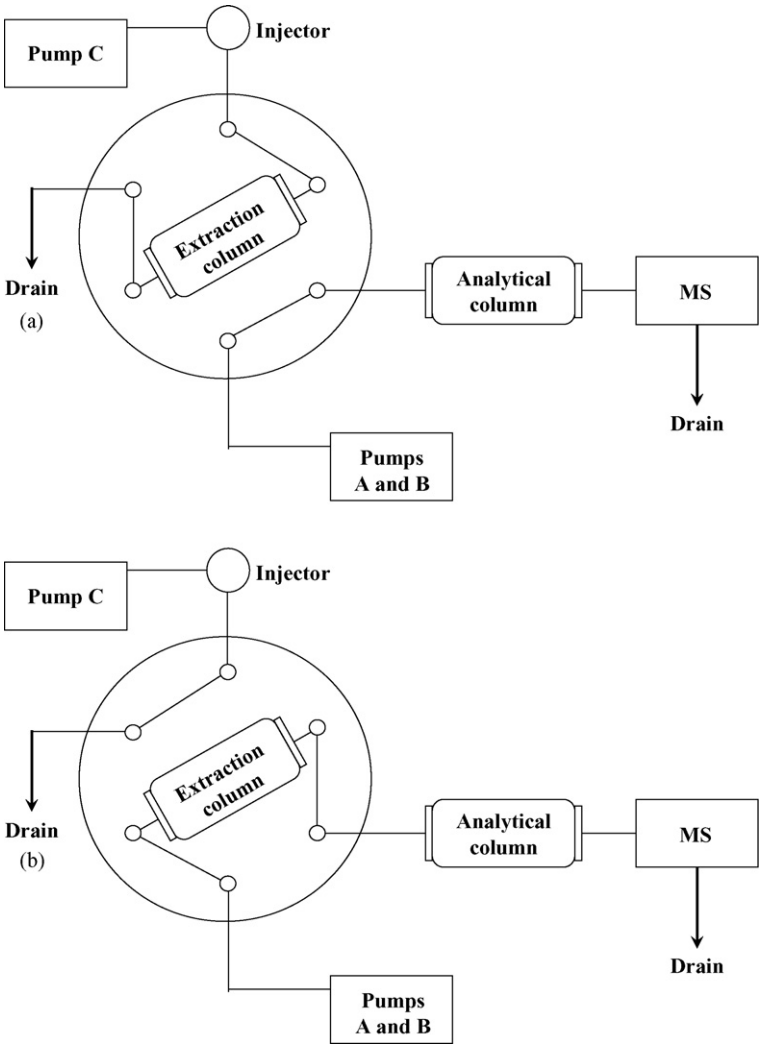

Fig. 1. Schematic representation of the CS-LC–ESI–MS system.

Kyoto, Japan) was used to induce flow through the extraction column to load and wash the sample and to equilibrate the extraction column. A Capcell Pak C18 MG II analytical column (2.0 mm × 150 mm, 5.0 μm, Shiseido Co., Ltd., Kyoto, Japan) was used for separation. An Inertsil Ph-3 column (4.6 mm × 50.0 mm, 5.0 μm, GL Sciences Inc., Tokyo, Japan) was used for cleanup and concentration as an extraction column.

In the dG and 8-OHdG assay, the UV detector and ECD used were a GILSON 234 (Middleton, WI) and an ESA Coulochem II (Bedford, MA). A Gynkotek 480 pump (Germering, Germany) was used to induce flow through the analytical column to load and elute the sample and to equilibrate the analytical column. An Ultrasphere ODS (4.6 mm × 150.0 mm, 5.0 μm, Beckman coulter Inc., CA, USA) was used for separation.

2.6. CS-LC–MS conditions for NO_2Gua analysis

The column-switching system depicted in Fig. 1 (configuration (a)) was used for the injection of liquid sample. After 100 μl of sample was injected with an auto sampler, it was loaded onto the extraction column by flowing acetonitrile–0.1% ammonium acetate (1.5/8.5, v/v) at a flow rate of 0.5 ml/min using pump C for 5 min. While the extraction column was directed to waste during the 5 min run, the sample was extracted and purified on the online extraction column. The impurities were removed and NO_2Gua was retained on the extraction column. After the online extraction for 5 min, the position of the switching valve was changed (see Fig. 1b). This configuration connected the back-flashing extraction column to the analytical column and the MS detector in the flow path of pumps A and B. The column oven was maintained at 40 °C for LC separation. The separation was carried out using a gradient mobile phase of 0.005% ammonium acetate in water–acetonitrile (v/v) at a flow rate of 0.2 ml/min. The gradient program is shown in Table 1. The effluent from the analytical column was directed to the ESI–MS. After elution for 40 min, the switching valve was returned to its original position (see Fig. 1b). The total run time was 50 min. The working conditions for ESI–MS were as follow: drying nitrogen gas was maintained at 350 °C and introduced into the capillary at a flow rate of 12 l/min; the capillary was held at a potential of 3500 V relative to the counter electrode in the positive and negative ion modes. The fragmentor voltage was 150 V for NO_2Gua during the chromatographic run. When working in the selected-ion monitoring (SIM) mode, *m/z* 391 and 394

Table 1
Time program for CS-LC–ESI–MS

Time (min)	Procedure	Column position	Mobile phase ammonium acetate in water:acetonitrile (v/v)
0.00	Sample injection	Configuration a	80:20
5.00	Column switching	Configuration b	80:20
15.00	Start analysis		
25.00			65:35
25.01			30:70
30.00	Stop analysis		
40.00		Configuration a	30:70
40.01			80:20
50.00	Next analysis		80:20

ions were assigned to $[M-H]^-$ of NO_2Gua-MTNG and [^{13}C, $^{15}N_2$]-NO_2Gua-MTNG in the negative ion mode, respectively.

2.7. HPLC–UV–ECD conditions for dG and 8-OHdG analysis

8-OHdG and dG were determined according to the method of Nakae et al. [6]. Briefly, an aliquot (50 μl) of the sample was injected into a ODS column maintained at 40 °C. The column was equilibrated with a mixture of 10 mM sodium phosphate/methanol (96/4, v/v). The compounds were eluted with an isocratically at a flow rate of 1.0 ml/min. The wavelength of the UV detector was set at 290 nm for the detection of dG. A Coulochem II ECD was used with a guard cell (Model 5020; +350 mV), an analytical cell (Model 5011; electrode 1, 150 mV; electrode 2, 300 mV).

2.8. Recovery

Recovery was evaluated by calculating the mean of the response at each concentration. The spiked concentrations of NO_2Gua were determined from the concentrations of NO_2Gua in calf thymus DNA, which were determined by CS-LC–ESI–MS. NO_2Gua standard was spiked into calf thymus DNA at 25, 50 and 250 nM.

2.9. NO_2Gua and 8-OHdG formation in DNA by the addition of $ONOO^-$

Two hundred and fifty micrograms of commercially available calf thymus DNA was dissolved in 1.0 mM DTPA containing 100 mM sodium phosphate buffer (pH 7.4). $ONOO^-$ at several concentrations was added to the DNA solutions and incubation was carried out at 37 °C after mixing for 1.0 h. After DNA digestion, a 50 μl portion of the digestion sample was subjected to HPLC–UV–ECD to determine 8-OHdG and dG levels. A 100 μl portion of the digestion sample was subjected to CS-LC–ESI–MS after MTNG derivatization for NO_2Gua analysis. The amount of NO_2Gua and 8-OHdG was calculated as NO_2Gua/10^5dG and 8-OHdG/10^5dG, respectively.

3. Results and discussion

3.1. Optimal condition for glyoxal derivatization

The buffer solutions at the high concentration used in the digestion process of DNA causes ion suppression in MS analysis along with the direct damage to MS detector. Therefore, retention of NO_2Gua on the column is requisite in order to separate from the buffer solution. First, we investigated commercially available reagents G, PG, HPG and MTNG for NO_2Gua pre-column derivatization. The derivatization by MTNG, but not G, PG or HPG, was able to improve the polarity (Fig. 2a). Then, the derivatization conditions for NO_2Gua by MTNG were investigated. It has been reported that the conditions for derivatization with glyoxal reagents for guanine compounds (guanine, 8-OHdG and ganciclovir) were

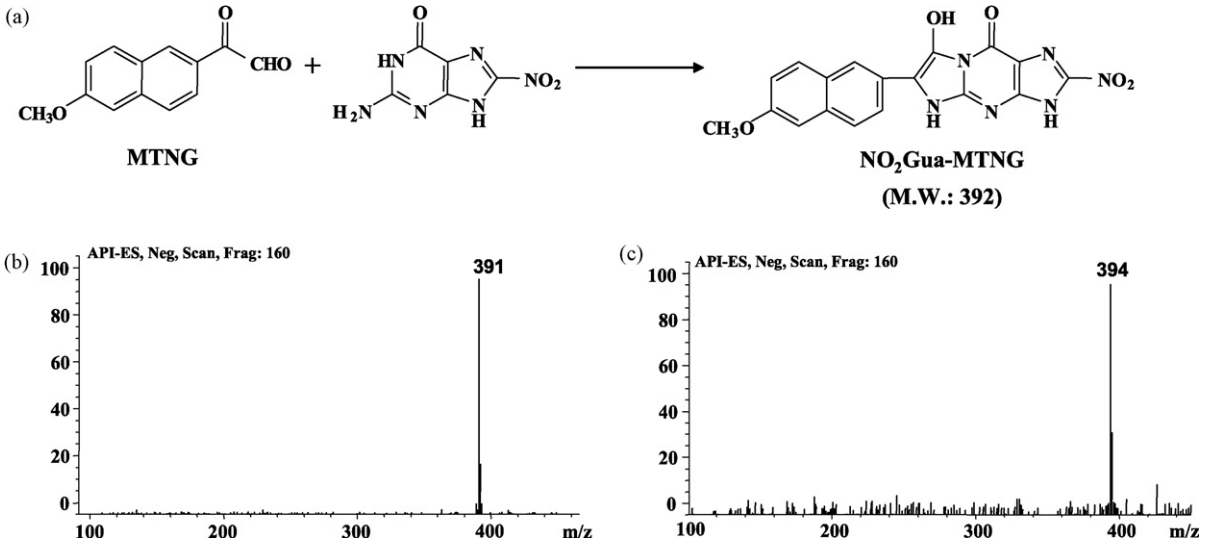

Fig. 2. (a) The reaction of NO_2Gua with the MTNG. MS spectra of NO_2Gua-MTNG (b) and [^{13}C, $^{15}N_2$]-NO_2Gua-MTNG (c).

phosphate buffer (pH 6.0–7.0)–DMSO [26,27], acetate buffer (pH 4.0 and 5.0)–DMSO [28] and acetic acid [29]. However, alternative optimal conditions were required to apply high-concentration acetate buffer widely used in the DNA digestion for 8-OHdG measurement. According to Nakae et al. [6], DNA digestion buffer consists of 20 mM sodium acetate buffer (pH 4.8), 1.0 M Tris–HCl buffer (pH 8.1) and 3.0 M sodium acetate buffer (pH 5.1). Based on each concentration used in the digestion process, for the optimization test we made the digestion buffer at the following rate; 20 mM sodium acetate buffer (pH 4.8)/1.0 M Tris–HCl buffer (pH 8.1)/3.0 M sodium acetate buffer (pH 5.1) = 10/1/1 (v/v/v). As a result, the addition of 10 μl of 2.5 M HCl and 40 μl of MTNG solution in DMSO to 100 μl of NO_2Gua in the digestion buffer gave the largest peak area after 1.5 h at room temperature. As MTNG derivatives were stable, the peak area (>95%) was sustained for at least 10 h in daylight at temperatures below room temperature (25 °C). In contrast, depreciation of reaction efficiency in the derivatization was observed at 40 °C. The optimal conditions for MTNG derivatives were confirmed in the DNA digestion buffer for 8-OHdG measurement.

3.2. Optimal conditions for LC–ESI–MS

In the ESI–MS analysis of standard solutions in the negative ion mode ($[M-H]^-$), NO_2Gua-MTNG and [^{13}C, $^{15}N_2$]-NO_2Gua-MTNG exhibited peaks at *m/z* 391 and 394, respectively (Fig. 2b and c). Then, the most important parameters affecting LC–ESI–MS, namely, fragmentor voltage and mobile phase, were investigated. In order to establish the optimum fragmentor voltage for the detection of NO_2Gua-MTNG, the signal of *m/z* 391 ion versus fragmentor voltage was investigated. The optimal fragmentor voltage was 150 V in the negative ion mode for NO_2Gua-MTNG. Moreover, as the ionization of samples at the LC–ESI–MS interface is affected by the mobile phase, a mobile phase containing a volatile acid or a salt is used frequently. In this case, the responses were measured using 0–0.1% ammonium acetate in water–acetonitrile (v/v) as the mobile phase. The responses of NO_2Gua-MTNG were increased by the addition of 0.005% ammonium acetate to the mobile phase. Optimization of LC–ESI–MS conditions gave a maximum response in 0.005% ammonium acetate at 150 V for NO_2Gua-MTNG in the negative ion mode. The gradient conditions and the CS time program are shown in Table 1.

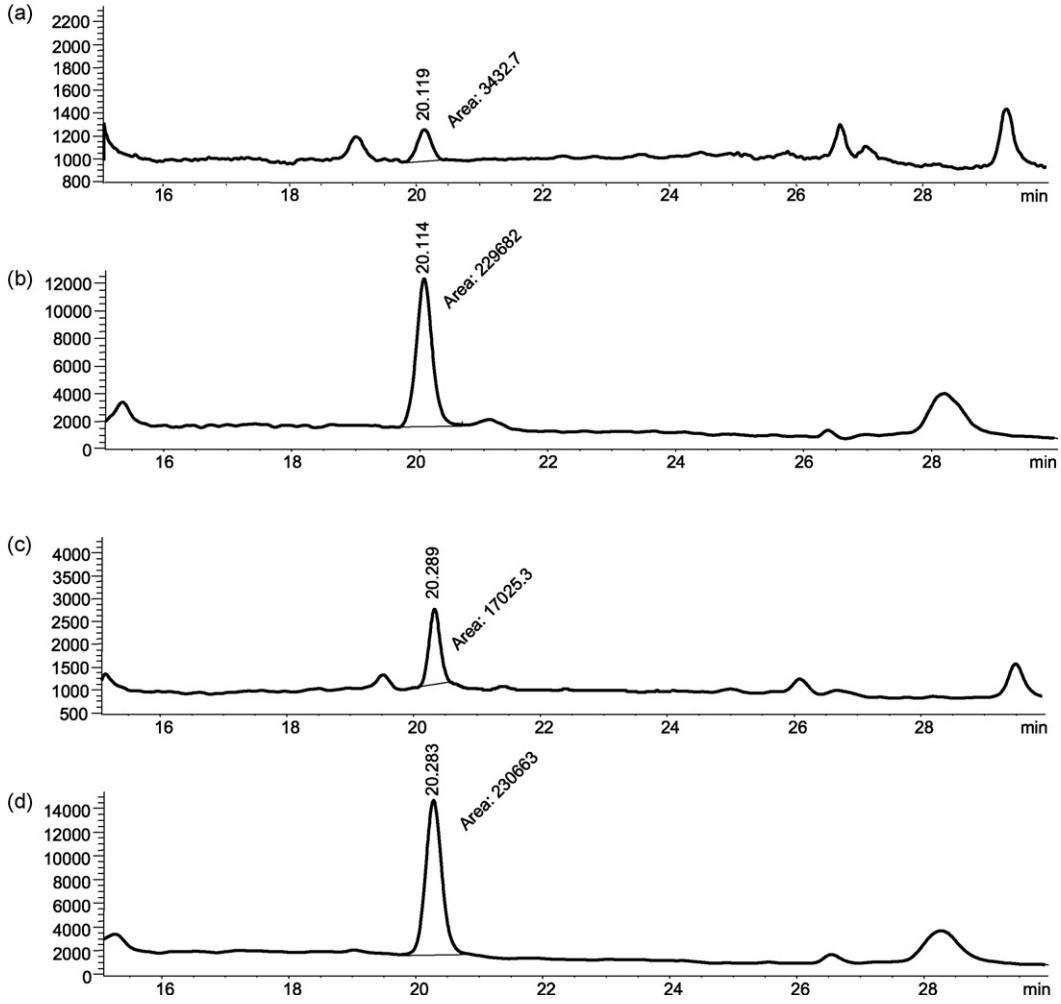

Fig. 3. CS-LC–ESI–MS chromatograms of NO_2Gua and surrogate standard in the DNA sample. (a) DNA sample (250 μg/ml) was spiked with 5.0 nM NO_2Gua; (b) surrogate standard; (c) DNA sample (250 μg/ml) was spiked with 25 nM NO_2Gua; (d) surrogate standard. CS-LC–ESI–MS conditions are described in Section 2.

3.3. Validation of MTNG derivatization followed by CS-LC–ESI–MS with SPE

The calculated limit of detection (LOD) of NO_2Gua-MTNG in the standard solutions was 1.0 nM, for CS-LC–ESI–MS detection at the ratio of the compound's signal to the background signal (S/N) of 3. In addition, the limit of quantification (LOQ) calculated when S/N = 10 was 3.0 nM for NO_2Gua-MTNG. The peak area ratio with respect to each surrogate standard was plotted, and the response was found to be linear over the calibration range with a correlation coefficient (r) of 0.999 (3–500 nM). The average retention time of NO_2Gua-MTNG standards was 20.18 min (R.S.D. = 1.19%, $n = 5$). Moreover, the accuracy of the R.S.D.s of the peak area ratios was 0.68%.

The recoveries (value ± R.S.D.%, $n = 5$) were 99.4 ± 0.53, 99.8 ± 0.94 and 99.1 ± 3.76% (25, 50 and 250 nM, respectively) for NO_2Gua-MTNG.

The chromatograms of DNA samples spiked with 5.0 and 25 nM NO_2Gua are shown in Fig. 3. The results indicate that the method enables the precise determination of NO_2Gua, and is applicable to the detection of NO_2Gua in DNA samples.

3.4. NO_2Gua and 8-OHdG formation in DNA by addition of $ONOO^-$

In order to confirm whether our method has the ability to estimate the two types of DNA damages, calf thymus DNA treated with $ONOO^-$ was analyzed. Typical CS-LC–ESI–MS chromatograms for NO_2Gua and LC–UV–ECD chromatograms for dG and 8-OHdG in calf thymus DNA sample are shown in Fig. 4. NO_2Gua and 8-OHdG levels in calf thymus DNA samples treated with $ONOO^-$ at several concentrations are shown in Fig. 5. In the control, no NO_2Gua was detected, whereas 8-OHdG was detected at 10.1 ± 2.15 (8-OHdG/10^5dG). Both

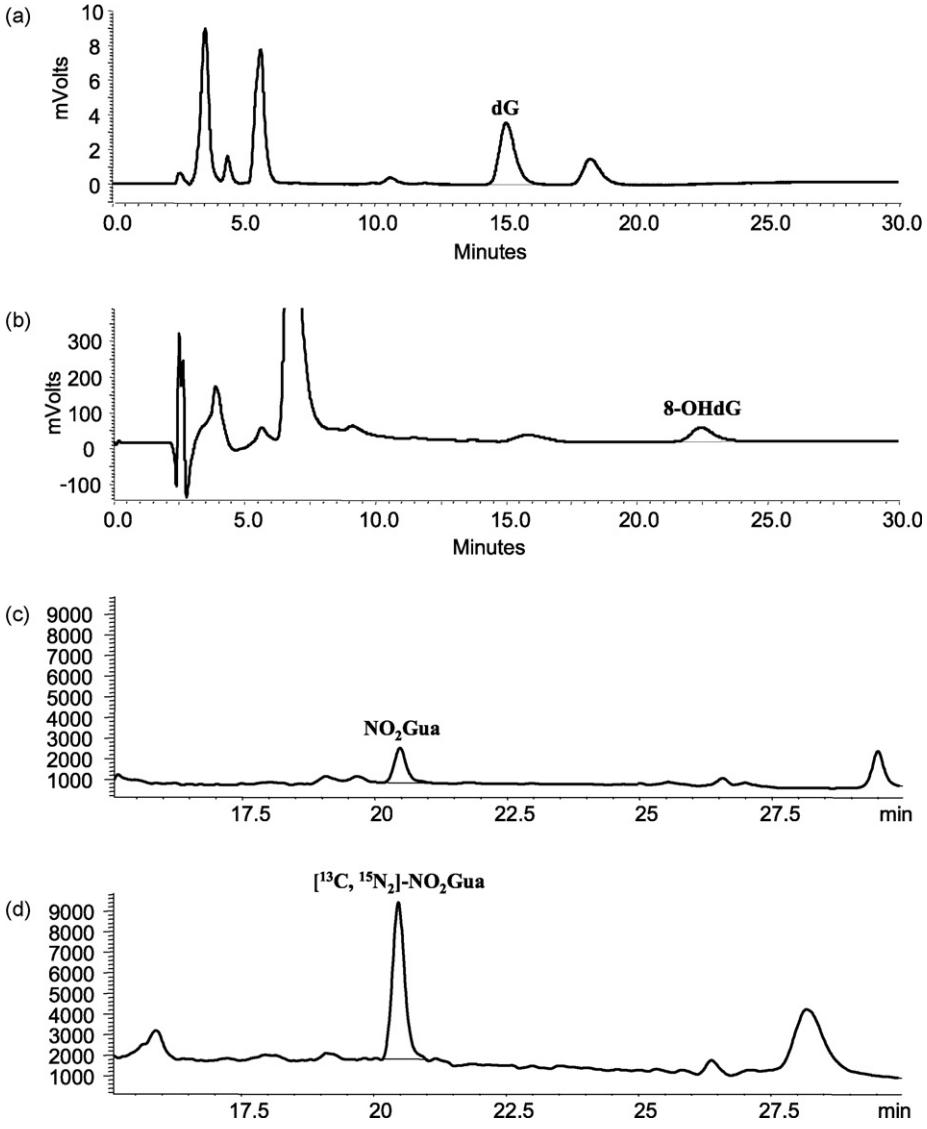

Fig. 4. LC–UV–ECD and CS-LC–ESI–MS chromatograms of calf thymus DNA treated with $ONOO^-$. (a) UV (290 nm) chromatogram of dG; (b) ECD (300 mV) chromatogram of 8-OHdG; (c) CS-LC–ESI–MS (SIM 391) chromatogram of NO_2Gua; (d) CS-LC–ESI–MS (SIM 394) chromatogram of [^{13}C, $^{15}N_2$]-NO_2Gua as a surrogate standard. Calf thymus DNA (250 μg/ml) dissolved in 1.0 mM DTPA containing sodium phosphate buffer (pH 7.4) was incubated with $ONOO^-$ at several concentrations for 60 min at 37 °C. DNA digestion procedure and LC–UV–ECD conditions are described in Section 2.

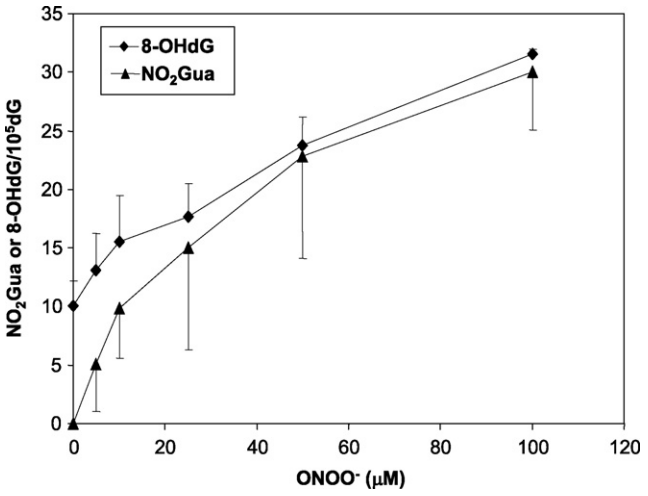

Fig. 5. NO_2Gua and 8-OHdG formation in calf thymus DNA treated with $ONOO^-$. Calf thymus DNA (250 μg/ml) dissolved in 1.0 mM DTPA containing sodium phosphate buffer (pH 7.4) was incubated with $ONOO^-$ at several concentrations for 60 min at 37 °C. After DNA digestion, 50 μl portion of the sample was subjected to HPLC–UV–ECD for 8-OHdG and dG analysis. One hundred microliters of the sample was subjected to CS-LC–ESI–MS after MTNG derivatization for NO_2Gua analysis. The amount of NO_2Gua and 8-OHdG was calculated as $NO_2Gua/10^5dG$ and 8-$OHdG/10^5dG$, respectively. Values are means ± S.D. of three separate experiments.

NO_2Gua and 8-OHdG levels were increased with increasing $ONOO^-$ (5–100 μM) concentration. After the addition of 50 or 100 μM $ONOO^-$, the amount of NO_2Gua (22.9 ± 8.74 or 30.1 ± 5.00 $NO_2Gua/10^5dG$, respectively) was almost the same as that of 8-OHdG (23.7 ± 2.45 or 31.5 ± 0.44 8-$OHdG/10^5dG$). In consideration of the fact that $ONOO^-$ is a frequently found intermediate in the reaction of NO with biomolecules, these results indicate that considerable amounts of NO_2Gua and 8-OHdG might be found under inflammatory conditions. Although these two types of damaged base on nucleotides have been reported to be detected by immunohistochemical analysis in the various infectious inflammatory diseases [29–32], our new method was able to provide precise quantitative data. This advantages involved in the new method were supported by simple pretreatment and the stable isotope as internal standard. In this respect, our newly developed method could be a powerful tool to understand the correlation between inflammation and carcinogenesis.

4. Conclusions

In the present study, we developed the CS-LC–ESI–MS method for the detection of NO_2Gua in which reducing the polarity of NO_2Gua by glyoxal derivatization made the use of the CS system possible, and use of the stable isotope of NO_2Gua gave high measurement precision. Additionally, the common procedures for digesting DNA enabled us to evaluate both DNA nitration and oxidation from one DNA sample. Our new method is expected to help us explore the mechanisms underlying NO-mediated toxicity and carcinogenesis. In order to clarify the functional role of the NO_2Gua *in vivo*, application of our methods for various NO-mediated carcinogenesis models is underway in our laboratory.

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