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SHORT COMMUNICATION

Room temperature derivatization of 5-hydroxy-2'-deoxycytidine and 5-hydroxymethyl-2'-deoxyuridine for analysis by GC/MS

FEI YU and ZORA DJURIC*

Barbara Ann Karmanos Cancer Institute, Wayne State University, 110 East Warren, Detroit, MI 48201, USA

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Hydroxylated DNA bases are one type of oxygen free radical-induced damage to DNA. Such damage has been implicated in the process of carcinogenesis, and the levels of hydroxylated DNA bases may serve as a marker of cancer risk in humans. Measurement of oxidative DNA damage can be hampered by the ease with which artifactual oxidative DNA damage can be induced via sample processing. In this report we describe convenient room temperature derivatization and stability of 5-hydroxy-2'-deoxycytidine (5-OHdCyd) and 5-hydroxymethyl-2'-deoxyuridine (5-OHmdU) using GC/MS analysis. The derivatization reagent was *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethylchloro-silane:acetonitrile, 2:1. This method avoids use of acid and is much milder than previously reported derivatization conditions which typically involve heating above 100 °C for at least 20 min. Although heating has been reported to be problematic, the calculated levels of 5-OHdCyd and 5-OHmdU in enzymatically-hydrolysed calf thymus DNA were very similar in our hands with and without heating the sample for 20 min. As an example of the technique, comparison of 5-OHdCyd and 5-OHmdU levels in calf thymus DNA indicated relatively higher endogenous levels of 5-OHdCyd. In DNA treated with hydrogen peroxide and ferric chloride, however, the levels of 5-OHmdU increased much more than that of 5-OHdCyd. In addition to these hydroxylated derivatives of deoxycytidine and thymidine, the method also appears to work well with 8-oxoguanine, 4,6-diamino-5-(formylamino)pyrimidine, and 5-methyl-2'-deoxycytidine. This method may therefore be useful with a variety of modified DNA bases and nucleosides.

Keywords: oxidative DNA damage, gas chromatography/mass spectrometry, 5-hydroxymethyluracil, 5-hydroxycytosine.

Introduction

Oxygen radical-induced DNA damage and oxidative stress have been linked to numerous disease processes including cancer, inflammatory disease, atherosclerosis and heart disease as well as the process of ageing (reviewed in Ames *et al.* 1993). Quantitation of such DNA damage therefore may be an important marker of susceptibility to disease. Methods that have been used to measure oxidative DNA damage include HPLC, GC/MS, GC with electron capture detection, immunoassay and ^{32}P-postlabelling (Cadet and Weinfeld 1993). Each method has its own advantages and disadvantages. The GC/MS method has the advantage that some structural confirmation of the analyte is obtained during the analysis. In our laboratory, we routinely use GC/MS after trimethylsilylation of enzymatically hydrolysed DNA (Djuric *et al.* 1991). Several other laboratories have used GC/MS

* To whom correspondence should be addressed.

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as well, typically with acid-hydrolysed DNA (for example Dizdaroglu *et al.* 1990, deRojas-Walker 1995, Hamburg and Zhang 1995, Douki *et al.* 1996).

One potential problem with GC/MS analysis of silylated DNA bases or nucleosides is that oxidative DNA damage may occur during heating with the silylation reagents (Hamburg and Zhang 1995, Ravanat *et al.* 1995, Douki *et al.* 1996). One group has avoided this problem for quantitation of 8-oxoguanine by using trifluoroacetic acid to dissolve DNA bases followed by derivatization at room temperature (Hamburg and Zhang 1995). We have avoided use of acid in this report, and we describe time- and temperature-dependent derivatization of the deoxynucleosides, 5-hydroxymethyl-2'-deoxyuridine (5-OHmdU) and 5-hydroxy-2'-deoxycytidine (5-OHdCyd), in enzymatically-hydrolysed DNA. The data show that levels of these two deoxynucleosides do not appear to increase after derivatization with heating; conversely, convenient room temperature derivatization appears to be sufficient for their quantitation.

Methods

Chemicals

Derivatization reagents were purchased from Pierce (Rockford, Il). Nucleosides, DNA bases, calf thymus DNA and laboratory reagents were purchased from Sigma Chemical Co. (St Louis, MO) except 8-oxoguanine which was from Aldrich Chemical Co. (Milwaukee, WI). 2'-Deoxycytidine-$^{15}N_3$ was purchased from Cambridge Isotope Laboratories (Andover, MA). Thymidine-d_4, uracil-2-^{13}C,6-d and paraformaldehyde-$^{13}Cd_2$ were purchased from MSD Isotopes (St Louis, MO). The derivatization reagents are toxic and were handled in a fume hood while wearing gloves. The synthesis of 5-OHdCyd was also carried out in a fume hood while wearing gloves.

Syntheses

5-Hydroxymethyl-2'-deoxyuridine-$^{13}C_2d_2$ was synthesized as previously described from uracil-2-^{13}C,6-d and paraformaldehyde-$^{13}Cd_2$, followed by enzymatic addition of the deoxysugar using thymidine phosphorylase which we described for synthesis of thymidine-d_4 (Djuric *et al.* 1991). 5-Hydroxy-2'-deoxycytidine and 5-hydroxy-2'-deoxycytidine-$^{15}N_3$ were synthesized from deoxycytidine or 2'-deoxycytidine-$^{15}N_3$, respectively, by oxidation with bromine using a slight modification of the method of Eaton and Hutchinson (Eaton and Hutchinson 1973). Deoxycytidine was dissolved in water (25 mg in 500 µl) and kept on ice while small amounts of saturated bromine water were added slowly until a yellow colour persisted. Air then was bubbled through the mixture to remove excess bromine and 250 µl collidine was added. The mixture was incubated at 37 °C for 2 h followed by extraction with ether four times. The products were purified by reverse phase HPLC (a Waters C-18 µ-Bondapack column, 3·9 ×300 mm, eluted with a linear gradient of 100 % water to 90 % water/10 % methanol over 15 min at 2 ml min^{-1}; the product retention time is 4 min and deoxycytidine elutes at 6·5 min).

The identity of the products was confirmed by UV (UV maxima at 220 and 292 nm in water for 5-OHdCyd, similar to that published by Luo *et al.* (Luo *et al.* 1996) and mass spectrometry. The product was derivatized with *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1 % trimethylchlorosilane:acetonitrile, 2:1, which results in cleavage of the deoxysugar from pyrimidine deoxynucleosides (Djuric *et al.* 1991). The expected molecular ion of *m/z* 343 was obtained for 5-OHdCyd with the following fragment ions: *m/z* 73 (100 %), 240 (21 %), 254 (19 %), 269 (6 %), 328 (72 %), 343 (30 %). The mass spectrum of the isotopically-labelled compound was similar and gave a molecular ion of *m/z* 346. This agrees with the previously published mass spectrum of silylated 5-OHdCyd, which also loses the deoxysugar upon derivatization (Feig *et al.* 1994). For the analysis of DNA samples by single-ion monitoring, *m/z* 328 and 331 (M-15) were monitored for analysis of 5-OHdCyd and 5-OHdCyd-$^{15}N_3$, respectively, to maximize sensitivity.

Oxidation of DNA

Calf thymus DNA was dissolved in 100 mM potassium phosphate buffer, pH 7·4 at a concentration of 1 mg ml^{-1}. Some samples had mannitol added at a final concentration of 1 or 10 mM. Hydrogen peroxide and iron were added quickly to final concentrations of 100 and 200 µM, respectively, and the reactions were allowed to proceed for 15 min. Reactions were stopped by adding an equal volume of isopropanol and 1/10 volume 6 M sodium chloride. After mixing, DNA was allowed to precipitate at

0 °C for 5 min. DNA was collected by centrifugation and then washed three times with 70 % ethanol. The DNA was re-dissolved in water and then processed as described in the next section.

GC/MS Analyses

DNA samples were hydrolysed enzymatically. A sample of 100 μg DNA in 100 μl water was treated with 10 μl DNase I (2 mg ml^{-1} stock prepared in 100 mM Tris pH 7·4, 10 mM $MgCl_2$) for 2 h at 37 °C. Then 10 μl nuclease P1 was added (280 U ml^{-1} stock in 1 mM $ZnCl_2$) and the incubation continued for 1 h. Alkaline phosphatase was added next for a 30 min incubation using 5 μl of a 500 U ml^{-1} stock (made in 10 mM NaAc pH 5, previously heated at 95 °C for 10 min). Finally, 11 μl of an enzyme mixture was added and the incubation continued overnight. The enzyme mixture was prepared by mixing the following: 400 μl DNase I (10 mg ml^{-1} in DNase buffer: 150 mM NaCl, 10 mM $MgCl_2$), 200 μl 1 M Tris pH 7·4, 400 μl PDE I, (0·5 U ml^{-1} in 50 mM Tris pH 7·4, previously dialysed against 50 mM Tris pH 7·4, and 10 % glycerol added), 400 μl PDE II (7·5 U ml^{-1} in 50 mM Tris pH 7·4, previously dialysed against 50 mM Tris pH 7·4 and 10 % glycerol added), 400 μl alkaline phosphatase (500 U ml^{-1}, previously heat treated at 95 °C for 10 min), 400 μl acid phosphatase (50 U ml^{-1}). The extent of DNA hydrolysis can vary widely for various enzymatic techniques, and we obtained relatively low hydrolysis efficiency for short incubations with limited types of enzymes, which is why we use a mixture of enzymes (Bowen and Djuric, unpublished data).

After hydrolysis, nucleosides were then isolated with use of water-wetted tC-18 Sep-Paks (Waters, Milford, MA) eluted with 3 ml pure methanol after a 750 μl water wash as previously described (Djuric *et al.* 1991). The internal standard contained 312·5 μg of thymidine-d_4 and 500 ng each of 5-OHdCyd-$^{15}N_3$ and 5-OHmdU-$^{13}C_2d_2$ in 2 ml of water, and 10 μl of this mixture was added to samples containing 100 μg DNA prior to the Sep-Pak procedure. For derivatization of single nucleosides, 1·5 μg of each compound and 1·5 μg of thymine-d_4 were used per vial.

Samples were derivatized in a final volume of 60 μl with BSTFA containing 1 % trimethylchlorosilane:acetonitrile, 2:1. Some samples were heated in sealed Reacti-vials (Pierce, St Louis, MO) while some samples were simply vortexed and kept at room temperature for 15 min before transferring to GC autosampler vials.

GC/MS was conducted with a Hewlett-Packard (Palo Alto, CA) 5890 series II GC with an Ultra 2 column (25 m ×0·2 mm ×0·011 μm film thickness) and a model 5971A mass-selective detector. For DNA samples, the temperature gradient was as follows: the initial temperature of 50 °C was increased to 167 °C at 40 °C min^{-1} followed by a gradient to 173°C at 1·5 °C min^{-1}, then a rapid increase to 280 °C and a 2 min final hold at 280 °C. The injector was operated in the splitless mode at 250 °C. The detector was kept at 290 °C, and the carrier gas was helium at a flow rate of 23 ml min^{-1}. The retention times of the derivatized nucleosides were: 4·1 min thymidine, 5·0 min 2'-deoxycytidine, 6·3 min 5-OHmdU and 6·6 min 5-OHdCyd. Note that for pyrimidine nucleosides the deoxysugar is cleaved during derivatization, so the products of derivatization that are actually analysed are derivatized DNA bases (Djuric *et al.* 1991). In DNA samples, deoxycytidine could not be quantified in most cases since some other component in the hydrolysate co-eluted with this peak, and this was present in such large quantity to cause a mass spectral shut-down at that retention time. Purified deoxycytidine did, however, derivatize well at room temperature and gave a strong GC peak.

The GC/MS peak areas were calculated for peaks of derivatized pyrimidine deoxynucleosides, which lose the deoxysugar upon derivatization to give the indicated molecular ions: 5-hydroxymethyluracil (5-OHmU) (*m/e* 358), 5-OHmU-$^{13}C_2d_2$ (*m/e* 362), 5-hydroxycytosine (5-OHCyt) (*m/e* 328), 5-OHCyt-$^{15}N_3$ (*m/e* 331), thymine (*m/e* 270) and thymine-*d4* (*m/e* 274). The relative amounts of 5-OHmdU, 5-OHdCyd and thymidine were calculated from standard curves created from analysis of samples with varying amounts of those deoxynucleosides (5–100 pg μl^{-1} for hydroxylated derivatives and 80-350 ng μl^{-1} for thymidine in 60 μl final volume). Since the curves were prepared using deoxynucleosides, not DNA bases, the levels are reported for deoxynucleosides in DNA. These curves were essentially the same using standards prepared with and without heating (120 °C, 20 min) during derivatization; therefore, the standards were combined to create one calibration curve for quantitation of all samples shown in table 1. The regression lines of the standards were $y = -0{\cdot}0404 + 0{\cdot}0174x$ for 5-OHmdU, $y = -0{\cdot}241 + 0{\cdot}0344x$ for 5-OHdCyd and $y = 0{\cdot}222 + 0{\cdot}0054x$ for thymidine.

The units used in table 1, fg hydroxylated nucleoside per ng thymidine, can be converted to number of modified nucleosides per 10^5 thymidine residues after multiplying with the appropriate mole ratio: 0·9959 for 5-OHdCyd and 0·9380 for 5-OHmdU. If it can be assumed that 25 % of the nucleosides in DNA are thymidine, then the number of modified nucleosides per 10^6 thymidine residues can be multiplied by 0·025 to yield number of modified nucleosides per 10^5 nucleotides. Thus, 72 fg 5-OHmdU per ng thymidine and 175 fg 5-OHdCyd per ng thymidine become 1·68 5-OHmdU/10^5 nucleotides and 4·36 5-OHdCyd/10^5 nucleotides.

For analysis of the compounds shown in table 2, the GC temperature was also 50 °C initially with an increase to 167°C at 40 °C min^{-1}. The temperature was then increased to 280 °C at a rate of 10 °C min^{-1}. With this programme, the retention times of silated DNA bases obtained from derivatization of the indicated compounds are: thymidine 4·2 min, 5-methyl-2'-deoxycytidine 4·9 min, FapyA 7·1 min and

Table 1. Levels of 5-hydroxymethyl-2'-deoxyuridine (5-OHmdU) and 5-hydroxy-2'-deoxycytidine (5-OHdCyd) in calf thymus DNA derivatized at various temperatures.

Derivatization conditions[a]		Area ratio[b] (mean SD)			Level[c] (fg-ng thymidine)	
		5-OHmdU	5-OHdCyd	Thymidine	5-OHmdU	5-OHdCyd
120 °C	20 min	0·368	2·491	1·680	64	238
		0·043	1·077	0·050	4	109
120 °C	10 min	0·429	3·312	1·860	74	300
		0·014	0·320	0·053	5	25
100 °C	10 min	0·381	2·942	1·732	70	285
		0·045	0·376	0·129	3	19
80 °C	10 min	0·421	2·247	1·766	76	204
		0·032	0·207	0·086	8	27
60 °C	10 min	0·527*	2·082	2·041*	82*	158
		0·047	0·100	0·132	5	17
Room temperature	10 min[d]	0·466	2·270	2·033*	72	175
		0·021	0·048	0·046	2	8
p-value[e]		0·0062	0·1760	0·0056	0·0423	0·0606

[a] Enzymatically-hydrolysed DNA samples were derivatized with *N*,*O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1 % trimethylchlorosilane:acetonitrile, 2:1, and heated at the indicated temperature and time prior to analysis by GC/MS with single-ion monitoring.

[b] The GC/MS area ratios for the peak area of the analyte divided by that of the isotopically-labelled internal standard (mean with standard deviation given underneath) were calculated for peaks corresponding to 5-OHmdU (*m*/*e* 358/362), 5-OHdCyd (*m*/*e* 328/331) and thymidine (*m*/*e*270/274). The number of replicates is three.

[c] The levels of 5-hydroxymethyl-2'-deoxyuridine (5-OHmdU), 5-hydroxy-2'-deoxycytidine (5-OHdCyd) and thymidine were calculated from standard curves created using pure nucleosides. The mean level is given with the standard deviation underneath.

[d] Samples were derivatized at room temperature for 10 min prior to transferring the samples to autosampler vials.

[e] The *p*-values were determined by ANOVA analyses and difference between pairs were evaluated by the Tukey–Kramer multiple comparisons procedure. The starred value (*) for the 358/362 ratio was significantly higher than either of the ratios obtained using 120 °C, 20 min or 100 °C, 10 min. The starred values for 270/274 were significantly higher than the values obtained with 120 °C, 20 min derivatization. The starred value for 5-OHmdU amount was significantly higher than the 5-OHmdU level after derivatization at 120 °C, 20 min.

8-oxoguanine 10·1 min. The mass spectrometer was operated in the full scan mode for these samples (*m*/*e* 50–550), and the total ion chromatograms were integrated.

Results

Enzymatically-hydrolysed DNA was analysed for levels of 5-OHmdU and 5-OHdCyd after derivatization at various temperatures (table 1). From 100 µg DNA, one would expect roughly 315 ng µl^{-1} thymidine in 60 µl final volume (assuming that 1/4 of the nucleosides are thymidine, and that there are 3·125 µmol nucleosides per mg DNA). This is close to what we observed (303 ± 42 ng µl^{-1}), indicating good hydrolysis. One must note, however, that DNA solutions such as ours that are vortexed are not homogeneous and they are difficult to pipette. This makes the actual amount hydrolysed variable and difficult to estimate accurately.

The levels of 5-OHmdU relative to thymidine were similar at the derivatization

Table 2. Derivatization of 8-oxoguanine, 5-methyl-2'-deoxycytidine and 4,5-diamino-5-(formylamino)pyrimidine (FapyA) at Room temperature.

	Mass spectral results[a]		
Sample	Heated	Room temperature	*p*-value[b]
8-Oxoguanine			
Ratio[c]	0·281 ± 0·016	0·281 ± 0·011	1·00
Area[d]	2·33 ± 0·34	1·99 ± 0·10	0·172
5-Methyl-2'-deoxycytidine			
Ratio	0·366 ± 0·009	0·359 ± 0·009	0·395
Area	2·84 ± 0·08	2·62 ± 0·13	0·067
FapyA			
Ratio	0·598 ± 0·012	0·568 ± 0·025	0·495
Area	4·73 ± 0·24	4·13 ± 0·58	0·173
Thymine-d_4			
Area[e]	7·97 ± 0·57	7·20 ± 0·41	0·130

[a] Samples containing were derivatized with TMS prior to GC/MS either at 120°C for 20 min or at room temperature for 15 min prior to analysis. The number of replicates is three in each case and data are mean ± standard deviation.

[b] The *p*-value was obtained from two-sample *t*-tests for the comparison of samples prepared either with heating or at room temperature.

[c] The ratio refers to the GC/MS peak area ratio of the analyte to the area of thymine-d_4, which was included as an internal standard in the samples prior to derivatization.

[d] The area refers to the peak area of the total ion chromatogram for the given analyte and is given in arbitrary units.

[e] The area calculated for thymine-d_4 in each sample (thymine-d_4 was the internal standard) is given separately here (n=9).

temperatures shown, although the level obtained using derivatization at 60 °C for 10 min was significantly higher than the level obtained after derivatization at 120 °C for 20 min. The levels of 5-OHdCyd were calculated relative to thymidine as well since there was chromatographic interference that did not allow us to quantify dCyd in enzymatically-hydrolysed DNA. Similar to 5-OHmdU levels, the levels 5-OHdCyd were not significantly different by derivatization temperature, although there was a trend for the levels to increase with derivatization temperature. This may be due to the calculated amount of thymidine, which decreased with increasing temperature. Raw area ratios of the analyte to internal standard were also measured in all cases (table 1). For thymidine, the area ratios of analyte to internal standard were significantly greater after derivatization at room temperature or at 60 °C than at 120 °C for 20 min. The only other statistically significant difference between derivatization at 120 °C for 20 min and any other temperature was for the 358/362 ratio which was higher in DNA derivatized at 60 °C for 10 min. Overall, the peak area ratios of analyte to internal standard and the calculated levels of 5-OHmdU or 5-OHdCyd relative to thymidine were similar at all the different derivatization temperatures investigated. The raw peak areas for all the ions monitored also showed little variation with temperature (data not shown).

The variability between replicate samples was generally low, and the standard deviation was generally less 10 % of the mean (table 1). This variability may be due to slight variations in sample handling as well as to variations in GC/MS response.

Variability in GC/MS response can be assessed by comparing results for duplicate injections. All the samples in table 1 were injected twice and the mean coefficient of variation (cv) for area ratios of the same samples injected twice was 10·1 % (data not shown). It also was of interest to determine if reproducibility was affected by derivatization temperature. There were no obvious trends in the cv with temperature. The only area ratios that yielded a statistically significant linear trend with temperature were those for 270/274 (p=0·0008), and this was due to higher cv values for samples derivatized at 100 °C or 120 °C for 10 min versus those derivatized at 60 °C or room temperature. The mean cv for the area ratios of all three DNA components derivatized at room temperature was 8 ± 4 %, which was somewhat lower than for derivatization at all other temperatures combined (10 ± 6 %). This indicates that variability between injections was acceptable using room temperature derivatization and that further derivatization did not appear to occur upon standing at room temperature for extended time periods.

Three other compounds, 8-oxoguanine, 5-methyl-2'-deoxycytidine and 4,5-diamino-5-(formylamino)pyrimidine (FapyA) also were derivatized at room temperature, and this was compared with results for derivatization at 120 °C for 20 min (table 2). Isotopically-labelled internal standards were not available for these compounds, therefore, thymidine-d_4 was used as an internal standard. Again there was little difference in the peak areas and area ratios of analyte to internal standard with derivatization temperature. None of the differences were statistically significant, although there was a trend for the peak areas to be somewhat smaller at room temperature. Thus, derivatization at room temperature may not be quite as efficient as at 120 °C for 20 min, but this did not affect the area ratio since the internal standard is subjected to the same conditions as the analyte. Note that although the GC programme was started at 50 °C, the injector was at 250 °C and some derivatization could have occurred during the vaporization of the sample.

We also examined levels of 5-OHmdU and 5-OHdCyd in calf thymus DNA before and after treatment with hydrogen peroxide and iron (table 3). Levels in the untreated calf thymus DNA in this experiment using room temperature derivatization were similar to that in table 1. It is important to note that although within-experiment reproducibility was reasonable, there is a fair amount of day-to-day variation that seems to be inherent in this technique. This may be due to the multi-step nature of sample preparation (DNA hydrolysis, nucleoside purification,

Table 3. Levels of Levels of 5-Hydroxymethyl-2'-deoxyuridine (5-OHmdU) and 5-Hydroxy-2'-deoxycytidine (5-OHdCyd) in Calf Thymus DNA Treated with Hydrogen Peroxide

Hydrogen peroxide[a]	Mannitol	Levels[b]	
		5-OHmdU	5-OHdCyd
0	0	59 ± 3	170 ± 1
100 μM	0	1616 ± 220*	397 ± 52*
100 μM	1 mM	783 ± 103*	258 ± 31
100 μM	10 mM	177 ± 71	170 ± 10

[a] Calf thymus DNA was treated with the indicated concentrations of hydrogen peroxide and mannitol in the presence of 200 μM $FeCl_2$ and 100 mM K_2PO_4, pH 7·4.

[b] Levels of each modified nucleoside are given in fg/ng thymidine. The starred values are significantly greater (p < 0·005) than un-oxidized DNA.

drying, derivatization) as well as to variability in the GC/MS response. For these reasons, we typically analyse calf thymus DNA samples with every set of samples for quality control, and use these to gauge how results on any given day compare with other experiments. In DNA treated with hydrogen peroxide, the levels of 5-OHmdU increased quite a bit more than that of 5-OHdCyd (table 3). Mannitol was effective in inhibiting formation of both products, which implicates hydroxyl radical in their formation (Minotti and Aust 1987).

Discussion

The results shown here indicate that derivatization of enzymatically-hydrolysed DNA with *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) containing 1% trimethylchloro-silane:acetonitrile, 2:1, may be carried out quite satisfactorily at room temperature (tables 1 and 2). There were some slight differences in the results when comparing samples derivatized with and without heating, but overall this did not appear to influence the results appreciably. The advantages are considerable, however, since room temperature derivatization minimizes possible degradation reactions, decreases processing time and decreases sample loss on heating if the cap/liner system fails. Oxidation may still occur during DNA purification, of course, and this is a separate issue that needs to be addressed.

The calculated amounts of 5-OHmdU or 5-OHdCyd in calf thymus DNA were not statistically different when enzymatically-hydrolysed and Sep-pak purified DNA was derivatized with and without use of heat. This is in contrast to previous reports, but in those published experiments, longer derivatization times and/or higher temperatures were used. The studies on artifactual formation of 8-oxoguanine were carried out using the same derivatization reagent that we have used, BSTFA, but heating of acid-hydrolysed DNA at 130 or 140 °C for 30 min was used (Hamburg and Zhang 1995). The studies showing formation of the DNA bases 5-hydroxycytosine and 5-hydroxymethyluracil during derivatization were performed by comparing derivatization times varying from 15 to 120 min while heating at 110 °C . Although we cannot exclude the possibility that artifactual formation of these compounds occurs when using room temperature derivatization, in our hands this does not appear to be the case since the levels of the derivatives are stable over several days at room temperature and heating for 10–20 min at various temperatures did not appreciably change their observed levels in DNA samples (table 1).

Interestingly, in laboratories that analyse for both 5-OHmdU and 5-OHdCyd, the levels of 5-OHdCyd in DNA are typically 2- to 10-fold higher than the levels of 5-OHmdU (Dizdaroglu *et al.* 1993, Toyokuni *et al.* 1994, Doetsch *et al.* 1995, Olinski *et al.* 1995, Jaruga and Dizdaroglu 1996). The results in table 1 are consistent with this. Measurement of the 5-OHdCyd lesion may be of importance not only because of its relatively high endogenous levels but also because of its relatively high mutagenic potential (Wagner *et al.* 1992, Feig and Loeb 1993). This lesion, however, may not be the best indicator of hydrogen peroxide-induced DNA damage since the increases in 5-OHmdU were relatively greater when DNA was treated with iron(II) and hydrogen peroxide (table 3). For the quantitation of both these modified DNA lesions in various investigations, our results suggest that when GC/MS is used for analysis of this and other nucleoside derivatives, room temperature derivatization may be the preferred procedure.

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