DOI 10.1002/mnfr.200700276 Mol. Nutr. Food Res. 2008, *52*, 472–482

Research Article

# Absence of 2′-deoxyguanosine-carbon 8-bound ochratoxin A adduct in rat kidney DNA monitored by isotope dilution LC-MS/MS

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The contribution of DNA adduct formation in the carcinogenic action of the mycotoxin ochratoxin A (OTA) has been subject to much debate. Recently, a carbon-bonded ochratoxin A-2′-deoxyguanosine adduct (dGuoOTA) formed by photochemical reaction *in vitro* has been shown by $^{32}$P-postlabeling/TLC to comigrate with a spot detected in DNA isolated from rat and pig kidney following exposure to OTA. Considering the large body of evidence arguing against covalent DNA binding of OTA and the poor resolution and specificity of postlabeling analysis, we developed a stable isotope dilution LC-MS/MS method to analyze dGuoOTA in kidney DNA isolated from rats treated with OTA. dGuoOTA and nitrogen-15-labeled dGuoOTA ($^{15}N_5$-dGuoOTA) were prepared by photoirradiation of OTA in the presence of dGuo or nitrogen-15-labeled dGuo. Conditions for DNA hydrolysis were optimized using a synthetic oligonucleotide containing dGuoOTA to ensure complete release of dGuoOTA. The LOD of the method (S/N > 3) was 10 fmol dGuoOTA on-column. However, dGuoOTA was not detected in DNA samples isolated from male F344 rats treated with OTA for up to 90 days at doses known to cause renal tumor formation. Detection limits, calculated for each individual sample based on the absolute LOD and the amount of DNA injected, were as low as 3.5 dGuoOTA/$10^9$ nucleotides. These data are consistent with previous results showing lack of DNA adduct formation by OTA and demonstrate that dGuoOTA is not formed in biologically relevant amounts under physiological conditions *in vivo*.

**Keywords:** Carcinogenicity / DNA adduct / Isotope dilution / Kidney / LC-MS/MS

Received: July 17, 2007; revised: August 28, 2007; accepted: September 8, 2007

## 1 Introduction

Ochratoxin A (OTA) is a naturally occurring mycotoxin produced by several fungus species belonging to the genera *Aspergillus* and *Penicillium* [1, 2]. It is widespread in various food commodities such as cereals, coffee, cocoa, wine, or dried fruits [3]. The actual effect of dietary exposure to OTA in humans remains unclear, and up to now, epidemiological studies have not allowed to assess the actual health impact of OTA exposure in humans [4, 5]. In animals, OTA was shown to produce a wide array of toxicological effects including nephrotoxicity, neurotoxicity, teratogenicity, immunotoxicity, and carcinogenicity [6, 7]. The toxicological data exhibit significant species and gender differences with regard to OTA-mediated nephrotoxicity and carcinogenicity. For instance, high incidences of renal adenoma

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**Abbreviations: ctDNA**, calf thymus DNA; **dGuo**, 2′-deoxyguanosine; **dGuoOTA**, carbon-bonded ochratoxin A-2′-deoxyguanosine adduct; **$^{15}N_2$-dGuo**, 1,2,3,7,9-$^{15}N_5$-2′-deoxyguanosine; **OTA**, ochratoxin A; **SPDE**, phosphodiesterase II from bovine spleen; **VPDE**, phosphodiesterase I from *Crotalus adamanteus*

* Both these authors contributed equally to this work.

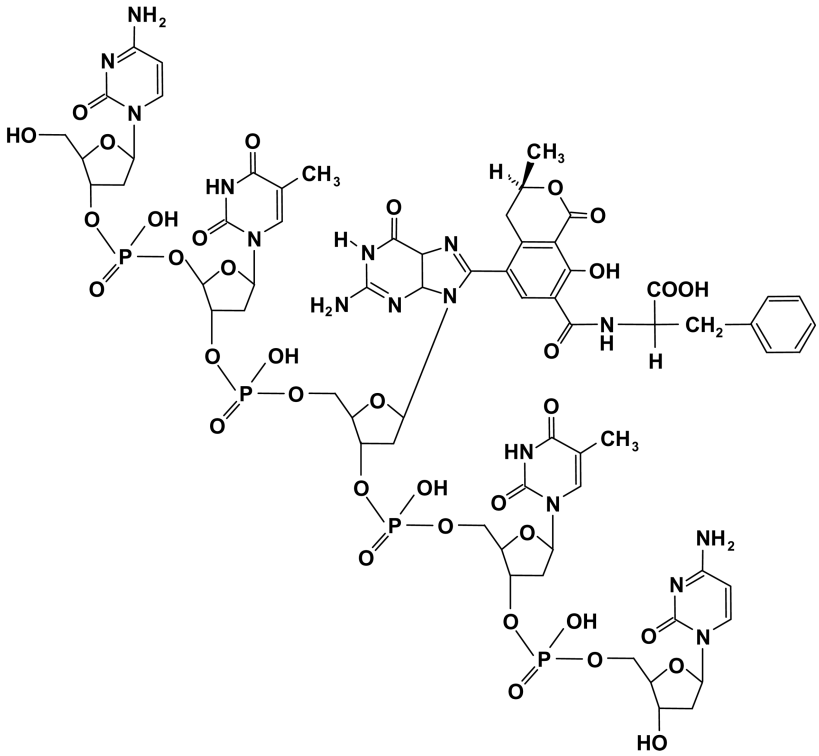

**Figure 1.** Chemical structure of the oligonucleotide CTG$^{OTA}$TC.

and carcinoma were observed in male rats treated with OTA at doses of 70 or 210 μg/kg b.w., while female rats and mice were found to be less sensitive. Male mice were approximately 20-fold less sensitive and female mice were completely refractive to OTA-induced toxicity and carcinogenicity [8 – 10]. Based on toxicological data in pig, the most susceptible species to the nephrotoxic effects of OTA, a provisional tolerable weekly human intake of 120 ng OTA *per* kg b.w. has been established [11, 12].

With regard to mechanism of toxicity, discrepant data have been reported in the literature. A genotoxic potential for OTA has been indicated *in vitro* [13, 14] as well as *in vivo* [15]. Mutagenicity studies appeared inconsistent, revealing mostly negative but also few positive responses to OTA [16, 17]. The nature of DNA damage and mutations induced by OTA is still unknown. Using $^{32}$P-postlabeling, the formation of spots interpreted as OTA-derived DNA adducts was observed in mice [18] and monkeys [19]; however, the identifed spots were not supported by a structural characterization and their presence, detected by $^{32}$P-postlabeling, were rationalized in terms of cytotoxicity rather than to direct adduct formation [20]. *In vitro*, incubation of rat kidney S-9 with NADPH in the presence of OTA did not reveal any detectable binding of OTA to DNA (detection limit at 0.2 adducts *per* $10^7$ nucleotides) [20]. This is in accordance with results demonstrating that either rat microsomes (liver and kidney) or human cytochromes P450 showed no or only very low activity to metabolize OTA [21]. Experiments performed *in vivo* showed that the level of covalent binding of [$^3$H]-OTA to DNA was below the detection limit of liquid scintillation counting in kidney and liver (<1.3 adducts *per* $10^{10}$ and 5.6 adducts *per* $10^{11}$ nucleotides, respectively) of rats 24 h-orally administrated with [$^3$H]-OTA (1000 mCi/mmol, 210 μg/kg b.w.) [22]. To further investigate the potential binding of OTA to DNA, the radiocarbon content of DNA isolated from liver and kidney male rats treated with a single dose of [$^{14}$C]-OTA (0.25 mCi/mmol, 500 μg/kg b.w.) was measured by accelerator MS (sensitivity approximately two orders of magnitude below those reported with $^{32}$P-postlabeling technique), and no difference was observed between control and treated animals [23]. Recently, the photoirradiation of a mixture containing OTA and 2′-deoxyguanosine (dGuo) in aqueous solution was reported to give rise to a carbon-bonded ochratoxin A-2′-deoxyguanosine adduct (dGuoOTA, Fig. 1). The adduct, comprehensively characterized by NMR ($^1$H-spectrum and $^1$H-$^{13}$C long range heteronuclear correlation) and MS, was described with a covalent bond between the carbon C-8 of the guanine moiety and the carbon C-5 of the coumarin structure of OTA [24]. This adduct was further analyzed by $^{32}$P-postlabeling and was found to comigrate with a spot detected in kidney DNA extracted from OTA-treated rat (12 wk-old Dark-Agouti males, 0.4 mg OTA *per* kg b.w. three times *per* week for *ca.* 2 years) and pig (20 μg OTA *per* kg b.w. in food for 3 wk). In rat kidney DNA, the level of dGuoOTA was estimated at 1.56 – 1.66 adducts *per* $10^8$ nucleotides, while 1.61 adducts *per* $10^8$ nucleotides were found in pig [25]. The $^{32}$P-postlabeling technique is very sensitive to detect DNA adducts, how-

ever it suffers from lack of specificity and resolution of the spots is poor in comparison to other analytical tools. Therefore, due to the fact that controversial data have been published regarding the potential adduction of OTA to DNA, the aim of this study was to improve an existing LC coupled with MS/MS (LC-MS/MS) method [23] and analyze potential formation of dGuoOTA in kidney DNA obtained from rats treated with OTA using isotope dilution LC-MS/MS, including optimized DNA digestion conditions to ensure complete release of dGuoOTA. To improve the previous method, particular focus was placed on the three following aspects. (i) Preparation and purification of dGuoOTA and a nitrogen-15-labeled dGuoOTA ($^{15}N_5$-dGuoOTA) to be used as internal standard to further ensure accurate quantification in the samples. (ii) Development and optimization of an enzymatic digestion, using the pentamer CTG$^{OTA}$TC (CTGTC with the adduct bond to the guanine nucleobase) and calf thymus DNA (ctDNA) irradiated in the presence of OTA, to ensure a complete release of dGuoOTA in kidney DNA of rats treated with OTA. (iii) Use of the column switching technique to facilitate injection of larger volumes, and ultimately decrease the LOD. After optimization, the method was applied on kidney DNA from rats treated with OTA.

## 2 Materials and methods

### 2.1 Chemicals and reagents

OTA and dGuo were obtained from Sigma (St Louis, MO, USA), while 1,2,3,7,9-$^{15}N_5$-2′-deoxyguanosine ($^{15}N_2$-dGuo) (isotopic purity at 96–98%) was from Cambridges Isotope Laboratories (Innerberg, Switzerland). ctDNA was purchased from Sigma–Aldrich (Steinheim, Germany). Triethylacetate ammonium buffer was provided by Fluka (Buchs, Switzerland) while ACN, sodium acetate, Tris-HCl, EDTA, and ammonia 30% were obtained from Merck (Darmstadt, Germany). Zinc sulphate (heptahydrate) was from Sigma. Phosphodiesterase I from *Crotalus adamanteus* (VPDE, for venom phosphodiesterase), phosphodiesterase II from bovine spleen (SPDE, for spleen phosphodiesterase), and alkaline phosphatase were purchased from Sigma. Nuclease P1 was from MP Biochemicals (Aurora, OH, USA). HPLC grade methanol and acetonitrile (ACN) were purchased from Sigma–Aldrich Fluka (Taufkirchen, Germany). Purified water for LC-MS/MS was purchased from Roth (Karlsruhe, Germany).

### 2.2 Synthesis of the CTGTC and CTG$^{OTA}$TC oligomers

Oligonucleotide CTGTC was prepared on an automated 392 DNA synthesizer (Applied Biosystems, Palo Alto, CA, USA) by phosphoramidite solid-phase synthesis [26], using the *iso*-butyryl group to protect the amino function of dGuo and 2′-deoxycytidine, and the benzoyl group for 2′-deoxyadenosine. Upon completion of the synthesis, the oligonucleotide was detached from the solid support and the amino functions were deprotected by treatment with concentrated aqueous ammonia (30%) at 50°C for 16 h. After evaporation of the solvent under vacuum, the crude oligomer was purified by RP HPLC on a Hypersil 5 µm C18 column (7 mm × 250 mm) by using the conditions described hereafter: linear gradient from 0 to 10% ACN (30 min) in 10 mM triethylacetate ammonium buffer at a flow rate of 2 mL/min (UV detection at 260 nm). The purity and homogeneity of the collected fractions were controlled by RP-HPLC analyses. Finally, MALDI-MS measurements confirmed the integrity of the purified pentamer. The CTGTC was further converted into the CTG$^{OTA}$TC (Fig. 1) by photoirradiation as described hereafter.

### 2.3 Photoinduced addition of OTA to nucleosides, CTGTC, and ctDNA

Irradiations (8 min) were carried out at room temperature and under aerobic conditions with a mercury high-pressure lamp (Heraeus TQ 150) through a cut-off filter (Schott WG 305, $\lambda > 305$ nm) in a mixture of aqueous potassium phosphate buffer (0.1 M, pH 7.4) and DMSO (3:1, v/v). For the nucleosides (dGuo and its $^{15}N$-labeled analog, $^{15}N_5$-dGuo) and DNA, 202 µg (0.5 µmol) of OTA were used, while 657 µg CTGTC (1.63 µmol) was added for photoirradiation of oligonucleotides. Experiments were conducted in a final volume of 1 mL for nucleosides and DNA, and 82 µL for CTGTC due to limited amounts available. The concentration of the substrates (dGuo, $^{15}N_5$-dGuo, CTGTC, and ctDNA) used for the irradiations was 20 mM in each case.

### 2.4 Synthesis of dGuoOTA and its nitrogen-15 labeled analog

dGuoOTA and its $^{15}N$-labelled analog were prepared by photoirradiation (for details, *vide supra*). dGuoOTA was isolated from the mixture by HPLC using a HP 1090 (Agilent, Waldbronn, Germany) equipped with a UV detector. dGuo, OTA, and dGuoOTA were separated on a Supelcosil LC-18-DB column (25 cm × 10 mm, 5 µm) from Supelco (Bellefonte, PA, USA) using a gradient ammonium formate 40 mM (solvent A) – ACN (solvent B) at a flow rate of 1.6 mL/min. The gradient was: 0–5 min: 10% B; 5–10 min: linear gradient to 50% B; 10–15 min: 50% B; 15–20 min: linear gradient to 100% B; 20–22 min: 100% B. Separation was monitored at 210 nm (to monitor nonspecific impurities), 260 nm (to monitor dGuo), and 330 nm (to monitor OTA and dGuoOTA). Under these conditions, retention times were at 10.7 min for dGuo, 17.8 min for dGuoOTA, and 19.4 min for OTA. Adduct standards were further characterized by MS. Enhanced product ion spectra for *m/z* 633 (dGuoOTA) and 638 ($^{15}N_5$-dGuoOTA) were recorded over the range of *m/z* 100–700 on a API Q-Trap

 

mass spectrometer (Applied Biosystems, Darmstadt, Germany) operating in the negative ion mode. A Turbo Ion Spray source was used with $N_2$ as heater gas at 400°C and a capillary voltage of −4.5 kV. Collision gas was $N_2$ at CAD = 4 and the collision energy was −30 V. The Q-Trap was coupled to an Agilent 1100 series HPLC/autosampler-system and LC-conditions were as previously described [23].

### 2.5 Animal treatment

Kidney samples were obtained from a 90-day toxicity study in which male F344 rats were treated with 210 μg/kg b.w. OTA in corn oil by oral gavage [27]. This dose is known to cause high incidences of renal tumors after long-term treatment [28]. Control rats received equal volumes of corn oil. In brief, male F344 Fischer rats (8–9 wk old) were purchased from Harlan-Winkelmann (Borchen, Germany), housed in Macrolon cages and allowed free access to standard laboratory chow (Altromin) and tap water. Room temperatures were maintained at 21 ± 2°C with a relative humidity of 55 ± 10% and a day/night cycle of 12 h. After 2, 4, and 13 wk of OTA treatment, rats were sacrificed by carbon dioxide asphyxiation and cervical dislocation, kidneys were excised, flash-frozen in liquid nitrogen and stored at −80°C. Additional kidney samples were obtained from a second experiment in which rats (three *per* group) were treated with OTA doses of 250, 500, 1000, or 2000 μg/kg b.w. in corn oil by oral gavage for two weeks [29]. Control rats received equal volumes of corn oil. Rats were sacrificied by carbon dioxide asphyxiation and cervical dislocation 72 h postdosing. This time-point was chosen as OTA is slowly eliminated and maximum DNA adduct concentrations have previously been reported in DNA extracted from kidneys 48–72 h after a single dose of OTA [18, 30]. Kidneys were removed, flash-frozen in liquid nitrogen, and stored at –80°C until further DNA extraction and dGuoOTA analysis.

### 2.6 DNA extraction and enzymatic digestion

DNA was isolated from 300 to 400 mg kidney tissue by the Nucleobond® method (Macherey-Nagel, Dueren, Germany) according to the manufacturers instructions with minor modifications. Briefly, tissue samples were homogenized using an ultra-turrax, treated with proteinase K and RNase and loaded onto a Nucleobond AX G 500 ion exchange cartridge. After washing, DNA was eluted from the cartridge using a modified elution buffer (1.5 M NaCl, 0.05 M Tris, 15% ethanol, pH 7.0). DNA was precipitated by the addition of 0.7 volumes isopropanol. After washing with 70% ethanol, DNA pellets were dissolved in $H_2O$ and hydrolyzed. DNA samples were further submitted to enzymatic digestion for release of the nucleosides. *Per* 50 μg DNA in 100 μL water, 20 μL of buffer P1 (sodium acetate 300 mM, $ZnSO_4$ 1 mM, pH 5.3), 30 μL of SPDE solution (0.0004 U/μL in water), and 10 μL of nuclease P1 (0.5 μg/μL in water) were added and the resulting solution was incubated 4 h at 37°C. Following incubation, 20 μL of buffer PA (Tris-HCl 500 mM, EDTA 1 mM, pH 8.0), 10 μL of VPDE solution (0.00026 U/μL in water), and 2.6 μL alkaline phosphatase (0.764 U/μL in water) were added and the mixture was incubated for 2 h at 37°C. Amounts of buffer and enzymes added to each sample were adjusted according to the amount of DNA available for each sample. DNA hydrolysates were filtered through a 5000 NMWL cut-off filter (Ultrafree, Millipore, Eschborn, Germany) to remove enzymes, evaporated to dryness and reconstituted in 450 μL $H_2O$.

### 2.7 Quantification by LC-MS/MS

For sensitive detection of dGuoOTA in DNA obtained from rats treated with OTA, a column switching technique was used to facilitate injection of larger volumes and therefore larger amounts of DNA. The liquid handling system consisted of an Agilent 1100 series autosampler, an automated switching valve and two Agilent 1100 HPLC pumps. DNA hydrolysates (containing up to 912 μg DNA, spiked with 1.25 pmol $^{15}N_5$-dGuoOTA in a total volume of 450 μL) were loaded onto an Oasis® HBL 25 μm 20 mm × 2.1 mm trap column (Waters, Milford, Massachusetts) using 10% methanol in 10 mM ammonium acetate, pH 4.3 at a flow rate of 1.5 mL/min for 2 min. The effluent of the trap column containing normal nucleosides was washed to waste, while dGuoOTA and $^{15}N_5$-dGuoOTA were retained on the trap column. After 2 min, the valve was switched and the concentrated sample zone was back flushed from the trap column onto the analytical column (Nucleosil 5 μm/100 Å 15 mm × 2 mm; Phenomenex, Aschaffenburg, Germany) with a flow rate of 0.3 mL/min using the following mobile phase: 80% A (water) for 1 min, followed by a linear gradient to 10% A/90% B (ACN) in 9 min. This was held for 3 min before returning to 80% A. The API 3000 mass spectrometer was operated with a Turbo Ion Spray source in the negative ion mode with a voltage of −4.0 kV. Spectral data were recorded with $N_2$ as heater gas at 400°C and as collision gas (CAD = 4) in the multiple reaction monitoring mode (MRM). The following mass transitions were analyzed: *m/z* 633.4–429.1 and *m/z* 633.4–517.3 as quantifier and qualifier, respectively, for dGuoOTA, and *m/z* 638.2–522.2 for $^{15}N_5$-dGuoOTA.

## 3 Results

### 3.1 Preparation, isolation, and characterization of dGuoOTA and $^{15}N_5$-dGuoOTA

Both dGuoOTA and $^{15}N_5$-dGuoOTA were prepared by photoirradiation, and the formation conditions were adapted

 

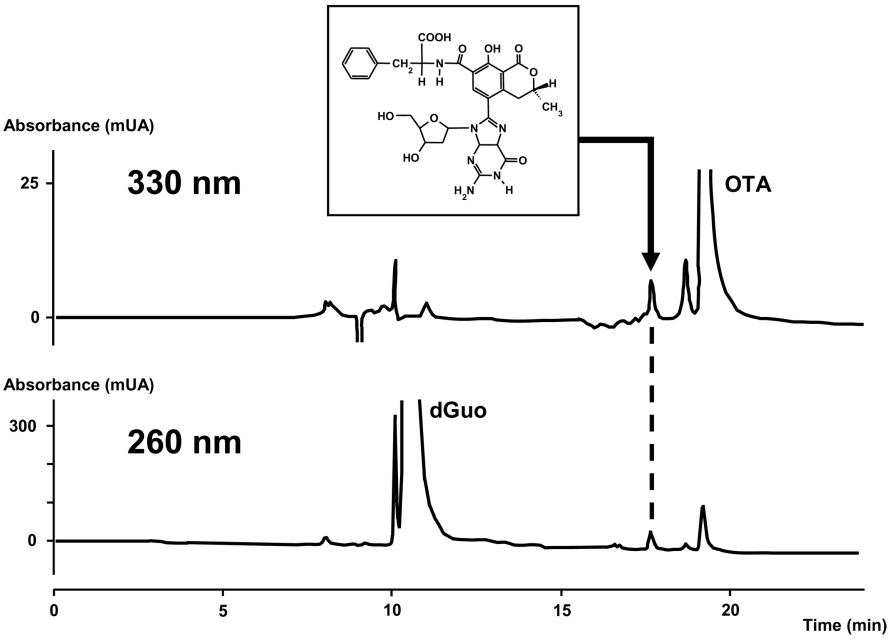


**Figure 2.** HPLC-UV chromatographic profile (260 and 330 nm) used for the isolation of dGuoOTA and $^{15}N_5$-dGuoOTA (retention time at 17.8 min) formed by photoirradiation $\lambda > 305$ nm in an aqueous mixture dGuo and OTA.

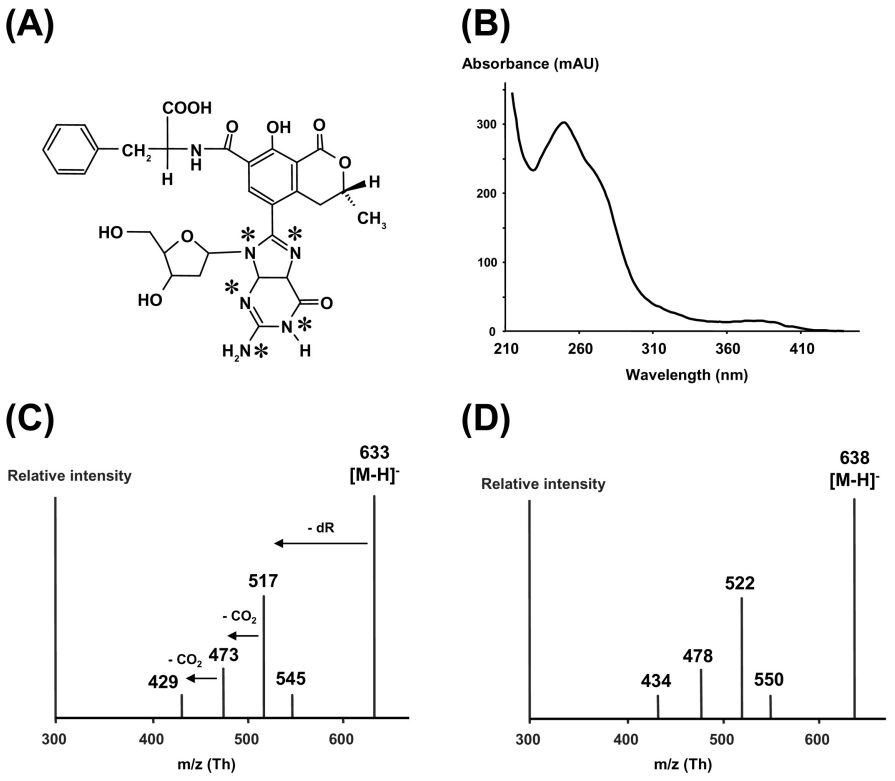


**Figure 3.** Spectroscopic features for the dGuo-carbon 8-bound ochratoxin A adduct (dGuoOTA) and the internal standard ($^{15}N_5$-dGuoOTA): (A) chemical structure of dGuoOTA (asteriks indicated the $^{15}$N-labelled nitrogen in $^{15}N_5$-dGuoOTA); (B) UV-spectrum of isolated dGuoOTA and $^{15}N_5$-dGuoOTA; (C) negative CID-spectrum of dGuoOTA (−30 eV); (D) negative CID-spectrum of $^{15}N_5$-dGuoOTA (−30 eV).

from those reported by Dai *et al.* [24]. Such an approach was convenient to obtain the compounds of interest in an efficient manner using an aqueous solution of OTA supplemented with either dGuo or $^{15}N_5$-dGuo. Due to the low formation yield of the adduct, an HPLC-UV-based method was optimized (Fig. 2) to separate the adduct from dGuo (or $^{15}N_5$-dGuo) and OTA, and obtain each standard at a purity compatible with analytical purposes. The chemical structure of the adduct was confirmed by UV and CID. The UV-spectra exhibited two absorption maxima at 258 and 380 nm approximately with a shoulder at *ca.* 270 nm (Fig. 3), and this profile was identical to the one reported by Faucet *et al.* [25]. The CID-spectrum of dGuoOTA, generated by the fragmentation of the negative *pseudo*-molecular ion

 

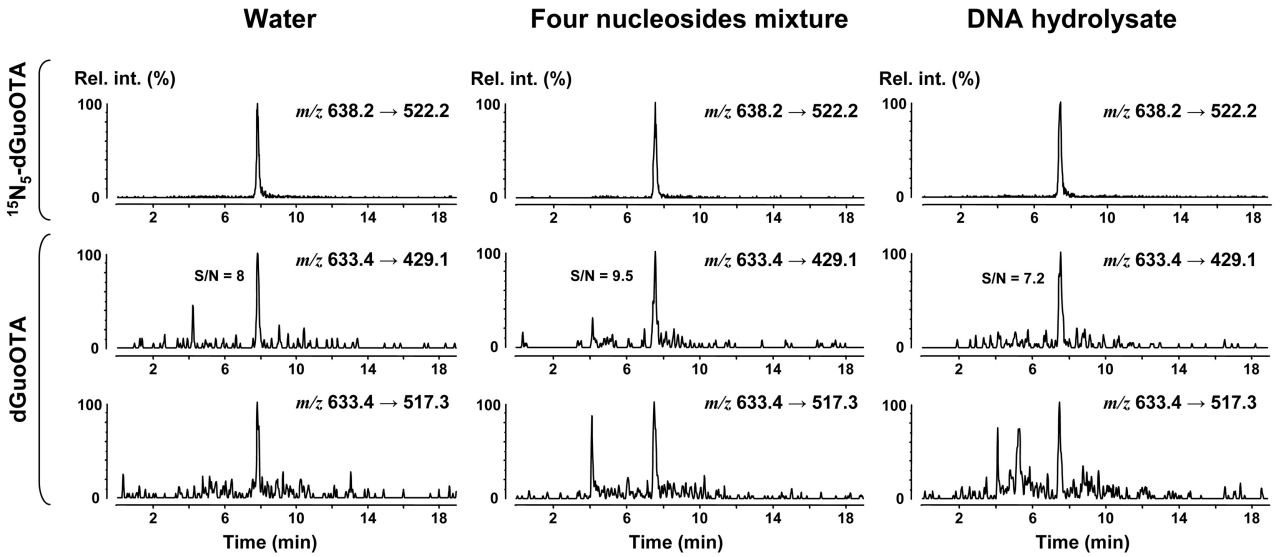


**Figure 4.** Selected LC-MS/MS chromatograms obtained by separation of 50 fmol dGuoOTA and 1.25 pmol $^{15}N_5$-dGuoOTA prepared in either water, a nucleoside mixture or DNA hydrolysate demonstrating lack of matrix effects on method performance.

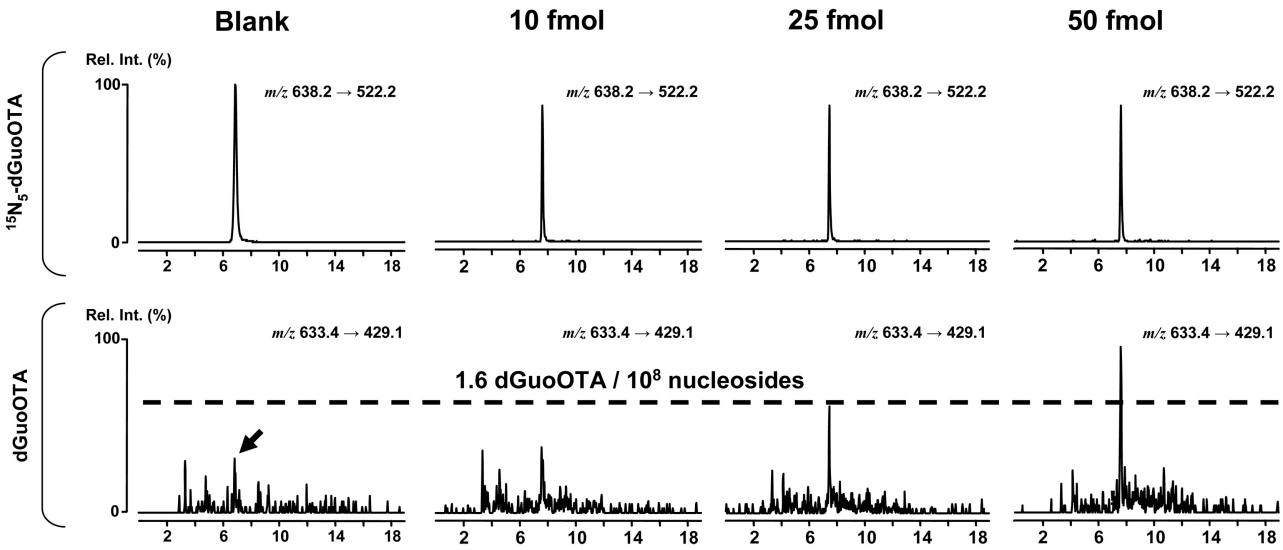


**Figure 5.** Selected LC-MS/MS chromatograms of DNA hydrolysates (500 μg) spiked with 10, 25, and 50 fmol dGuoOTA (1.25 pmol for $^{15}N_5$-dGuoOTA). The dotted line corresponds to a theoretical peak height associated with 1.6 dGuoOTA *per* $10^8$ nucleosides (*ca.* 25 fmol detected) detected by $^{32}$P-postlabeling [25], and the black arrow (blank sample) highlights the contribution of dGuoOTA as an impurity of $^{15}N_5$-dGuoOTA in the signal (for dGuoOTA, only transition *m/z* 633.4 → 429.1 is shown).

$[M-H]^-$ at *m/z* 633, gave rise to three main fragment ions at *m/z* 517, 473, and 429. The fragment ion at *m/z* 517 ($\Delta M$ = –116 amu) was assigned to the loss of 2-deoxy-β-D-*erythro*-pentofuranose, as typically observed with nucleosides [31, 32]. This assignment was confirmed with the CID-spectrum of $^{15}N_5$-dGuoOTA which also showed a 116 amu-mass loss with the transition *m/z* 638 → 522. The transitions *m/z* 517 → 473 and *m/z* 473 → 429 present in the CID-spectrum of dGuoOTA represent losses of carbon dioxide which occurred in the ochratoxin moiety [33, 34], but also possibly in the 6-membered ring of the guanine. Again, this proposed fragmentation pathway was consistent with the data provided in the CID-spectrum of $^{15}N_5$-dGuoOTA. Indeed, the transitions *m/z* 522 → 478 and 478 → 434 matched with the $M_r$ of carbon dioxide (44 Da).

### 3.2 Performance of LC-MS/MS detection

To exclude potential matrix effects, method performance was assessed using an artificial matrix consisting of

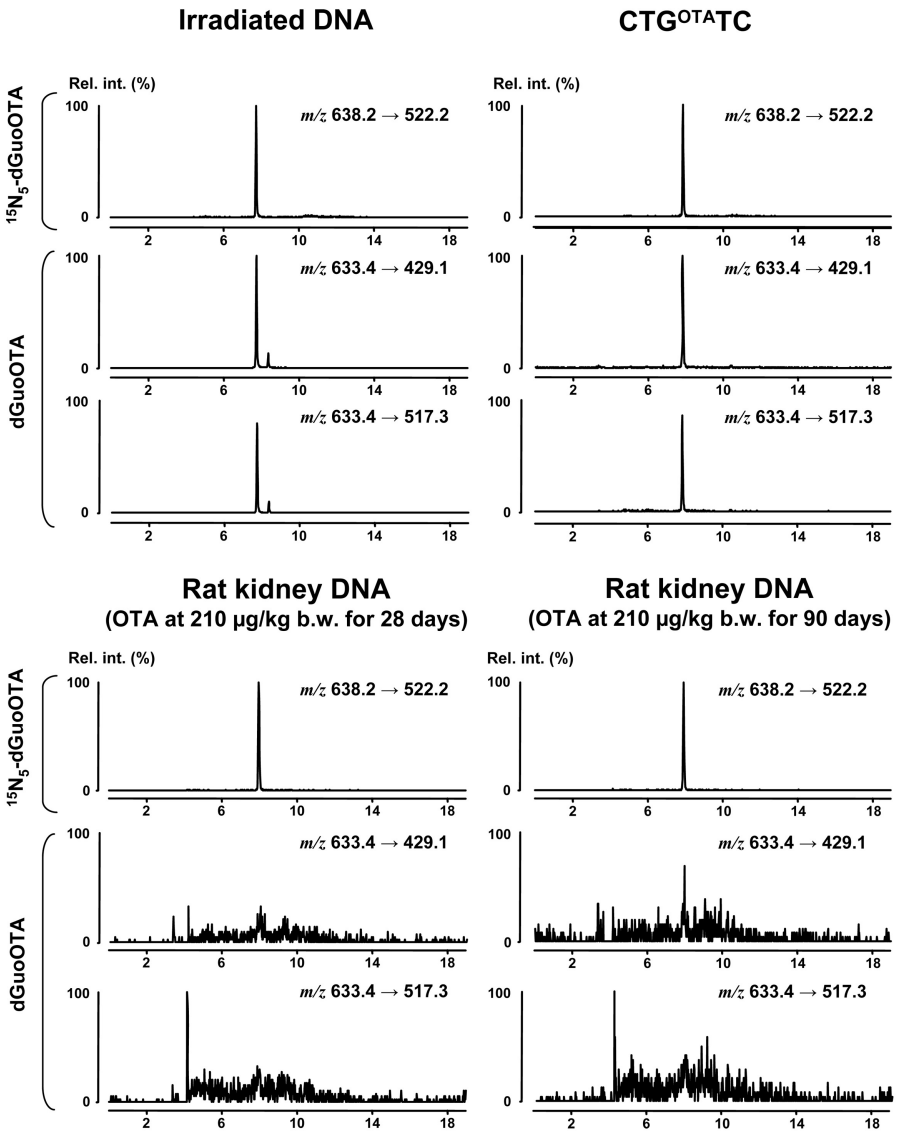


**Figure 6.** Representative reaction monitoring chromatograms of DNA hydrolysates prepared from DNA (300 µg) irradiated in the presence of OTA, oligonucleotide (136 µg) irradiated in the presence of OTA ($CTG^{OTA}TC$), kidney DNA (530 µg) isolated from a rat treated with 210 µg/kg b.w. OTA for 28 days, and kidney DNA (552 µg) isolated from a rat treated with 210 µg/kg b.w. for 90 days. dGuoOTA was not detectable in DNA obtained from kidneys of OTA exposed animals.

2′-deoxyribonucleosides (21% dGuo and 2′-deoxycytidine, and 29% 2′-deoxyadenosine and thymidine) and DNA hydrolysates generated from control rat liver. Samples equivalent to 200 µg DNA in a total volume of 450 µL were spiked with 1.25 pmol $^{15}N_5$-dGuoOTA and 50 fmol of dGuoOTA. Both the nucleosides mixture or hydrolyzed DNA did not influence method performance and particularly, the response factor of dGuoOTA was not influenced by the composition of the extract (Fig. 4). For the determination of the LOD, several hydrolysates equivalent to 500 µg DNA were spiked with 1.25 pmol $^{15}N_5$-dGuoOTA and various amounts of dGuoOTA (10, 25, and 50 fmol). The obtained chromatographic profiles show that, for the quantifier (*m/z* 638.2 → 429.1), the LOD is about 10 fmol (Fig. 5), corresponding to 6.4 dGuoOTA *per* $10^9$ nucleosides. In other words, with 500 µg DNA, 1.6 dGuoOTA *per* $10^8$ nucleosides would be detected with a signal of dGuoOTA corresponding to 25 fmol on-column. With rat kidney samples, the isolated amount of DNA ranged from 530 to 912 µg DNA *per* animal, which means that 1.6 dGuoOTA *per* $10^8$ nucleosides would be obtained with dGuoOTA signals within the range of 27 – 46 fmol detected. Calibration was linear ($R^2 = 0.999$) in the range of 50 fmol to 5 pmol OTA-dGuoOTA and accuracy was better than 85 – 115%.

### 3.3 Completeness of the digestion

To ensure a complete digestion of DNA and an efficient release of dGuoOTA as free nucleoside, cocktails of enzymes were tested on the model oligomer $CTG^{OTA}TC$ (pentamer CTGTC with the OTA bond to the C-8 position of the guanine, Fig. 1). It appeared that the highest amount of dGuoOTA was obtained with a two-step enzymatic diges-

 

**Table 1.** LC/MS-MS analysis of DNA and oligonucleotides irradiated in the presence of OTA, and of kidney DNA samples obtained from rats treated with OTA for up to 13 wk at doses known to cause kidney tumor formation (210 μg/kg b.w., 5 days *per* wk)

| Sample type | Amount of DNA (μg) | dGuoOTA/$10^9$ nucleotides |
|---|---|---|
| Kidney DNA from OTA-treated rats | | |
| Controls (2 wk) | 584 | <5.5 |
| | 524 | <6.1 |
| 2 wk treated | 640 | <5.0 |
| | 760 | <4.2 |
| 4 wk treated | 530 | <6.0 |
| | 560 | <5.7 |
| 13 wk treated | 552 | <5.8 |
| | 912 | <3.5 |
| Irradiated ctDNA | 300 | 13277 |
| $CTG^{OTA}TC$ | 136 | 4533 |

The relative LOD for each sample expressed as dGuoOTA/$10^9$ nucleotides was calculated based on the amount of DNA available and the absolute LOD of 10 fmol dGuoOTA on column.

tion. The first step encompassed an incubation in the presence of both VPDE and P1, while the second one was carried out with SPDE and alkaline phosphatase, the latter enzymes aimed at removing the phosphate groups to release the nucleobases as free nucleosides. A typical chromatogram obtained with $CTG^{OTA}TC$ is depicted in Fig. 6, and the LC-MS/MS quantification gave a concentration of 4.5 dGuoOTA/$10^6$ nucleotides in $CTG^{OTA}TC$ (Table 1). The efficiency of the digestion was confirmed with ctDNA preliminarly photoirradiated ($\lambda > 305$ nm) in the presence of OTA, and the LC-MS/MS analyses of the digested irradiated ctDNA led to the detection of dGuoOTA. Both enzyme amounts and incubation times were optimized to achieve maximum release of dGuoOTA with irradiated ctDNA. The optimal MS response was obtained with concentrations at 0.0004 U/μL, 0.5 μg/μL, 0.00026 U/μL, and 0.764 U/μL for SPDE, nuclease P1, VPDE, and alkaline phosphatase, respectively. When incubation times were set at 4 h for the first step (VPDE and P1) and 2 h for the second one (SPDE and alkaline phosphatase), the highest signal of dGuoOTA was achieved.

### 3.4 Analysis of dGuoOTA in kidney DNA extracted from rats treated with OTA

Potential formation of dGuoOTA in kidneys of rats exposed to OTA was assessed using the improved isotope dilution LC-MS/MS method, allowing injection of large quantities of DNA, thereby increasing the overall detection limit. However, dGuoOTA was not detected (S/N > 3) in kidney DNA extracted from rats treated with 210 μg/kg b.w. OTA for up to 90 days (Fig. 6). Detection limits were calculated based on samples size (DNA amount) and the absolute on-column LOD measured at 10 fmol dGuoOTA and ranged from 3.5 to 6.0 dGuoOTA/$10^9$ nucleotides (Table 1). Consistent with these findings, dGuoOTA was also not detectable in a set of kidney DNA samples obtained from an experiment in which male F344 rats were treated with up to 2 mg/kg b.w. OTA for 2 wk. However, it should be pointed out that the relative LOD for these samples was somewhat higher (1.4 – 6.8 dGuoOTA/$10^8$ nucleotides) due to limited amount of DNA available from this study.

## 4 Discussion

The potential of OTA to form covalent DNA adducts has been subject to much debate [35 – 38]. *In vivo* and *in vitro* studies using radiolabeled OTA consistently failed to detect DNA binding of OTA or OTA metabolites with detection limits ranging from 3 adducts/$10^9$ nucleotides to 1.3 adducts/$10^{10}$ nucleotides [20, 22, 23, 39]. Results from these studies are in good agreement with the lack of mutagenicity observed in standard mutagenicity assays [28, 40 – 42] and the poor metabolism of OTA observed in various systems [20, 21, 39].

In contrast, using postlabeling analysis, large numbers of putative OTA-derived DNA adducts have been reported to be present in a wide range of tissues from OTA treated rodents and pigs, and also in humans presumably exposed to OTA [19, 25, 43, 44]. However, these findings were not confirmed in another study [45], and the chemical nature of these "adduct spots" has not been identified. Based on DNA binding and biotransformation studies it appears that these DNA modifications are unlikely to contain OTA or parts of the OTA molecule, and the possibility that these "adduct spots" may constitute oxidative lesions rather than covalent DNA adducts derived from OTA is supported by recent findings which demonstrate that OTA causes oxidative DNA damage and alters expression of genes involved in chemical detoxification and antioxidant defense [46 – 48]. Furthermore, the implication of these "adduct spots" in the mechanism of OTA toxicology is questionable since their observation is not consistent with the species and target organ specific effects of OTA on animals. Up to one adduct *per* $10^7$ nucleotides was observed in kidney DNA of mice treated with a single OTA dose at 2.5 mg/kg b.w., and some of these "adduct spots" were already observed at a dose of 600 μg/kg b.w. [18, 49]. In contrast, no OTA-induced adducts were detected in kidney tissue of rats after 24 h-treatment with OTA at a dose of 1 mg/kg b.w. using $^{32}$P-postlabeling technique [20]. With rodents orally admistrated by gavage with identical doses of OTA, the abundance of the main "adduct spot" was shown to be slightly higher in mouse than in rat [37]. However, results indicate that mice are less susceptible to OTA-induced renal tumor than rats [13, 28, 50]. In addition, the participation of these

"adduct spots" observed in $^{32}$P-postlabeling experiments in the formation of renal tumors in rats may further be questioned by the fact that they are not only observed in the target tissue (kidney) but also in testis and liver [51]. In these organs, neither OTA-toxicity nor tumor induction has been observed.

It should be emphasized that the high number of different OTA-derived adducts obtained by $^{32}$P-postlabeling is puzzling, particularly in the case of a compound known to be poorly metabolized. Irrespective to the relative intensities, up to 31 spots were observed in both rat kidney, liver, and testis [51]. For comparison, in the case of aflatoxin $B_1$, a hepatocarcinogen acting by intercalation in DNA with a well established adduction mechanism with dGuo, only a single adduct was characterized as the *trans*-8,9-dihydro-8-($N^7$-guanyl)-9-hydroxyaflatoxin $B_1$ [52, 53].

Among the many and diverse "adduct spots" detected by $^{32}$P-postlabeling, one of them was reported to comigrate with dGuoOTA, a carbon-bound OTA-deoxyguanosine adduct which is formed *in vitro* by photochemical reaction of OTA in the presence of dGuo *via* a radical reaction [24, 25]. Adduct concentrations of dGuoOTA in rat kidney chronically treated with OTA at a dose of 0.4 mg/kg b.w. (3 days/wk), and in kidneys of pigs exposed to OTA at 20 µg/kg b.w. for 3 wk were reported in the range of 15 – 17 dGuoOTA/$10^9$ nucleotides, when analyzed by $^{32}$P-postlabeling. In view of the substantial evidence arguing against metabolic activation of OTA and covalent binding to DNA, as well as the poor resolution and low specificity of the postlabeling method, it is widely considered [12] that the results presented by Faucet *et al.* [25] do not provide convincing evidence for DNA adduct formation by OTA. Therefore, our aim was to try to detect the fully characterized 2′-deoxyguanosine-carbon 8-bound ochratoxin A adduct (dGuoOTA) in kidney DNA of rats treated with various doses of OTA, using LC-MS/MS to ensure the required high selectivity which cannot be achieved by $^{32}$P-postlabeling. The LC-MS/MS method reported in the present work is an improvement of an existing method, already used to attempt the detection of dGuoOTA in OTA-treated rat tissues [23]. The previous method failed to detect dGuoOTA, and it was concluded that this adduct was not formed *in vivo*. However, the lack of adducts could be challenged by uncomplete DNA digestion or insufficient sensitivity, since digestion efficiency was not tested in detail with modified oligonucelotides and/or model DNA, and no internal standard was employed to accurately estimate the matrix effect. To circumvent these limitations and provide unambiguous data, it was decided to improve the LC-MS/MS quantification of dGuoOTA by focusing on the three aspects listed hereafter: (i) use of column switching technique [54] to avoid loss of sensitivity induced by matrix effects [55] and to be able to load more sample and therefore decrease the LOD, (ii) use of the isotope dilution approach (with $^{15}N_5$-dGuoOTA as internal standard) to ensure an accurate quantification [56], and (iii) control and optimize the enzymatic digestion with $CTG^{OTA}TC$ and irradiated ctDNA to ensure a complete release of the adduct as nucleoside and therefore avoid false negative responses.

DNA samples were obtained from male F344 rats (which are most susceptible to renal tumor formation by OTA) treated with OTA for up to 90 days at a dose known to induce high incidences (74%) of renal tumors. Additional samples were obtained from rats repeatedly administered relatively high doses of OTA (up to 2 mg/kg b.w.), which were previously shown to cause DNA strand breakage [35]. However, dGuoOTA was not detected in any of these samples, despite detection limits as low as 3.5 dGuoOTA/$10^9$ nucleotides (Table 1). While our analyses clearly demonstrate that dGuoOTA was not formed under these treatment conditions, it needs to be emphasized that renal histopathological alterations thought to be critical for tumor formation were evident in both studies [27, 29], and that high OTA blood levels were determined. With respect to data from the literature which indicate that dGuoOTA was formed at a level of 7 adducts/$10^9$ nucleotides in kidney DNA from rats treated with a single OTA dose at 70 µg/kg b.w. [37], and according to the analytical performance of the method reported in the current work (sufficient LOD for detecting 3.5 adducts/$10^9$ nucleotides), the dGuoOTA adduct should have been detected in the rat samples of the present study.

Collectively, these data suggest that the mechanism by which OTA exerts its adverse effects in kidney, including carcinogenicity, does not involve formation of dGuoOTA, consistent with previous reports demonstrating lack of covalent DNA binding.

*Parts of the authors work were supported by the Fifth RTD Framework Programme of the European Union, Project No.: QLK1-2001-01614.*

*The authors have declared no conflict of interest.*

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