RESEARCH COMMUNICATION

Measurement of oxidized and methylated DNA bases by HPLC with electrochemical detection

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Oxidative DNA damage is thought to be an important contributor to cancer development and to be affected by dietary constituents, so its accurate measurement is important. DNA methylation is recognized as an important mechanism affecting gene expression. In the present paper we describe an HPLC-with-electrochemical-detection procedure that allows rapid and sen-

sitive measurement of four oxidized (2,6-diamino-4-hydroxy-5-formamidopyrimidine, 5-hydroxyuracil, 8-hydroxyguanine, 8-hydroxyadenine) and three methylated (7-methylguanine, 1-methylguanine, *O*⁶-methylguanine) bases in acid hydrolysates of DNA. Guanine was also detected, but was clearly separated from the other bases.

INTRODUCTION

Oxidative DNA damage appears to occur continuously *in vivo*, necessitating the presence of DNA repair enzymes [1–3]. Indeed, it has been suggested that the cumulative biological effects of oxidative DNA damage over the long human lifespan may contribute to the development of cancer [1,4–7]. It is therefore very important to be able to measure oxidative DNA damage accurately and, preferably, rapidly [1,6,8,9].

The most common procedure employed to assay oxidative DNA damage is the measurement of 8-hydroxydeoxyguanosine (8-OHdG) by HPLC with electrochemical detection (HPLC-ECD) after enzymic hydrolysis of DNA [10,11]. This procedure has the advantage that 8-OHdG is a major product of DNA damage, generated by attack of several reactive oxygen species [1,8–13]. However, problems can arise because of incomplete enzymic hydrolysis (e.g. oxidized bases can interfere with the action of nucleases [14]) and deglycosylation of 8-OHdG during sample handling (discussed in [15]). An alternative procedure is acidic hydrolysis of DNA, followed by derivatization of modified bases and analysis by GC/MS [16]. This method is highly sensitive, especially if selected ion monitoring is used, and has the advantage that it measures multiple base-damage products as well as 8-hydroxyguanine (8-OHG) [8,15,16]. In addition, the chemical pattern of base modification can give information about which reactive oxygen species caused the damage [8,15]. For example, reaction with hydroxyl radical ([•]OH) generates a multiplicity of products from all four DNA bases, whereas singlet oxygen (¹O₂) is selective for guanine [8,17]. However, the derivatization procedures required for GC/MS are slow and tedious, and in addition there is the possibility of 'artefactual' generation of some base damage products during the derivatization procedure if O₂ is not completely excluded [9,15,18,19]. The levels of 8-hydroxylated guanine in cell DNA determined by GC/MS are usually higher than those obtained using HPLC-ECD, although it is not clear as yet which result is correct (discussed in [15]).

In the present paper, we describe an HPLC-based assay (not requiring derivatization) that allows simultaneous measurement

of four major DNA damage products: 2,6-diamino-4-hydroxy-5-formamidopyrimidine (Fapy-guanine), 5-hydroxyuracil (isobarbituric acid, 5-OHU), 8-hydroxyguanine (2-amino-6,8-dihydroxypurine, 8-OHG) and 8-hydroxyadenine (6-amino-8-hydroxypurine, 8-OHAd), plus three methylated bases, namely 7-methylguanine (7-MeG), 1-methylguanine (1-MeG) and *O*⁶-methylguanine (*O*⁶-MeG). DNA methylation is now recognized as an important mechanism regulating gene expression and DNA replication, and altered methylation patterns may be implicated in cancer [20].

MATERIALS AND METHODS

Reagents

Calf thymus DNA, 8-bromoadenine, 5-OHU, 2,5,6-triamino-4-hydroxypyrimidine, 8-OHG, xanthine, guanine, 7-MeG, 1-MeG and *O*⁶-MeG were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). 8-OHAd was synthesized by treatment of 8-bromoadenine with concentrated formic acid (95%) at 150 °C for 45 min and purified by crystallization from water. Fapy-guanine was synthesized by treatment of 2,5,6-triamino-4-hydroxypyrimidine with concentrated formic acid (98%) at 150 °C for 1 h and recrystallized from water. Dialysis membranes with an *M_r* cut-off of 3500 were purchased from Spectrum [supplied by Pierce & Warriner (U.K.) Ltd., Chester, U.K.]. Purified water used for all preparations was from the ELGA MAXIMA unit (ELGA Ltd, High Wycombe, Bucks., U.K.). HPLC-grade methanol was obtained from Romel Ltd. (Cambridge, U.K.).

Irradiation of DNA

DNA (0.5 mg/ml in air-saturated 25 mM phosphate buffer KH₂PO₄/KOH, pH 7.4) was subjected to radiolysis by high-energy electrons from a linear accelerator (Paterson Laboratories, Christie Hospital, Manchester, U.K.) in a continuous pulsing mode (50 pulses/s). The total dose was 16 krad (equivalent to 50 μM [•]OH/s). The dose was calibrated using a Fricke dosimeter.

Abbreviations used: HPLC-ECD, HPLC with electrochemical detection; 8-OHdG, 8-hydroxydeoxyguanosine; 8-OHG, 8-hydroxyguanine; Fapy-guanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OHAd, 8-hydroxyadenine (6-amino-8-hydroxypurine); 5-OHU, 5-hydroxyuracil; 7-MeG, 7-methylguanine; 1-MeG, 1-methylguanine; *O*⁶-MeG, *O*⁶-methylguanine

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Hydrolysis of DNA with formic acid

DNA aliquots (1 ml of 50 mg DNA each; concentration determined at 260 nm; an A_{260} of 1.0 $\equiv$ 50 μ g of DNA/ml) were hydrolysed with 0.5 ml of formic acid (60%, v/v) in evacuated and sealed tubes (Pierce vacuum hydrolysis tubes) for 45 min at 150 °C. The tubes were allowed to cool. The contents were transferred to Pierce micro-vials, covered with Kleenex tissues cut to size (secured in place using a rubber band) and cooled in liquid nitrogen. Formic acid was then removed by freeze-drying. Samples were stored at -20 °C. Before analysis by HPLC they were redissolved in the eluent (final volume 1 ml).

HPLC analyses

DNA bases were separated using an HPLC Technology Nucleocil 3 μ m C_{18} column (30 cm $\times$ 4.6 mm) with a guard column (Hibar from Merck Ltd., Poole, Dorset, U.K.; with a C_{18} end-capped cartridge). The eluent was 20 mM sodium citrate/17 mM acetate buffer (pH 3.01)/methanol (9:1, v/v) at a flow rate of 1 ml/min through a Polymer Laboratories Ltd. isocratic pump equipped with an electrochemical detector (EDT LCA 15) containing a

glassy carbon working electrode and an Ag/AgCl reference electrode. The detector potential was set at +0.85 V for measuring the oxidized bases after determining the hydrodynamic voltammograms for each compound. Peaks were quantified by measuring peak heights after peak identity had been confirmed by 'spiking' with authentic standards and also by analyses using an HPLC/photodiode array UV detector (Gynkoteck model UVD-340 from HPLC Technology, Macclesfield, Cheshire, U.K.).

RESULTS

The HPLC technique we have developed gives good separation of hydroxypurines (8-OHG and 8-OHAd) from 5-OHU and Fapy-guanine (see Figure 1a. Figure 1(b) shows the separation of the three methylated bases from the four oxidized bases. The lower limit of detection of each product was 50 nM (detector sensitivity 10 nA), and calibration curves using standards were linear in the concentration range 0.5–10 μ M (detector sensitivity 300 nA). Fapy-guanine and 5-OHU are eluted close together and can overlap if shorter columns are used. This problem, if it occurs, can be solved by changing the oxidation potential from

Figure 1 HPLC separation of oxidized and methylated DNA bases

(a) HPLC separation of Fapy-guanine (FG; 10 μ M), 5-OHU (5 μ M), guanine (G, 5 μ M), 8-OHG (10 μ M) and 8-OHAd (10 μ M) using the HPLC conditions outlined in the Materials and methods section. (b) Separation of methylated bases 7-MeG (30 μ M), 1-MeG (5 μ M) and O⁶-MeG (10 μ M) from guanine (G) and the oxidized bases in (a). The ECD detector was set at +1.00 V.

Figure 2 Hydrodynamic voltammograms of oxidized and methylated DNA bases

(a) Voltammograms of Fapy-guanine (FG), 5-OHU, 8-OHG and 8-OHAd at 2.5 μ M each. (b) Voltammograms of xanthine and guanine (2.5 μ M each), 7-MeG, 1-MeG and O⁶-MeG at 30 μ M, 5 μ M and 10 μ M respectively.

Table 1 DNA base damage products measured by HPLC-ECD

A, analysis of oxidized bases in acid-hydrolysed calf thymus DNA. B, analysis of oxidised bases in calf-thymus DNA after γ -radiolysis under the conditions described in the Materials and methods section.

Sample	DNA base...	Amount detected (nmol/mg of DNA)			
		FG	5-OHU	8-OHG	8-OHAd
A		1.0	0.5	1.30	0.8
B		6.5	0.7	12.5	2.4

+0.85 V down to +0.67 V, since Fapy-guanine is not detected below +0.70 V. This is illustrated in Figure 2(a), which shows the hydrodynamic voltammograms of the four oxidized bases. Figure 2(b) shows the voltammograms of guanine, xanthine and the three methylated bases between the voltage range +0.4 to +1.2 V. Guanine is well separated from 8-OHG on HPLC (Figure 1) and is detected above +0.7 V (Figure 2b). Xanthine is co-eluted with 8-OHG using the chromatographic conditions given above. This complication can be overcome if necessary by working below +0.7 V to quantify 8-OHG only. Cytosine, thymine and adenine were not detected at the voltages used.

Table 1 illustrates application of the technique to an acid hydrolysate of DNA, before (A) and after (B) exposure of the DNA to $\cdot\text{OH}$ and other oxygen radicals generated by γ -radiolysis of aqueous solutions. The identity of the bases measured was confirmed by spiking with authentic standards, changing the voltage of the electrochemical detector and also by checking the absorbance spectra of the products as provided by the diode array detector. As expected for commercial DNA, significant levels of base damage products were found in the DNA before irradiation, and they increased markedly after irradiation.

DISCUSSION

The technique described in the present paper allows rapid (within 12 min) analysis of four major oxidative base damage products in acid hydrolysates of DNA (Figure 1a). HPLC-based techniques to measure individual base-damage products such as hydroxylated guanine [10,13] and 8-OHAd [21] have already been described in the literature, and GC/MS can measure multiple base products [8,17,18,22]. Our method is not as comprehensive as GC/MS, but avoids the slow and tedious derivatization step with its possible 'artefactual' oxidation of bases [15,18,19]. LC/MS systems are gaining in popularity [23–25], but are much more expensive than the HPLC system used here. There is indisputable need to measure oxidative damage to DNA both *in vivo* and *in vitro* with accuracy and reproducibility. In order to achieve this goal, Stadler et al. [26] have synthesized isotopically labelled analogues of 8-OHG and 8-OHAd for use as internal standards to compensate for potential artefacts formed during DNA work-up and derivatization for GC/MS. On the other hand, Douki et al. [19] have shown that adenine, cytosine,

thymine and thymidine can give rise to oxidized derivatives during derivatization if O_2 is not removed completely. Hence, they report an HPLC-based prepurification procedure to remove the precursors of the oxidized bases measured in DNA samples and emphasize the use of isotopically labelled internal standards to ensure that accurate and quantitative results are achieved. Our HPLC method seems an easier way of achieving the same result. The use of acid hydrolysis avoids complications due to incomplete enzymic hydrolysis of DNA, and it seems to be generally accepted that acidic hydrolysis is not a cause of 'artefactual' DNA oxidation provided that proper calibration with standards is carried out [15,18,19,27,28]. Of course, the number of modified bases that can be detected by this sensitive method is restricted to those that are electrochemically active.

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