**Simultaneous detection of five different 2-hydroxyethyl DNA
adducts formed by ethylene oxide exposure, using a high-
performance liquid chromatography/electrospray ionisation
tandem mass spectrometry assay.**

Elaine M. Tompkins, Donald J.L. Jones, John H. Lamb,
Debbie A. Marsden, Peter B. Farmer
and Karen Brown.*

Cancer Biomarkers and Prevention Group, Department of Cancer Studies and
Molecular Medicine, The Biocentre, University of Leicester, University Road,
Leicester LE1 7RH, UK.

*Corresponding Author. Fax: 0116 2231840

E-mail: kb20@le.ac.uk

ABSTRACT

A method has been developed for the simultaneous detection and quantitation of five different (2-hydroxyethyl)-DNA adducts (HE-DNA adducts) that could be formed as a result of exposure to ethylene oxide (EO). In addition to the major *N*7-HE-guanine (*N*7-HEG) adducts this assay can also measure the less prevalent but potentially more biologically significant *N*1-HE-2'-deoxyadenosine (*N*1-HEdA), *O*⁶-HE-2'-deoxyguanosine (*O*⁶-HEdG), *N*⁶-HE-2'-deoxyadenosine (*N*⁶-HEdA) and *N*3-HE-2'-deoxyuridine adducts (*N*3-HEdU). The method involves the isolation of HE-adducts from the unmodified nucleosides by either neutral thermal hydrolysis or enzymatic digestion, followed by HPLC purification, before detection and quantification by liquid-chromatography tandem mass spectrometry (LC-MS/MS) using selective reaction monitoring (SRM). The limits of detection were in the range 0.5 – 25 fmoles for each individual adduct, making this one of the most sensitive assays available for the detection of *N*7-HEG. To illustrate the possible applications of the assay, it has been employed in the measurement of endogenous/background and EO-induced HE-adducts in a variety of DNA samples.

KEYWORDS: LC-MS/MS, SRM, 2-hydroxyethyl, ethylene oxide, DNA adducts.

INTRODUCTION

Ethylene oxide (EO) is a widely used chemical intermediate and gaseous sterilant¹, which is also formed endogenously through the metabolism of ethylene^{2,3} by cytochrome P450-dependant monooxygenases *in vivo*^{4,5}. Possible endogenous sources of ethylene include lipid peroxidation⁶ and intestinal bacteria metabolism⁷, together with the oxidation of free methionine^{6,8} and haemoglobin products⁹. In contrast to ethylene, which has been designated as “not classifiable as to its carcinogenicity to humans”, EO is categorised as “carcinogenic to humans” by the International Agency for Research on Cancer (IARC), based largely on findings from animal studies and sufficient evidence to support a relevant mechanism of carcinogenicity in humans¹. For example, EO reacts directly with cellular macromolecules to form covalent 2-hydroxyethyl (HE) adducts with both proteins and DNA², without the need for prior metabolic activation and studies in various *in vitro* systems and animal models have shown that EO is capable of inducing mutations^{5,10,11}, sister chromatid exchanges and chromosomal aberrations¹². However, the current data is conflicting or inconclusive regarding whether exposure to EO actually increases the incidence of human cancers¹³⁻¹⁶. Consequently, there is much interest in elucidating the mechanisms through which EO causes these effects and identifying appropriate mechanism-based biomarkers that can be monitored for human risk assessment purposes.

Alkyl epoxides react predominantly with strong nucleophilic endocyclic ring nitrogens of DNA bases, via an S_N2 mechanism⁴. For EO, the principal site of 2'-hydroxyethylation is the N7-position of guanine, forming N7-hydroxyethylguanine (N7-HEG)¹⁷, which is illustrated in Figure 1 alongside the other possible DNA adducts formed by EO¹⁸. Alkylation of the N7 position of guanine produces a

quaternary ammonium ion, leading to cleavage of the *N*-glycosidic bond¹⁹, which if not repaired can result in an apurinic (AP) site. *N*7-HEG adducts are not considered to be promutagenic themselves, although presence of AP sites during DNA replication could result in miscoding^{20,21}. Being the predominant EO alkylation product however, measures of *N*7-HEG can provide a valuable biomarker of exposure. Very little is known about the properties of the other 2-hydroxyethyl DNA adducts, which together account for less than 5% of the reaction products when EO is incubated with DNA *in vitro*². It seems likely that if formed, they may contribute to the biological effects of EO, given that O⁶-alkylguanine adducts have been widely demonstrated to be mutagenic²². In addition, *N*3-HE-2'-deoxycytidine undergoes spontaneous hydrolytic deamination to form *N*3-HEdU^{23,24}, which has been suggested as a potentially mutagenic adduct²⁵ and adduction at the *N*1-position of adenine is proposed to be a miscoding lesion^{26,27}.

A variety of methods for the detection and quantitation of EO-derived DNA adducts have been reported, however, virtually all published assays have focused solely on measurement of the major *N*7-HEG adduct, excluding the lesser formed adducts^{28,29}. ³²P-Postlabelling³⁰⁻³⁴ approaches can detect *N*7-HEG in control rat liver at levels of 1 adduct per 10⁷ nucleotides³³ and similar amounts have been detected in the lymphocytes of unexposed humans³⁴. However, the assay procedure can be rather lengthy, it provides no structural information and the analysis is highly inefficient due to spontaneous depurination of the *N*7-HEG adduct, which must remain in the nucleotide form in order to be detectable. In a comparison of different immunoassay methods for *N*7-HEG detection³⁵ an immunoslot blot assay was found to be the most sensitive, although the limit of detection (LOD) was in the region of 0.3 adducts per

10⁶, considerably higher than other methods. Furthermore, immunoassays require antibodies specific to the adduct in question and these are not currently available for any other HE-adducts, limiting detection to the major N7-HEG lesion. HPLC with fluorescence detection has been used to measure the dosimetry of N7-HEG in rodents and could also detect O⁶-HEGuanine and N3-HEAdenine adducts in rats exposed to high doses (300 ppm) of EO, but the technique lacked the necessary sensitivity to detect O⁶-HEG and N3-HEA in unexposed animals³⁶. Mass spectrometry based methods afford greater specificity and the sensitivity of these systems is continually improving³⁷⁻³⁹. As very little is known about the frequency and biological effects of the lesser formed HE adducts, we have developed and validated a novel, highly sensitive, and specific LC-MS/MS method for the simultaneous detection and quantitation of five adducts that can potentially be derived from ethylene oxide exposure. This is useful to the study of HE-DNA adducts as previous assays could only detect the N7-HEG adduct. We also demonstrate various applications of the assay; for the detection of levels of endogenous/ background HE adducts as well as adducts induced by EO treatment in samples including DNA extracted from the liver of control and treated rats.

EXPERIMENTAL

Caution:

EO is classified as a human carcinogen and should be handled with the appropriate precaution.

Chemicals

Ethylene oxide gas (99.8 % purity), nucleosides and DNA bases for the synthesis of adduct standards and the enzymes snake venom phosphodiesterase and DNase I were purchased from Sigma (Poole, Dorset, UK). The O⁶-HEdG adduct standard was kindly provided by Dr Didier Gasparutto (Laboratoire des Lésions des Acides Nucléiques, CEA Grenoble, France). [¹⁵N₅]-Labelled 2'-deoxynucleosides were from Spectra Stable Isotopes (Columbia, MD, USA) and HPLC solvents from Fisher Scientific (Loughborough, UK). Shrimp alkaline phosphatase was obtained from Roche (East Sussex, UK).

Synthesis of DNA adduct standards

Hydroxyethyl (HE)-nucleoside and base adducts were required as authentic standards for the LC-MS assay. It was envisaged that nucleoside adducts which readily depurinate, such as the N7-HEdG, would be more accurately and reliably quantified in the free base form, liberated by hydrolysis of adducted DNA, whilst for the more chemically stable adducts it would be more appropriate to quantify the nucleoside lesion, which could be isolated after DNA digestion. Therefore, a method was established which permitted simultaneous detection of specific ethylene oxide derived adducts in either the base (N7-HEG) or nucleoside (N1-HEdA, N⁶-HEdA, O⁶-HEdG, N3-HEdU) form and these adducts were synthesised, along with two ¹⁵N-labelled internal standards ([¹⁵N₅]-N7-HEG and [¹⁵N₅]-N1-HEdA), for use in the development and validation of the assay.

For the production of nucleoside adducts dA, dU, and [¹⁵N₅]-dA, 2 mmoles of each were dissolved in H₂O (20 mL) and reacted with 2.3 mM EO for 18h at 37°C, then concentrated by freeze-drying. EO was condensed on ice and the required volume

added directly to the dissolved nucleosides to obtain the correct concentration. To generate the *N*7-HEG base standards, guanosine (Gua or [$^{15}\text{N}_5$]-Gua (1mmole)) was dissolved in 20 mL glacial acetic acid/ H_2O (1:1) and reacted with 2.3 mM EO for 18h at 37°C, then freeze-dried. The residue was redissolved in 1M HCl (10 mL) and heated at 100°C for 1h, then concentrated to dryness under vacuum. The sample was then dissolved in 1M HCl (4 mL) and the pH adjusted to 4.0, using 5 M NaOH and H_2O , in a final volume of 30 mL.

The products of each EO reaction (base and nucleosides) were separated by preparative HPLC, using a water:methanol gradient: 5-80 % MeOH over 25 min at a flow rate of 10 mL/ min, using a Hypersil BDS C_{18} column (250 x 21.2 mm, 5 μ , Phenomenex, Macclesfield, UK). Authentic standards were used to identify unreacted starting material and all other significant peaks were collected and evaporated to dryness for structural analysis, to identify those fractions containing 2-HE adducts. In some cases it was necessary to perform a second HPLC purification on the collected fractions and this was carried out using identical conditions.

Structural Characterisation of HE-DNA adducts

The UV spectrum was obtained for each potential adduct containing fraction (nucleoside and base) using a Hitachi U-3010 spectrophotometer (Hitachi, Wokingham, Berkshire) and compared to available published data for ethylene oxide DNA adducts¹⁸. Samples with UV spectra consistent with the addition of a hydroxyethyl group were subject to MS analysis.

HPLC fractions were analysed by mass spectrometry using a Micromass Quattro BioQ, equipped with a pneumatically assisted electrospray source (Waters, Hertfordshire, UK). The mass spectrometer was tuned using a thymidine standard in MeOH (5 pmoles/ μ L). Each HPLC peak residue was redissolved in methanol and analysed by continuous infusion. Analysis was carried out in the positive ion mode, and the molecular mass of the components of each peak was determined using a total ion scan (Figure 2). The structure of each potential HE-DNA adduct standard was further confirmed by MS/MS, and compared to the spectra for the fully characterized adducts published by Li *et al.*¹⁸ UV spectrophotometry was then used to determine the concentration of each of the standards, using published wavelengths and extinction coefficients¹⁸.

The reaction of EO with dA produced two different 2-hydroxyethyl adducts and MS/MS analysis of both fractions produced identical spectra, each containing the predicted $[M+H]^+$ ion at m/z 296. However, the fractions varied significantly in their chromatographic properties; the first eluted at 13.5 min and the second, at 18.2 min on the HPLC separation system, indicating different sites of hydroxyethylation. In order to identify the individual adducts isolated, a small aliquot of each HPLC fraction was removed and NaOH (1 M) was added to increase the pH of the solution to 12. After incubation at 37°C for 18 h the samples were neutralised with HCl (1 M) and the resulting products analysed by HPLC. The retention time of peak A remained at 13.5 min, however peak B appeared to have structurally rearranged into peak A, suggesting that peak B was N1-HEdA and peak A was N⁶-HEdA⁴⁰.

Isolation and LC-MS/MS analysis of HE-DNA adducts.

Typically, 1 pmole of a base adduct internal standard, [$^{15}\text{N}_5$]-*N*7-HEG, was added to each DNA sample (50-100 μg), which was then depurinated by heating to 100°C for 30 min. Preferentially released adducted purines were separated from the DNA by centrifugation through Microcon filters (3,000 MW, YM-3, Millipore, Watford, UK) for 30 min at 13,000 rpm (28,000g); *N*7-HEG passes through in the filtrate whilst intact DNA is retained and can be recovered from the filter, by inversion, for subsequent digestion. A second internal standard [$^{15}\text{N}_5$]-*N*1-HEdA (1 pmole) was then added to the recovered DNA, which was dried down under vacuum, redissolved in 40 mM tris-HCl, 10 mM MgCl_2 , pH 8.5 and digested with snake venom phosphodiesterase (0.05 U), DNase I (10 U) and shrimp alkaline phosphatase (2 U) for 2 h at 37°C. Enzymes were removed by centrifugation (30 min, 13,000 rpm, 28,000g) through Microcon YM-3 filters (Millipore), the samples were then concentrated to dryness prior to HPLC purification.

HE-DNA nucleoside adducts contained in the DNA digests were separated from the unmodified nucleosides by HPLC on a Phenomenex Luna C_{18} column (5 μ , 250 x 2.1 mm, Phenomenex, Macclesfield, UK), using a linear 0.1% acetic acid:methanol gradient (increasing from 0 - 35% MeOH over 25 min, 35 - 75% MeOH by 32.5 min, and finally 0% MeOH by 43 min, at a flow rate of 0.2 mL/min). Nucleoside adducts elute in the following fractions: 14-16 min (*N*⁶-HEdA), 21-23 min (*N*3-HEdU) and 25-32 min ([$^{15}\text{N}_5$]-*N*1-HEdA, *N*1-HEdA and *O*⁶-HEdG). All adducts are well resolved from the normal, unadducted nucleosides which elute around ~20-23 min, except for *N*3-HEdU which co-elutes with dT. This system was also used to isolate the *N*7-HEG (and [$^{15}\text{N}_5$]-*N*7-HEG) from other types of adducted purines which may be present in

the collected filtrate. This base adduct elutes between 5-8 min. A different HPLC solvent gradient was used to further separate *N3*-HEdU from the unmodified dT, consisting of a linear 0.1% acetic acid:methanol gradient (increasing from 0 - 25% MeOH over 50 min, at a flow rate of 0.2 mL/min). Using this system, dT elutes at 26 min and *N3*-HEdU at 27.5 min, allowing the hydroxyethylated dU to be collected separately. For each original DNA sample, all fractions containing the desired base and nucleoside HE-adduct fractions were then combined and evaporated to dryness prior to LC-MS/MS analysis. To ascertain whether stability of the nucleoside HE-adducts was affected by the depurination procedure employed to isolate the *N7*-HEG base lesions, untreated plasmid DNA (100µg) was spiked with 500 fmoles of each of nucleoside adduct standard. Each sample (n = 3) was divided in half and one aliquot was heated to 100°C for 30 min. Both samples were then digested, the adducts isolated by HPLC and then quantified by LC-MS/MS analysis as described below.

Samples were analysed for the presence of the five hydroxyethyl adducts (*N7*-HEG, *N3*-HEdU, *N*⁶-HEdA, *N1*-HEdA and *O*⁶-HEdG) in a single LC-MS/MS run, using a system consisting of a Waters Alliance 2695 separations module interfaced to a Waters Quattro Ultima Pt (Waters, Manchester, UK), operated in the positive ion mode. Samples were injected via a 100µL loop onto a Supelcosil C₁₈, 250 x 2.1mm, 5µ column (Sigma, Poole, UK) and separated using an isocratic system of 40 % MeOH in 0.5% acetic acid, at a flow rate of 0.12 mL/min. The eluate from the HPLC was introduced directly into the mass spectrometer. The source temperature was maintained at 120°C and the desolvation temperature at 350°C, nitrogen was used as the desolvating gas at a flow rate of 550 L/hr. The cone voltage was 98 V and the collision energy was 17 eV for all transitions measured. Each sample was dissolved in

40% MeOH in 0.5% acetic acid (40 μ L) and duplicate injections of 15 μ L were performed for each individual sample analysed. The mass spectrometer was operated in the selected reaction monitoring (SRM) mode and measured the following transitions simultaneously: m/z 312 to m/z 196 for O⁶-HEdG, m/z 301 to m/z 185 for [¹⁵N₅]-HEdA, m/z 296 to m/z 180 for both N1-HEdA and N⁶-HEdA, which all correspond to the loss of a 2-deoxyribose from positively ionised 2'-deoxyribonucleoside adducts *i.e.*, $[M+H]^+ \rightarrow [M+H-116]^+$ and m/z 295 to m/z 179 for N3-HEdU, corresponding to the loss of 2'-deoxyribose from the sodiated adduct $[M+Na]^+ \rightarrow [M+Na-116]^+$. The measurement of m/z 201 to m/z 157 for [¹⁵N₅]-N7-HEG and m/z 196 to m/z 152 for N7-HEG, corresponds to the measurement of the $[adducted\ base + H]^+$ to $[unadducted\ base + H]^+$.

The levels of nucleoside HE-DNA adducts in each sample were determined by measuring the ratio of the peak area of each individual HE-DNA adduct to that of the [¹⁵N₅]-N1-HEdA internal standard, whilst N7-HEG was measured relative to [¹⁵N₅]-N7-HEG⁴¹, then the values were converted to adducts/ 10⁸ nucleotides. Figure 3 gives an overview of the assay procedure for the isolation and quantitation of HE-adducts.

Validation Parameters

Linearity of calibration was determined by measuring the ratio of peak areas of the four different HE-adduct standards to the [¹⁵N₅]-N1-HEdA internal standard and the HE-base standard to the [¹⁵N₅]-N7-HEG internal standard. For validation of the assay, double the amount was prepared and half was injected onto the column. Calibration standards ranging from 0.5 - 500 fmoles were run three times each day over a three day period. The daily mean peak ratio of HE-adduct standard to internal standard was

used to construct a calibration line. A correlation coefficient was calculated to confirm linearity for each of the five different HE-adducts.

Intra-day precision was evaluated by analysis of calibration standards for the three injections of each concentration for each of the five HE-adducts. This was calculated in the same way over a three day period and determined by the relative standard deviation ($R.S.D = S.D./\text{mean concentration} \times 100$). The limit of quantitation (LOQ) was defined as a signal to noise baseline ratio of 7:1.

Measurement of HE-DNA adducts in DNA.

A variety of samples were analysed for the presence of HE-DNA adducts to demonstrate potential applications of the LC-MS/MS assay. Background levels of adducts were measured in commercially available control calf thymus (CT) DNA, and liver DNA from an untreated (control) male F-344 rat, provided from another study as described by Marsden, *et al.*⁴² In addition, adducts were measured in a pSP189 plasmid DNA isolated from *E.coli* bacteria⁴³ that had been exposed to EO *in vitro*, by reacting with 10mM EO for 24h at 37°C in water. HE-DNA adducts were isolated from the three types of DNA, purified by HPLC and quantitated by LC-MS/MS as detailed above.

RESULTS AND DISCUSSION

We have developed a sensitive and specific method allowing the simultaneous detection and quantitation of multiple HE-DNA adducts formed as a result of EO exposure. The standards were synthesised by reaction of nucleosides with EO and purification of the reaction products by HPLC. The reaction between dU and EO

resulted in one major product, in addition to unreacted dU, as detected by HPLC-UV (Figure 2A) and MS analysis indicated that this peak had the predicted m/z of 273, consistent with N3-HEdU. This structure was further confirmed by MS/MS analysis, containing fragment ions equivalent to N3-HEU and the 2'-deoxyribose. EO is known to react with both the N1- and N⁶- position of dA, as well as the N3-position, although this adduct is labile and can readily depurinate. On reaction of EO with dA, 2 different adduct peaks were evident by HPLC-UV analysis, which both resulted in molecules with m/z of 296 and produced the same dissociation spectra, when analysed by MS/MS (Figure 2B). Both are consistent with a hydroxyethylated dA adduct. Incubation of each adduct fraction with NaOH caused the later eluting peak B to convert into peak A. This confirmed that peak B was N1-HEdA and peak A was N⁶-HEdA, as in the presence of a strong base, a group at the N1- position can rearrange onto the N⁶- position, in a reaction known as the Dimroth rearrangement⁴⁴. [¹⁵N₅]-N1-HEdA was produced from an analogous reaction with [¹⁵N₅]-labelled dA by isolating the HPLC fraction with a similar retention time to that of N1-HEdA and the identity was confirmed by MS/MS. N7-HEG and [¹⁵N₅]-N7-HEG base adducts were synthesised by the reaction of guanosine with EO, then removal of the ribose group by acid hydrolysis. HPLC analyses of the reaction products revealed one major product peak in each reaction with similar retention times, which was then collected and the structures confirmed by ESI-MS, revealing the expected protonated molecules at m/z 196 and m/z 201 for the N7-HEG and [¹⁵N₅]-labelled standards, respectively. The structures of these two adducts were further confirmed by LC-MS/MS analysis, since the spectra produced by the dissociation of N7-HEG (m/z = 196) is comparable to that reported by Liao *et al.*⁴⁵ (Figure 2C).

Even though the assay utilised SRM as the analysis method, which allows the selective monitoring of a number of specific transitions, in any DNA sample HE-adducts would be present at extremely low levels relative to the normal, unmodified nucleosides. Therefore, enrichment procedures were necessary to minimise interference with adduct quantitation and obtain the maximum sensitivity. The labile nature of the *N*7-HEG adduct was exploited in the selective isolation of this adduct from the intact DNA by thermal depurination and filtration. In addition to the desired adduct, the filtrate from this procedure would be expected to contain other depurinating lesions present endogenously or produced by EO exposure, such as *N*3-HEA. Although other published assays analyse *N*7-HEG levels directly after hydrolysis and filtration^{43,45,48}, this approach resulted in inconsistent measures of the nucleoside adducts, probably due to the presence of other base lesions or contaminants in the filtrate causing interference or signal suppression during LC-MS/MS analysis. Therefore, in order to enable the accurate quantitation of multiple HE-adducts simultaneously, it was necessary to purify the *N*7-HEG and [¹⁵N₅]-labelled internal standard by HPLC prior to analysis.

The remaining HE-adducts were isolated by enzymatic digestion of the DNA recovered from the microcon filter, which is then separated by HPLC using a system capable of resolving the HE-adducts of interest from unmodified nucleosides (Figure 4), with the exception of *N*3-HEdU, which co-elutes with dT and is isolated by a further HPLC purification step. The addition of a nucleoside adduct internal standard [¹⁵N₅]-*N*1-HEdA prior to digestion accounts for losses due to subsequent sample handling of the unlabeled analogue, particularly during the HPLC separation step. LC-MS/MS analysis of nucleoside HE-adduct standards subjected to thermal

hydrolysis prior to going through the assay procedure, confirmed the depurination step did not cause loss of these adducts due to degradation. This was evidenced by the fact adduct levels for control and hydrolysed samples varied by no more than $\pm 5\%$.

The collected fractions from the preparative HPLC runs are then combined and analysed for the presence of the 5 different HE-adducts simultaneously by LC-MS/MS, by measuring a specific transition for each adduct. For nucleoside adducts, the optimum transition was found to be dissociation of the adducted nucleoside (or equivalent sodiated species for *N3*-HEdU) into adducted base through loss of the sugar moiety, whilst for base adducts, dissociation to the base as a consequence of loss of the 2-hydroxyethyl group was most suitable. The best possible separation conditions and solvent system were established using the synthetic standards, and a Supelcosil C₁₈, 250 x 2.1mm, 5 μ column, eluted with an isocratic mobile phase of 40% MeOH in 0.5% acetic acid, was found capable of providing the maximum overall sensitivity with optimum peak shape and retention time (Figure 5A). *N3*-HEA, *N3*-HEdT and *N3*-HEdC were not incorporated into this assay but could however be introduced if required.

Assay Validation

In order to validate this assay, the following criteria were determined: linearity, precision, repeatability, inter-day variability, intra-day variability and the LOQ. Linearity, inter-day variability, repeatability and the LOQ were determined by the construction of a series of calibration lines (Figure 5B). The calibration curves produced from data obtained over the 3 day period were linear in the range 0.5-500 fmoles with a correlation coefficient $R^2 \geq 0.99$ for each individual adduct. Calculation

of the R.S.D. for each concentration of individual HE-adducts was used to determine the inter-day precision of the developed assay, which was found to be $\leq 20\%$. The limits of quantitation were determined as 0.5 fmoles injected onto the column for *N*7-HEG and *N*1-HEdA, 5 fmoles for *O*⁶-HEdG and *N*⁶-HEdA and 25 fmoles for *N*3-HEdU. Measurement of *N*3-HEdU will always be an approximate value as an internal standard is ideally required to compensate for losses due to the additional HPLC step.

Quantitation of HE-DNA adduct levels in DNA.

Very little is known about the formation or biological consequences of HE-DNA adducts apart from the major *N*7-HEG lesion. Although, based on evidence from *in vitro* binding studies, the other potential HE-adducts account for a small proportion of total adduct formation by EO, and it is possible that under some circumstances they may be present at significant levels *in vivo*. The development of this assay provides, for the first time, a method that could potentially detect these adducts in DNA samples from exposed animals, humans or cultured cells, to determine whether they are formed and at what levels, using just a single DNA sample. The assay can also be applied to mechanistic studies, for example in combination with the *supF* forward mutation assay, which would allow mutagenic potency to be related to HE-DNA adduct profiles.

In order to demonstrate the possible applications of this assay, we have analysed levels of endogenous/background and EO-induced HE-adducts in various types of DNA as shown in Table 1 and Figure 6. The additional peaks present in the channels for *N*1-HEdA and *O*⁶-HEdG are artefactual. MS/MS analysis suggests that they are related to the adducts and it is possible that they could be corresponding residual

nucleoside *N*3-HEA and *N*7-HEG adducts. *N*7-HEG was detected in liver DNA from control rats at levels of ~ 8 adducts/ 10^8 nucleotides, which is comparable with that found by Wu *et al.*, who reported endogenous adduct levels in the range of 5-8 adducts/ 10^8 nucleotides⁴⁶ and similar to the 1-3.5 adducts/ 10^8 nucleotides described in the Marsden study⁴³. The DNA from the livers of rats treated with 1.5 mg/kg was also analysed (data not shown) but *N*7-HEG was still the only HE-adduct detected, suggesting that this dose is not high enough to induce the formation of *N*1-HEdA and *O*⁶-HEdG, or that they are formed but at a limit that is below the LOD of this assay. In untreated CT DNA, *N*7-HEG was the only HE-adduct detected, at background levels of ~ 10 adduct/ 10^8 nucleotides. For there to be endogenous/background adducts present in commercially available DNA is not unexpected, for example C8-(1-hydroxyethyl)-guanine adducts have previously been reported in CT DNA⁴⁷. *O*⁶-HEdG has previously been detected and measured in different rat tissues by fluorescence-HPLC at approximate levels of 3 adducts/ 10^7 nucleotides but only after 2 weeks of exposure to 300 ppm EO¹¹. In this study, *O*⁶-HEdG was not detected in any of the control DNA samples but exposure of pSP189 plasmid to a high concentration of EO (10mM) over a 24h period, induced measurable levels of *O*⁶-HEG ($\sim 575/10^8$ nucleotides), as shown in Figure 6, whilst also increasing the total *N*7-HEG and *N*1-HEdA to ~ 415166 and 24216 adducts/ 10^8 nucleotides, respectively. *N*1-HEdA has only previously been observed in DNA treated with ¹⁴C-EO *in vitro*¹⁷, and only quantitated after a large-scale 50 mM reaction of EO with CT DNA¹⁸.

More recently, the method of choice for the detection of *N*7-HEG adducts has been LC-MS. Leclercq *et al.*, described an assay using LC-MS with single ion monitoring which achieved an LOD of 1 pmole injected onto the column, and measured 3 *N*7-

HEG adducts/ 10^8 nucleotides in DNA from blood exposed to EO *in vitro*⁴⁸. A similar method was published by Liao *et al.*, with an LOD of 128 fmoles and the authors also demonstrated that using LC-MS/MS with SRM detection improved the sensitivity, decreasing the LOD further to 16 fmoles⁴⁵. In comparison, the highest LOD of the five HE-adducts analysed in this study is 25 fmoles injected onto the column. The most sensitive published method to date for the detection of *N*7-HEG appears to be the Gas Chromatography-Electron Capture-Mass Spectrometry method described by Wu *et al.*, which had an LOD of 600 amoles⁴⁶. The assay described here is slightly more sensitive, for both *N*7-HEG and *N*1-HEdA, with an LOD of 500 amoles, and has the advantage that it does not require the production of a pentafluorobenzyl-*N*7-HEG derivative as part of the sample preparation step.

In developing an LC-MS/MS method for the simultaneous measurement of a number of different DNA adducts there is inevitably a compromise in sensitivity in order to achieve detection of all five HE-adducts. However, the LOD for the *N*7-HEG adduct is still more sensitive than any previous published method and with advances in technology, for example nanospray or ultra high pressure HPLC (UPLC), the sensitivity may be further improved in the future. In addition, if one wished to study any single HE-adduct specifically then the current assay could be tailored in favour of that particular adduct by slight alteration of the MS conditions to maximise the sensitivity for the adduct of interest. Multi-adduct analysis is becoming a more common approach for the study of the effects of chemical carcinogens; recently, Brink *et al.*, have described a method for the determination of three promutagenic adducts formed as a result of exposure to dimethylnitrosamine,⁴⁹ and an assay for the quantification of four structurally different adducts caused by oxidative stress has

been reported by Churchwell *et al.*⁵⁰ Since most chemical carcinogens form a variety of lesions, it is more informative to quantitatively determine the adduct profile rather than focus on a single adduct and these methods make this approach possible in a rapid and sensitive manner.

Conclusion

In summary, a sensitive and selective LC-MS/MS assay has been developed and validated for the simultaneous detection and quantitation of five different HE-DNA adducts that can be formed as a result of EO exposure. This allows a more comprehensive study of HE-adduct formation which has not previously been possible. To demonstrate its utility, this assay has so far been employed in the measurement of endogenous/background levels of DNA adducts in unexposed calf thymus and rat liver DNA, as well as EO-induced lesions in pSP189 plasmid DNA, and has the potential to be applied to the measurement of HE-DNA adducts from a wide variety of *in vivo* and *in vitro* studies to further our understanding of the significance of endogenous/background and exogenous HE-DNA adducts.

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FIGURE CAPTIONS

Figure 1: The different possible HE-adducts that can be produced as a result of ethylene oxide reaction with DNA. Adducts are shown in their base form.

Figure 2: Characterisation of synthesised HE-DNA adducts. HPLC purification of the reaction products are shown, with the corresponding ESI-MS/MS spectra for the product peak inserted. The separation of N3-HEdU from unmodified nucleoside is shown in (A). (B) shows a typical ESI-MS/MS spectra for N⁶-HEdA, which is identical to that produced by N1-HEdA (not shown), (C) shows the purification and characterisation of N7-HEG.

Figure 3: Scheme depicting the sample preparation steps prior to LC-MS/MS analysis.

Figure 4: HPLC chromatogram demonstrating the separation of HE-nucleoside adducts from unmodified nucleosides, using the conditions employed for the isolation of nucleoside HE-adducts. Synthetic adduct standards have been used to produce this trace for illustrative purposes; in a typical digested DNA sample HE-adducts would not usually be present at detectable levels and fractions would be collected on the basis of retention time.

Figure 5: Panel A shows a typical LC-MS/MS chromatogram resulting from the simultaneous measurement of 500 fmoles of each individual adduct standard. Panel B illustrates calibration lines for each of the five HE-DNA adduct standards that were used to validate the LC-MS/MS assay.

Figure 6: Representative LC-MS/MS chromatograms from the analysis of liver DNA from a control F-344 rat (**A**) and pSP189 plasmid DNA reacted with 10 mM EO *in vitro* (**B**).

Figure 1

Figure 2

Figure 3:

Figure 4:

Figure 5:

Figure 6:

Table I. Representative adduct levels per 10^8 nucleotides present in calf thymus, pSP189 plasmid and rat liver DNA, as measured by the LC-MS/MS assay. Results presented are from the duplicate analysis of a single sample for each type of DNA, and are reported as the mean value.

DNA sample	Adducts/ 10^8 nucleotides		
	<i>N</i> 7-HEG	<i>N</i> 1-HEdA	<i>O</i> ⁶ -HEdG
Control rat liver	8 ± 1.5	< LOD*	< LOD
Calf thymus DNA	59 ± 1	< LOD	< LOD
EO exposed plasmid (10mM)**	415166 ± 19470	24216 ± 5967	575 ± 21

*Less than the limit of detection, this is approximately 1 adduct/ 10^9 for *N*1-HEdA and 1 adduct/ 10^8 for *O*⁶-HEdG. *N*⁶-HEdA and *N*3-HEdU adducts were not detectable in any samples analysed. **Treatment with higher concentrations of EO resulted in a dose dependent increase in these adducts (data not shown).

REFERENCES

1. IARC Monographs. *Some Industrial Chemicals*. 1994; **60**;73.
2. Segerbäck, D. *Chemico Biological Interactions* 1983; **45(2)**; 139.
3. Filser, J.G.; Denk, B.; Tornqvist, M.; Kessler, W.; Ehrenberg, L. *Archives in Toxicology* 1992; **66(3)**; 157.
4. Elfarra, A.A.; Duescher, R.J.; Pasch, C.M. *Archives in Biophysical Biochemistry* 1991; **286**; 244.
5. Ehrenberg, L.; Hussein, S. *Mutation Research* 1977; **46(3)**; 196.
6. Leibermann, M.; Mapson, L.W. *Nature*. 1964; **204**; 343.
7. Tornqvist, M.; Gustafsson, B.; Kautiainen, A.; Harms-Ringdahl, M.; Granath, F.; Ehrenberg, L. *Carcinogenesis*. 1989; **10**; 39.
8. Kessler, W.; Remmer, H. *Biochemical Pharmacology* 1990; **39**; 1347.
9. Clemens, M.R.; Einsele, H.; Frank, H.; Remmer, H.; Waller, H.D. *Biochemical Pharmacology* 1983; **32**; 3877.
10. Nivard, M.J.M.; Czene, K.; Segerbäck, D.; Vogel, E.W. *Mutation Research* 2003; **529**; 95.

11. Walker, V.E.; Fennell, T.R.; Boucheron, J.A.; Fedtke, N.; Ciroussel, F.; Swenberg, J.A. *Mutation Research* 1990; **233**; 151.
12. Kelsey, K.T.; Wiencke, J.K.; Eisen, E.A.; Lynch, D.W.; Lewis, T.R.; Little, J.B. *Cancer Research* 1988; **48(17)**; 5045.
13. Hogstedt, C.; Malmqvist, N.; Wadman, B. *Journal of the American Medical Association* 1979; **241**; 1132.
14. Hogstedt C.; Aringer, L.; Gustavsson, A. *Journal of the American Medical Association* 1986; **255(12)**; 1575.
15. Teta. M.J.; Sielken. R.L.; Valdez-Flores. C. *Risk Analysis* 1999; **19**; 1135.
16. Coggon, D.; Harris, E. C.; Poole, J.; Palmer K. T. *Occupational and Environmental Medicine* 2004; **61**; 358.
17. Segerbäck, D. *Carcinogenesis*, 1990; **11(2)**; 307.
18. Li, F.; Segal, A.; Solomon, J.J. *Chemico Biological Interactions* 1992; **83**; 35.
19. Lawley, P.D.; Brookes, P. *Biochemical Journal* 1963; **89**; 127.
20. Takeshita, M.; Chang, C-H.; Johnson, F.; Will, S.; Grollman, A.P. *Journal of Biological Chemistry* 1987; **262(21)**; 10171.
21. Loeb, L.A.; Preston, B.D. *Annual Reviews in Genetics* 1986; **20**; 201.

22. Lawley, P.D. *British Medical Bulletin* 1980; **36(1)**; 19.
23. Solomon, J.J.; Mukai, F.; Fedyk, J.; Segal, A. *Chemico Biological Interactions* 1988; **67**; 275.
24. Solomon, J.J.; Segal, A. *Environmental Health Perspectives* 1989; **81**; 19.
25. Bhanot, O.S.; Singh, U.S.; Soloman, J.J. *Journal of Biological Chemistry* 1994; **269(47)**; 30056.
26. Barlow, T.; Takeshita, J.; Dipple, D. *Chemical Research in Toxicology* 1998; **11**; 838.
27. Gamboa da Costa, G.; Churchwell, M.I.; Hamilton, L.P.; Von Tungeln, L.S.; Beland, F.A.; Marques, M.M.; Doerge, D.R. *Chemical Research in Toxicology* 2003; **16**; 1328.
28. Rusyn I.; Asakura, S.; Li, Y.; Kosyk, O.; Koc, H.; Nakamura, J.; Upton, P.B.; Swenberg, J.A. *DNA Repair* 2005; **4**; 1099.
29. Loeppky, R. N.; Ye, Q.; Goelzer, P.; Chen, Y. *Chemical Research in Toxicology* 2002; **15**; 470.

30. Eide, I.; Zhao, C.; Kumar, R.; Hemminki, K.; Wu, K-Y.; Swenberg, J.A. *Chemical Research in Toxicology* 1999; **12**; 979.
31. Koivisto, P.; Hemminki, K. *Carcinogenesis* 1990; **11(8)**; 1389.
32. Kumar, R.; Staffas, J.; Forsti, A.; Hemminki, K. *Carcinogenesis* 1995; **16(3)**; 483.
33. Kumar, R.; Hemminki, K. *Carcinogenesis* 1996; **17(3)**; 485.
34. Zhao, C.; Tyndyk, M.; Eide, I.; Hemminki, K. *Mutation Research* 1999; **424**; 117.
35. van Delft, J.H.M.; van Winden, M.J.M.; Luiten-Scuite, A.; Ribiero, L.R.; Baan, R.A. *Carcinogenesis* 1994; **15(9)**; 1867.
36. Walker, V.E.; Fennell, T.R.; Upton, P.B.; Skopek, T.R.; Prevost, V.; Shuker, D.E.G.; Swenberg, J.A. *Cancer Research* 1992; **52**; 4328.
37. Saha, M.; Abushamaa, A.; Giese, R.W. *Journal of Chromatography A* 1995; **712**; 345.
38. Singh, R.; Farmer, P.B. *Carcinogenesis*, 2006; **27(2)** 178.
39. Kao, C.-Y.; Geise, R.W. *Chemical Research in Toxicology* 2005; **18**; 70.

40. Plna, K.; Nilsson, R.; Koskinen, M.; Segerbäck, D. *Carcinogenesis* 1999; **20**(10); 2025.
41. Tretyakova, N.Y.; Burney, S.; Pamir, B.; Wishnok, J.S.; Dedon, P.C.; Wogan, G.N.; Tannenbaum S.R. *Mutation Research: Fundamental Molecular Mechanisms in Mutagenesis* 2000; **447**; 287.
42. Marsden, D.A.; Jones, D.J.L.; Lamb, J.H. Tompkins, E.M.; Farmer, P.B.; Brown, K. *Chemical Research in Toxicology* 2007, **20**; 290.
43. McLuckie, K.I.E.; Crookston R.J.R.; Gaskell, M.; Farmer, P.B.; Routledge, M.N.; Martin E.A.; Brown, K. *Biochemistry* 2005; **44**; 8198.
44. Brookes, P.; Lawley, P.D. *Journal of the Chemistry Society* 1960; 539.
45. Liao, P.-C.; Li, C-M.; Hung, C-W.; Chen, S-H. *Journal of Mass Spectrometry* 2001; **36**; 336.
46. Wu, K.-Y.; Scheller, N.; Ranasinghe, A.; Yen, T-Y.; Sangaiah, R.; Giese, R.; Swenberg, J.A. *Chemical Research in Toxicology* 1999; **12**; 722.
47. Nakao, L.S.; Fonseca, E.; Augusto, O. *Chemical Research in Toxicology* 2002; **15**; 1248.
48. Leclercq, L.; Laurent, C.; De Pauw, E. *Analytical Chemistry* 1997; **69**; 1952.

49. Brink, A.; Lutz, Y.; Volkel, W.; Lutz, W.K. *Journal of Chromatography B* 2006; **830**; 255.

50. Churchwell, M.I.; Beland, F.A.; Doerge, D.R. *Chemical Research in Toxicology* 2002; **15**; 1295.