# Detection and Characterization of Two Major Ethylated Deoxyguanosine Adducts by High Performance Liquid Chromatography, Electrospray Mass Spectrometry, and $^{32}$P-Postlabeling. Development of an Approach for Detection of Phosphotriesters

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Postlabeling can be one of the most sensitive methods for the measurement of DNA adducts. However, for the determination of alkylated adducts, essential requirements are standards which must be fully chemically characterized. In order to develop a postlabeling assay for monitoring exposure to genotoxic ethylating agents, the reaction of diethyl sulfate with 2′-deoxynucleoside 3′- and 5′-monophosphates was examined. The adducts generated were fully characterized using HPLC, electrospray tandem mass spectrometry, UV, and postlabeling. The major product was the phosphodiester derived from alkylation of the phosphate, and alkylation of the base occurred to a lesser extent. The phosphodiester standard, 2′-deoxyguanosine 3′-(mono-*O*-ethyl phosphate) (3′Et-pdG), was used to develop a postlabeling assay for the detection of this adduct in DNA samples. Since alkylated phosphodiesters in DNA are not susceptible to the actions of micrococcal nuclease and calf spleen phosphodiesterase, they can be obtained as alkylated phosphodiester dinucleosides from DNA. Nuclease $P_1$ was used as an enhancement step which allowed the separation of these adducted phosphotriesters from the unmodified nucleotides by HPLC. Subsequent hydrolysis of the phosphotriester dinucleosides in alkali yielded phosphodiesters, including 3′Et-pdG, which was efficiently postlabeled. This approach was shown to be capable of detecting this adduct in liver DNA from mice treated intraperitoneally with *N*-nitrosodiethylamine.

## Introduction

The formation of DNA adducts is believed to be a critical event in the initiation of carcinogenesis (*1, 2*). The measurement of DNA adducts has consequently become an increasingly popular approach for studying chemical mutagenesis and carcinogenesis and for indicating exposure to genotoxic compounds such as alkylating agents (*3, 4*). Exposure of man to these compounds occurs principally in the environment (*5, 6*) but may also occur following chemotherapy for treatment of tumors (*7*). In spite of the numerous reports on the detection of alkylated adducts in both nontarget and target organs (*8*), few studies have been designed to assess their value in predicting interindividual susceptibility to tumor formation. At the moment, DNA adducts are being used primarily as highly specific and sensitive indices of exposure in epidemiological studies (*9, 10*), the advantage of this approach being that they account for interindividual variations in carcinogen absorption, distribution, metabolism, and excretion.

One of the principal lesions produced *in vivo* following exposure to simple monoalkylating agents occurs at the nucleophilic *N*-7 position of guanine (*11*). This adduct is not considered to be promutagenic; nevertheless, it is relatively slowly repaired (*12*) and thus is widely used as an index of exposure. Further sites for DNA modification following exposures to simple alkylating agents include the exocyclic oxygens of guanine and thymine ($O^6$-alkylguanine and $O^4$-alkylthymine, respectively). Lesions at these sites are believed to be promutagenic. Although in many cases repair enzymes remove the alkyl groups relatively rapidly from these bases, long term exposure may result in steady state levels of some of these adducts (*13*).

Alkylating agents also react with the phosphate oxygens in DNA, yielding phosphotriesters (*14*). For simple alkylating agents, these adducts tend to be stable and not rapidly repaired, and hence are long-lived (*15, 16*). Mutation resulting from phosphotriester formation has

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[1] Abbreviations: *N*-7 EtG, *N*-7-ethylguanine; *N*-7 Et 3′pdG, *N*-7-ethyl-2′-deoxyguanosine 3′-monophosphate; 3′pdG, 2′-deoxyguanosine 3′-monophosphate; 3′pdN, 2′-deoxynucleoside 3′-monophosphate; 3′Et-pdG, 2′-deoxyguanosine 3′-mono(*O*-ethyl phosphate); 3′Et-pdN, 2′-deoxynucleotide 3′-mono(*O*-ethyl phosphate); 3′Et-pdGp, 2′-deoxyguanosine; 3′-(*O*-ethyl phosphate) 5′-phosphate; 3′Et-pdNp, 2′-deoxynucleoside 3′-(*O*-ethyl phosphate) 5′-phosphate; *N*-7 Et 5′pdG, *N*-7-ethyl-2′-deoxyguanosine 5′-monophosphate; 5′pdG, 2′-deoxyguanosine 5′-monophosphate; 5′pdN, 2′-deoxynucleoside 5′-monophosphate; 5′Et-pdG, 2′-deoxyguanosine 5′-mono(*O*-ethyl phosphate); 5′Et-pdN, 2′-deoxynucleoside 5′-mono(*O*-ethyl phosphate); pdGp, 2′-deoxyguanosine 3′,5′-bisphosphate; pdNp, 2′-deoxynucleoside 3′,5′-bisphosphate; 2′dN, 2′-deoxynucleoside; dNpdN, 2′-deoxynucleoside-(3′,5′)-2′-deoxynucleoside; dNp(Et)dN, 2′-deoxynucleoside-(3,′5′)-2′-deoxynucleoside mono-(*O*-ethyl phosphate); DES, diethyl sulfate; ES-MS, electrospray mass spectrometry; ES-MS/MS, electrospray tandem mass spectrometry; MN, micrococcal nuclease; CSPD, calf spleen phosphodiesterase; PNK, polynucleotide $T_4$ kinase, NDEA, *N*-nitrosodiethylamine; TEAF, triethylammonium formate; TEA, triethylammonium acetate; AF, ammonium formate; $P_i$, inorganic phosphate.

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not been fully addressed, but such adducts may influence cellular function by altering the binding of proteins to DNA through neutralization of the negative charge along the sugar−phosphate backbone. This may affect chromatin structure, leading to DNA strand breaks. Phosphotriester adducts hold considerable potential as valid surrogates for monitoring exposure to carcinogens and perhaps for risk assessment (*16, 17*).

*N*-Nitrosodiethylamine (NDEA)[1] is a simple alkylating agent that requires metabolic activation to generate its ethylating electrophile (*18, 19*), which is capable of interacting with DNA at many different sites (*20*). This compound is currently being used as a model carcinogen to investigate the predictive value of adducts for carcinogenic risk assessment in an animal model. In order to achieve this objective, we required sensitive assays for ethylated DNA, and in particular, for two of the products formed with 2′-deoxyguanosine 3′-monophosphate (3′pdG), *N*-7-ethyl-2′-deoxyguanosine 3′-monophosphate (*N*-7 Et 3′pdG), and 2′-deoxyguanosine 3′-mono(*O*-ethyl phosphate) (3′Et-pdG).

Numerous assays have been used to detect alkyl DNA adducts, such as high performance liquid chromatography (HPLC)/immunoassay (*21*), HPLC/fluorescence (*22*), HPLC/electrochemical detection (*23, 24*), or immunoslot blot or ELISA detection (*25*−*27*). Recent advances include the development of fluorescent postlabeling techniques where the adduct of interest is derivatized with highly fluorescent compounds for detection by HPLC (*28*). Mass spectrometry, though more useful as a tool for the structural characterization of adducts, has been employed to determine levels of DNA adducts (*29*). Although these methods have been successful, they may require relatively large quantities of DNA for analysis or rely upon specific antibodies and are often limited in their sensitivity. The technique which gives potentially the highest degree of sensitivity for detection of alkylated DNA adducts and which has the additional advantage of only requiring a few micrograms of DNA is $^{32}$P-postlabeling (*30*). Coupling of $^{32}$P-postlabeling with HPLC separation of adducts results in a highly specific detection method (*31*). However $^{32}$P-postlabeling of *N*-7 alkyl 3′pdG is not so successful because of its unstable nature, due to spontaneous depurination (*32*−*34*), and consequent low labeling efficiency (*35*). Attempts have been made to circumvent this problem by labeling the ring-opened derivatives (*36*). A further consideration for postlabeling of a low molecular weight adduct such as ethyl is that the introduction of an enhancement step (e.g., HPLC or immunoaffinity purification) to separate the adduct from similar unmodified 2′-deoxynucleotides may result in some loss of adduct.

In contrast to the situation for *N*-7-alkylguanine, there have only been limited attempts made to analyze alkyl phosphotriesters in DNA (*37*), although the potential for $^{32}$P-postlabeling of such adducts has, however, recently been demonstrated by Saris *et al.* (*38*). Phosphate alkylation in DNA will yield a phosphotriester, which is not a substrate for postlabeling. However, alkaline hydrolysis of alkyl phosphotriesters yields 3′-phosphodiesters. One of the products is the 2′-deoxynucleoside 3′-mono(*O*-alkyl phosphate), which may be postlabeled and could be used as a marker of the extent of phosphate alkylation.

For development of postlabeling assays, it is necessary to obtain standards of the adduct of interest, which should be fully structurally characterized. This paper describes the characterization of two major adducts derived from reaction of 3′pdG with the model ethylating agent diethyl sulfate (DES), *N*-7 Et 3′pdG, and 3′Et-pdG. Conditions were optimized for their detection by HPLC and $^{32}$P-postlabeling, and labeling efficiencies were determined. The possibility that 3′Et-pdG would be liberated following enzymic digestion of DNA in the postlabeling procedure was also investigated. A new approach for the detection of alkyl phosphotriesters in DNA was developed, and the possibility for the formation of ethyl phosphotriesters in DNA *in vivo* was investigated in mice treated intraperitoneally with NDEA.

## Materials and Methods

**Chemicals.** 2′-Deoxynucleoside 3′- or 5′-monophosphate (3′pdN or 5′pdN), ATP, DES, dithiothreitol, nuclease $P_1$, spermidine, micrococcal nuclease (MN), calf thymus DNA (Type 1), and potato apyrase, grade VI, were purchased from the Sigma Chemical Co. Ltd. (Poole, Dorset, U.K.). **Caution:** *DES is a mutagen and carcinogen. Protective clothing should be worn and appropriate safety procedures followed when working with this compound.* Polynucleotide $T_4$ kinase (3′-phosphatase free) (PNK) and calf spleen phosphodiesterase (CSPD) were purchased from Boehringer Mannheim (Lewes, East Sussex, U.K.). [$\gamma$-$^{32}$P]ATP, >5000 Ci/mmol, was purchased from Amersham International (Amersham, Buckinghamshire, U.K.). **Caution:** *Appropriate procedures should be used to minimize exposure to $\gamma$ - radiation when using [$\gamma$-$^{32}$P] ATP.* All other reagents and solvents of analytical grade were purchased from either Merck Ltd. (Poole, Dorset, U.K.) or Fisher Scientific Ltd. (Loughborough, Leicestershire, U.K.). Authentic *N*-7 ethylguanine (*N*-7 EtG) was kindly provided by Professor M. Jarman (Institute of Cancer Research, Sutton, Surrey, U.K.).

Ethylated nucleotide standards were generated by reacting the 2′-deoxynucleotide (2 mg) with 100 mM DES in 0.5 M sodium phosphate buffer (pH 6.0) at room temperature for 8 h as reported previously (*35*) and were then separated by HPLC. Calf thymus DNA (ca. 50 mg) was ethylated by treatment with DES (1−100 mM) in 0.5 M sodium phosphate buffer (pH 6.0) at room temperature for 8 h and precipitated by the addition of 3 M sodium acetate (1:10, v/v) and 2-propanol (0.8 volume).

**HPLC Separation Procedures.** HPLC was routinely performed using LKB (Pharmacia Biotech Ltd., Milton Keynes, U.K.) or Gilson (Gilson Medical Electronics Inc., Middleton, WI) pumps connected to a fixed wavelength detector or diode array UV detector. Absorbance of eluate was monitored at 254 nm. All analyses were performed at room temperature, using a reverse-phase $C_{18}$ column (Apex, 5 $\mu$m; 4.6 × 250 mm; Jones Chromatography) fitted with a $C_{18}$ guard column (Techsphere, 5 $\mu$m; 3 × 10 mm; HPLC Technology). The flow rate was 1 mL/min. Three different buffered mobile phase systems were utilized for gradient elution as described below:

(1) Triethylammonium formate (TEAF). Solvent A: methanol/0.1 M TEAF (1:99 v/v) (pH 6.0); solvent B: methanol/0.1 M TEAF (20:80 v/v) (pH 6.0); gradient: 0 min: 0% B, 20 min: 45% B, 28 min: 100% B, 35 min: 100% B, 40 min: 0% B. (2) Triethylammonium acetate (TEA). Solvent A: 0.1 M TEA (pH 7.0); solvent B: acetonitrile; gradient: 0 min: 1% B, 20 min: 1% B, 45 min: 2% B, 55 min: 95% B, 60 min: 1% B. (3) Ammonium formate (AF). Solvent A: 0.05 M AF, (pH 5.4); solvent B: methanol; gradient: 0 min: 0% B, 15 min: 20% B, 20 min: 0% B.

HPLC fractions were collected, and either evaporated to dryness using a DNA centrifugal evaporator (DNA 110, Savant, Farmingdale, NY) or freeze-dried (IEC Hyoprep 3000, International Equipment Co., Dunstable, U.K.) for $^{32}$P-postlabeling or mass spectrometry analysis.

**Analysis of *N*-7-Ethylguanine by HPLC-Electrochemical Detection.** The DES-treated calf thymus DNA samples were hydrolyzed in 0.1 M formic acid (pH 2.3) at 70 °C for 1 h to release adducted bases. An enhancement step was performed

on the sample using reverse-phase HPLC (AF mobile phase) to separate *N*-7 EtG from guanine and adenine, which were also generated by the hydrolysis conditions utilized. The HPLC fraction containing *N*-7 EtG was lyophilized, redissolved in water, and analyzed by electrochemical detection (Antec EC detector, Antec Analytical Technology, Leiden, The Netherlands). The optimum oxidation potential of 1.10 V for the detector cell was determined by chromatographing a standard sample of *N*-7 EtG over a range of settings. *N*-7 EtG was isocratically separated on a reverse-phase $C_{18}$ column (Hypersil, 5 $\mu$m; 4.6 × 250 mm; Shandon HPLC) using 25 mM potassium phosphate (pH 6.0)/methanol (90:10 v/v). Determination of the levels of *N*-7 EtG following the treatment of calf thymus DNA with various concentrations of DES demonstrated a linear dose-dependent increase in the level of adduct produced, and subsequently these samples were used in the development of a postlabeling method for 3′Et-pdG. The limit of detection of the method was 6 *N*-7 EtG adducts per $10^6$ nucleotides (2.0 pmol) per 100 $\mu$g of DNA.

**Mass Spectrometry.** Mass spectrometry was carried out using a Quattro BQ tandem quadrupole instrument (Micromass, Manchester, U.K.) fitted with an electrospray source operating in the negative ion mode. The carrier solvent used in all the experiments was acetonitrile and water (1:1 v/v) maintained at a constant flow rate of 50 $\mu$L/min. Dried HPLC fractions were resuspended in 20 $\mu$L of water prior to MS analysis. Samples were introduced into the solvent flow using a Rheodyne 7125 injector, fitted with a 10 $\mu$L loop, and the instrument ($MS_1$) was scanned over a mass range of 300−450. Product ion spectra were obtained with a collision energy of 110 eV and argon collision gas at a pressure of $2.0 \times 10^{-4}$ mbar and scanning $MS_2$ over a mass range of 50−400.

**Postlabeling of *N*-7 Et 3′pdG and 3′Et-pdG with Non-radioactive ATP.** Labeling of *N*-7 Et 3′pdG and 3′Et-pdG with cold ATP was carried out by incubation with labeling buffer (12 $\mu$L, 200 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM dithiothreitol, 10 mM spermidine, pH 7.6), 2 mM ATP (15 $\mu$L), HPLC grade water (6 $\mu$L), and PNK (2 $\mu$L, 10 U/$\mu$L) at 37 °C for 1 h. The total mixture (55 $\mu$L) was separated by using the TEAF mobile phase. The HPLC fraction containing the ethylated 2′-deoxynucleoside bisphosphate product of 3′Et-pdG was evaporated to dryness, redissolved in 15 $\mu$L of HPLC grade water, and incubated with nuclease $P_1$ (5 $\mu$L, 2 U/$\mu$L) at 37 °C for 30 min prior to HPLC.

**$^{32}$P-Postlabeling. Labeling Efficiencies of *N*-7 Et 3′pdG and 3′Et-pdG.** The labeling efficiency of each adduct, dissolved in HPLC grade water (5 $\mu$L), was investigated by incubation with 500 fmol of 3′pdG (as the internal standard, 1 $\mu$L), labeling buffer (4 $\mu$L, 200 mM Tris-HCl, 100 mM $MgCl_2$, 100mM dithiothreitol, 10 mM spermidine, pH 7.6), 10 $\mu$Ci of [$\gamma$-$^{32}$P]ATP (specific activity >5000 Ci/mmol), and PNK (1 $\mu$L, 10 U/$\mu$L) for 1 h at 37 °C. The postlabeling mixture was then applied to prewashed (with water) poly(ethylenimine) (PEI) cellulose TLC plates (20 × 20 cm, Macherey-Nagel, Camlab, Cambridge, U.K.). One-dimensional chromatography was performed using 1 M ammonium formate (pH 3.5). Radiolabeled spots were visualized by storage phosphor image analysis (ImageQuant software (v. 3.3) and Phosphoimager, Molecular Dynamics, Sunnyvale, CA) and adduct levels quantified as (fmol of 3′pdG) × (screen volume postlabeled *N*-7 Et 3′pdG or 3′Et-pdG/screen volume postlabeled 3′pdG).

**Determination of 3′Et-pdG in DNA Samples.** DNA (50 $\mu$g) extracted from animal tissue or calf thymus DNA was incubated with MN (5 $\mu$L, 0.4 U/$\mu$L), CSPD, (35 $\mu$L, 1 mU/$\mu$L), and digestion buffer (10 $\mu$L, 100 mM sodium succinate, 50 mM $CaC1_2$, pH 6.0) at 37 °C overnight. The mixture was further incubated with nuclease $P_1$ (30 $\mu$L, 2 U/$\mu$L) at 37 °C for 6 h and then with 25% ammonium hydroxide solution (70 $\mu$L) for 24 h at 70°C. The fraction corresponding to 3′Et-pdG was collected, following HPLC separation of the reaction mixture using 50 mM ammonium formate/methanol gradient and evaporation to dryness, and $^{32}$P-postlabeled after resuspension in 5 $\mu$L of water. Labeling buffer (5 $\mu$L, 200 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM dithiothreitol, 10 mM spermidine, pH 7.6), [$\gamma$-$^{32}$P]ATP (10 $\mu$Ci, specific activity >5000 Ci/mmol), and PNK (1 $\mu$L, 10 U/$\mu$L) were added, and the mixture was incubated at 37 °C for 1 h. Following a further incubation with apyrase (4 $\mu$L, 40 mU/$\mu$L), the postlabeled mixture was spotted onto cellulose-coated glass plates (20 × 20 cm, 0.1 mm, UV 254 nm, Macherey-Nagel, Camlab, Cambridge) at a position 2 cm from each edge in the lower left-hand corner. These plates were prespotted with an unlabeled UV marker 5′pdG which runs in the same position as the postlabeled 3′Et-pdG adduct. The plates were developed in the first dimension (D1: isobutyric acid/water/concentrated $NH_4OH$, 66:20:1 v/v), rotated through 90°, and developed similarly in the second dimension (D2: saturated $(NH_4)_2SO_4$/2-propanol/1 M sodium acetate, 80:2:18 v/v) (*39*). Each chromatographic run took 7−8 h for the solvent front to reach the top of the plate. The UV marker was visualized under UV light (254 nm) and the $^{32}$P-postlabeled 3′Et-pdG adduct detected by storage phosphor image analysis.

**Table 1. Comparison of HPLC Phase Systems for the Separation of Ethylated Guanine Products Expressed as Capacity Ratios[a]**

| mobile phase nucleotide/base | capacity ratio ($k'$) | | |
|---|---|---|---|
| | TEAF | TEA | AF |
| *N*-7 Et 3′pdG | 6.24 | 11.48 | 4.00 |
| 3′Et-pdG | 11.48 | 18.96 | 5.80 |
| *N*-7 Et 5′pdG | 4.44 | 5.64 | 3.88 |
| 5′Et-pdG | 10.20 | 19.00 | 4.96 |
| *N*-7 EtG | 8.16 | 12.96 | 6.19 |

[a] $(t_R - t_0)/t_0$, where $t_R$ is the retention time of the ethylated product and $t_0$ is the elution time of an unretained component.

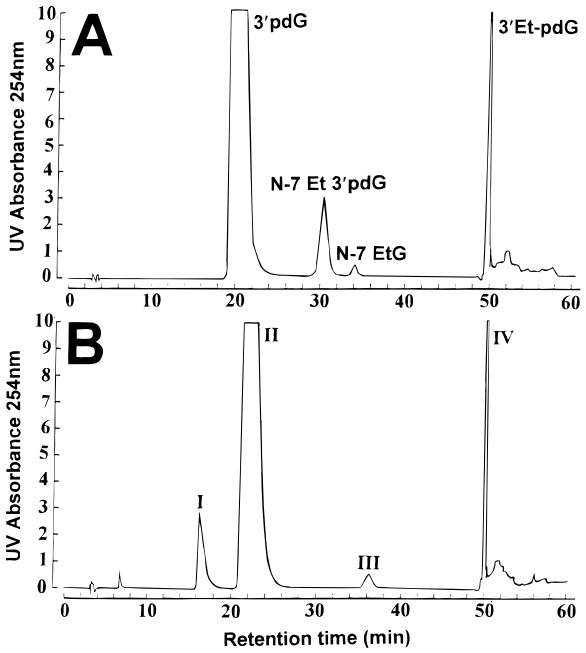



**Figure 1.** Typical reverse-phase HPLC chromatograms obtained for the reaction of (A) 3′pdG (5.76 $\mu$mol) and (B) 5′pdG (5.76 $\mu$mol) with 100 mM DES in 0.5 M sodium phosphate buffer, pH 6.0 (1.0 mL). A 50 $\mu$L aliquot of the reaction mixture was separated using the TEA mobile phase. (I, *N*-7 Et 5′pdG; II, 5′pdG; III, *N*-7 EtG; IV, 5′Et-pdG).

## Results

**Synthesis and Characterization of Ethylated Guanine Deoxynucleotides.** The ultimate requirement to carry out $^{32}$P-postlabeling necessitated the generation of ethylated standards from 3′pdG. However, for reasons of greater accessibility of the 2′-deoxynucleotide, initial optimization experiments of the HPLC and ES-MS methods used standards generated from 5′pdG. The

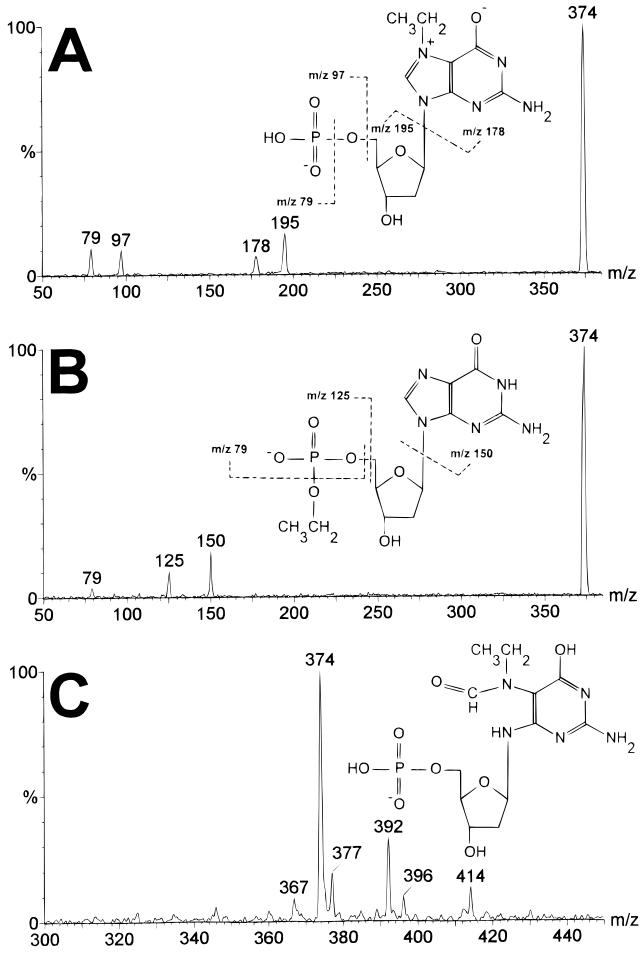



**Figure 2.** (A) Typical ES-MS/MS spectrum obtained for peak I, *N*-7 Et 5′pdG; fragments *m*/*z* 178 and 97 $(M - H)^-$ represent the product ions of ethylated guanine and the phosphate group, respectively, formed from the molecular ion *m*/*z* 374 of *N*-7 Et 5′pdG. (B) Typical ES-MS/MS spectrum obtained for peak IV, 5′Et-pdG; fragments *m*/*z* 150 and 125 $(M - H)^-$ represent the product ions of guanine and the ethylated phosphate group, respectively, formed from the molecular ion *m*/*z* 374 of 5′Et-pdG. (C) ES-MS spectrum of peak I following imidazole ring-opening with ammonium hydroxide/water (30:70 v/v) for 24 h; ions *m*/*z* 392 and 374 $(M - H)^-$ represent the molecular ions of imidazole ring-opened *N*-7 Et 5′pdG (2′-deoxy-$N^5$-ethyl-$N^5$-formyl-2,5,6-triamino-4-oxopyrimidine 5′-monophosphate) and unreacted *N*-7 Et 5′pdG, respectively.

retention times of analogous 5′pdG or 3′pdG products were different using the three different mobile phase systems (Table 1).

Two major and one minor product resulted from the reaction of DES with 3′pdG, as shown in the HPLC chromatogram (Figure 1A). Analogous products were produced from 5′pdG (Figure 1B). Peak II from the latter reaction mixture was confirmed as unreacted starting material following ES-MS analysis (*m*/*z* 346) and by coelution with an authentic standard. Peak I was identified as *N*-7-ethyl 2′-deoxyguanosine 5′-monophosphate (*N*-7 Et 5′pdG); peak III as the spontaneous depurination product of peak I, *N*-7 EtG; peak IV as 2′-deoxyguanosine 5′-mono(*O*-ethyl phosphate) (5′Et-pdG). The variation in retention time of *N*-7 EtG observed between these two experiments (Figures 1A and 1B) was within the standard deviation for repeated analyses carried out using this system. In each case, the structure of *N*-7 EtG was confirmed by ES-MS and cochromatography with the authentic standard.

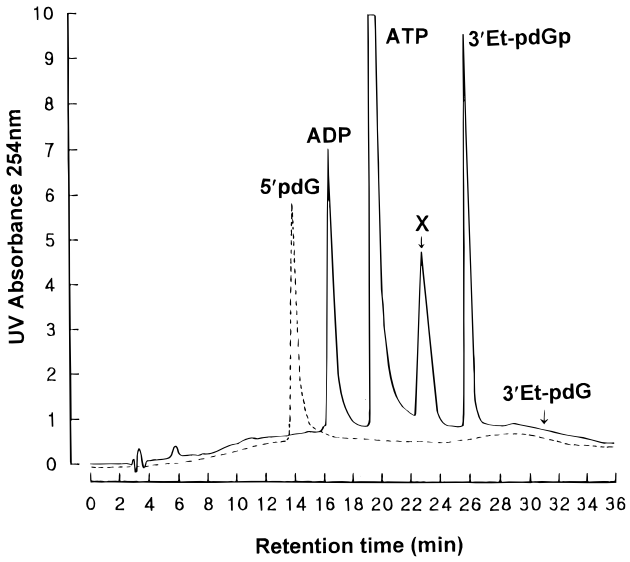



**Figure 3.** Typical HPLC chromatograms obtained for the postlabeling of 3′Et-pdG ($t_R$: 31.2 min) using nonradioactive ATP (—), and after nuclease $P_1$ treatment (- - -). The incubation mixture was separated using the TEAF mobile phase. (X denotes the peak observed for enzyme/labeling buffer blank.)

ES-MS analysis of peaks I and IV gave ions of *m*/*z* 374 $(M - H)^-$ for both products, consistent with the mass of ethylated derivatives of 5′pdG. The site of ethylation for each of these products was further investigated by ES-MS/MS.

Peak I gave a parent ion of *m*/*z* 374 $(M - H)^-$ and product ions of *m*/*z* 195 ([sugar + phosphate] $- H)^-$, 178 ([ethylated guanine] $- H)^-$, 97 $(H_2PO_4)^-$, and 79 $(PO_3)^-$ consistent with an ethylated guanine moiety, probably at the *N*-7 position (Figure 2A). ES-MS/MS does not allow an assignation site for the ethyl group on the guanine base. To confirm that the ethyl moiety was at the *N*-7 position, the product was depurinated by heating to 70 °C. The heat-treated fraction was subsequently shown to coelute with an authentic *N*-7 EtG standard, and further analysis of this HPLC fraction by ES-MS confirmed its identity as that of an ethylated guanine species (data not shown). Treatment of the product in peak I with 30% ammonium hydroxide in water for 24 h at room temperature produced the imidazole ring-opened *N*-7 Et 5′pdG, as confirmed by ES-MS analysis, *m*/*z* 392 $(M - H)^-$ (Figure 2C). However, this treatment was not sufficient to allow complete conversion to the ring-opened form, as indicated by the presence of an ion of *m*/*z* 374 in the ES-MS spectrum. The identity of peak I was further confirmed by performing UV absorption spectroscopy at neutral, acidic, and alkaline pH, resulting in the predictable change of $\lambda_{max}$ from 254 to 266 nm at alkaline pH (*40*).

The ES-MS/MS analysis of peak IV from the reaction of 5′pdG with DES gave a parent ion of *m*/*z* 374 and product ions of *m*/*z* 150 ([guanine] $- H)^-$ and *m*/*z* 125 $(C_2H_6PO_4)^-$ consistent with ethylation of the phosphate group of the nucleotide (Figure 2B).

**$^{32}$P-Postlabeling of *N*-7 Et 3′pdG and 3′Et-pdG.** Analogous analytical approaches were applied to the products from 3′pdG. Initial attempts to postlabel (with nonradioactive ATP) and perform ES-MS on *N*-7 Et 3′pdG were unsuccessful. Although ATP was dephosphorylated to ADP by PNK as evidenced by HPLC of the reaction mixture, there was no peak corresponding to either *N*-7 ethyl-2′-deoxyguanosine 3′,5′-bisphosphate or

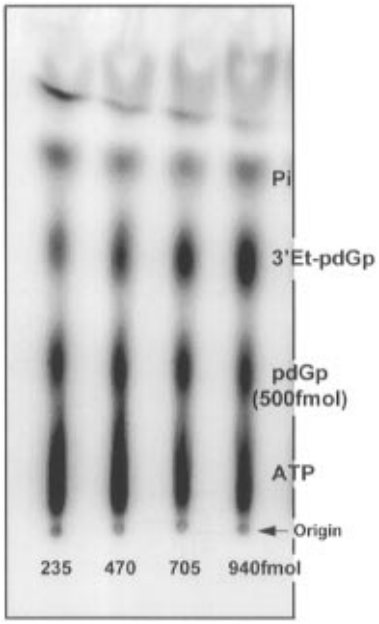



**Figure 4.** Typical storage phosphor image following the one-dimensional TLC separation of $^{32}$P-postlabeled 3′Et-pdG, with 3′pdG as the internal standard. The amounts of 3′Et-pdG labeled are shown at the bottom of the image. Adducts were separated using 1 M ammonium formate (pH 3.5); 15 min exposure.

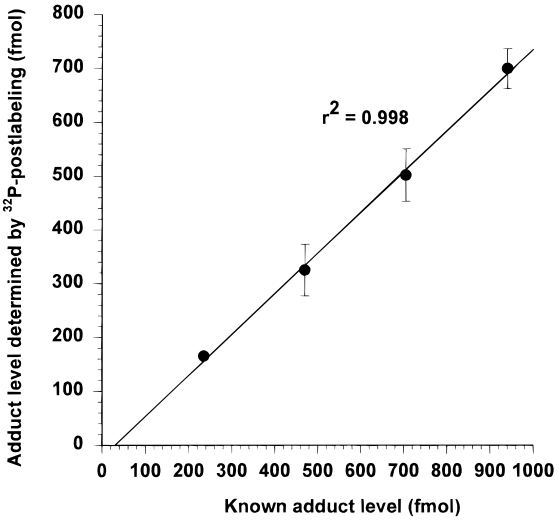



**Figure 5.** Standard curve for determination of 3′Et-pdG by $^{32}$P-postlabeling using 500 fmol of 3′pdG as the internal standard.

of the parent compound. The parent compound had depurinated during evaporation in the DNA centrifugal evaporator, probably as a result of the increase in temperature generated during centrifugation and of the apparent greater chemical instability of the 3′ relative to the 5′ derivative. Freeze-drying of samples thereafter rectified this problem. Postlabeling of *N*-7 Et 3′pdG with nonradioactive ATP gave one major product which was separated by HPLC from unreacted ATP and ADP produced as a result of the transfer of the terminal phosphate group from ATP to the nucleotide. This had a parent ion of *m*/*z* 454 $(M - H)^-$ and product ions at *m*/*z* 97 and 79 when further analyzed by ES-MS/MS consistent with an ethylated 2′-deoxyguanosine bisphosphate product with ethylation of the guanine moiety.

Postlabeling of 3′Et-pdG with cold ATP gave one major product with an HPLC retention time of 26.0 min and a molecular ion of *m*/*z* 454 upon ES-MS analysis. Subse-

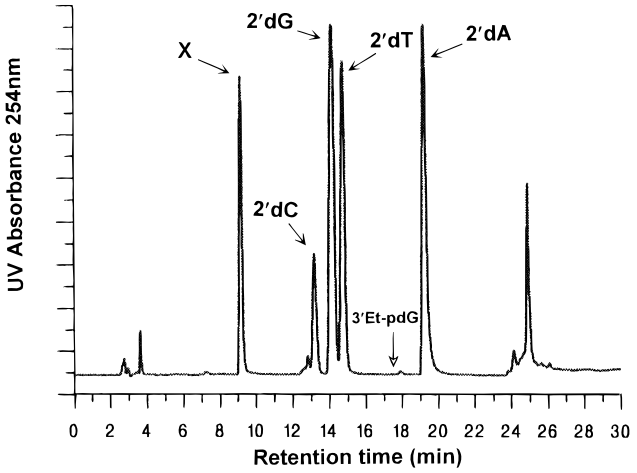
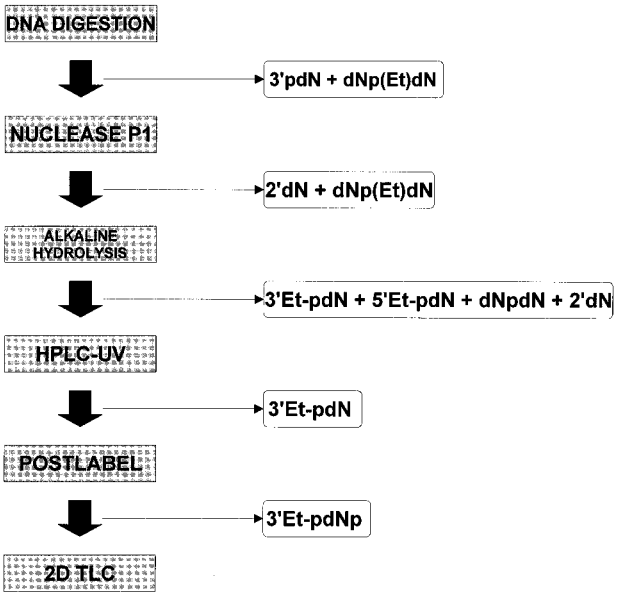



**Figure 6.** Typical HPLC chromatogram of the products formed when DNA is digested with MN and CSPD, followed by a further incubation with nuclease $P_1$ and hydrolyzed in 25% ammonium hydroxide (as shown in Scheme 1). Products were separated using the AF mobile phase: 0.0 min: 0% B, 15.0 min: 20% B, 20.0 min: 0% B, 25.0 min: 100% B, 30.0 min: 0% B. The arrow indicates the retention time of 3′Et-pdG (X denotes an unidentified peak).

**Scheme 1**




quent treatment of this 2′-deoxyguanosine bisphosphate with nuclease $P_1$, which acts as a 3′-phosphatase, resulted in the formation of the unethylated 2′-deoxynucleoside 5′-monophosphate. The structural identity of this was confirmed by ES-MS, which gave an ion at *m*/*z* 346 $(M - H)^-$ representing the molecular ion of 5′pdG, thus unequivocally demonstrating original ethylation of the 3′-phosphate group (Figure 3).

Incubations of DES with the 2′-deoxynucleotides of the other three DNA bases yielded similar results to those obtained with 3′ or 5′pdG, in that the major product of reaction was the ethylated phosphodiester (data not shown).

Once characterized, the two 3′pdG adduct standards were investigated for their postlabeling efficiencies using 3′pdG as an internal standard. The concentration of *N*-7 Et 3′pdG was determined by HPLC−electrochemical detection following neutral depurination at 70 °C for 1 h. The concentration of the 3′Et-pdG standard was determined using a standard curve of 3′pdG, where

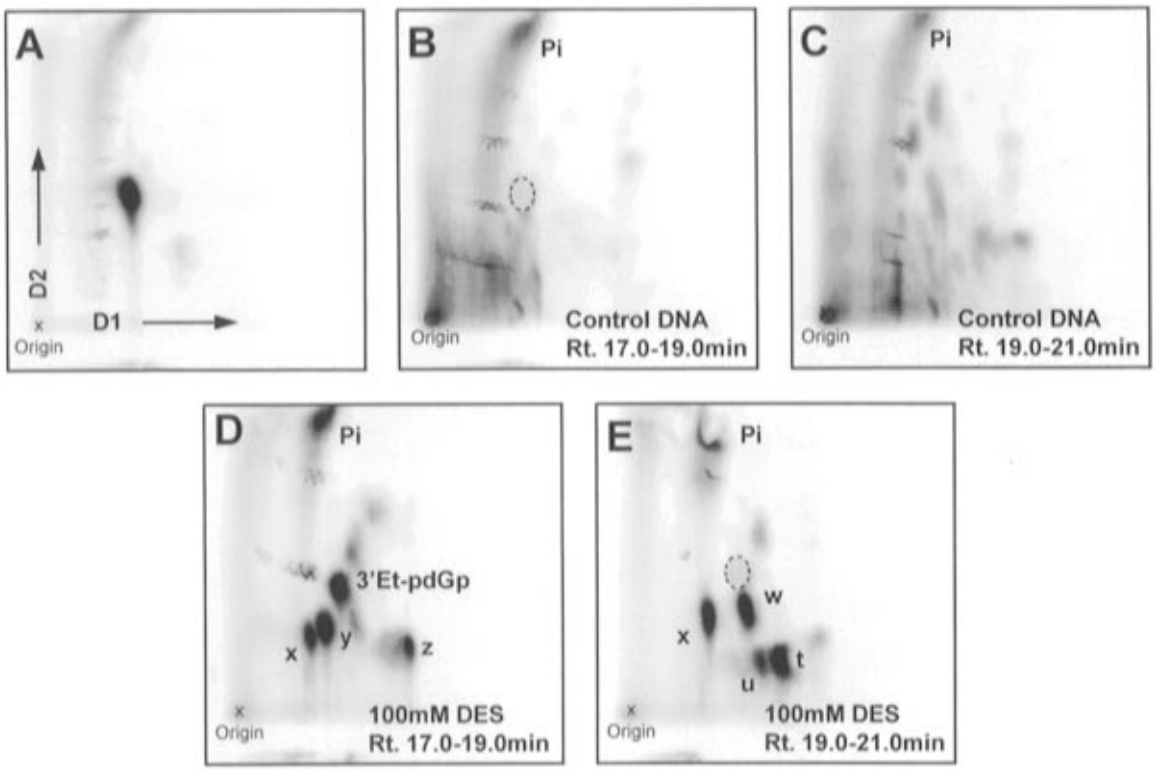



**Figure 7.** Typical storage phosphor images following the two-dimensional TLC separation of $^{32}$P-postlabeled: (A) HPLC fraction corresponding to authentic 3′Et-pdG; 20 min exposure. (B) HPLC fraction corresponding to 3′Et-pdG (17.0−19.0 min) derived from untreated calf thymus DNA. The dotted circle represents the position of the UV marker (unlabeled 5′pdG) which comigrates with postlabeled 3′Et-pdG; 20 min exposure. (C) HPLC fraction (19.0−21.0 min) derived from untreated calf thymus DNA; 30 min exposure. (D) HPLC fraction corresponding to 3′Et-pdG (17.0−19.0 min, Figure 6) derived from calf thymus DNA treated with 100 mM DES; 20 min exposure; x, y, and z denote unidentified spots. (E) HPLC fraction following the 3′Et-pdG fraction (19.0−21.0 min, Figure 6) derived from the same DNA sample; 20 min exposure; t, u, w, and x denote unidentified spots.

absorbance was measured at 253 nm. The postlabeled mixture was applied to prewashed PEI cellulose TLC plates and developed one-dimensionally in 1 M ammonium formate, pH 3.5 (3′Et-pdG plate depicted in Figure 4). Radiolabeled spots were visualized and quantitated by storage phosphor image analysis. The labeling efficiency for 3′ Et-pdG was determined to be 71.3 ± 2.3% compared to 3′pdG, the standard curve being shown in Figure 5. The labeling efficiency for *N*-7 Et 3′pdG could not be determined because the postlabeled adduct was undetectable using *N*-7 Et 3′pdG in the range 170−679 fmol.

**Detection of 3′Et-pdG in DNA.** Attempts to detect 3′Et-pdG in calf thymus DNA treated with 100 mM DES following digestion with MN and CSPD, by using a two-step HPLC purification procedure to remove unmodified nucleotides, followed by postlabeling, were unsuccessful. This was expected, since the 3′Et-pdG adduct is not generated following digestion of DNA with MN and CSPD alone, which generates ethylated 2′-deoxynucleoside phosphotriesters. The approach therefore followed for analysis of 3′Et-pdG involved digestion of DNA with MN and CSPD to 3′pdNs, incubation with nuclease $P_1$, and alkaline hydrolysis according to Scheme 1. Figure 6 shows a typical HPLC elution profile of the products from such an approach, and Figure 7D shows the typical TLC images that are obtained for alkylated DNA following postlabeling of the HPLC fraction corresponding to 3′Et-pdG marked with an arrow in Figure 6. Postlabeling of a fraction collected following the 3′Et-pdG fraction (Figure 7E) reveals a different pattern. The spot for 3′Et-pdG was not present, confirming that this was not an artifact from the postlabeling process. Three new spots from compounds of unknown identity appear (spots w, u, and t); however, one spot appears to be unchanged from the previous fraction (spot x). None of these spots was present in postlabeling maps from corresponding fractions from untreated DNA (Figures 7B and 7C). The method has initially been applied to tissues at various time points generated from an animal study in which mice were dosed with NDEA, with results showing the presence of 3′Et-pdG (Figures 8A and 8B). This adduct, which has so far not been quantified, is detectable up to 56 days following dosing in Balb/c and SWR mice.[2]

## Discussion

The major products formed when DES reacts with 3′pdN have been shown to be the phosphodiesters, e.g., 3′Et-pdG, which has been chemically characterized using, in particular, ES-MS/MS. For reactions with 3′pdG, the ring substituted product *N*-7 Et 3′pdG was similarly characterized, and postlabeling conditions were established using routine procedures.

Alkylated phosphotriesters are not substrates for PNK, and consequently, they have to be converted into compounds that are substrates, namely, phosphodiester dinucleosides or 2′-deoxynucleoside 3′-mono(*O*-alkyl phosphates). Saris *et al.* (*38*) have attempted to determine the whole range of adducted phosphotriesters present in DNA following exposure to alkylating agents. Their method relies on the conversion of the adducted phosphodiester dinucleosides to normal dinucleosides and this may be disadvantageous, since contamination from nor-

[2] Singh *et al.*, unpublished results.

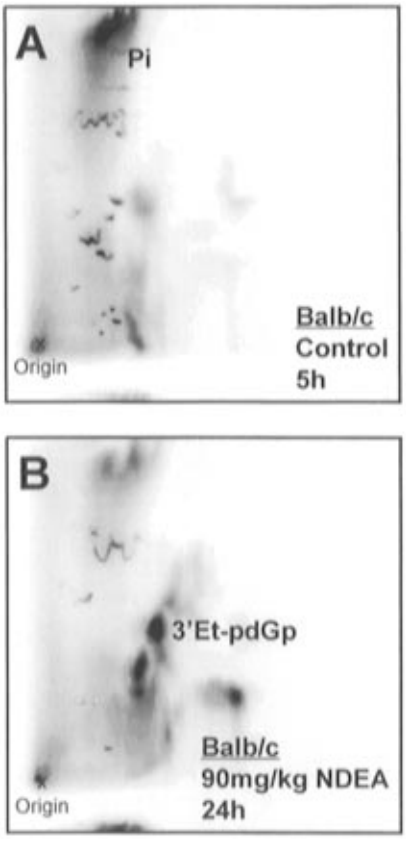



**Figure 8.** Typical storage phosphor images following the two-dimensional TLC separation of $^{32}$P-postlabeled: (A) liver DNA from Balb/c mice, 5 h after ip injection with saline; 30 min exposure; (B) liver DNA from Balb/c mice, 24 h after ip injection with NDEA (90 mg/kg); 30 min exposure.

mal dinucleosides may occur if these are not adequately resolved from the adducted phosphodiesters during the workup procedures of the method. We have used nuclease $P_1$ treatment as an enhancement step for alkylated phosphodiesters. Unmodified nucleotides and base alkylated nucleotide adducts are converted to their corresponding 2′-deoxynucleosides following treatment, rendering them no longer substrates for phosphorylation by PNK. This eliminates contamination of the adduct fraction by unmodified nucleotides that occurs as a result of peak tailing, which is normally a problem encountered when HPLC is used as an enhancement step for the separation of alkylated adducts from unmodified nucleotides, due to their similar chromatographic properties.

As 3′Et-pdG will not be generated from ethylated DNA by digestion with MN and CSPD, the overall extent of ethylation of DNA may be seriously underestimated by simply measuring by postlabeling the extent of base alkylation, especially since many alkylating agents such as nitrosamines tend to ethylate the phosphate backbone of DNA to a greater extent than the base of the nucleotide (*11*).

A further advantage of studying 3′Et-pdG as a marker for ethylation damage of DNA is that it is a very stable adduct and has been shown to withstand heating to 100 °C (*16*), in contrast to adducts formed at the *N*-7 position of guanine, which are very labile and can easily be lost during the lengthy workup procedures involved in postlabeling. In comparison to the $O^6$-ethylguanine adduct, a well-characterized repair mechanism does not exist for 3′Et-pdG; hence measurement of the latter provides a longer-term marker of exposure.

In addition to 3′Et-pdG, one would theoretically expect to find 16 dinucleoside products as well as the ethylated phosphodiester nucleosides for thymine, adenine, and cytosine being generated in our analytical procedure. Since the elution times for these products are not known, a 100% methanol wash step is included at the end of the HPLC gradient, ensuring no carry-over of these products into the next run. In the TLC images that were obtained for alkylated DNA following postlabeling of the HPLC fraction corresponding to 3′Et-pdG (Figure 7D), and the subsequent fraction (Figure 7E), there were unidentified spots which could represent the reaction products described above. This suggestion is supported by the observation that these spots are only present after treatment of DNA with the ethylating agent (Figures 7B−7E). Further work clearly is required to determine the yield of 3′Et-pdG following each of the steps in the method, for instance, by using a radiolabeled standard of 3′Et-pdG. The possibility of using an internal standard also needs to be investigated, as this would allow more accurate quantitation of the adduct and define the sensitivity of the method. We have demonstrated that 3′Et-pdG has a labeling efficiency that is very much greater than that for *N*-7 Et 3′pdG. The low labeling efficiency of *N*-7 Et 3′pdG may be explained by chemical instability of the adduct and loss by depurination during the postlabeling procedure, or by it being a poor substrate for PNK. Similar results were obtained by Koivisto and Hemminki (*35*) who determined a labeling efficiency of 1.5% for *N*-7 Et 3′pdG. The adduct 3′Et-pdG shows both chemical stability and persistence *in vivo*, its presence being determined in DNA extracted from the livers of mice treated with NDEA. Although the biological consequences of phosphotriester formation remain unknown, the determination of this lesion may provide valuable information on earlier exposure to genotoxic compounds.

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