ELSEVIER

Journal of Chromatography B, 710 (1998) 67–74

JOURNAL OF CHROMATOGRAPHY B

Gas chromatography–mass spectrometry with high-performance liquid chromatography prepurification for monitoring the endonuclease III-mediated excision of 5-hydroxy-5,6-dihydrothymine and 5,6-dihydrothymine from γ-irradiated DNA

Cédric D'Ham, Jean-Luc Ravanat, Jean Cadet*

Département de Recherche Fondamentale sur la Matière Condensée/SCIB, Laboratoire Lésions des Acides Nucléiques, CEA/Grenoble, 17 Avenue des Martyrs, F-38 054 Grenoble Cedex 9, France

Received 13 January 1998; received in revised form 3 March 1998; accepted 9 March 1998

Abstract

The endonuclease III from *Escherichia coli* is a repair enzyme which exhibits both a glycosylase and an endonuclease function. The activity of the enzyme can be assayed by measuring the released targeted bases in solution from a sample of modified DNA. In the present study, gas chromatography–mass spectrometry was used together with an HPLC prepurification step in order to single out the released bases. The prepurification was found to enhance the specificity and the sensitivity of the assay. Thus, the overall method allowed us to analyze separately 5-hydroxy-5,6-dihydrothymine from the *cis* and *trans* isomers of 6-hydroxy-5,6-dihydrothymine. Examples of application of the assay are provided with the measurement of the *E. coli* endonuclease III-mediated excision of 5-hydroxy-5,6-dihydrothymine and 5,6-dihydrothymine from samples of γ-irradiated DNA in the presence of cysteine.

Keywords: Endonuclease III; 5-Hydroxy-5,6-dihydrothymine; 5,6-Dihydrothymine; DNA

1. Introduction

Cellular DNA is subject to permanent damage and repair processes. The repair of radiation-induced pyrimidine and purine single DNA base lesions is mostly accounted for by the base excision repair (BER) pathway. Several assays have been applied to evaluate the enzymes acting through the BER mechanism. These include electrophoresis on polyacrylamide gels for oligonucleotides, and the single cell gel electrophoresis technique, the so-called ''COMET assay'' for isolated cells. It should be noted that both methods reveal the strand breaks induced by the endonuclease activity of the repair enzymes. Another approach involves the use of sensitive and specific analytical tools aimed at singling out free modified bases released by the glycosylases activity. In the latter respect, gas chromatography–mass spectrometry (GC–MS) and HPLC-electrochemical detection assays, were applied for determining the substrate specificity of the endonuclease III [1,2], and formamidopyrimidine glycosylase (Fpg) proteins [3–5]. Interestingly, the GC–MS approach provided the requested sensitivity to allow the measurement of low amounts of the released modified bases.

*Corresponding author.

0378-4347/98/$19.00
PII: S0378-4347(98)00133-9

We report in this study, an improved GC–MS assay which allows the measurement of 5-hydroxy-5,6-dihydrothymine (5-OHDHT) in the presence of the *cis* and the *trans* isomers of 6-hydroxy-5,6-dihydrothymine (6-OHDHT): HPLC prepurification was found to be required in order to separate the three 5,6-dihydropyrimidine products that exhibit similar GC–MS properties. Furthermore, by eliminating salts and other contaminant products, the prepurification step was found to enhance the sensitivity and the selectivity of the GC–MS assay.

Examples of application of the optimized GC–MS assay, are provided with the measurement of *Escherichia coli* endonuclease III-mediated excision of 5,6-dihydrothymine compounds. This concerns 5-OHDHT and 5,6-dihydrothymine (DHT) which were generated by γ-radiolysis of oxygen-free aqueous solutions of DNA in the presence of cysteine. It should be noted that the release of 6-OHDHT was not observed under the present conditions of analysis.

2. Experimental

2.1. Chemicals

Thymine, cysteine, hydrogen peroxide and formic acid were purchased from Merck (Darmstadt, Germany). Tetradeutero-thymine (α,α,α,6-d_4) was provided by MSD Isotopes (Montreal, Canada). Hydrochloric acid and acetic acid were obtained from Carlo Erba (Milan, Italy). Zinc, potassium iodide and sodium acetate were from Prolabo (Paris, France). Bis(trimethylsilyl)trifluoroacetamide (BSTFA) was obtained from Aldrich (Steinheim, Germany). Salmon testis DNA was purchased from Sigma (St. Louis, MO, USA).

2.2. Synthesis of model compounds and isotopically labeled analogs

2.2.1. 5-OHDHT and DHT

The two 5,6-saturated thymine compounds were prepared by γ-irradiation of thymine (or thymine-d_4 for the internal standards) in the presence of cysteine, as described previously for thymidine [6]. Typically, an aqueous solution of 700 ml of 1 m*M* thymine and 0.5 m*M* cysteine was deoxygenated by bubbling a stream of nitrogen for 15 min. The oxygen-free aqueous solution was then exposed to the gamma rays emitted by a ^{60}Co source (overall dose of irradiation=8 kGy). Radiation-induced decomposition products were subsequently purified on a N5C18-25F Nucleosil C_{18} 5 μm (250×6.5 mm I.D.) column (Interchim, Montluçon, France) using water as the eluent. 5-OHDHT (k=2.1) and DHT (k=7.2) were obtained in 31% and 8% yields, respectively. The labeled standards were characterized by ^{1}H NMR and electron impact mass spectrometry.

2.2.2. Cis and trans isomers of 6-OHDHT

The *cis* isomer of 6-OHDHT was synthesized by reduction of 30 mg of *trans* 5-bromo-6-hydroxy-5,6-dihydrothymine [7] in 0.75 ml water by adding 78 mg of zinc together with 35 μl of acetic acid [8]. After filtration, the product was purified by reversed-phase HPLC under the above reported conditions (k=2.4, yield=50%).

The *trans* isomer of 6-OHDHT was obtained by oxidation of *cis* 6-OHDHT into the corresponding hydroperoxides, i.e., *cis* and *trans* isomers of 6-hydroperoxy-5,6-dihydrothymine. Reduction of the hydroperoxides yielded a mixture of the *cis* and *trans* isomers of 6-OHDHT [9]: typically, 10 mg of *cis* 6-OHDHT were placed for 3 h in 5 ml of 30% hydrogen peroxide and 40 μl of 12.4 *M* HCl. The solution was then evaporated to dryness. HPLC separation of the reaction mixture gave a major peak which contained *cis* 6-OHDHT together with the *cis* and *trans* isomers of 6-hydroperoxy-5,6-dihydrothymine. The three products were collected and the reduction of the hydroperoxides was achieved by adding 10 mg of KI and 2.5 μl of acetic acid. Iodine was extracted by chloroform and the *cis* and *trans* isomers of 6-OHDHT were separated by HPLC using water as the eluent.

2.3. DNA irradiation

A 2 ml volume of a solution of salmon testis DNA (500 μg/ml) and 0.5 m*M* cysteine were placed in a glass tube (irradiated beforehand). The solution was purged for oxygen by maintaining a stream of nitrogen for 15 min. Irradiation was delivered by four barrels of ^{60}Co providing γ-rays at a dose rate

of 30 Gy/min. After irradiation (5, 10 or 25 min), DNA was precipitated and was then washed once with ethanol.

2.4. Enzymatic assay

The standard reaction mixture (100 μl final volume) contained 50 μg of DNA, 20 ng of the internal standards of 5-OHDHT and DHT, 20 m*M* Tris HCl buffer (pH 7.5), 100 m*M* KCl and 2 μg of endonuclease III. The enzymatic reaction was carried out at 37°C for 40 min and then stopped by DNA precipitation with cold ethanol. The supernatant was then evaporated to dryness under reduced pressure while pellets were kept for further acid hydrolysis.

2.5. Acid hydrolysis

DNA pellets were dissolved in 1 ml 88% formic acid with the internal standards of 5-OHDHT and DHT (20 ng of each). Hydrolysis was carried at 130°C for 40 min and the formic acid was then evaporated under reduced pressure.

2.6. HPLC prepurification

The prepurification was achieved using a Gilson HPLC apparatus (Middleton, MI, USA). This consisted of three pumps (model 305), a dynamic mixer (811 C), a sampling injector (231 XL) together with an automatic fraction collector (FC 204). The separations were carried out on a H5C18-25F Hypersil C_{18} 5 μm (250×4.6 mm I.D.) analytical column (Interchim). The isocratic eluent consisted of 25 m*M* ammonium formate. The column was washed with 30% acetonitrile between each run.

In order to determine the retention times of the products under the conditions described above, low amounts (to avoid contamination) of targeted products were injected. Typically, 150 pmol of each product was injected and blind collection was made with fractions of 30 s. After lyophilisation, derivatization and GC–MS analysis, the collection time intervals for the blind prepurification of each product were determined: 5.3 to 7.6 min for 5-OHDHT, 7.7 to 10.2 min for the *cis* and *trans* isomers of 6-OHDHT, and 13.2 to 15.5 min for DHT. The fractions to be prepurified (supernatants and hydrolyzed pellets) were dissolved in 150 μl 25 m*M* ammonium formate aqueous solution and subsequently analyzed as described above.

2.7. GC–MS analyses

Prior to GC–MS injection, each dried sample was derivatized in a 50:50 (v/v) mixture of acetonitrile and BSTFA for 20 min at 120°C in order to form the volatile trimethylsilyl (TMS) derivatives. GC–MS analyses were performed on a HP 5890 Series II gas chromatograph (Hewlett–Packard, Les Ulis, France) equipped with a capillary column (0.25 mm, 30 m) coated with a 0.25 μm film of methylsiloxane substituted by 5% phenylsiloxane (HP5-MS; Hewlett–Packard). Helium was used as the carrier gas. Injections (injection volume=1 μl) were performed in the splitless mode with the temperature of the injection port set at 210°C. The temperature of the GC oven was maintained at 70°C for 1 min and raised from 70°C to 300°C at a rate of 20°C/min. Detection of positive ions was provided by an HP 5972 mass detector (Hewlett–Packard) using electron impact ionization. MS analysis were carried out in the single ion monitoring mode. The retention times of the compounds and the recorded ions were respectively the following:

DHT–TMS: 6.82 min; m/z 257.1 [M + 2TMS − CH_3], 271.1 [M + 2TMS]

DHT–d_4–TMS: 6.81 min, m/z 261.1 [M + 2TMS − CH_3], 275.1 [M + 2TMS].

5–OHDHT–TMS, *cis* and *trans* 6–OHDHT–TMS: 7.76 min; m/z 345.1 [M+3TMS − CH_3], 360.1[M + 3TMS]

5–OHDHT–d_4–TMS: 7.75 min, m/z 349.1 [M + 3TMS − CH_3], 364.1 [M + 3TMS].

3. Results and discussion

E. coli endonuclease III is a repair enzyme involved in the BER pathway [10]. It has been

shown to possess both DNA N-glycosylase and abasic site nicking activities [11]. The corresponding gene is the *n*th gene which has been cloned [12], and the crystallographic structure of the protein is now available [13]. Numerous studies aimed at delineating the substrate specificity of the enzyme have been performed [1,2,14–16]. It was found that endonuclease III is able to excise several thymine and cytosine derived lesions. This particularly applied to modified residues which have lost their aromaticity. DHT is one of the lesions that are included in the substrate specificity spectrum of the enzyme. However, the situation remains confusing for 5-OHDHT and 6-OHDHT, since no structural assignment of the latter compounds was achieved using the earlier GC–MS analysis [17]. Our aim was to determine if the *cis* and *trans* isomers of 6-OHDHT were other modified thymine residues that could be recognized by endonuclease III. For this purpose, it was a requisite to develop an assay capable of singling out the 6-OHDHT isomers and 5-OHDHT.

3.1. GC–MS analyses of 5-OHDHT and cis and trans isomers of 6-OHDHT

5-OHDHT and the *cis* and *trans* isomers of 6-OHDHT were synthesized in order to determine their chromatographic properties. In addition, the isotopically labeled analogs of 5-OHDHT and DHT were synthesized for GC–MS quantitation using the isotope dilution technique [18]. The isotopically labeled analogs of the *cis* and *trans* isomers of 6-OHDHT were not synthesized due to their instability as they readily decompose into thymine through a dehydration process.

Tentative electron impact mass spectra of 5-OHDHT–TMS, *cis* and *trans* 6-OHDHT–TMS have been previously reported through the GC–MS analysis of a mixture of radiation-induced decomposition products of thymine [17]. However, the presently used GC–MS analytical system was found to be ineffective in separating the TMS derivatives of the three radiation-induced decomposition products that coeluted in a single peak. As the ion fragmentation of the three DHT derivatives is similar (Fig. 1), it was not possible to carry out individual measurement of the products using the GC–MS analysis. Similar results were obtained irrespective of the conditions of derivatization which were applied, including the use of either BSTFA or MtBSTFA.

It should be noted here that the GC–MS analysis of the *cis* and *trans* isomers of 6-OHDHT induced a partial decomposition of these products into thymine. This is likely to happen during either the silylation reaction or during the injection. In order to minimize the thermal decomposition of the latter compounds, the temperature of the injection port was lowered by 50°C with respect to usual conditions of analysis.

3.2. HPLC prepurification of DHT compounds

The necessity to prepurify targeted oxidized DNA bases has recently been highlighted in order to avoid artifactual oxidation of the overwhelming normal DNA bases during the silylation step [19–21]. In the present experiments, the latter side reaction is not expected to occur, since 5-OHDHT, 6-OHDHT and DHT are not oxidation products of thymine. Nevertheless, prepurification was found to be necessary in order to separate 5-OHDHT from the *cis* and *trans* isomers of 6-OHDHT. The HPLC separation of the latter compounds and thymine on an Hypersil ODS column is illustrated in Fig. 2. The mixture of the four compounds is well resolved using 25 m*M* ammonium formate aqueous solution as the isocratic eluent. However, the two isomers of 6-OHDHT exhibit very close retention times [22].

Furthermore, the prepurification step was shown to induce a significant gain in the sensitivity of the GC–MS analysis: HPLC prepurification of 5-OHDHT and DHT provided GC–MS elution profiles with lower background levels. Therefore, integration of peaks was more accurate in the case of small amounts of analyzed products.

The advantages of applying the prepurification and isotope dilution techniques for GC–MS analysis of modified DNA bases are summarized in Table 1.

3.3. Application of the optimized GC–MS assay to the E. coli endonuclease III-mediated release of DHT derivatives from γ-irradiated DNA

Cysteine is a radiation modifier which enhances the formation of 5-OHDHT, DHT and *cis* and *trans* 6-OHDHT upon exposure of thymine to γ-irradiation in oxygen-free aqueous solutions [6]. A similar

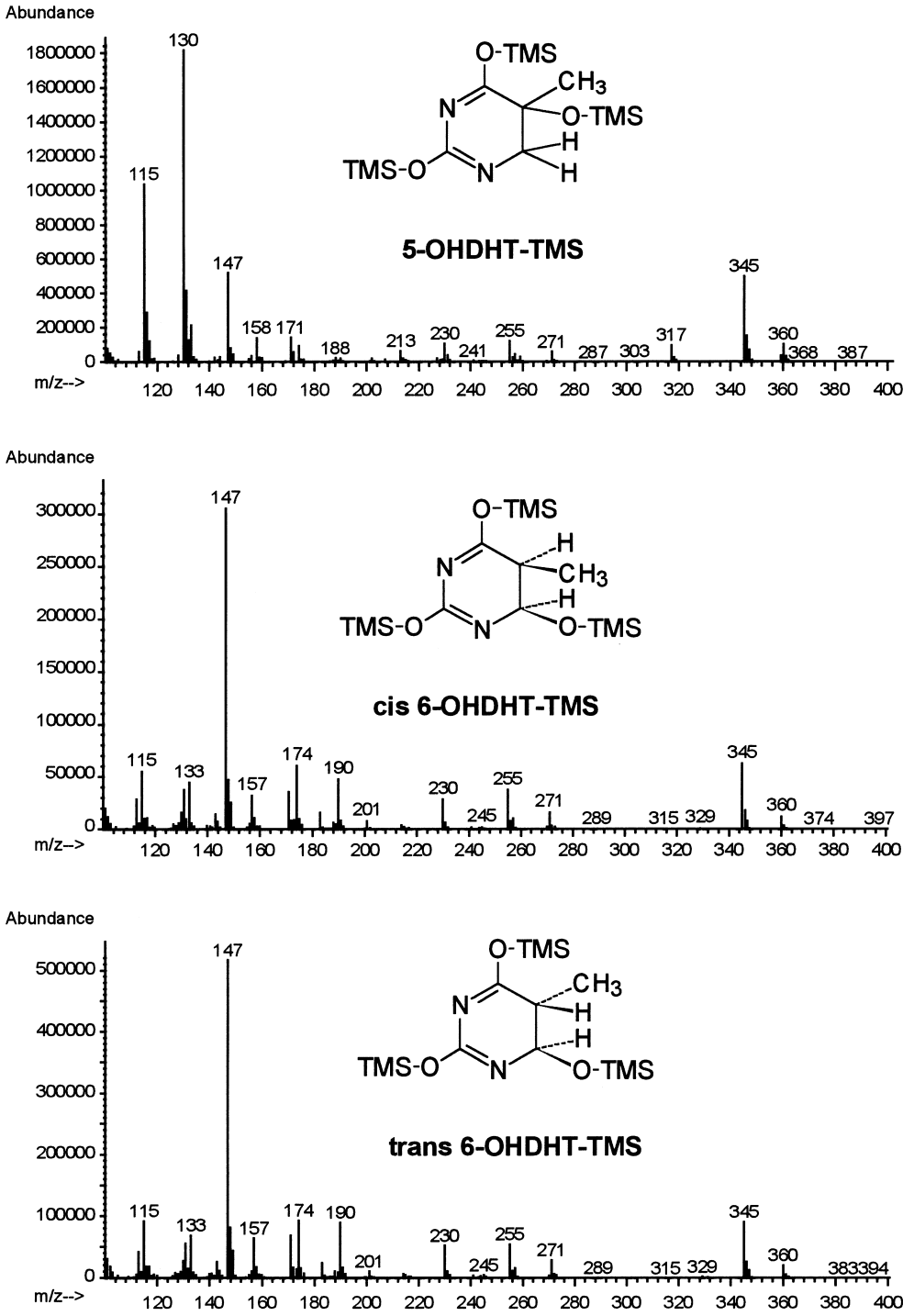

Fig. 1. Electron impact ionization mode mass spectra of the TMS derivatives of the 5,6-dihydrothymine compounds: 5-OHDHT–TMS, *cis* 6-OHDHT–TMS and *trans* 6-OHDHT–TMS.

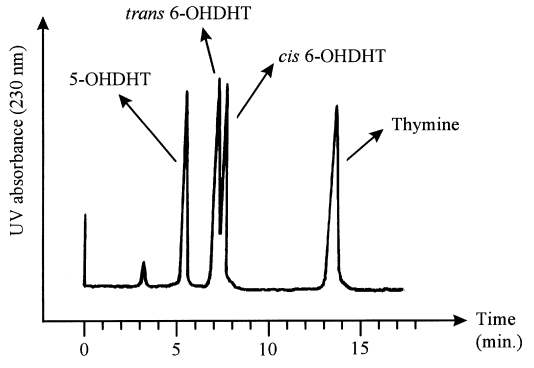

Fig. 2. HPLC elution profile of 5-OHDHT, *cis* and *trans* isomers of 6-OHDHT and thymine. Eluent: 25 m*M* ammonium formate, flow-rate: 1.0 ml/min, column: Hypersil C_{18} 5 μm. Detection by UV absorbance at $\lambda = 230$ nm.

approach was used to generate the four latter DHT derivatives within isolated DNA. It should be added that cysteine protects the DNA backbone against strand break formation through the H-donation mechanism. This is expected to favor the enzymatic activity of *E. coli* endonuclease III which requires a double-stranded DNA structure [23].

DNA was exposed to γ-rays for different periods and then was incubated with *E. coli* endonuclease III. After precipitation, the DNA was hydrolyzed in order to determine the level of radiation-induced 5-OHDHT and DHT (Fig. 3). Experiments have been made to confirm that 5-OHDHT and DHT are sufficiently stable upon formic acid treatment (unpublished results). In contrast, the *cis* and *trans* isomers of 6-OHDHT were found to quantatively decompose into thymine under acidic hydrolysis conditions.

The present results confirmed that 5-OHDHT and DHT are substrates for endonuclease III, as shown in Fig. 4. Our results are in agreement with others [1] showing that endonuclease III is an enzyme with a specific activity rather low for DHT and 5-OHDHT by comparison with other repair enzymes, i.e., the specific activity of Fpg for substrates such as 8-oxo-

Table 1
Requested analytical conditions for an accurate GC–MS measurement of DNA base lesions

Step of the assay	Possible problem	Solution
Acid hydrolysis	Stability of the lesion	Internal standard
Prepurification	Loss of product during blind collection	Internal standard
Derivatization	Stability of the lesion	Internal standard
	Autoxidation[a]	Prepurification
GC–MS analysis	GC separation	Prepurification
	Contamination by other products	Prepurification

[a] This would apply to the search of oxidative thymine lesions such as 5,6-dihydroxy-5,6-dihydrothymine.

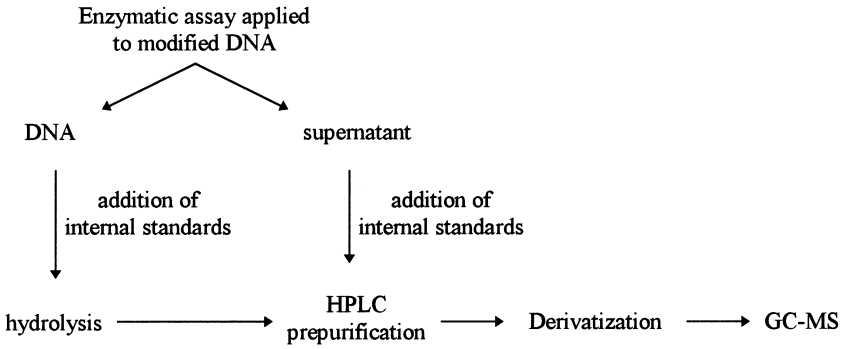

Fig. 3. Scheme of the enzymatic assay.

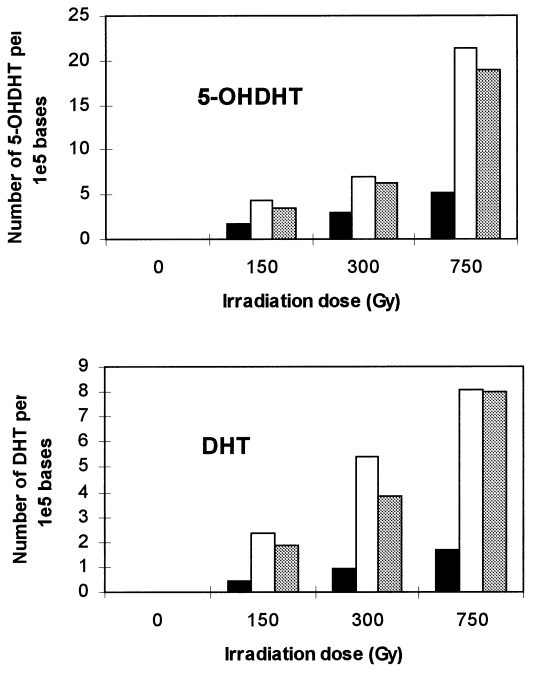

Fig. 4. Excision of 5-OHDHT and DHT from γ-irradiated DNA by *Escherichia coli* endonuclease III (37°C, 40 min) as determined by HPLC–GC–MS measurement. (black bars) Supernatant after endonuclease III treatment. (white bars) Hydrolysis of DNA without endonuclease III treatment. (grey bars) Hydrolysis of DNA after endonuclease III treatment.

7,8-dihydroguanine [5]. Excision of each of the modified bases is dependent on the substrate concentration (irradiation dose) in a linear way. This suggests that our reaction conditions are situated in the linear part of the Michaelis–Menten kinetic curve. It may be added that the concentrations of DHT and 5-OHDHT (between 50 n*M* and 350 n*M*) are lower than the K_M values of endonuclease III for these substrates.

It is also likely that 6-OHDHT should be a substrate for endonuclease III since it was shown that a related modified base, 6-hydroxy-5,6-dihydrocytosine, is released from UV-irradiated DNA upon incubation with *E. coli* endonuclease III [24]. However, the presence of either isomer of *cis* and *trans* 6-OHDHT was not detected in the supernatants of the endonuclease III-mediated digestion of γ-irradiated DNA. This may be due to lower rates of radiation-induced formation of the latter products whose limit of detection by the GC–MS analysis is higher than for DHT and 5-OHDHT.

4. Conclusion

Prepurification and the use of isotopically labeled internal standards, provide significant improvements in the GC–MS method in terms of selectivity and quantitative measurement. Interesting application of the assay deals with the measurement of the repair of 5-OHDHT and DHT by endonuclease III in a sample of modified DNA. In particular, the enzymatic release of 5-OHDHT was specifically measured without any contribution of 6-OHDHT. It should be added that the optimized HPLC–GC–MS assay may be applied to the evaluation of other DNA repair glycosylases activities and other potential DNA substrates for the latter enzymes.

Acknowledgements

We are grateful to Dr. Serge Boiteux for the gift of endonuclease III. The project was partly supported by grants from the French Ministry of Science and Research (ACC-SV N°8-MESR 1995) and Electricité de France (Comité de Radioprotection).

References

[1] M. Dizdaroglu, J. Laval, S. Boiteux, Biochemistry 32 (1993) 12105.
[2] J.R. Wagner, B.C. Blount, M. Weinfield, Anal. Biochem. 233 (1996) 76.
[3] S. Boiteux, E. Gajewski, J. Laval, M. Dizdaroglu, Biochemistry 31 (1992) 106.
[4] A. Karakaya, P. Jaruga, V.A. Bohr, A.P. Grollman, M. Dizdaroglu, Nucleic Acids Res. 25 (1997) 474.
[5] J.L. Ravanat, M. Berger, S. Boiteux, J. Laval, J. Cadet, J. Chim. Phys. 90 (1992) 871.
[6] J. Cadet, M. Berger, P. Demonchaux, J. Lhomme, Radiat. Phys. Chem. 32 (1988) 197.
[7] J. Cadet, J. Ulrich, R. Téoule, Tetrahedron 31 (1975) 2057.
[8] J. Cadet, R. Ducolomb, R. Téoule, Tetrahedron 33 (1977) 1603.
[9] J. Cadet, R. Téoule, Int. J. Appl. Rad. Isotop. 22 (1971) 273.
[10] L. Breimer, T. Lindhal, Nucleic Acids Res. 8 (1980) 6199.
[11] Y. Kow, S.S. Wallace, Biochemistry 26 (1987) 8200.

[12] H. Asahara, P.M. Wistort, J.F. Bank, R.H. Bakerian, R.P. Cunningham, Biochemistry 28 (1989) 4444.
[13] C.-F. Kuo, D.E. McRee, C.L. Fisher, S.F. O'Handley, R.P. Cunningham, J.A. Tainer, Science 258 (1992) 434.
[14] M.F. Laspia, S.S. Wallace, J. Bacteriol. 170 (1988) 3359.
[15] Z. Hatahet, Y.W. Kow, A.A. Purmal, R.P. Cunningham, S.S. Wallace, J. Biol. Chem. 269 (1994) 18814.
[16] D. Wang, J.M. Essigmann, Biochemistry 36 (1997) 8628.
[17] M. Dizdaroglu, J. Chromatogr. 295 (1984) 103.
[18] O.A. Mamer, J. Chromatogr. B 682 (1996) 182.
[19] T. Douki, T. Delatour, F. Bianchiní, J. Cadet, Carcinogenesis 17 (1996) 347.
[20] J.L. Ravanat, R.J. Turesky, E. Gremaud, L.J. Trudel, R.H. Stadler, Chem. Res. Toxicol. 8 (1995) 1039.
[21] J. Cadet, T. Douki, J.L. Ravanat, Environ. Health Perspect. 105 (1997) 1034.
[22] J. Cadet, M. Berger, L. Voituriez, J. Chromatogr. 238 (1982) 488.
[23] S. Boiteux, J. Photochem. Photobiol. B Biol. 19 (1993) 87.
[24] R.J. Boorstein, T.P. Hilbert, J. Cadet, R.P. Cunningham, G.W. Teebor, Biochemistry 28 (1989) 6164.