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Quantification of Thiazolidine-4-carboxylic Acid in Toxicant-Exposed Cells by Isotope-Dilution Liquid Chromatography−Mass Spectrometry Reveals an Intrinsic Antagonistic Response to Oxidative Stress-Induced Toxicity

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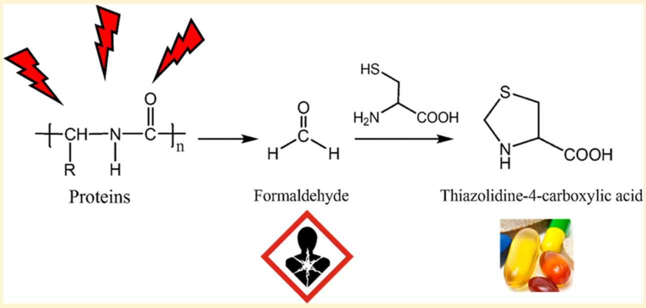
Supporting Information

ABSTRACT: Carcinogenic formaldehyde is produced by endogenous protein oxidation and various exogenous sources. With formaldehyde being both ubiquitous in the ambient environment and one of the most common reactive carbonyls produced from endogenous metabolism, quantifying formaldehyde exposure is an essential step in risk assessments. We present in this study an approach to assess the risk of exposure to oxidative stress by quantifying thiazolidine-4-carboxylic acid (TA), a cysteine-conjugated metabolite of formaldehyde in toxicant-exposed *Escherichia coli*. The method entails TA derivatization with ethyl chloroformate, addition of isotope-labeled TA derivatives as internal standards, solid-phase extraction of the derivatives, and quantification by liquid chromatography−mass spectrometry (LC−MS). After validating for accuracy and precision, the developed method was used to detect TA in oxidizing agent-exposed *E. coli* samples. Dose-dependent TA formation was observed in *E. coli* exposed to hydroxyl radical mediators Fe^{2+}-EDTA, H_2O_2, and NaOCl, indicating the potential use of TA as a biomarker of exposure to oxidative stress and disease risk.



■ INTRODUCTION

Chronic inflammation and oxidative stress lead to the formation of reactive oxygen and nitrogen (RONS) species that are linked to many human diseases such as cancer and atherosclerosis as well as Parkinson's and Alzheimer's diseases.[1,2] These mediators of oxidative stress attack endogenous biomolecules, such as proteins, lipids, nucleic acids, and carbohydrates, forming a broad spectrum of reactive carbonyl-containing species.[2−4] These damaged molecules represent a potential source of biomarkers for defining mechanisms of pathology and for quantifying the risk of human disease.

For example, as one of the most common reactive carbonyls, formaldehyde is generated from both endogenous oxidative stress and a wide variety of exogenous sources[5] and has been classified as a probable cause of cancer by the National Cancer Institute of the National Institutes of Health (June, 2011). Thus, reactive carbonyls and their metabolites are ideal biomarker candidates because they are generated during oxidative stress and inflammation and are directly involved in the pathology of these conditions.

Metabolism is important for the disposal and detoxification of endogenous and exogenous xenobiotics.[6,7] Among the reactive carbonyl metabolites, thiazolidine-4-carboxylic acid (TA; Scheme 1), a condensation product of formaldehyde reacting with cysteine, has been extensively investigated with regard to its pharmaceutical properties.[8−13] Some of the observed biological activities of TA include anticancer,[12,13] hepatoprotective,[9] anti-inflammatory,[10] and anti-diabetes.[11] Currently, TA is being marketed as a dietary supplement sold under the trade name thioproline.

Recent observations have also revealed that TA is one of the urinary metabolites of formaldehyde in formaldehyde-exposed rats,[14] establishing thiazolidine as a metabolic product of endogenous aldehydes. However, the association between excreted TA and oxidative stress has not been established, which prompted us to develop a highly sensitive and noninvasive approach for quantifying TA as a biomarker of mechanism and risk.

Analytical methods based on ion-exchange and ion-pairing chromatographic techniques have been developed in previous quantitative studies of TA in biological fluids.[15−17] However, these methods suffer from the inherent drawback of poor sensitivity for the quantification of low-level TA in the complex matrix of urine and blood samples. A gas chromatography−

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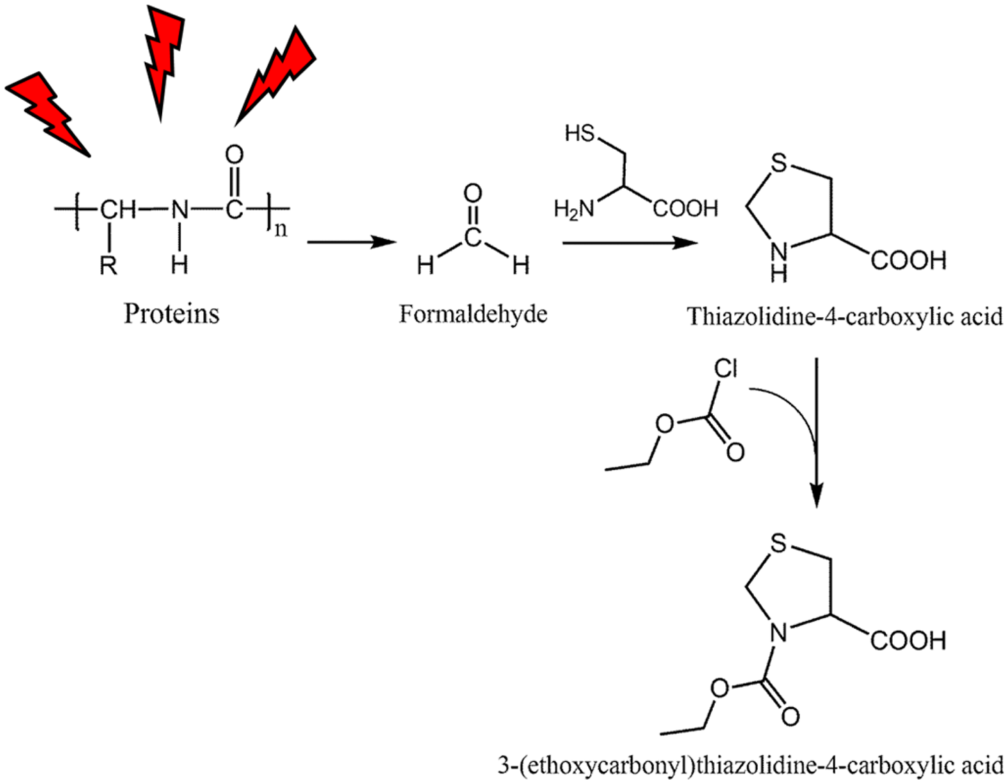
Scheme 1. Formation of Thiazolidine-4-carboxylic Acid from the Reaction of Protein Oxidation-Induced Formaldehyde with Cysteine and the Generation of an Ethyl Chloroformate Derivative for UPLC−MS Analysis



mass spectrometric (GC−MS) method with two derivatization reactions that convert the polar carboxylic acid and amino groups to methyl ester and ethylcarbamate forms, respectively, has been developed to quantify urinary TA.[14] Although selective, the double derivatization used in the developed GC−MS method produced multiple products, which reduced analytical sensitivity. To the best of our knowledge, the identification of TA by liquid chromatography−mass spectrometry (LC−MS) has not been reported. We report in this study the use of a quadrupole time-of-flight mass spectrometer (QTOF) with high resolution and high mass accuracy for the sensitive and selective quantification of TA in toxicant-exposed cells.

The study aimed to develop an LC−MS method for the rigorous quantification of TA in biological fluids. Given the high polarity of TA and its poor retention on reversed-phase liquid chromatography (RP-LC), chemical derivatization was utilized to increase the hydrophobicity and thus the chromatographic behavior of TA on RP-LC. The isotopic-dilution mass spectrometric method, known to be the highest metrological in quantitative analysis, was adopted in this study to minimize experimental error arising from sample processing and instrumental instability. Another aim was to develop TA as a biomarker of oxidative stress and disease risk. Compared with previous studies on TA that are descriptive in nature, we sought to define the formation of TA in *Escherichia coli* exposed to hydroxyl radicals generated by hydrogen peroxide (H_2O_2) and Fe^{2+}-EDTA as well as oxidative stress-inducing sodium hypochlorite (NaOCl).

■ MATERIALS AND METHODS

Chemicals. All chemicals were of the highest purity available and used without further purification unless noted otherwise. TA, formaldehyde-d_2, ethyl chloroformate (ECF), H_2O_2, and L-cysteine were obtained from Sigma-Aldrich (St. Louis, MO). LC−MS grade methanol and acetonitrile were purchased from J.T. Baker (Philisburg, NJ). Deionized water was further purified with a Milli-Q Ultrapure Water System (Billerica, MA) and used in all experiments.

Synthesis of 3-(Ethoxycarbonyl)thiazolidine-4-carboxylic Acids. 3-(Ethoxycarbonyl)thiazolidine-4-carboxylic acid (EC-TA) was prepared at 40 °C through the reaction of TA with a 10-fold molar excess of ECF for 30 min. The product after solid-phase extraction (SPE) using a C18 cartridge (500 mg, Grace) to remove excess ECF was resolved by RP-LC on a Grace VisionHT C18 column (150 × 2.1 mm, 3 μm) using water and methanol as the mobile phase. The chromatographically pure product was characterized by UV absorption spectrophotometry, high mass accuracy mass spectrometry, and tandem mass spectrometry.

The d_2-labeled form of TA (d_2-TA) was synthesized in an overnight reaction of formaldehyde-d_2 with L-cysteine. In brief, 10 μL of formaldehyde-d_2 was added to 1 mg of L-cysteine in 0.1 mL of D_2O and allowed to react at 4 °C overnight with occasional vortex mixing. The reaction mixture containing d_2-TA was then converted to its ethylcarbamate form (d_2-EC-TA) and HPLC purified using the protocol described above. The chromatographically pure internal standard was then dissolved in acetonitrile, quantified spectrophotometrically using the excitation coefficient of EC-TA, and stored at −80 °C until use for analysis.

Exposure of *E. coli*. A culture of *E. coli* (DH5α, ATCC) was grown to mid log phase and harvested by centrifugation at 4000*g* for 15 min. After washing three times with potassium phosphate-buffered saline (PBS, 100 mM, pH 7.4), the cellular pellet was resuspended in the same buffer for toxicant exposure. To ~0.8 g of *E. coli* in 10 mL of PBS were added oxidative stress generators in the following final concentrations: Fe^{2+}-EDTA, 0.5, 1, 2, or 2.5 mM; NaOCl, 0.1, 0.3, 0.5, or 1.0 μM (The Chlorox Co., Oakland, CA); and H_2O_2, 1.25, 2.5, 5.0, or 8.5 μM. After 1 h of exposure, extracellular PBS was separated from the cells by centrifugation, derivatized with ECF, enriched by SPE, and analyzed using LC−MS.

Sample Preparation. To 9.0 mL of the extracellular fluid from toxicant-exposed *E. coli*, was added 900 μL of ECF, and the cells were incubated at 40 °C. After 30 min of incubation, 50 μL of d_2-EC-TA (500 ng/mL) was added to the sample mixture as internal standard, vortex mixed, and SPE enriched using a C18 SPE cartridge (500 mg; Grace, Columbia, MD), with columns activated by a 5 mL methanol wash followed by reconditioning with 5 mL of water. After loading the sample, the SPE columns were washed with 0.5 mL of water and then eluted with 1.5 mL of methanol. The methanolic eluate was collected and evaporated to dryness under nitrogen at 30 °C. The residue was dissolved in 100 μL of acetonitrile for LC−MS analysis.

LC−MS Analysis. Chromatographic separation was performed on a Waters UPLC system (Milford, MA). A 10 μL aliquot of the sample extract was injected into a Waters HSS C18 column (100 mm × 2.1 mm, 1.8 μm) eluted with the following gradient of methanol in 0.1% formic acid at a flow rate of 350 μL/min at 40 °C: 0−2 min, 1%; 2−4.5 min, 1−40%; 5.5−6.5 min, 100%; 7.5−10 min, 1%.

The UPLC was coupled with a Waters Xevo G2 QTOF (Manchester, UK) mass analyzer for mass spectrometric analysis. MS and MS/MS data were acquired in positive ion mode using an electrospray ion source. The source temperature was set at 100 °C with a cone gas flow of 50 L/h; the desolvation gas temperature was 400 °C with a desolvation gas flow of 800 L/h. The capillary and cone voltage was set to 3.0 kV and 10 V, respectively.

Calibration. The isotope-dilution mass spectrometric method was used for the rigorous quantification of TA in toxicant-exposed *E. coli* samples. Into 9 mL of PBS (100 mM, pH 7.4) was spiked TA at final concentrations of 0.1, 0.5, 1, 5, and 10 ng/mL (n = 3). The solutions were derivatized, spiked with EC-TA-d_2, SPE enriched, and analyzed using LC−MS as described above. A calibration curve for quantifying TA was established by plotting the peak area ratios of the pseudomolecular ion ($[M + H]^+$) of EC-TA (m/z 206.05) to that of the isotope-labeled internal standard (m/z 208.06).

Method Validation. The developed method was validated for sensitivity, accuracy, and precision. The limit of detection (LOD) was established as the amount of analyte in the blank sample extract that generated a signal 3 times the signal-to-noise ratio.[18−20] Method accuracy was determined by spiking TA to cultured *E. coli* (n = 3) at 0.5, 1.0, and 5.0 ng/mL, processed, and analyzed using the developed LC−MS method. Method precision was evaluated by analyzing *E. coli* spiked with TA at the three stated concentrations on the same day (n = 3) and over three different days of a week.

■ RESULTS AND DISCUSSION

Characterization of 3-(Ethoxycarbonyl)thiazolidine-4-carboxylic Acid. Hydrophilic TA shows poor chromatographic retention in RP-LC (Figure 1A).[17] The incorporation of a hydrophobic ethoxycarbonyl moiety into TA reduced its polarity (Scheme 1), facilitating its analysis by RP-LC (Figure 1A). UV absorption spectrometric analysis of 3-(ethoxycarbonyl)thiazolidine-4-carboxylic acid in acetonitrile showed a local absorption maximum at 238 nm, with a molar extinction coefficient (ε_{238}) of 160 M^{-1} cm^{-1}.

EC-TA is amphoteric, containing both an acidic and a basic group that can either donate or accept a proton (H^+), respectively. Therefore, EC-TA should favor ESI−MS detection in negative and positive modes, respectively. The mass spectrometric response of EC-TA under both ESI modes was investigated. Our results showed that positive mode ESI−MS produced better (∼9-fold) MS signal than that in negative mode ESI−MS. A possible reason for this is the easier protonation of the amino group in EC-TA in positive mode ESI−MS than the deprotonation process in negative mode ESI−MS analyses, especially in an acidic environment with the added formic acid in the mobile phase that is used in this study. Accordingly, positive mode ESI−MS was used throughout the entire study.

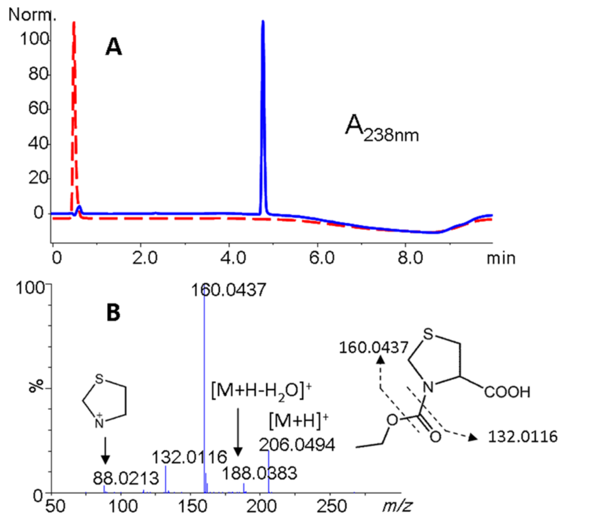


Figure 1. (A) Chromatograms obtained from reversed-phase HPLC analysis of thiazolidine-4-carboxylic acid (dashed red line) and 3-(ethoxycarbonyl)thiazolidine-4-carboxylic acid (solid blue line). (B) ESI−MS/MS spectrum of the pseudomolecular ion $[M + H]^+$ ion of 3-(ethoxycarbonyl)thiazolidine-4-carboxylic acid at m/z 206 and the cleavage reactions for the formation of major fragment ions found in MS/MS analysis.

High-accuracy MS analysis of the EC-TA derivative revealed a close correlation between the measured (206.0494) and theoretical (206.0487) m/z values of the $[M + H]^+$ ion, with a mass error of 3.4 ppm. Collision-induced dissociation of the pseudomolecular ion at m/z 206.0494 led to the formation of a base peak at m/z 160.0437 (Figure 1B), originating from de-ethoxylation of the adduct ion. The corresponding daughter ion at m/z 162.0551 was also identified when the EC-TA-d_2 internal standard (m/z 208.0585) was analyzed under identical conditions (Figure S1).

Optimization of Reaction Conditions for TA Derivatization. ECF is an ethoxycarbonyl agent that is frequently used in organic synthesis to protect amino groups.[21,22] The reagent has also been used to derivatize amino acids, amine-containing illicit drugs, and nitrogenous urinary metabolites for gas chromatography and liquid chromatography analyses.[23−26]

As the basis of our method, Shin et al. developed a GC−MS method quantifying urinary TA after the polar carboxylic acid and amine groups were derivatized by esterification and ethoxycarbonylation, respectively.[14] However, as illustrated in Figure S2, the double derivatization was incomplete and produced a mixture of monoderivatized byproducts that reduced analytical sensitivity. We sought to develop a sensitive method for the quantification of TA by combining precolumn ECF derivatization and LC−MS detection.

The derivatization conditions were optimized for the ethoxycarbonylation of TA. Specifically, the formation of EC-TA at different ECF concentrations, temperatures, and reaction times was investigated. At a fixed TA concentration, increasing the amount of ECF from 1 to 10% (v/v) was found to gradually increase the analytical signal of EC-TA. Further increasing the ECF content negatively affected the ethoxycarbonylation reaction. Thus, 10% ECF was used in subsequent studies on TA derivatization. A similar phenomenon has also been observed in our previous study on derivatizing urinary creatinine.[18]

We next studied the reaction yield by varying the reaction temperature (25−70 °C) and time (1−60 min). In contrast to the previous observation that elevated reaction temperature favors ethoxycarbonylation,[18] the mild derivatization conditions of incubating the reaction mixture at 40 °C for 30 min was the most efficient for the ethoxycarbonylation reaction of TA in this study (Figure 2). We believe that the electron-donating carboxylate group in TA increased its reactivity toward the *N*-ethoxycarbonyl reaction. As a result, the derivatization conditions of incubating with 10% of ECF (v/v) at 40 °C for 30 min were used throughout the entire study.

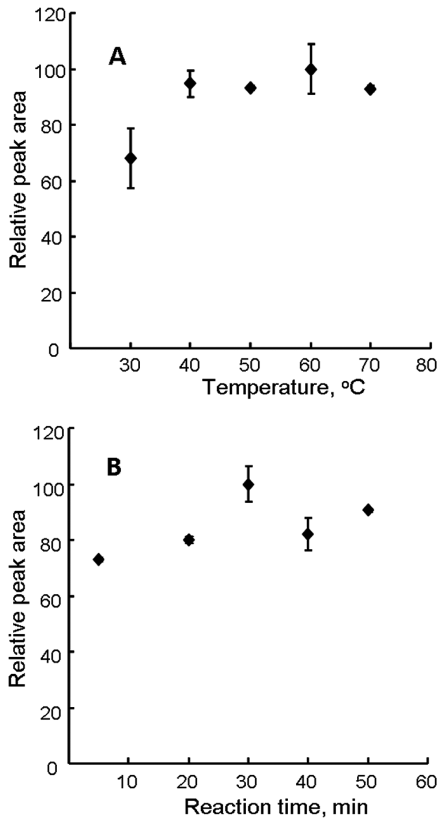


Figure 2. Influence of (A) temperature and (B) time on the yield of the derivatization reaction. The data represent the mean ± SD of three independent experiments.

Method Calibration and Validation. A calibration curve was generated by plotting the peak area ratios between EC-TA and EC-TA-d_2 versus the concentrations of TA in the sample solutions. The peak area ratios of TA linearly increased within the tested concentration range (Figure S3), with the calibration slope, intercept, and coefficient of determination (r^2) being 0.3828, −0.0429, and 0.9984, respectively. Figure 3A shows a typical chromatogram obtained from LC−MS analysis of the sample solution containing TA at 1 ng/mL.

The LOD, defined as the concentration of TA that generates a signal three times the signal-to-noise ratio, was 0.03 ng/mL, which was significantly lower than that obtained using existing methods (1.0−665 ng/mL).[14−17] We believed that the high selectivity brought about by combination of precolumn derivatization and high-resolution QTOF-MS measurements allowed sensitive TA determination in the complex matrix of the biological samples.

The precision of the developed method was evaluated by analyzing PBS-washed *E. coli* samples (0.8 g of *E. coli* in 10 mL of PBS) spiked with TA at three different concentrations (0.5, 1.0, and 5.0 ng/mL), derivatized by ECF, purified by SPE, and analyzed by LC−MS, as described above. The relative standard deviations for the intra- and interday reproducibility were less than 5.7 and 8.4%, respectively, signifying the reproducibility of the developed assay.

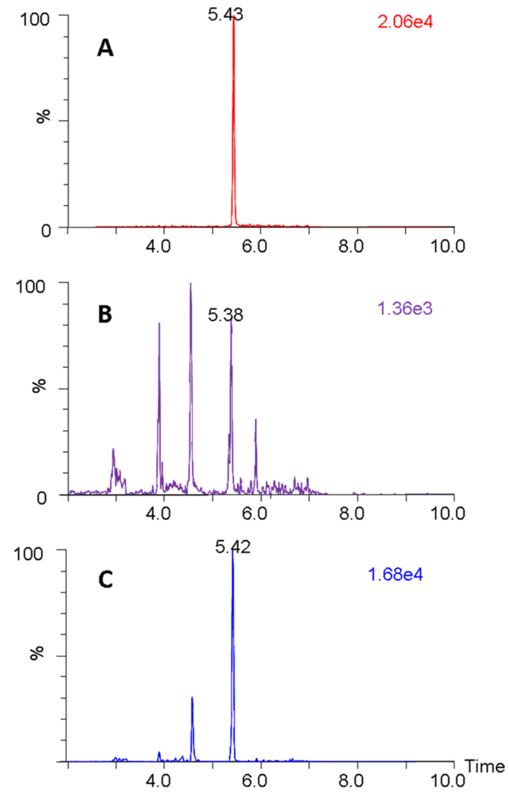


Figure 3. Extracted ion chromatograms of thiazolidine-4-carboxylic acid derivative (*m*/*z* 206.0−206.1) obtained from UPLC−MS analyses of (A) blank PBS spiked with 1 ng/mL thiazolidine-4-carboxylic acid, (B) untreated *E. coli*, and (C) *E. coli* treated with 2.5 mM Fe^{2+}-EDTA.

The method accuracy was determined by spiking TA at the three stated concentrations into blank *E. coli* samples, derivatizing, SPE enriching, and analyzing by LC−MS. The overall efficiency of the analytical method (at 0.5−5.0 ng/mL), calculated as the measured quantities of TA divided by the added quantity, was found to range from 92.9 to 108.7% of the theoretical value, indicating that the developed method was highly quantitative. The results of the accuracy and precision of the developed LC−MS method are summarized in Table 1. The high precision and accuracy of the data indicate that the developed method is highly reproducible and accurate for quantifying TA in biological systems.

Table 1. Accuracy and Precision of the Developed UPLC−MS Method for the Determination of Thiazolidine-4-carboxylic Acid (n = 3)

	accuracy		precision	
concentration spiked (ng/mL)	concentration found (ng/mL)	% recovery	intraday (RSD) (%)	interday (RSD) (%)
0.5	0.54 ± 0.04	108.7	1.6	5.3
1.0	1.00 ± 0.10	99.9	2.6	8.4
5.0	4.64 ± 0.30	92.9	5.7	3.2

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UPLC–MS Quantification of TA in Toxicant-Exposed *E. coli*. The developed isotope-dilution UPLC–MS method was used to determine TA formation in toxicant-exposed *E. coli*. The well-studied chemical toxicants Fe^{2+}-EDTA, H_2O_2, and NaOCl, which are known to produce oxidative stress *in vivo*, were used in these experiments.[27−29] The chromatographic peak of EC-TA in *E. coli* was identified by comparing the retention time with that of the reference compound prepared by reacting ECF with TA using high mass accuracy mass spectrometry and collision-induced dissociation MS/MS analyses.

Our studies with hydroxyl radical generators Fe^{2+}-EDTA and H_2O_2 revealed the dose-dependent formation of TA by *E. coli*. As shown in Figure 4, Fe^{2+}-EDTA and H_2O_2 produced TA at 0.72 and 28.7 ng/mL per mM toxicant, respectively. The results showed that H_2O_2 was a prominent oxidative stress inducer, in reasonable agreement with the higher rate of hydroxyl radical generation by H_2O_2 than that by Fe^{2+}-EDTA, as determined by Jiang et al.[30] A typical chromatogram obtained from the analyses of Fe^{2+}-EDTA-exposed *E. coli* is shown in Figure 3.

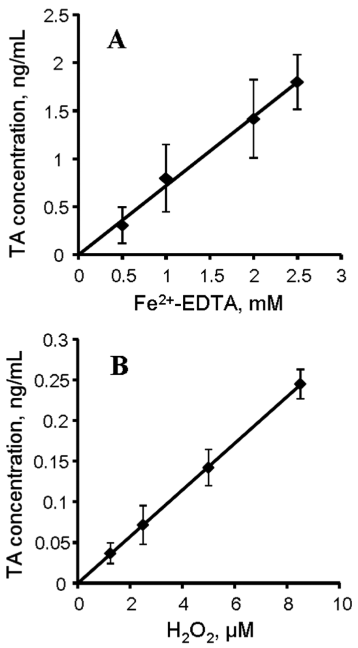

Figure 4. Dose-dependent formation of thiazolidine-4-carboxylic acid in *E. coli* upon exposure to (A) Fe^{2+}-EDTA (r^2 = 0.9927) or (B) H_2O_2 (r^2 = 0.9998). The values represent the mean ± SD from three independent measurements and were corrected for background levels of TA.

Notably, TA was also detected in untreated *E. coli* samples at a concentration of 0.17 ± 0.001 ng/mL, corresponding to the signal generated from reacting endogenous formaldehyde with cysteine. Therefore, data from toxicant-exposed *E. coli* samples were corrected for the measured background levels of TA. Conversely, no detectable signal for TA was identified in the control experiment with only the isotopic internal standard in PBS buffer, indicating that no observable deuterium/hydrogen exchange of the isotope-labeled internal standard took place during sample preparation and LC–MS analysis.

Studies with NaOCl, another oxidizing agent that releases hydroxyl radicals upon dissolving in water, showed dose-dependent formation of TA at a response rate of 1384.8 ng/mL per mM NaOCl (Figure 5), which is the highest among the three oxidizing agents tested. The detailed mechanism underlying this observation is under investigation, but the result agreed with the LD_{50} that was estimated for H_2O_2 (9.4 mM) and NaOCl (0.66 μM).[31] We suspected that the high yield of TA that was observed for NaOCl could be due to the higher yield of hydroxyl radicals from NaOCl. Another potential reason for the observed discrepancy was that NaOCl, upon dissolving in water, also produced singlet oxygen,[32] a reactive oxygen species demonstrated to cause lipid peroxidation.[2]

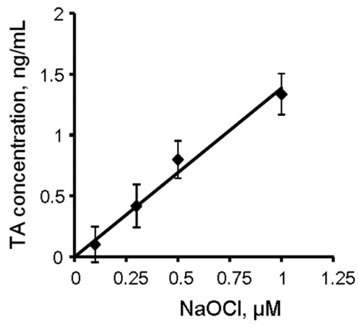

Figure 5. Dose-dependent formation of thiazolidine-4-carboxylic acid in *E. coli* upon NaOCl exposure (r^2 = 0.9824). The values represent the mean ± SD from three independent measurements and were corrected for background levels of TA.

■ CONCLUSIONS

We have developed a novel isotope-dilution LC–MS method for the analysis of TA in biological samples by combining precolumn derivatization with ECF and RP-LC coupled with mass spectrometric analysis. For the first time, our study revealed the dose-dependent formation of TA in *E. coli* exposed to various hydroxyl generators, indicating the potential use of TA as a biomarker of oxidative stress and disease risk. Given that TA is generated *in vivo* upon reacting formaldehyde with L-cysteine and that carcinogenic formaldehyde is produced both endogenously from oxidative stress-induced damage to biomacromolecules and exogenously from a wide variety of sources, we believe that the method can enable the development of TA as a general biomarker for assessing cancer risk. We also expect that the developed analytical method can facilitate TA metabolism and disposal studies.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

ESI–MS/MS spectrum of 3-(ethoxycarbonyl)thiazolidine-4-carboxylic acid-d_2; chromatograms obtained from reversed-phase HPLC analysis of the reaction mixtures derivatized using two different methods; calibration curve for the LC–MS analysis of thiazolidine-4-carboxylic acid; and chromatogram and ESI–MS/MS spectrum obtained from LC–MS/MS analysis of ECF-derivatized 2-methyl-1,3-thiazolidine-4-carboxylic acid. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS

RP-LC, reversed-phase liquid chromatography; LC−MS, liquid chromatography−mass spectrometry; GC−MS, gas chromatography−mass spectrometry; TA, thiazolidine-4-carboxylic acid; MTA, 2-methyl-1,3-thiazolidine-4-carboxylic acid; SPE, solid-phase extraction; QTOF-MS, quadrupole time-of-flight mass spectrometry; UPLC, ultraperformance liquid chromatography; ECF, ethyl chloroformate

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