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The 1-Carbamoyl-2-oxo-4,5-dihydroxyimidazolidine Component of ROS-Induced DNA Damage in White Blood Cells

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Abstract

The 1-carbamoyl-2-oxo-4,5-dihydroxyimidazolidine modification of cytosine is a known base modification produced *in vitro* by oxidative stress. However, the presence of this modification *in vivo* has not been established. In this study the introduction of this base modification into dinucleoside monophosphates was accomplished using the Fenton reaction. Subsequently, the modification was produced in short isotopically labeled oligomers. Labeled tetramers bearing the lesion were used as internal standards for LC-MS/MS determinations of the base modification in the DNA of white blood cells from healthy donors. The background level of the 1-carbamoyl-2-oxo-4,5-dihydroxyimidazolidine modification of cytosine was found to be larger than the levels of the formamide and thymine glycol base modifications.

INTRODUCTION

Cells exhibit a surprisingly high level of oxidatively induced DNA damage. Presumably this damage is caused primarily by reactive oxygen species (ROS) generated *in vivo* (1). Superoxide radical and hydrogen peroxide are ROS generated in cells during synthesis of ATP. The level of the 8-oxo-7,8-dihydroguanine lesion in DNA is often taken as an indicator of oxidative stress. This indicator is used despite the fact that measurements of background levels of the 8-oxo-7,8-dihydroguanine in the DNA of white blood cells (WBC) disagree widely among different laboratories (2–7). The cited reports used either electrochemical detection or ³²P postlabeling to measure the 8-oxo-7,8-dihydroguanine lesion.

Exposure of DNA *in vitro* to ROS produces a variety of base modifications in addition to the 8-oxo-7,8-dihydroguanine lesion. Being less subject to the vagaries associated with measurements of 8-oxo-7,8-dihydroguanine, other base modifications would likely be more reliable indicators of oxidative stress. Studies of the radiation chemistry of DNA and its constituents suggest many alternative modifications for study. For example, exposure of d(CpApTpG) to ionizing radiation in aqueous solution generates multiple products separable by chromatography, as shown in Fig. 1. Several of the products have been characterized by mass spectrometry and by proton NMR spectroscopy (8). The major base modifications identified included 8-oxo-7,8-dihydroguanine, the formamide remnant from the breakdown of either of the pyrimidine bases, thymine glycol derived from thymine, and an imidazolidine modification derived from cytosine. With the notable exception of the last mentioned, these modifications have also been detected in cells exposed to oxidative stress. The neglected cytosine modification, 1-carbamoyl-2-oxo-4,5-dihydroxyimidazolidine, is the

focus of this report. The isomers of this modification are off scale in Fig. 1. The structure of 1-carbamoyl-2-oxo-4,5-dihydroxyimidazolidine derived from irradiated cytosine was determined by Hahn *et al.* (9). This base modification has also been characterized at the nucleoside level (10).

In this study, the 1-carbamoyl-2-oxo-4,5-dihydroxyimidazolidine modification was characterized in dinucleoside monophosphates, for example d(CⁱpA). An isotopically labeled tetramer containing the modification, d(GpCⁱpA^{*}pT), where A^{*} stands for [¹⁵N]-labeled 2'-deoxyadenosine, was synthesized for use as an internal standard for mass spectrometry measurements. The imidazolidine modification was quantified in the DNA of WBC from human blood samples and was found to be comparable to the baseline levels of the formamide and thymine glycol modifications. The simultaneous measurement of the formamide, thymine glycol and imidazolidine base damages is proffered as a more reliable assessment of oxidative stress. For reasons enumerated below, DNA damage is measured in our assay in the form of modified dinucleoside monophosphates, as shown in Fig. 2 (11, 12). Modified dinucleoside monophosphates with 2'-deoxyadenosine as the 3' nucleoside are preferred because overall these sequences chromatograph most advantageously.

MATERIALS AND METHODS

The modification of short oligomers to contain the imidazolidine lesion used the Fenton reaction. For example, a solution containing 500 μ l 0.1 M Tris, pH 7.4, 120 μ l 1 mM CuSO₄, 30 μ l H₂O₂, 30% and 2 mg d(CpA) (650 μ l total volume) was maintained at room temperature for 24 h. Products were isolated by HPLC and characterized by mass spectrometry and by proton NMR spectroscopy.

The synthesis of isotopically labeled d(GpCⁱpA^{*}pT), where Cⁱ stands for deoxycytosine bearing the imidazolidine modification and A^{*} stands for [¹⁵N]-labeled 2'-deoxyadenosine, was a three-step process. Universally [¹⁵N]-labeled 2'-deoxyadenosine phosphoramidite was procured from Cambridge Isotopes Laboratories. The phosphoramidite was incorporated into the tetramer d(GpCpA^{*}pT) by PrimeSyn Inc. The labeled tetramer was modified in our laboratory using the Fenton reaction. The imidazolidine modifications were isolated by HPLC analogous to Fig. 1. The labeled tetramer was enzymatically hydrolyzed and run on HPLC along with the modified dinucleoside monophosphate characterized in Fig. 3. The labeled and unlabeled dinucleoside monophosphates eluted identically. Internal standards are added to the DNA sample prior to enzymatic digest. In addition to d(GpCⁱpA^{*}pT), we employed internal standards of the form d(GpApApT^fpA^{*}) and d(GpCpApT^gpA^{*}) for quantifying the formamide and thymine glycol modifications (Fig. 2b and c, respectively) (12). The internal standards and the DNA sample are hydrolyzed together and the modifications were detected at the dimer level.

Blood samples were drawn with EDTA as anticoagulant under an IRB-approved protocol. The sample was centrifuged and the buffy coat was collected. DNA was extracted from the cells using a kit designed to minimize spurious oxidation reactions (ZeptoMetrix, Inc., Buffalo, NY). The kit uses chaotropic precipitation of the DNA together with desferol in the extraction procedure. One hundred micrograms of DNA was hydrolyzed and dephosphorylated using nuclease P1 and alkaline phosphatase. A solution containing 15 μ l sodium acetate buffer (0.25 M, pH 5.2), 50 μ l 3.0 mM Zn Cl₂, 50 μ l water, and either 1.0 or 3.0 U nuclease P1 (Sigma N8630) together with the DNA and isotopically labeled internal standards was incubated at 37°C for 2 h at pH 5.2. After addition of 25 μ l of Tris-HCl (1 M, pH 9.0) and 70 U of alkaline phosphatase, the sample was incubated for an additional 2 h at pH 8.2.

RESULTS

The 1-carbamoyl-2-oxo-4,5-dihydroxyimidazolidine modification has not been included previously in assays of oxidatively induced DNA damage in WBC. Characterization of this modification at the dimer level was a necessary preliminary step in this study. The dinucleoside monophosphate d(CpA) was treated with hydrogen peroxide in the presence of Cu^{++} (Fenton reaction) as described above. Separation of products by HPLC yielded the elution profile of Fig. 3a. Product peaks were isolated, desalted and analyzed by mass spectrometry. Peaks 7 and 8 had identical molecular weights, 589, corresponding to the negative ion weight of the product of interest, d(CⁱpA) of Fig. 2a. The C4 and C5 carbon atoms of 1-carbamoyl-2-oxo-4,5-dihydroxyimidazolidine are chiral, and the two products can be accounted stereoisomers. These products were further characterized by proton NMR spectroscopy. The low-field portions of the proton NMR spectra are shown in Fig. 3b and c. The chemical shifts associated with the C4-H and C5-H resonances distinguish between the two stereoisomers. The negligible spin-spin coupling between the C4 and C5 protons indicates that these are the *trans* stereoisomers. The modified dimers d(CⁱpC), d(CⁱpG) and d(CⁱpT) were synthesized similarly.

Our assay takes advantage of the fact that base modifications typically inhibit the hydrolysis by nuclease P1 of the phosphoester bond 3' to the modified nucleoside. Consequently, the nuclease P1 (plus alkaline phosphatase) hydrolysate tends to accumulate base modifications in the form of modified dinucleoside monophosphates as shown, for example, in Fig. 2. Detection by LC-MS/MS using electrospray ionization and operating in the negative ion mode is more sensitive for dinucleoside monophosphates than for nucleosides due to the presence of the phosphodiester bond. Furthermore, the fragmentation pattern of dinucleoside monophosphates reliably generates the 3' fragment formed by breakage of the C5'-O bond of the 5' nucleoside. The MS/MS values for detection of d(CⁱpA), for example, are 589/330.

Two samples containing 100 μg of DNA derived from human cells in culture were prepared for LC-MS/MS analysis as described above. Synthetic d(CⁱpC), d(CⁱpG), d(CⁱpT) and d(CⁱpA) reference compounds were added to one of the samples. The spectrometer was programmed for MS/MS values: 565/306, 605/346, 580/321 and 589/330. The elution profiles of the two samples are compared in Fig. 4. In the left-hand set of elution profiles, the signal arises from the DNA of untreated cells; in the right-hand set of elution profiles, the signals arise predominantly from the added reference compounds. The latter profile was substantially unchanged whether it was prepared from the early- or late-eluting components of each pair of isomers. Evidently, the imidazolidine modification is present in the DNA of untreated human cells. The sequence of elutions in Fig. 4 is as expected. The same sequence of elution is observed for this chromatography applied to unmodified dinucleoside monophosphates (14). The stereoisomers of d(CⁱpG) and d(CⁱpA) are not resolved in Fig. 4.

The imidazolidine modification was incorporated into a larger assay that also included the formamide and thymine glycol modifications. A typical set of LC-MS/MS profiles for the three modifications displayed in Fig. 2 from the DNA of untreated WBC from a healthy donor is shown in Fig. 5. A compilation of results for measurements of the three modifications in the DNA of WBC of healthy donors is given in Table 1. These measurements are a determination of the background levels of the three modifications of Fig. 2 in the DNA of healthy individuals. The measurements show that the level of the 1-carbamoyl-2-oxo-4,5-dihydroxyimidazolidine modification is of the same order of magnitude as the formamide or thymine glycol modifications. The key value in Table 1, 7.5 fmol d(CⁱpA)/ μg of DNA, is equivalent to $7.7 \text{ dC}^i/10^6$ nucleotides.

The base modifications discussed herein occur as stereoisomers. The occurrence of isomers in the case of the imidazolidene modification has already been noted. It is generally preferable to quantify the product from DNA by referencing it to the sum of the isomers in the internal standard because of the possibility of interchange between isomers. In the case of the formamide modification, rotomers are present due to restricted rotation about the amide bond (15). The rotor states are unequally populated and are stable enough to yield distinct resonances in proton NMR spectra of nucleosides and oligomers containing this base modification (8, 16). Our measurements of the thymine glycol modification pertain to the cis stereoisomers.

The MS/MS values for d(C¹pA) differ from the values for d(T^εpA) by a single mass unit. Furthermore, the later eluting isomer of d(C¹pA) and an earlier-eluting isomer of d(T^εpA) tend to elute simultaneously. The natural abundances of isotopes ¹³C, ¹⁵N and ¹⁷O of d(T^εpA) enhance the signal arising from d(C¹pA). Quantification of the imidazolidene modification was accomplished in this study by comparing the signals from the earlier-eluting components of the sample and internal standard signals.

DISCUSSION

Sequencing studies have shown that breast tumors and colorectal tumors accumulate of the order of 100 mutations during tumorigenesis (17, 18). Oxidative stress is a plausible global contributor to mutagenesis. Levels of oxidative DNA damage, inferred from the measurements of 8-oxo-7,8-dihydroguanine, are reported to be higher in patients with lung, leukemia, colorectal, bladder, breast and esophageal cancers than in controls (2–7), although the reported control levels vary widely. The level of the thymine glycol modification is higher in ovarian cancer patients than in controls (19). These data contribute to the interest in assessing and monitoring oxidatively induced DNA damage as an indicator of oxidative stress.

Hydroxyl radical is the ROS mainly responsible for generating oxidative stress. A further reservation concerning 8-oxo-7,8-dihydroguanine as an indicator of oxidative stress is that hydroxyl radical is relatively unreactive toward deoxyguanosine (20). On the other hand, the formation of 1-carbamoyl-4,5-dihydroxyimidazolidine is a classic example of a hydroxyl radical-induced damage product. The first step in the reaction of hydroxyl radical is to produce a free radical adduct, in this case 5(6)-hydroxy-6(5)-yl-cytosine. Under aerated conditions, addition of oxygen to the carbon atom bearing the unpaired electron generates the peroxy radical. In the reducing environment of a cell, formation of 5(6)-hydroxy-6(5)-hydroperoxide is probable. The final product, 1-carbamoyl-2-oxo-4,5-dihydroxyimidazolidine, follows upon rearrangement. The increase in molecular weight is 50, consistent with observed properties of the modification in question.

The main result of this study is to establish the 1-carbamoyl-2-oxo-4,5-dihydroxyimidazolidine modification of cytosine as a significant oxidatively induced modification of DNA. This conclusion is based on measurements of the level of the modification in the DNA from WBC of healthy donors. Typical basal levels of oxidative base damage in DNA are high, of the order of 1–10 per 10⁶ nucleotides, although lower levels have been reported (2–7, 19, 21, 22). The levels of the imidazolidine modification are no exception. Our results show the abundance of the imidazolidine modification to be comparable to the formamide or thymine glycol modifications.

This study is another instance of a base modification identified initially in bare DNA exposed to ionizing radiation that was subsequently shown to be present at significant levels in human DNA. Oxidative stress generates a multiplicity of DNA modifications, most of

which have not been quantified in the DNA from human donors. Oxidatively induced DNA damage is likely to be a risk factor for cancer because it is present in DNA as many types of damage, frequently at high levels. Levels of 1–10 base modifications per 10^6 nucleotides may be compared with, for example, smoking-induced DNA damage, which is typically an order of magnitude smaller (23–25).

The development of LC-MS/MS spectroscopy provides unique opportunities for DNA damage studies. Detection by LC-MS/MS is very sensitive, is highly selective, and has the potential for measuring multiple products simultaneously. It may be noted that previous assays of DNA damage in this laboratory used an HPLC step that removed unmodified nucleosides, constituting the bulk of the sample, from the hydrolyzed DNA before LC-MS/MS analysis (11, 12, 19). This labor-intensive step was omitted in the current protocol.

DNA damage analysis at the dimer level has advantages. Detection of modified dinucleoside monophosphates by LC-MS/MS operating in the negative ion mode is particularly sensitive due to the presence of the phosphodiester bond. Furthermore, it is often convenient to synthesize an isotopically labeled internal standard, needed for quantification of a lesion, by incorporating the isotopic label in the unmodified 3' nucleoside.

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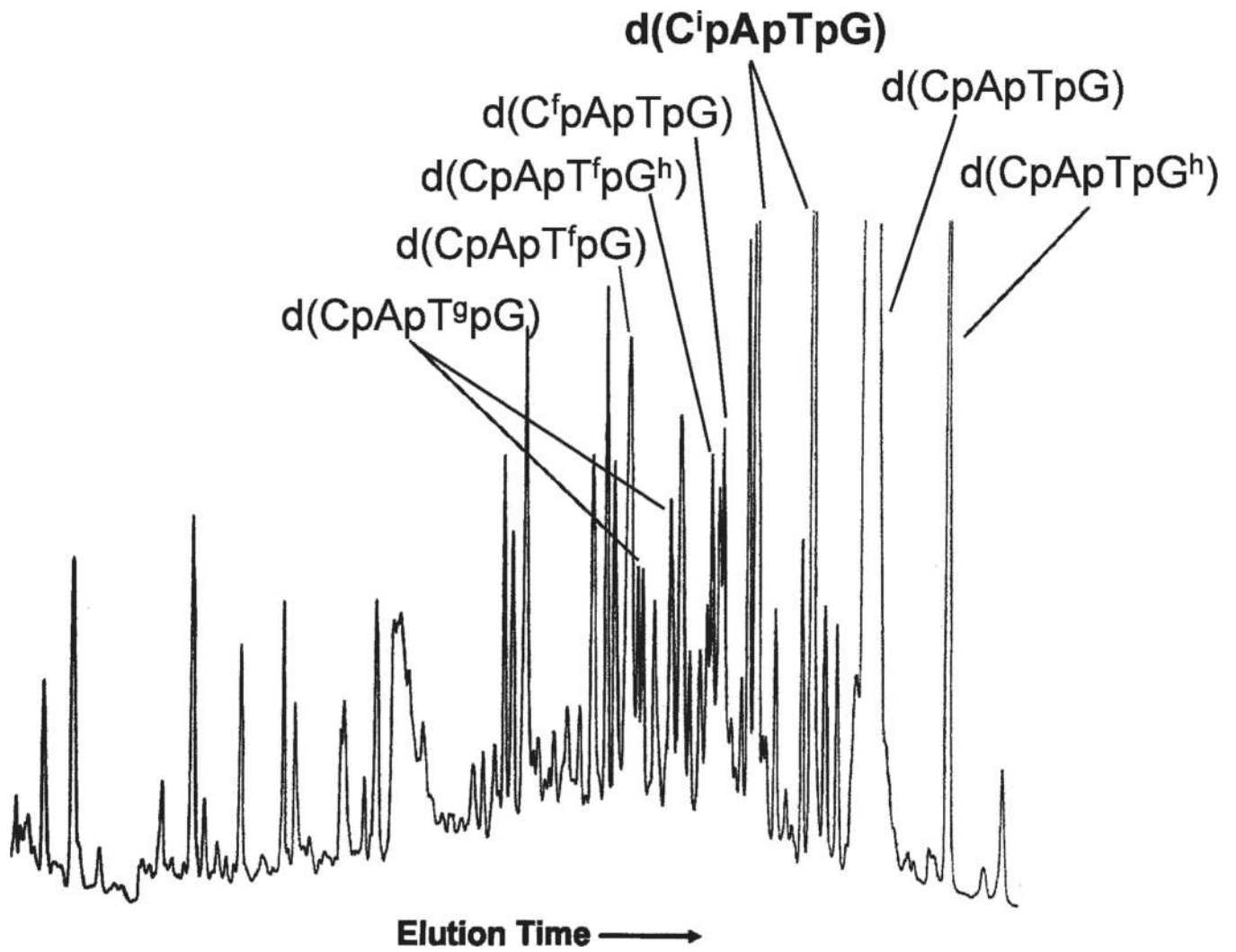


FIG. 1. HPLC elution profile of $d(CpApTpG)$ after exposure to X rays in oxygenated aqueous solution at a concentration of 0.028 mM. The dose was 240 Gy (8). The products of interest for this study are labeled $d(C'pApTpG)$ and are off scale.

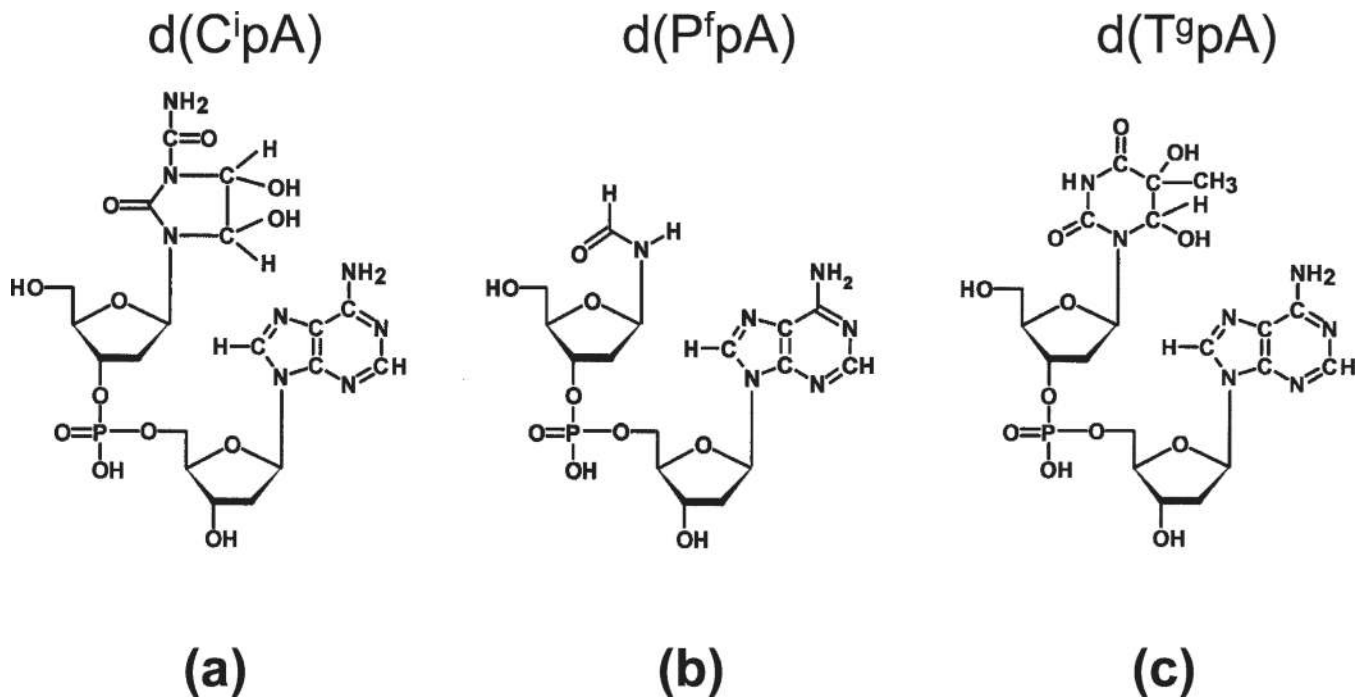


FIG. 2. The imidazolidine, formamide and thymine glycol base modifications were measured in the DNA of WBC in the form of modified dinucleoside monophosphates, a, b and c, respectively.

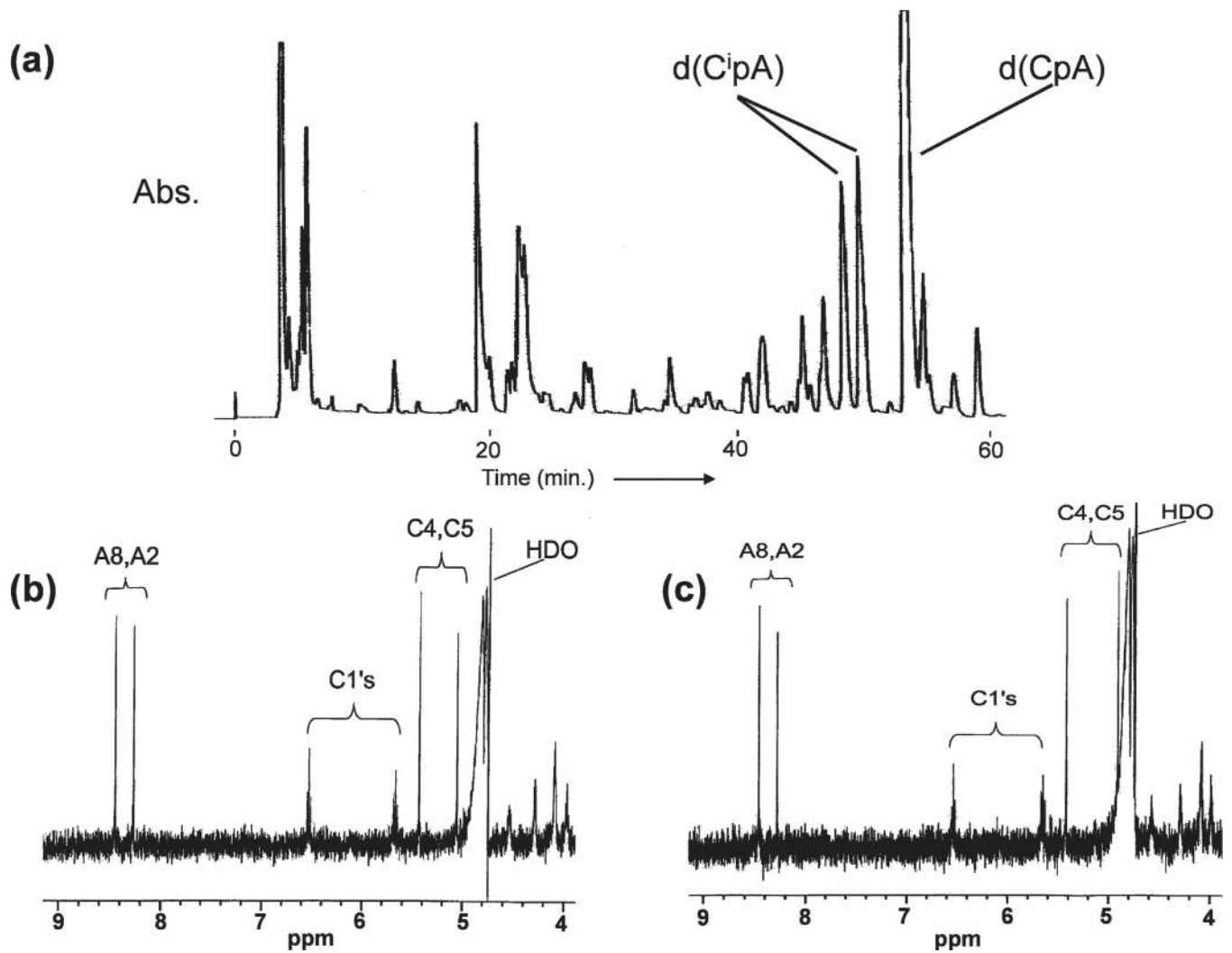


FIG. 3. Panel a: HPLC elution profile of d(CpA) after treatment with Cu^{++} and H_2O_2 . Peaks labeled d(CⁱpA) contain imidazolidine base modifications. Panels b and c: Proton NMR spectra of early- and late-eluting stereoisomers, respectively, of d(CⁱpA).

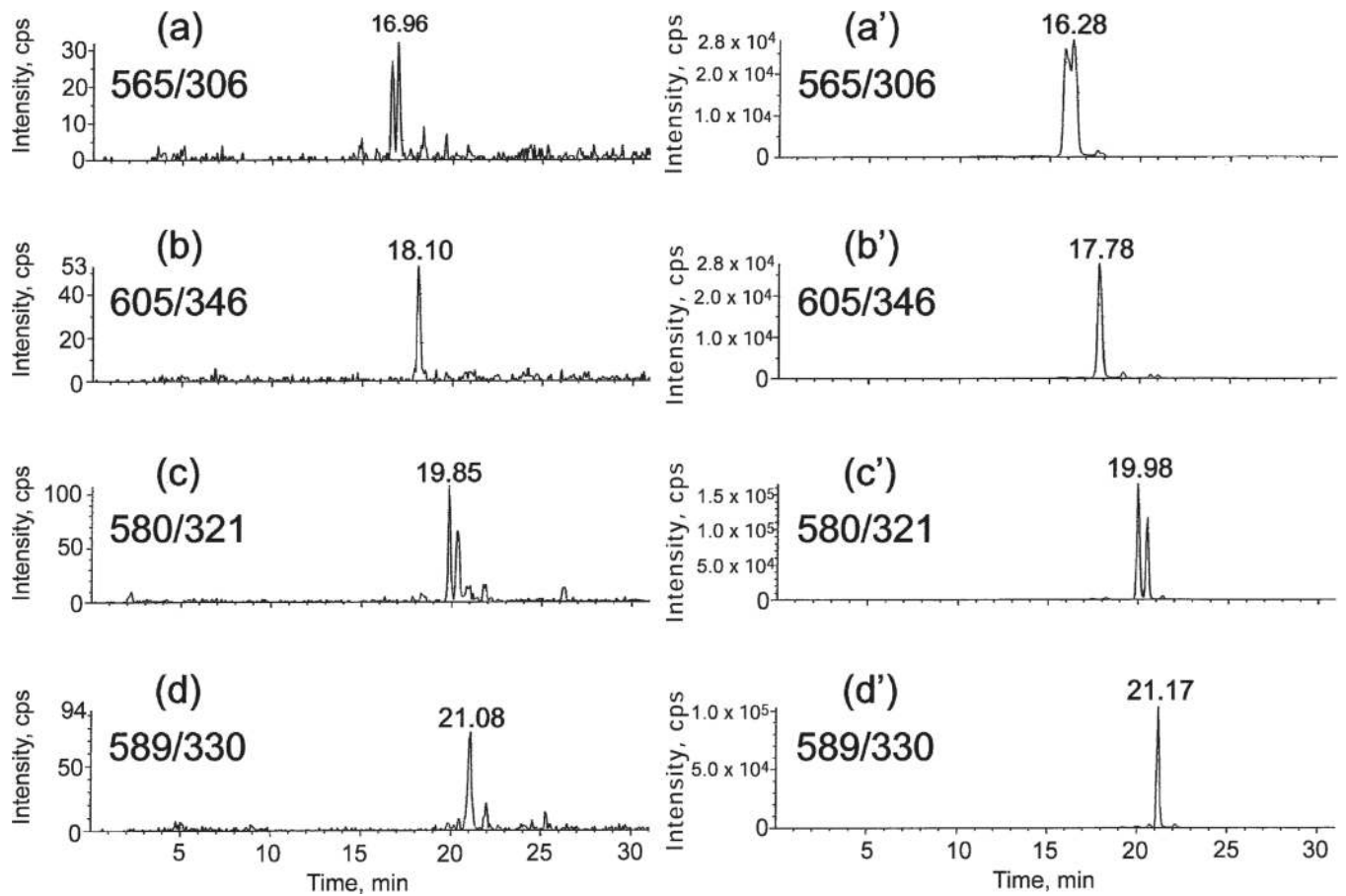


FIG. 4.
 LC-MS/MS elution profiles for DNA samples with MS/MS values set to detect $d(C^{13}pC)$, $d(C^{13}pG)$, $d(C^{13}pT)$ and $d(C^{13}pA)$. Right-hand profiles were spiked with reference materials.

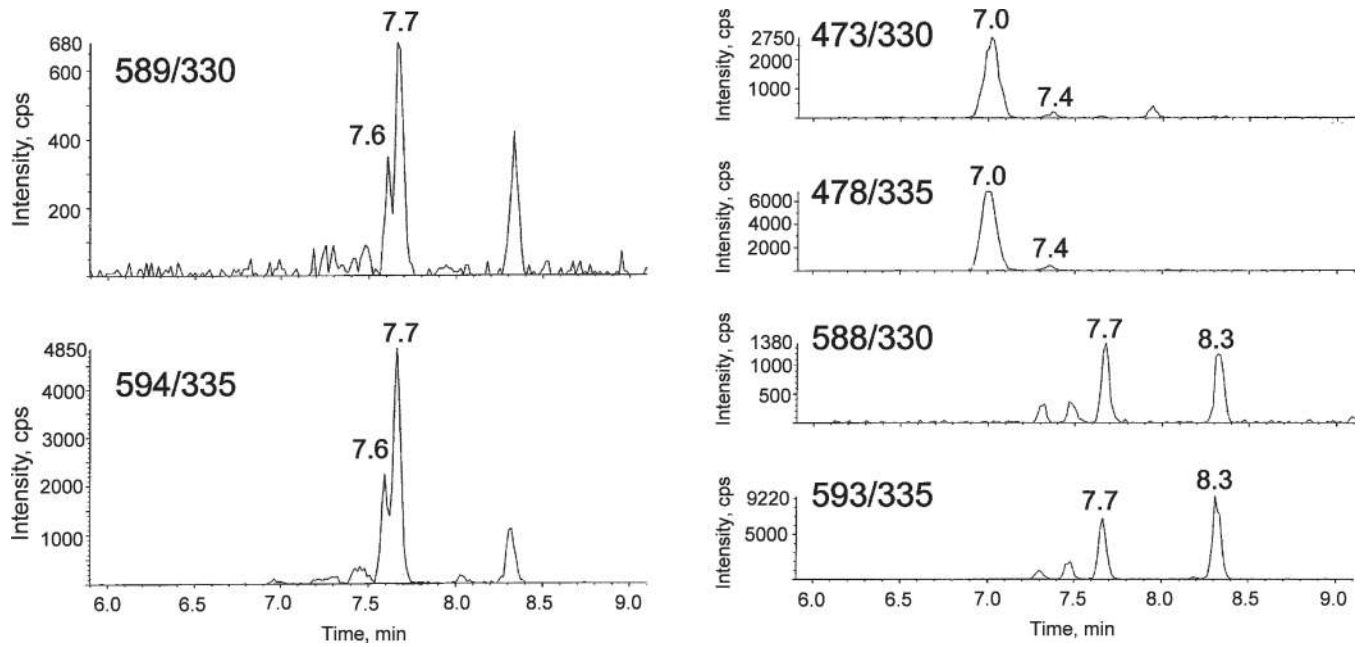


FIG. 5. Typical LC-MS/MS elution profiles from a hydrolysate of DNA obtained from WBC. Elution profiles are shown for dinucleoside monophosphates containing the imidazolidine (left-hand profiles), formamide (upper right profiles), and thymine glycol (lower right profiles) modifications and for their respective internal standards. Internal standards were added to the DNA sample before enzymatic hydrolysis.

TABLE 1

Background Levels of Imidazolidene, Formamide and Thymine Glycol in WBC of Healthy Donors

	d(C¹pA) (fmol/μg of DNA)	d(T^fpA) (fmol/μg of DNA)	d(T^gpA) (fmol/μg of DNA)
Group 1, 3 U (4)	8.3 ± 2.5	5.0 ± 0.8	4.4 ± 0.9
Group 2, 1 U (4)	6.7 ± 2.0	6.0 ± 1.0	3.2 ± 0.8
Mean (8)	7.5 ± 1.5	5.5 ± 0.6	3.8 ± 0.6

Notes. Mean values are given in terms of fm/μg of DNA ± SEM. The amount of endonuclease used in processing the two groups was either 1 U or 3 U (see the Materials and Methods).