Determination of \(O^6\)-Butylguanine in DNA by Immunoaffinity Extraction/Gas Chromatography-Mass Spectrometry

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ABSTRACT

A sensitive, specific, and rapid method for quantitating the minor adduct \(O^6\)-butylguanine (\(O^6\)BuG) in hydrolyzed DNA has been developed by combining immunoaffinity chromatography and high resolution gas chromatography-negative ion chemical ionization-mass-spectrometry. Polyclonal antibodies raised against \(O^6\)BuG were coupled to CNBr-activated Sepharose 4B and used for sample clean-up and extraction of the specific \(O^6\)-alkylguanine. After addition of \(O^6\)BuG and its deuterium labeled analogue (\(O^6\)BuG-D\(_2\)), used as internal standard, hydrolyzed DNA was applied on the immunoaffinity column and washed with water, and the immunoadsorbed butylated guanines were eluted with acetone/water/ctome/water (95/5) before gas chromatographic derivatization. \(O^6\)BuG and \(O^6\)BuG-D\(_2\) were analyzed and quantitated by high resolution gas chromatography-negative ion chemical ionization-mass-spectrometry as their pentafluorobenzyl-trimethylsilyl derivatives. Immunoaffinity column capacity and \(O^6\)BuG recovery from this column were 1.53 mmol \(O^6\)BuG/column and 62 ± 5%, respectively.

The method was applied to evaluate \(O^6\)BuG levels in DNA butylated in vitro with 10 mm \(N\)-nitroso-\(N\)-butylurea or isolated from rats given an i.p. dose of 185 mg/kg \(N\)-nitroso-\(N\)-butylurea or \(N\)-nitrosodibutylamine. In the first case the level of modifications present in calf thymus DNA was 104 mmol \(O^6\)BuG/mol guanine, and in the second case \(O^6\)BuG in liver DNA was about 6 times higher after \(N\)-nitroso-\(N\)-butylurea (2.11 mmol \(O^6\)BuG/mol guanine) than after \(N\)-nitrosodibutylamine (0.34 mmol \(O^6\)BuG/mol guanine) treatment.

These results indicate that \(O^6\)BuG formed in vivo can be isolated and quantitated by this method, which may also be useful for studying DNA damage and repair mechanisms.

INTRODUCTION

Alkylating agents interact with DNA to yield a variety of reaction products. Various alkylating sites have been identified, the predominant one being in the nucleophilic \(N^\)position of guanine and, to a lesser extent, at the \(O^6\) and \(O^4\) atoms of guanine and thymine, respectively (1). While \(N\)-alkylguanine does not appear to be directly mutagenic, the minor adducts, \(O^6\)-alkylguanine and \(O^6\)-alkylthymine, have been shown to lead to point mutations in DNA after replication (1, 2).

\(N\)-Nitroso compounds represent a class of alkylating agents shown to induce tumors in several animal species (3). Their carcinogenic effect is tissue specific and depends on the \(N\)-nitroso compound used (3, 4). Although most work has focused on \(N\)-nitroso compounds producing methylation and ethylation species with the consequent formation of \(O^6\)-methyl and \(O^6\)-ethyldguanine adducts, other higher alkylating compounds such as butylating agents, although rarer, are also important, since they are carcinogens in animals and have been detected in various foods products and other constituents of the human environment (1, 5–7).

Butylating agents can be very specific in their site of tumor production; in fact NDBA (4) and its \(O^6\)-oxidized metabolites \(N\)-nitrosobutyric(4-hydroxybutyl)amine and \(N\)-nitrosobutyric(3-carboxypropyl)amine are potent and selective urinary bladder carcinogens in rats and mice (3, 8). According to the most widely accepted nitrosamine activation pathway, biotransformation of these compounds would lead to the formation of butylating species that react with cellular macromolecules, such as proteins or DNA (9–11). Because butylation at the \(O^6\) atom of guanine would be a promutagenic lesion, \(O^6\)BuG identification and quantification can be used for studies of DNA damage, for estimating the steady state level of such damage, and for assessing its possible significance.

In general, the detection of modified bases is hindered mainly by the analytical obstacle involved in measuring such a low concentration. Consequently, sensitivity and selectivity are the two main factors required for the development of an analytical method suitable for this purpose.

During the past few years several antibody-based methods such as radioimmunoassay and ELISA have been used for measuring alkylations products in hydrolyzed DNA (12–15). However, this approach has some limitations. A critical point is the degree of antibody cross-reactivity, which cannot be avoided, with other DNA adducts. Moreover, the purification of samples, usually involving HPLC separation, is often laborious and time-consuming (14, 15).

GC-MS is also used to detect and measure DNA adducts in biological samples but this technique, which has been applied mainly to urine (16), requires even more extensive sample purification.

In order to overcome these problems, we exploited the specificity of polyclonal antibodies raised against \(O^6\)BuG for purification purposes, developing immunoaffinity chromatography, which makes the clean-up of samples efficient and simple. The subsequent use of HRGC-MS for instrumental analysis, preferably with NICI, achieves high selectivity and sensitivity.

Although monoclonal antibodies to \(O^6\)BudG were previously produced by Saffhill et al. (12) and used to set up a sensitive radioimmunoassay for the detection of \(O^6\)BudG, the purpose of our work was to utilize antibodies for the extraction-purification step rather than the quantitation of \(O^6\)BudG.

We present here a new, specific, and sensitive method for the one-step immunoaffinity extraction of \(O^6\)BuG followed by GC-MS.
MS analysis. The method allows O\textsuperscript{6}BuG quantification in DNA butylated in vitro with NBU or isolated from rats given NDBA or NBU. O\textsuperscript{6}BuG and its deuterium-labeled analogue, used as internal standard (O\textsuperscript{6}BuG-D\textsubscript{7}), were analyzed as their PFB-TMS derivatives.

The development and characterization of polyclonal antibodies raised against O\textsuperscript{6}BuG is discussed.

MATERIALS AND METHODS

Chemicals. NDBA was from Eastman Kodak Co. (Rochester, NY) and NBU was from Serva Feinbiochemica GMBH & Co. (Heidelberg, FRG). 2-Amino-6-chloropurine, BSA, HC from \textit{Limum polysiphonum} hemolymph, complete Freund’s adjuvant, sodium ethylmercurithiosalicylate (thimerosal), Tween 20, and calf thymus DNA were from Sigma Chemical Co. (St. Louis, MO). [2,3,3,4,4,4,\textsuperscript{2}H]butanol was from Cambridge Isotope Laboratories (Woburn, MA). PFBBr was from Aldrich (Milwaukee, WI). N\textsubscript{6}-O-Bis(trimethylsilyl)trifluoroacetamide was from Fluka (Buchs, Switzerland). Protein A, Sepharose, and CNBr-Sepharose 4B were from Pharmacia (Uppsala, Sweden). ELISA microtitration plates were from Flow Laboratories (Irvine, Scotland, UK). Horse serum was from Gibco (Paisley, Scotland, UK). Peroxidase-labeled anti-rabbit IgG and peroxidase substrate, 2,2\textsuperscript{,3}-azinobis(3-ethylbenzothiazoline-6-sulfonic acid), were from Kirkegaard & Perry Laboratories Inc. (Gaithersburg, MD). All solvents, of analytical grade, were from Merck (Darmstadt, FRG) or Farmitalia Carlo Erba (Milan, Italy).

Synthesis of O\textsuperscript{6}-Alkylguanines. Synthesis of O\textsuperscript{6}alkylguanine was by a modification of the method of Balsiger and Montgomery (17). Briefly, metallic sodium (55 mg, 2.4 mmol) was dissolved in 2 ml of the appropriate alcohol (methanol, ethanol, propanol, butanol, and deuterated butanol), which was previously dried by distillation over sodium. Anhydrous toluene (2 ml) and 2-amino-6-chloropurine (50 mg, 0.30 mmol) were added to the alkoxide solution and the reaction mixture was refluxed for 30 min under a dry nitrogen atmosphere. After cooling to room temperature, the reaction was quenched with the stoichiometric amount of 2 N HCl. The organic layer was removed \textit{in vacuo} and the product was recrystallized from aqueous ethanol. Reaction yield ranged from 90 to 95%.

Preparation of O\textsuperscript{6}-Butylguanosine Conjugates and Anti-O\textsuperscript{6}BuG Antiserum. To obtain an immunizing antigen, O\textsuperscript{6}BuG-GA, synthesized as described by Farmer et al. (18), was conjugated to BSA or HC, following the procedure of Erlanger and Beiser (19). The conjugates were characterized by UV spectroscopy (20).

One mg of the immunizing agent, O\textsuperscript{6}BuG-BSA, was suspended in 0.5 ml PBS and emulsified with 0.5 ml of complete Freund’s adjuvant. The emulsion was then injected s.c. into the shaved backs of male New Zealand White rabbits (1.5 kg; Charles River, Calco, Como, Italy) at 10 different sites in 100-μl aliquots. The animals were boosted after 1 week with 0.5 mg of conjugate in complete Freund’s adjuvant and then with 0.1 mg every 2 months to increase the immune response. One week after booster injections, blood was taken from the ear artery and serum was prepared for ELISA determination of antibody production.

ELISA and Competitive ELISA. Immunoglobulin production was checked by ELISA, using O\textsuperscript{6}BuG-BSA, and HC, as coating antigens, and peroxidase-labeled anti-rabbit IgG as second antibody. Briefly, the plates were coated by incubating them with O\textsuperscript{6}BuG-BSA or HC, in 0.1 M sodium carbonate buffer, pH 9.6, at 4°C overnight. The plates were then washed 3 times with PBS containing 0.05% Tween 20. A similar wash was done after each incubation. Nonspecific binding to the plates was minimized by addition of 200 μl/well of 1% horse serum in PBS, followed by 60 min of incubation at 37°C. Serial dilutions of rabbit antisera were added, and the plates were incubated for 60 min at 37°C before adding peroxidase-labeled anti-rabbit IgG (diluted 1:1000). After 60 min of incubation at 37°C, 100 μl peroxidase substrate was added to each well and left for 60 min at room temperature. The color was read at 405 nm with a TITERTEK (Flow Laboratories) microplate reader. Competitive ELISA was performed to check antibody sensitivity and specificity, using O\textsuperscript{6}BuG-BSA as coating antigen.

Preparation and Use of Immunoadfinity Columns. Immunoglobulins were purified from other serum proteins using a Protein A-Sepharose column, following the manufacturer’s instructions. About 9 mg of IgG were recovered per ml of rabbit serum.

The IgG fraction was coupled (98% yield) to CNBr-activated Sepharose 4B (5 mg of IgG/ml of gel), as recommended by the manufacturer. Immunoadfinity columns were prepared by introducing 0.25 ml of coupled gel, diluted 1:1 with uncoupled matrix, into an empty Bond Elut reservoir (0.5-cm diameter, 6-cm long) with a porous polyethylene frit and a Teflon stopcock at the bottom. For extraction, 0.1–5 mg of DNA (10 mg/ml 0.1 N HCl) were hydrolyzed at 80°C for 1 h; after hydrolysis, the pH was adjusted to 7–7.4 with 1 M K\textsubscript{2}HPO\textsubscript{4}, and the samples were diluted to 2 ml with PBS. A known amount of deuterated internal standard (O\textsuperscript{6}BuG-D\textsubscript{7}) was added and samples were loaded on the column, left in contact with the gel for 10 min, under mixing, and then percolated from the gel. The columns were washed with 10 ml of distilled water, which was pooled with the 2 ml of percolate for determination of guanine content. Immunoadsorbed O\textsuperscript{6}BuG and O\textsuperscript{6}BuG-D\textsubscript{7} were eluted with 3 ml of acetonitrewater (95/5). After washing with water (20 ml) and 95% acetonitrile (10 ml) and regeneration with distilled water (20 ml) and 0.05 M phosphate buffer, pH 7.4, containing 0.02% of thimerosal (10 ml), the columns were stored at 4°C.

Immunoadfinity columns were characterized by determining retention capacity and recovery from reconstituted blank samples of hydrolyzed DNA. Column capacity was measured by extracting known amounts of O\textsuperscript{6}BuG. For recovery studies, samples of hydrolyzed blank DNA were buffered to pH 7–7.4 with 1 M K\textsubscript{2}HPO\textsubscript{4}, diluted to 2 ml with PBS, spiked with known amounts of O\textsuperscript{6}BuG, and loaded on the immunoadfinity column. A known amount of O\textsuperscript{6}BuG-D\textsubscript{7} was added after elution and before derivation and was taken as a reference compound. Recovery was calculated by comparing the HRGC-SIR peak area ratios (O\textsuperscript{6}BuG peak area/O\textsuperscript{6}BuG-D\textsubscript{7} peak area) of extracted samples with those of standards containing known amounts of the derivatized compounds.

Derivatization, GC-MS Characterization, and Quantitation. The immunoadfinity column eluate was dried under vacuum and directly derivatized. PFB-TMS derivatives of O\textsuperscript{6}BuG and O\textsuperscript{6}BuG-D\textsubscript{7} were obtained using the following experimental conditions: 20 μl of a solution of PFBBr in anhydrous ethanol (1/50, v/v) and 50 μl of a solution of KOH in anhydrous ethanol (8 mg/ml) were added to the dried samples in a conical tube and heated at 60°C for 1 h. The reaction products were characterized by \textsuperscript{1}H NMR (300 MHz; CD\textsubscript{3}OD). \textsuperscript{1}H NMR spectra were obtained on a Varian VXR-300 NMR spectrometer.

After evaporation of the solvent the samples were redissolved in 50 μl of N\textsubscript{6},O-bis(trimethylsilyl)trifluoroacetamide, heated at 60°C for 1 h and then analyzed by GC-MS.

Instrumental analysis was performed on a VG TS-250 mass spectrometer, equipped with a HP 5890 gas chromatograph and with a modified ion source developed in the laboratory for better performances in negative ion chemical ionization. GC conditions were as follows: oven temperature from 120°C (1 min) to 160°C at 20°C/min and from 160°C to 250°C at 5°C/min; injector temperature 240°C, in splitless mode. The column was a CP Sil 5 CB (Chrompack Italia, Cornaredo, Milan) (length, 15 m; i.d., 0.25 mm; film thickness, 0.12 μm) operated with a head pressure of 40 kPa of helium. The mass spectrometer was used in the NICI mode with the following conditions: source temperature, 180°C; electron energy, 80 eV; emission current, 400 μA. Isobutane was used as the reacting gas for the formation of negative ions by electron capture. Samples were quantitated by an isotope dilution method, adding a constant amount of O\textsuperscript{6}BuG-D\textsubscript{7} before extraction. A calibration curve was obtained from standards containing different amounts of O\textsuperscript{6}BuG (25–5000 fmol) and a constant amount of O\textsuperscript{6}BuG-D\textsubscript{7} (500 fmol).

Guanine Quantitation. Fractions collected from the immunoadfinity column before elution of O\textsuperscript{6}BuG were lyophilized and resuspended in 2.5% (v/v) methanol in 0.05 M K\textsubscript{2}HPO\textsubscript{4}, pH 4.5, before quantitative analysis of guanine by HPLC. Analyses were done on a Beckman System Gold HPLC. A 25-cm x 5-mm Hilmar C\textsubscript{18} reverse phase column was used, with an isocratic system of 2.5% (v/v) methanol in 0.05 M
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KH$_2$PO$_4$, pH 4.5, at a flow rate of 1.2 ml/min. The UV monitoring wavelength was 254 nm. Using these conditions, the retention time of guanine was 6.3 min.

Butylation of Calf Thymus DNA and Liver DNA. The method was validated by analyzing DNA treated in vitro and in vivo with butylating agents. Calf thymus DNA was alkylated in vitro with 10 mM NBU, as described by Saf/hile (21). One mg of butylated DNA was chemically digested in 0.1 N HCl at 80°C for 1 h, buffered to pH 7-7.4 with 1 M K$_2$HPO$_4$, and analyzed by immunoaffinity chromatography-HRGC-NICI-MS.

Liver DNA samples were obtained from male CD rats (body weight, 250-300 g; Charles River, Calco, Como, Italy) given a single i.p. dose of 185 mg/kg NDBA or NBU in dimethyl sulfoxide. One h after treatment rats were sacrificed and liver DNA was extracted as described by Montesano et al. (22). Two mg of butylated DNA were chemically digested in 0.1 N HCl at 80°C for 1 h, buffered to pH 7-7.4 with 1 M K$_2$HPO$_4$, and analyzed by the method previously described.

RESULTS AND DISCUSSION

The identification and quantitation of DNA adducts such as O6-alkylguanines can be used for studies of DNA damage and repair (1, 23). However, the very low concentrations of damaged residues in comparison to normal bases limits this quantitation.

In the last few years several methods have been developed for this purpose, and the coupling of a HPLC purification step to immunoassay has resulted in one of the most sensitive analytical systems (14, 15). Nevertheless, chromatographic isolation and purification of DNA alkyl adducts is laborious and time-consuming, and cross-reactivity of a specific antibody with other alkylated bases often cannot be avoided.

In order to overcome these problems, we have developed a method for detection of O6BuG that couples immunoaffinity chromatography, which makes the purification of a sample simple, with high efficiency and recovery, with HRGC-NICI-MS analysis, which achieves high selectivity and sensitivity.

This new methodological approach involves different separation factors: binding to an antibody, separation by high resolution gas chromatography, and detection of a specific ion in the mass spectrometer. The first separation factor is very important and has to be efficient, since HRGC-MS has the serious drawback of requiring thorough purification procedures before sophisticated analysis. The selectivity of the purification step by immunoaffinity chromatography depends on the specificity of the antibody employed. The antibody raised against O6BuG used in this study seems to meet this requirement.

O6BuG-BSA and O6BuG-HC conjugates obtained for the production of the specific polyclonal antibody to O6BuG had O6BuG to protein molar ratios of 16:1 and 8:1, respectively. O6BuG-HC was used as coating antigen in immunoassays.

Table 1. Specificity of the antibody raised against O6-butyIguanine

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>pmol required for 50% inhibition of antibody binding in ELISA</th>
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<tbody>
<tr>
<td>O6BuG</td>
<td>1.45</td>
</tr>
<tr>
<td>O6BuG</td>
<td>3.38</td>
</tr>
<tr>
<td>O4PrG</td>
<td>3.12 x 10$^4$</td>
</tr>
<tr>
<td>O6PrG</td>
<td>6.40 x 10$^4$</td>
</tr>
<tr>
<td>O6MeG</td>
<td>&gt;5.0 x 10$^4$</td>
</tr>
<tr>
<td>Guanosine</td>
<td>&gt;1.0 x 10$^4$</td>
</tr>
<tr>
<td>Guanine</td>
<td>&gt;1.0 x 10$^4$</td>
</tr>
<tr>
<td>Adenine</td>
<td>&gt;1.0 x 10$^4$</td>
</tr>
</tbody>
</table>

* Inhibition produced by 5.0 x 10$^4$ O6MeG, 34%.

** Inhibition produced by 1.0 x 10$^4$ guanosine, 20%; guanine, 20%; adenine, 20%.

The compounds were tested at the highest possible concentration.

Table 2. NICI mass spectra and structures of pentafluorobenzyl-trimethylsilyl derivatives of the O6-alkylguanines indicated. Common to all NICI spectra of PFB-TMS derivatives is the loss of the PFB group, which gives the most intense peak (M – 181).

<table>
<thead>
<tr>
<th>m/z</th>
<th>H NMR signals of O6BuG-N7-PFB and O6BuG-N9-PFB derivatives</th>
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<tbody>
<tr>
<td></td>
<td>Spectra were obtained in CD$_3$OD. Shifts are expressed in ppm downfield from internal trimethylsilane. PFBBr can react with N7 or N9 atoms of guanine, giving rise to the formation of two isomers for each alkylated guanine, O6BuG-N7-PFB and O6BuG-N9-PFB. Multiplicity and J values are in parentheses.</td>
</tr>
</tbody>
</table>

Table 2. Competitive ELISA standard curve. Percentage of inhibition of O6BuG-HC binding to polyclonal antibody by increasing amounts of O6BuG.

![Fig. 1. Competitive ELISA standard curve. Percentage of inhibition of O6BuG-HC binding to polyclonal antibody by increasing amounts of O6BuG.](image)

![Fig. 2. NICI mass spectra and structures of pentafluorobenzyl-trimethylsilyl derivatives of the O6-alkylguanines indicated. Common to all NICI spectra of PFB-TMS derivatives is the loss of the PFB group, which gives the most intense peak (M – 181).](image)
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Fig. 3. Typical HRGC-SIR chromatograms of pentafluorobenzyl-trimethylsilyl derivatives of the O6-alkylguanines indicated. The most intense NICI fragment (M - 181) of each compound was recorded. Chromatograms show the presence of two isomers for each alkylated guanine (1 and 2). The amount of compound injected was 250 fmol for each O6-alkylguanine. GC conditions were as follows: oven temperature from 120°C (1 min) to 250°C at 15°C/min; injector temperature of 240°C, in splitless mode.

Fig. 4. Typical HRGC-SIR analysis of pentafluorobenzyl-trimethylsilyl derivatives of O6BuG extracted from DNA of 2.5-g liver samples, processed as described in “Materials and Methods.” O6BuG-D7 was used as internal standard to quantitate O6BuG. Chromatograms show the presence of two isomers (1 and 2) for O6BuG (m/z 278) and O6BuG-D7 (m/z 285). The final and injected volumes per sample were 50 and 2 µl, respectively. A, hydrolyzed liver DNA from treated rat. B, hydrolyzed liver DNA from control rat. C, hydrolyzed liver DNA from control rat in which no internal standard was added.

because of the high cross-reactivity with BSA of the antibody found with O4BuG-BSA.

Antibody production reached a plateau after the fourth immunization. At this time the dilution of antisera that gave positive results, coating ELISA plates with 50 ng/well O4BuG-HC, was 1:50,000.

Competitive ELISA was used to determine the specificity of the antibody. A standard inhibition curve obtained with polyclonal antibody raised against O6BuG is shown in Fig. 1. The response was linear over a range of 0.1-50 pmol O6BuG, with 1.45 pmol/well being required to produce 50% inhibition of binding.

The specificity of the antibody was examined by comparing the amounts of normal or modified free bases required to produce 50% inhibition of antibody-antigen binding in ELISA. The effectiveness of inhibitors is shown in Table 1. The antibody primarily recognized O6BuG and the inhibition caused by bases modified at this site decreased in the order O6BuG > O6PrG > O6EtG > O6MeG. Guanosine, guanine, and adenine produced very little inhibition when used singly. On account of their very low buffer solubility, O6MeG, guanosine, guanine, and adenine were tested at the highest possible concentrations.

The low affinity for the normal bases of the polyclonal antibodies used meant guanine could be collected during the immunoaffinity column washing step (about 95% of recovery) and subsequently quantified. It was thus possible to relate the number of O6BuG modifications directly to the amount of parent base in the same sample.

Unfortunately, because of unidentified interferences affecting antibody binding, competitive ELISA was not suitable for measuring O6BuG in samples processed by immunoaffinity chrom
matography. This problem probably arose because the reacting substances retained by the column were able to interfere with ELISA.

Column capacity was 1.53 nmol of O6BuG/1.25 mg of antibody, and the recovery of O6BuG from the immunoaffinity column was 62 ± 5% (mean ± SD). A characteristic of the immunoaffinity columns is that they can be reused after regeneration as described above. To date, a set of 10 columns has been repeatedly used with no detectable loss of extraction efficiency. Column capacity, checked throughout the whole procedure after the columns had been used 20 times, was 1.33 nmol O6BuG/1.25 mg of antibody/column, with a loss of about 13%. Despite this small loss, column capacity remained high in comparison to the levels of O6BuG that could be found in biological samples.

The identity of the synthesized standard compounds was verified by GC-MS.

NICI was chosen as the ionization mode for HRGC-MS quantitation analysis because of its high sensitivity, based on the low HRGC-MS background noise and the predominant formation of a single negative ion, which carries most of the total ion current (see Fig. 2).

The NICI mass spectra and the structures of O6MeG, O6EtG, O6PrG, and of O6BuG and O6BuG-D7, as their PFB-TMS derivatives, are shown in Fig. 2. The capacity of PFBBR to react with the N7 or the N9 atoms of guanine (due to delocalization of the hydrogen atom) gives rise to the formation of two chromatographically distinguishable isomers for each alkylated guanine (O6BuG-N7-PFB and O6BuG-N9-PFB) (24).

The structure of O6BuG-PFB was confirmed by 'H NMR Overhauser effect (25) measurement showed a cross-peak between protons at δ 5.69 and δ 4.50 of the major regioisomer, indicating their spatial proximity. No similar interaction was observed in the case of O6BuG-D7, because of the formation of a single regioisomer.

Common to all the NICI spectra of PFB-TMS derivatives was the loss of the PFBB group, which always gave the most intense peak (M − 181). The spectra of the two isomers of each alkylguanine were identical (data not shown).

SIR was done on the most intense NICI fragment of each compound: m/z 278 for O6BuG, m/z 285 for O6BuG-D7, m/z 236 for O6MeG, m/z 250 for O6EtG, and m/z 264 for O6PrG, as shown in Fig. 3. The relative intensity of O6-alkylguanine isomers was variable, but the sum of the areas of the two peaks generated during derivatization was always constant, thus permitting quantitation.

The formation of two isomeric peaks after derivatization with PFBBR does not appreciably lower the absolute sensitivity of the method and indeed may serve as further confirmation for the identification of modified guanines present in DNA from biological samples.

The calibration curve obtained plotting the HRGC-SIR peak area ratio O6BuG/O6BuG-D7 against the amount of O6BuG analyzed showed a correlation coefficient r = 0.999. O6BuG DNA content was expressed as μmol of O6BuG/mol guanine, measured as specified in "Materials and Methods." The absolute sensitivity for O6BuG-PFB-TMS using this type of ionization was about 10 fmol injected into the gas chromatograph. In order to establish whether the technique was suitable for assessing DNA damage, DNA samples were treated in vitro or in vivo with butylating agents. The results showed that calf thymus DNA, alkylated in vitro with 10 mM NBU, contained 104 ± 17 (SD) μmol O6BuG/mol guanine.

Liver DNA samples, analyzed for O6BuG content 1 h after injection of 185 mg/kg NBU or NDBA to rats, indicated that butylation at the O6-position of guanine was detectable in liver DNA from rats treated either with NBU or with NDBA. The levels of modification were 2.11 μmol O6BuG/mol guanine and 0.34 μmol O6BuG/mol guanine for NBU and NDBA, respectively. A typical HRGC-SIR chromatogram obtained analyzing O6BuG extracted from liver DNA is shown in Fig. 4.

O6-Alkylguanine levels in DNA are reported to depend on the formation of alkylating species and on the efficiency of the DNA repair system, which governs the persistence of damage (1, 23). O6BuG levels after NBU treatment were 6 times higher than after NDBA, reflecting the fact that NBU is a direct alkylating agent, while NDBA requires metabolic activation to generate butylating species that bind to DNA (8, 9, 21). The lowest level of O6BuG detectable in DNA extracted from biological samples with the method described was about 0.15 μmol/mol guanine, using HRGC-NICI-SIR as the detection system.

In conclusion, a sensitive, specific, and rapid method for quantitating the minor adduct O6BuG in hydrolyzed DNA has been developed. Antibody-mediated extraction represents a very important improvement in purifying biological samples containing DNA adducts. The results indicate that this method permits the quantitation of different O6-alkylguanines at the same time, providing that specific antibodies are available for the preparation of a single mixed immunoaffinity column (26).

It has been shown for the first time that liver DNA can be modified by i.p. administration of NBU and NDBA, indicating that O6BuG formed in vivo can be isolated and quantitated by this method.

Furthermore, since during the past few years our interests have focused particularly on the activation of the bladder carcinogens NDBA, N-nitrosobutyl(4-hydroxybutyl)amine, and N-nitrosobutyl(3-carboxypropyl)amine in rats (9, 27), this method will be applied to assessing DNA damage and repair mechanisms in the urinary bladder of animals exposed to these alkylating agents.

ACKNOWLEDGMENTS

We thank Judy Baggott and the staff of the G. A. Pfeiffer Memorial Library who helped prepare the manuscript.

REFERENCES

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Determination of $\text{O}^6$-Butylguanine in DNA by Immunoaffinity Extraction/Gas Chromatography-Mass Spectrometry

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