ABSTRACT

The 32P-postlabeling procedure, developed originally by Randerath and coworkers, has been modified for the detection and analytical quantitation of \( O^6 \)-alkyl-2'-deoxyguanosine residues in DNA. Chromatographic techniques were developed to resolve individually the normal deoxyribonucleotide-3'-monophosphates and the \( O^6 \)-alkyldeoxyguanosine-3'-monophosphates by high-pressure liquid chromatography. Selective deoxyribonucleotide-3'-monophosphates (e.g., \( O^6 \)-alkyldeoxyguanosine-3'-monophosphates) were then converted to labeled deoxyribonucleotide-5'-32P-monophosphates by 32P-postlabeling and nucleoside P1 treatment and separated by two-dimensional thin layer chromatography. The \( O^6 \)-methyl- and \( O^6 \)-ethyl-2'-deoxyguanosine-3'-monophosphate nucleotides, and the respective 5'-monophosphates, were chemically synthesized for standardization of these quantitative procedures. The quantitation of \( O^6 \)-methyl- and \( O^6 \)-ethyl-2'-deoxyguanosine was observed to be analytically adequate between one \( O^6 \)-alkyl-2'-deoxyguanosine residue per 1000 and 10 \( O^6 \)-2'-deoxyguanosines. The limit of detection was less than one \( O^6 \)-alkyl-2'-deoxyguanosine in 10 \( O^6 \)-2'-deoxyguanosine residues in a sample size of 100 \( \mu \)g of DNA, i.e., approximately 10 pg of adduct. The quantitation of \( O^6 \)-methyl-2'-deoxyguanosine in the liver DNAs of rats treated with \( ^{13}C \)-MeN-nitrosodimethylamine compared well with values obtained by both \(^{14}C \) and high-pressure liquid chromatography coupled with fluorescence detection. Thus, these 32P-postlabeling and nucleotide chromatographic procedures should be useful in monitoring human exposure to methyating and ethylating carcinogens.

INTRODUCTION

The determination of chemical carcinogens as etiological factors in the occurrence of selected human cancers would be facilitated by the ability to assess not only the extent of exposure but also the biological damage (e.g., carcinogen-DNA adducts) subsequently produced in the tissues and cells of people exposed to carcinogens (1). Many techniques have been developed to detect minute levels of aromatic carcinogens in biological fluids and in cellular DNA. Immunoassays systems comprise the majority of these sensitive methods for the detection of polycyclic aromatic hydrocarbons (2-6), acetylaminofluorene (7), and aflatoxin B1 (8, 9). The assessment of polycyclic aromatic hydrocarbon-type carcinogens, other arylating carcinogens, and aflatoxin B1 exposure has, however, been enhanced by the use of synchronous fluorescence spectrophotometric (10-12) and 32P-postlabeling methods (13, 14), which complement the radioimmunological techniques.

The detection of alkyl-type DNA adducts such as \( O^6 \)-alkylGdG has been performed by high pressure liquid chromatography (HPLC) with fluorescence detection (15) and radioimmunological techniques (16-18). The determination of the levels of \( O^6 \)-alkylGdG residues in human DNAs may be important due to the mutagenic potential of this adduct (19-21) and the strong correlation of DNA oxygen alkylation and the carcinogenic potency of agents (22-25). Human exposure to N-nitroso compounds and other alkylating agents occurs in tobacco smoke (24-26), industrial work environments and fossil fuels (27), and cancer chemotherapy (28).

One problem that has arisen is that even monoclonal antibodies generally recognize, at least to some extent, adducts other than the major adduct under study. Clearly, alternative and supporting methodologies that may corroborate the radioimmunoassay results are needed for the quantitation of alkyl DNA adducts. In response to these needs, we have modified the 32P-postlabeling and nucleotide chromatographic methodology developed by Randerath and coworkers (29) for the detection and analytical quantitation of \( O^6 \)-mdG and \( O^6 \)-etdG.

MATERIALS AND METHODS

Chemicals. The deoxyribonucleotides 5'S-mdC, 5'dC, 5'dT, 5'dG, 5'dAp, 3'dCp, 3'dTp, 3'dGp, and 3'dAp were obtained from Pharmacal Biochemicals (Piscataway, NJ). \( N \)-Nitrosodimethylamine was purchased from Sigma (St. Louis, MO) and \( [\text{14}C \]-MeN-nitrosodimethylamine (14 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). Radiolabeled \( [\text{32}P \]ATP (5000 Ci/mmol) was obtained from Amersham. 5'-Dimethoxytrityl-A'-isobutyryl-2'-deoxyguanosine phosphates was purchased from Cruachem, Inc. (Herndon, VA). Hydrazine hydrate, \( N,N \)-dicyclohexylcarbodiimide (DCCI), 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU), levulinic acid, triisopropylbenzene-sulfonfyl chloride (TPS-CI), anhydrous trimethylamine (Me,N), anhydrous pyridine, and 2-cyanoethyl phosphate (barium salt dihydrate) were purchased from Aldrich Chemical Co. (Milwaukee, WI).

Preparation of 3'- and 5'-Monophosphate \( O^6 \)-Methyl- and \( O^6 \)-Ethyl-2'-deoxyguanosine. The general synthetic route for 3'- and 5'-monophosphates of \( O^6 \)-methyl- and \( O^6 \)-ethyl-2'-deoxyguanosine is shown in Fig. 1. Completely protected derivatives of \( O^6 \)-alkyl-2'-deoxyguanosine (II) were prepared by a synthetic procedure developed by Jones and coworkers (30-32); facile sulfonylation of the 6 position of guanine by trisopropylbenzenesulfonfyl chloride, followed by its displacement by anhydrous trimethylamine, generated an unstable 6-trimethylamino compound which, in turn, was converted to the \( O^6 \)-alkyl derivative (II) with a high yield. After purification, a portion of the nucleoside derivative (II) was detritylated by aqueous acid treatment to form III, whereas the rest of the derivative II was treated with hydrazine to hydrolyze the 3'-levaline group yielding IV. Phosphorylation of the 3'-hydroxyl functionality was performed under anhydrous conditions by pyridinium-\( \beta \)-cyanoethyl phosphate in the presence of \( N,N \)-dicyclohexyl-carbodiimide in pyridine following the method of Tener (33). Deprotection of III was carried out by the alkoxide ion in the respective alcohol in 1,8-diazabicyclo[5.4.0]undec-7-ene for 2 days (31); deblocking of IV, in addition, required detritylation. The \( O^6 \)-alkyl-2'-deoxyguanosine phosphates, thus produced, were purified on a Whatman Partisil SAX column (linear gradient of 1-300 mM potassium phosphate buffer (pH 5.3) over 60 min, at a flow rate of 1.5 ml/min) followed by desalting on a C18 Sep-Pak (Waters) cartridge. The Sep-Pak cartridge was prepared by washing sequentially with 50% acetonitrile.
Wilson et al. (34). The resultant 3‘dNps were then fractionated by HPLC and the appropriate fractions for 3‘dGP and 3‘O6-mdGP (or 3‘O6-etdGP) were pooled and lyophilized to concentrate and remove the elution buffer. One-hundredth to one-thousandth of the 3‘dGP peak was added to the 3‘O6-mdGP fraction and the mixture was analyzed by 32P-postlabeling as described previously (34). Briefly, 32P was transferred to the 5′ position of the 3‘dNps by the action of T4 polynucleotide kinase in the presence of [γ-32P]ATP and the 3′-phosphate moiety removed by nuclease P1 treatment. The labeled [5′-32P]dNps were then separated by two-dimensional thin-layer chromatography on cellulose plates (20 x 20 cm plates without fluorescent indicator, Curtis Matheson Corp.) in the presence of UV detectable levels of unlabeled 5′pdG and 5′pO6-mdG. The first and second dimension solvents were isobutyric acid:H2O:NH4OH (66:20:1, v/v) and saturated (NH4)2SO4 isopropanol:1 m NaOAc (80:2:18, v/v), respectively (34). The spots were scraped and counted for radioactivity and the ratio of 5′pO6-mdG to 5′pdG was determined.

RESULTS

We have previously demonstrated that the limit of detection of a rare nucleotide (e.g., 5-methyl-2′-deoxycytidine-5′-monophosphate) was only 0.01% of 5′pdC (or 5′pdG) in a genomic DNA sample, using a modified Randerath 32P-postlabeling procedure (34, 35). However, the detection of DNA adducts in human and animal tissues and cells requires more sensitive methods. These procedures were, therefore, further modified for the detection of O6-alkylG adducts by isolating the 3′O6-mdGp and 3′O6-etdGp nucleotides from the normal nucleotides by HPLC prior to 32P-postlabeling. The HPLC elution profile of O6-mdGp was determined using postlabeled [5′32P]dG and [5′32P]pO6-mdG (Fig. 2), since only limited quantities of 3′O6-mdGp were available and the 5′pdNs elute in the same relative positions as the respective 3′dNps under the HPLC conditions used here. The elution solvent was a weak buffer of the volatile solute NH4OAc, so that the isolated nucleotides could be concentrated and the salt removed by lyophilization. The elution of [5′32P]O6-etdG (or 3’O6-etdGp) from the C18 reversed-phase...
column required, however, a methanol wash (Fig. 3).

These techniques provided an efficient means of isolating and detecting of O'-alkyldG from a mixture of deoxyribonucleotides (Fig. 4). A mixture of 3′dAp, 3′dCp, 3′dGp, 3′dTp, and 3′O'-etdGp was prepared with a known 3′O'-etdGp:3′dGp ratio of 1:1000. The 3′dGp and the 3′O'-etdGp were resolved from the other deoxyribonucleotides by HPLC and isolated (Fig. 4, top). One-hundredth of the HPLC eluted 3′dGp was combined with the 3′O'-etdGp, 32P-postlabeled, and separated by two-dimensional TLC (Fig. 4, bottom). The two-dimensional Rf values for both 5′pO'-mdG and 5′pO'-etdG are listed in Table 1.

Using contrived mixtures of 3′O'-mdGp or 3′etdGp to 3′dGp, the quantitation of O'-mdG and O'-etdG was found to be accurate and precise over three orders of magnitude (Fig. 5). Quantitation of these alkylated nucleotide residues was performed by scraping and counting the radioactivity in the TLC spots. The chromatographic recovery of O'-alkyldG residues was excellent as judged by the fact that greater than 90% of the radioactivity of [5′-32P]O'-mdG (and [5′-32P]O'-etdG) applied to the HPLC column was recovered in the appropriate location on the subsequent TLC plate (data not shown).

The usefulness of these procedures for the analysis of tissue DNA samples was demonstrated in the quantitation of O'-mdG in liver DNA isolated from a rat treated with 10 mg/kg of N-nitrosodimethylamine (Fig. 6). Following enzymatic digestion of the liver DNA, only one-hundredth of the HPLC-isolated 3′dGp was added to the pooled fractions containing the 3′O'-mdGp nucleotide in order to prevent the chromatogram from being overwhelmed by labeled [5′-32P]dG. The level of O'-mdG in the rat liver DNA was determined to be approximately two adducts per 10^6 2′-deoxyguanosine residues.

Quantitation of O'-mdG in rat liver DNA by 32P-postlabeling analysis was also observed to compare well with other physical methods of detection (Table 2). Liver DNA was isolated 4 h after treatment of rats with various doses of [14C-Me]N-nitrosodimethylamine. Following the acid hydrolysis of 100 μg of DNA, the level of O'-mG was measured by ion exchange HPLC and fluorescence detection according to the method of Herron and Shank (15). The limit of detection by fluorescence was observed to be approximately five O'-mG (or about 140 pg O'-mG) per 10^6 guanine residues, as previously reported (15). The amount of radioactivity (14C) eluting with the fluorescent O'-mG nucleoside was also determined, so that the level of O'-mG could be calculated based on the specific activity of the [14C-Me]N-nitrosodimethylamine the rat received. From these results, approximately 3% of the bound 14C was present as O'-mG in the DNA. Enzymatic digestion of another 100 μg portion of DNA and subsequent HPLC and 32P-postlabeling analysis provided levels of O'-mdG similar to the 14C and fluorescent methods (Table 2). Even if ribonucleotides contaminate the

![Fig. 3](image-url) HPLC elution profile of standard nucleotides (—) detected by absorbance at 260 nm and the 32P-labeled products of postlabeling analysis of a mixture of 3′dGp and 3′O'-etdGp (— —) as described in text.

![Fig. 4](image-url) Separation and detection of O'-etdG from a mixture of 3′dCp, 3′dGp, 3′dTp, and 3′dAp spiked with 3′O'-etdGp. Top, HPLC elution profile of the mixture with detection by absorbance at 260 nm; bottom, autoradiogram of the two-dimensional TLC separation of the 32P-postlabeled mixture of the pooled HPLC fractions containing 3′O'-etdGp and one-hundredth of the 3′dGp HPLC fractions, as described in text. All unreacted [γ-32P]ATP was degraded to 32Pi by apyrase during the postlabeling procedure (see "Materials and Methods"). T2P, migrates to the top of the TLC plate as the spot in the top left of the autoradiogram demonstrates. X-ray film exposure time was 20 min.

<table>
<thead>
<tr>
<th>Dimension</th>
<th>5′pdG</th>
<th>5′pO'-mdG</th>
<th>5′pO'-etdG</th>
<th>5′pdA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1D</td>
<td>0.27</td>
<td>0.69</td>
<td>0.91</td>
<td>0.79</td>
</tr>
<tr>
<td>2D</td>
<td>0.40</td>
<td>0.17</td>
<td>0.17</td>
<td>0.24</td>
</tr>
</tbody>
</table>
Fig. 5. The HPLC/32P-postlabeling determination of standard mixtures of very low levels of 3′O6-mdGp (left) and 3′O6-etdGp (right) in the presence of 3′dGp as described in text. Points, mean of at least three separate determinations ± SD. Linear regression analyses provided the slopes and correlation coefficients shown for each curve.

HPLC elutants of these 2′-deoxyribonucleotides and DNA adducts, ribonucleotide-5′-monophosphates migrate dramatically different from the 5′pdNs (34). Thus, one reason the HPLC/32P-postlabeling O6-mdG values are consistently lower than those observed with the other detection methods may have been due to small amounts of contaminating RNA in the isolated rat liver DNAs. The limit of detection by HPLC and 32P-postlabeling was less than one O6-mdG per 107 2′-deoxyguanosine residues, or about 10 pg O6-mdG in 100 µg of DNA. Although the dose-response curve was not linear, the values compare well with those previously reported by Pegg and Hui (36).

DISCUSSION

The inability to measure low levels of DNA adducts has hampered efforts to determine biologically effective doses of carcinogens received by individuals. The present work demonstrates that low levels of DNA O6-alkylG residues can be quantitated in human tissues and cells by HPLC/32P-postlabeling techniques. One adduct per 107 2′-deoxyguanosine residues was detectable in DNA, so that these techniques provided a sensitivity of O6-alkylG detection equivalent to that reported for radioimmunological methods (16, 17). These physical methods may, however, be more selective than radioimmunoassay techniques, due to both HPLC and two-dimensional TLC separation and isolation of the adducts. Thus, the HPLC/32P-postlabeling assay will complement the radioimmunoassay and other techniques for O6-alkylG detection, and the assessment of biological damage inflicted by methylating carcinogens recently received by an individual.

Although, a loss of less than 10% of 3′O6-mdGp, or 3′O6-etdGp, may have been incurred during the chromatographic separation and detection of this adduct from DNA, the determined ratio of 3′O6-mdGp to 3′dGp was apparently unaltered. This may have been due to the equivalent loss of 3′dGp during the isolation and detection procedure, so that the determined levels of O6-mdG matched well with known standard mixtures of 3′O6-alkylGp and 3′dGp. The suggestion that the quantitation of O6-mdG truly reflects the actual level of this adduct in DNA is further supported by the similar values obtained using other detection techniques. Equivalent O6-mdG levels in rat liver DNAs, following an i.p. dose of [14C-Me]N-nitrosodimethylamine, were obtained using HPLC/fluorescence (15), HPLC/14C radioactivity, or HPLC/32P-postlabeling detection. These values were also in accord with those reported by Pegg and Hui (36). The slightly lower values of O6-mdG determined by HPLC/32P-postlabeling may be accounted for by some RNA contamination of the DNA preparations, since the ribonucleotides migrate at a much slower rate than the equivalent 2′-deoxyribonucleotides under these two-dimensional TLC conditions (34). The lower limit of O6-mdG detection in the control rat (less than one adduct in 107 2′-deoxyguanosine residues) also suggests that O6-mdG formation by endogenous methylation by S-adenosylmethionine (37, 38) may be well below the level of O6-mdG present in the DNA of carcinogen-exposed people (17). Further clarification of endogenous O6-mdG formation will require the analyses of human DNAs by a variety of sensitive methods.

DNA O6-alkylG are adducts of well-known mutagenic po-
tential (19-21), so that mammalian and procaroy cells alike have evolved enzymatic systems for the removal and repair of these lesions from their DNAs (39, 40). Thus, the level of O6-alkyG detected will be the resultant balance between the amount of O6-alkyG formed from carcinogen exposure and the amount removed by DNA repair processes, which vary with organ tissue or cell type (23, 41-43). However, other DNA adducts such as O4-alkyl-2'-deoxythymidine and O2-alkyl-2'-deoxythymidine, are formed by exposure to alkylating carcinogens and are generally removed and repaired at a slower rate (22, 44, 45). Although less O4-alkyl-2'-deoxythymidine and O2-alkyl-2'-deoxythymidine are initially formed (44, 46), the mutagenic and carcinogenic potential of these lesions may be greater than that of O6-alkyG lesion due to their persistence in DNA (45). The majority of the mutagenic activity of methylating N-nitroso compounds has, however, been proposed to be due to O6-mdG residues (21, 47). Thus, the monitoring of a number of DNA adducts such as O6-alkyl-2'-deoxyguanosine and O4-alkyl-2'-deoxyguanosine, will be present in DNA at higher levels than O6-mdG (22, 36), and selective adducts formed by chemotherapeutic agents, so that HPLC/I3P-postlabeling procedures should prove to be valuable for monitoring both human exposure to environmental carcinogens and the treatment of cancer patients.

People are exposed to numerous methylating carcinogens capable of forming O6-mdG in the individual's tissues and cellular DNA. N-Nitrosamines comprise the majority of these carcinogens in the food, work, and living environments (27, 48, 49), and from individual habits such as tobacco smoking (24-26, 50). The epidemiological characterization of an individual N-nitrosamine or other carcinogen as a major etiological agent in selective cancers will require the combined use of more than one method of analysis due to the loss of selectivity with increased detection sensitivity observed in these emerging technologies. The ability to monitor for the actual level of a small alkylated product in the DNA of a human subject by a physical detection method, such as the method described here, represents a significant contribution toward the eventual goal of understanding the relationship between carcinogen exposure and the incidence of human cancer.

REFERENCES


Table 2 Comparison of methods for the quantitation of O6-mdG in rat liver DNA isolated 4 h after [14C]-MeV-nitrosodimethylamine treatment

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>&quot;C&quot;</th>
<th>[O6-mdG]/[dG] × 10^6 HPLC/fluorescence</th>
<th>HPLC/I3P-postlabeling</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>&lt;0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td>6.7</td>
<td>7.9</td>
<td>6.0 ± 3.6 (4)</td>
</tr>
<tr>
<td>5</td>
<td>22.3</td>
<td>27.8 ± 1.1 (4)</td>
<td>16.8 ± 3.0 (4)</td>
</tr>
<tr>
<td>10</td>
<td>202.0</td>
<td>217.0 ± 75.0 (4)</td>
<td>186.0 ± 28.0 (4)</td>
</tr>
</tbody>
</table>

* Calculated from the specific activity of the i.p. injection and the radioactive recovered in the HPLC elution of O6-mdG from 100 μg of DNA. Values, average of two determinations.

† Determined by the method of Herron and Shank (15). Values, mean ± SD of (N) samples.

‡ Determined by HPLC/I3P-postlabeling as described in "Materials and Methods." Values, mean ± SD of (N) samples.

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AalkyldG DETECTION BY 32P-POSTLABELING


O\textsuperscript{6}-Alkyldeoxyguanosine Detection by \textsuperscript{32}P-Postlabeling and Nucleotide Chromatographic Analysis


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