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Simultaneous measurement of 3-chlorotyrosine and 3,5-dichlorotyrosine in whole blood, serum, and plasma by isotope dilution HPLC-MS/MS

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Abstract

Chlorine is a public health concern and potential threat due to its high reactivity, ease and scale of production, widespread industrial use, bulk transportation, massive stockpiles, and history as a chemical weapon. This work describes a new, sensitive, and rapid stable isotope dilution method for the retrospective detection and quantitation of two chlorine adducts. The biomarkers 3-chlorotyrosine (Cl-Tyr) and 3,5-dichlorotyrosine (Cl_2-Tyr) were isolated from the pronase digest of chlorine exposed whole blood, serum, or plasma by solid phase extraction (SPE), separated by reversed-phase HPLC, and detected by tandem mass spectrometry (MS/MS). The calibration range is 2.50–1000 ng/ml ($R^2 \geq 0.998$) with a lowest reportable limit (LRL) of 2.50 ng/mL for both analytes, an accuracy of ≥ 93%, and an LOD of 0.443 ng/mL for Cl-Tyr and 0.396 ng/mL for Cl_2-Tyr. Inter- and intra-day precision of quality control samples had coefficients of variation of ≤ 10% and ≤ 7.0%, respectively. Blood and serum samples from 200 healthy individuals and 175 individuals with chronic inflammatory disease were analyzed using this method to assess background levels of chlorinated tyrosine adducts. Results from patients with no known inflammatory disease history (healthy) showed baseline levels of <LRL-4.26 ng/mL Cl-Tyr and <LRL Cl_2-Tyr. Patients with inflammatory disease had baseline levels of <LRL-15.4 ng/mL Cl-Tyr and <LRL-5.22 ng/mL Cl_2-Tyr. Blood exposed to 2.02 ppm chlorine gas for 15 min produced 941 ng/mL Cl-Tyr and 223 ng/mL Cl_2-Tyr. This high-throughput method has been developed and analytically validated for the diagnosis of human exposure to chlorine.

Keywords

Chlorine gas; chlorine exposure; chlorine biomarkers; chlorinated tyrosine; LC-MS/MS; inflammatory disease; chronic inflammation

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Introduction

Molecular chlorine (Cl_2) is a yellow-green, dense gas that can be lethal when inhaled. Toxicity is due to formation of hydrochloric acid (HCl) and hypochlorous acid (HOCl) through hydrolysis in the moist linings of the airways (1). Low dose exposures (1–50 ppm) will cause mild to moderate irritation of the airways while higher doses (>500 ppm) are fatal (2). Chlorine gas is classified as a choking agent and a toxic dual-use chemical by the Organisation for the Prohibition of Chemical Weapons (OPCW) (3). Despite its vast industrial and commercial use, chlorine can be defined as a chemical weapon if its production, stockpiling, or use violates the Chemical Weapons Convention (CWC) (4).

Chlorine was the first chemical warfare agent successfully deployed on a massive scale 100 years ago. During World War I, the German Army released the dense gas to displace entrenched French division forces in Ypres on April 22nd, 1915 (5,6). Over 1000 soldiers were killed and thousands more were critically injured as a result of this single gas attack. There has been no reported use of chlorine gas as a chemical weapon since WWI until recent events in the Middle East. These events include the use of chlorine tanker trucks as bombs to release gas in 2007 during Operation Iraqi Freedom (7), and in 2014 the OPCW reported the use of chlorine as a chemical weapon in Syria (8).

Despite the significant threat of chlorine use in warfare, the greatest public health concern for acute exposure may be the industrial scale production and transportation of chlorine (9,10). Nearly 1400 accidental releases of chlorine gas were reported to the U.S. National Response Center in the last decade (11). The majority of these accidents were small scale and at fixed locations, however large scale, difficult to contain releases are most concerning and associated with the bulk quantities of chlorine transported by rail (7). Each year, several million tons of chlorine gas are produced by the electrolysis of brine and transferred by rail for industrial purposes in the United States (12). One of the worst recorded railway disasters released 40–60 tons of chlorine gas in Graniteville, SC, resulted in 9 fatalities, and overwhelmed local hospitals (13). Approximately 600 people sought medical attention within the first week and many required repeated emergency room visits (14). In order to effectively identify, triage, and monitor possibly affected individuals in these large-scale emergencies, an appropriate assay to confirm exposure should be rapid and measure a persistent biomarker.

Chlorine is known to form stable adducts to tyrosine (Tyr) (15,16). These adducts can be used as biomarkers to confirm exposure in specimens collected days after a large-scale chlorine release. Oxidation of Tyr with chlorine can occur via electrophilic aromatic substitution and *N*-chlorination. The phenol group directs aromatic substitution at *ortho* positions resulting in 3-chlorotyrosine (Cl-Tyr) (Figure 1A) as a stable intermediate and 3,5-dichlorotyrosine (Cl_2-Tyr) (Figure 1B) as the final aromatic substitution product. *N*-Chlorination can occur on the amine (free circulating Tyr) or on the amide (protein incorporated Tyr). The former produces a complex mixture of reactive and stable products including chloramines, chloro-imines, nitriles, and aldehydes (15,17) while the latter is significantly less reactive by comparison (15,18).

Cl-Tyr has been extensively studied as a biomarker for inflammation and oxidative tissue damage resulting from neutrophil myeloperoxidase in cases of chronic inflammatory disease (16,19–22). Specifically, increased levels of Cl-Tyr have been linked to renal failure (23), atherosclerosis (22), myocardial infarction (24), and cystic fibrosis (25). The higher order chlorination product Cl_2-Tyr is produced at lower concentrations but may prove to be the more specific biomarker when separating acute chlorine exposure from chronic inflammatory disease (26). Both biomarkers were readily detected in rat nasal tissue following exposure to chorine gas (27). Cl-Tyr has been predominately measured by GC-MS (19,21–27) and HPLC-UV (17,20) though one study compared GC-MS/MS and HPLC-MS/MS (28); however, Cl_2-Tyr has only been measured by GC-MS (25–27). To date, there has been no report of the simultaneous measurement of Cl-Tyr and Cl_2-Tyr in whole blood, serum, or plasma by HPLC-MS/MS. We present a newly developed and fully validated method for the simultaneous detection and quantitation of Cl-Tyr and Cl_2-Tyr in whole blood, serum, and plasma with a run time of only 5 min. The chlorotyrosine adducts are readily detected by HPLC-MS/MS using only 50 μL of sample following digestion by pronase and purification by solid phase extraction (SPE). This method was designed to be rugged and transferable to increase the capabilities of U.S. regional response laboratories.

Experimental

Reagents and Supplies

The following materials were purchased from Fisher Scientific (Hanover Park, IL): ammonium bicarbonate, HPLC-grade acetonitrile, HPLC-grade methanol, heat sealing foil, 96-well conical bottom plates, and Optima grade trifluoroacetic acid (TFA). The following materials were purchased from Sigma-Aldrich (St. Louis, MO): Protease Type XIV from *Streptomyces griseus* (pronase E, lot no. 128K1388, SLBH4482V, SLBD5482V, and SLBP0477V), LCMS grade formic acid, 3-chloro-L-tyrosine (Cl-Tyr), and sodium hypochlorite solution (active chlorine: 4.00–4.99 wt.%). Oasis® HLB 96-well (60 mg, 60 μm) SPE plates were purchased from Waters (Milford, MA). Adhesive PCR foil and 96-well PCR plates were purchased from Eppendorf (Hauppauge, NY). The internal standard $^{13}C_6$-3-chloro-L-tyrosine ($^{13}C_6$-Cl-Tyr) was purchased from Cambridge Isotopic Laboratories (Andover, MA). Synthetically prepared 3,5-dichloro-L-tyrosine (Cl_2-Tyr) and $^{13}C_9$,^{15}N-3,5-dichloro-L-tyrosine ($^{13}C_9$,^{15}N-Cl_2-Tyr) were purchased from IsoSciences, LLC (King of Prussia, PA). Pooled plasma for preparing quality control (QC) materials was purchased from Tennessee Blood Services (Memphis, TN). All samples used for preparing QC materials were screened by each vendor for hepatitis B, hepatitis C, *Treponema pallidum*, and HIV in agreement with FDA regulations.

Preparation of Calibrators and Quality Controls

Individual stock solutions for each native and isotopically labeled standard (1.00 mg/mL) were prepared in HPLC-grade water and stored at −70 °C. Native stock solutions were combined and diluted to prepare eight calibration levels at a final concentration for each analyte of 2.50, 5.00, 10.0, 25.0, 50.0, 100, 500 and 1000 ng/mL in HPLC-grade water. Isotopically labeled stock solutions were combined to prepare a single internal standard

(ISTD) solution at a final concentration of 250 ng/mL for $^{13}C_6$-Cl-Tyr, and 500 ng/mL for $^{13}C_9$,^{15}N-Cl_2-Tyr. Calibration and ISTD materials were stored at −70 °C until use.

Six QC samples, Cl-Tyr QC low (Cl-Tyr QCL), Cl-Tyr QC medium (Cl-Tyr QCM), Cl-Tyr QC high (Cl-Tyr QCH), Cl_2-Tyr QCL, Cl_2-Tyr QCM, and Cl_2-Tyr QCH, were prepared by spiking serum with a sodium hypochlorite (NaOCl) solution containing 5.22% active chlorine. The percentage of active chlorine in the NaOCl solution was determined using a previously published protocol (29). The amount of NaOCl solution added to prepare the Cl-Tyr QCL, QCM, and QCH was 8.0, 16, and 40 μL, respectively. To prepare the Cl_2-Tyr QCL, QCM, and QCH materials, 56, 80, and 160 μL of NaOCl solution was added, respectively. For all QCs, deionized water was added for a total added volume of 160 μL. QC materials were stored at −70 °C until use. Twenty independent measurements were used to characterize the Cl-Tyr and Cl_2-Tyr analytes in the QC materials.

Preparation of Chlorine Gas Exposed Materials for use as Quality Control Samples

Cl_2 gassed materials were produced in-house by exposing 1 mL of blood, serum, or plasma to 2.02 ppm, 670 ppb, or 330 ppb Cl_2 gas for 15 min. Gas generation was carried out inside of a small, open chamber located in a larger sealed vessel which contained serum, plasma, or blood. Cl_2 gas was created by addition of excess HCl to an uncapped 2.0 mL Nalgene cryotube containing 5.0 μL of 0.03%, 0.01%, or 0.005% active chlorine NaOCl suspended above the sample inside a 15 mL centrifuge tube. The cryotube served as gas-generation chamber keeping all the reactant (NaOCl and HCl) from directly contacting the sample. The 15 mL centrifuge tube was capped, and a 22 gauge syringe was used to pierce the side of the centrifuge tube and inject 100 μL of 2M HCl into the uncapped cryotube. The syringe was immediately removed and the hole from the piercing was covered with Parafilm. The liberated Cl_2 gas filled the cryotube and overflowed into the sealed centrifuge tube, reacting with the sample. The reaction proceeded for 15 min after which the exposed sample was transferred to an empty cryotube. Exposure levels were determined using the fixed volume of the sealed vessel and the complete conversion of NaOCl to Cl_2.

Sample Preparation

Fifty microliters of sample (plasma, serum, or whole blood), calibrator, or QC was transferred to a 2 mL conical bottom 96-well plate. Twenty microliters of ISTD, 200 μL of ammonium bicarbonate (50 mM, pH 7.9), and 100 μL of a pronase solution (10 mg/mL in ammonium bicarbonate, pH 7.9) were then added to each well containing sample, calibrator, or QC. The plate was sealed with adhesive foil and incubated at 50 °C for 75 min with shaking at 1000 rpm. The digested samples were adjusted to pH 1 with 100 μL of TFA and shaken for 5 min at 500 rpm. The entire sample (~470 μL) was added to an HLB SPE 96-well plate that was first conditioned with methanol (1 mL) followed by water (1 mL). The sample was washed with 2% methanol in water (1 mL) and eluted with methanol (1 mL) into a clean 96-well receiving plate. All samples were evaporated to dryness under nitrogen at 50 °C using a Porvair Ultravap (Porvair Sciences, Leatherhead, U.K.). The dried samples were reconstituted with 0.1% formic acid in HPLC-grade water (100 μL), transferred to a 96-well PCR plate, and heat sealed with pierceable foil.

UHPLC-MS/MS

The chromatographic system used was an Agilent 1290 with a binary pump, refrigerated autosampler, and temperature controlled column compartment (Santa Clara, CA). Separation was performed on a Hypercarb 3 μm, 2.1 × 30 mm analytical column (Thermo Scientific, Hanover Park, IL) at 60 °C. Initial chromatographic conditions were 98% mobile phase A (MPA, 0.1% formic acid in HPLC-grade water) and 2% mobile phase B (MPB, 0.1% formic acid in acetonitrile) at a flow rate of 250 μL/min. Following a needle wash of 6s with MPB, samples were injected (1 μL). A 1 min hold was followed by a 3 min linear gradient to 2% MPA and 98% MPB and held for 1 min. Initial mobile phase solvent conditions were then returned at the end of the 5.01 min chromatographic method. The gradient delay volume was determined to be 105 μL in loop mainpass mode and 65 μL in loop bypass mode. To reduce the gradient delay volume and potential for loop carryover, the autosampler state was set to bypass mode at 5 s (20 × injection volume, 20 μL) following sample injection.

The HPLC was coupled with an Agilent 6490 triple quadrupole mass analyzer with a Jet Stream ESI source operating in positive ionization mode. Data were acquired with Mass Hunter 6.0 in selected reaction monitoring (SRM) mode recording the two most selective and sensitive transitions for the native product (quantitation [quant] and confirmation [conf] transitions) and a single transition for the corresponding isotopically labeled products. Ionization source parameters were optimized by post column infusion of a 100 ng/mL solution of Cl-Tyr in HPLC-grade water at a flow rate of 5 μL/min. During the infusion experiment, the LC pump was set to deliver 53% MPB at 245 μL/min, corresponding to the approximate solvent composition in source at the elution time of Cl-Tyr, factoring in gradient delay, column void, and post column volume. The optimized source settings were: 200 °C sheath gas at 11 L/min, 290 °C drying gas at 11 L/min, nebulizer gas at 60 psi, nozzle voltage at 300 V, capillary voltage at 1000 V, and fragmentor voltage at 280 V. The ion funnel low and high pressure RF voltages were 80 and 125 V, respectively. Transition specific parameters were individually optimized for each native and labeled Tyr adduct. A cell accelerator voltage of 2 V was optimal for all transitions while the collision energy (CE) was dependent on the product ion produced (Table 1). All transitions were recorded at 20 ms, resulting in 12–20 points across each chromatographic peak.

Calibration curves were constructed by plotting the response ratio (analyte area divided by ISTD area) versus the expected calibrator concentration. Linear regression was used to fit the data with 1/x weighting as determined by residual analysis; the origin was not forced through zero. The peak area ratio of the quant to conf transitions were used to calculate the confirmation ion ratio (CIR), a value established for each analyte and used to confirm detection.

Method Validation

Data from 20 replicate calibration curves and QC sample sets were evaluated to assess accuracy, precision, linearity, lowest reportable limit (LRL), and limits of detection (LOD) for each analyte. Three scientists prepared and analyzed no more than two runs per day over the span of two months using two separate lots of pronase. Inter-day precision was calculated from 20 individual preparations of QC samples over two months. Intra-day

precision was calculated from eight individual preparations of each QC level within a single batch. The LOD was calculated for each analyte as described by Taylor (30). Briefly, a plot of the analyte concentration versus the standard deviation of the four lowest calibrator concentrations was used to extrapolate the standard deviation of a blank sample. Method ruggedness was investigated for five digestion parameters; temperature, time, pronase concentration, buffer concentration, and buffer pH, which are expected to have the greatest impact on method variance. Cl-Tyr and Cl_2-Tyr synthetic standards and QC materials stored at −70 °C were monitored for five months to assess stability. Additionally, an accelerated stability study was conducted for two weeks on QC materials stored at 37, 23, 4, and −20 °C and included an experiment to test three freeze and thaw cycles. Finally, extracted samples were stored in the instrument autosampler at 4 °C and analyzed over five days.

Matrix Effects and Extraction Recovery

Matrix effects and extraction recovery were evaluated by a pre/post-SPE spiking experiment performed in triplicate. Blank serum was processed through the digestion steps and split into two samples prior to SPE, identified as the pre-and post-spiked samples. Prior to SPE, the pre-spike sample was fortified with 50 μL of the 10.0 ng/mL standard, and the post-pike sample was fortified with 50 μL of water. Both samples were processed by SPE, then dried. The pre-spike extract was reconstituted in 50 μL of water and 50 μL of a 10.0 ng/mL ISTD solution. The post-spike extract was reconstituted in 50 μL of the 10.0 ng/mL standard and 50 μL of a 10.0 ng/mL ISTD solution. These samples were analyzed and compared to a matrix-free solution containing 50 μL of the 10.0 ng/mL standard and 50 μL of a 10.0 ng/mL ISTD solution, unprocessed by the method. The average of the response ratio of the pre-spike sample was compared to that of the post-spike sample to assess extraction recovery:

$$\text{extraction recovery (\%)} = \frac{\text{av response ratio of pre-spike sample}}{\text{av response ratio of post-spike sample}} \times 100$$

The average peak areas of the native and isotopically labeled post-spike samples were compared to that of the matrix free solution to assess the matrix effect in terms of relative ionization efficiency:

$$\text{relative ionization efficiency (\%)} = \frac{\text{av peak area of post-spike sample}}{\text{av peak area of matrix free sample}} \times 100$$

This process was used to evaluate conditioning, loading, washing, and elution on several SPE phases during method development.

Convenience Samples

A convenience set of 100 serum and 100 blood samples was purchased from Tennessee Blood Services to assess background levels of each analyte in volunteers with no reported inflammatory disease (healthy). A total of 175 serum and blood samples collected from patients with declared disease states were purchased from BioreclamationIVT (Baltimore, MD), Bioserve (Beltsville, MD), Precision-Med (Solana Beach, CA), and Discovery Life

Sciences (Los Osos, CA). Thirty-five samples for each of the following disease states were purchased: rheumatoid arthritis (RA), atherosclerosis (ATH), cystic fibrosis (CF), cardiovascular disease (CVD), and inflammatory bowel disease (IBD). This study used de-identified serum, plasma, and blood acquired from commercial sources, and thus, the work was determined not human subjects research as specified in 45 CFR 46.102(f).

Safety Considerations

Chlorine adducts to tyrosine are not expected to pose a risk greater than that of free tyrosine. Universal safety precautions for handling biological samples should be strictly adhered to at all times when handling blood products. Chlorine gas generation should be performed in a chemical fume hood by trained personnel.

Results and Discussion

Sample Preparation Optimization

In order to capture the greatest amount of Cl-Tyr and Cl_2-Tyr from a specimen, protein isolation procedures were eliminated from the sample preparation. Removal of these steps provided access to free circulating biomarkers and allowed for addition of the ISTD directly to the pre-processed specimen thereby normalizing all subsequent sample handling steps.

We previously developed a robust protein digestion protocol using pronase to release organophosphorus nerve agent-tyrosine adducts (OPNA-Tyr) in whole blood, serum, and plasma (31). The same protocol proved to be near optimal for releasing chlorotyrosine adducts. Digestion time point studies of serum exposed to Cl_2 gas *in vitro* indicated that digestion was complete in 75 min, slightly faster than observed for OPNA-Tyr. No changes to the enzyme concentration, digest temperature, pH, or buffer concentration were necessary.

Unmodified, digested samples extracted by anion exchange or mixed mode anion exchange SPE had low recovery. Extraction by hydrophilic interaction chromatography (HILIC) on silica worked well but required a lengthy solvent exchange step by evaporating the aqueous buffered digest and reconstituting in acetonitrile prior to extraction. Alternatively, lowering the pH of the digested samples from 8 to 1 with TFA and extracting the acidified samples on a hydrophilic lipophilic balance (HLB) sorbent produced comparable recoveries to HILIC at a significant time savings.

Total sample digestion relies on reproducibly releasing all potential Cl-Tyr and Cl_2-Tyr from a wide variety of proteins and peptides in any blood matrix. Therefore, the reproducibility of digestion was extensively studied to determine rugged and robust conditions. QC materials were processed in triplicate at ±20% of the nominal digestion temperature, pH, time, enzyme concentration, and buffer concentrations. The resulting values were compared to the QC values established during method validation. All variable conditions produced QC values within a single standard deviation of the mean characterized value for Cl_2-Tyr and within acceptable QC limits for Cl-Tyr. Furthermore, the calculated QC values obtained from digestion with four separate lots of pronase were indistinguishable. These findings indicate a reproducible and robust sample digestion.

In order to receive and process all blood product samples with a single method, a comparison of the sensitivity and quantitation equivalency was conducted. Matrix matched calibration curves were prepared in triplicate for whole blood, serum, plasma, and water by fortifying blank matrix or water with Cl-Tyr and Cl_2-Tyr at known concentrations. These samples, along with QC materials, were digested and extracted according to the validated method and processed as individual matrix calibration curves and as unknowns calculated from the calibration curve in water. The mean calibration curve slopes from serum (Cl-Tyr = 0.00756, Cl_2-Tyr = 0.0110), plasma (Cl-Tyr = 0.00738, Cl_2-Tyr = 0.0106), and whole blood (Cl-Tyr= 0.00716, Cl_2-Tyr= 0.0108) were found to be within 6% of the mean slope of the matrix free (water) curve (Cl-Tyr: 0.00750, Cl_2-Tyr: 0.106). Despite low levels of endogenous Cl-Tyr in each matrix, the accuracy of the individual levels was within 15% of the expected value for all levels in all matrices when calculated from the matrix free curve. Finally, the QC materials were quantified separately from each curve, and results were within the acceptable limits established during the characterization of the materials. As a result, whole blood, serum, and plasma can be reliably processed and quantified in a single batch from a calibration curve prepared in any of the tested matrices provided the endogenous Cl-Tyr in the chosen matrix is < 1.00 ng/mL. For simplicity, the final calibration curve of the method was prepared in water.

Matrix Effects and SPE Recovery

Matrix effects were evaluated by examining the relative ionization efficiency under varying source, chromatographic, and extraction conditions. SPE recovery along with the relative ionization efficiency were assessed to determine optimal method performance. After digestion with pronase, pooled serum was fortified with 10.0 ng/mL Cl-Tyr and Cl_2-Tyr either before or after SPE and 10.0 ng/mL ISTD after SPE to evaluate the SPE recoveries and relative ionization efficiencies. SPE recoveries for Cl-Tyr and Cl_2-Tyr were 67% and 77% while relative ionization efficiencies were 16% and 54%, respectively. Despite the low relative ionization efficiency for Cl-Tyr, the validated assay could detect Cl-Tyr in nearly every individual sample tested, regardless of health status or blood matrix format.

HPLC-MS/MS Optimization

Retention and separation of Cl-Tyr and Cl_2-Tyr was achieved on pentafluorophenyl (PFP) and porous graphite carbon (PGC) columns. The PGC column was selected due to the extended lifetime under final analytical conditions and stable retention times (Cl-Tyr, 2.51 min [2.01% CV]; Cl_2-Tyr, 2.99 min [2.80% CV]. A reversed phase gradient reaching a final concentration of 98% acetonitrile effectively cleaned the column between samples. Figure 2A shows an example of the resulting chromatographic separation of the 50.0 ng/mL calibration standard. The final separation produced a gradient retention factor (k^*) of 2.8 and provided baseline separation between Cl-Tyr and a closely eluting isobaric interferent in the 216.0 → 135.1 transition (Figures 2B–F). The total time from injection to injection was 5.5 min.

Product ion scans were collected for the native and isotopically enriched Cl-Tyr and Cl_2-Tyr at collision energies of 5, 10, and 30 eV, and the resulting spectra were summed for representation (Figure 3). The precursor ion for each compound was the ^{35}Cl isotope(s) of

the protonated molecular ion. Fragmentation was consistent between Cl-Tyr and Cl_2-Tyr with loss of HCOOH producing the most abundant fragments 170.0 m/z (Figure 3A) and 204.0 m/z (Figure 3C), respectively. Additional loss of ^{35}Cl gives the more specific product ions used for quantitation (135.1 m/z for Cl-Tyr and 169.0 m/z for Cl_2-Tyr). In both cases, the transitions associated with the more abundant loss of HCOOH had significantly higher backgrounds and were not ideal for quantitation at low concentrations. The isotopically enriched chlorotyrosines (Figures 3B and 3D) produced fragmentation spectra equivalent to their corresponding native analogues. We verified loss of chlorine radical by fragmenting the $[M+H]^+$ ions for ^{37}Cl-Tyr, $^{37}Cl^{35}Cl$-Tyr, and $^{37}Cl_2$-Tyr. Fragmentation of ^{35}Cl-Tyr (216.0 m/z) (Figure 3A) and ^{37}Cl-Tyr (218.0 m/z) both yield the 135.1 product ion. Fragmentation of $^{37}Cl_2$-Tyr (254.0 m/z) gives the 171.0 product ion while fragmentation of $^{37}Cl^{35}Cl$-Tyr (252.0 m/z) shows both 171.0 and 169.0 product ions, and fragmentation of Cl_2-Tyr (m/z 250.0) yields the 169.0 product ion (Figure 3C).

Linearity, Accuracy, Precision, and LOD

Twenty replicate calibration curves were prepared and processed over the course of two months for method validation (Table 2). The linear range of 2.50 to 1000 ng/mL had a coefficient of determination value (R^2) ≥0.998 for Cl-Tyr and Cl_2-Tyr. The mean accuracies were within 7% at 5.00 ng/mL, 3% at 50.0 ng/mL, and 1% at 500. ng/mL of the expected value. Inter-day precision was within 9% CV at 5.00 ng/mL, 8% CV at 50.0 ng/mL, and 5% CV at 500. ng/mL. The inter-day precision was determined for the QC materials (Table 3) and found to be between 6 - 11% for both analytes. Intra-day precision was determined with the same QC material and determined to be ≤ 7.5% for all levels. The LOD values (Table 2) were calculated using a modified Taylor method of extrapolating the standard deviation of the blank from a plot of measured standard deviations at low concentration levels. The LRL was above the LOD thereby providing accurate and reliable quantitation down to the lowest calibration standard (2.50 ng/mL). The LRL corresponds to 2.5 pg on column (11.6 fmol for Cl-Tyr and 10.0 fmol for Cl_2-Tyr) at the injection volume of the method. Confirmation ions were recorded, and the CIRs were calculated to be 2.27 (± 0.24) and 2.60 (± 0.22) for Cl-Tyr and Cl_2-Tyr, respectively. Confirmation of Cl-Tyr was unreliable below 10.0 ng/mL due to the high background in the 216.0 → 170.0 transition in blood, serum, and plasma; however, Cl_2-Tyr was confirmed throughout the entire quantitation range.

Stability

QC materials were stored for two weeks at 37, 23, 4, and −20 °C and compared to the characterized QC lot stored at −70 °C. The concentrations of Cl_2-Tyr were within the characterized limits for the QC materials, regardless of storage condition. Cl-Tyr stored at 37 °C for two weeks was 25% lower than the expected value while all other storage temperatures were within the characterized limits. This suggests that Cl_2-Tyr may be the more reliable biomarker for exposure confirmation for samples not immediately collected following an acute exposure to chlorine. There was no change in calculated concentration of Cl-Tyr or Cl_2-Tyr after three freeze/thaw cycles. Additionally, prepared samples stored in the HPLC autosampler at 4 °C were re-injected daily for five days with no significant change in signal or concentration for either analyte. Finally, synthetic standards used for calibration

and samples from the characterized QC lot used for these stability studies have been routinely analyzed for five months and show no signs of instability.

Chlorine Exposed Materials and Convenience Samples

Inflammatory diseases have previously been associated with elevated levels of Cl-Tyr and Cl_2-Tyr,(16,19–25) a result of HOCl produced by neutrophil myeloperoxidase (Figure 2D–F). Blood and serum from healthy donors were analyzed by the reported method and compared to blood and serum from patients with select inflammatory diseases. The resulting data was ranked by quartile for each group (Figure 4). Samples with detectable levels of Cl-Tyr and/or Cl_2-Tyr between the LOD and LRL were included for the purpose of this ranking comparison. Within the healthy group (n=200), Cl-Tyr was detected in >90% of the samples yet only one specimen had a detectable amount of Cl_2-Tyr. Concentrations of Cl-Tyr and Cl_2-Tyr were elevated among individuals with selected inflammatory disease and statistically different from the healthy group. The average Cl-Tyr:Cl_2-Tyr ratio was 3.97 ± 3.27 and consistent with previous reports.(17,26).

Pooled blood, serum, and plasma were exposed to Cl_2 gas and NaOCl *in vitro* to produce QC materials for method development and validation. The calculated concentrations of Cl-Tyr and Cl_2-Tyr and the Cl-Tyr:Cl_2-Tyr ratio varied considerably among matrix, exposure concentrations, and exposure methods for these materials. Generally, the concentrations of Cl-Tyr and Cl_2-Tyr were two orders of magnitude higher in matrix with added NaOCl compared to matrix exposed to chlorine gas (normalized to % active chlorine). The concentration of Cl-Tyr exceeded that of Cl_2-Tyr for all samples indicating chlorine was consumed by other reactions and/or not in excess of available Tyr. Representative chromatograms for pooled serum before and after a 15 min exposure to 370 ppb Cl_2 are shown in Figure 2B and C, respectively. Low levels of Cl-Tyr were detected in the matrix pools prior to addition of Cl_2; this was later confirmed to be consistent with the convenience set of specimens from 200 individuals with no reported inflammatory disease. Cl_2-Tyr was not detected in the matrix pools prior to chlorine exposure.

In vivo exposure studies in rats have previously indicated that Cl-Tyr and Cl_2-Tyr are readily observed in nasal tissue following 90 min exposure to 0.5–2.5 ppm chlorine gas (27). We simulated an acute Cl_2 exposure in human clinical specimens by combining NaOCl with excess HCl in a sealed chamber containing a separate vessel of serum, plasma, or blood. The samples were exposed to either 370 ppb, 670 ppb, or 2.02 ppm chlorine gas for 15 min and produced values of Cl-Tyr and Cl_2-Tyr well above those observed in the disease state samples. Cl-Tyr was measured at 593 ng/mL in plasma, 941 ng/mL in blood, and estimated at 2580 ng/mL in serum following exposure to 2.02 ppm Cl_2, a level associated with the onset of symptoms (2). Cl_2-Tyr was measured at 273 ng/mL in plasma, 223 ng/mL in blood, and 527 ng/mL in serum for the same samples. The average Cl-Tyr:Cl_2-Tyr ratio was 3.77 ± 1.40. Although these samples only represent a simulated exposure and cannot account for such variables as uptake and metabolism, we anticipate acute exposure to chlorine to produce levels of Cl-Tyr and Cl_2-Tyr that are readily distinguishable from healthy and chronic inflammatory disease specimens.

Conclusion

A new method for the simultaneous detection of Cl-Tyr and Cl_2-Tyr as biomarkers of potential chlorine exposure has been developed and analytically validated. This method was designed and ruggedized for transferability and intended for use by emergency response laboratories. The method eliminates protein enrichment while providing high accuracy, high precision, and low limits of quantitation using only 50 μL of whole blood, plasma, or serum. Preliminary testing indicates this method should be amenable to bronchoalveolar lavage fluid, saliva, and other clinical tissues. A set of 96 samples can be processed and ready for analysis in less than 2 hours. Synthetic calibrators and chlorine exposed QC materials are stable for at least five months when stored at −70 °C. The method was used to characterize concentrations of Cl-Tyr and Cl_2-Tyr in clinical samples exposed to Cl_2 gas and NaOCl *in vitro*, samples from healthy donors, and samples from patients with chronic inflammatory disease.

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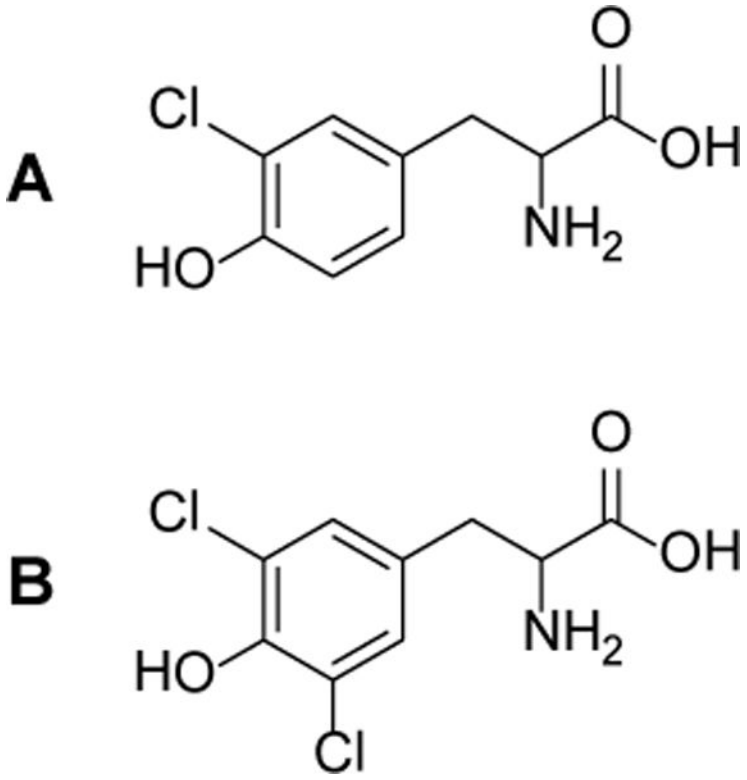

Figure 1.
Structures of 3-chlorotyrosine (Cl-Tyr) (**A**), 3,5-dichlorotyrosine (Cl_2-Tyr) (**B**)

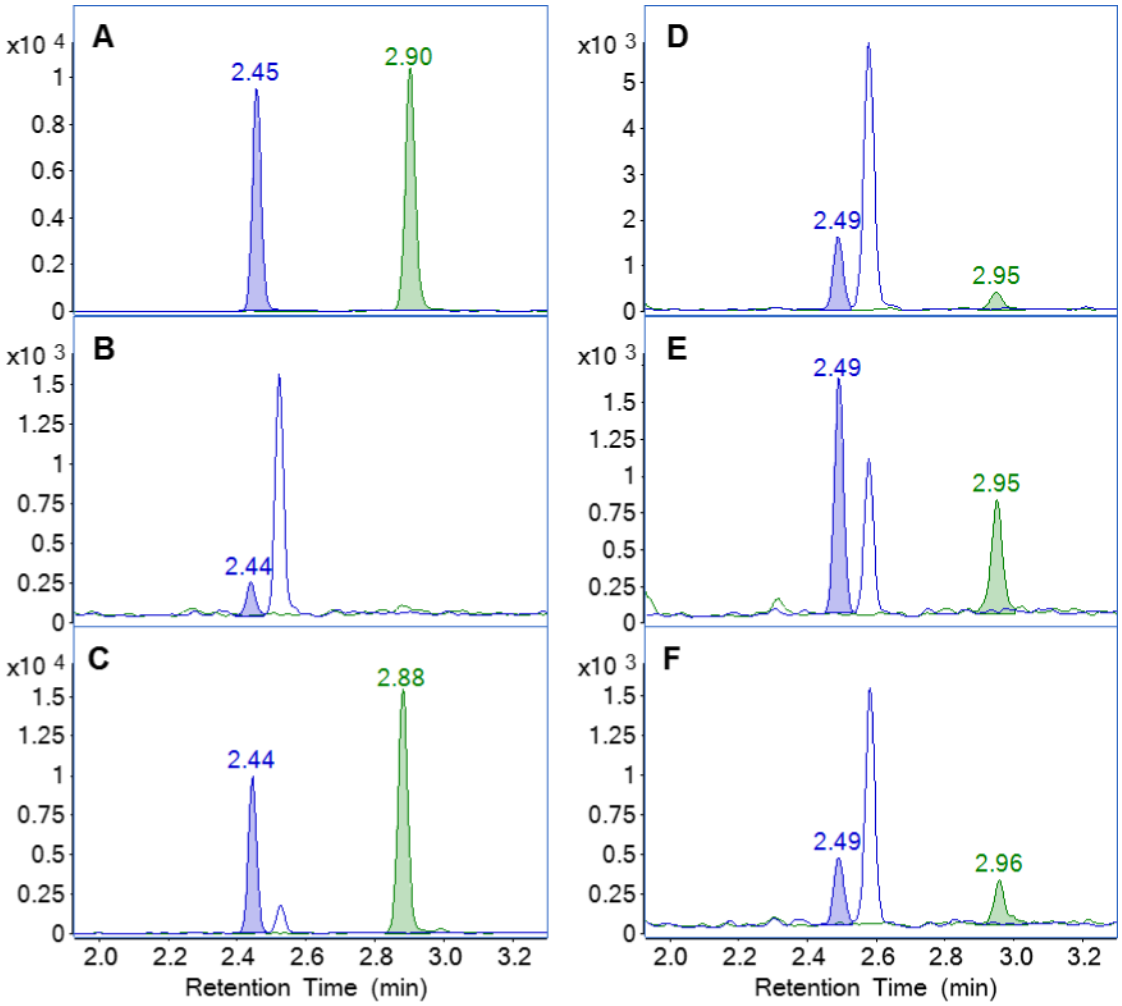

Figure 2.

Overlay of extracted ion chromatograms for Cl-Tyr (blue) and Cl_2-Tyr (green). Chromatogram of the 50.0 ng/mL synthetic calibration standard (**A**). Pooled blank serum before (**B**) and after (**C**) exposure to 370 ppb Cl_2 gas for 15 min. Serum collected from de-identified, individual patients with cystic fibrosis (**D**), ulcerative colitis (**E**), and atherosclerosis (**F**).

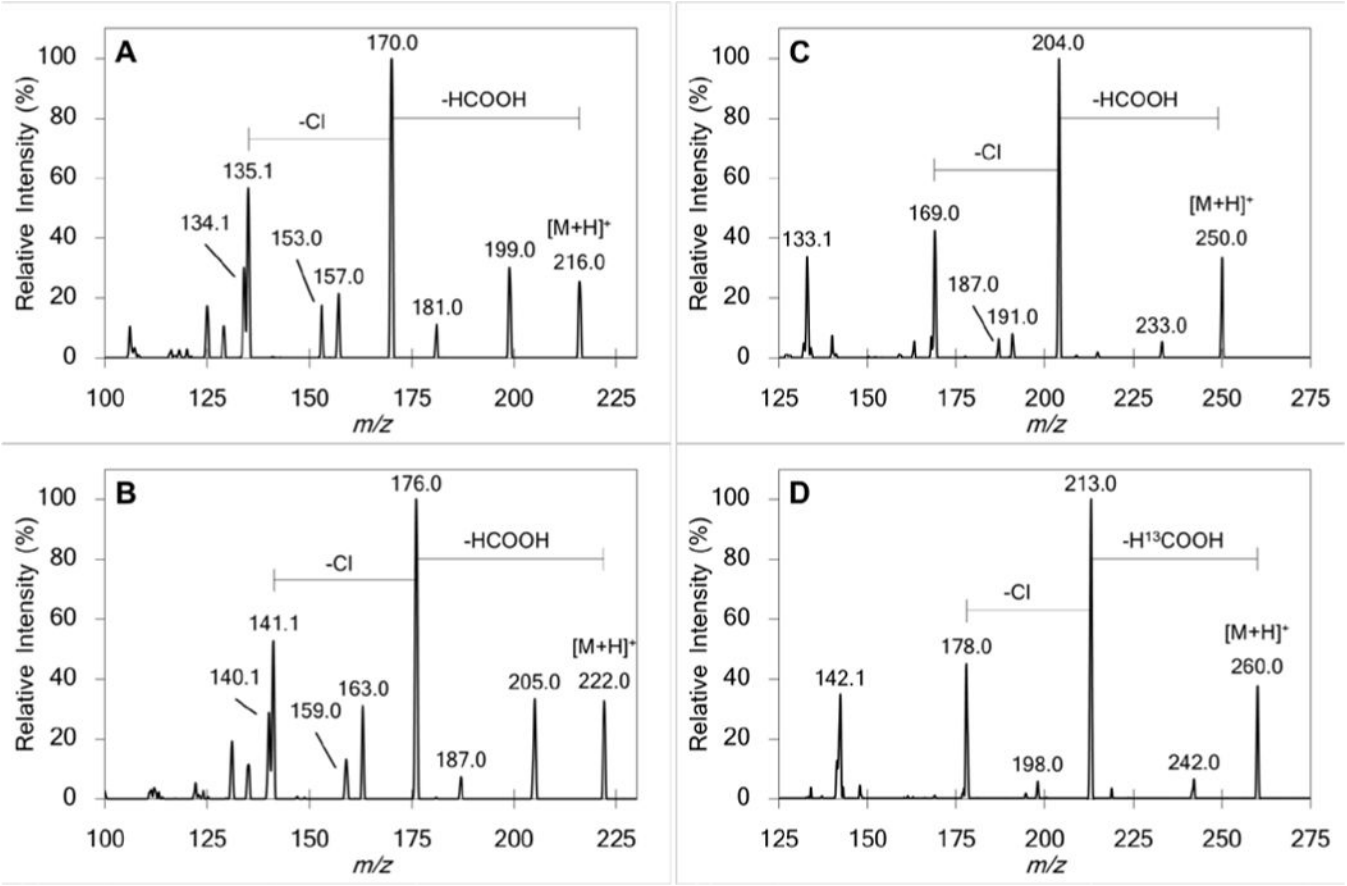

Figure 3.

Product ion scans for Cl-Tyr (**A**), $^{13}C_6$-Cl-Tyr (**B**), Cl_2-Tyr (**C**), and $^{13}C_9$,^{15}N-Cl_2-Tyr (**D**).

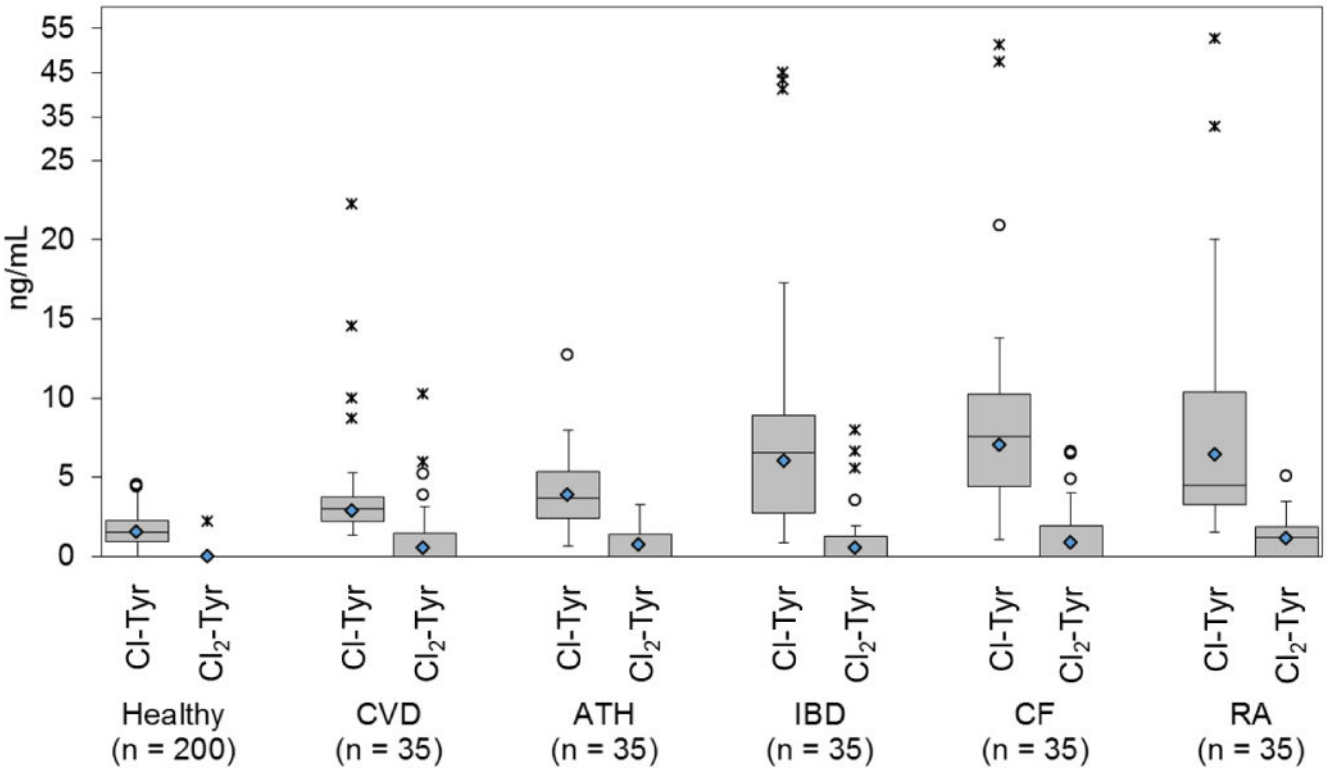

Figure 4.

Distribution of Cl-Tyr and Cl_2-Tyr concentration in blood products from patients with no known inflammatory disease (healthy) and those with cardiovascular disease (CVD), atherosclerosis (ATH), inflammatory bowel disease (IBD), cystic fibrosis (CF), or rheumatoid arthritis (RA). The mean is represented by a blue diamond, outliers by an open circle, and extreme outliers by an x.

Table 1

Optimized ESI-MS/MS parameters for detection of native and labeled chlorotyrosines.

compound		precursor ion (m/z)	product ion (m/z)	CE (V)
Cl-Tyr	quant	216.0	135.1	30
	conf	216.0	170.0	10
$^{13}C_6$-Cl-Tyr	ISTD	222.0	141.0	30
Cl_2-Tyr	quant	250.0	169.0	30
	conf	250.0	204.0	10
$^{13}C_9$,^{15}N-Cl_2-Tyr	ISTD	260.0	178.0	30

Table 2

Accuracy, inter-day precision, reporting limits, and quantitation limits of chlorotyrosine adduct calibrators ($n = 20$).

compd	expected concn (ng/mL)	calcd concn (ng/mL)	accuracy of mean (%)	precision (%CV)	LRL (ng/mL)[a]	URL (ng/mL)[b]	LOD (ng/mL)[c]
Cl-Tyr	5.00	4.67	93.4	6.87	2.50	1000	0.443
	50.0	51.3	103	7.75			
	500.	496	99.2	4.23			
Cl_2-Tyr	5.00	4.91	98.2	8.24	2.50	1000	0.396
	50.0	50.7	101	4.94			
	500.	501	100	4.92			

[a] Lowest reportable limit.

[b] Upper reportable limit.

[c] Limit of detection calculated by Taylor method.

Table 3

Inter-day ($n = 20$) and intra-day ($n = 8$) precision of quality control materials.

		inter-day precision		intra-day precision	
compd	**QC material**	**calcd concn (ng/mL)**	**%CV**	**calcd concn (ng/mL)**	**%CV**
Cl-Tyr	Cl-Tyr QCL	10.3	8.04	11.9	5.67
	Cl-Tyr QCM	111	6.92	116	3.20
	Cl-Tyr QCH	687	9.09	685	7.48
Cl_2-Tyr	Cl_2-Tyr QCL	10.5	8.84	11.3	4.39
	Cl_2-Tyr QCM	84.5	6.03	85.0	2.85
	Cl_2-Tyr QCH	450	10.5	474	4.95

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