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Measurement of formamidopyrimidines in DNA

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ABSTRACT

Formamidopyrimidines, 4,6-diamino-5-formamidopyrimidine (FapyAde) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), are among major lesions in DNA generated by hydroxyl radical attack, UV radiation, or photosensitization in vitro and in vivo. FapyAde and FapyGua exist in living cells at detectable background levels and are formed by exposure of cells to DNA-damaging agents. Numerous prokaryotic and eukaryotic DNA glycosylases exist for the repair of formamidopyrimidines by base excision repair pathways in cells, indicating their biological significance. Moreover, they are premutagenic lesions, albeit to different extents, revealing a possible role in disease processes. Methodologies using gas chromatography/mass spectrometry (GC/MS) with capillary columns have been developed to accurately measure FapyAde and FapyGua in DNA in vitro and in vivo. Stable isotope-labeled analogues of these compounds have been synthesized and are commercially available to be used as internal standards for accurate quantification. GC/MS with isotope dilution provides excellent sensitivity and selectivity for positive identification and accurate quantification, and has widely been applied in the past to the measurement of formamidopyrimidines under numerous experimental conditions. This paper reports on the details of this GC/MS methodology.

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Introduction

Oxygen-derived species including free radicals are produced in living cells by normal metabolism and by exogenous sources such as ionizing radiations and redox-cycling drugs (reviewed in [1]). Of the free radicals, the highly reactive hydroxyl radical (·OH) reacts with DNA and other biological molecules near or at diffusion-controlled reaction rates (reviewed in [2]). Reactions of ·OH with DNA constituents result in formation of a plethora of products that include modified bases and sugars, 8,5′-cyclopurine 2′-deoxynucleosides, base-free sites, strand breaks, clustered lesions, and DNA–protein cross-links (reviewed in [3–6]). In addition, UV radiation, singlet oxygen, and photosensitization generate some of these compounds in DNA (reviewed in [6,7]). This type of damage, also called oxidatively induced DNA damage, is subject to repair in living cells by a variety of elaborate mechanisms (reviewed in [8]). Unrepaired and accumulated DNA damage may lead to mutagenesis, carcinogenesis, aging, and other biological effects (reviewed in [8,9]).

Of the numerous products of oxidatively induced damage to DNA, formamidopyrimidines, i.e., 4,6-diamino-5-formamidopyrimidine (FapyAde) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine (FapyGua), are unique in that they are derived from adenine and guanine, respectively, and contain an open imidazole ring. Unlike other pyrimidines, they are connected to the sugar moiety of DNA through the amino group attached to C6 of the pyrimidine ring (reviewed in [10]). In early studies, formamidopyrimidines have been identified as ionizing radiation-induced products of purines [11–18]. Subsequent studies demonstrated that these compounds exist in living cells at detectable background levels, and are generated by ·OH, UV radiation, or photosensitization in vitro and in vivo under a variety of conditions (reviewed in [10]). Formamidopyrimidines are produced together with 8-hydroxypurines [8-hydroxyadenine (8-OH-Ade) and 8-hydroxyguanine (8-OH-Gua)] from a common precursor on one-electron reduction and one-electron oxidation, respectively. The details of mechanisms of formation of formamidopyrimidines along with those of 8-hydroxypurines can be found in a recent review [10]. The background levels and yields of these lesions are comparable to those of other major DNA lesions. Various prokaryotic and eukaryotic DNA glycosylases efficiently remove FapyAde and FapyGua from DNA, indicating their biological importance. FapyGua and FapyAde possess mutagenic properties, albeit to different extents, pointing to a possible role in disease processes (reviewed in [10]).

Abbreviations: FapyAde, 4,6-diamino-5-formamidopyrimidine; FapyGua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; GC/MS, gas chromatography/mass spectrometry; ·OH, hydroxyl radical; 8-OH-Gua, 8-hydroxyguanine; 8-OH-Ade, 8-hydroxyadenine; HPLC-ECD, high-performance liquid chromatography with electrochemical detection; EI, electron ionization; Me_3Si, trimethylsilyl; SIM, selected-ion monitoring; ESCODD, European Standards Committee for Oxidative DNA Damage; 8-OH-dG, 8-hydroxy-2′-deoxyguanosine; LC/MS/MS, HPLC-tandem mass spectrometry; Fpg, *E. coli* formamidopyrimidine DNA glycosylase; FapydA, 4-amino-5-formylamino-6-(2′-deoxyribosyl)aminopyrimidine; FapydG, 2-amino-4-hydroxy-5-formylamino-6-(2′-deoxyribosyl)aminopyrimidine; FMF, familial Mediterranean fever; AP site, base-free site.

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Accurate measurement of FapyAde and FapyGua in vitro and in vivo is essential for an understanding of their formation mechanisms, cellular repair, and biological effects. This paper reviews the measurement of formamidopyrimidines in DNA by various techniques with the emphasis given to those that employ mass spectrometry.

Measurement of formamidopyrimidines

Various chromatographic techniques have been described to isolate and characterize formamidopyrimidines, including thin-layer chromatography, paper chromatography, ion-exchange chromatography, high-performance liquid chromatography (HPLC), and HPLC with electrochemical detection (HPLC-ECD) [11–19]. These techniques provide no structural evidence for analytes and generally measure a single product at a time. In contrast, the techniques that use mass spectrometry provide structural evidence, thus unequivocal identification and accurate quantification of constituents of nucleic acids [20]. The use of mass spectrometry with electron ionization (EI) coupled to gas chromatography (GC/MS) has been introduced for the measurement in DNA of formamidopyrimidines and other oxidatively induced products as free bases or as nucleosides [21,22]. Subsequently, the use of isotope-dilution mass spectrometry has been employed for the improved accurate quantification of FapyAde and FapyGua, and other DNA lesions [23]. The advantage of GC/MS over other techniques is that numerous modified bases from all four DNA bases can be measured with structural evidence in a single DNA sample and in a single analysis. A wealth of data demonstrated the advantage of measuring multiple DNA products, as the measurement of a single product such as 8-OH-Gua may be misleading for use as a biomarker and/or for determination of the rate of DNA damage (reviewed in [1,6]).

For GC/MS analysis, DNA has been hydrolyzed to bases by acid or to nucleosides by a combination of endo- and exonucleases plus a phosphatase, followed by derivatization and analysis. Trimethylsilylation has been used as the method of choice for derivatization [24]. Trimethylsilyl (Me_3Si) derivatives of formamidopyrimidines and their nucleosides have provided mass spectra with intense characteristic ions to be used for unequivocal identification and quantification [21,22]. Stable isotope (^{13}C and ^{15}N)-labeled analogues of FapyAde and FapyGua have been synthesized [25], and used as internal standards for this purpose. Cambridge Isotope Laboratories (Cambridge, MA) also synthesized the ^{13}C- and ^{15}N-labeled analogues of FapyAde and FapyGua, and other modified bases and nucleosides. Thus, they are now commercially available. During GC/MS analysis, selected-ion monitoring (SIM) is used for selective and sensitive identification of analyzed compounds. With SIM, characteristic ions of a compound and its stable isotope-labeled analog as an internal standard are monitored during a short time period where the compound elutes, achieving an excellent level of sensitivity and selectivity for GC/MS analysis [26]. Using SIM, high levels of sensitivity for the measurement of Me_3Si derivatives of modified DNA bases and nucleosides have been achieved [27].

Artifactual formation of several modified bases from corresponding intact DNA bases has been alleged to occur during derivatization at high temperatures [19]. In general, the main concern has been about the formation of additional 8-OH-Gua from intact Gua in acid hydrolysates of DNA during derivatization. This product has been regarded incorrectly as "the most important and abundant product" of oxidatively induced DNA damage, and thus measured by many laboratories, mainly using HPLC-EDC. A "European Standards Committee for Oxidative DNA Damage" (ESCODD) has been established to resolve the problems associated with the measurement of 8-OH-Gua by different techniques and methodologies [28]. Trials by EDCODD showed significant variations in the levels of 8-OH-Gua and its nucleoside 8-hydroxy-2′-deoxyguanosine (8-OH-dG) between laboratories and techniques used for measurement [29–32]. However, no attention has been directed to other products of oxidatively induced DNA damage that are formed in DNA as abundantly as 8-OH-Gua in most cases, and may be as important as 8-OH-Gua in terms of biological effects. Prevention of artifact formation not only in terms of 8-OH-Gua but also in terms of other products has been described by various laboratories (see, e.g., [33,34]). Facts about the artifacts in GC/MS analysis have also been discussed in detail [27,35,36].

In the case of formamidopyrimidines, a complete destruction of FapyAde and FapyGua during hydrolysis with formic acid at high temperature (140°C) prior to GC/MS analysis has been reported [37]. Instead, hydrofluoric acid in pyridine has been used for hydrolysis. This method has been applied to the measurement of FapyGua only in DNA in aqueous solution exposed to ionizing radiation under various gassing conditions. Another study contrasted this finding and showed the destruction of these compounds to some extent, but not to completion, depending on hydrolysis conditions [38]. The complete destruction of formamidopyrimidines by hot acid is in contrast to the fact that the original paper on the use of GC/MS for the measurement of FapyAde and FapyGua [21] and subsequent papers (see, e.g., [27]) demonstrated successful use of formic acid for hydrolysis of formamidopyrimidines prior to GC/MS analysis. Differences in experimental conditions such as removing the acid in vacuum [37] vs by lyophilization [21] may have caused these discrepancies. It should be pointed out that the use of stable isotope-labeled analogues of FapyAde and FapyGua as internal standards may compensate for any loss during acidic hydrolysis or any other step of analysis.

Alternative methodologies have been developed for the measurement of formamidopyrimidines. Thus, a GC/MS methodology has been described that uses hydrolysis of DNA to nucleotides by nuclease P1, followed by formic acid hydrolysis of nucleotides at room temperature to FapyAde and FapyGua (and other modified bases), trimethylsilylation, and GC/MS analysis [39,40]. Another methodology added a purification step by HPLC after nuclease P1 and formic acid hydrolysis, but prior to derivatization [41]. This HPLC-GC/MS methodology has been applied to the measurement of FapyAde and FapyGua in human monocytes exposed to γ-radiation or UV radiation. The detection of formamidopyrimidines by this method required approximately 350 μg of DNA per analysis. The limit of sensitivity has been found to be 3 FapyGua/10^6 DNA bases. The disadvantage of this method has been the failure of the detection of FapyAde. Only the use of very high radiation doses between 250 and 1000 Gy has made it possible to detect formation of FapyGua in DNA of monocytes. The detection of FapyAde or FapyGua in unirradiated cells has not been possible. The same approach has also been used in another instance to measure formamidopyrimidines in isolated and cellular DNA [42]. In addition to GC/MS, HPLC-tandem mass spectrometry (LC/MS/MS) with isotope dilution has been applied to the detection of FapyAde and FapyGua, following hydrolysis of DNA to nucleotides and then hydrolysis by formic acid [42,43]. The levels of these products measured by HPLC-GC/MS and LC/MS/MS correlated well with one another. However, it has been stated that the time-consuming HPLC-GC/MS assay was not sensitive enough to detect these compounds in cellular DNA. The detection thresholds of LC/MS/MS for pure FapyAde and FapyGua have been found to be 5 and 60 fmol, respectively [42,43]. These values are much higher than that (~1 fmol for both) achieved by GC/MS [27]. LC/MS/MS with formic acid hydrolysis of nucleotides released from DNA by nuclease P1 has also been used for detection of formamidopyrimidines in cells exposed to ionizing radiation or high linear-energy transfer particles [44]. High doses up to 1000 Gy have been used to be able to detect formation of FapyAde and FapyGua in cells. The same methodology has been applied in the case of exposure of isolated DNA to H_2O_2 in the presence of Cu ions [45]. Attempts have also been made to apply HPLC-ECD to the measurement of FapyAde and FapyGua; however, this has not been achieved as these compounds eluted in the void volume [46]. HPLC-ECD has been used almost exclusively for the measurement of 8-hydroxy-2′-deoxyguanosine only [47].

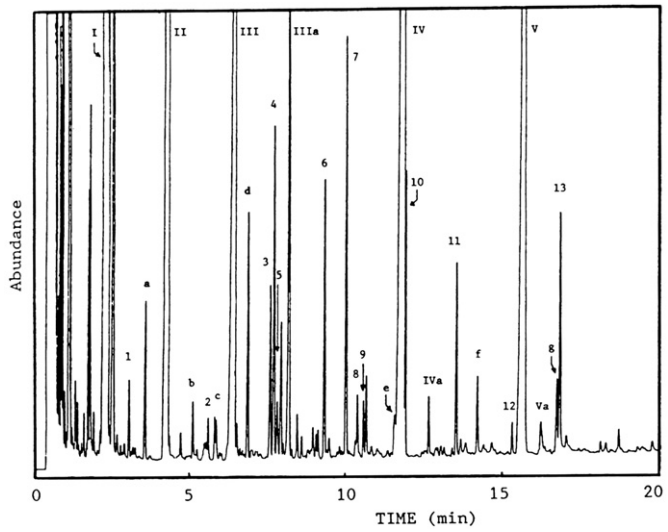


Fig. 1. GC separation of a trimethylsilylated acid hydrolysate of DNA exposed to ionizing radiation (from [21]). Peaks 10 and 12 represent the Me_3Si derivatives of FapyAde and FapyGua, respectively. The experimental details and peak identification can be found in the original paper.

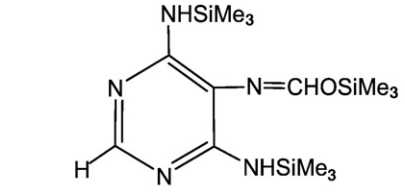
A fluorescence assay has been developed to detect and quantify FapyGua along with 8-OH-Gua in DNA [48]. A spermine derivative has been used for tagging the oxidation products of these lesions in the presence of Na_2IrCl_6, followed by the use of amplex red oxidized to fluorescent resorufin for quantification. When $K_3Fe(CN)_6$ has been used as the oxidant, 8-OH-Gua has been trapped only. The amounts of FapyGua and 8-OH-Gua have been determined in γ-irradiated DNA. This method, however, could not detect FapyAde or 8-OH-Ade because of the lack of formation of adducts with the spermine derivative. This method may be applicable in the future to the measurement of FapyGua and perhaps also other products in DNA.

A recent paper reported the measurement of FapyGua along with 8-OH-Gua and 8-OH-dG in human urine by LC/MS/MS [49]. ^{13}C- and ^{15}N-labeled analogues of these compounds have been used as internal standards. This method has been tested in urine samples from smokers and nonsmokers with chronic kidney disease to assess the level of oxidative damage to nucleic acids. It has been found to be suitable for detection of significantly higher levels of FapyGua and other lesions in smokers when compared to nonsmokers. This method may be useful for the measurement of oxidative stress in humans in terms of oxidatively induced DNA lesions including FapyGua in relation to various disease states.

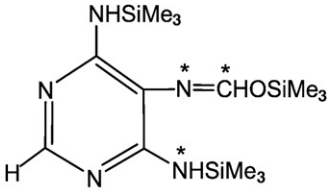
Recently, we introduced a modified version of the GC/MS methodology that uses the DNA repair enzyme, *E. coli* formamidopyrimidine DNA glycosylase (Fpg), instead of acid to remove FapyAde and FapyGua along with 8-OH-Gua from DNA prior to GC/MS analysis [27,50–52]. This concept resulted from the application of GC/MS for the first time to the determination of the substrate specificity of a DNA glycosylase, namely Fpg using DNA containing multiple lesions [53]. Subsequently, GC/MS has been applied to the determination of substrate specificities and excision kinetics of numerous other DNA glycosylases (reviewed in [54]). Fpg is specific for FapyAde, FapyGua, and 8-OH-Gua, and efficiently removes them from DNA with similar excision kinetics [53,55]. This modified GC/MS methodology has the advantage of preventing possible artifacts that may occur during acid hydrolysis of DNA and subsequent derivatization of hydrolysates prior to GC/MS analysis, because no intact Ade and Gua are released from DNA by Fpg. The identification and quantification of levels such as less than 1 formamidopyrimidine/10^6 DNA bases have been achieved. In recent years, this methodology has been successfully applied to the measurement of formamidopyrimidines in isolated or cellular DNA [10,56–60].

Principles of the methodology

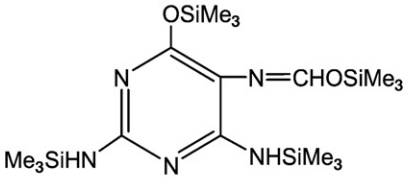
The GC/MS methodology consists of several steps that include the release of FapyAde and FapyGua from DNA by hydrolysis, derivatization of hydrolysates, gas chromatographic separation, and mass spectrometric identification and quantification. For GC/MS analysis, FapyAde and FapyGua must be removed from DNA as free bases in the



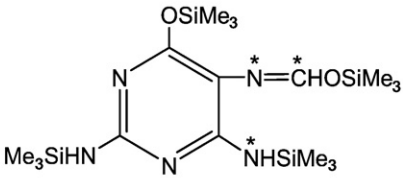
m/z 369 ($M^{+\bullet}$)
m/z 368 [$(M - {}^{\bullet}H)^+$]
m/z 354 [$(M - {}^{\bullet}CH_3)^+$]
m/z 280 [$(M - {}^{\bullet}OSiCH_3)^+$]



m/z 372 ($M^{+\bullet}$)
m/z 371 [$(M - {}^{\bullet}H)^+$]
m/z 357 [$(M - {}^{\bullet}CH_3)^+$]
m/z 283 [$(M - {}^{\bullet}OSiCH_3)^+$]



m/z 457 ($M^{+\bullet}$)
m/z 442 [$(M - {}^{\bullet}CH_3)^+$]
m/z 368 [$(M - {}^{\bullet}OSiCH_3)^+$]



m/z 460 ($M^{+\bullet}$)
m/z 445 [$(M - {}^{\bullet}CH_3)^+$]
m/z 371 [$(M - {}^{\bullet}OSiCH_3)^+$]

Fig. 2. Structures of the Me_3Si derivatives of FapyAde, FapyAde-$^{13}C,^{15}N_2$, FapyGua, and FapyGua-$^{13}C,^{15}N_2$ with characteristic ions. Stars denote ^{13}C and ^{15}N atoms.

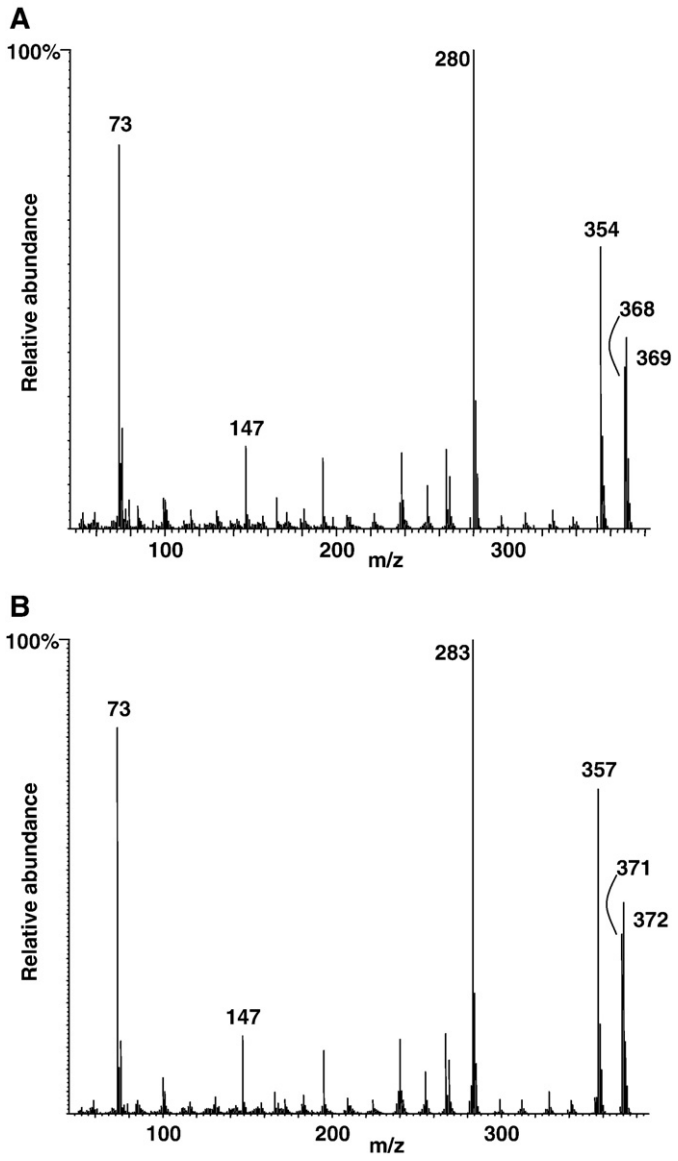


Fig. 3. EI-mass spectra of the Me_3Si derivatives of FapyAde (A) and FapyAde-$^{13}C,^{15}N_2$ (B).

first step. This is achieved by acidic hydrolysis. Formic acid has been used for this purpose in many studies in the past. As an alternative, the use of the *E. coli* DNA repair enzyme, Fpg, has been suggested for the release of formamidopyrimidines from DNA because of its specificity for these compounds (see above). The released bases must be converted to volatile derivatives for GC-separation. This is achieved by trimethylsilylation to obtain volatile Me_3Si derivatives of bases and nucleosides. Hydrolysates resulting from acid or Fpg-hydrolysis are dried by lyophilization and then trimethylsilylated and analyzed by GC/MS. Fused silica capillary columns are used for this purpose, offering excellent properties for the separation of Me_3Si derivatives of DNA bases. Such columns permit the measurement of retention times with great accuracy and precision, facilitating unequivocal identification, in addition to the simultaneous measurement of masses of characteristic ions by the mass spectrometer. EI-mass spectra of the Me_3Si derivatives of FapyAde, FapyGua, and other products provide characteristic mass spectra for unequivocal identification [21,22,61]. Quantitative measurement of modified bases is achieved by isotope-dilution mass spectrometry using their stable isotope-labeled analogues as internal standards that are added to DNA samples prior to hydrolysis [23].

Fig. 1 illustrates a typical GC separation of trimethylsilylated acid hydrolysate of DNA containing multiple lesions. This chromatogram is from the original paper that introduced the GC/MS analysis of oxidatively induced base lesions in DNA [21]. As Fig. 1 shows, the products of four bases (peaks 1–13) are separated from one another and from four intact bases (peaks II–V). The peak assignments can be found in the original paper. Peaks 10 and 12 represent the Me_3Si derivatives of FapyAde and FapyGua, respectively. Fig. 2 illustrates the structures of these compounds with characteristic ions and those of their ^{13}C- and ^{15}N-labeled analogues used as internal standards for quantification. Figs. 3 and 4 illustrate the mass spectra, which have recently been recorded in our laboratory. Figs. 3A and 4A are essentially identical to the mass spectra published in the original paper [21], clearly indicating the reproducibility of the results. The mass spectra of the Me_3Si derivatives of FapyAde-$^{13}C,^{15}N_2$ and FapyGua-$^{13}C,^{15}N_2$ are shown in Figs. 3B and 4B, respectively. These mass spectra are dominated by an abundant molecular ion ($M^{+\cdot}$) and an abundant $(M - {}^{\cdot}CH_3)^+$ ion, which results from loss of a methyl radical from $M^{+\cdot}$. In the case of FapyAde and FapyAde-$^{13}C,^{15}N_2$, an abundant $(M - {}^{\cdot}H)^+$ ion (*m/z* 368 and *m/z* 371, respectively) is also observed, which is due to loss of an H atom from $M^{+\cdot}$. As expected, the masses of $M^{+\cdot}$, $(M - {}^{\cdot}H)^+$, and $(M - {}^{\cdot}CH_3)^+$ ions in the mass spectra in Figs. 3A and 4A are shifted by 3 Da to higher masses in Figs. 3B and 4B, due to the presence of one ^{13}C plus two ^{15}N atoms in the

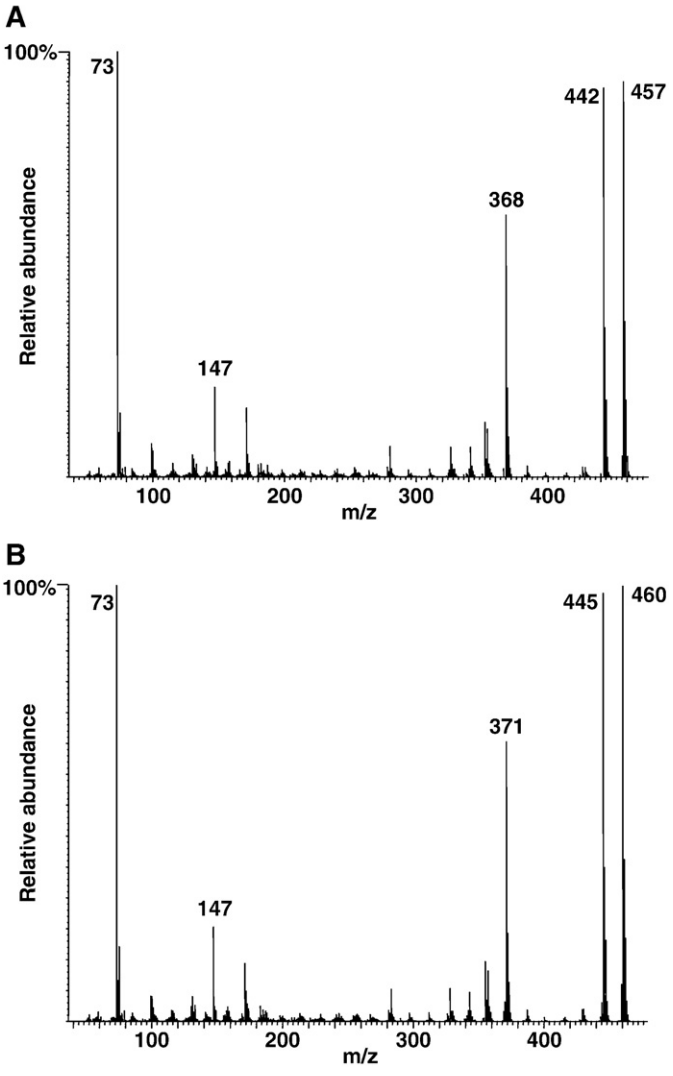


Fig. 4. EI-mass spectra of the Me_3Si derivatives of FapyGua (A) and FapyGua-$^{13}C,^{15}N_2$ (B).

corresponding molecules. The abundant ions at *m/z* 280, 283, 368, and 371 in the mass spectra result from loss of $^{\cdot}OSiCH_3$ from $M^{+\cdot}$ [21]. The ions at *m/z* 73 and 147 are not characteristic and commonly appear in mass spectra of trimethylsilylated modified or intact DNA bases, thus serving no diagnostic purpose [21,62].

The GC/MS analysis of the 2′-deoxyribonucleside forms of FapyAde and FapyGua [4-amino-5-formylamino-6-(2′-deoxyribosyl)aminopyrimidine (FapydA) and 2-amino-4-hydroxy-5-formylamino-6-(2′-deoxyribosyl)aminopyrimidine (FapydG), respectively] as Me_3Si derivatives has also been described [22]. For this purpose, DNA has been hydrolyzed to 2′-deoxyribonuclesides using a combination of endo- and exonucleases plus a phosphatase. Fig. 5 illustrates the structures and fragmentation patterns of these compounds leading to characteristic ions in the mass spectra [22]. These spectra contain $M^{+\cdot}$ and $(M - {}^{\cdot}CH_3)^+$ ions with low abundance, probably due to partial loss of aromaticity because of the open imidazole ring. In contrast, Me_3Si derivatives of other modified 2′-deoxynucleosides with an intact purine ring such as 8-OH-dG and 8,5′-cyclopurine-2′-deoxynucleosides provide abundant $M^{+\cdot}$ and $(M - {}^{\cdot}CH_3)^+$ ions [22,63–65]. In the case of FapydA and FapydG, major ions with high abundance result from fragmentation of the molecules (Fig. 5). The most abundant ions result from the cleavage of the glycosidic bond with an H atom transfer followed by loss of $^{\cdot}OSiCH_3$. Fragmentation of the sugar moiety followed by loss of $^{\cdot}OSiCH_3$ also leads to abundant ions. Because of the lack of stable isotope-labeled analogues of FapydA and FapydG, this method has not been applied to the measurement of formamidopyrimidines. We hope that future work will attempt to obtain stable isotope-labeled analogues and that this method will be tested for its potential.

For the measurement by GC/MS of low levels (low fmol) of compounds in a complex mixture such as DNA hydrolysates, SIM by the mass spectrometer is used [26]. The application of SIM requires knowledge of the mass spectrum, characteristic ions, relative intensities of ions, and the GC retention time of compounds to be analyzed. Having established these parameters, several characteristic and abundant ions of a compound and its isotope-labeled analog are simultaneously recorded by the mass spectrometer during the time period, in which the compound elutes from the GC column and enters the ion source of the mass spectrometer. Identification is achieved by the signals of the monitored characteristic ions with typical relative intensities all lined up at the retention time of the compound. The measured areas of the ion-current profiles of the monitored ions of the compound and its internal standard are used to determine the level of a modified base in a given DNA sample. GC/MS with SIM provides

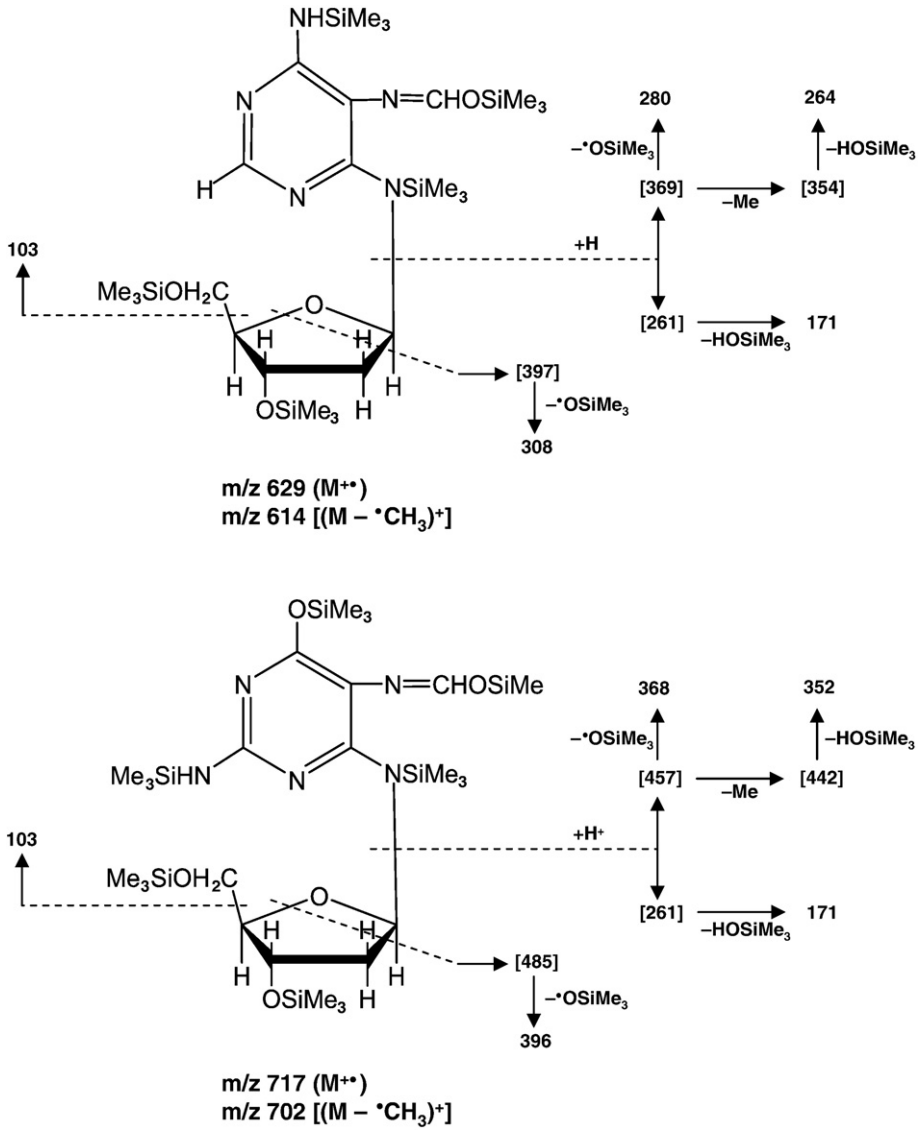


Fig. 5. Structures of the Me_3Si derivatives of FapydA and FapydG with fragmentation patterns leading to characteristic ions.

excellent selectivity and sensitivity, because a few selected characteristic ions of a compound within a short time period are monitored. The level of sensitivity of this mode for Me_3Si derivatives of modified DNA bases and nucleosides has been determined to be ~1–2 fmol of a compound eluting from the GC column [27,66]. With the split mode of injection, only ~0.5 μg of DNA is introduced onto the GC column after injection and splitting to achieve this level of sensitivity. However, it should be noted that the level of sensitivity might depend on the GC/MS instrument and the column used, and other experimental conditions.

Applications

Since its introduction, the GC/MS methodology has extensively been used for the measurement of FapyAde and FapyGua in DNA under various experimental conditions. These products have been identified and quantified in DNA in vitro, and in cultured mammalian cells and tissues at detectable background levels, after exposure to DNA-damaging agents or depending on the pathological conditions. There are multiple articles published on this subject. It is beyond the scope of this paper to review all the studies. An extensive review on formamidopyrimidines in DNA can be found elsewhere [10]. As an example, Fig. 6 demonstrates the measurement of endogenous levels of FapyAde in DNA of lymphocytes from patients with familial Mediterranean fever (FMF) [60]. This figure illustrates ion-current profiles of the $(M - {}^{\cdot}CH_3)^+$ ions of Me_3Si derivatives of FapyAde (*m/z* 354) and FapyAde-$^{13}C,^{15}N_2$ (*m/z* 357), obtained during GC/MS-SIM analysis of a DNA sample after hydrolysis with Fpg and subsequent trimethylsilylation. Fig. 7 shows the levels of FapyAde in DNA of control individuals and FMF patients, indicating the significantly greater level of this lesion in patients than in controls. This study showed significant accumulation of FapyAde and other oxidatively induced DNA lesions in polymorphonuclear leukocytes of FMF patients, pointing to a possible role of oxidatively induced DNA damage in this disease.

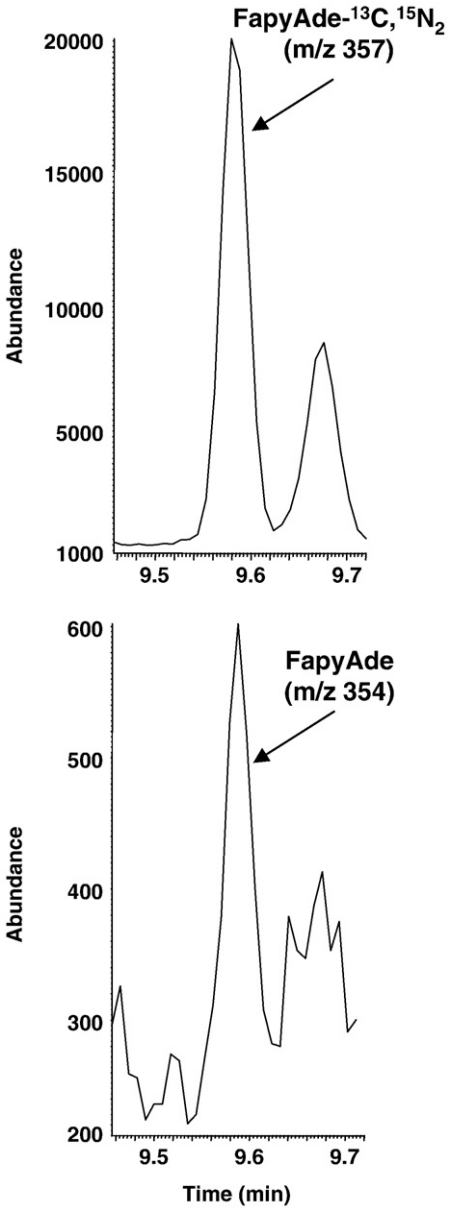


Fig. 6. Ion-current profiles of the ions at *m/z* 354 and *m/z* 357 of the Me_3Si derivatives of FapyAde and FapyAde-$^{13}C,^{15}N_2$, respectively, obtained during GC/MS-SIM analysis of a DNA sample from an FMF patient (from [60]). DNA was hydrolyzed with Fpg.

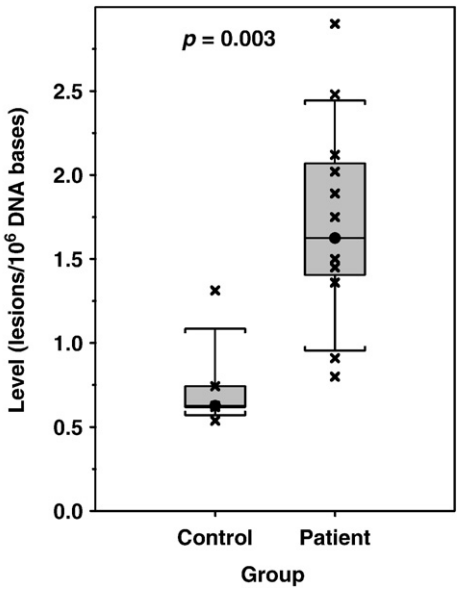


Fig. 7. Box plots of the levels of FapyAde in DNA of control individuals and FMF patients. The *P* value indicates statistical significance between the control group ($n=5$) and the patient group ($n=10$) (from [60]). The "whiskers" of the plots represent the uncertainties.

Another major application of GC/MS has been the determination of substrate specificities of DNA glycosylases in terms of FapyAde and FapyGua, and other lesions (reviewed in [54,67]). In addition, kinetic parameters of excision have been measured. DNA glycosylases are involved in the first step of the base excision repair pathway, which is highly conserved from bacteria to humans (reviewed in [8]). A DNA glycosylase hydrolyzes the *N*-glycosidic bond between a modified base and the sugar moiety of DNA, releasing the modified base and generating a base-free site (AP site). The removal of the modified base is followed by processing the AP sites by several AP endonucleases, polymerases, and ligases to fully repair damaged DNA. DNA glycosylases are highly specific and remove various types of purine- and pyrimidine-derived lesions from DNA. A plethora of modified bases can concurrently be measured in a given DNA sample by GC/MS with isotope dilution as discussed above. This ability of GC/MS permits the identification and quantification of modified bases that are excised or not excised from DNA by a given DNA glycosylase. For this purpose, damaged DNA is treated by the DNA glycosylase and then precipitated by ethanol. DNA pellets and supernatant fractions that contain released modified bases are separated and then measured by GC/MS. The details of this methodology can be found elsewhere [54]. An

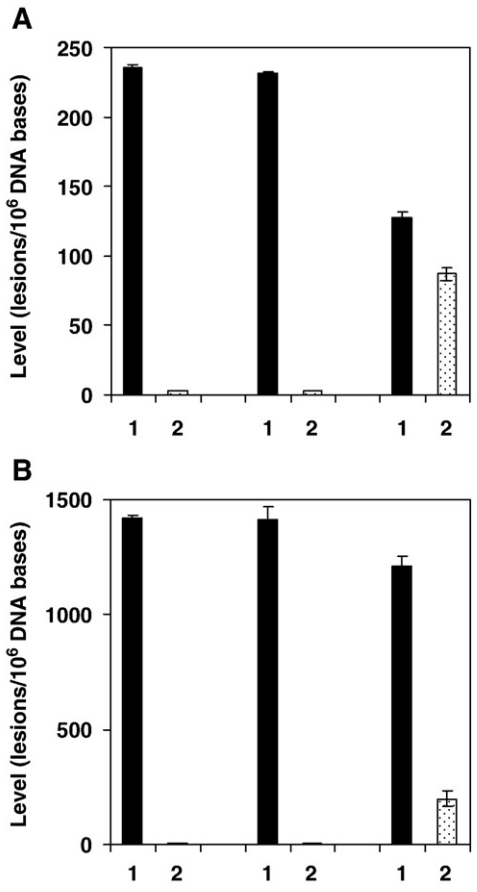


Fig. 8. Excision of FapyAde (A) and FapyGua (B) by mouse NEIL1 from DNA exposed to ionizing radiation. Dark columns (1), DNA pellets; light columns (2), supernatant fractions. Left: DNA incubated with no enzyme; middle: DNA incubated with heat-inactivated enzyme; right: DNA incubated with active enzyme (from [68]). The uncertainties are standard deviations.

example of this concept is illustrated in Fig. 8. Shown are the levels of FapyAde (A) and FapyGua (B) in DNA pellets (1) and supernatant fractions (2) of two control DNA samples and of a DNA sample treated with active mouse DNA glycosylase NEIL1 [68]. The lower levels of FapyAde and FapyGua in the DNA pellets and their appearance in the supernatant fractions of the samples treated with the active enzyme prove the specificity of mouse NEIL1 for these lesions. Human and mouse NEIL1 proteins have been determined to be specific for FapyAde and FapyGua as the major substrates when damaged DNA has been used as the substrate [68–70]. Using this concept and GC/MS methodology, the substrate specificities and excision kinetics of a variety of DNA glycosylases have been determined (reviewed in [54,67]).

Materials

The materials are as follows: dithiotreitol (99+%, Cat. No. D9779), ethanol (anhydrous, 200 proof, 99.5+%, Cat. No. 459836), formic acid (88% A.C.S reagent, Cat. No. 399388), KCl (for molecular biology, Cat. No. P9541), pyridine (anhydrous 99.8%, Cat. No. 270970), sodium phosphate (dibasic, SigmaUltra, min. 99+%, Cat. No. D-9779), sodium phosphate (monobasic, SigmaUltra, min. 99.0%, Cat. No. S8282) all from Sigma-Aldrich (St. Louis, MO); 0.5 M EDTA (pH 8.0 UltraPure, Cat. No. 15575-0380) (Gibco, Invitrogen Corporation, Grand Island, NY); 4,6-diamino-5-formamidopyrimidine-$^{13}C,^{15}N_2$ (FapyAde-$^{13}C,^{15}N_2$, Cat. No. CNLM-3752-25) and 2,6-diamino-4-hydroxy-5-formamidopyrimidine-$^{13}C,^{15}N_2$ (FapyGua-$^{13}C,^{15}N_2$, Cat. No. CNLM-3858-25) (Cambridge Isotope Laboratories, Andover, MA); *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (Cat. No. 38833) (Pierce, Rockford, IL); compressed, ultrahigh purity carrier grade nitrogen (UN1066) and helium (UN1046) (Roberts Oxygen Company, Rockville, MD).

Instrumentation

The instrumentation included a gas chromatograph/mass spectrometer (6890N Network GC System with 7683 Series Injector, coupled with 5973 Network Mass Selective Detector) (Agilent Technologies, Inc., Rockville, MD); a high-resolution fused-silica capillary column (HP-Ultra 2, i.d. 0.2 mm, film thickness 0.33 μm, Agilent Technologies, Inc., Rockville, MD); a UV-VIS spectrophotometer (DU-640, Beckman Coulter); a microcentrifuge (Mikro 22R Hettich Zentrifuge) (Andreas Hettich GmbH & Co KG, Tuttingen, Germany); a SpeedVac (SC210A SpeedVac Plus, with RVT 4104 Refrigerated Vapor Trap) (Thermo-Savant, Holbrook, NY); and a lyophilizer (Flexi-Dry μP Freez-Dryer, Kinetic Systems, Inc.) (Durham, NC).

Protocols

DNA isolation and quantification

DNA must be first isolated from cultured cells or tissues. Different types of methods for DNA isolation have been described in the literature. The advantages and shortcomings of various methods have been discussed in detail in terms of artifactual formation of DNA lesions during isolation. Most of them dealt with the artifactual formation of 8-OH-Gua and its prevention. It is beyond the scope of this article to review all the papers describing different methods of DNA isolation. Several papers can be referred to as examples [31,71–75].

As the last step of the isolation procedure, DNA is precipitated with ethanol and then washed with 70% ethanol several times. After centrifugation, the supernatant fraction is removed and DNA pellet is dried in a SpeedVac. Subsequently, DNA is dissolved in water, usually overnight at 4°C. DNA amount in the solution is measured by recording the UV spectrum of each sample using an absorption spectrophotometer between the wavelengths of 200 and 350 nm, both to ascertain the quality of DNA and to accurately determine the DNA concentration. The absorbance at 260 nm is used to measure the DNA concentration (absorbance of 1 = 50 μg of DNA/ml). Subsequently, 50-μg aliquots of DNA samples are dried in a SpeedVac under vacuum.

Hydrolysis of DNA with acid

DNA samples (50 μg) are supplemented with aliquots of FapyAde-$^{13}C,^{15}N_2$ and FapyGua-$^{13}C,^{15}N_2$ as internal standards, and dried in a SpeedVac under vacuum. The amount of the internal standards can be adjusted according to the levels of FapyAde and FapyGua. Samples are then hydrolyzed with 0.5 ml of 60% formic acid in evacuated and sealed tubes for 30 min at 140°C. The hydrolysates are frozen in liquid nitrogen and then lyophilized to dryness for 18 h. At this stage, lyophilization, not drying in a SpeedVac, is essential to obtain efficient removal of acid at low freezing temperature. Removal of acid in a SpeedVac at room temperature may cause formation of artifacts. Lyophilization is also important for achieving sufficient dryness of samples for derivatization. Traces of water can react with derivatization reagents. A lyophilizer equipped with a −90°C cooling system should be used.

Hydrolysis of DNA with Fpg

DNA samples (50 μg) are dissolved in 50 μl of an incubation buffer consisting of 50 mM Na_2HPO_2 (pH 7.4), 100 mM KCl, 1 mM EDTA, and 0.1 mM dithiotreitol. Aliquots of FapyAde-$^{13}C,^{15}N_2$ and FapyGua-

$^{13}C,^{15}N_2$ are added as internal standards. Samples are incubated with 1 μg of Fpg protein for 1 h at 37°C in a water bath. After incubation, 125 μl of cold ethanol (kept at −20°C) is added to the samples to stop the reaction and to precipitate DNA. The samples are kept at −20°C for 1 h, and then centrifuged at 15,000*g* for 30 min at 4°C. DNA pellets and supernatant fractions are separated. Ethanol is removed from the supernatant fractions under vacuum in a SpeedVac. Aqueous supernatant fractions are frozen in liquid nitrogen and lyophilized to dryness for 18 h.

Derivatization

An aliquot of 60 μl of a mixture of nitrogen-bubbled bis (trimethylsilyl)trifluoroacetic acid [containing trimethylchlorosilane (1%; v/v)] (BSTFA) and pyridine (1:1, v/v) is added to lyophilized supernatant fractions from Fpg hydrolysis of DNA. Samples are vortexed and purged individually with ultrahigh-purity nitrogen, tightly sealed under nitrogen with Teflon-coated septa, and then heated at 120°C for 30 min. After cooling, the clear supernatant fractions are removed and placed in vials used for injection of samples onto the GC column. Vials are purged with ultrahigh-purity nitrogen and tightly sealed with Teflon-coated septa. Lyophilized acidic hydrolysates of DNA samples are derivatized with 60 μl of a mixture of BSTFA and pyridine (1:1, v/v) for 3 h at room temperature. Aliquots (4 μl) of derivatized samples are analyzed by GC/MS-SIM with split-mode injection under the experimental conditions described below.

GC/MS analysis

For GC/MS measurements, a fused silica capillary column (12.5 m, 0.2 mm i.d.) coated with cross-linked 5% phenylmethylsilicone gum phase (film thickness, 0.33 μm) is used. Ultrahigh purity helium is used as the carrier gas. The injection port and the GC/MS interface are kept at 250 and 280°C, respectively, with a column head pressure at 65 kPa. The ion source temperature of the mass spectrometer is kept at 230°C. Aliquots of 4 μl of the derivatized samples are injected onto the GC column by means of an automatic sampler. The split mode of injection with a split ratio of 10 to 1 is generally used. The split ratio is adjusted electronically. Analyses by the mass spectrometer are performed in the EI-mode at 70 eV. The oven temperature of the gas chromatograph is programmed from 130 to 280°C at a rate of 8°C/min after 2 min at 130°C. For identification and quantification, SIM is used to monitor the characteristic ions of the Me_3Si derivatives of FapyAde, FapyAde-$^{13}C,^{15}N_2$, FapyGua, and FapyGua-$^{13}C,^{15}N_2$ as described above. The quantification of the monitored compounds is achieved using integrated areas of the signals of their ions. It should be noted that these experimental conditions may be adjusted according to the needs of each laboratory.

Conclusions

The GC/MS methodology described here is well suited for the measurement of FapyAde and FapyGua in DNA in vitro and in vivo. The level of sensitivity of GC/MS with SIM amounts to ~1 fmol of the Me_3Si derivatives of FapyAde and FapyGua eluting from the GC column. The selectivity and sensitivity of mass spectrometry with isotope dilution permit the positive identification and accurate quantification of these lesions in DNA of living cells at levels less than 1 lesion/10^6 DNA bases. This methodology is also applied to the determination of the substrate specificities and excision kinetics of DNA glycosylases that are specific for the removal from DNA of FapyAde and FapyGua, and other oxidatively induced DNA lesions. There is no doubt that the GC/MS methodology will be applied in future as well to the measurement of these lesions in DNA in vivo, and thus will contribute to the understanding of their role in disease processes.

Acknowledgments

Certain commercial equipment or materials are identified in this paper in order to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

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