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Detection of chlorinated DNA and RNA nucleosides by HPLC coupled to tandem mass spectrometry as potential biomarkers of inflammation

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Abstract

Upon inflammation, activated neutrophils secrete myeloperoxidase, an enzyme able to generate hypochlorous acid (HOCl) from hydrogen peroxide and chloride ions. An analytical method, involving HPLC coupled to electrospray tandem mass spectrometry, has been set-up to detect low levels of HOCl-induced nucleic acids lesions, including both ribo and 2′-deoxyribonucleoside derivatives of 8-chloroguanine, 8-chloroadenine and 5-chlorocytosine. Validation of the developed method was achieved using isolated cells treated with HOCl. The method was found to be sensitive enough to allow the measurement of background levels of 5-chloro-2′-deoxycytidine in the DNA of human white blood cells isolated from 7 mL of blood.

Keywords: Oxidative stress; DNA damage; RNA damage; Hypochlorous acid; Tandem mass spectrometry; Human biomarkers

1. Introduction

Following infection and inflammation, various cells of the immune systems are activated with subsequent induction of several enzymes, including inducible nitric oxide synthase, NAD(P)H oxidase and myeloperoxidase (MPO). A consequence of this activation is the production of several reactive oxygen and nitrogen species such as superoxide anion ($O_2^{\bullet -}$), nitric oxide, peroxynitrite and hypohalous acids (HOCl and HOBr) [1]. The resultant oxidant species can oxidize, nitrate, chlorinate or brominate several cellular constituents including lipids, proteins and nucleic acids [2]. Such processes are recognized as risk factors for human cancers since the bactericidal agents could also damage host tissues.

Among the different activated species, it has been shown that HOCl is able to chlorinate several biological molecules [3] and among them, nucleic acids represent one of the most studied biomolecules due to its key role in cell evolution. Reaction of HOCl with isolated DNA and RNA has been found to generate several chlorinated base modifications [4,5]. The mechanism of formation of such DNA and RNA damage has been proposed to involve the transient formation of base radicals [6].

Oxidized DNA lesions, and among them mostly 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo) have been extensively used as in vivo biomarkers of oxidative stress [7]. For this purpose, several physico-chemical and biological approaches have been developed to allow the determination of the cellular level of 8-oxodGuo [8]. During the last two decades, the difficulty of measuring oxidized DNA lesions, mostly 8-oxodGuo, has been highlighted [9,10]. The background level of 8-oxodGuo in different untreated cell lines was found to vary by, at least, three orders of magnitude, depending on the method used. The main goals of the European network European Standards Committee on Oxidative DNA Damage (ESCODD) that was recently set-up, were to determine the origins of the discrepancies and to propose

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optimized protocols of measurement. It seems now well established that the main drawback was the artifactual DNA oxidation that may occur during sample preparation, which mainly implies DNA extraction [11–14]. However, there is still an incomplete agreement between the results obtained using methods requiring DNA extraction and indirect approaches such as the comet assay [15]. Therefore, the use of oxidized DNA lesions as biomarkers of oxidative stress still remains a challenging issue [16]. The use of chlorinated DNA bases as indicators of inflammation could represent a suitable alternative since artifactual formation of chlorinated DNA bases is not expected to occur, at least significantly, during sample preparation. However, the cellular background level of such DNA modifications in human leukocytes has not been yet determined. In addition, it has not been clearly shown that significant amounts of chlorinated DNA lesions could be produced in cells incubated in the presence of HOCl.

In this work, a HPLC coupled to electrospray tandem mass spectrometry (HPLC–MS/MS) based method has been developed for the measurement of both chlorinated DNA lesions including 5-chloro-2′-deoxycytidine (5-CldCyd), 8-chloro-2′-deoxyguanosine (8-CldGuo), 8-chloro-2′-deoxyadenosine (8-CldAdo) and chlorinated RNA nucleosides including 5-chlorocytidine (5-ClCyd), 8-chloroguanosine (8-ClGuo) and 8-chloroadenosine (8-ClAdo). Such a method has been used to measure the level of chlorinated DNA and RNA lesions in cells incubated with HOCl. Thereafter, the assay has been applied for monitoring the extent of the different chlorinated nucleosides in the DNA of freshly isolated human white blood cells.

2. Material and methods

2.1. HPLC–MS/MS measurements

The chlorinated nucleosides were obtained as previously described [4]. On-line HPLC–MS/MS measurements were carried out using a Agilent (Massy, France) 1100 HPLC system, equipped with a thermostated autosampler, a binary HPLC pumping system, an oven and a UV detector. Separations were performed using a reversed phase Uptisphere-ODB (3 μm, 0.2 cm × 15 cm) column from Interchim (Montluçon, France). The elution was achieved at a flow rate of 0.2 mL/min in the gradient mode, the column being maintained at 28 °C. The proportion of acetonitrile in 5 mM ammonium formate (pH 6.5), starting from 0%, reached either 25% (conditions A) or 20% (conditions B) within 30 min for the measurement of chlorinated 2′-deoxyribonucleosides and ribonucleosides, respectively. After the completion of the HPLC analysis (30 min), the column was washed with 50% acetonitrile for 5 min and an additional 15 min period was required to equilibrate the HPLC column between two injections. At the output of the column, the eluent was directed first to a UV detector set at 260 nm for monitoring the overwhelming normal nucleosides. Then, after addition of MeOH (0.1 mL/min), the eluent was directed onto a API3000 tandem mass spectrometer (Applied Biosystems) through a "Turbospray" electrospray source (Sciex Thornil Canada) as described in details elsewhere [17–19]. The system was entirely controlled by Analyst software 1.2. To improve the sensitivity of detection a low resolution (±1.2 amu) was used. In addition, for each of the ribo- and 2′-deoxyribonucleosides, the different parameters of ionization and fragmentation were optimized by infusing a 20 μM solution of the nucleoside directly onto the mass spectrometer, as previously described [17,18]. For all the studied DNA lesions, a higher sensitivity was obtained in the positive ionization mode for an ion spray voltage of 5500 V and a temperature of 450 °C for the turbo gas (nitrogen), the acquisition dwell time of each transition being 750 ms.

A sensitive MRM method was applied to measure the different chlorinated nucleosides. The different transitions used for the detection of the chlorinated nucleosides, together with their retention time and the collision energy for fragmentation are given in Table 1. The most abundant daughter ion was found to correspond, for all the chlorinated nucleosides studied, to the loss of the (2-deoxy)ribose moiety. To improve the specificity of detection, a second transition, corresponding to the loss of the (2-deoxy)ribose moiety for the molecule containing the ^{37}Cl isotope, was also monitored for all chlorinated DNA and RNA lesions. The limit of quantification, determined for a S/N = 10, for the detection of the studied chlorinated nucleosides varies between 2 and 25 fmol injected (Table 1). Quantification of the amount of the different DNA lesions was performed by external calibration. For that purpose prior and after each series of samples, three different standards containing either the three chlorinated nucleosides or 2′-deoxyribonucleosides were injected. Similarly, an external quantification was used to quantify the amount

Table 1
Mass spectrometric and HPLC features of the different chlorinated ribo and 2′-deoxyribonucleosides detected by HPLC–MS/MS under HPLC conditions A or B used for optimal separation of 2′-deoxyribonucleosides and ribonucleosides, respectively (conditions detailed in material and methods)

Product	Molecular weight	Retention time (min)	Main transition	Collision energy (eV)	Limit of quantification (fmol)
5-CldCyd	261	14.5 (A)	262 → 146	13	5
8-CldGuo	301	19.1 (A)	302 → 186	16	25
8-CldAdo	285	22.5 (A)	286 → 170	19	2
5-ClCyd	277	11.7 (B)	278 → 146	15	5
8-ClGuo	317	17.8 (B)	318 → 186	21	15
8-ClAdo	301	22.0 (B)	302 → 170	31	2

of hydrolyzed DNA [18] or RNA samples, using the area of the peak of a standard of dGuo or Guo, respectively. Results, given as the number of lesions per million normal nucleosides, represent, if not specified, the average and standard deviation of four independent determinations.

2.2. Cell treatment with HOCl

A SKM-1 cell line was used. Cells in growth phase were collected by centrifugation before to be washed. Then, 10 million of cells were suspended in 10 mL of HBSS buffer and an adequate volume of HOCl (prepared as previously described [4]) was added to the buffer solution to give a 300 μM final concentration. Then, the cells were incubated at 37 °C for 10 min. After incubation, *N*-acetylcysteine was added to stop the reaction [4]. Then, cells were washed and in a subsequent step DNA and RNA were isolated using Qiagen extraction kit following the recommendations of the manufacturer. Nucleic acids were digested to nucleosides as described previously [20]. The results reported in Fig. 3, represent the average and standard deviation of four independent determinations.

2.3. Isolation of human leukocytes

Leukocytes obtained from 10 human volunteers were rapidly isolated from 7 mL of blood using citrate CPT vacutainers obtained from Beckton Dickinson (Pont de Claix, France) and a single centrifugation step according to manufacturer's recommendations. Cells were then washed twice in PBS buffer and stored frozen until DNA extraction. The DNA was extracted and digested using the recently optimized protocol (chaotropic method) that minimizes adventitious DNA oxidation to occur during the work-up [20]. Practically, the so-called "chaotropic method" consists in the transient isolation of nuclei followed by precipitation of DNA using NaI subsequently to protease and RNases treatments, as described in detailed elsewhere [18,20].

3. Results

The newly designed HPLC–MS/MS method was found to be sensitive with a limit of quantification for different chlorinated ribo- and 2′-deoxyribonucleosides within a few fmole range (Table 1). In addition, for all the measured nucleosides, including 5-CldCyd, 8-CldGuo, 8-CldAdo, 5-ClCyd, 8-ClGuo and 8-ClAdo, the intensity of detection was found to increase linearly ($R^2 > 0.98$) with the amount of nucleosides injected over, at least, three orders of magnitude, starting from 5 fmoles up to 5 pmol injected (not shown). Using the relatively high abundance of ^{37}Cl isotope, a second transition corresponding to the loss of either the 2-deoxyribose or ribose moieties was used to improve the specificity of detection of the 2′-deoxyribonucleosides and ribonucleosides, respectively. As shown in Figs. 1 and 2, the second transition, as expected, has an intensity only three times lower

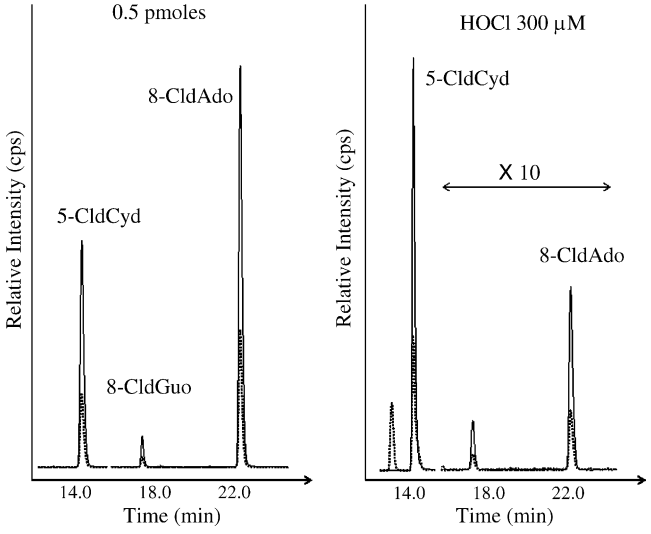

Fig. 1. Typical chromatograms obtained for the determination of chlorinated nucleosides in DNA samples. Left panel represents the chromatogram obtained by injection of 0.5 pmol each of 5-CldCyd, 8-CldGuo and 8-CldAdo. Right panel represents the HPLC–MS/MS chromatogram obtained by analysis of a DNA sample extracted from cells treated with 300 μM HOCl. For clarity, signals for 8-CldGuo and 8-CldAdo have been enhanced by a factor of 10. For each lesion, the two monitored transitions correspond to the loss of the 2-deoxyribose moiety (loss of 116) from the molecule containing the ^{35}Cl (full line) or ^{37}Cl (dashed line) isotope.

than that of the major transition (natural abundance of ^{35}Cl and ^{37}Cl are 75% and 25%, respectively). However, a high background is observed for the second transition 280 → 148 selected for 5-ClCyd (Fig. 2). The background level of the different chlorinated DNA and RNA lesions in untreated SKM-

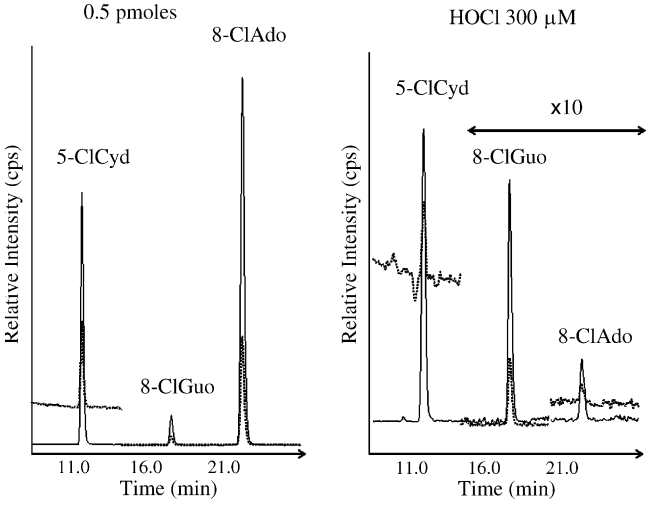

Fig. 2. Typical chromatograms obtained for the determination of chlorinated bases in RNA samples. Left panel represents the chromatogram obtained by injection of 0.5 pmol each of 5-ClCyd, 8-ClGuo and 8-ClAdo. Right panel represents the HPLC–MS/MS chromatogram obtained by the analysis of a RNA samples extracted from cells treated with 300 μM HOCl. For clarity, signals for 8-CldGuo and 8-CldAdo have been enhanced by a factor 10. For each lesion, the two monitored transitions correspond to the loss of the ribose moiety (loss of 132) from the molecule containing the ^{35}Cl (full line) or ^{37}Cl (dashed line) isotope.

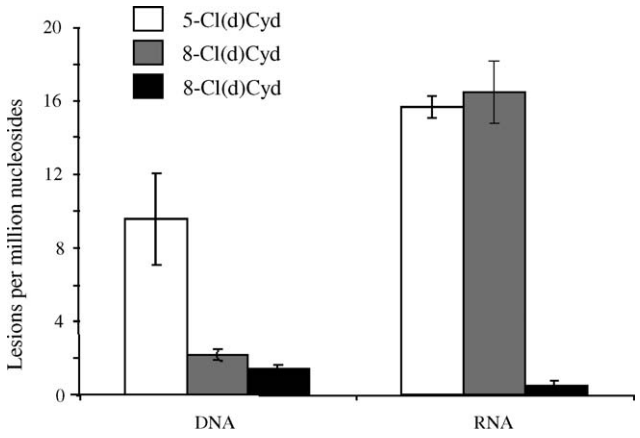

Fig. 3. Formation of chlorinated DNA and RNA bases, as determined by HPLC–MS/MS, in the DNA and RNA of cells treated for 10 min in the presence of 300 μM HOCl. Results, expressed as the number of modification per million nucleosides represent the average ± standard deviation of four independent experiments.

1 cells was found to be close to, or even below, the limit of quantification of the HPLC–MS/MS assay. On the other hand, a significant amount of the six studied DNA and RNA lesions was detected in HOCl treated cells (Fig. 3). Interestingly, HOCl induces mainly the formation of 5-CldCyd in DNA and, under our experimental conditions, a concentration of 300 μM HOCl was found to give rise to 9.8 ± 2.3 5-CldCyd per 10^6 nucleosides (Fig. 3). In the mean time, the induced levels of 8-chloropurines were found to be much lower since 2.0 ± 0.4 8-CldGuo and 1.5 ± 0.4 8-CldAdo per 10^6 nucleosides were generated. A somewhat different quantitative formation of chlorinated lesions was observed in RNA (Fig. 3). Thus, high amounts of both 5-ClCyd and 8-ClGuo were found to be generated in the RNA of treated cells, with values of 15.8 ± 0.5 and 16.2 ± 1.8 lesions per 10^6 nucleosides, respectively. As observed in DNA, chlorination of the adenine moiety in RNA appears to be a minor process, leading to the formation of 0.5 ± 0.4 lesion per 10^6 nucleosides. Interestingly, chlorination of the bases was found to occur three-fold more efficiently in RNA than in DNA.

About 70 μg of DNA was obtained using our DNA extraction protocol after isolation of human white blood cells utilizing CPT vacutainers starting from 7 mL of human blood. Among the three different chlorinated DNA lesions that may be expected, only 5-CldCyd was found to be present in significant amounts as determined by HPLC–MS/MS analysis of the enzymatic DNA hydrolysate. No detectable amounts of 8-ClGuo were observed indicating that the level of the latter chlorinated nucleoside is below the limit of detection of the assay, namely 0.2 lesion per 10^6 nucleosides. A small peak of 8-CldAdo with a signal to noise ratio around 3 was detected (not shown) in the DNA hydrolysate. However, the levels of the lesion were not high enough to enable a precise quantification (S/N > 10). According to the limit of sensitivity of our assay we could estimate the level of 8-CldAdo in the DNA of human white blood cells at around 0.02 lesion per 10^6 nucleosides. The background level of 5-CldCyd was determined to be around 0.15 lesions per 10^6 nucleosides, a level much higher than our limit of quantification as shown in Fig. 4. Unfortunately, the second transition used to improve the specificity of detection of 5-CldCyd was found to be useless, since a large contaminating peak was found to elute just after 5-CldCyd in the samples (not shown). Interestingly, significant variations were observed between the different healthy volunteers with levels ranging between 0.06 and 0.4 5-CldCyd per 10^6 nucleosides ($n = 10$).

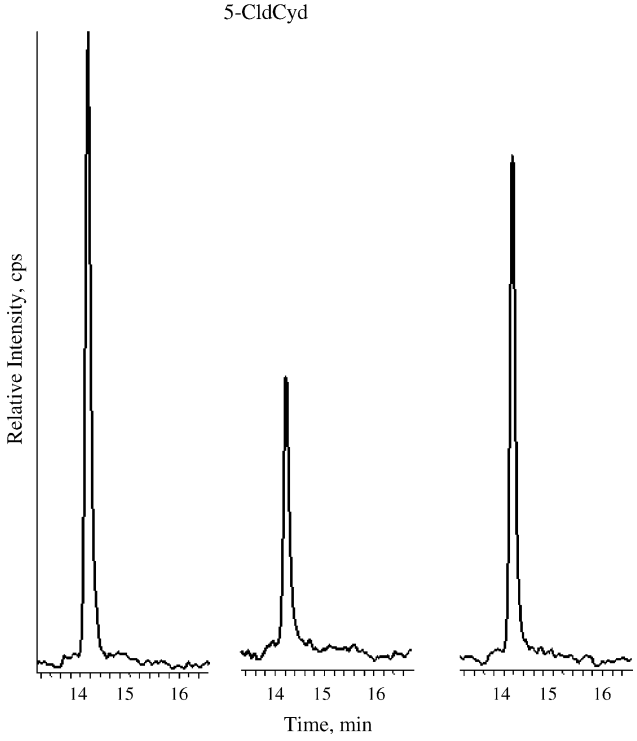

Fig. 4. Typical chromatograms obtained for the determination of 5-CldCyd in three different DNA samples extracted from human white blood cells. The HPLC–MS/MS detected peaks (transition 262 → 146) have a signal-to-noise ratio ranging from 42 (left chromatogram) to 20 (middle chromatograms) representing levels of detected 5-CldCyd as low as 12 fmol (middle chromatogram), obtained following the injection of 15 μg hydrolyzed DNA.

4. Discussion

DNA lesions, and among them mainly 8-oxodGuo, have been extensively used as in vivo biomarkers of oxidative stress. However, an overview of the literature indicates that some of the methodologies used to measure 8-oxodGuo were not accurate and in numerous cases have significantly overestimated the cellular level of the latter oxidized purine nucleoside. The difficulties of assessing low amounts of the DNA lesion are not due to the sole necessity to have a sensitive and specific assay that should allow an accurate determination of levels around 1 lesion per 10^6 nucleosides. In fact, one of the main limitations is due to the possible occurrence of adventitious DNA oxidation during samples preparation [21].

During the last few years, mainly in Europe through the ESCODD research network, significant efforts have been made to overcome the above mentioned difficulties [15]. However, nowadays the background levels of 8-oxodGuo measured by either HPLC–EC or HPLC–MS/MS in DNA extracted with an optimized protocol that minimizes the occurrence of spurious DNA oxidation during the work-up [20] are still three to five times higher than those determined by the comet assay or alkaline elution associated with DNA repair enzymes. Additional efforts have to be made in order to determine the origin of the gap between the two different approaches.

Another alternative to be considered is the measurement of DNA lesions that could not be produced artifactually during sample preparation. In this respect, DNA adducts resulting from the reaction of reactive aldehydes derived from lipid peroxidation, such as malondialdehyde or 4-hydroxy-2-nonenal represent interesting biomarkers [22,23]. In the present work, we have evaluated if chlorinated DNA or RNA bases could be of interest as well. For this purpose, we have designed a HPLC–MS/MS based method, as previously described for 8-oxodGuo [17] and other oxidized DNA bases [18]. After optimization of the parameters of electrospray ionization and fragmentation, it appears that the method exhibits a limit of sensitivity of a few fmoles. This should allow an accurate determination with a level of detection of about 0.1 lesion per 10^6 nucleosides in 50 μg DNA. Concerning the fragmentation, it is interesting to note that, in the most sensitive positive ionization mode, the loss of either the 2-deoxyribose or ribose moiety is the predominant daughter ions for the 2′-deoxyribo- and ribonucleosides, respectively. This feature that has been already observed for several normal and modified nucleosides [24] has been used for the search of unknown DNA lesions [25]. In addition, in order to improve the specificity of detection we have taken advantage of the relatively high natural abundance of ^{37}Cl isotope (33% compared to ^{35}Cl) to apply a second transition for the monitoring of the different chlorinated derivatives.

The appropriate HPLC behavior of the different chlorinated DNA or RNA nucleosides allows an efficient separation of the lesions on the C18 reversed phase used (Figs. 1 and 2). Therefore, the three chlorinated nucleosides could be detected simultaneously both in DNA or RNA hydrolysates, and in the mean time the normal overwhelming nucleosides could be quantified using an on-line UV detector set-up at 260 nm. To check the efficacy of the HPLC–MS/MS method and the efficiency of HOCl to halogenate nucleic acids, isolated cells were treated with 300 μM of HOCl. Thereafter, both DNA and RNA were isolated from the cells using a commercially available kit. Hydrolysis of RNA was performed under conditions initially developed for DNA since the enzymes used are also able to digest RNA into ribonucleosides. Then, the levels of chlorinated DNA and RNA nucleosides were assessed by HPLC–MS/MS using an external calibration. The level of chlorinated purine nucleosides in the nucleic acids of untreated cells was found to be below the limit of sensitivity. In contrast, the presence of 5-CldCyd and 5-ClCyd was detected in the DNA and RNA of untreated cells, with a signal to noise ratio of about 3 (not shown). Thus, the level of the latter chlorinated pyrimidine nucleoside was estimated to be around 0.05 lesion per 10^6 nucleosides. Incubation of the cells with a concentration of 300 μM HOCl for 10 min was found to induce a significant increase in the amounts of the chlorinated DNA and RNA nucleosides (Fig. 3). Interestingly, it was found that 5-CldCyd is the main lesion produced in DNA, whereas 8-ClGuo and 5-ClCyd were shown to be more efficiently generated in the RNA of treated cells. It may be pointed out that chlorination of adenine was found to be a minor process, both in RNA and DNA. Another point of major interest deals with the fact that RNA is a much better substrate for chlorination than DNA. Altogether, under the present experimental conditions, about 14 chlorinated DNA bases per 10^6 nucleosides are generated in the cells treated with 300 μM HOCl, while about 34 lesions per 10^6 nucleosides are detected in RNA. This could be explained by the important cytoplasmic localization of RNA that is more accessible than DNA. Another possibility deals with the fact that RNA is mostly single-stranded and unpacked compared to the double-stranded DNA which is highly condensed in the nucleus. Since it is well known that RNA is not, or at the best, partly repaired, it could be hypothesized that RNA lesions could represent better biomarkers in comparison with DNA lesions, even if the rapid turnover of RNA is expected to limit accumulation of lesions.

As demonstrated with the experiments involving HOCl treated cells, the HPLC–MS/MS method possesses the required sensitivity to measure low levels of chlorinated lesions. In addition, the already excellent specificity of tandem mass spectrometry could be increased for the detection of chlorinated DNA nucleosides since a second transition corresponding to the detection of the molecules containing the ^{37}Cl isotope could be monitored. As shown in Figs. 1 and 2, the presence of the chlorinated DNA lesions is further confirmed by the detection of the two transitions in the same HPLC peak at the expected retention time of the different nucleosides. This is observed for both DNA and RNA lesions.

Attempts were then made to determine the background levels of chlorinated nucleosides in the DNA isolated from human white blood cells. For this purpose, white blood cells were first separated using CPT vacutainers and the DNA was subsequently extracted using an optimized protocol developed for the measurement of 8-oxodGuo [20], in order to be able, in the near future, to simultaneously quantify both chlorinated and oxidized DNA lesions. One limitation of such an approach is that the latter protocol does not enable isolation of RNA; however work is in progress to resolve this limitation and to allow the detection of both oxidized and chlorinated RNA lesions in human lymphocytes. It should be noted that the Qiagen DNA extraction method used in the experiment performed with isolated cells (vide supra) has been shown to induce significant DNA oxidation during its isolation [20]. Unfortunately, the sensitivity of the present

assay was not good enough to allow the determination of the 8-chloropurine nucleosides, even if small peaks, corresponding to the limit of sensitivity of the assay (S/N = 3), were detected for 8-CldAdo. Therefore, the levels of 8-CldGuo and 8-CldAdo could be estimated below 0.2 and close to 0.02 lesions per 10^6 nucleosides, respectively, whereas, the level of 5-CldCyd is about 0.15 lesions per 10^6 nucleosides. The detected level of 5-CldCyd is significantly higher than that of chlorinated purine nucleosides, and above the limit of quantification of the assay (Fig. 4). The higher level of 5-CldCyd could be correlated to the fact that the chlorinated pyrimidine is more efficiently generated in cells treated with HOCl (vide supra). It may be noted that notable variations in the level of 5-CldCyd are observed between the different volunteers, with levels ranging from 0.06 up to 0.4 lesions per 10^6 nucleosides (not shown). However, the second transition used for the detection of 5-CldCyd (264 → 148) shows the presence of an intense peak detected eluting just after 5-CldCyd. This prevents the use of the latter transition to unambiguously confirm the presence of 5-CldCyd (not shown). Efforts will be made to overcome this limitation by improving the DNA extraction protocol. Such an improvement is also required in order to enable the simultaneous isolation of both RNA and DNA for the quantification of chlorinated and oxidized bases in human lymphocytes.

In conclusion, a very sensitive and specific HPLC–MS/MS assay has been set-up for the measurement of chlorinated DNA and RNA nucleosides. The developed method allowed us to show that incubation of cells in presence of HOCl induced chlorination of both DNA and RNA. 5-CldCyd was the main generated DNA lesion, whereas, chlorination of both Guo and Cyd occurs notably in RNA. Finally, the level of 5-CldCyd could be accurately determined in the DNA extracted from human white blood cells that were obtained from only 7 mL of blood. In the near future, attempts will be made to check whether the different chlorinated DNA and RNA lesions could be used as biomarkers of inflammation. The main objective of the work will be to simultaneously determine several nucleic acids lesions, including oxidized, chlorinated and lipid peroxidation adducts both in RNA and DNA and to use them, in human biomonitoring studies as potential biomarkers of oxidative stress and inflammation. For that purpose, efforts are currently made to improve protocols for RNA isolation.

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