RESEARCH COMMUNICATION

Measurement of oxidative DNA damage by gas chromatography–mass spectrometry: ethanethiol prevents artifactual generation of oxidized DNA bases

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Analysis of oxidative damage to DNA bases by GC-MS enables identification of a range of base oxidation products, but requires a derivatization procedure. However, derivatization at high temperature in the presence of air can cause 'artifactual' oxidation of some undamaged bases, leading to an overestimation of their oxidation products, including 8-hydroxyguanine. Therefore derivatization conditions that could minimize this problem were investigated. Decreasing derivatization temperature to 23 °C lowered levels of 8-hydroxyguanine, 8-hydroxyadenine, 5-hydroxycytosine and 5-(hydroxymethyl)uracil measured by GC–MS in hydrolysed calf thymus DNA. Addition of the reducing agent ethanethiol (5 %, v/v) to DNA samples during trimethylsilylation at 90 °C also decreased levels of these four oxidized DNA bases as well as 5-hydroxyuracil. Removal of guanine from hydrolysed DNA samples by treatment with guanase, prior to derivatization, resulted in 8-hydroxyguanine levels (54–59 pmol/mg of DNA) that were significantly lower than samples not pretreated with guanase, independent of the derivatization conditions used. Only hydrolysed DNA samples that were derivatized at 23 °C in the presence of ethanethiol produced 8-hydroxyguanine levels (56 ± 8 pmol/mg of DNA) that were as low as those of guanase-pretreated samples. Levels of other oxidized bases were similar to samples derivatized at 23 °C without ethanethiol, except for 5-hydroxycytosine and 5-hydroxyuracil, which were further decreased by ethanethiol. Levels of 8-hydroxyguanine, 8-hydroxyadenine and 5-hydroxycytosine measured in hydrolysed calf thymus DNA by the improved procedures described here were comparable with those reported previously by HPLC with electrochemical detection and by GC–MS with prepurification to remove undamaged base. We conclude that artifactual oxidation of DNA bases during derivatization can be prevented by decreasing the temperature to 23 °C, removing air from the derivatization reaction and adding ethanethiol.

INTRODUCTION

Biomolecules are susceptible to damage by a variety of reactive oxygen (ROS), chlorine (RCS) and nitrogen species (RNS) such as hypochlorous acid (HOCl), hydroxyl radical ($^{\bullet}OH$), singlet oxygen ($^1O_2$), nitryl chloride (NO_2Cl) and peroxynitrite ($ONOO^-$) (reviewed in [1]). Many of these species are capable of causing oxidative damage to DNA, culminating in chemical changes to both pyrimidine and purine bases [2–4] as well as a variety of other lesions, such as single- and double-strand breaks, abasic sites, DNA–protein cross-links and modified sugars [5–6]. The frequency of these lesions and the effectiveness of repair systems appear to be important factors in the development of mutations and carcinogenic states, and in some degenerative diseases [3,7–9]. The accurate assessment of oxidative DNA damage in human samples should be a valuable marker not only of the risk of occurrence of carcinogenic events, but of the overall level of oxidative stress within the body [1,3]. Identification of the pattern of DNA base damage and the alternative products that arise from the same DNA base radical intermediate under different chemical environments, e.g. the 8-hydroxyguanine (8-OH-guanine) radical, can help to identify the damaging species and its mechanism of action, e.g. the patterns of oxidative damage to DNA by $^1O_2$ [10], $^{\bullet}OH$ [1,2,11], $ONOO^-$ [12] and HOCl [13] are markedly different. Therefore development of a simple, reliable method for analysis of multiple DNA base damage products is important. Indeed measurement of only a single oxidation product can give misleading results about the extent of DNA damage, as illustrated by recent studies in Parkinson's disease [14].

GC–MS is becoming widely used to quantify DNA damage because of its ability to identify a wide range of DNA base products [1,2,15,16]. Baseline levels of DNA oxidation in healthy individuals have yet to be unequivocally established. Most study has been devoted to the quantification of 8-OH-guanine, either as the nucleoside (8-hydroxy-2′-deoxyguanosine) after enzymic hydrolysis [3,17] or as the base after acid hydrolysis [2,16,18]. Levels of 8-OH-guanine measured in cellular DNA are often, but not always, greater when measured by GC–MS compared with other methods such as HPLC with electrochemical detection (HPLC-ECD) (reviewed in [15]).

After DNA hydrolysis, GC–MS requires a derivatization procedure in order to convert the polar nucleosides/bases and

Abbreviations used: HPLC-ECD, HPLC with electrochemical detection; ROS, reactive oxygen species; RCS, reactive chlorine species; RNS, reactive nitrogen species; FAPy adenine, 4,6-diamino-5-formamidopyrimidine; FAPy guanine, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 8-OH-guanine, 8-hydroxyguanine; 2-OH-adenine, 2-hydroxyadenine; 8-OH-adenine, 8-hydroxyadenine; 5-(OH,Me)-uracil, 5-(hydroxymethyl) uracil; 5-OH,Me-hydantoin, 5-hydroxy-5-methylhydantoin; 5-OH-hydantoin, 5-hydroxyhydantoin; 5-OH-uracil, 5-hydroxyuracil; 5-OH-cytosine, 5-hydroxycytosine; BSTFA, bis(trimethylsilyl)trifluoracetamide; TMCS, trimethylchlorosilane; TFA, trifluoroacetic acid.

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internal standards to volatile, thermally stable derivatives which possess characteristic mass spectra. Trimethylsilylation is the most common derivatization reaction used [2,6,11,16]. It is often carried out at a high temperature (90–140 °C) and under nitrogen to prevent artifactual oxidation of samples. Recent studies of derivatization have demonstrated that, at high temperatures and in the presence of air, levels of 8-OH-guanine and some other oxidized bases can be elevated due to 'artifactual' oxidation of undamaged base, and it has been suggested that the higher levels of 8-OH-guanine and some other base-modification products detected in DNA by GC–MS may be artifacts of the derivatization process [19–22]. It has been further shown that removal of undamaged base by HPLC or immunoaffinity column pre-purification prior to derivatization results in levels of various oxidized bases similar to those obtained by HPLC in the same study [19–21]. These extra precautionary steps are time-consuming and require expensive, isotopically labelled oxidized bases as internal standards to compensate for any loss of material in the work up [20]. Derivatization at room temperature has been suggested to minimize the artifactual oxidation of DNA bases, diminishing the discrepancy between GC–MS and HPLC [22].

In the present paper we provide data showing that addition of ethanethiol to the derivatization mixture appears to eliminate derivatization artifacts. Ethanethiol has already been used to prevent the oxidation of anabolic steroids during trimethylsilylation reactions prior to GC–MS analysis [23] and comparable concentrations were used in the present study. The present paper reports simple and reliable derivatization conditions for the accurate GC–MS determination of several damaged DNA bases, including 8-OH-guanine.

METHODS

Materials

Calf thymus DNA, 6-azathymine, 2,6-diaminopurine, 8-bromoadenine, 5-hydroxyuracil (isobarbituric acid) (5-OH-uracil), 4,6-diamino-5-formamidopyrimidine (FAPy adenine), 2,5,6-triamino-4-hydroxypyrimidine, 5-(hydroxymethyl)uracil [5-(OH, Me)-uracil] and guanase were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.). 8-Hydroxyguanine (8-OH-guanine) and ethanethiol were purchased from Aldrich. 8-Hydroxyadenine (8-OH-adenine) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FAPy guanine) were synthesized (courtesy of Dr. H. Kaur, Kings College, London) by, respectively, treatment of 8-bromoadenine with concentrated formic acid (95 %) at 150 °C for 45 min with purification by crystallization from water [24], and treatment of 2,5,6-triamino-4-hydroxypyrimidine with concentrated formic acid with purification by crystallization from water [25]. Thymine glycol was synthesized by reaction of 5-methyluracil with OsO_4 for 1 h at 60 °C, and excess OsO_4 was removed by freeze-drying [26]. Purity of standards (> 99 %) was assessed by MS. 2-Hydroxyadenine (2-OH-adenine), 5-hydroxycytosine (5-OH-cytosine), 5-hydroxyhydantoin (5-OH-hydantoin) and 5-hydroxy-5-methylhydantoin [5-(OH,Me)-hydantoin] were kindly given by Dr. M. Dizdaroglu (National Institute of Standards and Technology, Gaithersberg, MD, U.S.A.).

Cellu.Sep dialysis membranes with an M_r cut-off of 3500, silylation-grade acetonitrile, and bis(trimethylsilyl)trifluoracetamide (BSTFA) [containing 1 % trimethylchlorosilane (TMCS)] were obtained from Pierce Chemical Co., Rockford, IL, U.S.A. A Centrifree micropartition system with an M_r cut-off of 30000 (product no. 4104) was supplied by Amicon. Distilled water passed through a purification system (Elga, High Wycombe, Bucks.) was used to make up all solutions.

Acid hydrolysis.

Calf thymus DNA (100 μg) containing the internal standards 6-azathymine and 2,6-diaminopurine (0.5 nmol of each) was hydrolysed by addition of 0.5 ml of 60 % formic acid and heating at 140 °C for 45 min in an evacuated, sealed hydrolysis tube. Samples were cooled and freeze-dried.

Guanase treatment

Hydrolysed and freeze-dried DNA samples were incubated with guanase (4 munits/100 μg of DNA) in 1 ml of 20 mM phosphate buffer, pH 8.0, at 37 °C for 1 h. Protein was then removed by filtration through a Centrifree micropartitition system (M_r cut-off 30000), followed by centrifugation at 4000 ***g*** for 10 min. Samples were freeze-dried prior to subsequent derivatization. GC–MS analysis of hydrolysed DNA treated with guanase confirmed that all guanine was converted into xanthine and that the other purines studied, including 2,6-diaminopurine, were unaffected by this enzyme.

Derivatization

Caution: Ethanethiol has a very unpleasant odour. Samples were derivatized in poly(tetrafluoroethylene)-capped glass vials after purging with nitrogen. Either 100 μl of a BSTFA (+1 % TMCS)/acetonitrile (4:1, v/v) mixture was added to samples or 100μl of a BSTFA (+1 % TMCS)/acetonitrile/ethanethiol (16:3:1, by vol.) mixture was added. Samples were derivatized at either 90 °C or 23 °C for 1 h and analysed by GC–MS as described previously [13] after a further 1 h at 23 °C.

Time course of derivatization

Mixtures containing 1 nmol of guanine, thymine, base-damage products and 0.5 nmol of internal standards (6-azathymine and 2,6-diaminopurine) were derivatized at 23 °C as described above with 100 μl of BSTFA + 1 % TMCS/acetonitrile/ethanethiol (16:3:1, by vol.) for varying periods of time and analysed by GC–MS over increasing periods of time. Octadecane, which is stable and does not undergo derivatization, was added to all samples (100 nmol) prior to derivatization as a reference [22] in order to assess the effect of time on the extent of derivatization

Statistical analysis

Data points are means ± S.D. from four or more experiments. Analysis of variance and Student's t test were carried out as appropriate. Differences of $P < 0.05$ were considered statistically significant.

RESULTS

The effect of derivatization conditions on the levels of oxidized bases measured in an acid hydrolysate of commercial calf thymus DNA was investigated. Addition of 5 % ethanethiol to the derivatization mixture did not significantly alter the rate of DNA base derivatization at 23 °C. Formation of base derivatives had ceased after 2 h incubation (Figures 1A–1C), and this incubation time was selected for further experiments.

Decreasing derivatization temperature from 90 °C to 23 °C caused lower levels of 8-OH-guanine, 8-OH-adenine, 5-OH-cytosine and 5-(OH,Me)-uracil to be measured in hydrolysed DNA (Table 1). Addition of ethanethiol to samples derivatized at 90 °C resulted in lower levels of 8-OH-guanine, 8-OH-adenine, 5-OH-cytosine, 5-OH-uracil and 5-(OH,Me)-uracil. (Table 1).

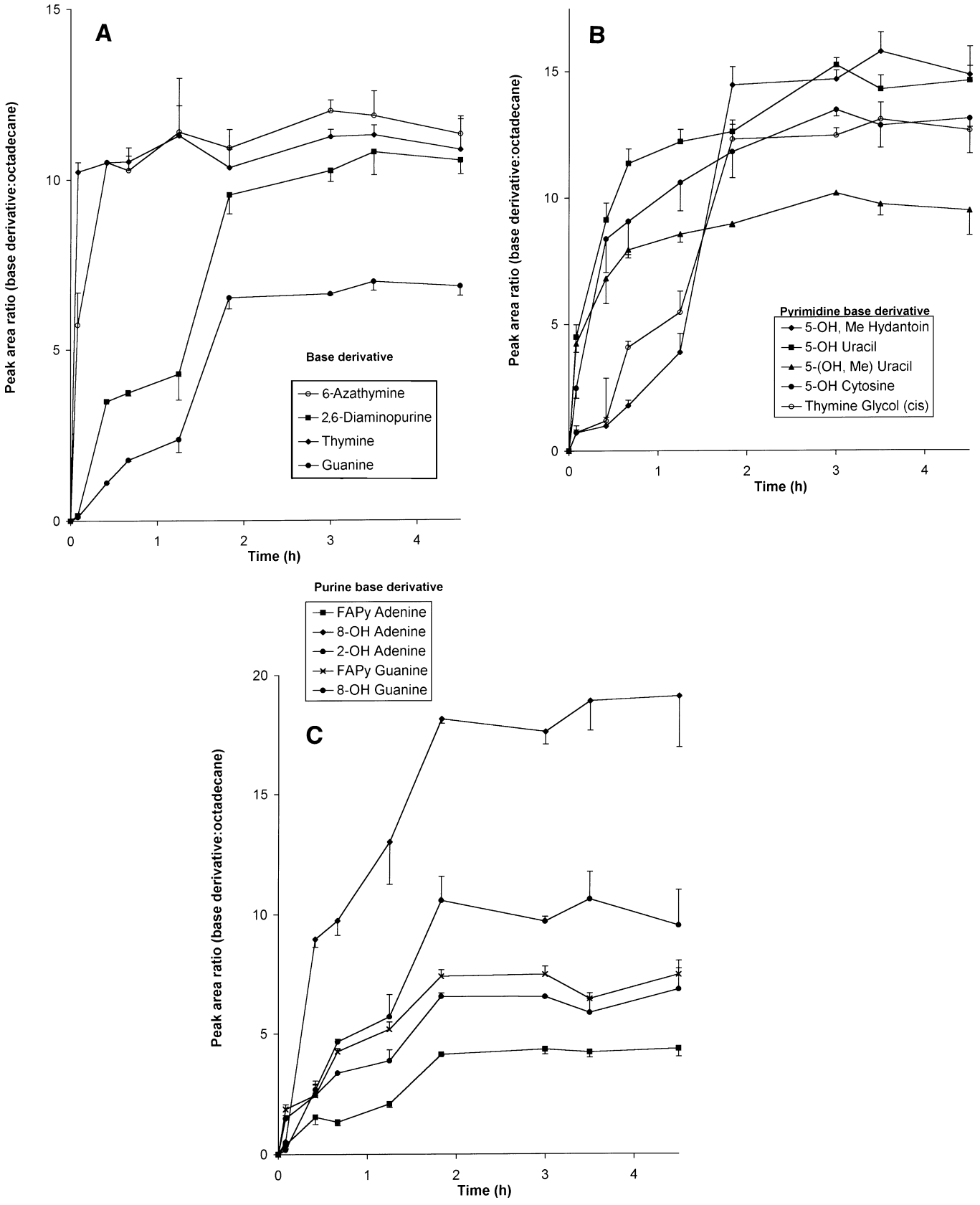

Figure 1 Time course of DNA base derivatization at 23 °C using 100 μl of a BSTFA + 1% TMCS/acetonitrile/ethanethiol (16:3:1, by vol.) mixture (5% ethanethiol)

Derivative concentration was calculated by comparison with octadecane. Data points are means ± S.D. for four or more experiments. (**A**) Guanine, thymine and internal standards; (**B**) pyrimidines; (**C**) purines.

Table 1 Influence of derivatization reaction temperature and ethanethiol on the levels of oxidized DNA bases measured in calf thymus DNA by GC–MS

Samples were purged with nitrogen before derivatization at each temperature for 1 h and then kept at 23 °C for 1 h prior to GC–MS analysis. Data points are mean ± S.D. for four or more experiments.

		Damaged DNA base (pmol/mg of DNA)			
		Control		Ethanethiol	
Base	Reaction temperature (°C) ...	90	23	90	23
5-(OH,Me)-hydantoin		39 ± 5	41 ± 9	47 ± 2	40 ± 8
5-OH-hydantoin		27 ± 6	26 ± 4	20 ± 4	20 ± 3
5-OH-uracil		39 ± 4	34 ± 5	14 ± 3	5 ± 1
5-(OH,Me)-uracil		37 ± 9	19 ± 1	22 ± 1	23 ± 4
5-OH-cytosine		226 ± 9	82 ± 18	119 ± 9	58 ± 7
Thymine glycol		136 ± 17	110 ± 18	107 ± 16	92 ± 16
FAPy adenine		97 ± 4	78 ± 16	93 ± 15	81 ± 9
8-OH-adenine		172 ± 20	97 ± 7	120 ± 17	88 ± 11
2-OH-adenine		43 ± 12	46 ± 3	42 ± 4	43 ± 1
FAPy guanine		121 ± 13	111 ± 6	123 ± 12	122 ± 2
8-OH-guanine		214 ± 33	114 ± 9	100 ± 22	56 ± 8

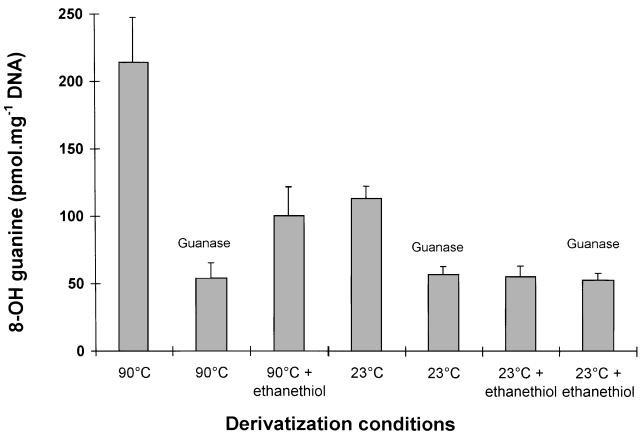

Figure 2 Influence of guanase treatment, derivatization reaction temperature and ethanethiol on the level of 8-OH-guanine measured in calf thymus DNA by GC-MS

Samples were purged with nitrogen before derivatization at each temperature for 1 h and then kept at 23 °C for 1 h prior to GC–MS analysis. Data points are means ± S.D. for four or more experiments

Ethanethiol also significantly lowered levels of 8-OH-guanine, 5-OH-cytosine and 5-OH-uracil when samples were derivatized at 23 °C (Table 1). Removal of guanine from hydrolysed DNA samples, by guanase treatment, prior to derivatization, eliminated the effect of temperature or ethanethiol on the levels of 8-OH-guanine measured (Figure 2). 8-OH-guanine levels in guanase-pretreated samples were all identical, independent of the derivatization conditions used (Figure 2). Removal of guanine from hydrolysed DNA significantly decreased 8-OH-guanine levels, except for samples that were derivatized at 23 °C with ethanethiol (Figure 2). Using these derivatization conditions, levels of 8-OH-guanine were the same with or without guanase pretreatment.

DISCUSSION

When measuring oxidative DNA damage it is important to know whether an artifact is being introduced while preparing samples for analysis [15,19–22]. The present study indicates that levels of several bases, especially 8-OH-guanine, 8-OH-adenine and 5-OH-cytosine, are artifactually increased during derivatization of hydrolysed DNA at high temperatures, leading to an overestimation of oxidative DNA damage. These results agree with several other recent reports that have also identified the source of these artifacts as oxidation of undamaged base during derivatization [19–22]. Formation of these four oxidized bases is potentiated when air is not removed prior to derivatization [19–22]. There are considerable differences in the magnitude of the artifacts measured between different studies, particularly for 8-OH-guanine. This is probably due to differences in the amount and types of derivatization solvents used, as well as the exact time and temperature conditions used and the efficiency with which air is excluded; some studies failed to exclude air at all [19–21]. Owing to the large excess of undamaged compared with damaged bases in cellular DNA, as little as 0.01 % artifactual base oxidation will confound the detection of small changes in levels of oxidized DNA bases.

The present paper shows that the reducing agent ethanethiol prevents artifactual oxidation of guanine when the derivatization is performed at room temperature, in that levels of 8-OH-guanine are identical whether or not guanine is first removed. If guanine is removed, then it cannot be artifactually oxidized during derivatization. Since guanine is the most readily oxidizable DNA base, these derivatization conditions are likely to prevent artifactual oxidation of adenine, thymine and cytosine also, as indicated in Table 1. The routine use of guanase treatment for GC–MS analysis is not recommended, since the extra manipulation is time-consuming and affects the analysis of certain pyrimidine compounds. In addition, guanase treatment would not eliminate artifactual oxidation of other DNA bases and would prevent measurement of xanthine, an important product of attack by RNS [12]. Trifluoroacetic acid (TFA) has been reported to improve room-temperature derivatization of guanine and 8-OH-guanine [22], but we have found that TFA is also unsuitable for quantification of pyrimidines by GC–MS. The great advantage of GC–MS is that it can measure the whole profile of DNA damage products [1,15,16], and several cases are known where measurement of 8-OH-guanine alone can give misleading information about the extent of DNA damage [12–14].

Ethanethiol has no detrimental effect on the derivatization of other bases, demonstrated by the sensitivity and linearity of the calibration curves ($r > 0.96$, range 20–1000 pmol; results not shown). Interestingly, levels of 5-OH-uracil, unaffected by derivatization temperature, were significantly decreased by ethanethiol. This suggests that artifactual formation of this base can be prevented by a reducing agent, but not solely by lowering derivatization temperature. Ethanethiol thus provides a protection against oxidation of undamaged base during derivatization, additional to that of temperature reduction, which is necessary for complete prevention of artifactual 8-OH-guanine, 5-OH-cytosine and 5-OH-uracil generation. Artifactual formation of 8-OH-adenine and 5-(OH,Me)-uracil seems to be completely prevented by reducing the derivatization temperature to 23 °C, since addition of ethanethiol contributes no further decrease at this temperature.

Table 2 GC–MS and HPLC measurement of 8-OH-guanine in commercial calf thymus DNA after acid hydrolysis

Values from these publications were converted on the basis of 1 nmol/mg of DNA = $318/10^6$ bases, 1 ng of 8-OH guanine = 5.98 pmol and that each base is 25% of the total in DNA. HPLC determination of 8-hydroxy-2′-deoxyguanosine after enzymic hydrolysis of commercial calf thymus DNA resulted in values of 23.4–1006 pmol/mg of DNA (reviewed in [15]).

Analytical technique	8-OH-guanine (pmol/mg of DNA)	Derivatization conditions	References
GC–MS	43	30 min, Ar, 23 °C, TFA	[22]
	336	30 min, Ar, 140 °C, TFA	[22]
	500–1000	30 min, N_2, 130 °C	[11,15]
	45–53	30 min, 130 °C, pre-purification	[19,21]
	2426	30 min, 130 °C no degassing,	[19]
	210	60 min, N_2, 90 °C	[12]
	56	2 h, N_2, 23 °C, ethanethiol	Present study
HPLC	53	No derivatization, ECD	[19,21]

There has been much debate about the accurate measurement of oxidative DNA base damage and, in particular, 8-OH-guanine. Levels of this commonly used biomarker vary considerably between different laboratories, with GC–MS generally estimating levels greater than those estimated by HPLC [15]. The present study demonstrates that ‘artifactual’ oxidation during derivatization can be prevented by decreasing derivatization temperature, removing as much air as possible from the derivatization reaction and by adding ethanethiol as an antioxidant. When this is achieved, levels of oxidized base measured by GC–MS are comparable with those measured by HPLC-EC (Table 2).

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