Determination of malondialdehyde-induced DNA damage in human tissues using an immunoslot blot assay

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Malondialdehyde (MDA) is a product of lipid peroxidation and prostaglandin biosynthesis. It is mutagenic and carcinogenic and the major adduct formed by reaction with DNA, a highly fluorescent pyrimidopurine (M\(_1\)-dG), has been detected in healthy human liver and leukocyte DNA. Analytical methods used so far for the detection of M\(_1\)-dG have not been applied to a large number of individuals or variety of samples. Often, only a few µg of DNA from human tissues are available for analysis and a very sensitive assay is needed in order to detect background levels of M\(_1\)-dG in very small amounts of DNA. In this paper, the development of an immunoslot blot (ISB) assay for the measurement of M\(_1\)-dG in 1 µg of DNA is described. The limit of detection of the assay is 2.5 adducts per 10\(^8\) bases. A series of human samples were analysed and levels of 5.6–9.5 (n = 8) and 3.1–64.3 (n = 42) of M\(_1\)-dG per 10\(^8\) normal bases were detected in white blood cell and gastric biopsy DNA, respectively. Results on four human samples were compared with those obtained using an HPLC/PPL-post-labelling (HPLC/PPL) method previously developed and indicated a high correlation between M\(_1\)-dG levels measured by the two assays. The advantages of ISB over other assays including HPLC/PPL, such as the possibility of analysing 1 µg DNA/sample and the fact that it is less time-consuming and laborious, means that it can be more easily used for routine analysis of a large number of samples in biomonitoring studies.

**Introduction**

The wide variation in incidence of common cancers and other chronic diseases throughout the world suggests that environmental and lifestyle factors are potent modifiers of overall risk. One key feature of lifestyle is diet and results from epidemiological studies have suggested an important role of diet in the aetiology of certain cancers or chronic diseases. Environmental and lifestyle factors are potent modifiers of other chronic diseases throughout the world suggests that the role of diet in cancer, we have become interested in the endogenous production of genotoxic agents from dietary precursors.

Malondialdehyde (MDA) is a carbonyl compound generated by lipid peroxidation (1) and during arachidonic acid metabolism for the synthesis of prostaglandins (2). Malondialdehyde is mutagenic in bacterial and mammalian systems (3–5) and carcinogenic to rats (6). MDA reacts with DNA at neutral pH forming adducts to deoxyguanosine (dG), deoxyadenosine (dA), deoxycytidine (dC) (4) and cross-links (7). The main adduct, a highly fluorescent cyclic pyrimidopurine (M\(_1\)-dG) (Figure 1), has been detected by gas chromatography/electron capture–negative chemical ionization/mass spectrometry (GC/EC–NCI/MS) in disease-free human liver and leukocytes DNA at levels of 4.8–110 adducts in 10\(^8\) normal nucleotides (8,9). The adduct also has been measured in human white blood cell, breast tissue and gastric DNA using \(^32\)P-post-labelling techniques (10–12). Levels of M\(_1\)-dG adducts in leukocytes have been shown to depend on dietary fatty acid composition (13).

The spectrum of mutations induced by reaction of MDA with M13 phage DNA and subsequent transformation of *Escherichia coli* includes frameshift mutations (e.g. additions) and base-pair substitutions (76%), with G→T transversions (35%) and also A→G (44%) and C→T (56%) transitions (14). In a site-specific mutagenicity study, M\(_1\)-dG was shown to induce mainly transversions to T and transitions to A (15).

The endogenous formation of malondialdehyde in humans and the data on its genotoxicity and mutagenicity suggest that it may play a significant role in human carcinogenesis. It is therefore important to estimate background levels of M\(_1\)-dG in different human tissues and examine whether and how these levels can be modified by diet. For this purpose, very sensitive and specific analytical methods are required in order to be able to evaluate background variations in the adduct levels using small amounts of DNA from human tissues. Methods used so far for M\(_1\)-dG detection are sensitive and/or specific, but often require large amounts of DNA for analysis or are time-consuming and laborious. For these reasons, they have not as yet been applied to a large number of subjects or to a wide variety of tissues.

In the present study, an immunoslot blot (ISB) assay for the measurement of M\(_1\)-dG in small amounts (≤5 µg) of DNA was developed. Synthetic nucleoside standard, MDA-modified calf thymus DNA and oligonucleotides were prepared and used for the optimization of the method. The assay can detect background levels of the adduct in white blood cells and gastric biopsies of humans on normal diets.

**Materials and methods**

**Materials**

Tetramethoxypropane was obtained from Aldrich (Dorset, UK). Guanine, calf thymus DNA (CT-DNA), micrococcal nuclease (MN), nuclease P1, thymidine,
purine nucleoside phosphorylase, thymidine phosphorylase, proteinase K and ribonuclease A (from bovine pancreas) were purchased from Sigma (Dorset, UK). Calf spleen phosphodiesterase (SPDE) was purchased from Boehringer Mannheim (Lewes, UK). Oxoid phosphate buffered saline tablets were purchased from Unipath (Hampshire, UK). All other reagents and solvents of analytical or HPLC grade were obtained from either BDH or Fisher Scientific (Loughborough, UK).

Synthesis of M1−guanine

1N2-malondialdehyde–guanine (pyrimido[1,2-a]purin-10(3H)-one base) (M1-G) was synthesized following the procedure of Seto et al. (16) with modifications. Briefly, tetramethoxypropane (25 mmol; Sigma) was hydrolysed to malondialdehyde in 25 ml 1 M HCl at 40°C for 40 min, mixed with a solution of guanine (750 mg in 25 ml 1 M HCl) and incubated in a shaking water bath at 40°C for 1 h. The mixture was centrifuged at 1000 r.p.m. for 10 min and the pellet was extracted several times with hot water (60°C). The product was purified by preparative HPLC using a Gilson 306 HPLC pump and Dynamax Rainin UV detector, model UV-1 (254 nm). The column was a S5 Spherisorb ODS (250 × 20 mm). The eluent (0.1 M TEA, pH 7, 1% methanol) was kept constant for 10 min. The proportion of methanol was then increased to 30% over 40 min. The flow rate was 10 ml/min. M1−G was purified by HPLC using a water:methanol gradient and finally characterized by electrospray–mass spectrometry (ES–MS) and 1H-nuclear magnetic resonance (NMR).

Synthesis of M1−deoxyguanosine

1N2-M1−deoxyguanosine (pyrimido[1,2-a]purin-10(3H)-one deoxy nucleoside) (M1−dG) was synthesized by enzyme-catalysed transfer of deoxyribose from thymidine to 1N2-M1−guanine as described by Chapeau and Marnett (17). The reaction mixture was analysed by HPLC and purified by preparative HPLC with the instruments described above. The HPLC conditions were similar to those described above, except the methanol was increased to 15% between 10 and 40 min. M1−dG was identified by fluorescence and UV spectra, and characterized by ES–MS and 1H-NMR. The yield was ~80%.

In vitro MDA-modified DNA

CT-DNA (0.26 mg/ml) was incubated with malondialdehyde (2 mM or 20 mM, generated by hydrolysis of tetramethoxypropane) in 0.1 M potassium dihydrogen orthophosphate, pH 4.5, for 4 days at 37°C. After precipitation with ethanol, DNA was redissolved in water and kept for ISB or HPLC/PPL analysis. CT-DNA was also spiked with different amounts of synthetic M1−dG to check the recovery of the adduct in the HPLC/PPL assay. Aliquots of 10 µg of DNA (10 µl) were digested to 2′-deoxynucleoside-3′-monophosphates (dNp) by incubation with MN (1.6 U) and SPDE (24 mU) in SSCC buffer (final concentration 10 mM sodium succinate, 5 mM CaCl2, pH 6) in a final volume of 24 µl. The samples were dried, redissolved in water and analysed by HPLC for DNA quantitation (Figure 2) and HPLC/PPL as described below.

For determination of adduct level in DNA to be used as a standard in the ISB assay, MDA-modified calf thymus DNA (0.5 µg) was digested to deoxynucleosides by incubation with MN (1.6 U) and SPDE (24 µU) for 2 h at 37°C followed by overnight incubation with nuclease P1 (5 U) at 37°C. The adduct was quantified by HPLC-fluorescence by comparing the peak area with a calibration curve previously generated using synthetic M1−dG. CT-DNA was also modified with malondialdehyde as described by Rouzer et al. and the amount of M1−dG determined by LC–MS as previously described (9).

HPLC

HPLC separations were performed using a Waters 600E system equipped with a Waters 484 UV and a Waters 470 fluorescence detector, a narrow-bore Hypersil BDS C18 column (5 µm, 100×2.1 mm) and isocratic conditions with 0.1 M triethylammonium acetate (TEA), pH 5, containing 4% methanol as the eluent. The flow rate was 0.2 ml/min.

Synthesis of oligonucleotides and their modification by MDA

A random 100-base-long oligonucleotide was synthesized using standard phosphoramidite procedure and an Applied Biosystems Model 394-08 DNA/RNA synthesizer. The oligonucleotide was precipitated using absolute ethanol (~20°C), washed with 70% ethanol and dissolved in 0.1 M potassium dihydrogen orthophosphate, pH 4.5. The final concentration of oligonucleotide was 2 µg/μl as determined by UV absorption at 260 nm. MDA (2 mM), generated as previously described, was added and the solution incubated at 37°C for 4 days. The modified oligonucleotide was precipitated with ethanol, washed with 70% ethanol and resuspended in ultrapure water for analysis. The GC/CT:A composition was determined after enzymatic digestion to 2′-deoxynucleosides and HPLC analysis. The amount of adduct present was also determined by HPLC as described for CT-DNA.

Antibody

A murine monoclonal antibody (D10A1) specific for the M1−dG adduct has been prepared and characterized by Sevia et al. (18). In a direct ELISA assay, the antibody has been shown to bind to RNA or DNA that have been reacted with MDA, but not to unmodified proteins, RNA or DNA. In a competitive ELISA, the antibody showed an affinity for M1−G ribonucleoside that was 2-fold higher than its affinity for the deoxyribonucleoside and 10-fold higher than its affinity for the base. Competitive inhibition studies also showed much lower specificity (100- to >100 000-fold) of D10A1 binding to other related exocyclic adducts (18), e.g. H2-M1G-R, etheno–dG, H2-M1G-R and glyoxal–dG.

Fig. 1. M1−dG is formed in DNA by reaction of malondialdehyde with deoxyguanosine.

Fig. 2. A typical HPLC-UV (260 nm) separation of DNA samples for DNA quantitation, after digestion with MN and SPDE. The retention time of M1−dG, which was collected for PPL, is indicated with an arrow.
Immunoslot blot

Standard MDA-modified CT-DNA and oligonucleotides were prepared as described above and digested to deoxynucleotides. The amount of M₁–dG was measured by HPLC-fluorescence using a calibration curve obtained with synthetic M₁–dG. The total amount of DNA and oligonucleotides was quantitated by HPLC-UV (Figure 2) after digestion to monodeoxynucleotides as previously described.

Modified DNA and oligonucleotides were diluted with unmodified DNA and oligonucleotides to obtain decreasing amounts (fmol to attomol) of adduct and used for generating standard curves in the immunoslot blot assay (IBS). Standard curves obtained by dilutions of the MDA-modified CT-DNA that were prepared in Leicester, UK and Nashville, USA, which had different initial levels of M₁–dG adduct, gave very similar values when analysed on the same immunoslot blot.

Immunoslot blots were carried out essentially as described by Mientjes et al. (19) with modifications. Briefly, CT-DNA containing various amounts of M₁–dG, control CT-DNA and human DNA samples (3.5 μg in 300 μl of 10 mM diopotassium hydrogen orthophosphate, pH 7) were sonicated for 5 s (or 10 min in a water bath sonicator) to obtain fragments of ~100 base pairs. DNA was then heat-denatured for 5 min in a boiling water-bath, cooled with ice for 10 min and mixed with an equal volume of 2 M ammonium acetate. When the oligonucleotide was used, sonication and denaturing steps were omitted. Single-stranded DNA and oligonucleotides (88 μl containing 1 μg DNA/sample, in triplicate) were immobilized on nitrocellulose (NC) filters (0.45 μm, BA797; Schleicher and Schuell, Dassel, Germany) using a Minifold II, 72-well slot blot microfiltration apparatus (Schleicher and Schuell). The slots were rinsed with 200 μl 1 M ammonium acetate. The filters were subsequently removed from the support and baked at 80°C for 90 min. They were washed twice for 5 min with PBS–Tween 20 (PT, 0.1%) containing 0.5% fat-free milk powder. The filters were then incubated overnight at 4°C with the anti-M₁–dG monoclonal antibody D10A1 (18) diluted 1:150 000 or 1:300 000 in 20 ml PT containing 0.5% fat-free milk powder. The NC filters were washed with PT for 1 min and then twice for 5 min. The filters were then incubated with goat anti-mouse IgG horseradish peroxidase conjugate (#PO447; Dako, Glostrup, Denmark), diluted 1:2000 or 1:4000 in 16 ml PT containing 0.5% non-fat milk powder for 2 h at room temperature. The filters were washed with 30 ml PT for 15 min and then twice for 5 min. Enzymatic activity was visualized by boiling the NC filter either for 1 min in ECL chemiluminescence reagent (Amersham Life Sciences, Buckinghamshire, UK), or for 5 min in SuperSignal Ultra (Pierce and Warriner, Chester, UK), prepared just prior to use. Filters were then exposed to a film (Hyperfilm-ECL; Amersham) and developed.

Densitometry analysis of X-ray films was performed using a Molecular Dynamics Densimeter and was carried out by integrating the whole area of each band. Standard curves were generated using modified CT-DNA and oligomers (optical density signal against amount M₁–dG). Adduct levels in human sample DNA were quantified by referring to the standard curve.

Human samples

DNA samples were obtained from blood of human volunteers on standardized diets (in collaboration with Dr S.Bingham, MRC Dunn Clinical Nutrition Centre, Cambridge, UK). Human gastric biopsies were kindly provided by Prof. A.R.Axon (Royal Institute, Leeds University, UK). DNA was extracted from whole blood and biopsy tissues using Qiagen genomic DNA extraction kit (Qiagen, Crawley, UK) and digested by Proteinase K and RNase A. DNA purity was assessed by 260/280 ratio using a Perkin-Elmer UV spectrophotometer and by reverse phase (RP)-HPLC after digestion to deoxynucleotides. Adduct recovery after DNA extraction was 96% as determined by subjecting CT-DNA containing known amounts of M₁–dG to the purification procedure (incubation with Proteinase K and RNase A and extraction using the Qiagen kit).

HPLC²³⁵P-post-labelling

M₁–dGp was synthesized following the procedure of Seto et al. (16), with modifications, characterized by ES-MS and used for the development of an HPLC²³⁵P-post-labelling assay for the detection of the adduct in human samples. The HPLC enrichment of the adduct before PPL was performed using the Waters system and the conditions described above. Fractions eluting at the retention time corresponding to M₁–dGp (Figure 2) were collected, evaporated to dryness and retained for PPL. Synthetic M₁–dGp and the corresponding adduct from DNA samples were dissolved in water and converted to²³⁵P-post-labelled deoxyxoside bisphosphates as previously described (20). Apyrase (2 μl; 0.4 mU/μl) was then added and the samples incubated for 30 min at 37°C. After adding NPI (4 μl; 2 μg/μl in 0.28 M sodium acetate, 0.5 mM zinc chloride, pH 5) the mixture was incubated for a further 2 min at 37°C and then spotted on to 10×10 cm PEI-cellulose plates (#801053; Machery–Nagel, Camlab, Cambridge, UK). The plates were pre-developed in water to the origin and then run in the same direction with 2.1 M lithium formate, 3.75 M urea, pH 3.35. Plates were washed for 10 min in water, allowed to dry and chromatographed in the second direction (90° to the first direction) with 0.14 M sodium phosphate, pH 6.35, to 2 cm above the origin and then run completely on to a filter paper wick (Whatman paper no. 1, 10×10 cm) for 2 h with 0.14 M sodium phosphate, pH 6.35, 1.4 M urea (modified from ref. 11). After removal of the wick, plates were washed for 5 min in water and then dried. Normal nucleotides were labelled with ²³⁵P-ATP as described by Jones et al. (21) and spotted on Merck PEI-cellulose TLC plates (20×20 cm) which were run in one direction with 0.12 M sodium phosphate, pH 6.5. Adducts were quantified using a Molecular Dynamics Image Analysrer. Plates were exposed for 1 h in the presence of a ²³⁵P-ATP standard strip to convert phosphorimage analysis signals to d.p.m. The amount of adducts was determined by (adduct d.p.m. – background d.p.m.)/dilution factor. Background radioactivity was determined on a specific part of the TLC plate in an area identical to that corresponding to the adduct spot.

Results

CT-DNA modified by MDA in vitro was used to set up an immunoslot blot assay for the detection of M₁–dG in intact DNA samples. A prerequisite of the assay was the ability to precisely determine the amount of M₁–dG modification in intact DNA. Thus, M₁–G was synthesized and converted to M₁–dG as described in Materials and methods. The identity of M₁–G and M₁–dG was confirmed by MS and NMR analysis, which gave identical results to literature data (16). An HPLC calibration curve for M₁–dG was then generated and used for exact quantitation of the adduct in modified CT-DNA after enzymatic digestion to 2’-deoxynucleotides.

CT-DNA containing 6.75 pmol adduct/μg DNA was used in the IBS assay. Figure 3a shows a typical IBS with constant amounts of DNA (1 μg/slot) but containing decreasing amounts of M₁–dG (3.4–0.65 fmol). In Figure 3b, the optical density values obtained by densitometric evaluation of the IBS X-ray film (shown in Figure 3a) are plotted against M₁–dG content. As can be seen in Figure 3a, ~0.65 fmol can still be quantitated above background in a total amount of 1 μg DNA, which
to 2.5 adducts in 10^8 nucleotides, was thus obtained with the nucleotides. A limit of detection of 0.08 fmol/µg corresponds to 17 adduct per 10^8 nucleosides. The limit of detection for M1–dG and human white blood cell DNA samples (in triplicate). Adducts were visualized using SuperSignal Ultra (Pierce) chemiluminescent reagent and autoradiography.

Fig. 4. ISB analysis of an oligonucleotide containing decreasing amounts of M1–dG and human white blood cell DNA samples (in triplicate). Adducts were visualized using SuperSignal Ultra (Pierce) chemiluminescent reagent and autoradiography.

corresponds to 17 adduct per 10^8 nucleosides. The limit of detection was ~0.3 fmol/µg when SuperSignal Ultra (Pierce) was used as the chemiluminescent reagent (instead of ECL) because the signal was more intense and longer lasting. The binding of the antibody to control DNA, shown in Figure 3, is probably caused by either non-specific binding of the antibody or to the presence of background levels of M1–dG in CT-DNA. Background levels of M1–dG were also measured in control CT-DNA by HPLC/32P-post-labelling (HPLC/PPL) (data not shown). The absolute detection limit of the ISB assay appeared therefore to be lower than the lowest detected value, when CT-DNA was used as a reference standard.

To examine this possibility, a 100-base oligomer containing the four deoxynucleosides in equal proportions was synthesized and modified by MDA. The amount of M1–dG per µg oligonucleotide was determined by HPLC after digestion to deoxynucleosides as described for CT-DNA. Figure 4 shows a typical immunoslot blot using constant amounts of oligonucleotide (1 µg) containing different amounts of M1–dG (0–2.5 fmol/µg oligonucleotide). The assay was identical to that used for CT-DNA except that sonication and denaturing steps were omitted. Standard curves generated with modified CT-DNA and oligonucleotides spotted on the same slot blot gave identical signal intensities indicating that no adduct loss occurred in CT-DNA during the denaturing step. No background levels of M1–dG were detected in untreated oligonucleotides. A limit of detection of 0.08 fmol/µg, corresponding to 2.5 adducts in 10^8 nucleotides, was thus obtained with the modified oligomer when the SuperSignal Ultra chemiluminescent reagent was used for visualization.

To assess the specificity of D10A1 antibody binding to M1–dG in intact DNA in the ISB assay, competitive inhibition studies were conducted. CT-DNA containing decreasing amounts of adduct was immobilized on nitrocellulose membranes and incubated overnight with the antibody in the presence of free M1–dG. Control samples were also run at the same time. M1–dG at a concentration of 50 pg/ml PBS gave between 60 and 90% signal inhibition. The antibody binding to the membrane was completely inhibited when free M1–dG was present at 5 ng/ml PBS (data not shown).

Figure 4 also shows a typical ISB analysis of DNA from human samples using SuperSignal Ultra chemiluminescence reagent. Slots 1–8 contained standard oligo samples in triplicate for the generation of a reference calibration curve for each experiment. In this way a direct correlation of the ISB signal with the amount of M1–dG/slot for the human samples containing unknown amounts of adduct was obtained. The method was applied to a series of human samples. Levels ranging from 3.1 to 64.3 (n = 42, average 19.35 ± 15.83) adducts in 10^8 normal bases were detected in human gastric biopsy DNA, indicating a wide interindividual variation. For comparison, levels of 5.6–9.5 M1–dG in 10^8 bases were measured in eight samples of white blood cell DNA from volunteers on a standardized diet. Addition of free M1–dG as a competitive inhibitor suppressed the signal completely in all human samples.

In order to compare the immunoslot blot with the previously established HPLC/PPL (Figure 5), four human white blood cell (WBC) samples, for which enough DNA was available, were analysed using the two methods. The results obtained showed a good correlation between the two assays in adduct levels measured, with HPLC/PPL showing slightly higher values for three samples, after correction for 50% recovery (Table I).

Table I. Levels of M1–dG (adducts per 10^8 normal nucleotides) in human white blood cell DNA measured by HPLC/32P-post-labelling (PPL) and immunoslot blot (ISB)

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>HPLC/PPL (corrected for recovery)</th>
<th>ISB</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>5.2</td>
<td>5.60 ± 0.26</td>
</tr>
<tr>
<td>30</td>
<td>9.2</td>
<td>7.10 ± 0.35</td>
</tr>
<tr>
<td>33</td>
<td>11.4</td>
<td>9.10 ± 0.4</td>
</tr>
<tr>
<td>34</td>
<td>10.6</td>
<td>8.24 ± 0.41</td>
</tr>
</tbody>
</table>

Discussion

Malondialdehyde (MDA) is a mutagenic and carcinogenic product of two metabolic processes, lipid peroxidation and prostaglandin biosynthesis. MDA forms endogenous adducts on DNA and the major adduct, a fluorescent pyrimidopurinone (M1–dG), has been measured in healthy human liver, leukocyte, breast tissue and gastric biopsy DNA by GC/EC–NCI/MS and 32P-post-labelling (8–12). Although mutagenicity studies indicate a direct involvement of M1–dG in inducing mutations, further investigations are needed to elucidate the importance of this adduct in human carcinogenesis and other chronic diseases. It is important to evaluate the distribution of M1–dG in different tissues or cell types and whether adduct levels can be modulated by dietary intake or other environmental factors. The GC/MS and 32P-post-labelling methods used in previous studies have not been applied to a large number of subjects or to a wide variety of tissues. Although these methods are highly sensitive,
they require relatively large amounts of DNA (10 µg–1 mg) for analysis and often only a few micrograms of DNA are available from small samples of human tissues. A very sensitive and specific assay is therefore needed to be able to detect very low levels of the adduct in very small amounts of DNA (<5 µg).

The availability of a new monoclonal antibody (D10A1) produced and characterized by Sevilla et al. (18) made it possible to develop an immunoslot blot assay for the detection of M1–dG in intact DNA using only 1 µg of DNA per slot. Synthetic standards were used for the optimization of the procedure. MDA-modified CT-DNA and oligonucleotides containing known amounts of adduct were used to generate calibration curves for each ISB analysis and binding of D10A1 antibody was proportional to the amount of M1–dG in the samples. The assay has a limit of detection of ~80 attomol M1–dG/µg intact DNA (2.5 adducts/10^8 normal bases), thus giving sensitivity comparable to that of previously described methods but using much less DNA. Human WBC and gastric biopsy DNA were analysed and levels of M1–dG were quantitated using the MDA-modified oligonucleotide as a reference standard. A wide interindividual variation in adduct levels was observed in gastric biopsies as also previously reported for other tissues (8–13). However, M1–dG values detected in white blood cells were not too different among individuals, possibly because DNA was extracted from blood of volunteers on a standardized diet. These samples are part of collaborative studies and full results will be presented elsewhere.

Comparison between HPLC/32P-post-labelling (HPLC/PPL) and ISB was made by analysing four human WBC DNAs by both methods and a good correlation was obtained between the two assays, although the HPLC/PPL showed slightly higher values for three samples after correction for 50% recovery (Table 1). One of the problems with 32P-post-labelling is to determine the exact recovery for each sample individually in order to correct for all potential sources of error in such a multiple step procedure. Nair et al. (22) and Povey et al. (23) developed PPL assays, which included known amounts of internal standard in the 32P-phosphorylation reaction mixture for all samples analysed. Quantitation of the adducts was made by using the internal standard as a reference, and the values measured for the adducts were corrected based on the recovery of the internal standard after PPL and TLC separation. We investigated the use of several internal standards to be included in the HPLC/PPL analysis of M1–dG, but unfortunately all nucleotides tested (dUp, Up, dIp, Ip) were washed off the TLC plate using the optimal conditions for M1–dG separation. Therefore precise recovery for each sample is not known and an average recovery of 50% was based on results with spiked CT-DNA.

Levels of adducts detected in human WBC were quantitatively very similar to results obtained by Rouzet et al. (9) who used the same monoclonal antibody D10A1 for immunoaffinity purification of the adduct before GC/EC–NCI/MS. This latter method is highly sensitive and reliable and provides a higher level of specificity than other immunochemical assays (ISB or ELISA) because detection depends not only on antibody recognition in the immunoaffinity step but also on correct GC retention time and molecular mass. The limit of detection of the assay is similar to that of the ISB described in this paper but 1 mg of DNA is required to achieve that sensitivity.

ISB presents many advantages over other assays including 32P-post-labelling. Only 1 µg DNA/well is needed and in our assay all samples are analysed in triplicate on the same ISB. This assay is much less laborious and time-consuming than other assays, thus allowing routine analyses of a large number of samples in short times. Quantitation of the adduct in human samples is based on a calibration curve obtained using a standard present on the same filter as the real samples, thus standardizing results obtained on different ISB. However, when using an assay based on adduct detection by direct antibody binding, the possibility of non-specific binding has to be taken into consideration. The monoclonal antibody used in this work has been completely characterized by Sevilla et al. (18) and shown to recognize M1–G ribonucleoside with higher specificity than M1–dG. In the present work all human DNA samples analysed by ISB were checked by HPLC and no RNA contamination was detected. The only normal nucleic acid components reported to show cross-reactivity with the antibody were free deoxyguanosine and riboguanosine but at much higher levels. However, Sevilla et al. (18) have shown that D10A1 does not bind to unmodified intact RNA or DNA. In our study, no binding was detected for unmodified oligonucleotide. Very low background binding to control DNA was probably caused by the presence of endogenous levels of the adduct in CT-DNA. Previously described competitive inhibition studies showed that cross-reactivity of antibody binding to other related endogenous exocyclic adducts was very low (18). In our study, antibody binding to CT-DNA, both control and MDA-modified, as well as human DNA samples was completely inhibited by free M1–dG in a competitive ISB.

In conclusion, an immunoslot blot assay for the analysis of M1–dG in DNA was developed. The method can detect background levels of the adduct in WBC and gastric biopsy DNA and showed sensitivity similar to that of previously published methods, including 32P-post-labelling and GC/EC–NCI/MS. The advantages of ISB, such as the possibility of analysing 1 µg DNA/well and the fact that it is less time-consuming and laborious than other assays, means that it can be more easily used for analysing a large number of samples, for example in biomonitoring studies in relation to dietary fat or antioxidant intake and for investigating M1–dG distribution in different human tissues.

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C. Leuratti et al.


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