Simultaneous determination of N⁷-alkylguanines in DNA by isotope-dilution LC-tandem MS coupled with automated solid-phase extraction and its application to a small fish model

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In the present study, we report the development of a sensitive and selective assay based on LC (liquid chromatography)–MS/MS (tandem MS) to simultaneously measure N⁷-MeG (N⁷-methylguanine) and N⁷-EtG (N⁷-ethylguanine) in DNA hydrolysates. With the use of isotope internal standards (¹⁵N₅-N⁷-MeG and ¹⁵N₅-N⁷-EtG) and on-line SPE (solid-phase extraction), the detection limit of this method was estimated as 0.42 fmol and 0.17 fmol for N⁷-MeG and N⁷-EtG respectively. The high sensitivity achieved here makes this method applicable to small experimental animals. This method was applied to measure N⁷-alkylguanines in liver DNA from mosquito fish (*Gambusia affinis*) that were exposed to NDMA (*N*-nitrosodimethylamine) and NDEA (*N*-nitrosodiethylamine) alone or their combination over a wide range of concentrations (1–100 mg/l). Results showed

that the background level of N⁷-MeG in liver of control fish was $7.89 \pm 1.38 \mu\text{mol/mol}$ of guanine, while N⁷-EtG was detectable in most of the control fish with a range of 0.05–0.19 $\mu\text{mol/mol}$ of guanine. N⁷-MeG and N⁷-EtG were significantly induced by NDMA and NDEA respectively, at a concentration as low as 1 mg/l and increased in a dose-dependent manner. Taken together, this LC-MS/MS assay provides the sensitivity and high throughput required to evaluate the extent of alkylated DNA lesions in small animal models of cancer induced by alkylating agents.

Key words: DNA alkylation, liquid chromatography–tandem MS (LC-MS/MS), *N*-nitrosamine, on-line solid-phase extraction, small fish model.

INTRODUCTION

Exposure to carcinogenic agents can lead to the formation of covalently bound adducts in DNA. Alkylating agents, including *N*-nitrosamines, are a well-characterized group of DNA-damaging agents that are widespread in the environment. Alkylation at the N⁷ position of guanine in DNA represents a good biomarker for determining exposure to alkylating agents, since it is the predominant reaction site and is slowly repaired by a base-excision repair pathway involving DNA glycosylases [1]. Although N⁷-alkylguanine adducts are not considered to be promutagenic, they are hydrolytically unstable and undergo spontaneous depurination to produce apurinic sites and single strand breaks in DNA. If not repaired, apurinic sites can potentially cause G to T transversions [2].

A variety of analytical techniques has been developed for quantification of alkylated DNA adducts, including HPLC with UV [3], electrochemical [4] or fluorescence detection [5], immunochemical methods [6], GC/MS [7] and ³²P-post-labelling [8,9]. Although these methods have been successful they have drawbacks, such as being labour-intensive or requiring chemical derivatization, and can have low sensitivity or limited specificity due to possible interferences arising from the complex biological matrix. Recently, LC (liquid chromatography)–MS/MS (tandem MS) has become a powerful technology to overcome the sensitivity and specificity issues in analysis of DNA adducts [10]. Accurate quantification of adducted bases at extremely low concentrations has frequently relied on the use of non-radioactive isotope-label-

led standards to compensate for the loss of analyte during sample preparation, which has been the most critical step in eliminating the matrix effect for analysis of modified bases by MS [11]. Moreover, the on-line sample extraction using a column-switching device is an extremely useful technique to prepare biological samples automatically for LC-MS methods [12]. Its advantages include less ion-suppression, relatively short run-time as well as higher sensitivity and selectivity, especially for the biological samples containing a considerable amount of co-eluting interferences.

Over the past decade, small fish species have been proven useful both as environmental sentinels and as versatile animals in toxicity and carcinogenicity bioassays [13–15]. The use of small fish models in cancer research has the obvious advantages of economy, rapid response, low background incidence of tumours and the opportunity to work large numbers of specimens when compared with the more traditional rodent models [16]. However, only a few studies have attempted to measure specific adduct levels in small fish species exposed to alkylating carcinogens [i.e. NDMA (*N*-nitrosodimethylamine) and NDEA (*N*-nitrosodiethylamine)], which are widely utilized for liver tumour induction. This paucity is perhaps due to the fact that most of the existing analytical methods were not sensitive or reliable enough to utilize such small amounts of tissue available from these species [17].

In order to analyse the trace levels of N⁷-alkylguanines in the limited amounts of DNA samples available, an analytical method with high sensitivity and specificity is required. In the present study, an isotope-dilution LC-MS/MS method coupled

Abbreviations used: CAD, collision-assisted dissociation; CV, coefficient of variation; ESI, electrospray ionization; Gua, guanine; LC, liquid chromatography; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; MS/MS, tandem MS; N⁷-EtG, N⁷-ethylguanine; N⁷-MeG, N⁷-methylguanine; NDEA, *N*-nitrosodiethylamine; NDMA, *N*-nitrosodimethylamine; SPE, solid-phase extraction.

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Figure 1 Timing scheme for the column-switching system

with an on-line SPE (solid-phase extraction) system was first developed for the rapid and simultaneous analysis of N7-MeG (N⁷-methylguanine) and N7-EtG (N⁷-ethylguanine) in DNA. This method was then applied to quantitate the amounts of N7-MeG and N7-EtG in liver of small fish exposed to NDMA and NDEA alone or in combination under controlled laboratory conditions. Mosquito fish (*Gambusia affinis*) was chosen as a test species, because it is sensitive to chemical induction of liver neoplasia and can be cultured easily in the laboratory [18].

EXPERIMENTAL

Chemicals

Solvents and salts were of analytical grade. Reagents were purchased from the indicated sources: Triton X-100, desferrioxamine mesylate, SDS, proteinase K, dimethyl sulfate, diethyl sulfate, *N,N*-dimethylacetamide, NDMA and NDEA (Sigma-Aldrich); RNase A and RNase T1 (Roche); ¹⁵N₅-dG (¹⁵N₅-deoxyguanosine; Cambridge Isotope Laboratories); guanine, N7-MeG N7-EtG (Merck). The internal standards ¹⁵N₅-N7-MeG (¹⁵N₅-N7-methylguanine) and ¹⁵N₅-N7-EtG (¹⁵N₅-N7-ethylguanine) were synthesized as described previously [19,20].

Automated on-line extraction system

The column-switching system used in this study was as described in detail previously [21]. In this study, it consisted of a switching valve (two-position microelectric actuator; Valco) and a Nucleosil NH₂ cartridge (35 mm long × 4.6 mm internal diameter, 10 μm particle size). The switching valve function was controlled using PE-SCIEX control software (AnalystTM; Applied Biosystems). Figure 1 summarizes the detailed column-switching operation sequence. When the switching valve was at position A, 100 μl of prepared DNA sample was loaded on to the trap cartridge using an autosampler (PE series 200; PerkinElmer) and a quaternary pump (PE series 200; PerkinElmer) delivered in 0.1 % (v/v) formic acid in 96 % (v/v) acetonitrile at a flow rate of 1 ml/min as the loading and washing buffer. The trap cartridge was flushed with the loading buffer for 2 min, followed by valve switching to injection position B to inject the sample into the LC system. At 4 min after injection, the valve was switched back to the loading position A, and the trap cartridge was washed using a mobile phase with linear gradient from 100 % of 0.1 % formic acid in 96 % (v/v) acetonitrile to 100 % of 0.1 % formic acid in 50 % (v/v) acetonitrile for 6 min, followed by 100 % of 0.1 % formic acid in 96 % (v/v) acetonitrile for 1 min for equilibration of the trap cartridge and preparation for the next analysis. The total run time was 15 min.

LC

After automatic sample clean-up (see Figure 1 at the 2 min time point), the sample was automatically transferred on to a Poly-

amine-II endcapped HPLC column (150 mm × 4.6 mm internal diameter, 5 μm; YMC) in backwash mode. The gradient mode was used to achieve the desired sample separation using solvent A [0.1 % formic acid in 90 % (v/v) acetonitrile] and solvent B [0.1 % formic acid in 80 % (v/v) acetonitrile] at a flow rate of 1 ml/min. The following gradient was run: 0 to 3.0 min, 0 % solvent B; 3.0 to 13.0 min, 100 % solvent B; 13.0 to 14.0 min, 0 % solvent B; 14.0 to 15.0 min, 0 % solvent B.

ESI (electrospray ionization)-MS/MS

The sample eluted from the HPLC system was introduced into a TurboIonSpray[®] source installed on an API 3000TM triple-quadrupole mass spectrometer (Applied Biosystems), operated in positive mode with a needle voltage of 5.5 kV, using nitrogen as the nebulizing gas and with the turbogas temperature set at 500 °C. Data acquisition and quantitative processing were accomplished using AnalystTM software, version 1.1 (Applied Biosystems). Optimal MRM (multiple reaction monitoring) conditions were obtained for six channels: N7-MeG (*m/z* 166–149 and 166–124), ¹⁵N₅-N7-MeG (*m/z* 171–153), N7-EtG (*m/z* 180–152 and 180–135) and ¹⁵N₅-N7-EtG (*m/z* 185–157). The dwell times per channel were set to 150 ms for the analytes and 100 ms for the internal standards. Nebulizer and curtain gas flow-rates were set to 15 (arbitrary units). CAD (collision-assisted dissociation) gas and turbogas were set at 12 and eight (arbitrary units) respectively. The collision energy was set at 30 eV for N7-MeG and 25 eV for N7-EtG with nitrogen as the collision gas. Peak widths were set at 0.7 Th (full width at half maximum) for both Q1 and Q3.

Fish treatment

Adult, 30–35-mm-long, laboratory-reared mosquito fish were maintained at 25 ± 1 °C with a 12-h light/dark cycle for at least 2 weeks. Fish were fed commercial flake food twice a day, except for the 24 h before treatment. Fish were then respectively exposed to 1.5 litres of 0, 1, 10, 50 and 100 mg/l NDMA or NDEA alone and mixtures containing equal concentrations of NDMA and NDEA (0, 10 and 50 mg/l of each). Each dose was performed in triplicate and each treatment contained four fish. Exposures were static and conducted in 2 litre beakers for 96 h with daily renewal of the test medium. Beakers were placed in a water bath that was maintained at 25 ± 1 °C, and fish were not fed during the exposure period. At the end of the exposure period, fish were rinsed in clean water three times, and then killed. The livers were immediately removed, pooled at four per sample, and stored at –80 °C until DNA isolation. The total wet weight of the four pooled mosquito fish livers ranged from 40 to 80 mg.

Isolation and preparation of DNA samples for N7-alkylguanine analysis

DNA was isolated from liver tissue according to the procedures described by Ravanat et al. [22] with several modifications. Briefly, approx. 40 mg of liver tissue was homogenized with 3 ml of buffer A [320 mM sucrose, 5 mM MgCl₂, 10 mM Tris/HCl, pH 7.5, 0.1 mM desferrioxamine and 1 % (v/v) Triton X-100]. After homogenization, the sample was centrifuged at 1500 *g* for 10 min. The resultant pellet was washed with 1.5 ml of buffer A and recovered by centrifugation (1500 *g* for 10 min). A total of 600 μl of buffer B (10 mM Tris/HCl, pH 8, 5 mM EDTA and 0.15 mM desferrioxamine) and 35 μl of 10 % (w/v) SDS was added, and the samples agitated vigorously. After 30 μl of RNase A (1 mg/ml) in RNase buffer (10 mM Tris/HCl, pH 7.4, 1 mM EDTA, and 2.5 mM desferrioxamine) and 8 μl of RNase T1 (1 unit/μl in RNase buffer) were added and the samples incubated

at 37°C for 1 h to remove contaminating RNA from the DNA. After the removal of RNA, 30 µl of proteinase K (20 mg/ml) was added and the samples were incubated at 37°C for 1 h. Subsequently, 1.2 ml of NaI solution (7.6 M NaI, 40 mM Tris/HCl, pH 8.0, 20 mM EDTA and 0.3 mM desferrioxamine) and 2 ml of propan-2-ol were added. The sample was gently shaken until the DNA had precipitated completely and then centrifuged at 5000 g for 15 min. The DNA pellet was washed with 1 ml of 40% (v/v) propan-2-ol. After centrifugation (5000 g for 15 min) the DNA pellet was washed with 1 ml of 70% (v/v) ethanol. Finally, the DNA pellet was collected by centrifugation (5000 g for 15 min) and dissolved in 200 µl of 0.1 mM desferrioxamine overnight. DNA concentration was measured by the absorbance at 260 nm. Routinely, 40–80 µg of DNA was obtained per 40 mg of liver tissue. Protein contamination was checked using the A_{260}/A_{280} ratio, where an absorbance ratio over 1.6 was acceptable. RNA contamination was checked by HPLC with UV detection after digestion to nucleosides [23] and was found to be less than 0.1%.

DNA samples (10 µg) were spiked with 376 fmol of ¹⁵N₅-N⁷-MeG and 240 fmol of ¹⁵N₅-N⁷-EtG. The DNA solutions were subjected to neutral thermal hydrolysis at 100°C for 30 min to release N⁷-alkylguanines by cleavage of the glycosidic bond [24]. The partially depurinated DNA backbone was precipitated by addition of 2 vol. of ice-cold ethanol and was centrifuged at 5000 g for 15 min. The supernatant was dried under vacuum and redissolved in 200 µl of 96% (v/v) acetonitrile with 0.1% formic acid for N⁷-alkylguanine adduct analysis. The N⁷-alkylguanine adduct standard stock solution was prepared by dissolving an equal amount of N⁷-MeG and N⁷-EtG in 96% (v/v) acetonitrile/0.1% formic acid; it was then serially diluted 1:1 with 96% (v/v) acetonitrile/0.1% formic acid to yield aqueous standard solutions for establishing the linear calibration curve. Two linear ranges were determined for both N⁷-MeG and N⁷-EtG from 0.24–7.81 pg (low range: 0.24, 0.49, 0.98, 1.95, 3.91 and 7.81 pg) and 7.81–250 pg (high range: 7.81, 15.6, 31.3, 62.5, 125 and 250 pg); each calibrator contained 376 fmol of ¹⁵N₅-N⁷-MeG and 240 fmol of ¹⁵N₅-N⁷-EtG. The levels of N⁷-alkylguanines in liver DNA were expressed as µmol/mol of guanine. The analysis of guanine was performed as described below.

Determination of guanine

Guanine concentrations were determined from aliquots (< 1 µg) obtained from each DNA sample prior to the addition of the ¹⁵N₅-N⁷-alkylguanine internal standards. The guanine content was determined by an isotope-dilution LC-MS/MS method. Stable isotope internal standard, ¹⁵N₅-Gua (¹⁵N₅-guanine), was simply obtained by treatment of ¹⁵N₅-dG with 1 M HCl at 80°C for 30 min, and was purified by a semi-preparative HPLC system. Guanine was released from fish DNA by mild acid hydrolysis (80°C for 30 min in 200 µl of 0.1 M HCl). Aliquots (20 µl) of the guanine samples were diluted 50 times with 5% (v/v) methanol containing 20 mM ammonium acetate. A 100 µl aliquot of diluted guanine sample was spiked with 4.64 pmol of ¹⁵N₅-Gua as internal standard, and then vortex-mixed for approx. 5 s; 20 µl of the sample solution was then injected into the same LC-MS/MS as described above.

The analytical column was a Supelco Discovery C₁₈ (150 mm × 2.1 mm internal diameter, 5 µm; Bellefont). The gradient mode was used to achieve the separation of analytes using mixtures of mobile phase A [5% (v/v) methanol with 20 mM ammonium acetate] and mobile phase B (100% methanol) at a flow rate of 250 µl/min. The following gradient was run: 0 to 6.0 min, 0% mobile phase B; 6.0 to 10.0 min, 100% mobile phase B; 10.0 to 11.0 min, 0% mobile phase B; 11.0 to 13.0 min, 0%

mobile phase B. The fragmentation pattern of protonated guanine observed in the present study (results not shown) was consistent with that reported by Weimann et al. [25]. The transition of the $[M + H]^+$ precursor ion of guanine to $[MH - NH_3]^+$ resulted in the product ion with highest intensity. Therefore, for the MRM analysis, the transition of m/z 152–135 was chosen for guanine and the corresponding transition of m/z 157–139 for ¹⁵N₅-Gua. The dwell times were set to 150 ms. Nebulizer and curtain gas flow-rates were set to 12. CAD gas and turbogas were set at 6 and 8 respectively and the source heater probe temperature was set at 350°C.

RESULTS

LC-MS/MS characteristics of N⁷-MeG and N⁷-EtG

Product ion spectra of N⁷-alkylguanines and their isotope internal standards are shown in Figure 2. The spectra were recorded by selecting the protonated ion ($[M + H]^+$) in the first quadrupole (Q1). After collision activation of the selected ions in the collision cell, the daughter ion spectra were recorded by scanning the last quadrupole (Q3). The transition of the $[M + H]^+$ precursor ion of methylated guanine to $[MH - NH_3]^+$ resulted in the product ion with highest intensity, whereas the dominant product ion of ethylated guanine was observed at the transition of the $[M + H]^+$ precursor ion to guanine. In the present study, the most abundant fragment ion (quantifier ion) was used for quantification and the second abundant fragment ion (qualifier ion) for confirmation of the identity of analyte. For the isotope internal standards only one fragment ion was selected. Therefore, the samples were analysed in the positive ion MRM mode and the transitions of the precursors to the product ions were as follows: m/z 166–149 (quantifier) and 166–124 (qualifier) for N⁷-MeG, m/z 171–153 for ¹⁵N₅-N⁷-MeG, m/z 180–152 (quantifier) and 180–135 (qualifier) for N⁷-EtG, and m/z 185–157 for ¹⁵N₅-N⁷-EtG.

Figure 3 shows a typical on-line SPE LC-MS/MS chromatogram for a liver DNA sample from mosquito fish. This fish sample was exposed to a mixture of 10 mg/l NDMA and 10 mg/l NDEA for 4 days before extraction. The retention times were 9.0 and 7.5 min for N⁷-MeG and N⁷-EtG respectively, with a total analysis time of 15 min per sample.

LOQ (limit of quantification) and LOD (limit of detection)

The LOQ was defined as the lowest concentration of N⁷-alkylguanine adducts that could be reliably and reproducibly measured with values for accuracy, intra- and inter-day imprecision [CV (coefficient of variation)] < 20%. Using the present method, the LOQ of N⁷-MeG and N⁷-EtG were determined to be 2.0 pg/ml on-column (1.21 fmol in an injection volume of 100 µl) and 1.0 pg/ml on-column (0.56 fmol in an injection volume of 100 µl) respectively, based on the direct measurement of diluted calibration solutions. The LOD, defined as the lowest concentration that gave a signal-to-noise ratio of at least three, were found to be 0.7 pg/ml on-column (0.42 fmol) and 0.3 pg/ml on-column (0.17 fmol) for N⁷-MeG and N⁷-EtG respectively, which corresponds to 0.055 and 0.022 µmol adducts/mol of guanine when using 10 µg of DNA per analysis.

Linearity, precision and recovery

Calibration of the assay was performed by the addition of a fixed amount of ¹⁵N₅-N⁷-MeG (376 fmol) and ¹⁵N₅-N⁷-EtG (240 fmol) internal standards with various amounts of N⁷-MeG and N⁷-EtG standard solutions ranging from 0.24 to 250 pg (corresponds to 1.48 fmol to 1.52 pmol for N⁷-MeG and 1.36 fmol to 1.40 pmol for N⁷-EtG). As shown in Figure 4, the on-line SPE LC-MS/MS

Figure 2 Product ion spectra

Product ion spectra of $[M + H]^+$ of N7-MeG (**A**), $^{15}\text{N}_5$ -N7-MeG (**B**), N7-EtG (**C**) and $^{15}\text{N}_5$ -N7-EtG (**D**).

standard curves for N7-MeG and N7-EtG showed excellent linearity ($r^2 > 0.999$) at both the high and low ranges. Over the entire concentration range of the calibration curves, the mean observed percentage deviation of back-calculated concentrations was between -4.1% and $+6.2\%$ for N7-MeG and -3.4% and $+5.3\%$ for N7-EtG, with an imprecision (CV) $< 10\%$.

The precision of the present method was evaluated by performing replicate determinations of N⁷-alkylguanines in pooled liver DNA of fish, which were exposed to 10 mg/l of NDEA (Table 1). The intra-day imprecision was 4.8% for N7-MeG and 6.9% for N7-EtG. The inter-day test was carried out by assaying the same sample on five different days over a period of 120 days. The inter-day imprecision of N7-MeG and N7-EtG were determined to be 4.8 and 7.1% respectively. Recovery was evaluated by spiking 20–500 fmol of the unlabelled N7-MeG and N7-EtG standards with 10 μg aliquots of the pooled DNA and measuring three replicates of these samples. All of the standards were added prior to DNA hydrolysis. As shown in Table 1, the recovery of the present method, as calculated from the slope of the regression, was 99.3% for N7-MeG and 101.7% for N7-EtG ($r^2 > 0.99$), and the mean recovery was 98.5% and 99.8% for N7-MeG and N7-EtG respectively, as estimated from the increase in the measured amount after addition of N⁷-alkylguanine divided by the amount added.

Matrix effects

Matrix effects were calculated from the peak areas of the internal standard added to the calibrator solutions and compared with the

peak areas of the internal standard that was added to each DNA sample [26,27]. The relative change in peak area of the internal standard was attributed to matrix effects, which reflect both on-line extraction losses and ion suppression due to the DNA matrix. In the present study, the matrix effects were found to be less than 15% for both N7-MeG and N7-EtG in all DNA samples. Although, the use of stable isotope-labelled internal standards could have compensated for different matrix effects, a low matrix effect achieved in this study ensures a high sensitivity of the method.

N⁷-alkylguanines in liver DNA of mosquito fish treated with NDMA and NDEA

The present method was applied to quantify the levels of N⁷-alkylguanine adducts in liver DNA from a series of NDMA-, NDEA- or combination-treated fish. The background level of N7-MeG in liver of control fish was $7.89 \pm 1.38 \mu\text{mol/mol}$ of guanine (equivalent to 2.96 ± 0.55 per 10^6 bp) and NDMA treatment alone induced a linear dose-response increase in N7-MeG levels (Figure 5A). The amount of N7-EtG in liver of control fish was detectable in seven out of nine samples, with a range of 0.05–0.19 $\mu\text{mol/mol}$ of guanine (equivalent to 0.02–0.076 per 10^6 bp). N7-EtG was clearly detectable in liver DNA at an NDEA concentration as low as 1 mg/l and a sublinear dose-response for N7-EtG levels was found with increasing concentrations of NDEA (Figure 5B). When fish received combined treatments of NDMA and NDEA, both N7-MeG and N7-EtG were dose-dependently increased (Figure 5C).

Figure 3 Chromatograms of a liver DNA sample from mosquito fish that were exposed to a mixture of 10 mg/l NDMA and 10 mg/l NDEA for 4 days

N7-MeG was monitored at (A) m/z 166 → 149 and (B) m/z 166 → 124, and the internal standard $^{15}\text{N}_5$ -N7-MeG monitored at (C) m/z 171 → 153. N7-EtG monitored at (D) m/z 180 → 152 and (E) m/z 180 → 135, and the internal standard $^{15}\text{N}_5$ -N7-EtG was monitored at (F) m/z 185 → 157. cps, counts per second.

DISCUSSION

The present study describes a sensitive and reliable LC-MS/MS method for the simultaneous determination of N7-MeG and N7-EtG in small amounts of DNA. With the use of isotope internal standards and on-line SPE, this method exhibited remarkably low LODs of 0.42 fmol and 0.17 fmol on-column for N7-MeG and N7-EtG respectively, and a total analysis time per sample as short as 15 min. The use of LC-MS/MS has been proposed

Figure 4 Calibration curve obtained by plotting the ratio between the area of the analyte peak divided by the area of the internal standard peak as a function of the amount of analyte

Linear regression was calculated with non-weighting and non-zero-forced. (A) Calibration curve for N7-MeG: the main panel shows the high-range calibration (0.0473–1.515 pmol; $y = 2.5741x - 0.0015$, $r^2 = 1$), and the inset shows the low-range calibration (0.00148–0.0473 pmol; $y = 2.4886x + 0.0002$, $r^2 = 0.9994$). (B) Calibration curve for N7-EtG: the main panel shows the high-range calibration (0.0436–1.397 pmol; $y = 4.5391x - 0.0218$, $r^2 = 0.9998$), and the inset shows the low-range calibration (0.00136–0.0436 pmol; $y = 4.3423x - 0.0027$, $r^2 = 0.9993$).

previously for the analysis of N7-MeG in DNA by Yang et al. [28] and Zhang et al. [29], who reported LODs of 64 and 76 fmol respectively. Singh et al. [30] developed an LC-MS/MS method to quantify the N7-EtG in DNA, which resulted in an LOD of 2.0 fmol. Apparently, our newly developed on-line sample purification/enrichment system coupled with isotope-dilution LC-MS/MS has an even better sensitivity than LC-MS/MS methods published previously. A lower amount of DNA needed would be expected when this method was applied in the determination of N⁷-alkylguanines.

The fragmentation pattern of N7-MeG and N7-EtG observed in the present study (Figure 2) was consistent with that reported by Zhang et al. [29] and Singh et al. [30] respectively. Previous methods have not adopted a qualifier ion, probably because of the lack of sensitivity for the qualifier ion. In the present study, the most abundant fragment ion was used for quantification,

Table 1 Precision and recovery of on-line SPE LC-MS/MS method for N⁷-alkylguanine analysis

For precision, the values are means $\pm$ S.D., with CV (%) in parentheses. The mean recovery, with the range in parentheses is shown. Each value was based on five repeated analyses of the pooled liver DNA from mosquito fish exposed to 10 mg/l of NDEA; inter-day test was carried out by five freeze/thaw cycles over a period of 120 days. Recovery was estimated by the addition of N⁷-alkylguanines in five different amounts (20, 50, 100, 200 and 500 fmol) to 10 μ g aliquots of the pooled DNA. The recovery was estimated from (a) the slope of the regression of measured N⁷-alkylguanine versus added N⁷-alkylguanine and (b) the increase in measured amount after addition of N⁷-alkylguanine divided by the amount that was added.

	Precision		Recovery	
	Intra-day variation (fmol)	Inter-day variation (fmol)	Slope of regression	Mean recovery (%)
N ⁷ -MeG	62 $\pm$ 3 (4.8)	62 $\pm$ 3 (4.8)	0.993	98.5 (91.4–103.7)
N7-EtG	29 $\pm$ 2 (6.9)	28 $\pm$ 2 (7.1)	1.017	99.8 (92.9–105.8)

whereas a second, less abundant, ion was used for confirmation, since the LODs of N7-MeG and N7-EtG were low enough to include a qualifier ion.

In addition to LC-MS/MS, several attempts have been made previously to monitor the formation of N7-MeG or N7-EtG in biological samples [9,24,31]. Among these, the ³²P-post-labelling approach, despite being time consuming and requiring large amounts of radioactivity, has the advantage of high sensitivity. Haque et al. [8] used ³²P-post-labelling assays for quantification of N7-MeG in human DNA and obtained a 1.3 fmol detection limit, whereas Kato et al. [32] reported an LOD of 0.76 fmol for N7-EtG by HPLC combined with ³²P-post-labelling. The on-line SPE LC-MS/MS method in the present study provided a comparable sensitivity with ³²P-post-labelling. Furthermore, our method offers numerous advantages over the ³²P-postlabelling assays for the detection of N⁷-alkylguanine adducts (i.e. high selectivity and fast analysis) and avoids problems of dealing with radioactive materials and low labelling efficiencies due to depurination. It has been suggested that there were background levels of $\sim$ 2.5 N7-MeG adducts/10⁶ dGp [33] and $\sim$ 0.2 N7-EtG adducts/10⁶ dGp [34] in human tissues, which were equivalent to $\sim$ 19 fmol N7-MeG and $\sim$ 1.5 fmol N7-EtG in 10 μ g of DNA. This suggests that our newly developed on-line SPE LC-MS/MS method (LODs: 0.42 fmol for N7-MeG and 0.17 fmol for N7-EtG) is also capable of quantifying the level of N⁷-alkylguanines in small amounts of human DNA (<10 μ g) and could be a useful tool for surveillance of alkylating agent exposure.

Detection of the base adduct is particularly suited to the analysis of chemical carcinogens that form DNA adducts that are chemically labile. The labile base adducts (i.e. N⁷-alkylguanine and N³-alkyladenine) can be released from DNA by neutral thermal hydrolysis and then separated from the depurinated DNA by filtration/centrifugation [11]. It was argued that acid thermal hydrolysis would lead to the cleavage of unmodified DNA bases, which can cause suppression of ionization for the analytes during LC-MS/MS analysis [35]. Hence, a purification step is normally required before the LC-MS/MS analysis. In the present study, acid thermal hydrolysis of DNA using 0.1 M HCl at 80°C for 30 min was also attempted, but interestingly direct injection of the acid hydrolysates on to the on-line SPE LC-MS/MS had a comparable sensitivity and selectivity with the DNA samples subjected to neutral thermal hydrolysis (results not shown). This can be attributed to those matrix effects resulting from the presence of the unmodified bases that were largely eliminated by the automatic SPE system.

It has been suggested that methods relying on the analysis of free nucleic acid bases cannot distinguish between bases derived from DNA and RNA. However, N⁷-alkyldeoxyguanosine adducts are unstable and prone to spontaneous depurination. To prevent decomposition of N⁷-alkyldeoxyguanosine, DNA samples must

be kept cold and processed quickly [28]. Even though RNA contamination of DNA has the potential to interfere with DNA analyses, the analysis of N⁷-alkylguanine has the advantage of greater stability over that of N⁷-alkyldeoxyguanosine. This is mainly attributed to the fact that, regardless of the rates of depurination on storage, the alkylated purines cleaved from DNA are stable and can still be quantified in DNA solution [36]. In the present study, the stability of N7-alkylguanine in DNA solutions can be shown from a low inter-day CV obtained (Table 1), indicating that both N7-MeG and N7-EtG remained stable after five freeze/thaw cycles over 120 days. Moreover, the use of stable isotope-labelled internal standards in combination with tandem MS adds increased reliability and reproducibility to the present method.

The on-line SPE LC-MS/MS method was validated in liver DNA of mosquito fish that were exposed to a series of NDMA and/or NDEA for 4 days (Figure 5). In the control group, the background level of N7-MeG can be easily detected in 10 μ g of DNA. N7-MeG has been frequently detected in untreated rat liver DNA [33,37,38] and this may be attributed to endogenous or environmental methylation. N7-EtG was detectable in most of the control fish, with a range of 0.05–0.19 μ mol/mol of guanine observed in this study. Interestingly, van Delft et al. [37] also found the possible presence of $\sim$ 0.3 μ mol N7-EtG/mol of nucleotides in salmon sperm DNA by HPLC with electrochemical detection. Reasons for the presence of very tiny amounts of N7-EtG in control fish are unknown at present.

N7-MeG and N7-EtG were induced significantly by NDMA and NDEA respectively, at concentrations as low as 1 mg/l and increased in a dose-dependent manner (Figures 5A and 5B). This suggests that the present method is suitable for evaluating molecular mechanisms of carcinogenesis from chronic low-level exposure to alkylating agents in small animal models. It was noted that NDEA formed less alkyl DNA adducts than NDMA when compared on the basis of adducts formed per micromolar concentration. Meanwhile, N7-MeG in liver was proportional to the NDMA dose, whereas N7-EtG increased faster and more dramatically with increasing NDEA (Figures 5A and 5B). These results tend to confirm the previous reports that ethylating agents are less reactive than methylating agents and that ethyl purines are removed less efficiently than methyl purines [39,40]. Furthermore, when fish received combined treatments of NDMA and NDEA (Figure 5C), the combined treatment of both agents at 10 mg/l each did not generate significantly more N7-MeG and N7-EtG than either treatment alone at 10 mg/l (Figures 5A and 5B). However, interestingly, as compared with those treated with 50 mg/l NDMA or NDEA alone, the combined treatment of both agents at 50 mg/l each resulted in statistically significant increases in N7-MeG and N7-EtG ($P < 0.05$) by 36 % (from 100.6 to 136.5 μ mol/mol of guanine) and 44 % (from 17.3 to

Figure 5 Formation of N⁷-alkylguanines in liver DNA of mosquito fish

Formation of N⁷-alkylguanines in liver DNA of mosquito fish exposed for 4 days to 0, 1, 10, 50 and 100 mg/l of NDMA (**A**); 0, 1, 10, 50 and 100 mg/l of NDEA (**B**); and mixtures containing equal concentrations of NDMA and NDEA (0, 10 and 50 mg/l of each) (**C**). The results are presented as means $\pm$ S.D. ($n = 3$). All treatment groups had significantly higher corresponding adduct levels compared with controls ($P < 0.01$). Gua, guanine.

25.1 $\mu\text{mol/mol}$ of guanine) respectively. This may be explained by the saturation of detoxification and DNA repair mechanisms in the livers of fish when exposed simultaneously to both agents at high concentrations (i.e. 50 mg/l).

In conclusion, a rapid, specific and sensitive isotope-dilution LC-MS/MS method for the simultaneous detection of N7-MeG and N7-EtG in DNA has been established for the first time in the present study. When combined with on-line SPE and isotope dilution, this method could allow for high-throughput analysis of N7-MeG and N7-EtG without compromising quality and validation criteria. The developed method enables the detection

of less than one alkylated adduct per 10^7 unmodified bases using 10 μg of DNA. Despite the increased use of small fish models in cancer research, little emphasis has been placed on examining early biochemical events of carcinogenesis in small fish species. This method now makes it possible to measure alkylated DNA in small fish species treated with low concentrations of alkylating agents (as low as 1 mg/l). This method could be useful in future mechanistic studies evaluating the relationship between DNA alkylation and biological response from exposure to alkylating agents.

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