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Short communication

Quantification of cyanuric acid residue in human urine using high performance liquid chromatography–tandem mass spectrometry☆

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ABSTRACT

Concern has increased about the resulting health effects of exposure to melamine and its metabolic contaminant, cyanuric acid, after infants in China were fed baby formula milk products contaminated with these compounds. We have developed a selective and sensitive analytical method to quantify the amount of cyanuric acid in human urine. The sample preparation involved extracting free-form cyanuric acid in human urine using anion exchange solid phase extraction. Cyanuric acid was separated from its urinary matrix components on the polymeric strong anion exchange analytical column; the analysis was performed by high performance liquid chromatography–tandem mass spectrometry using negative mode electrospray ionization interface. Quantification was performed using isotope dilution calibration covering the concentration range of 1.00–200 ng/mL. The limit of detection was 0.60 ng/mL and the relative standard deviations were 2.8–10.5% across the calibration range. The relative recovery of cyanuric acid was 100–104%. Our method is suitable to detect urinary concentrations of cyanuric acid caused by either environmental exposures or emerging poisoning events.

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1. Introduction

Cyanuric acid (1,3,5-triazine-2,4,6-triol: CAS No 108-80-5; Fig. 1) is a structural analogue of melamine, a common component of plastics, which is nitrogen-rich and may be found as an impurity in its formulations. Cyanuric acid is approved by the U.S. Food and Drug Administration (FDA) as a component of feed-grade biuret, a ruminant feed additive. It is also found in swimming pool water as the dissociation product of dichloroisocyanurates used for water disinfection [1,2]. Additionally, cyanuric acid is a metabolic intermediate during the bacterial metabolism of s-triazine pesticides in the environment [3,4].

Melamine and cyanuric acid garnered worldwide attention after the contamination of animal feed in the United States in 2007 and the contamination of infant formula in China in 2008. Melamine and cyanuric acid were implicated in the development of kidney stones and renal toxicity in animals and children when concurrent, but not separate, exposures occurred. After these incidents, the FDA set a tolerable daily intake of 0.63 mg/kg of body weight per day for both melamine and cyanuric acid for health and safety purposes [2]. Concurrent exposure to melamine and cyanuric acid induces the formation of melamine–cyanurate crystals that cause progressive tubular blockage, degeneration, and subsequent renal failure [5–15].

In response to these contamination incidents, several analytical methods were quickly developed to investigate occurrences of melamine and cyanuric acid in food supplies and milk products [16–25]. Despite the availability of a few analytical methods that can directly quantify the amount of cyanuric acid in biological samples, most of those methods are unsuitable to determine human exposure to cyanuric acid. Those methods are unsuitable likely because of their lack of sensitivity or selectivity to identify concentrations at levels as low as usually found in complex biological matrices such as urine [26–31].

In this study, we aimed to develop a highly selective and sensitive analytical method capable of detecting low levels of cyanuric acid in human urine caused by exposures ranging from background exposures to acute poisonings.

☆ *Disclaimer*: The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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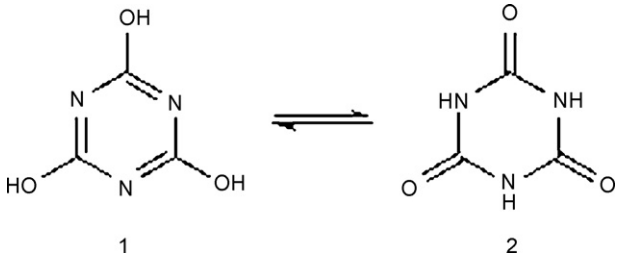

Fig. 1. Chemical structure of cyanuric acid.

2. Experimental

2.1. Chemicals

All solvents we used were of analytical grade. Methanol was purchased from Tedia Company Inc. (Fairfield, OH, USA). Formic acid (99%), ammonium hydroxide, and pyridine were purchased from Fisher Scientific (Phillipsburg, NJ, USA). Ammonium acetate (98%) and acetic acid (99%) were from Sigma–Aldrich (St. Louis, MO, USA). Deionized water was organically and biologically purified with a NANO pure® Infinity UF purchased from Barnstead International (Dubuque, IA, USA).

The native standard of cyanuric acid (98%) was purchased from TCI America (Portland, OR, USA), while its isotopically labeled analogue [($^{13}C_3$-$^{15}N_3$)-cyanuric acid] (90%) was purchased from Cambridge Isotope Laboratories (Andover, MA, USA).

2.2. Preparation of standards solution and quality control materials

Nine cyanuric acid standard spiking solutions were prepared by serial dilution of the stock solution with pyridine to cover the concentration ranges of 0.05–10.0 μg/mL. Cyanuric acid was less soluble in common solvents such as methanol or acetonitrile, thus pyridine was used as a solvent to make a concentrated stock solution. The labeled standard spiking solution was also prepared in pyridine, giving an approximate concentration of 1.25 μg/mL. Two quality control (QC) spiking solutions containing the cyanuric acid were prepared with standard spiking solutions by serial dilution of the initial stock solutions with pyridine. The designated concentrations of these QC spiking solutions were 0.75 μg/mL for medium-level material (QCM) and 1.50 μg/mL for high-level material (QCH). All standard stock solutions and spiking solutions were dispensed into amber vials and stored at −5 °C until used.

A 100-fold dilution of urine with deionized water was used as a matrix for calibration set and blank samples. A 10-fold dilution was used as a matrix for QC materials. At this dilution ratio, background levels of cyanuric acid were still present and, therefore, these solutions were used as the low-level QC material (QCL).

To prepare a calibration set and QC materials, we added 20 μL each of standard solution and isotope-labeled standard to each 1 mL of designated matrix (100-fold diluted urine for calibration set and 10-fold diluted urine for QC materials). Based upon this procedure, the fortified concentration range of calibration set was 1.00–200 ng/mL, while the fortified concentrations of QC materials were 15.0 ng/mL and 30.0 ng/mL, respectively.

2.3. Urine collection and storage

All urine used for calibration plots, blank samples, and QC materials were collected from multiple anonymous donors, combined and mixed overnight at 4 °C, pressure filtered with a 0.45 μm filter capsule (Whatman Inc., Florham Park, NJ, USA), and diluted 10- or 100-fold with deionized water, depending on its intended use. Diluted urine was pipetted into capped vials and stored at −20 °C until use.

2.4. Sample preparation and injection

To prepare the sample, 1 mL of working matrix was mixed with 20 μL of internal standard. The urine sample was then diluted with 2 mL of 2% ammonium hydroxide, mixed, and loaded onto a mixed-mode solid phase extraction cartridge (Oasis MAX, 500 mg/6 cc, Waters, Milford, MA, USA) that had been conditioned with 3 mL of methanol followed by 3 mL of water. The cartridge was then washed with 3 mL of 2% ammonium hydroxide in deionized water and 3 mL of methanol to eliminate interfering components. The cartridge was vacuum-dried before elution with 3 mL of 5% formic acid in methanol. The eluate was evaporated to dryness using a TurboVap LV Evaporator (Zymark, Framingham, MA, USA) with a water temperature of 60 °C and nitrogen pressure of 15 psi. Dried sample was kept in freezer to minimize potential degradation and was reconstituted with 100 μL of water only before injection. The total injection volume was 10 μL.

2.5. Sample analysis

Chromatographic separation was performed by using high performance liquid chromatography (HPLC) (Agilent 1200, Agilent Technologies, Waldbronn, Germany); the HPLC consisted of a binary pump, a degasser, an auto sampler, and a temperature-stable column compartment. All of the HPLC modules were programmed and controlled using Analyst software version 1.4.2 (Applied Biosystem, Foster City, CA, USA). The analytical column used was ZirChrome SAX (150 mm × 4.6 mm, 3 μm particle size, 110 Å pore size, Separations Inc., Anoka MN, USA), which was placed in column compartment with the temperature set at 60 °C during analysis. The isocratic elution using 10 mM ammonium acetate in methanol:water (70:30, v/v) was used for optimum separation of cyanuric acid from other urine components. The column was washed with 5 column volumes of 0.1% acetic acid in methanol:water (30:70, v/v) before each subsequent injection to eliminate matrix interferences, including retained salts. The total run time including the wash was 20 min.

2.6. Mass spectrometry operating conditions

Tandem mass spectrometer (API 5000 MS/MS, AppliedBiosystem/MDS Sciex, Foster City, CA, USA) with a negative ion mode electrospray ionization interface was used to analyze the samples. The analysis of the cyanuric acid was optimized to achieve the best overall sensitivity and selectivity. The MS/MS setting parameters were: 20 psi Curtain Gas; 65 psi Nebulizer Gas (GS1); 80 psi Turbo Gas (GS2); −3500 V Ion Spray Voltage; 600 °C, 5 arbitrary unit CAD; −5.0 V Entrance Potential; and 150 ms Dwell Time. The mass spectrometer was programmed and controlled using Analyst software version 1.4.2 (AppliedBiosystem/MDS Sciex, Foster City, CA, USA).

A total of three precursor→product ion pairs were identified. A single time segment containing specific multiple reaction monitoring (MRM) experiment was created and used during MS/MS acquisition. Within a month, we selected both the quantification and confirmation ions by monitoring the intensity, peak shape, background level, and potential interferences in different urine samples. The same procedure was applied for the isotopically labeled internal standard.

2.6.1. Method validation

2.6.1.1. Limit of detection (LOD). LOD value was calculated as three times the concentration of blank matrix used in the calibration set to ensure that the lowest concentration of an analyte can be

reliably differentiated from background noise in our bioanalytical procedure [32].

2.6.1.2. Extraction efficiency. The extraction recovery of the method was determined by repeated analysis of water and non-diluted urine samples spiked with two concentrations (5.00 ng/mL and 50.0 ng/mL). Because levels of endogenous cyanuric acid were found in the urine pool, the subsequent concentrations of urine samples after enrichment with these amounts were ~25 ng/mL and ~70 ng/mL, respectively. To begin this experiment, we spiked each of the five urine samples with the designated standard concentration, including the internal standard, and extracted according to the method (total $n = 10$). Concurrently, we extracted 10 additional urine samples (spiked only with internal standard). However, before the evaporation steps, we spiked each of the five additional extracts with the designated standard concentration to represent the 100% recovery. We analyzed the samples after they evaporated and were reconstituted. We calculated the recovery by comparing the responses of the urine samples spiked before extraction to the responses of the urine samples spiked after extraction. We used the same procedure to determine extraction recovery of a target compound in water samples.

2.6.1.3. Precision. We determined the method precision by calculating the relative standard deviation (RSD) of repeat measurements of samples from the QC materials (10-fold diluted urine) at three different concentrations (~2.00 ng/mL, 15.0 ng/mL, and 30.0 ng/mL). We prepared and analyzed two samples from each of the QC materials daily during a 10-day period ($n = 20$); the results were used to determine the inter- and intra-day precision.

2.6.1.4. Accuracy and linearity. We determined the method accuracy by repeated measurements of spiked samples ($n = 10$) at three concentrations (1.00 ng/mL, 10.0 ng/mL, and 50.0 ng/mL). A 100-fold dilution of urine with deionized water was used as matrix to avoid the contribution of endogenous levels of cyanuric acid. We calculated the percent deviation of the observed mean concentrations from the nominal spiked concentrations. We assessed the linearity of the calibration plot by determining an average r^2 value (20 analytical runs) of linear regression plots between nominal concentration values versus calculated concentration values of each cyanuric acid across the entire range of calibration curve.

2.6.1.5. Storage stability. We determined stability of cyanuric acid in urine by repeated analysis ($n = 10$) of non-spiked urine pool samples (non-diluted urine, ~20 ng/mL) and spiked urine pool samples (non-diluted urine, ~200 ng/mL) that were stored at two different temperatures (4 °C and −70 °C). The urine extracts were analyzed at days 0 and 30. We demonstrated the stability of cyanuric acid (%loss) by comparing the responses of the cyanuric acid in urine samples analyzed at day 0 to the responses of the cyanuric acid in urine samples analyzed at day 30, both at the same storage conditions.

2.6.1.6. Matrix effects. In isotope dilution technique, the labeled internal standard improves method accuracy and precision. The internal standard provides the denominator for the calculation of a response ratio factor and is thus used to calculate the final concentration against the calibration curve. The labeled standard accounts for differences in extraction efficiency, instrument response, and other sources of error among samples, thus allowing data from multiple samples to be easily compared. For optimal performance of the method, the internal standard should be free of interfering components among matrices, and its signal should be easily readable for each sample. If matrix components affect the intensity of the internal standard peak, they should similarly affect the intensity of the target analyte. However, wide variations in the internal standard signal can greatly affect the LOD for each individual sample. Thus, observing the possible matrix effects that may affect the internal standard intensity is crucial. We spiked a known amount of internal standard (25.0 ng/mL) into 10 different samples derived from the following matrices: 1 mL of 100-fold diluted urine, 1 mL of 10-fold diluted urine, 0.5 mL of non-diluted urine, and 1 mL of non-diluted urine. Samples were then prepared, extracted according to our proposed method, and analyzed. The RSDs (%RSD) were then calculated to represent the variation of internal standard intensity in each matrix.

3. Result and discussion

3.1. Solid phase extraction

Initially, we compared the extraction recovery of cyanuric acid using three commercially available anion exchange cartridges. The recovery of cyanuric acid was much greater when we used Oasis MAX cartridges, however, the recovery was strongly dependent on the amount of sorbent available. Our primary results indicated that <50% of cyanuric acid was recovered when we used 200-mg Oasis MAX cartridges (data not shown). This obtained result disagreed with the previous data published by Smoker and Krynitsky [20] primarily because our working matrix, human urine, has much higher ionic strength and therefore needs more sorbent amount compared to other non-biological matrices. Therefore, when we used custom-ordered 500-mg Oasis MAX cartridges, we obtained an average 83% extraction recovery of cyanuric acid in undiluted urine at both low and high concentrations (~25 ng/mL and ~70 ng/mL). Similar results were found for water at both low and high concentrations (5.00 ng/mL and 50.0 ng/mL) where an average 88% extraction recovery was obtained. Even though a slight reduction of recovery in urine matrix was observed, typically due to higher ionic strength than water, the recoveries were fairly similar across matrices and urine dilutions.

3.2. Separation and analysis

We performed the chromatographic separation by using HPLC with an isocratic anion exchange technique. For anion exchange separation, the pH and the ionic strength of the mobile phases are the most critical parameters. We chose to work with 10 mM ammonium acetate buffer that typically provides a pH of ~7–8 in aqueous solution. At this level, which is >2 units higher than the pK_a of cyanuric acid, it should be fully ionized (negatively charged) and would thus be retained on the strong anion exchange column. Cyanuric acid was successfully separated from other matrix components and eluted from the column based upon the ionic strength of the mobile phase. Although the retention mechanism provided by ion exchange chromatography is normally thought to be almost completely matrix-insensitive, we however observed a slight shift in the retention time of target compound. This shift likely occurred because of an unexpected change of pH in the buffer used, the amount of target compound eluted, and the ionic strength of buffer and sample matrix. Urinary salts may cause shifts in the retention time of target compound, which is particularly relevant during analysis of urine with high ionic strength. In this case, isotopic internal standard was invaluable in identifying and confirming the target peak of interest. Even though retention time tended to shift, the observed difference was <1 min among individual samples analyzed as well as among different sets of sample analysis. Despite this phenomenon, we found that our current HPLC conditions provided better chromatographic results of cyanuric acid than was

reported in the literature [22,26,33], even though our targeted concentrations were much lower than those previously reported (e.g., 10–100 ng/mL).

We chose to work with a polymeric strong anion exchange column, ZirChrome SAX, with a pH resistance range of 1–12 instead of silica-based column with a smaller working pH range (2–8). Although we could have used the same pH on either a silica-based or polymer-based column, we found that the polymer-based column provided better long-term separation and remained usable for a longer period of time, likely because we were operating on the upper-end range of the silica-based column. We found the polymer-based column more amenable to the acidic wash after the basic mobile phase separation. The acidic wash was necessary to remove the unwanted matrix components retained on the column after each injection. Without the wash, the efficiency of the separation diminished after only a few injections. We found that washing the column with 0.1 acetic acid in water:methanol (70:30, v/v) with at least five times the column volume drastically prolonged the col-

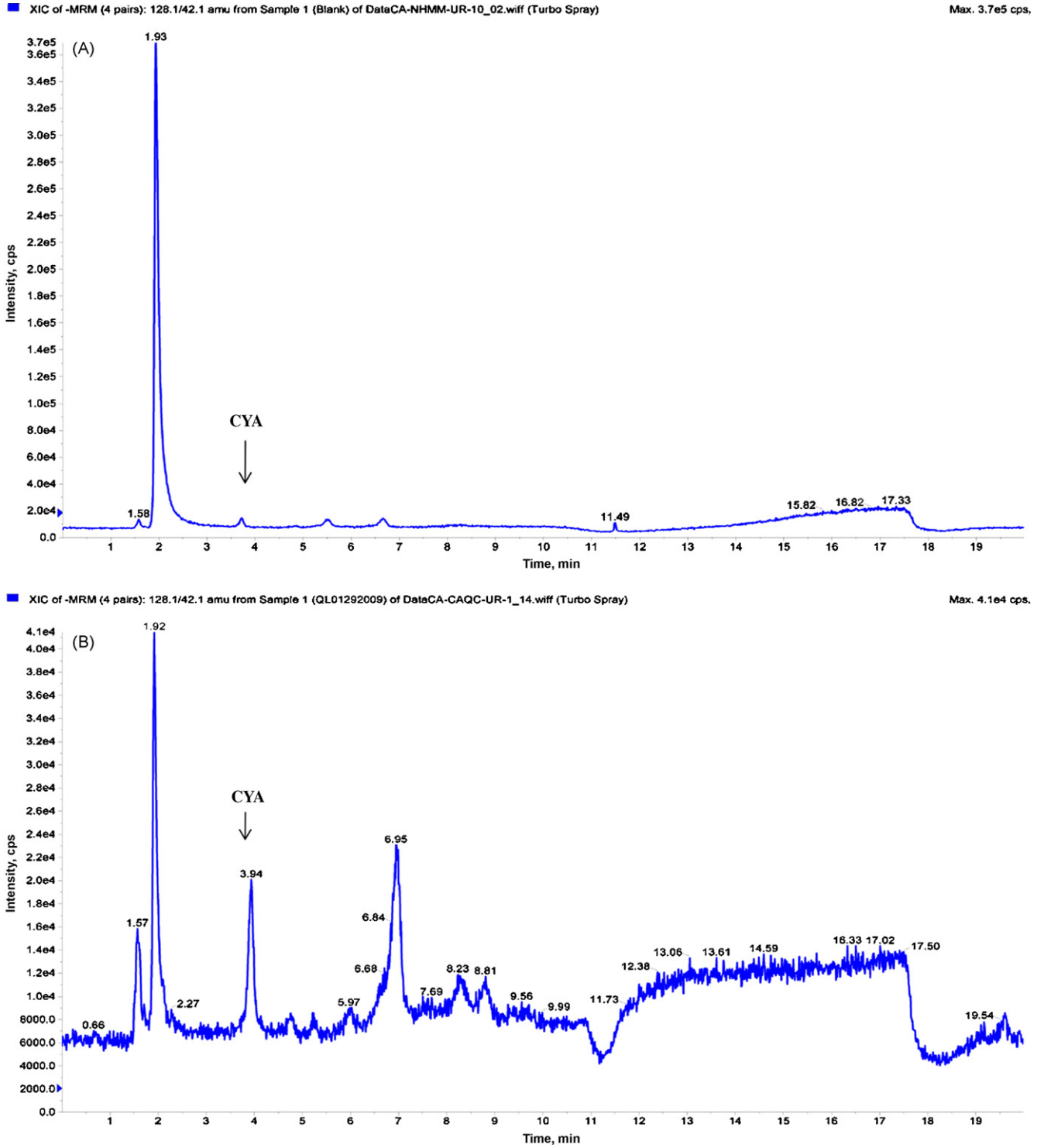

Fig. 2. Extracted ion chromatogram of cyanuric acid (CYA) and its isotopic internal standard in different type of samples; (A) a 100-fold diluted urine sample (Blank), (B) a 10-fold diluted urine sample (QCL), (C) an actual unknown urine sample, and (D) internal standard peak presents in unknown urine sample.

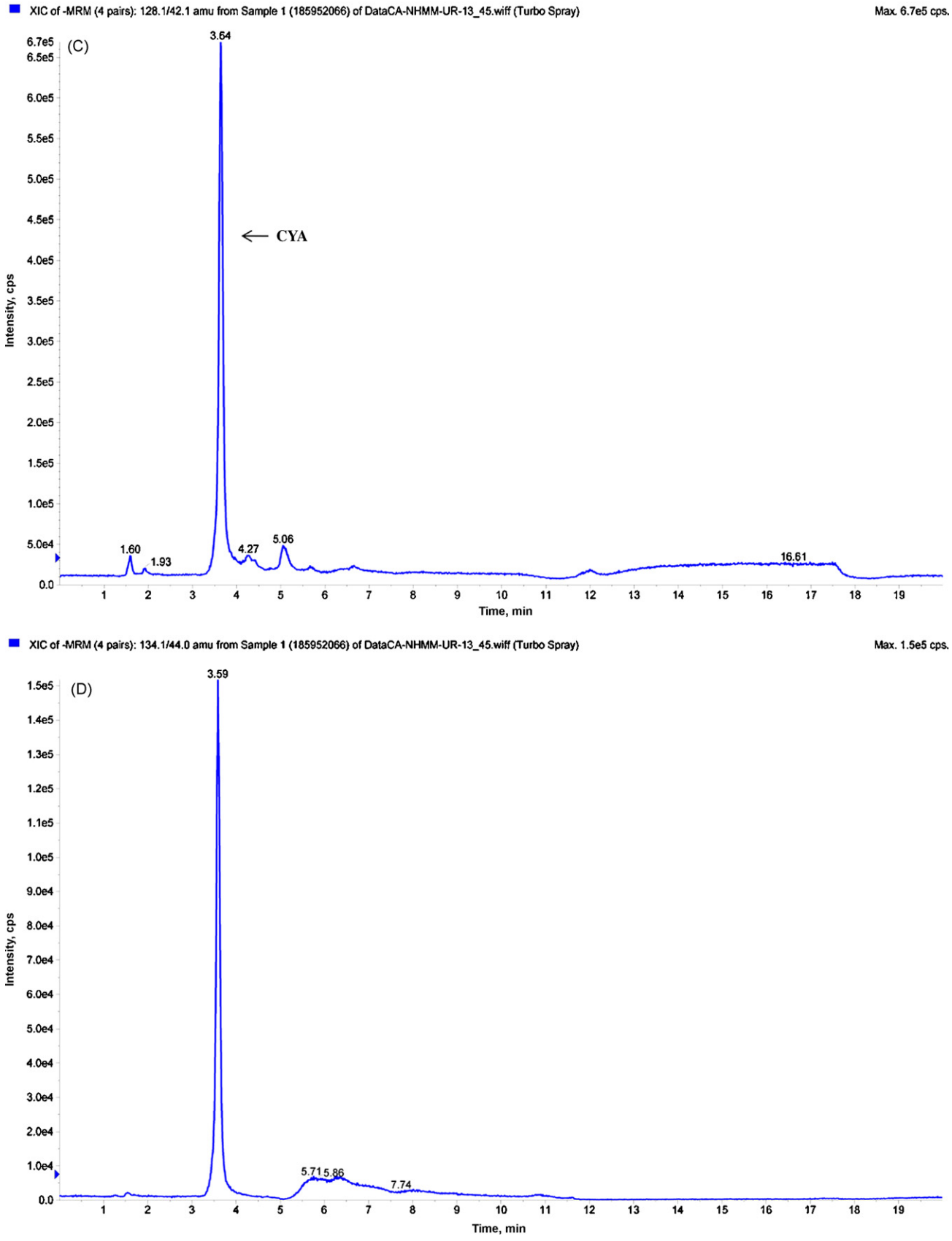

Fig. 2. (*Continued*).

Table 1
Summary of method performance.

Accuracy		Precision		
Expected conc.	Measured conc.[a]	Expected conc.	%RSD inter-day	%RSD intra-day
1.00	1.04 ± 0.06 (104)	2.00	10.5	10.2
10.0	10.0 ± 0.37 (100)	17.0	5.55	2.40
50.0	50.0 ± 1.31 (100)	32.0	2.77	2.48

Concentration unit is presented in ng/mL; $n = 10$ was used for accuracy result, and $n = 20$ for precision. A 100-fold dilution of urine with deionized water was used as matrix for accuracy study, and a 10-fold dilution for precision study. Expected concentrations in precision study were those that included background levels of cyanuric acid.

[a] Expressed as mean ± SD (% of expected conc.).

umn lifetime and increased separation efficiency. A mostly aqueous acidic mobile phase provided the most comprehensive cleaning of the column.

During our method optimization, we found that gas temperature played a significant role in achieving the best intensity of cyanuric acid. Our preliminary results indicated the highest intensity was obtained for cyanuric acid when temperature was set at 600 °C; a substantially reduced intensity was observed at more common lower temperatures. We chose the quantification ion (Q) and the confirmation ion (C) based primarily on chromatographic behavior and intensity. In the MRM table, precursor→product ion pairs Q [m/z] 128→42 (−24 V) and C [m/z] 128→85 (−12 V) were used for native cyanuric acid, whereas L [m/z] 134→44 (−22 V) was used for its isotopic internal standard. Although [m/z] 128→85 (12 V) transition showed the best intensity during the precursor→product ion pair characterization (matrix-free condition); we later found that this transition was heavily affected, primarily intermittent interferences, by the urine matrix, and resulted in lower intensity compared to another transition. Therefore [m/z] 128→42 (−24 V) transition was instead used as our quantitative ion. Fig. 2 shows a selected ion chromatogram for cyanuric acid and its internal standard in a blank sample, QCL material, and an unknown urine sample.

3.3. Method performance

Our findings on background concentration of cyanuric acid were in agreement with the recent published work by Zhang et al. [33] except that the background concentration we observed was higher.

To reduce the impact of background levels of cyanuric acid in the urine pool on calibration and quantification, we employed pseudo-matrix matching analysis. We could not use water as a matrix because the calibration slope was significantly different from that in urine. However, our data indicated that urine diluted as much as 100:1 has a slope very similar to one derived from undiluted urine (data not shown). The 100-fold dilution, which completely diluted the endogenous to achieve an unquantifiable concentration, was used for the calibration matrix; a 10-fold dilution was used for QC materials because the smaller dilution preserved matrix components similar to the undiluted urine.

Table 1 summarizes our method performance. In general, accuracies (also called relative recoveries) ranged from 100% to 104%. Our method precision was expressed as the percent RSD values that ranged from 2.77% to 10.5% for inter-day variation, and from 2.40% to 10.24% for intra-day variation. All reported RSD values were <15%, which is desirable in bioanalytical analysis [32]. The results of linearity evaluations were excellent. The average correlation coefficient of the calibration plots ($n = 20$) was 1.000 with an error about the slope of <1%. Also, using the data from the same calibration plots ($n = 20$), the average values for intercept (±SD) and slope (±SD) were 0.030 ± 0.014 and 0.017 ± 0.00, respectively. Because the approximate concentration of cyanuric acid in our undiluted urine pool was ∼20 ng/mL, by diluting the matrix 100-fold, we should have had an endogenous concentration of ∼0.2 ng/mL. Using this concentration, we then re-calculated the method LOD as three times this value and, therefore, reported the LOD as 0.6 ng/mL for our method. With suitable extraction recoveries leading to elevated overall intensity, we obtained a much lower LOD than previously reported methods [20–23,26,29,33]. The improved LOD was also the result of better chromatography and reduced matrix interferences.

Results from matrix effect studies indicated that RSDs of internal standard intensity decreased with urine dilution or with amount of urine used. In each matrix, the obtained RSD value was reported as the followings: 7.7% from 1 mL of 100-fold diluted urine, 9.2% from 1 mL of 10-fold diluted urine, 10.8% from 0.5 mL of non-diluted urine, and 33.1% from 1 mL of non-diluted urine. Based on these results, we suggest that 0.5 mL instead of 1 mL of urine sample should be used for subsequent analysis to reduce variations in internal standard intensity; variations would negatively affect the LODs. For subsequent analysis, for 0.5 mL of urine a dilution factor of 2 should be applied to obtain the final concentration.

3.4. Sample stability

We developed this method in anticipation of a post-event investigation for immediate use, thus the stability of compound in urine was studied for only a short period of time. Our results indicate ∼38–42% loss of endogenous cyanuric acid in the non-spiked urine samples (background concentration ∼20.0 ng/mL) stored in refrigerator and freezer, whereas <5–8% loss was observed in spiked samples (subsequent concentration after enrichment: ∼220 ng/mL) stored in similar conditions for one month. Our results were not totally in agreement with previous report [29,33] because we found that endogenous cyanuric acid may undergo further degradation, reaction, or change in its molecular structure at a relatively faster rate. We believe that matrix components in certain urine samples contribute to this observation, particularly when a lower amount is present. This observed difference may limit our ability to accurately quantify the cyanuric acid in stored urine samples and may indicate that cyanuric acid should be measured soon after urine collection.

4. Conclusions

We successfully developed an HPLC–MS/MS method that is rapid, selective, and reproducible for the quantification of cyanuric acid in human urine after different exposure scenarios (from background exposure to poisoning).

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