3-Chlorotyrosine and 3,5-Dichlorotyrosine as Biomarkers of Respiratory Tract Exposure to Chlorine Gas*

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Abstract

Modification of tyrosine by reactive chlorine can produce both 3-chlorotyrosine (CY) and 3,5-dichlorotyrosine (dCY). Both of these amino acids have proven to be promising biomarkers for assessing the extent of myeloperoxidase-catalyzed chlorine stress in a number of adverse physiological conditions. To date, there has been no application of these biomarkers for determining the extent of exposure to environmentally present gaseous chlorinating chemicals. In this manuscript, we present a method using selective ion monitoring gas chromatography for the simultaneous analysis of both CY and dCY in nasal tissue excised from Fisher 344 rats exposed to varying concentrations of chlorine gas. Using this method, we were able to demonstrate the following: 1. a dose-dependent increase in the conversion of tyrosine to CY and dCY in the respiratory epithelium tissue; 2. preferential formation of CY and dCY in the respiratory and transitional epithelium versus the olfactory epithelium of the nasal cavity of the rat; and 3. similar rates of formation for CY and dCY when exposed to chlorine gas based on a strong [CY] versus [dCY] correlation (slope = 1.001, r^2 = 0.912).

Introduction

Environmental and occupational exposure to chlorine or chlorinating compounds occurs by various routes of administration including inhalation. Inhalation of chlorinating compounds at low doses will result in mild to moderate airway irritation (coughing, sore throat, etc.), and high to severe exposure may result in chest tightening, dyspnea, bronchospasm, non-cardiogenic pulmonary edema, and death (1,2). The mode of action for these chlorinating compounds in the respiratory tract results primarily from the corrosive nature of the secondary products [hydrochloric acid and hypochlorous acid (HOCl)] formed when chlorine undergoes hydrolysis with the water present in the respiratory tract lining (1,3,4). In addition to the listed physiological effects, permanent cellular and tissue damage may also occur. Modification by the addition of chlorine to a molecule occurs with every class of biologically important compound, resulting in a loss, decrease, or change in primary biochemical function of that compound. Although the biochemical and physiological modes of action for chlorinating compounds are fairly well recognized, information on the developmental, reproductive, or carcinogenic effects from long-term chronic inhalation exposures in humans is limited. Despite the need for these additional data, there are currently no specific clinical tests available that can determine the extent of environmental or occupational exposure to chlorine or chlorinating compounds.

Two known biomarkers of in vivo generated chlorine stress are the chlorine modified forms of tyrosine: 3-chlorotyrosine (CY) (5,6) and 3,5-dichlorotyrosine (dCY) (7). These biomarkers form through the electrophilic addition of chlorine to the meta position(s) of the tyrosine aromatic ring. These modified amino acids are ideal biomarkers of chlorine stress as they are known to be chemically stable to the heated acidic conditions required for their analysis (7). Because of their chemical stability, these biomarkers have been used for investigating the role myeloperoxidase-generated HOCl plays in the onset and/or progression of tissue damage associated with Parkinson's disease (8), Alzheimer's disease (9), atherosclerosis (10), asthma (11), and acute respiratory distress syndrome (12). Despite the broad range of applications researchers have found for CY (and to a lesser extent dCY), there are very little, if any, data where it is used to assess the extent or duration of an environmental or occupational exposure to chlorinating compounds.

As part of a larger project for assessing the distribution of in-

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haled reactive gasses within the nasal passage, we have developed a method with a sensitivity sufficient to detect an increase in CY (and dCY) levels above those observed in control samples. In this manuscript we present a sample work-up procedure coupled to a sensitive gas chromatography–mass spectrometric (GC–MS) method with isotope dilution internal standardization for the analysis of CY and dCY in respiratory (lateral wall and septum) and olfactory (septum and ethmoid turbinate) nasal tissues of rats exposed to chlorine gas (Cl_2). This method documented a statistically significant dose-dependent increase in CY and dCY in the nasal tissues of all Cl_2 exposure groups. In addition, a spatial distribution of CY and dCY was observed within the nasal cavity with preferential formation occurring in the lateral and septum respiratory epithelium tissues after inhalation exposures of rats to several parts-per-million of chlorine.

Materials and Methods

Apparatus

Processed samples were analyzed using an Agilent 6890 GC coupled to an Agilent 5973 inert mass selective detector (Agilent, Santa Clara, CA). Chromatographic separations were accomplished through the use of a capillary column (RTX-5MS, 30 m, 0.25-mm i.d., 0.25-µm film thickness, Restek, Bellefonte, PA) with the carrier gas, electronics-grade helium, set to 1.2 mL/min. The injection port was set to splitless, lined with a 2.0-mm internal diameter cyclo double gooseneck liner (Restek), and maintained at a temperature of 275°C. The GC temperature gradient used for the analysis was as follows: 1. initial temperature was set to 100°C and held for 0.5 min; 2. at 0.5 min, temperature was increased at a rate of 20°C/min; and 3. the final temperature, 300°C, was held for 2 min. Total run time was 13.75 min.

The mass selective detector was run in negative ion chemical ionization mode using ultra-high purity methane as the reagent gas. Temperature settings for the transfer-line heater, ion source, and quadrupole of the MS were 250°C, 150°C, and 150°C, respectively. Selective ion monitoring (SIM) of the derivatized amino acids was carried out on the following ions: tyrosine, 595 amu (M^--HF); [$^{13}C_9$]tyrosine, 604 amu (M^--HF); 3-chlorotyrosine, 629 amu (M^--HF); 3-[$^{13}C_9$]chlorotyrosine, 638 amu (M^--HF); and 3,5-dichlorotyrosine, 683 amu (M^--HF). The MS was programmed to conduct a SIM scan for tyrosine and its internal standard with an electron multiplier voltage (EMV) adjustment of –300V before switching over to the chlorinated tyrosine analytes with an increase in the EMV of +200.

Methods

Unless stated otherwise, all reagents purchased were of the highest purity available from Sigma Chemical (St. Louis, MO). All derivatization reagents used were of the highest purity available from Pierce Chemical (Rockford, IL). Isotopically labeled tyrosine ($^{13}C_9$) was purchased from Cambridge Isotope Laboratories (Andover, MA) (98% purity). Both the external standard for 3,5-dichlorotyrosine and the internal standard 3-[$^{13}C_9$]chlorotyrosine were synthesized at The Hamner Institutes for Health Sciences [95% purity by high-performance liquid chromatography (HPLC)].

Synthesis of 3-[$^{13}C_9$]chlorotyrosine and 3,5-dichlorotyrosine was based on a previously published method (7,10) with some modifications. Briefly, sodium hypochlorite is added to a solution of tyrosine [5 mL deionized (DI) water containing 10 mg of tyrosine] at a molar ratio of 1:1 for the 3-[$^{13}C_9$]chlorotyrosine and 2:1 for the 3,5-dichlorotyrosine. Both solutions are incubated at room temperature overnight. Following incubation, solutions are diluted 1:1 with 0.2% (v/v) trifluoroacetic acid in water and applied to a cation exchange

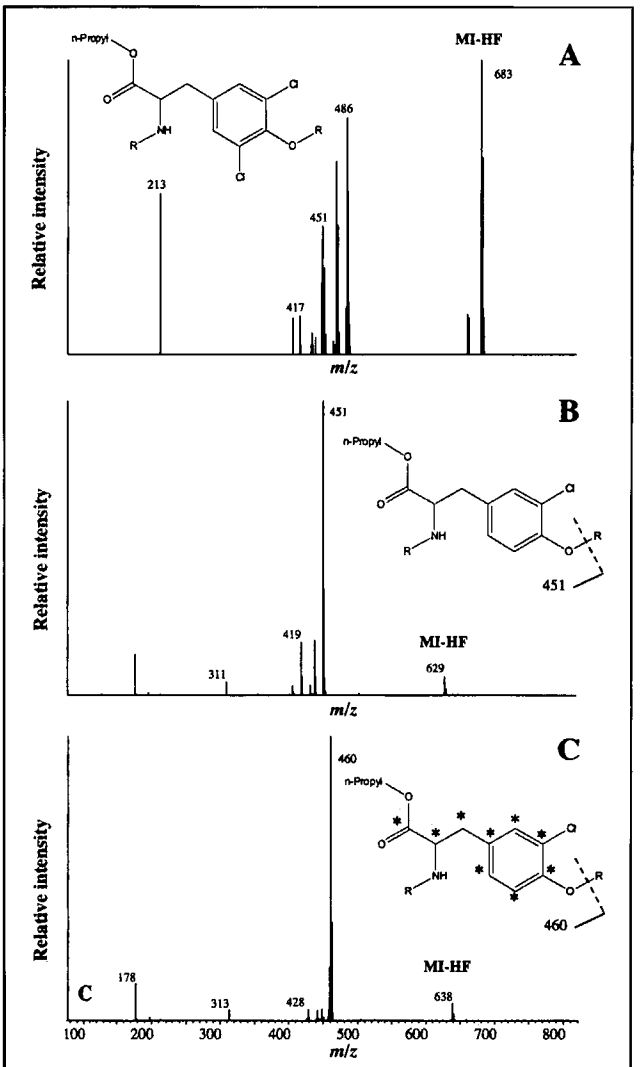

Figure 1. Mass spectral fragmentation pattern for 3,5-dichlorotyrosine (dCY) (A), 3-chlorotyrosine (CY) (B), and [$^{13}C_9$]-3-chlorotyrosine (IS) (C) using negative chemical ionization mass spectrometry with methane as the reagent gas. The most prominent ions were those resulting either from the loss of HF or the loss of the heptafluorobutyryl group from the molecular ion. Because selective ion monitoring of the *m/z* 451 ion produced noisy chromatograms (not shown), it was only used to confirm analyte elution times, and the *m/z* 629, 638, and 683 ions were used as the quantitative ions for CY, IS, and dCY, respectively. Quantitative ions for the analysis of tyrosine and [$^{13}C_9$]-tyrosine also corresponded to the M-HF ions.

column (Waters MCX Oasis, Waters, Milford, MA) previously equilibrated with methanol and 0.1% TFA. The loaded columns are washed twice with 1 mL of 0.1% TFA and eluted with 5 mL of 50% methanol. Eluents are dried under nitrogen, reconstituted in 4 mL of 30:70:0.1 acetonitrile/water/TFA, and further purified by preparative HPLC (Waters X-Terra C_{18}, 10 µm, 19 × 150 mm). Purified product was dried down in a centrifuge evaporator, reconstituted in 0.1% formic acid, and quantified by UV-vis spectroscopy (ε_{274} = 1879 $M^{-1}cm^{-1}$ for 3-chlorotyrosine and ε_{279} = 1368 $M^{-1}cm^{-1}$ for 3,5-dichlorotyrosine) (13,14). A sample of the suspected analyte peak(s) from the preparative HPLC step was derivatized (see Tissue collection and preparation section) and analyzed by GC–MS. The fragmentation pattern (negative chemical ionization, NCI) for commercially available 3-chlorotyrosine was compared with that of isotopically labeled 3-chlorotyrosine in order to confirm the identity of the internal standard (Figure 1). The identity of 3,5-dichlorotyrosine was inferred from the NCI fragmentation pattern of the preparative HPLC isolated peak believed to be the analyte of interest (Figure 1).

Tissue collection and preparation

The isolated upper respiratory tract (URET) of anesthetized male and female Fisher 344 (F344) rats were exposed for 90 min to various combination sets of exposure concentrations and unidirectional flow rates (15). Concentrations included 0.5, 1.0, and 2.5 ppm, which correspond to concentrations used in a two-year bioassay of chlorine (16). Flow rates were 100, 200, and 400 mL/min which bracket the typical ventilation rates for these rats (15). Nasal tissue samples were isolated from the septal respiratory epithelium (SRE), lateral wall respiratory epithelium (LRE), septal olfactory epithelium (SOE), and ethmoid olfactory epithelium (ETH) sections of the nose (15), placed in polypropylene screw-cap microcentrifuge tubes (1500 µL), and frozen immediately after necropsy in liquid nitrogen. Samples were stored at –80°C until analysis. Proteins isolated from the samples were obtained by transferring tissue samples to labeled polypropylene screw-cap microcentrifuge tubes (1500 µL) and adding 150 µL of DI water, 300 µL of methanol, and two 3-mm stainless steel bearings. Samples were then homogenized using a ball mill tissue homogenizer (MM 301, Retsch, Newtown, PA) set at 30 Hz for 3 min. Following homogenization, 700 µL of chloroform was added to each sample and vortex mixed. Samples were cooled for 15 min at ~4°C and centrifuged at 6000 × *g* and 4°C for 5 min. The protein in the sample precipitates and forms a solid disc suspended between the aqueous (top) and organic (bottom) layers. The protein disc was isolated by removing both the aqueous and organic layers using a glass pipette. Diethyl ether (700 µL) was added to each sample (to remove any additional lipophilic compounds), vortex mixed briefly, and centrifuged at 6000 × *g* and 4°C for 5 min. The diethyl ether layer was poured off the top and the resulting protein pellet was dried in a nitrogen evaporator. Dried protein samples were stored at –80°C until hydrolyzed.

Protein hydrolysis was carried out by first adding 500 µL of DI water and vortex mixing in order to create a protein suspension. Aliquots (250 µL) of reconstituted protein samples were transferred into 1-mL Reactivials (Pierce Chemical) along with 250 µL methane sulfonic acid (MSA, 6M) and internal standards ([$^{13}C_9$]tyrosine at 3000 ng/sample and 3-[$^{13}C_9$]chlorotyrosine at 30 ng/sample). Samples were purged with nitrogen, tightly capped, and placed in a 120°C heating block for 24 h. Upon completion, samples were diluted 1:10 with DI water and applied to a 96-well mixed mode cation exchange plate (Waters Oasis MCX LP, 60 µm, 60 mg) previously equilibrated with methanol followed by 0.3M MSA. The loaded column wells were washed twice with 1 mL of DI water and eluted with 2 mL of 2M pyridine in water into a 96-well deep plate. The deep well plate containing the eluent was dried at 40°C overnight in a 96-well plate nitrogen evaporator (GlasCol, Terre Haute, IN). Amino acid residues were then reconstituted in 200 µL of 2M pyridine in DI water, transferred to a GC vial, and dried in a centrifuge evaporator.

N-Propyl/heptafluorobutyryl derivatives of the amino acids were prepared in two steps. Hydrogen bromide (3.5M) in *n*-propanol was prepared by slowly adding 5.2 mL of acetyl bromide to 20 mL of ice cold anhydrous *n*-propanol while gently swirling the solution. Each sample was spiked with 150 µL of this solution, capped tightly, and incubated at 65°C for 1 h. Once the incubation time has elapsed, samples were dried in a centrifuge evaporator and spiked with 50 µL of a 1:3 solution of heptafluorobutyric acid anhydride in ethyl acetate. Samples were capped and allowed to incubate at room temperature for 1 h. After incubation, samples were completely dried down in a centrifuge evaporator, reconstituted in 100 µL of ethyl acetate, and analyzed by selective ion monitoring (SIM) GC–MS.

Data analysis and calculations

CY and dCY were quantified using an external calibration curve with $^{13}C_9$-CY as an internal standard for both analytes, whereas tyrosine was quantified using an external calibration curve and $^{13}C_9$-tyrosine as internal standard. Chromatograms for both standards and samples were integrated using Chemstation software (Agilent). Once all of the integrated sample areas were converted to analyte concentrations (represented as moles of each analyte), CY and dCY values were converted to percent of total tyrosine present using the following equations:

$$\%CY = \frac{[CY]}{[CY] + [dCY] + [Tyr]} \times 100\% \quad \text{(Eq. 1)}$$

$$\%CY = \frac{[dCY]}{[CY] + [dCY] + [Tyr]} \times 100\% \quad \text{(Eq. 2)}$$

Results and Discussion

A number of derivatization schemes involving combinations of esterification reactions (methyl, ethyl, and propyl alcohols) followed by various acetic anhydrides (trifluoroacetic, pentafluoroacetic, and heptafluoroacetic anhydride) were investigated. Although all of the alcohols investigated were essentially identical in derivatization efficacy, there was a noticeable difference in the acetic anhydrides selected. The

higher molecular weight acetic anhydrides produced chromatograms with fewer interfering peaks. As a result, the final derivatization procedure used in this method was very similar to a previously developed method (7) for the analysis of chlorotyrosine in human tissue samples. Figures 1A–1C illustrates the negative chemical ionization mass spectral fragmentation patterns for 3,5-dichlorotyrosine (dCY) (A), 3-chlorotyrosine (CY) (B), and [$^{13}C_9$]-3-chlorotyrosine (IS) (C). Additional, higher intensity ions representing the M^--HFB species of both the mono and dichlorotyrosine were observed and considered for this method; however, because there was a considerable amount of chromatographic interference associated with this ion (not shown), it was not selected as the analytical ion for quantitative analysis of the nasal tissue samples. Figure 2A illustrates typical chromatogram obtained using the selective ion monitoring mode on the GC–MS along with the sample work-up procedure outlined to analyze LRE tissue from a chlorine gas-exposed F344 rat. Figures 2B and 2C are enlarged views of the *m/z* 629 (CY) and 638 (dCY) extracted ion chromatograms superimposed onto a control LRE sample.

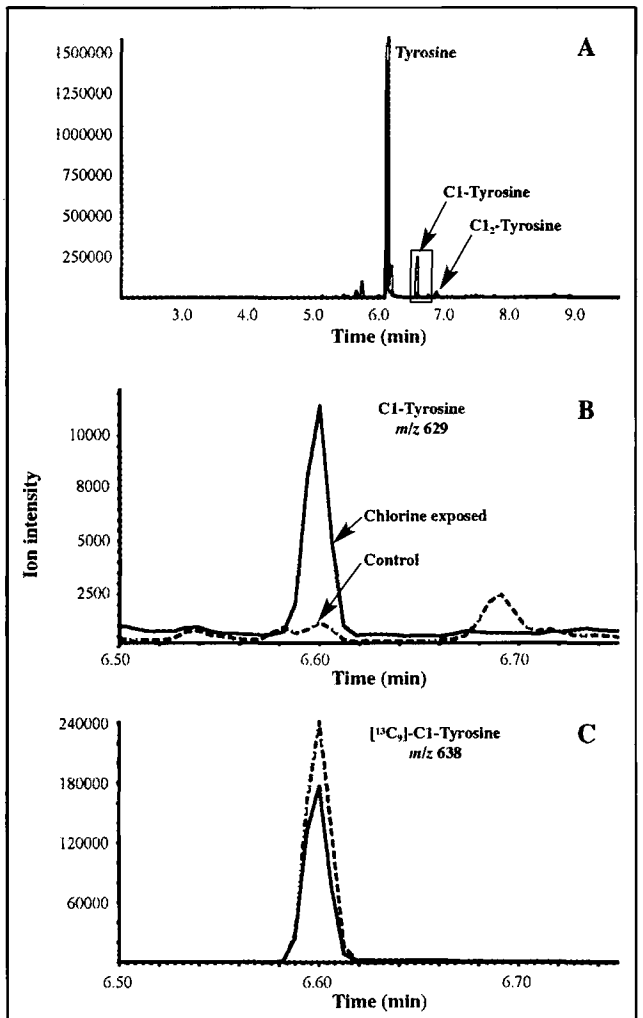

Figure 2. GC–MS chromatogram of a typical total ion chromatogram of a tissue sample (LRE) removed from a Fisher F344 rat exposed to 0.36 µmol (cumulative exposure) chlorine gas (A); select ion monitoring overlap of the *m/z* 629 ion (CY) in a treated (solid line) and control sample (dashed line) illustrates the specificity of this method as well as the lack of artifactual generation of CY during sample workup (B); and select ion monitoring overlap of the *m/z* 638 ion (IS) for the same samples (C). DCY (not shown) has a retention time of 6.9 min and does not have any interfering peaks present in control tissue. The peak present at ~6.69 min is a non-interfering peak of unknown identity.

Because there was no observable CY or dCY present in any of the control tissues, we assessed whether or not there was a need to work up the tissue samples using the outlined protein extraction method. In a separate study (data not shown) using B6C3F1 mice, samples were analyzed without the protein isolation step because of prohibitively small sample sizes. The chromatograms obtained from the mouse samples were markedly noisier compared to F344 rat samples analyzed for this manuscript, although the CY and dCY were still baseline resolved from any neighboring peaks. In addition, there was no CY or dCY artifacts formed in the control tissues of the F344 samples (Figures 2B and 2C) or the B6C3F1 samples (data not shown) as a result of sample work-up and the CY/tyrosine ratios calculated were comparable in range to previous investigations (10). Although previous experiments have shown that hydrolysis of proteins in hydrochloric acid forms CY as a by-product (10), the levels of free chlorine present in small tissue samples (such as nasal tissue from mice) do not appear to contribute significantly to the formation of CY or dCY. This observation implies that the current method, as outlined here, can be modified for the analysis of CY and dCY in much smaller sample sizes, such as those obtained from human nasal skin scrapings.

The limit of detection for both CY and dCY was determined using a propagation of error formula for chromatography (17),

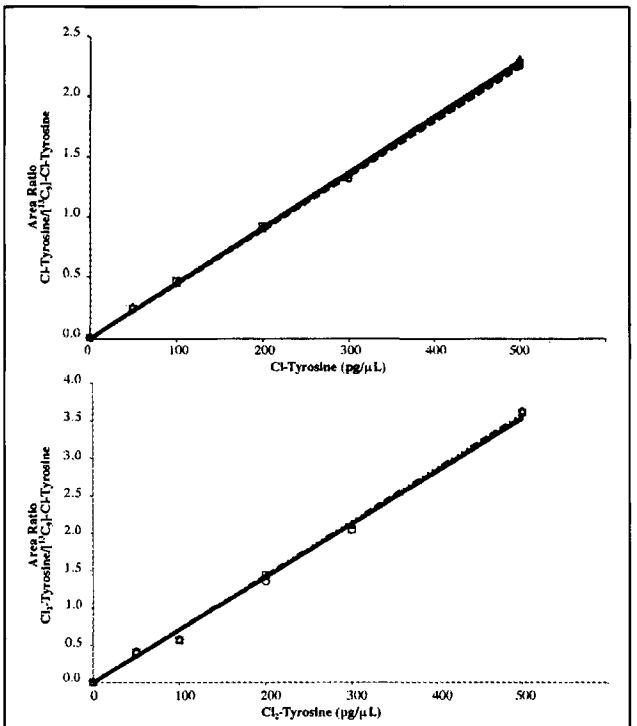

Figure 3. Triplicate analysis of calibration curves for CY and dCY. Standards were independently prepared (as described in the Methods section) and carried through the entire sample workup process prior to the analysis and data work-up represented in this figure.

the LOD for tyrosine was not determined. Calibration curves were prepared in triplicate for both CY and dCY with an operational range of 50–500 pg/µL along with a constant amount of $^{13}C_9$-CY. Using this technique, we calculated the LOD for CY to be 500 fg/µL (2.3 fmol on column) and for dCY 1100 fg/µL (4.4 fmol on column). These values were far below the lowest CY and dCY levels observed in any of the LRE or SRE rat tissues exposed to chlorine gas. Method linearity and ranges were assessed for CY, dCY, and tyrosine. Calibration curves were linear up to a 500 pg/µL for both CY and dCY and up to a 100 ng/µL for tyrosine. Replicate calibration samples prepared on different days produced a precision for the low middle and high end of the calibration curve below 5% (Figure 3).

Figure 4 shows percentage CY values obtained from the analysis of LRE and SRE tissues from rats exposed to known cumulative doses of chlorine. CY was detected in all of the chlorine exposed LRE tissue samples, while some of the samples at the low end of the chlorine exposure spectrum did not show any measurable amount of CY in the SRE. Observable amounts of CY were not present in either the SOE or the ETH tissues except in the rats with the exposures to the highest concentrations of chlorine (data not shown), and even then only SOE showed a small increase. This observation is consistent with previously investigated chlorine gas exposures (16). The likely mechanism involved in the removal of chlorine from the inhaled air stream is the airflow transfer and diffusion of chlorine gas into the moist mucous lining of the nasal passage followed by hydrolysis to hydrochloric and hypochlourous acid (14–16). Hypochlorous acid is believed to be the form of reactive chlorine which converts tyrosine to the mono and dichlorinated tyrosine species in addition to reactions of HOCl with many other biologically important compounds (18). Early re-

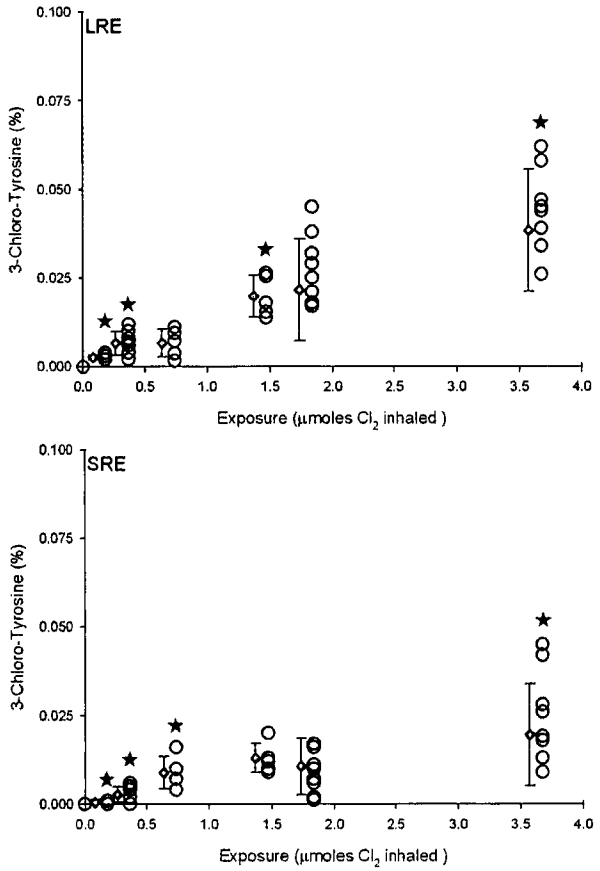

Figure 4. Analysis of CY in the LRE and SRE tissue samples of Fisher F344 rats exposed to known total chlorine gas concentrations. Cumulative chlorine exposures for the two tissue types were 0 (n = 5, 5 for LRE, SRE), 0.18 µmol (n = 4, 4), 0.36 µmol (n = 9, 9), 0.72 µmol (n = 5, 5), 1.44 µmol (n = 5, 5), 1.88 µmol (n = 10, 8), and 3.76 µmol (n = 10, 9) of chlorine gas inhaled per rat. Comparison of means was done through the use of a students t-test. The open-faced diamond symbols represent mean values with the error bars representing ± 1 SD. Values for the t-test were as follows for LRE: < 0.001 (0.18 µmol), 0.049 (0.36 µmol), 0.460 (0.72 µmol), 0.002 (1.44 µmol), 0.070 (1.88 µmol), 0.002 (3.76 µmol). Values for the t-test were as follows for SRE: 0.045 (0.18 µmol), 0.022 (0.36 µmol), 0.003 (0.72 µmol), 0.096 (1.44 µmol), 0.107 (1.88 µmol), 0.003 (3.76 µmol). Sample groups which are significantly higher ($p < 0.05$) than the previous group are marked with a star.

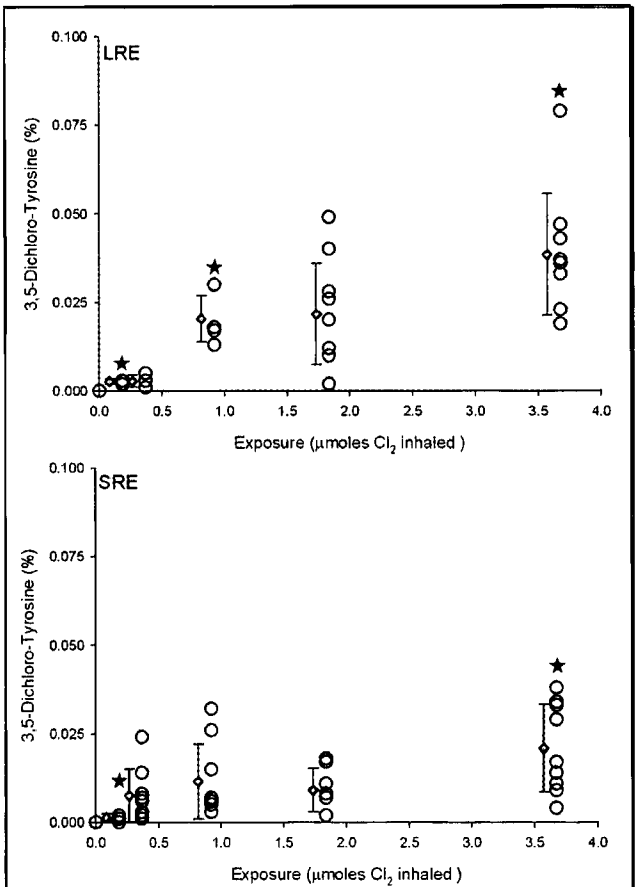

Figure 5. Analysis of dCY in the LRE and SRE tissue samples of Fisher F344 rats exposed to known total chlorine gas concentrations. Cumulative chlorine exposures for the two tissue types were 0 (n = 5, 5 for LRE, SRE), 0.18 µmol (n = 4, 4), 0.36 µmol (n = 4, 9), 0.92 µmol (n = 4, 9), 1.88 µmol (n = 10, 8), and 3.76 µmol (n = 10, 9) of chlorine gas inhaled per rat. Comparison of means was done through the use of a students t-test. The open faced diamond symbols represent mean values with the error bars representing ± 1 SD. Values for the t-test were as follows for LRE: < 0.001 (0.18 µmol), 0.404 (0.36 µmol), 0.002 (0.92 µmol), 0.391 (1.88 µmol), 0.016 (3.76 µmol). Values for the t-test were as follows for SRE: 0.010 (0.18 µmol), 0.066 (0.36 µmol), 0.177 (0.92 µmol), 0.288 (1.88 µmol), 0.014 (3.76 µmol). Sample groups which are significantly higher ($p < 0.05$) than the previous group are marked with a star.

moval of reactive chlorine (as well as any reactive gases) in the nasal passage would be advantageous from a diagnostic testing perspective. Obtaining tissue scrapings from the LRE or SRE sections of the nasal passage provides less of a problem compared to sampling from the SOE or ETH tissues in humans.

Figure 5 shows the dCY values for both LRE and SRE tissues under the same conditions as in Figure 4. Once again, there was no significant increase in the presence of dCY in the SOE and ETH samples until the highest concentrations (data not shown). The dCY values observed in the LRE and SRE were considerably higher than we were anticipating. Based on review literature (18) and previously published kinetic data (19), our original hypothesis was that dCY would take longer to form compared with CY and therefore we would see the rate of formation of dCY lag behind that of CY. Hypothetically, this would have allowed us to use CY for assessing acute chlorine exposures, while using dCY for assessing chronic or high dose exposures. However, it appears as though the dCY is formed at a similar rate as CY. In fact, plotting the CY data versus the dCY data in the LRE samples yielded a high correlation between both data sets (slope = 1.001, r^2 = 0.912, control samples were excluded).

Conclusions

Both analytes investigated in this paper appear to be good biomarkers of environmental exposure to chlorinating chemicals, with CY appearing to be slightly better than dCY based on its ability to consistently detect the effects of chlorine at the lowest exposures investigated. Increases of CY and dCY at the exposure concentrations of these studies appeared to be localized to the LRE and SRE sections of the nose making these areas an important focus for further methods development. Although there were some observable levels of CY in both the SOE and ETH tissues, in general these areas are not likely to play an important future role in the development of new and better methods for assessing acute chlorine exposure, although lesions in these regions are important for chronic risk assessment.

The method presented in this paper is only applicable to the use of determining exposure levels in laboratory animals, as the collection of samples for assessing human exposure using the current technique would be too invasive. Additional investigations need to be conducted on making this method less invasive and more "user friendly" for diagnostic purposes. Future work will involve identifying and quantifying new biomarkers of chlorine exposure from nasal mucous samples or skin scrapings; determining how persistent and versatile these new biomarkers of chlorine exposure are; and finding biomarkers of natural inflammation (e.g., chlorination resulting from polymorphonuclear leukocyte activity). Ideally, these yet to be identified biomarkers will be measured without the currently used time consuming steps of delipidation, acid hydrolysis, and derivatization. Although the method presented in this manuscript is not likely to be used as a diagnostic for human chlorine gas exposures, it does represent a good starting point for future method development and has value for applications in research of chlorine absorption in various regions of the respiratory tract.

Acknowledgments

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