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Determination of *cis*-thymine glycol in DNA by gas chromatography–mass spectrometry with selected ion recording and multiple reaction monitoring

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Abstract

A novel method for the determination of *cis*-thymine glycol in DNA has been developed, using gas chromatography–mass spectrometry with selected ion recording or multiple reaction monitoring. The procedure involves acidic hydrolysis of DNA in the presence of the internal standard *cis*-[$^2H_3$]thymine glycol, followed by derivatisation with N-methyl-N-(*tert.*-butyldimethylsilyl)trifluoroacetamide. The method was validated on DNA that had been oxidatively modified in vitro by radiation treatment, and was then applied to determine *cis*-thymine glycol in human placental DNA. Background levels of 5.45±2.98 ng *cis*-thymine glycol/mg DNA were observed in the human samples. © 1997 Elsevier Science B.V.

Keywords: *cis*-Thymine glycol; DNA

1. Introduction

The generation of free radicals in vivo is believed to be associated with a variety of adverse biological effects. Such radicals are capable of causing chemical modifications of, and damage to, proteins, lipids, carbohydrates and nucleic acids. A radical of particular toxicity is the highly reactive hydroxyl radical (·OH) which may be formed by endogenous sources or by exposure to exogenous chemicals, such as cytostatic drugs and non-genotoxic carcinogens, or radiation. The interaction of ·OH with DNA results in modified bases, modified sugars, strand breaks and DNA-protein cross-links and oxidative damage to DNA may result in cytotoxicity or mutation (and the potential for carcinogenicity) (reviewed in [1]).

Because of these possible toxicological problems many approaches have been developed in recent years to assess the extent of hydroxyl radical damage ("oxidative damage") to DNA. The number of chemical products formed in such reactions is extensive and extremely sensitive systems are required for their analysis in human samples, whose accessibility

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is normally limited [2]. The problem is further complicated by the fact that artefactual production of some of these oxidised products has been shown to occur during DNA isolation, handling and the analytical work-up procedures, which results in artificially high levels of modification being observed.

Of the possible oxidative DNA damage products that have been examined, most attention has been paid to 8-oxoguanine. The analytical methods that have been employed for its determination have included high-performance liquid chromatography (HPLC) coupled with electrochemical detection (ED) [3], gas chromatography–mass spectrometry (GC–MS) [4,5], immunochemical techniques [6–8] and "postlabelling" using radioactive [9–11] or fluorescent [12] derivatives. The results obtained have shown considerable inconsistencies with GC–MS determinations showing levels as much as 50-times higher than those obtained by HPLC–ED. Part of the reason for this was shown by Hamberg and Zhang [5] and Ravanat et al. [13] to be artefactual production of 8-oxoguanine from guanine during derivatisation for GC–MS. Other artefactual sources of 8-oxoguanine have also been well reported ([14] and references therein).

Two other important interaction products of DNA with hydroxyl radicals, 5-hydroxymethyluracil (5-OHMeU) and *cis*-5,6-dihydroxy-5,6-dihydrothymine (*cis*-thymine glycol) (Tg), are formed with thymine (Fig. 1). Methods for the analysis of 5-OHMeU have included ^{32}P-postlabelling [15], and GC–MS or LC–MS of the free base after derivatisation [16–19]. High levels of 5-OHMeU have been found in human DNA (e.g., 9.3 5-OHMeU/10^4 thymines in DNA from nucleated peripheral blood cells [19]), but there is little information concerning artefactual formation of this base.

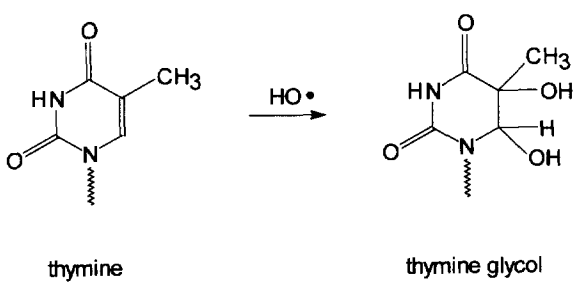


Fig. 1. Reaction of thymine with hydroxyl radicals, yielding thymine glycol.

Previous methods for detection and/or determination of Tg have been more extensive than those for 5-OHMeU and have included thin-layer chromatography (TLC) [20], HPLC with UV detection after reduction to thymine [21] or acetylation [22], immunochemical methods using polyclonal [23] or monoclonal [24] antibodies, fluorescent postlabelling [12], ^{32}P-postlabelling [25–27], GC–MS of the free base after derivatisation of a DNA hydrolysate [4,28,29], GC–MS of a methanolysed [30,31] or a pentafluorobenzyl-*tert.*-butyldimethylsilylated product [32] after alkaline cleavage/borodeuteride reduction of DNA, thermospray MS [33], and laser desorption Fourier–Transform MS [34]. Despite the abundance of these methods for Tg there is still only limited data on background levels of this modified base in human DNA samples and some uncertainty concerning the relevance of some of the published data because of lack of demonstration that any Tg observed was not generated artefactually. However, of particular note is the fact that Nackerdien et al. [28] showed that several oxidised DNA bases (including Tg) are not significantly formed from DNA bases during formic acid treatment.

The levels of Tg in DNA have been reported as ca. 0.01–0.06 nmol/mg DNA (equivalent to ca. 3–19 Tg molecules/10^6 total DNA bases) in human lung tissue [35] and 1 Tg/10^5 thymines in calf thymus DNA [25]. These levels (like those that have been reported for other oxidised bases such as 8-oxoguanine [14]) are considerably in excess of those that have been reported for covalently bound alkyl adducts, such as those caused by exposure to methylating and ethylating agents, aflatoxin B1 and PAH epoxides ([36] and references contained therein) and their mutagenic significance is unknown.

The purpose of the work reported here was to develop a specific and sensitive method for Tg determination in DNA, which is free of problems caused by artefactual formation of this modified base. The method was first validated on DNA samples oxidatively modified in vitro and then applied to the determination of Tg in control human samples. The effects of antioxidant therapy on the level of Tg in human samples was also studied.

2. Experimental

2.1. Chemicals

All solvents were obtained from Fisons (Loughborough, UK); HPLC grade solvents were used for the quantitative assay and analytical grade was employed in the synthesis of the analyte standards. The derivatising agent, a mixture of N-methyl-N-(*tert.*-butyldimethylsilyl) trifluoroacetamide (MTBSTFA) and 1% *tert.*-butyldimethylchlorosilane (TBDMCS) and trimethylchlorosilane (TCMS) for silylation of glassware were purchased from Pierce and Warriner (Chester, UK). Calf thymus DNA was obtained from Sigma (Poole, UK).

3-[$^2H_3$]Methyl malic acid was a gift (MRC Toxicology Unit, Carshalton, UK, I. Bird). All other materials were obtained from Sigma or Aldrich (Gillingham, UK).

2.2. Synthesis of cis-thymine glycol and cis-[$^2H_3$]thymine glycol

cis-Thymine glycol was synthesised by the oxidation of thymine with $KMnO_4$ essentially according to the method of Iida and Hayatsu [37]. Thymine (1.0 g) was fully dissolved in warm water (300 ml) at 50–60°C. The solution was then cooled in iced water to 5–10°C, and the pH was adjusted to 8.6 using NH_3 solution (5%, ~1 ml). $KMnO_4$ (0.07 *M*, 150 ml) was then added to the aqueous thymine solution (final pH 9.3). After 5 min, saturated H_2SO_3 was added dropwise until MnO_2 was precipitated and the purple colour just disappeared (pH~2.3); the solution was immediately re-basified using NH_3 solution (5%, ~12 ml) to pH 8.6.

The solution was then filtered and evaporated down to ca. 5 ml on a rotary evaporator (40–50°C). Acetone (500 ml) was added to the mixture which, on shaking, produced a white precipitate which was removed by filtration. The filtrate was concentrated to ~5–10 ml using a rotary evaporator (40–50°C), cooled and the product crystallised (yield: 63%, m.p. 215°C). Identification and purity were confirmed by NMR (ARX Bruker 250 Mhz), which was determined in both [$^2H_6$]dimethylsulphoxide and [$^2H_2$]O; the spectra were consistent with those reported by Frenkel et al. [38]. Fast atom bombardment (FAB) MS in positive ion mode with glycerol matrix yielded the expected $[M+H]^+$ pseudomolecular ion at *m/z* 161. The NMR spectrum showed no evidence for *trans*-Tg in the product.

The internal standard, *cis*-[$^2H_3$]Tg was prepared from [$^2H_3$]thymine (methyl labelled) by a similar procedure to that used for the synthesis of the unlabelled compound. [$^2H_3$]Thymine was synthesised from the reaction of 3-[$^2H_3$]methyl malic acid with urea in oleum solution [39]. The FAB mass spectrum of *cis*-[$^2H_3$]Tg was consistent with that obtained for the unlabelled compound, with a pseudomolecular ion at *m/z* 164. The amount of the unlabelled isotopomer present was below the limit of detection using mass spectral scanning.

2.3. In vitro samples and irradiation procedure

Aliquots (1 ml) of a solution of calf thymus DNA in deionised water (1 mg/ml) were irradiated in a ^{60}Co–γ source at a dose rate of 1.5 kgy/h; the total dose ranged from 0 gy to 400 gy or from 0 gy to 100 gy. After irradiation, 100 μl of a 10 pg/μl aqueous [$^2H_3$]Tg solution was added to each aliquot. Samples were taken to dryness by vacuum centrifugation (SpeedVac, Savant Instruments, Farmingdale, USA), and 100 mg aliquots hydrolysed, derivatised and assayed by GC–MS-SIR as described below.

2.4. Placental samples and extraction procedure

Placental samples were obtained from forty women resident in Teplice (Czech Republic). Vitamins were supplemented in twenty subjects who were administered antioxidants (300 mg vitamin C, 200 mg vitamin E and 10 mg β-carotene daily, divided between two doses in the morning and evening) from the third month to the end of the pregnancy. The control group ($n=20$) did not receive antioxidant supplementation. The study was approved by the Ethical Committee of the Reg. Institute of Hygiene of Central Bohemia.

Tissue samples were isolated from the middle of the placenta and the DNA isolated from 10% tissue homogenates in the extraction buffer (10 m*M* Tris–HCl, 100 m*M* EDTA, 0.5% SDS, pH 8.0). Following treatment with RNAses A and T_1, and proteinase K, DNA was extracted with phenol–chloroform–iso-

amyl alcohol and precipitated with ethanol as previously described [40]. Final DNA concentrations were determined by UV spectrophotometry ($A_{260,\ 280}$). Samples were supplied in aqueous solution and kept frozen at −20°C until analysis. Aliquots, containing a total of 75 μg DNA, to which 1 ng [$^2H_3$]Tg was added as internal standard were taken for analysis.

2.5. Hydrolysis of DNA

Glassware for hydrolysis and derivatisation was washed with chromic acid and silylated using a 5% TCMS solution in toluene prior to use. DNA samples (70–120 μg) were dried by vacuum centrifugation and subjected to acid hydrolysis. Initial experiments used a variety of concentrations of formic acid (60–88%) and times of reaction (15–60 min). The final choice of conditions (see Section 3.1) were 60% formic acid (500 μl) under vacuum at 140°C for 40 min, similar to the method of Nackerdien et al. [28]. Samples were then dried in a vacuum centrifuge prior to derivatisation.

2.6. Derivatisation procedure

After hydrolysis, the Tg samples were derivatised to their *tert.*-BDMS derivatives using MTBSTFA–1% TBDMCS and pyridine (1:1, total volume 60 μl) at 60°C for 30 min. The derivatised samples were then dried in a centrifugal evaporator and reconstituted in 20 μl ethyl acetate.

2.7. Quantitative analysis

Quantitation was achieved using isotope-dilution MS with [$^2H_3$]Tg as the internal standard. The principles of this approach have been described by Dizdaroglu [4]. Calibration standards utilised calf thymus DNA, [75-μg aliquots from a 1 mg/ml solution in deionised water (concentration confirmed by UV)], to which known amounts of unlabelled Tg and the internal standard were added prior to acid hydrolysis and derivatisation. Samples (to which the internal standard had been added) were analysed in batches, up to a total of 6–8 samples and calibration lines were determined separately for each batch. Calibration standards and samples were assayed on the same day. Linear regression analysis was used to construct calibration plots of ion peak area ratio (determined manually) versus the amount of added Tg (ng, total). Total Tg levels in the calibration samples were calculated by adding the "background" level, determined from the calibration plot. Using this relationship between ion peak area ratio and total Tg levels it was then possible to calculate Tg levels (in units of ng/mg DNA) in human and calf thymus DNA samples.

2.8. Gas chromatography–mass spectrometry

GC–MS instrumentation comprised a CE8060 gas chromatograph (Carlo Erba, Fisons Instruments, Loughborough, UK) interfaced to a VG AutoSpec Ultima-Q mass spectrometer (VG Organic, Manchester, UK). The in vitro samples and initial SIR assay of the placental samples utilized a DB-5, 15 m×0.25 mm, 0.25 μm film thickness column (J&W Scientific, Fisons, Loughborough, UK). The column used for GC–MS multiple reaction monitoring (MRM) analysis of the placental samples was a PTE-5, 30 m×0.25 mm, 0.25 μm film thickness (Supelco, Poole, UK). For both columns, the initial oven temperature was 80°C (1 min) followed by a temperature ramp of 15°C/min to 280°C. Helium carrier column flow-rate was determined to be 1 ml/min at 80°C. Split injection (1 μl) was employed with split ratios between 10:1 and 25:1 and the injector temperature was 280°C.

All data was obtained using electron impact (EI) mode. Electron energy and trap current were 70 eV and 200 μA, respectively. The source temperature was 250°C and the transfer line was held at 290°C.

2.9. Selected ion recording (SIR)

The monitored ions were *m/z* 331.2 ([$^2H_0$]Tg) and *m/z* 334.2 ([$^2H_3$]Tg). Dwell and delay times were 40 ms and 10 ms, respectively, to give a total scan time of 0.1 s. Ions were selected at 1000 resolution.

2.10. Multiple reaction monitoring

The MRM transitions used were, for [$^2H_0$]Tg, *m/z* 331.2→*m/z* 288.1 and for [$^2H_3$]Tg, *m/z* 334.2→*m/z* 291.1. Dwell and delay times were 300 ms and 20 ms to give a total scan time of 0.64 s. Precursor

ions were selected at 1000 resolution and quadrupole resolution was 1.5 m/z units at base. Collision energy was optimised using the m/z 326 ion from heptacosa (septum inlet) as the precursor ion. The beam attenuation was ca. 50% and the product ions were used to check quadrupole calibration. The collision energy was in the range 5–10 eV with no added collision gas.

3. Results and discussion

3.1. Hydrolysis

Optimisation of hydrolysis conditions have been reported previously [28,41]. The use of 88% formic acid with a hydrolysis temperature of 150°C was shown by Fuciarelli et al. [41] to result in complete base release from irradiated (100 gy) DNA in 40 min. We have conducted a similar experiment using 60% formic acid at 140°C with irradiated calf thymus DNA (400 gy) and obtained the same result (data not shown) – the level of Tg reaching a plateau in 40 min and no evidence of Tg degradation on heating for longer periods. In another experiment, the effect of temperature on base release from irradiated DNA was investigated (other conditions were: 60% formic acid, 1 h hydrolysis time). Tg release continued to increase with temperature up to 150°C (upper limit of study). Our results were consistent with those of Nackerdien et al. [28] and Fuciarelli et al. [41]. It has been reported that acid hydrolysis of Tg standards leads to losses of 20–25% of the base [28]. For the assay described here, it was expected that the use of an isotopically-labelled internal standard would compensate for losses on hydrolysis.

3.2. Thymine glycol bis-tert.-butyldimethylsilyl derivative

Most of the previous quantitative GC–MS assays developed for Tg have used the trimethylsilylated derivative [4,28,41–43]. The use of the *tert.*-BDMS derivative has also been described for qualitative studies [44,45]. The *tert.*-BDMS derivatives have a number of advantages over TMS derivatives – the most important of which include greater resistance to hydrolysis and the formation, in EI mode, of intense $[M-57]^+$ ions (due to loss of a tertiary butyl group) which are suitable for SIR [46]. Crain and McCloskey [47] have described conditions for the formation of the *tert.*-BDMS derivative of 5-methylcytosine which were subsequently employed by Dizdaroglu [44,45] to generate (*tert.*-BDMS)$_4$-Tg. We found that the derivatisation conditions described by Crain and McCloskey produced bis- and tris-*tert.*-BDMS Tg in addition to the fully-derivatised base. Further heating (60 min at 120°C) after the derivatisation mixture had been allowed to stand overnight was required in order to convert most of the bis- and tris-derivative to the fully-derivatised base. Injection in ethyl acetate, rather than the derivatising agent, appeared to cause hydrolysis of tris- and tetra-*tert.*-BDMS-Tg to yield the bis-*tert.*-BDMS derivative (ca. 95% of the detected derivatives). Further optimisation was not pursued as a deuterated internal standard was to be used in the assay. The derivatisation conditions described in Section 2.6 yielded the bis-*tert.*-BDMS derivatives of Tg and its deuterated analogue. The chromatographic properties/instrument response were adequate for quantitation and derivatisation yields were not determined.

It was not possible, from the proton NMR spectrum of derivatised thymine glycol, to determine the position of the two *tert.*-BDMS groups, although the EI mass spectrum was consistent with derivatisation of the two hydroxyl groups (C-5 and C-6).

The major higher mass ion in the EI mass spectrum is the $[M-57]^+$ ion at m/z 331 for the $[^2H_0]$ derivative (Fig. 2a, Scheme 1). The molecular ion (m/z 388) and m/z 373 (loss of a methyl radical) were observed but were of low intensity. Consequently, the m/z 331 ion was used for SIR and as the precursor ion for MRM. The ion at m/z 288 is thought to be produced by loss of HNCO from m/z 331, although this is not an abundant fragmentation. Although this type of fragmentation occurs readily for 2,4-dioxypyrimidines, where it occurs by a reverse Diels–Alder mechanism, saturation of the 5,6 bond (as in thymine glycol derivatives) blocks this mechanism, making loss of HNCO more difficult [48,49]. The MRM product ion, m/z 288 ($[^2H_0]$), is also assumed to be due to loss of HNCO (−43 u) from the $[M-57]^+$ ion. Other major ions in the EI mass spectrum may possibly be attributed to loss of *tert.*-butyldimethylsilanol (*tert.*-BDMS-OH, −132

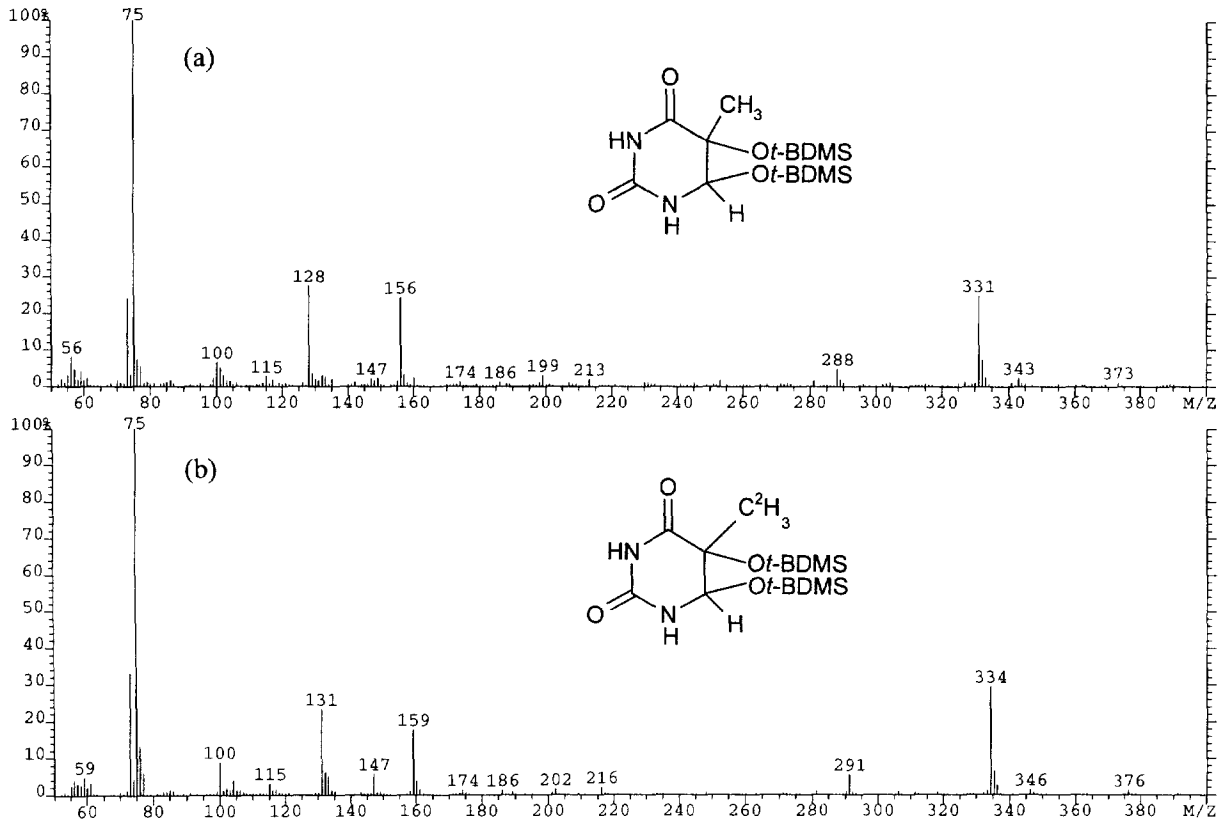
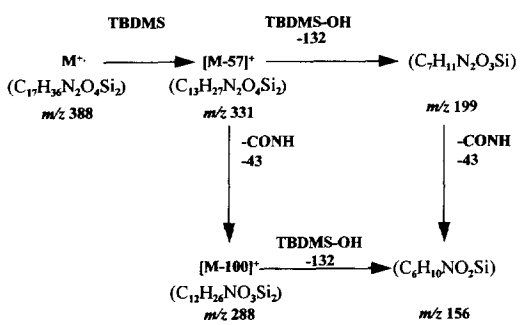


Fig. 2. EI mass spectra: (a) bis-*tert.*-BDMS [$^2H_0$]Tg; (b) bis-*tert.*-BDMS [$^2H_3$]Tg.

u): *m/z* 199 (from *m/z* 331) and *m/z* 156 (from *m/z* 288). Alternatively, the ion at *m/z* 156 may be the product ion from loss of HNCO from the ion at *m/z* 199, by a reverse Diels–Alder mechanism.



Scheme 1. Fragmentation pathway for bis-*tert.*-BDMS [$^2H_0$]Tg. All of the indicated fragments were 3 *m/z* units higher for bis-*tert.*-BDMS [$^2H_3$]Tg.

Ions at *m/z* 147, 75 and 73 were attributed to silyl fragments reported previously [46]. With the exception of the silyl fragments and *m/z* 186, 174 and 160, [$^2H_3$]Tg produced the expected fragment ions at 3 *m/z* units higher than those observed for [$^2H_0$]Tg (Fig. 2b). Monitored channels for the internal standard were *m/z* 334 (SIR) and the transition *m/z* 334 to *m/z* 291 (MRM).

3.3. Quantitation and background levels

Prior to assaying the in vitro and placental samples, the calibration method was evaluated. Fig. 3 shows an example of a calibration line which was obtained using GC–MS-SIR. [$^2H_0$]Tg was added at levels of 0–400 pg, together with 2 ng of the internal standard ([$^2H_3$]Tg), to calf thymus DNA (120 μg). Fig. 4 shows the SIR trace for the calibration sample

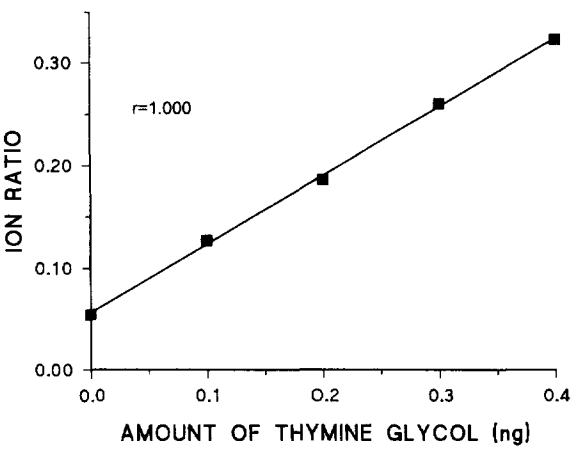


Fig. 3. Internal standard calibration plot obtained by GC–MS-SIR with calf thymus DNA matrix. Levels of bis-*tert.*-BDMS [$^2H_0$] and [$^2H_3$]Tg are given in Section 3.3.

containing 100 pg [$^2H_0$]Tg. Hydrolysis and derivatisation were as described previously.

A background level of Tg is apparent from the calibration. By treating the calibration as "method of standard addition" data [50], the background level of Tg in the calf thymus DNA was determined to be 0.692±0.17 ng/mg DNA (equivalent to 5.33±1.31 Tg molecules/10^6 thymine molecules, assuming thymine to account for one quarter of the deoxynucleotides in DNA). This background level may be due to contributions from Tg already present in the calf thymus DNA. Floyd et al. [51] reported that background levels of 8-hydroxy-2′-deoxyguanosine determined from commercially available DNA could vary significantly from batch to batch. Other sources may be the work-up procedure and derivatisation which may lead to the oxidation of thymine or the formation of artefacts. However, Nackerdien et al. [28] have concluded that thymine is not converted to Tg on acid hydrolysis and derivatisation, which suggests that modified base level in calf thymus DNA is the most important source of Tg background. In terms of quantitation, Tg levels are corrected for this background level such that reported values represent the total amount of Tg detected.

3.4. Irradiation of in vitro samples

Fig. 5 shows the dose response obtained from irradiation of calf thymus DNA (0–400 gy) using the GC–MS-SIR assay described above. A linear dose response was observed. For the 0–100 gy dose range

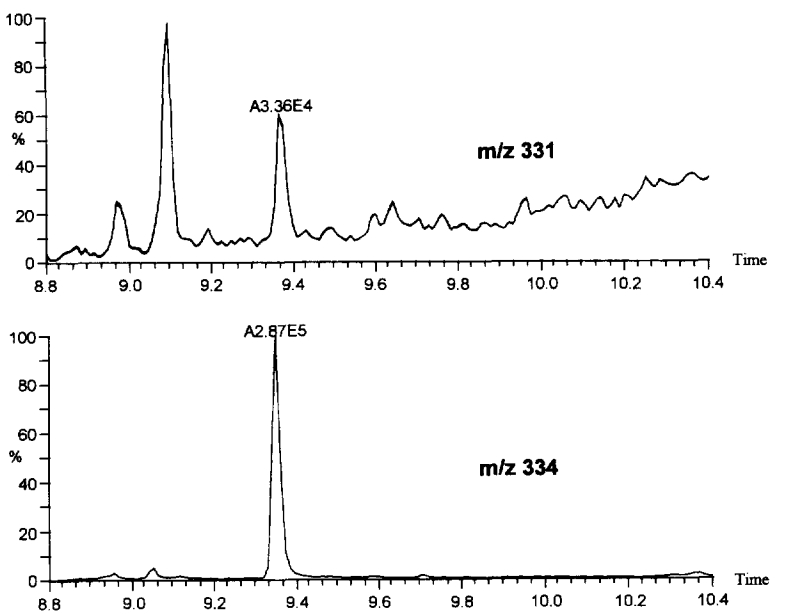


Fig. 4. SIR trace of calibration sample (100 pg [$^2H_0$]Tg, 2 ng [$^2H_3$]Tg, 120 μg calf thymus DNA). The upper trace is of *m/z* 331 for detection of [$^2H_0$]Tg and the lower trace is of *m/z* 334 for detection of [$^2H_3$]Tg.

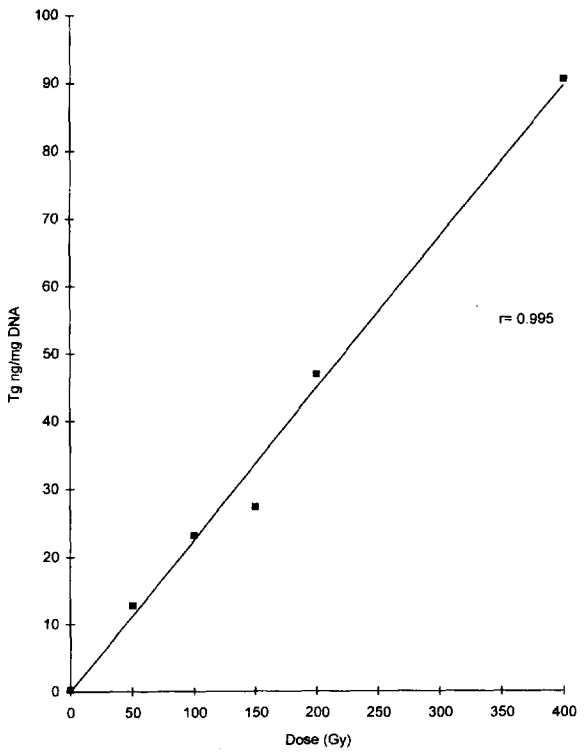


Fig. 5. Irradiation of calf thymus DNA–dose response plot obtained by GC–MS-SIR for bis-*tert.*-BDMS [$^2H_0$]Tg.

samples were analysed in duplicate, one week apart. Absolute levels of Tg were not determined, but the linear response obtained from SIR calibration plots (see Fig. 3) indicates that the increase in Tg from irradiated DNA in this dose range is again linear. The dose response observed is in agreement with similar studies on irradiated DNA [41] and chromatin. [28,43].

3.5. GC–MS: placental samples

Having demonstrated that the GC–MS-SIR assay could be used to quantitate biologically-significant levels of (added) Tg in samples of calf thymus DNA in vitro (control and radiation-treated), the method was applied to placental tissue samples. It was anticipated that levels of Tg would be reduced in the samples from women treated with antioxidants during their pregnancy compared with the control sample group. Hydrolysis and derivatisation were as described in Sections 2.5 and 2.6 and the calibration range was 0–500 pg [$^2H_0$]Tg added to the DNA. The amount of internal standard ([$^2H_3$]Tg) added to each sample aliquot (75 μg) was 1 ng.

Initially, the GC–MS-SIR assay was employed. However, it was found that the SIR scan mode lacked the necessary specificity for reliable quantitation as can be seen from Fig. 6a, which shows a typical trace from one of the control samples. Numerous (additional) chromatographic peaks were obtained close to the expected retention time for the analyte and internal standard. This effect was more pronounced in the *m/z* 331 channel. The level of potential interferents in both the analyte and internal standard channels was assumed to be due to co-extraction of other components in the tissue samples which may have lead directly to the observed responses. Alternatively, the hydrolysis and derivatisation procedure may have generated components with the correct *m/z* values for detection in the monitored SIR channels. Artefactual responses may also have been possible, although it has been suggested that the origin of such responses could be due to the elution of matrix components not removed by the work-up procedure [52]. Although the extraction and hydrolysis procedure employed for the placental samples were similar to those commonly used for quantitation of constituents of DNA from other tissues by MS, the presence of any co-extracted material from the placenta might reduce the specificity of the assay.

Whilst an improved work-up procedure was desirable (see later discussion), another possibility was to alter the mode of detection of the GC assay by using MS–MS with MRM. A number of placental samples were analysed by GC–MS-MRM in order to determine whether the level of interferents detected was reduced sufficiently (compared to the SIR traces) to allow quantitation. Fig. 6b shows the same control sample trace as Fig. 6a acquired in MRM mode. Additional chromatographic peaks were still observed but at much lower levels relative to the analyte peaks, such that quantitation was possible.

The calibration lines obtained from MRM and SIR scan modes were compared (Fig. 7). From the slope of the lines, it was apparent that MRM gave the greater response and that background levels of Tg were not significantly different: 2.25±1.29 ng/mg DNA (SIR) and 2.33±0.88 ng/mg DNA (MRM). It

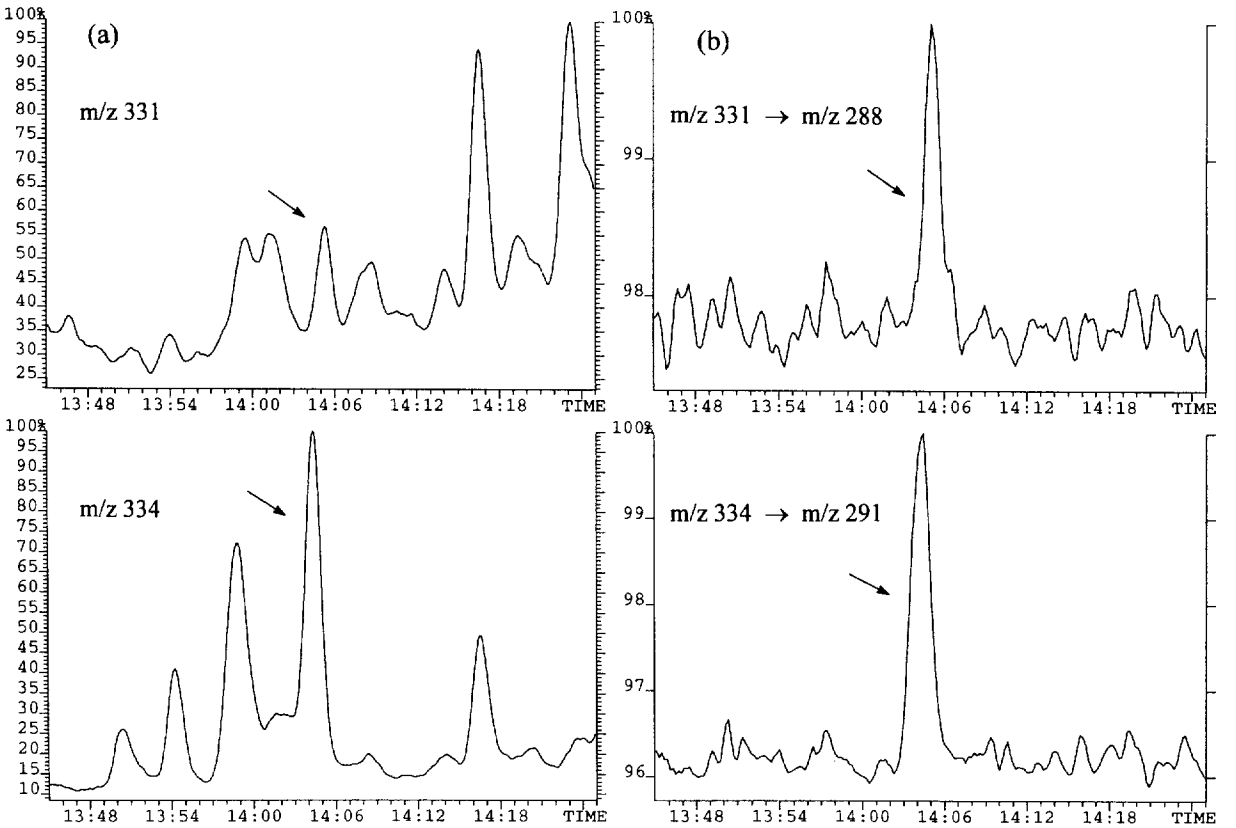


Fig. 6. Comparison of SIR and MRM scan modes for the same control sample. The retention times of bis-*tert.*-BDMS [$^2H_0$]Tg and the internal standard were 14.05 min and 14.04 min, respectively (indicated peaks). (a) SIR: traces from the monitored channels; (b) MRM: traces from the monitored transition channels.

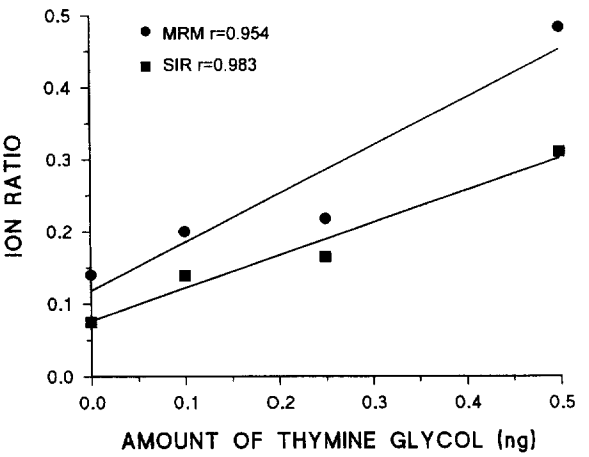


Fig. 7. Comparison of calibration plots obtained by SIR and MRM with calf thymus DNA matrix. Levels of bis-*tert.*-BDMS [$^2H_0$] and [$^2H_3$]Tg are given in Section 3.5.

is anticipated that the limit of detection for a pure Tg standard would be lower for SIR than MRM, but in assaying samples, the "limit of detection" (in this case, the lowest level of the analyte that is significantly above background) is determined by the background level of Tg and the precision with which this level can be measured [28]. (The signal-to-noise ratio for our SIR detection of the background level of Tg was acceptable, being typically ca. 3:1.)

The placental samples were assayed using MRM and the results for the two sample groups are shown in Fig. 8. In the antioxidant-treated group, three samples had not yielded a signal in the analyte channel ([$^2H_0$]Tg); these were not included in the statistical analysis. Given that a background level of Tg was expected, together with the variation of that level between samples, it was concluded that for the

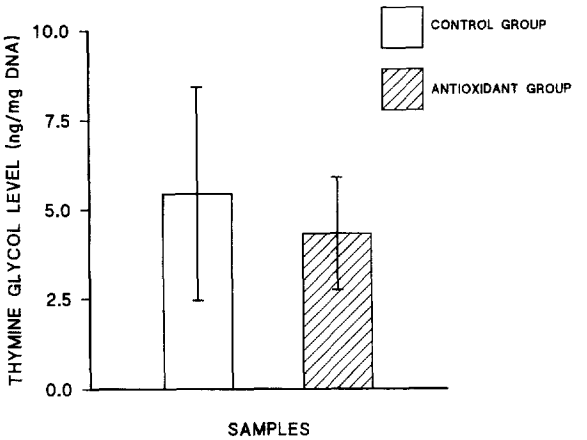


Fig. 8. Placental samples: antioxidant ($n=17$) versus control ($n=20$) group. Mean values of Tg for the two groups are given in Section 3.5.

three "zero" samples, the analyte level was below the limit of detection of the assay.

Application of a two sample t-test at the 95% level indicated that there was no significant difference between the control ($n=20$) and antioxidant ($n=17$) groups, which had mean levels of Tg of 5.45 ± 2.98 ng/mg DNA and 4.33 ± 1.58 ng/mg DNA, respectively. Although the food intake of the control group was not controlled, the availability to them of other rich sources of antioxidant vitamins, such as fizzy drinks, was limited. We are not aware of any other studies that are directly comparable to the one described here, but in two other studies which reported the quantitation of oxidised bases in human samples (cancerous versus normal tissue), levels of Tg were either not significantly elevated [35] or could not be detected [53].

3.6. Comparison of scan modes

Although MRM has greater specificity than SIR, the MS–MS scan mode does have a number of disadvantages. The limit of detection is higher than SIR due to reduced ion transmission in MS–MS experiments. Additionally, in our experience, ion ratio precision is less than that obtainable by SIR. It is possible that both of these factors were important in: (i) the apparent zero level of Tg in three control samples; (ii) the absence of a significant difference between control and antioxidant samples.

A separate calibration line was determined for each batch of placental samples and the calibration standards and samples were analysed on the same day, so the effect of day-to-day variation of instrument tuning and response on the quantitative results was minimised. However, a combined plot of four MRM calibration lines (Fig. 9), which were obtained over a period of fourteen days, shows that the precision of ion ratio measurement over this time period was poor. Given that the combined calibration line was obtained from four sets of calf thymus DNA calibration standards, the variation in ion ratios also includes differences between calibration standards introduced during the work-up procedure. As a deuterated internal standard was used, the most significant factors in the work-up procedure were likely to have been volumetric and gravimetric precision. Additionally, degradation of calf thymus DNA may have contributed to the overall precision. Although the calf thymus DNA used for calibration was taken from the same batch, the standard was stored frozen in aqueous solution. It has recently been reported [31] that an increase in the level of Tg in DNA can result from storage in aqueous solution for extended periods of time.

It is not possible to ascertain which aspect of the

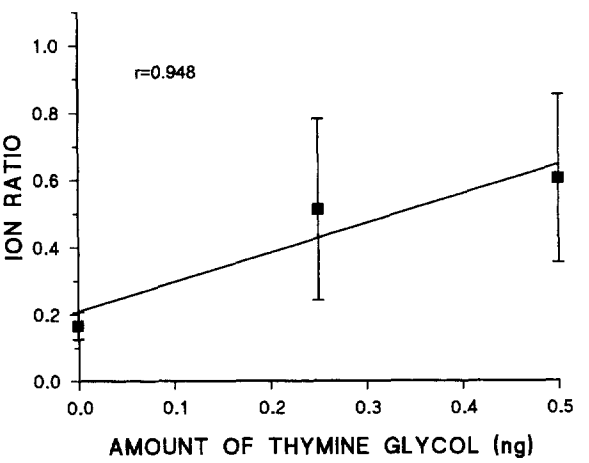


Fig. 9. Averaged MRM calibration plot. Each point is the mean of four separate sample preparation/calibration experiments, conducted over a 14-day period.

assay – work-up procedure or irreproducibility in ion ratio measurement in MRM – had the greatest influence on the overall precision. We suggest that with MRM there is a greater need for repeat measurement of calibration standards and samples. From the results presented above, it is apparent that calibration lines need to be obtained for each batch of samples which further increases the overall analysis time. The use of MRM, however, is preferable in terms of assay specificity especially in the analysis of tissue samples.

3.7. General comments

In common with Dizdaroglu [44], we have found that the quantitation of oxidised nucleobases by GC–MS requires a high level of column and injector maintenance. Sensitivity was found to decrease after analysis of approximately twenty hydrolysed DNA samples which we attributed to the build-up of partially-derivatised nucleobases and the sugar–phosphate backbone in the injector and at the top of the column. Removal of approximately 50 cm of column and thorough silylation of the injector liner was required in order to improve sensitivity. It is possible that injection in the derivatising agent would have reduced the frequency of injector/column maintenance. Additionally, the yield of *tert.*-BDMS-Tg may have increased but it is possible that the tri- and tetra-derivatised base would have been produced. Injection in the derivatising agent may also have lead to more derivatised co-extracted material being introduced on to the column. We suggest that the assay could be improved, both with respect to signal-to-noise ratio and specificity, by introducing an additional clean-up stage to remove (or reduce the amount of) the sugar–phosphate backbone after hydrolysis. This could be achieved, for example, by the use of a boronate affinity column [21].

4. Conclusions

The analytical procedure that we have developed for Tg has sufficient sensitivity to detect background levels of this oxidised base in placental DNA (ca. 5 ng/mg DNA or ca. 1 Tg/10^5 nucleotides). This level of modification is similar to that reported by Hegi et al. [25], using ^{32}P-postlabelling of commercial calf thymus DNA. The placental background level of Tg appears to be unchanged by antioxidant treatment. It has been supposed for some time that antioxidants may reduce the extent of oxidative damage, although the evidence for any extension of lifespan potential by antioxidant therapy is not clear [54].

In our procedure there is no purification of Tg between the DNA hydrolysis and GC–MS. A successful analysis was only achieved by the use of tandem MS which greatly increased the selectivity of the determination. Although this gives the advantage that it leads to a rapid analysis, care did have to be taken to avoid contamination problems in the GC. An intended improvement for future analyses is to include a further purification stage for Tg to remove contaminants rather than rely simply on the use of tandem MS.

With any analytical procedure for oxidative DNA damage there is the question as to whether or not there is any artefactual formation of the analyte during the analytical procedure. For the above described procedure for Tg, evidence has been presented to show that the DNA hydrolysis stage is not responsible for any such effect. The possibility that Tg could be produced during DNA isolation, or the final *tert.*-BDMS-derivatisation stage, will be investigated using a synthetic oligonucleotide containing Tg. However, the consistency of our results with those derived from other determinations using techniques other than GC–MS suggests that the derivatisation stage is not causing significant artefactual formation of Tg. In future, we intend to use the method for determining environmental influences on the amount of oxidative DNA damage in human samples.

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