Toxicology Letters 213 (2012) 309–315

Contents lists available at SciVerse ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

In vivo formation of *N*7-guanine DNA adduct by safrole 2′,3′-oxide in mice

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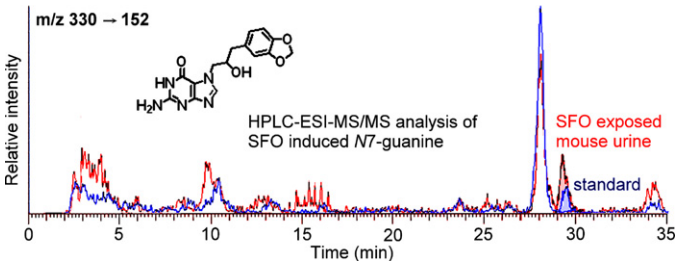
HIGHLIGHTS

- *N*7-(3-benzo[1,3]dioxol-5-yl-2-hydroxypropyl)guanine (*N*7γ-SFO-Gua) was characterized.
- An HPLC–ESI-MS/MS method was first developed to measure *N*7γ-SFO-Gua.
- *N*7γ-SFO-Gua was detected in urine of mice treated with safrole 2′,3′-oxide (SFO).
- This is the first study to suggest the formation of *N*7γ-SFO-Gua in SFO-treated mice.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 1 May 2012
Received in revised form 6 July 2012
Accepted 9 July 2012
Available online 20 July 2012

Keywords:
Safrole 2′,3′-oxide
*N*7-(3-Benzo[1,3]dioxol-5-yl-2-hydroxypropyl)guanine (*N*7γ-SFO-Gua)
High performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS)

ABSTRACT

Safrole, a naturally occurring product derived from spices and herbs, has been shown to be associated with the development of hepatocellular carcinoma in rodents. Safrole 2′,3′-oxide (SFO), an electrophilic metabolite of safrole, was shown to react with DNA bases to form detectable DNA adducts *in vitro*, but not detected *in vivo*. Therefore, the objective of this study was to investigate the formation of *N*7-(3-benzo[1,3]dioxol-5-yl-2-hydroxypropyl)guanine (*N*7γ-SFO-Gua) resulting from the reaction of SFO with the most nucleophilic site of guanine *in vitro* and *in vivo* with a newly developed isotope-dilution high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS) method. *N*7γ-SFO-Gua and [$^{15}N_5$]-*N*7-(3-benzo[1,3]dioxol-5-yl-2-hydroxypropyl)guanine ([$^{15}N_5$]-*N*7γ-SFO-Gua) were first synthesized, purified, and characterized. The HPLC–ESI-MS/MS method was developed to measure *N*7γ-SFO-Gua in calf thymus DNA treated with 60 μmol of SFO for 72 h and in urine samples of mice treated with a single dose of SFO (30 mg/kg body weight, intraperitoneally). In calf thymus DNA, the level of *N*7γ-SFO-Gua was 2670 adducts per 10^6 nucleotides. In urine of SFO-treated mice, the levels of *N*7γ-SFO-Gua were 1.02 ± 0.14 ng/mg creatinine ($n = 4$) on day 1, 0.73 ± 0.68 ng/mg creatinine ($n = 4$) on day 2, and below the limit of quantitation on day 3. These results suggest that SFO can cause *in vivo* formation of *N*7γ-SFO-Gua, which may then be rapidly depurinated from the DNA backbone and excreted through urine.

1. Introduction

Safrole, a phenylpropene found in dietary plants, such as basil, cinnamon, nutmeg, and black pepper (Daimon et al., 1998; Lee et al., 2005), causes hepatocellular carcinoma in rodents (Abbott et al., 1961; Long et al., 1963), and is classified as a hepatocarcinogen by the International Agency for Research on Cancer (IARC, 1976). This phenylpropene is also present in high concentrations (15 mg/g) in

Abbreviations: SFO, safrole 2′,3′-oxide; *N*7γ-SFO-Gua, *N*7-(3-benzo[1,3]dioxol-5-yl-2-hydroxypropyl)guanine; [$^{15}N_5$]-*N*7γ-SFO-Gua, [$^{15}N_5$]-*N*7-(3-benzo[1,3]dioxol- 5-yl-2-hydroxypropyl)guanine.

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http://dx.doi.org/10.1016/j.toxlet.2012.07.006

Piper betel inflorescence, a common ingredient along with areca nut and slaked lime paste in betel quid formulations (Chang et al., 2002; Lee et al., 2005). Studies have shown that chewing of *Piper betel* inflorescence-containing quids is highly associated with increased risk of oral squamous cell carcinoma (Chen et al., 1999), oral submucous fibrosis (Chen et al., 1999), and esophageal cancer (Lee et al., 2005). However, the mechanisms associated with safrole carcinogenicity have not been well studied.

Upon uptake, safrole is metabolized by cytochrome P450 to 1,2-dihydroxy-4-allylbenzene, 3′-hydroxysafrole (Benedetti et al., 1977), 1′-hydroxysafrole (Borchert et al., 1973; Wislocki et al., 1976), and safrole 2′,3′-oxide (SFO) (Luo and Guenthner, 1996). Of the four metabolites, 1′-hydroxysafrole is further metabolized by sulfotransferase to 1′-hydroxysafrole sulfate (Boberg et al., 1983), which subsequently reacts with 2′-deoxyguanosine to form DNA adducts, namely N^2-(*trans*-isosafrol-3′-yl)-2′-deoxyguanosine, N^2-(safrole-1′-yl)-2′-deoxyguanosine, C8-(*trans*-isosafrole-3′-yl)-2′-deoxyguanosine, and *N*7-(*trans*-isosafrol-3′-yl)guanine (Wiseman et al., 1985). ^{32}P-postlabelling analysis revealed that N^2-(*trans*-isosafrol-3′-yl)-2′-deoxyguanosine and N^2-(safrole-1′-yl)-2′-deoxyguanosine were present in DNA in oral cancer tissue (Lee et al., 2005) and that N^2-(*trans*-isosafrol-3′-yl)-2′-deoxyguanosine was present in DNA of hepatocellular carcinoma tissue from a patient with a history of chewing areca quid (Chung et al., 2008).

SFO has been demonstrated to cause moderate mutagenicity in *Salmonella typhimurium* strains TA1535 and TA100 (Wislocki et al., 1977; Swanson et al., 1979). In addition, SFO was shown to cause the formation of various tumors in female CD-1 mice (Miller et al., 1983). Our recent study found that SFO resulted in a significant increase in the frequency of DNA strand breaks and micronucleus formation in HepG2 cells and in mice (Chiang et al., 2011). Qato et al. reported that SFO-DNA adducts identified from incubation of calf thymus DNA with SFO were not detectable in liver tissue of Balb/C mice at 24 h after intraperitoneal treatment with SFO (106.9 mg/kg body wt) or safrole (97.3 mg/kg body wt) using a ^{32}P-postlabelling method (Qato and Guenthner, 1995). A possible reason might be because SFO is rapidly detoxified by glutathione *S*-transferases (GSTs) and epoxide hydrolases (EHs) (Luo et al., 1992; Luo and Guenthner, 1994, 1995). Studies have shown that a series of epoxides, such as ethylene oxide, propylene oxide, and styrene oxide can cause the *in vivo* formation of DNA adducts (Koskinen and Plna, 2000; Boysen et al., 2009; Wu et al., 2011), and that these epoxides are rapidly detoxified through the same mechanisms as SFO (Krause et al., 1997; Faller et al., 2001; Csanady et al., 2003). Epoxides are likely to attack the most nucleophilic *N*7-position of guanine through the S_N2 reactions to form *N*7-guanine adducts, which could be enzymatically and spontaneously depurinated from the DNA backbone (Koskinen and Plna, 2000; Boysen et al., 2009). These depurinated DNA adducts can be excreted through urine; therefore, analysis of urinary *N*7-guanine adducts may serve as biomarkers to confirm exposures to their corresponding genotoxic chemicals (Groopman et al., 1993; Vodicka et al., 2006; Huang et al., 2011).

The objective of this study was to study the *in vitro* and *in vivo* formation of the *N*7-guanine adduct induced by SFO with our newly developed isotope-dilution liquid chromatography electrospray ionization tandem mass (HPLC–ESI-MS/MS) method. This adduct was synthesized, purified, and characterized as *N*7-(3-benzo[1,3]dioxol-5-yl-2-hydroxypropyl)guanine (*N*7γ-SFO-Gua). An HPLC–ESI-MS/MS was developed to measure the levels of the *N*7-guanine adduct in calf thymus DNA and in urine of mice treated with SFO. This adduct was detected in all urine samples of SFO-treated mice. This is the first study to demonstrate the *in vivo* formation of *N*7γ-SFO-Gua in SFO-treated mice.

2. Materials and methods

2.1. Chemicals and reagents

2′-Deoxyguanosine and calf thymus DNA were purchased from Sigma–Aldrich Company Ltd (St. Louis, MO). [$^{15}N_5$]-2′-deoxyguanosine was purchased from Medical Isotope Inc. (Pelham, NH). Safrole 2′,3′-oxide (SFO) was prepared as our previously published work (Shen et al., 2012). HPLC-grade acetonitrile was purchased from Mallinkrodt Baker Inc (Kentucky, USA). Ammonium formate was obtained from Fluka Biochemika (Steinheim, Germany). Formic acid was purchased from Riedel-de Haën (Seelze, Germany). Water was purified using a Milli-RO/Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Synthesis, purification, and characterization of *N*7γ-SFO-Gua and [$^{15}N_5$]-*N*7γ-SFO-Gua

SFO (42.7 mg, 0.24 mmol) was reacted with 2′-deoxyguanosine (31.5 mg, 0.12 mmol) at a 2:1 molar ratio in 0.2 N K_2HPO_4 (pH 7.4) and incubated at 37 °C for 72 h. *N*7γ-SFO-Gua was isolated from the reaction mixtures using a Prodigy ODS (3) column (4.6 mm × 250 mm, 5 μm) and a Hitachi L-7000 pump equipped with a Hitachi L-7450A diode array detector. The mobile phase consisted of 50 mM ammonium formate, pH 5.5 (A) and acetonitrile (B). A gradient of 0–20% B from 0% to 20 min, 20% B from 20 min to 30 min, and 20–80% B from 30 min to 45 min was employed. The fraction with a peak retention time (t_R) of 26.8 min was collected. The isolated compound was further purified and desalted by changing eluent A to deionized water with a linear gradient from 0% B to 50% B over 30 min. The fraction with a peak retention time (t_R) of 20.5 min was collected and dried under vacuum. A white solid (6.8 mg, 0.02 mmol) with 16.7% yield was obtained.

The high-resolution mass spectrum was recorded by electrospray ionization-quadrupole ion trap/time-of-flight mass spectrometry (IT-TOF, Shimadzu, Kyoto, Japan) operated under positive ion mode. The purified product was dissolved in a 1:1 (v/v) mixture of 0.1% formic acid/acetonitrile and introduced to the ion source with a syringe pump at 5 μL/min. The spectrum revealed the following mass-electron ratios: *m/z* 330 ([M+H]$^+$), 152 ([M−$C_{10}O_3H_{11}$+H]$^+$). HRMS (ESI$^+$): *m/z* calcd. for $C_{20}H_{24}N_5O_6$ [M+H]$^+$ 330.1202; found 330.1193. UV–vis spectra of the adduct at pH 1, pH 7, and pH 13 were recorded on a HP-8453 spectrophotometer with diode array detection. The maximum absorbance (λ_{max}) was 279 nm at pH 1, 286 nm at pH 7, and 283 nm at pH 13.

^{1}H NMR spectra were recorded on either a Bruker DRX-300 or Varian-Unity INOVA-500 MHz spectrometer. NMR spectra of naturally occurring ^{13}C were obtained using pulse Fourier transform techniques with a 300 or 500 MHz NMR spectrometer operating at 75.4 or 125.7 MHz, respectively. Broadband decoupling, H, H-COSY (Correlated Spectroscopy), HMQC (Heteronuclear Multiple Quantum Coherence), and HMBC (Heteronuclear Multiple Bond Coherence) were carried out to simplify spectra and to aid peak identification. Samples were dissolved in d_6-DMSO for NMR analysis. The alkylation positions of DNA adducts were mainly determined by long-range H,C correlations of HMBC spectra. The ^{1}H NMR (500 MHz, in d_6-DMSO) spectrum showed δ = 2.64 (dd, 1H, α′-H, J_1 = 2.2 Hz, J_2 = 2.7 Hz, J_3 = −13.8 Hz), 2.52 (m, 1H, α″-H overlap with solvent), 3.97–4.01 (m, 2H, β-H, γ′-H), 4.24 (dd, 1H, γ″-H, J_1 = 7.4 Hz, J_2 = −17.6 Hz), 5.94 (s, 2H, g-H), 6.27 (s, 2H, NH_2), 6.65 (d, 1H, e-H, J = 8.0 Hz), 6.78 (s, 1H, d-H), 6.79 (s, 1H, a-H), and 7.79 (s, 1H, 8-H) ppm. The ^{13}C NMR (125.7 MHz, in d_6-DMSO) spectrum showed: δ = 40.4 (C-α), 52.0 (C-γ), 70.2 (C-β), 100.6 (C-g), 107.9 (C-a), 108.2 (C-5), 109.7 (C-d), 122.2 (C-e), 132.4 (C-f), 143.8 (C-8), 145.4 (C-c), 146.9 (C-b), 152.8 (C-2), 154.8 (C-6), 159.8 (C-4) ppm. After the NMR measurements, *N*7γ-SFO-Gua was recovered from the NMR tube and subjected to HPLC purification with desalting condition as mentioned above to eliminate d_6-DMSO. The recovered *N*7γ-SFO-Gua (6.37 mg) was dissolved into 2.12 mL of H_2O (3000 ppm) as a stock solution.

To synthesize [$^{15}N_5$]-labeled *N*7γ-SFO-Gua to serve as an internal standard, [$^{15}N_5$]-2′-deoxyguanosine (5 mg) was dissolved into H_2O (1 mL) to serve as the stock solution. SFO (14 μmol) was added to [$^{15}N_5$]-2′-deoxyguanosine (400 μL, 7 μmol) in 0.2 N K_2HPO_4 solution (600 μL) and reacted at 37 °C for 72 h. The reaction mixture was subjected to HPLC separation as mentioned above. The eluent was collected at retention time from 26.0 min to 27.5 min and concentrated to serve as a stock solution without further purification. *N*7γ-SFO-Gua was used as a standard to quantify the [$^{15}N_5$]-*N*7γ-SFO-Gua stock solution with HPLC–ESI-MS/MS, assuming a negligible ^{15}N isotope effect.

2.3. Development of an HPLC–ESI-MS/MS method for analysis of *N*7γ-SFO-Gua

A reversed-phase HPLC system with an autosampler (ACCELA, Thermo Scientific, MA) was used for quantification. Sample (20 μL) was injected into a C_{18} column (150 mm × 2.1 mm, 3 μm, HyPURITY® C_{18}; Thermo Hypersil-Keystone). The mobile phase consisted of 10 mM ammonium formate buffer pH 5.1 (solution A) and 0.1% formic acid in acetonitrile (solution B) and was delivered at 200 μL/min. For analysis of calf thymus DNA, a gradient of 0–42% B from 0 min to 25 min, 42–100% B from 25 min to 28 min, and 100–0% B from 28 min to 32 min was employed. For the analysis of mouse urine, a gradient of 0% B from 0 min to 3 min, 0–18% B from 3 min to 28 min, 18–100% B from 28 min to 31 min, 100–0% B from 31 min to 32 min, and 0% B from 32 min to 35 min was applied. A triple-quadrupole tandem mass spectrometer

(TSQ Quantum ACCESS, Thermo Scientific, MA) comprising a heated-electrospray ionization (H-ESI) source was operated in the positive ion mode. Quantification of *N*7γ-SFO-Gua was carried out by monitoring the ion pairs *m/z* 330 → 152 and [$^{15}N_5$]-*N*7γ-SFO-Gua was quantified by monitoring the ion pairs *m/z* 335 → 157 in selected reaction monitoring (SRM) mode. The vaporizer temperature was set at 300 °C, nitrogen sheath gas was set at 35, auxiliary gas was set at 15, and the heated capillary temperature was set at 300 °C. A collision energy of 23 eV was applied. The argon gas pressure was set at 0.2 Pa for collision-induced dissociation (CID), and the discharge current was set at 5 μA. Total ion chromatograms and mass spectra were recorded on a personal computer with Xcalibur software (Version 2.0.7, Thermo Fisher Scientific Inc., MA).

2.4. Analysis of *N*7γ-SFO-Gua adduct in calf thymus DNA

Calf thymus DNA (10 mg) was dissolved in 10 mL of Tris–HCl buffer (pH 7.5–8.5) containing 1 mM EDTA and reacted with SFO (60 μmol) at 37 °C for 72 h. The reaction mixture was extracted with Et_2O (10 mL × 3) to remove unreacted SFO. The solution was then kept on ice for a few hours to evaporate the Et_2O and processed using two methods. Method 1: A 400 μL-aliquot of solution, spiked with [$^{15}N_5$]-*N*7γ-SFO-Gua (4 ng), filtered through a 0.22 μm PVDF syringe filter (Receanttec, Tokyo, Japan) to remove the DNA backbone, was analyzed with our newly developed isotope-dilution HPLC–ESI-MS/MS method. Method 2: Another 400 μL of the solution was spiked with [$^{15}N_5$]-*N*7γ-SFO-Gua (4 ng) and subjected to neutral thermal hydrolysis (70 °C for 1 h) to release *N*7γ-SFO-Gua from the DNA backbone. After removal of the DNA backbone with a 0.22 μm PVDF syringe filter, the resulting solution was analyzed with the HPLC–ESI-MS/MS method. Non-treated calf thymus DNA was processed according to the protocol in method 1 and served as the control. The calibration curve of *N*7γ-SFO-Gua (5 ng/mL, 10 ng/mL, 25 ng/mL, 50 ng/mL, and 100 ng/mL) prepared in H_2O spiked with [$^{15}N_5$]-*N*7γ-SFO-Gua (4 ng) was established for quantitative analysis with the HPLC–ESI-MS/MS method.

2.5. Animal experiments

The animal experiment was approved by and conducted in accordance with the China Medical University Animal Ethics Committee guidelines on animal care. Sixteen male FVB mice aged 6–7 weeks and weighing 20–25 g were purchased from the National Laboratory Animal Center (Taipei, Taiwan). Animals were acclimatized for seven days prior to SFO treatment. SFO was dissolved in olive oil and the final concentration was 9 mg/mL of SFO. Mice were divided into control (5 mice) and exposed groups (four groups with a total of 11 mice). For the exposed group, mice were intraperitoneally injected with 30 mg/kg body wt of SFO in olive oil and then housed in 4 metabolic cages. There were 2, 3, 3, and 3 mice in each group. For the control group, mice were intraperitoneally injected with the same volume of olive oil as the exposed group. Urine samples were collected at 24 h, 48 h, and 72 h after treatment. During urine collection, the mice were housed in individual stainless steel metabolic cages. The collected urine samples were mixed with 10 μL of sodium azide (final concentration 0.05%) and centrifuged at 3000 rpm for 10 min at room temperature. The supernatant was collected and stored at −80 °C until used for analysis.

2.6. Analysis of *N*7γ-SFO-Gua in urines of mouse treated with SFO

Urine samples (200 μL) of mice were spiked with 17 μL of [$^{15}N_5$]-*N*7γ-SFO-Gua (13 pg/μL) with a final concentration of 1.1 ng/mL. These samples were filtered through a 0.22 μm PVDF membrane filter, and then analyzed by using the newly developed isotope-dilution HPLC–ESI-MS/MS method. Creatinine in each urine sample was analyzed at a local hospital (Taipei, Taiwan). The concentration of *N*7γ-SFO-Gua in urine was adjusted to the level of creatinine and expressed as ng/mg creatinine.

*N*7γ-SFO-Gua standard solutions (0.25 ng/mL, 0.5 ng/mL, 1 ng/mL, 1.5 ng/mL, 2 ng/mL, 4 ng/mL, and 5 ng/mL) were prepared in H_2O (set 1) and in urine from control mice (set 2). Both sets were spiked with 17 μL of [$^{15}N_5$]-*N*7γ-SFO-Gua (13 pg/μL) with a final concentration of 1.1 ng/mL, follow by filtration through a 0.22 μm PVDF membrane filter. Linear calibration curve were constructed by plotting the ratios of the peak areas for *N*7γ-SFO-Gua and [$^{15}N_5$]-*N*7γ-SFO-Gua *versus* the concentrations of the standards. The matrix effect was evaluated by comparing the slope of set 2 with that of set 1 (Matuszewski et al., 2003). The precision was evaluated by repeatedly analyzing of the second set of calibration standard solutions 5 times on three different days.

3. Results

3.1. Synthesis, purification, and characterization of *N*7γ-SFO-Gua

The reaction mixtures of SFO with 2′-deoxyguanosine were separated with HPLC (Fig. 1) and the fraction at retention time at 26.8 min was collected and characterized by UV, mass spectrometry, and NMR analyses. The product ion-scan spectrum showed

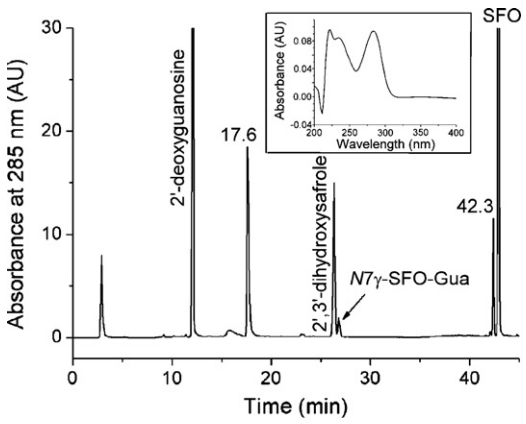

Fig. 1. A representative HPLC chromatogram for purification of *N*7γ-SFO-Gua from the product of 2′-deoxyguanosine reacted with SFO for 72 h at 37 °C. *N*7γ-SFO-Gua is shown to be the peak at retention time 26.8 min.

that fragmentation of this adduct produced a major fragment ion with *m/z* = 152, which corresponds to the loss of the 2-hydroxy-(3-benzo[1,3]dioxol-5-yl)-propane moiety from the protonated molecular ion (*m/z* = 330) (Fig. 2a). The UV spectrum of the pure adduct showed a lambda absorption maximum (λ_{max}) at 285 nm (pH 7), which corresponds to a *N*7-guanine adduct (Tretyakova et al., 1997).

In the ^{1}H NMR spectrum of *N*7γ-SFO-Gua, the signals at 6.27 and 7.79 ppm correspond to NH_2 and H-8 which are in agreement with those previously reported for 2-amino-7-(2-hydroxy-2-phenylethyl)-purin-6-one (a *N*7-guanine adduct of styrene oxide) (Novák et al., 2004). Besides, the absence of the proton signals corresponding to the deoxyribose portion of 2′-deoxyguanosine used as starting material indicates that the formation of a depurinated adduct. In addition, the cross peak of 8-H in the HMQC (Fig. S1, Supporting information) indicates that the methine carbon at 148.3 ppm is C-8 (see DEPT in Fig. S2, Supporting information). In the HMBC spectrum (Fig. 3), the proton 8-H couples to two quaternary carbons at 159.8 and 108.2 ppm. The signals of 159.8 and 108.2 ppm could be assigned to carbons that are next to two nitrogen atoms and a nitrogen atom, respectively, and are assigned to C-4 and C-5. The assignments of α′-H (2.64 ppm) and α″-H (2.52–2.55 ppm) to the 2-hydroxy-(3-benzo[1,3]dioxol-5-yl)-propane moiety were based on the correlation of C-f (132.4 ppm, $^2J_{CH}$) with C-a (107.9 ppm, $^3J_{CH}$) and C-e (122.2 ppm, $^3J_{CH}$) in the HMBC spectrum. According to the correlation of α-H in the COSY spectrum (Fig. S3, Supporting information), the proton signal at 3.97–4.01 ppm, with an integration value of 2 in ^{1}H NMR spectroscopy, could be assigned as β-H. Therefore, the other proton signals at 3.97–4.01 and 4.24 ppm correspond with β-H in the COSY spectrum, indicating that they belong to γ′-H and γ″-H. Moreover, the secondary carbon (52.0 ppm) correlated with γ′-H and γ″-H in the HMQC spectrum, and was, therefore, assigned to C-γ. Thus, the separated γ′-H and γ″-H correlated with both C-5 ($^3J_{CH}$) and C-8 ($^3J_{CH}$), suggesting that this compound results from the reaction of *N*7-guanine on the γ position of SFO, namely, *N*7γ-SFO-Gua. The yield of *N*7γ-SFO-Gua from the reaction of SFO with 2′-deoxyguanosine was estimated to be 16.7% after 72 h.

3.2. Analysis of *N*7γ-SFO-Gua adduct in calf thymus DNA

In order to study spontaneous depurination of the *N*7γ-SFO-Gua from DNA backbone, after being reacted with SFO, calf thymus DNA was processed with two methods (Fig. S4). *N*7γ-SFO-Gua adduct in the reaction solution, was measured at 1400 *N*7γ-SFO-Gua adduct per 10^6 nucleotides at 72 h. After the neutral thermal hydrolysis,

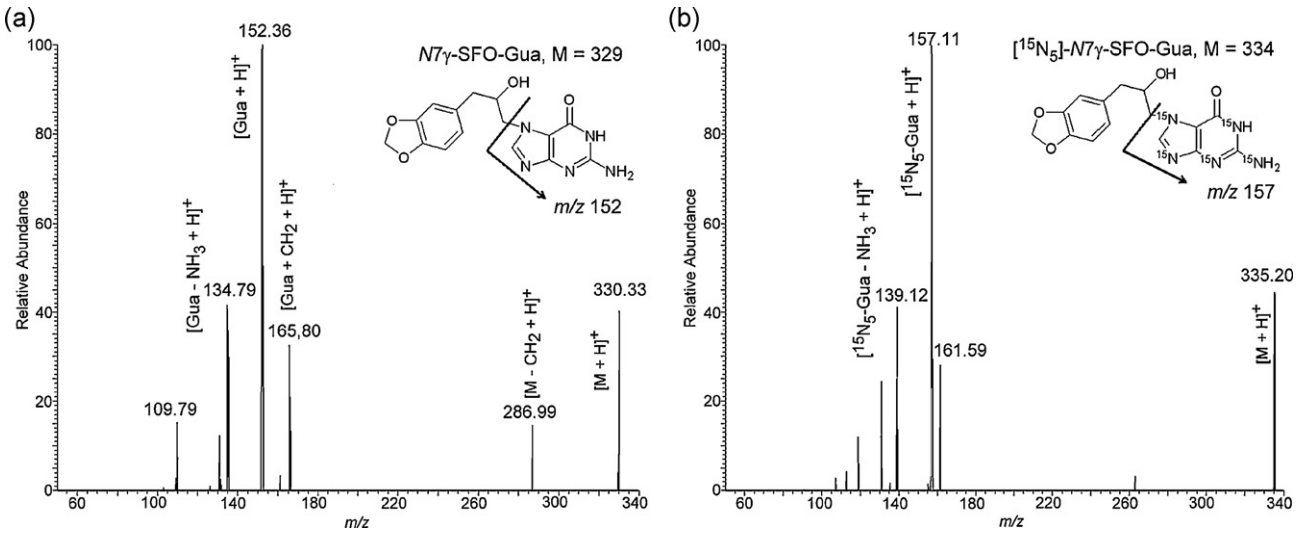

Fig. 2. The product-ion spectra of (a) *N*7γ-SFO-Gua (330 → 152) and (b) [$^{15}N_5$]-*N*7γ-SFO-Gua (335 → 157) due to the loss of 2-hydroxy-(3-benzo[1,3] dioxol-5-yl)propane from their corresponding protonated molecular ions $[M+H]^+$.

the *N*7γ-SFO-Gua adduct in DNA hydrolysate was determined to be 2670 per 10^6 nucleotides. Therefore, the spontaneously depurinated *N*7γ-SFO-Gua adducts accounted for 52.4% of total formation of this adduct at DNA backbones at room temperature.

3.3. Analysis of *N7γ-SFO-Gua* in urine of mouse treated with SFO

An isotope-dilution HPLC–ESI-MS/MS method was successfully developed to specifically analyze *N*7γ-SFO-Gua in urine with a high degree of sensitivity. The mass spectrometer was operated in the selected reaction mode (SRM) to monitor the ion transition *m/z* 330 → 152 for *N*7γ-SFO-Gua and *m/z* 335 → 157 for [$^{15}N_5$]-*N*7γ-SFO-Gua. When 22 μL of the sample was injected, the limit of detection (LOD) for *N*7γ-SFO-Gua was determined to be 5.5 pg (0.25 ng/mL) on-column with a signal-to-noise (S/N) ratio greater than 3. When *N*7γ-SFO-Gua and [$^{15}N_5$]-*N*7γ-SFO-Gua were spiked into urine collected from control mice and analyzed with the stable isotope dilution HPLC–ESI-MS/MS method, the chromatograms demonstrated that *N*7γ-SFO-Gua was separated from the interfering constituents in the urine. The intra-day precision

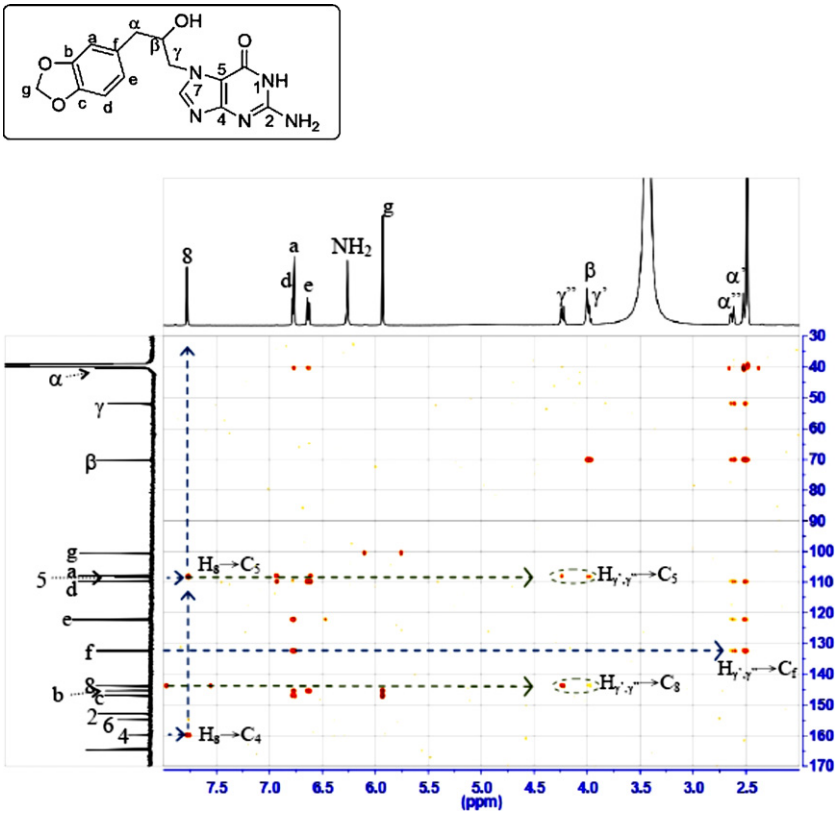

Fig. 3. HMBC NMR spectrum of *N*7γ-SFO-Gua (500 MHz in d_6-DMSO). The correlations of γ'-H and γ"-H with both C-5 ($^3J_{CH}$) and C-8 ($^3J_{CH}$) indicates that the alkylation was at *N*7-guanine position.

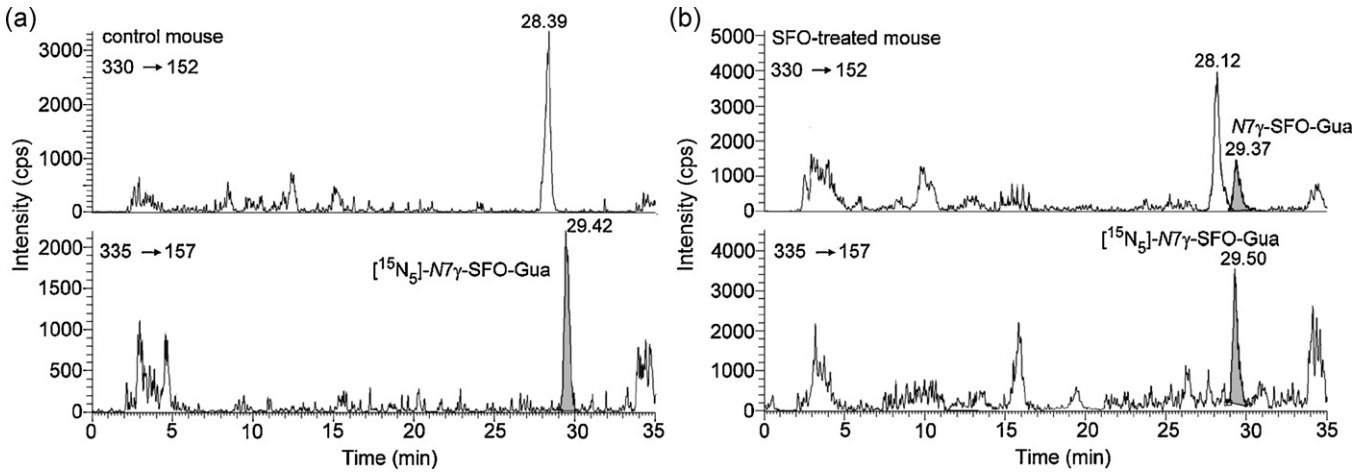

Fig. 4. Representative HPLC–ESI-MS/MS chromatograms generated from the analysis of (a) a urine sample of control mouse and (b) SFO-treated mouse spiked with [$^{15}N_5$]-*N*7γ-SFO-Gua (all with a final concentration of 1.1 ng/mL).

was 2–10% and the inter-day precision was 7–13%. The mean accuracy of the method, defined as ratios of measured concentrations of *N*7γ-SFO-Gua *versus* the corresponding spiked concentrations, was calculated to be 99 ± 1.5%.

The calibration curves of *N*7γ-SFO-Gua for sets 1 and 2 were $y = 1.05x - 0.27$ ($r^2 = 0.996$) and $y = 0.73x - 0.14$ ($r^2 = 0.998$) (Fig. 5), respectively. The unknown matrix component may potentially reduce or enhance the ion intensity of the analyte, and was evaluated by comparing the slope of set 2 with that of set 1 (Matuszewski et al., 2003). From our experiments, 70% matrix effect was obtained, indicating that 30% ion suppression occurred in the urine matrix, and was compensated with the use of isotope-labeled standard. The calibration curve was established by analyzing the set 2 standard solutions for quantitation of *N*7γ-SFO-Gua in urine of mice. The limit of quantitation (LOQ) of this adduct in urine was 0.5 ng/mL (or 1.5 nM) with a S/N ratio greater than 10.

Urinary *N*7γ-SFO-Gua was detectable in all treatment groups but not in the control group. Representative HPLC–ESI-MS/MS chromatograms generated from the analysis of urine collected from control and from SFO-treated mice are shown in Fig. 4a and b, respectively. The levels of urinary *N*7γ-SFO-Gua were 1.02 ± 0.14 and 0.73 ± 0.68 ng/mg creatinine ($n = 4$) on day 1 and day 2, respectively. However, it was below the limit of quantitation on day 3.

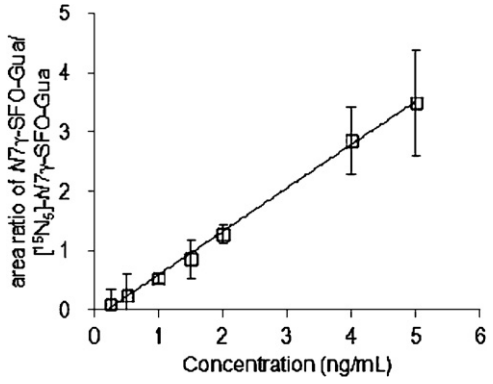

Fig. 5. Calibration curves of *N*7γ-SFO-Gua constructed by analysis of *N*7γ-SFO-Gua and [$^{15}N_5$]-*N*7γ-SFO-Gua prepared in urine of control mice. The concentration of *N*7γ-SFO-Gua is plotted against to the ratio of peak areas of *N*7γ-SFO-Gua to the peak area of [$^{15}N_5$]-*N*7γ-SFO-Gua. The points represent the average value with triplicate measurements and the error bars represent the standard deviation of each point. A regression line $y = 0.73x - 0.14$ ($r^2 = 0.998$) was obtained.

4. Discussion

Formation of the *N*7-guanine DNA adduct induced by genotoxic chemicals including ethylene oxide, propylene oxide, 1,3-butadiene oxide, and styrene oxide has been well studied (Tretyakova et al., 1997; Koskinen and Plna, 2000; Munter et al., 2002; Swenberg et al., 2007; Boysen et al., 2009). The *N*7-guanine DNA adduct does not participate in the Watson–Crick base pairing and is not promutagenic, however, studies have shown that epoxides not only induce *N*7-guanine DNA adducts but also N^2- and O^6-guanine DNA adducts, which are promutagenic (Boysen et al., 2009). Similar to other epoxides, SFO can also cause the formation of promutagenic adducts that probably led to SFO genotoxicity (Chiang et al., 2011; Shen et al., 2012). In addition, the *N*7-guanine adduct can be spontaneously and enzymatically depurinated from the DNA backbone and excreted through urine. This adduct in urine is considered as a biomarker of exposure to specific chemicals and can be used to identify its corresponding active metabolites (Boysen et al., 2009; Wu et al., 2011). However, the formation of SFO-induced DNA adducts has not been detected *in vivo*.

Numerous methods have been developed to analyze DNA adducts (Wu et al., 2011). The HPLC–MS/MS-based method exhibits excellent sensitivity and provides information on chemical structure, which makes it a good modality for analyzing and identifying DNA adducts (Wu et al., 2011). This study was designed to study *N*7-guanine adduct formation caused by SFO *in vitro* and *in vivo*. The adduct caused by SFO was synthesized, purified, and characterized by UV–vis, mass spectrometry, NMR, and 2D NMR. The spectra obtained from these characterizations revealed that the purified adduct was *N*7γ-SFO-Gua. [$^{15}N_5$]-*N*7γ-SFO-Gua was synthesized, purified, and characterized in a similar manner to serves as an internal standard to analyze *N*7γ-SFO-Gua in urine of SFO-treated mice. Using our method, we were able to detect *N*7-guanine adduct in urine of mice exposed to a single dose of SFO (30 mg/kg), providing valuable evidence on the formation of SFO-induced DNA adducts *in vivo*.

In order to determine whether *N*7γ-SFO-Gua is excreted through urine like the other *N*7-guanine adducts, calf thymus DNA was reacted with SFO at room temperature for 72 h. Analysis of *N*7γ-SFO-Gua in the calf thymus DNA backbone and in solution revealed that 52.4% of the total formation of *N*7γ-SFO-Gua at calf thymus DNA that was reacted with 60 μmol of SFO for 72 h (2670 adducts per 10^6 nucleotides) was depurinated, thereby validating our hypothesis that *N*7γ-SFO-Gua could be spontaneously depurinated from the DNA backbone. A similar observation was

reported in the reaction of allylbenzene 2′,3′-oxide with [^{14}C-8]-2′-deoxyguanonsine (Luo and Guenthner, 1996). Moreover, the *N*7-guanine adduct can also be removed by repair enzymes, suggesting that the majority of *N*7γ-SFO-Gua formed in DNA could be depurinated (Shuker and Farmer, 1992; Hemminki et al., 2000). Hence, the presence of *N*7γ-SFO-Gua in the urine of mouse strongly suggests that SFO reacts with DNA to form DNA adducts *in vivo*.

Qato and Guenthner (1995) reported that SFO-DNA adducts were not detectable in liver tissue of Balb/C mice treated with SFO (106.9 mg/kg body wt) or safrole (97.3 mg/kg body wt). One of the reasons that Qato et al. did not detect SFO-DNA adducts with their analytical method might be that the majority of the most abundant *N*7γ-SFO-Gua were depurinated, and therefore, undetectable. In this study, we were able to detect *N*7γ-SFO-Gua in urine of SFO-treated mice (30 mg/kg) with our newly developed HPLC–ESI-MS/MS method. Furthermore, we found that the levels of *N*7γ-SFO-Gua in the urine of mouse had decreased by 28.4% on day 2 after treatment, and were below the limit of quantitation on day 3. The half-life of urinary *N*7γ-SFO-Gua in mice was approximately 60 h. These findings suggest that SFO could cause *in vivo* formation of *N*7γ-SFO-Gua, and that this adduct could be subjected to spontaneous and enzymatic depurination. These results are consistent with our previous findings that SFO is genotoxic in mice, as evidenced by the increased frequencies of micronuclei and DNA strand breaks in the blood cells of SFO-treated mice (Chiang et al., 2011).

In summary, *N*7γ-SFO-Gua was well characterized in chemical structures and detected in the urine of mice treated with SFO with our newly developed HPLC–ESI-MS/MS method. These results suggest that this adduct may be formed *in vivo* and excreted through urine with a half-life of approximate 60 h. Further studies are needed to elucidate the mechanisms associated with SFO genotoxicity.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported in parts by the Environmental and Occupational Center grant of National Taiwan University, grant no. 98HP0022 and the National Science Council (project to WSC, NSC-99-2119-M-009-001-MY2).

Appendix A. Supplementary data

HMQC, DEPT, and COSY spectra of *N*7γ-SFO-Gua, and HPLC–ESI-MS/MS chromatograms for analysis of *N*7γ-SFO-Gua in Calf thymus DNA. Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.toxlet.2012.07.006.

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