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Integrated preservation and sample clean up procedures for studying water ingestion by recreational swimmers via urinary biomarker determination

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Abstract

The use of cyanuric acid as a biomarker for ingestion of swimming pool water may lead to quantitative knowledge of the volume of water ingested during swimming, contributing to a better understanding of disease resulting from ingestion of environmental contaminants. When swimming pool water containing chlorinated cyanurates is inadvertently ingested, cyanuric acid is excreted quantitatively within 24 h as a urinary biomarker of ingestion. Because the volume of water ingested can be quantitatively estimated by calculation from the concentration of cyanuric acid in 24 h urine samples, a procedure for preservation, cleanup, and analysis of cyanuric acid was developed to meet the logistical demands of large scale studies. From a practical stand point, urine collected from swimmers cannot be analyzed immediately, given requirements of sample collection, shipping, handling, etc. Thus, to maintain quality control to allow confidence in the results, it is necessary to preserve the samples in a manner that ensures as quantitative analysis as possible. The preservation and clean-up of cyanuric acid in urine is complicated because typical approaches often are incompatible with the keto-enol tautomerization of cyanuric acid, interfering with cyanuric acid sample preparation, chromatography, and detection. Therefore, this paper presents a novel integration of sample preservation, clean-up, chromatography, and detection to determine cyanuric acid in 24 h urine samples. Fortification of urine with cyanuric acid (0.3–3.0 mg/L) demonstrated accuracy (86–93% recovery) and high reproducibility (RSD < 7%). Holding time studies in unpreserved urine suggested sufficient cyanuric acid stability for sample collection procedures, while longer holding times suggested instability of the unpreserved urine. Preserved urine exhibited a loss of around 0.5% after 22 days at refrigerated storage conditions of 4°C.

Graphical abstract

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Appendix A. Supplementary data: Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.aca.2017.06.012>.

Swimmers ingesting the urinary biomarker, cyanuric acid (CA)

Keywords

Cyanuric acid; Biomarker; Urine; Recreational water; Sample preservation; Large scale application

1. Introduction

Ingestion of water is a pathway for exposure to environmental chemical, biological, and radiological contaminants. Exposure to contaminants may result as a byproduct of their intended use, through malicious intent (such as criminal or terrorist activities), or unintentionally (such as accidents and natural disasters). Estimating the potential dose from water ingestion requires information about the quantity of water consumed [1]. In addition to ingestion of water as a beverage or in other liquids, ingestion may occur during swimming or diving during recreational use [2]. Recreational waters include swimming pools, hot tubs, water parks, water play areas, interactive fountains, lakes, rivers, or oceans. One recreational water of great interest is swimming pool water because exposure to pathogenic organisms via water ingestion has caused high visibility cases of illness and death, especially in infants and children [3]. A significant uncertainty in recreational water risk assessment is the relationship between the actual exposure level resulting from ingestion of contaminated water and the corresponding level of illness. Although the frequency of illness can be determined through epidemiological studies, estimating the exposure factors that contribute to these adverse health effects, such as the volume of water ingested, requires detailed study [2].

One way to estimate the volume of swimming pool water ingested is to measure the level of a suitable chemical biomarker in the swimming pool and also in the total urine sample obtained from the swimmer. From these measurements, the volume of water ingested can be quantitatively estimated. For this purpose, cyanuric acid [87-90-1] can be a chemical biomarker because it is widely used as a stabilizer for the chlorine disinfectant in many outdoor swimming pools [4,5]. Toxicological studies revealed this compound is not metabolized in the human body and that it is excreted quantitatively in urine [6,7]. For a large scale study of water ingestion, an efficient and accurate method of determining cyanuric acid in both swimming pool water and in urine is required. Briggles and co-workers developed a method for the determination of cyanuric acid in urine. However, this method requires elaborate and lengthy sample cleanup, followed by a difficult HPLC separation [8], precluding its use for the analysis of hundreds of samples that would be generated by a

large-scale study. More recently, other reports have documented methods for analyzing cyanuric acid in swimming pool water and/or urine [9–14].

While these reported methods are efficient in their intended application, additional work is required to confidently apply such methods in studies of large number of swimmers. For example, one inherent challenge in applying such methods to large numbers of swimmers is that significant delays may exist between collection and analysis of samples. Cyanuric acid is excreted over a 24 h collection period, so urine samples are collected over that period. Preservation procedures for cyanuric acid in urine are reported to affect quantitative results as a result of degradation, reaction, or change in molecular structure related to urine matrix components, leading to the suggestion that cyanuric acid be measured “soon after urine collection” [13,15].

From a practical standpoint, however, urine collected from large numbers of swimmers cannot be analyzed immediately, given requirements of sample collection, shipping, and handling. Thus, to increase confidence in results of urine analysis, it is desirable to preserve samples in a manner to ensure as quantitative recovery analysis as possible. Because of the nature of cyanuric acid, including its pH dependent keto-enol tautomerization and the complexities of the urine matrix, sample preservation is not trivial, nor, as summarized above, has it been reported previously to the author's knowledge [9–15]. The objective of this paper is to present the development of analytical methodology that integrates sample preservation, pre-treatment, chromatography, and detection that allow the confident determination of cyanuric acid in urine. Holding time experiments will investigate cyanuric acid stability following sample preservation, leading to methodology compatible with logistical demands of large scale studies of water ingestion during swimming activities.

2. Materials and methods

2.1. Reagents, solutions, pool, and urine samples

Cyanuric acid (98%), metaphosphoric acid (99.99%), K₂HPO₄ (98+%), perchloric acid (60%, v/v), formic acid (96%, v/v), 1.0 M hydrochloric acid, 1.0 M sodium hydroxide, HPLC grade methanol and methylene chloride were purchased from Aldrich Chemical (Milwaukee, WI). A stock solution of cyanuric acid (122 mg/L) was prepared by sonicating the cyanuric acid solid in de-ionized water for 30 min. All aqueous solutions were filtered through 0.45 µm cellulose filters to remove insoluble impurities. A 10 mg/L stock cyanuric acid solution was prepared and a set of 0.1, 0.5, 1.0, 3.0, and 5.0 mg/L dilutions were made each in the following: de-ionized water, 13 mM K₂HPO₄ buffer, and a mixture of 0.25% perchloric acid (v/v) and 0.025% metaphosphoric acid (w/v). Representative human urine samples were prepared from human urinary metabolite lyophilizate (part number U6378, lot 95H7010) and urinary protein lyophilizate (part number U8126, lot 127F7045) purchased from Sigma (St. Louis, MO). The urine samples were prepared according to the manufacturer's instructions where 1 g of metabolites represents 30–35 mL of urine, and 1 mg of protein represents 40 mL of urine.

2.2. Integrated sample preservation, preparation, and analysis of pool water and urine

The pool water samples were collected, stored refrigerated at 4°C, and then filtered using a 0.2 µm cellulose syringe filtration disk. The filtrate was then refrigerated at 4°C and analyzed within one week by direct injection using the HPLC parameters in Table 1.

The urine samples required a clean-up procedure to remove proteins and other urinary interfering substances. The optimization and development of the clean-up procedure is described in the Supplement Information. In summary, within 6 h of collection of a 24 h urine sample, a 10 mL aliquot of the bulk urine sample was preserved by the addition of 1 mL of the acid preservation reagent composed of 10% perchloric acid (% v/v) and 1% metaphosphoric acid (% w/v). The acidified urine sample was stored at 4°C.

Subsequently, a 1.5 mL of the preserved urine sample was centrifuged at 14,000 g for 15 min. A 1.0 mL of the supernatant was then cleaned up by solid phase extraction using three solid phase extraction cartridges (C18, SCX, Polymer as described below) connected in series, after each conditioned separately. During conditioning, all sorbents were kept moist by leaving 1.5 mL above the sorbent bed during conditioning. First in the sequence was a 3 mL cartridge containing 500 mg of octadecyl (C18) silica (JT Baker, Phillipsburg, NJ), which was cleaned and activated by passing 15 mL each of methanol and de-ionized water. The second cartridge contained 1.8–2.0 meq of a strong cation exchange (SCX) resin (On Guard-H, Dionex Sunnyvale, CA). It was attached to the bottom of the first cartridge after being cleaned and activated by passing 30 mL of de-ionized water. The third cartridge contained 6 meq of polyvinylpyrrolidone (On Guard-P, Dionex, Sunnyvale, CA), was conditioned by passing 30 mL of de-ionized water and then attached to the bottom of the second cartridge. The urine was filtered at gravity filtration rate (about 10 drops/min or slower) through the series of connected cartridges, using 4 mL of 0.05 M hydrochloric acid solution as the eluent with the first one mL discarded.

As a final clean-up step, a volume of 1.5 mL of cleaned urine was extracted by shaking with 2.5 mL of methylene chloride in a glass vial for about 1 min. After allowing the phases to separate for 30 min, the aqueous phase was drawn off with a pipette, placed in an HPLC autosampler vial, refrigerated at 4 °C, and analyzed within a month using the instrumental parameters in Table 1.

2.3. Identification of urinary interferences and confirmation of cyanuric acid

Identification of interferences was done using a Thermo-Finnigan TSQ (San Jose, CA) triple quadrupole mass spectrometer in the positive ion electrospray ionization (ESI) mode. It was calibrated using a caffeine/MRFA/Ultramark 1621 mixture according to the manufacturer. The instrument was first operated in LC/MS mode, scanning from m/z 110 to 600, which indicated that m/z 114 was of interest. This m/z was hypothesized to arise from creatinine, a well known substance in urine. LC/MS/MS experiments were performed by selecting m/z 114 and scanning the second mass analyzer from m/z 10–116 for both the urine sample and authentic sample of creatinine.

To help confirm that cyanuric acid was quantitated in the samples and not a co-eluting interference, an analytical approach was used in which cyanuric acid is selectively extracted

from solution without relying on a chromatographic separation. Specifically, stable association complex electrospray mass spectrometry (cESI/MS) for the analysis of cyanuric acid in urine is described in more detail elsewhere [16]. Briefly, one mL of the sample and 3.0 mL of methyl t-butyl ether (MTBE) were combined in a disposable 4 mL polytetrafluoroethylene (PTFE) faced stopper vial and shaken vigorously for 2 min. A volume of 2.5 mL of the organic solvent was drawn off quantitatively and evaporated to dryness with a gentle stream of argon. The sample was reconstituted in 0.100 mL of aqueous 10 mM decyltrimethylammonium bromide (Fluka, Buchs, Switzerland). Aliquots (10 μ L) of this solution are then injected.

2.4. Fortification of urine samples and holding time studies

The holding times mentioned above and discussed in more detail below were determined through studies with and without the preservation described, but using the sample cleanup steps described, as appropriate. Briefly, a volume of 60 mL of urine was prepared and acidified as described above. A volume of 35 mL was fortified at 5.00 mg/L (high level), and 10 mL was fortified at 1.00 mg/L (low level) cyanuric acid. Approximately 10 mL were reserved for the blank determinations. The fortified and blank urine samples were transferred as 1.5 mL aliquots into 3 mL sample tubes (part # LX5013AT, A. Daigger, Vernon Hills, IL). The samples were refrigerated at 4 °C.

For the unpreserved urine sample holding time studies, five urine samples were donated from laboratory personnel, not exposed to swimming pool waters containing cyanuric acid. A composite urine of 500 mL was placed in a 0.5 L graduated cylinder. The urine was fortified at the 5.00 mg/L cyanuric acid level. Aliquots were drawn, and acidified at the specified holding times, and refrigerated. The samples were cleaned within 1 week and then analyzed.

3. Results and discussion

3.1. Deproteinization of urine

The deproteinization of urine and precipitation of other high molecular weight compounds is necessary to avoid high concentrations of these substances from interfering with the analysis of cyanuric acid, e.g., by clogging the chromatography or by co-precipitation of cyanuric acid with proteins over time. Deproteinization requires a suitable reagent, and common deproteinization reagents for urine and/or plasma are metaphosphoric acid [17], perchloric acid [17], trichloroacetic acid [17], tribromoacetic acid [18], methanol [19], and acetonitrile [20]. Use of inorganic acidic reagents seemed proper as cyanuric acid is both soluble and stable in 96% sulfuric acid [21], 37% hydrochloric acid [21], pH 7 solution of perchloric acid [22], and pH 1 solution of phosphoric acid [23]. Organic solvents were ruled out because their use resulted in distorted chromatographic peaks, presumably from dissimilarity of the sample composition and the mobile phase. In the current study, the proteins were precipitated from the urine samples with a 10:1 mixture of perchloric acid and metaphosphoric acid (section 2.2). The metaphosphoric acid was added to prevent potential decomposition of cyanuric acid, as discussed in more detail in the holding time section 3.5

below. This ratio was also used to overcome the high ionic strength of metaphosphoric acid, thus preventing elution of cyanuric acid in the chromatographic void volume [24].

3.2. Initial gradient vs. isocratic elution for HPLC separations

For biological samples, such as urine, gradient elution is often recommended as the best starting point for HPLC method development because of the greater potential to separate the analyte from the many interfering compounds that are typically found in biological samples [25]. Fig. 1 shows the preliminary isocratic (top) and gradient elution (bottom) analyses of a urine sample fortified with cyanuric acid. In this preliminary case, no sample cleanup procedure was used. Both chromatograms show clusters of interferences that surround the cyanuric acid peak, prohibiting accurate quantitation. Nonetheless, the two chromatograms denote obvious differences in the two separation modes. Most notable is the severe baseline distortion occurring in the gradient elution mode because of slow column equilibration, characteristic of the porous graphitic carbon (PGC) stationary phase [26]. Baseline stability is very important to attain reliable low-level quantitation [25]. Therefore, it was necessary to use an isocratic HPLC method and to apply sample cleanup procedures.

3.3. Sample cleanup

The partitioning of a hydrophobic organic compound from the aqueous phase to an organic phase is related to its water-octanol partition coefficient (K_{oct}), although the exact partitioning is determined by the specific organic liquid [27]. Hennion and coworkers [22,28] reported $\log K_{\text{oct}} = -0.2$ for cyanuric acid. This represents very low hydrophobicity as compared to other triazine compounds such as hydroxyatrazine ($\log K = 1.4$) [22] and atrazine ($\log K = 2.7$) [22]. Consequently, the extraction of cyanuric acid from aqueous matrices is problematic using most extraction sorbents or resins of different functionalities (e.g., nonpolar, anion exchange, polymeric, etc.). Instead of attempting to extract cyanuric acid from urine, our strategy focused on developing a straight forward cleanup procedure using commercially available sorbents or resins to remove most urinary interferences while allowing the cyanuric acid analyte to pass through unretained. For this purpose, it is fortunate that after addition of the acid preservation reagent, the pH of the urine sample is $\sim$ pH 1, which forces the keto-enol equilibrium to the keto structure [29] of cyanuric acid which should not interact significantly with the solid phase extraction sorbents under investigation. This lack of interaction is expected because the aromatic structure of the enol form of cyanuric acid prefers to strongly interact with other aromatic structures, such as porous graphitic carbon, which forms extended and orderly layers of hexagonal ring aromatic structures [30].

The development of the cleanup procedure is described in detail in the Supplemental Information. In brief, a series of three solid phase exchange cartridges (C_{18} , SCX, Polymer) were selected and utilized to remove a variety of interferences. Removal of interferences improved method performance for quantitative determination of cyanuric acid, as well as increased lifetime of the chromatographic column, reducing the logistics, complexity, and resulting error of utilizing HPLC during large scale study of ingestion, involving hundreds of swimmers. During the course of this work, creatinine was identified by LC/MS/MS as major

contributor to solid loading on the column, potentially leading to reduced column lifetimes and resulting maintenance complexities.

Fig. 2 (middle) shows a typical chromatogram after application of the cleanup procedure which was based on existing methods in the literature [31,32]. The acidification for deproteinization promoted full protonation of the creatinine base which was then removed by the SCX resin. In addition, the procedure removed or minimized other common urinary interferences such as urea [33], purine metabolites [34], creatine [31], uric acid [31], xanthines [31], aromatic amino acids [35], bioactive amines [32], nucleic acid [32], and other guanadino compounds [36] which are also known to be extracted via ion exchange mechanisms. A urine blank is included for comparison (top). A standard of cyanuric acid prepared in de-ionized water is shown to show retention time matching (bottom). Final conditions for the method are summarized in Table 1. As noted, the late eluting and previous most dominant peak corresponding to the creatinine interference was significantly reduced in intensity and, in fact, nearly completely removed. The baseline enabled low-level quantitation. Also, the column life was significantly extended. Prior to implementing the SCX cleanup, 50 urine injections made the column unusable. The SCX cleanup procedure enabled the analysis of approximately 92 urine samples, even with a 50 μ L sample loop to compensate for dilution effects. Additionally, over 176 injections of aqueous type of samples were performed with the same column as part of an independent study [16]. The improved column lifetime could be attributed to the removal of the large amounts of creatinine and related substances which would otherwise not be removed even by repetitive column back-flushings. As a good laboratory practice, the use of a guard column also assisted in extending the life of the analytical column. It was necessary, however, to replace the guard column after every 40 injections in order to maintain good reproducibility.

While the cleanup procedure removes creatinine, a major component of urine, as well as other urinary substances mentioned above, there are a number of unknown compounds that were not removed. In practice, after 9e10 runs using a 2 cm guard column or 5e6 runs using a 1 cm guard column, it was necessary to backflush the guard column and analytical column to remove these impurities. As a result of the first backflush of a new column, we noted that the retention time of cyanuric acid changed by 20%, probably due to physical changes in the column packing. After the initial backflush, subsequent backflushes did not affect the retention time, leading us to surmise that the first backflush changes the packing of the column. Although no significant peak shifting or change in peak characteristics was observed during subsequent backflushes, a recommendation for quality control measure is to run certified reference material of a cyanuric acid standard to ensure chromatographic peak identification and reproducibility. In order to increase the life of the guard column, the urine sample may be further cleaned by liquid-liquid extraction as described in section 2.2, with no change in method performance (Fig. 2). This resulted in doubling the lifetime of the guard column (i.e., 12 injections per 1 cm guard column). To be conservative, however, we elected to replace the guard column every 7 samples, which reduced the frequency of the time-consuming backflushing described above, increasing sample throughput. It also increases confidence in the peak identification because excess impurities tended to cause irreproducibility in the cyanuric acid retention time, which would be important if a large number of particularly impure urine samples happen to be analyzed sequentially.

3.4. Analysis of urine samples

Because the sample preparation procedure results in an injected sample with pH ~ 1 , the effect of sample pH was investigated for chromatographic effects. If the sample did not equilibrate to the basic pH of the aqueous mobile phase during analysis, the degree of ionization of the cyanuric acid would be affected, and, as documented, its sensitivity using UV detection would change dramatically [37]. The retention time of cyanuric acid proved sufficient to provide adequate equilibration between the sample pH of 1.1 and the alkaline eluent pH of 9.1. It is known that the ultraviolet absorption spectra of cyanuric acid progressively changes from a non-absorbing form (keto form) to a strongly absorbing molecule (enol form) [38]. This was confirmed by preparing the calibration curves in the 0.1–5 ppm range in de-ionized water, 13 mM K_2HPO_4 buffer (pH 9.1), and de-proteinization acidic reagents. The statistical analysis of the slopes of the calibration curves were not significantly different, with $R^2 > 0.9999$ for all curves. These experiments provide useful information for the analysis of urine samples. First, it is sufficient to use cyanuric acid standards in water, which are easily prepared and are stable for 43 days [37]. Thus, the preparation of standards of cyanuric acid in phosphate salts and other acids is avoided. Second, it is possible to adjust the pH of the cleaned urine sample to pH 7–9 by equimolar addition of 1.0 M sodium hydroxide prior to analysis. Without pH adjustment, some unassigned peaks tended to shift irreproducibly resulting in interference with the cyanuric acid determination.

To determine method performance, a composite urine sample was obtained by pooling the urine from 6 swimmers. The composite urine was spiked at the 0.3 and 3.0 mg/L level and subjected to the sample clean up procedure (section 2.2). The percent recoveries and standard deviations were $86\% \pm 7\%$ ($n=14; 3$) and $93\% \pm 3\%$ ($n=14; 3$) for the low and high level spikes, respectively. As an estimate of the detection limit, we used 10 times the standard deviation of the low level spike, which calculates to 0.02 mg/L. The precision, accuracy, and sensitivity of this technique meet the requirements for large scale studies of water ingestion by swimmers, resulting in the results of an initial study [12] being of sufficient quality and quantity to be utilized in an exposure estimation handbook [1].

Application of the method to other urine samples yielded a few urine samples where the cyanuric acid was not well resolved from impurities unique to these samples. The addition of 0.5% acetonitrile to the eluent helped to resolve the cyanuric acid and did not affect the linearity of the calibration curve. As an example, the percent recovery of these urine samples spiked with a 3.0 mg/L was $98\% \pm 5\%$.

In addition to the good recoveries from fortified solutions, the retention times of the urine sample matched those of authentic cyanuric acid standards, as illustrated in the chromatogram of Fig. 2 (bottom). This provides confidence in the cyanuric acid determination. In order to further increase confidence in the quantification of cyanuric acid, data from two independent confirmatory procedures were used. The first procedure consisted of HPLC detection of the extract using a confirmatory silica-based column [9,37], and the retention time in the urine sample matched that of the cyanuric acid standard. The second approach used to increase confidence for cyanuric acid analysis in urine was stable association complexation electrospray mass spectrometry (cESI/MS), in which cyanuric acid

is quantified as a stable complex with a quaternary ammonium salt. This technique has proven advantageous for the determination of several anionic environmental analytes [39,40]. Details of its application for cyanuric acid determination are described elsewhere [16]. The application of cESI/MS technique to a cleaned urine fortified with 5.00 mg/L cyanuric acid resulted in a calculated cyanuric acid recovery of $100 \pm 7\%$.

3.5. Holding time studies

From a practical standpoint, urine collected from swimmers cannot be analyzed immediately, given requirements of sample collection, shipping, and handling. In order to fulfill the goal of a large-scale study, it was necessary to study the holding time of the cyanuric acid in urine. Long-term stability has been characteristic of urinary substances containing an amide moiety such as urea preserved in acid for 100 days [41]. The perchloric acid and meta-phosphoric acid added to precipitate urinary proteins may also result in preservation of cyanuric acid in urine for two reasons. First, sample pH ~ 1 may cause unfavorable conditions for survival of bacteria, thus inhibiting the microbiological decomposition of cyanuric acid. Second, the use of metaphosphoric acid has been reported to prevent oxidation of certain organic acids (e.g., ascorbic acid converting to dehydroascorbate) via catalysis of ferric ions [17,42].

Three distinct classes of holding time studies were performed to investigate separately the effects of holding time on the cyanuric acid recovery in both acid-preserved and unpreserved urine samples. First, sample holding time studies were conducted by monitoring replicates of acid-preserved urine samples fortified at 5.00 mg/L cyanuric acid. The recovery was measured after holding the cleaned-up samples for 1 day, 22 days, and 5 months. Second, in order to determine the effect of delaying sample clean-up, recoveries were determined for acid-preserved replicates held for 22 days before being cleaned-up. Third, because sample preservation by acidification may not always be possible immediately after collection, recoveries of cyanuric acid from replicate unpreserved samples were determined after holding them 0, 6, 21, 30, 45, or 69 h. The results of these studies are summarized in Table 2.

In Table 2, the observed difference between samples cleaned on day 1 and samples cleaned on day 22 was a decline of 0.3%; at the 95% confidence level, the amount of degradation in these samples over the 22 days was no more than 2.6%. Even after 5 months, samples that were subjected to immediate preservation and cleaning showed little degradation within experimental error. Recovery observed among these samples was 1.3% higher than that seen initially, although this may be an artifact of sampling error because the 95% confidence limit indicates that a gain (or loss) of as much as 7%. However, a substantial, and statistically significant ($p < 0.05$), increase in standard deviation is noted among samples held for 5 months when compared to those held no longer than 22 days. Among the samples for which clean-up was delayed until day 22, the observed decline amounted to 0.9%, not significantly different from the decline seen in samples that were cleaned on day 1. Confidence limits at the 95% level indicate that any degradation that may have occurred among these samples was no greater than 4.3%. In samples that remained unpreserved, only minor differences from initial recovery levels were observed for up to 30 h in the unpreserved state. At 30 h,

these samples showed a decline of approximately 2%; the 95% confidence level was 10%. Beyond this, however, significant loss of analyte occurred, reaching about 15% at 69 h.

The results of the holding time studies suggest that cyanuric acid is stable in urine when preserved in the described manner. This increases confidence in the use of the sample cleanup procedure for the large-scale determination of cyanuric acid in the urine of swimmers resulting from ingestion of pool water [12].

4. Conclusion

From a scientific standpoint, the preservation and sample clean up approaches of our study may avoid association of cyanuric acid with urine matrix elements. The tendency for cyanuric acid to form such associations is suggested by earlier studies [16], and related, mechanistic association studies have been detailed recently [43–45]. Also, note that all steps are systematically integrated into the overall method. Thus, if one step is changed, e.g., through the choice of a different type of detector or chromatography column, the entire method may need to be re-integrated and re-optimized. Accordingly, the steps in the method presented here might not be directly, incrementally included in previously reported analytical procedures developed for specific purposes [9–15].

From a practical standpoint, a procedure for preservation, cleanup, and analysis of cyanuric acid was developed to meet the logistical and cost demands of large scale studies. First, holding time data from this study support short term stability of cyanuric acid in unpreserved urine, i.e., the 24 h necessary for sample collection purposes. Second, whereas cyanuric acid in unpreserved urine beyond 30 h suggested degradation and is deemed to be unreliable, data from this study also support long term stability of cyanuric acid in acid preserved urine for up to twenty two days and longer stability for urine samples cleaned within one day and stored for up to five months at refrigerated storage (4 °C) conditions. This long term stability allows for shipping and handling of samples, which is needed to fulfill the logistic challenges of a large-scale study involving hundreds of participants. Third, while the guard column and analytical column requires replacement at the frequency discussed above, the cost of the columns are estimated to be less than 2% of the total cost of a quality controlled, full-scale study.

From the standpoint of broader application of the methodology presented, the results of a study of the volume of ingested water by a large group of volunteers from a broad range of age groups during recreational swimming activities, using the methodology described for the first time here, are presented in Refs. [12,46], which represent pilot-scale and full-scale studies, respectively. An additional broader application is that while the development of the methodology discussed was predicated on ingestion of water during swimming, the results may also have implications for other studies of ingestion of cyanuric acid through other means as has occurred, perhaps most notably through contamination or adulteration of animal feed or infant formula in the United States and China, respectively [47,48]. These concerns emerged in the mid 2000's, and continue to garner attention usually through detection of adulterants in commodities such as milk and milk products [49–66]. The present study may enable analysis of urine from individuals who have ingested such adulterants.

Namely, such studies could involve a diverse range of individuals and species, and the preservation/clean-up procedure described, by design, robustly removes a large number of substances which tend to challenge even highly specific detection techniques.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- USEPA, U.S. EPA. U.S. Environmental Protection Agency; Washington, DC: 2011. Exposure Factors Handbook 2011 Edition (Final). EPA/600/R-09/052F, https://cfpub.epa.gov/si/si_public_record_report.cfm?dirEntryId=236252 [last accessed Feb 2017]
- USEPA. [last accessed Feb 2017] Technical Information about Beaches. 2016. <https://www.epa.gov/beach-tech>
- CDC. [last accessed Feb 2017] Recreational Water Illnesses. 2016. <http://www.cdc.gov/healthywater/swimming/swimmers/rwi.html>
- Kowalsky, L., editor. NSPF. Certified Pool-SPA Operator. National Swimming Pool Foundation; San Antonio, TX: 1992. p. 46
- CDC. [last accessed Feb 2017] The Model Aquatic Health Code (MAHC): an All-inclusive Model Public Swimming Pool and Spa Code. 2014. <http://www.cdc.gov/mahc/currentedition/index.html>
- Allen LM, Briggles TV, Pfaffenberger CD. Absorption and excretion of cyanuric acid in long-distance swimmers. *Drug Metab Rev.* 1982; 13:499–516. [PubMed: 7105972]
- Hammond BG, Barbee SJ, Inoue T, Ishida N, Levinskas GJ, Stevens MW, Wheeler AG, Cascieri T. A review of toxicology studies on cyanurate and its chlorinated derivatives. *Environ Health Perspect.* 1986; 69:287–292. [PubMed: 3545805]
- Briggles TV, Allen LM, Duncan RC, Pfaffenberger CD. High performance liquid chromatographic determination of cyanuric acid in human urine and pool water. *J Assoc Off Anal Chem.* 1981; 64:1222–1226. [PubMed: 7287619]
- Cantu R, Evans O, Kawahara FK, Wymer LJ, Dufour AP. HPLC determination of cyanuric acid in swimming pool waters using phenyl and confirmatory porous graphitic carbon columns. *Anal Chem.* 2001; 73:3358–3364. [PubMed: 11476236]
- Cantu R, Evans O, Magnuson ML. Rapid analysis of cyanuric acid in swim pool waters by high performance liquid chromatography using porous graphitic carbon. *Chromatographia.* 2001; 53:454–456.
- Dorevitch S, Panthi S, Huang Y, Li H, Michalek AM, Pratap P, Wroblewski M, Liu L, Scheff PA, Li A. Water ingestion during water recreation. *Water Res.* 2011; 45:2020–2028. [PubMed: 21227479]
- Dufour AP, Evans O, Behymer TD, Cantu R. Water ingestion during swimming activities in a pool: a pilot study. *J Water Health.* 2006; 4:425–430. [PubMed: 17176813]
- Panuwet P, Wade EL, Nguyen JV, Montesano MA, Needham LL, Barr DB. Quantification of cyanuric acid residue in human urine using high performance liquid chromatography-tandem mass spectrometry. *J Chromatogr B-Analytical Technol Biomed Life Sci.* 2010; 878:2916–2922.
- Zhang M, Li S, Yu C, Liu G, Jia J, Lu C, He J, Ma Y, Zhu J, Yu C. Determination of melamine and cyanuric acid in human urine by a liquid chromatography tandem mass spectrometry. *J Chromatogr B-Analytical Technol Biomed Life Sci.* 2010; 878:758–762.

15. Patel K, Jones K. Analytical method for the quantitative determination of cyanuric acid as the degradation product of sodium dichloroisocyanurate in urine by liquid chromatography mass spectrometry. *J Chromatogr B, Anal Technol Biomed life Sci.* 2007; 853:360–363.
16. Magnuson ML, Kelty CA, Cantu R. Stable association complex electrospray mass spectrometry for the determination of cyanuric acid. *J Am Soc Mass Spectrom.* 2001; 12:1085–1091. [PubMed: 11605969]
17. Koshiishi I, Mamura Y, Liu J, Imanari T. Evaluation of an acidic deproteinization for the measurement of ascorbate and dehydroascorbate in plasma samples. *Clin Chem.* 1998; 44:863–868. [PubMed: 9554500]
18. Yuen KH, Peh KK. Simple high-performance liquid chromatographic method for determination of ketoconazole in human plasma. *J Chromatogr B Biomed Sci Appl.* 1998; 715:436–440. [PubMed: 9792531]
19. Romanova D, Grancai D, Jozova B, Bozek P, Vachalkova A. Determination of apigenin in rat plasma by high-performance liquid chromatography. *J Chromatogr A.* 2000; 870:463–467. [PubMed: 10722103]
20. Shihabi ZK. Sample matrix effects in capillary electrophoresis: II. Acetonitrile deproteinization. *J Chromatogr A.* 1993; 652:471–475.
21. Merck-Index. 12th.
22. Pichon V, Chen L, Guenu S, Hennion MC. Comparison of sorbents for the solid-phase extraction of the highly polar degradation products of atrazine (including ammeline, ammelide and cyanuric acid). *J Chromatogr A.* 1995; 711:257–267.
23. Farber H, Nick K, Schoöler HF. Determination of hydroxy-s-triazines in water using HPLC or GC-MS. *Fresenius' J Anal Chem.* 1994; 350:145–149.
24. Washko PW, Welch RW, Dhariwal KR, Wang Y, Levine M. Ascorbic acid and dehydroascorbic acid analyses in biological samples. *Anal Biochem.* 1992; 204:1–14. [PubMed: 1514674]
25. Snyder, LR., JJ, K., Glajch, JL. *Practical HPLC Method Development.* second. John Wiley & Sons, Inc; 1997.
26. Hennion MC. Graphitized carbons for solid-phase extraction. *J Chromatogr A.* 2000; 885:73–95. [PubMed: 10941668]
27. Lyman, WJ., Reehl, WF., Rosenblatt, DH. *Handbook of Chemical Property Estimation Methods : Environmental Behavior of Organic Compounds.* McGraw-Hill; New York: 1982.
28. Guenu S, Hennion MC. On-line sample handling of water-soluble organic pollutants in aqueous samples using porous graphitic carbon. *J Chromatogr A.* 1994; 665:243–51.
29. Geith J, Klapoötke TM. Ab initio calculations of the polymerization pathways of isocyanic acid HNCO. *J Mol Struct THEOCHEM.* 2001; 538:29–39.
30. Kawahara FK, Michalakos PM. Base-catalyzed destruction of PCBs New donors, new transfer agents/catalysts. *Industrial Eng Chem Res.* 1997; 36:1580–1585.
31. Kochansky CJ, Strein TG. Determination of uremic toxins in biofluids: creatinine, creatine, uric acid and xanthines. *J Chromatogr B Biomed Sci Appl.* 2000; 747:217–227. [PubMed: 11103907]
32. Yokoyama Y, Ozaki O, Sato H. Separation and determination of amino acids, creatinine, bioactive amines and nucleic acid bases by dual-mode gradient ion-pair chromatography. *J Chromatogr A.* 1996; 739:333–342. [PubMed: 8765852]
33. Wolff SD, Yancey PH, Stanton TS, Balaban RS. A simple HPLC method for quantitating major organic solutes of renal medulla. *Am J Physiology - Ren Physiology.* 1989; 256:F954–F956.
34. Shingfield KJ, Offer NW. Simultaneous determination of purine metabolites, creatinine and pseudouridine in ruminant urine by reversed-phase high-performance liquid chromatography. *J Chromatogr B Biomed Sci Appl.* 1999; 723:81–94. [PubMed: 10080636]
35. Yokoyama Y, Sato H, Tsuchiya M, Kakinuma H. Simultaneous determination of urinary creatinine and aromatic amino acids by cation-exchange chromatography with ultraviolet detection. *J Chromatogr B Biomed Sci Appl.* 1991; 566:19–28.
36. Inamoto Y, Inamoto S, Hanai T, Tokuda M, Hatase O, Yoshii K, Sugiyama N, Kinoshita T. Chemical characterization of guanidino compounds in serum. *Am Clin laboratory.* 2000; 19:12–14.

37. Cantu R, Evans O, Kawahara FK, Shoemaker JA, Dufour AP. An HPLC method with UV detection, pH control, and reductive ascorbic acid for cyanuric acid analysis in water. *Anal Chem.* 2000; 72:5820–5828. [PubMed: 11128942]
38. Klotz IM, Askounis T. Absorption spectra and tautomerism of cyanuric acid, melamine and some related compounds. *J Am Chem Soc.* 1947; 69:801–803. [PubMed: 20292463]
39. Magnuson ML, Kelty CA. Microextraction of nine haloacetic acids in drinking water at microgram per liter levels with electrospray-mass spectrometry of stable association complexes. *Anal Chem.* 2000; 72:2308–2312. [PubMed: 10845379]
40. Magnuson ML, Urbansky ET, Kelty CA. Microscale extraction of perchlorate in drinking water with low level detection by electrospray-mass spectrometry. *Talanta.* 2000; 52:285–291. [PubMed: 18967987]
41. Hellström D, Johansson E, Grennberg K. Storage of human urine: acidification as a method to inhibit decomposition of urea. *Ecol Eng.* 1999; 12:253–269.
42. Rumelin A, Fauth U, Halmagyi M. Determination of ascorbic acid in plasma and urine by high performance liquid chromatography with ultraviolet detection. *Clin Chem laboratory Med.* 1999; 37:533–536.
43. Lam CK, Hau SCK, Yau CW, Mak TCW. Hydrogen-bonded anionic host lattices constructed with isocyanurate and thiourea/urea. *Cryst Growth & Des.* 2016; 16:759–773.
44. Ma M, Bong D. Determinants of cyanuric acid and melamine assembly in water. *Langmuir ACS J surfaces colloids.* 2011; 27:8841–8853.
45. Yeom S, Mutlu BR, Aksan A, Wackett LP. Bacterial cyanuric acid hydrolase for water treatment. *Appl Environ Microbiol.* 2015; 81:6660–6668. [PubMed: 26187963]
46. Dufour A, Behymer T, Cantu R, Magnuson M, Wymer L. Ingestion of swimming pool water by recreational swimmers. *J Water Health.* Jun; 2017 15(3):429–437. <http://dx.doi.org/10.2166/wh.2017.255>. [PubMed: 28598347]
47. FDA. [last accessed Feb 2017] Update: Interim Safety and Risk Assessment of Melamine and its Analogues in Food for Humans. 2008. <http://www.fda.gov/Food/FoodborneIllnessContaminants/ChemicalContaminants/ucm164520.htm>
48. WHO. [last accessed Feb 2017] Melamine and Cyanuric Acid: Toxicity. Preliminary Risk Assessment and Guidance on Levels in Food. 2008. http://who.int/foodsafety/fs_management/Melamine.pdf
49. Borase HP, Patil CD, Salunkhe RB, Suryawanshi RK, Salunke BK, Patil SV. Biofunctionalized silver nanoparticles as a novel colorimetric probe for melamine detection in raw milk. *Biotechnol Appl Biochem.* 2015; 62:652–662. [PubMed: 25322814]
50. Domingo Edo C, Tireli AA, Nunes CA, Batista AV, Guerreiro MC, Pinto SM. Rapid extraction of melamine in powdered milk for direct electrospray ionization tandem mass spectrometry analysis. *Talanta.* 2015; 132:535–540. [PubMed: 25476341]
51. Draher J, Ehling S, Cellar N, Reddy T, Henion J, Sousou N. Determination of emerging nitrogenous economic adulterants in milk proteins by high-performance liquid chromatography/compact mass spectrometry. *Rapid Commun mass Spectrom RCM.* 2016; 30:1265–1272. [PubMed: 27173108]
52. Ellis DI, Brewster VL, Dunn WB, Allwood JW, Golovanov AP, Goodacre R. Fingerprinting food: current technologies for the detection of food adulteration and contamination. *Chem Soc Rev.* 2012; 41:5706–5727. [PubMed: 22729179]
53. Guo Z, Cheng Z, Li R, Chen L, Lv H, Zhao B, Choo J. One-step detection of melamine in milk by hollow gold chip based on surface-enhanced Raman scattering. *Talanta.* 2014; 122:80–84. [PubMed: 24720965]
54. Hu Y, Feng S, Gao F, Li-Chan EC, Grant E, Lu X. Detection of melamine in milk using molecularly imprinted polymers-surface enhanced Raman spectroscopy. *Food Chem.* 2015; 176:123–129. [PubMed: 25624214]
55. Hu Y, Lu X. Rapid detection of melamine in tap water and milk using conjugated “One-Step” molecularly imprinted polymers-surface enhanced raman spectroscopic sensor. *J food Sci.* 2016; 81:N1272–N1280. [PubMed: 27061315]

56. Kumar N, Kumar H, Mann B, Seth R. Colorimetric determination of melamine in milk using unmodified silver nanoparticles, *Spectrochimica acta. Part A. Mol Biomol Spectrosc.* 2016; 156:89–97.
57. Lim J, Kim G, Mo C, Kim MS, Chao K, Qin J, Fu X, Baek I, Cho BK. Detection of melamine in milk powders using near-infrared hyperspectral imaging combined with regression coefficient of partial least square regression model. *Talanta.* 2016; 151:183–191. [PubMed: 26946026]
58. MacMahon S, Begley TH, Diachenko GW, Stromgren SA. A liquid chromatography-tandem mass spectrometry method for the detection of economically motivated adulteration in protein-containing foods. *J Chromatogr A.* 2012; 1220:101–107. [PubMed: 22197251]
59. Moore JC, Spink J, Lipp M. Development and application of a database of food ingredient fraud and economically motivated adulteration from 1980 to 2010. *J food Sci.* 2012; 77:R118–R126. [PubMed: 22486545]
60. Nieuwoudt MK, Holroyd SE, McGoverin CM, Simpson MC, Williams DE. Raman spectroscopy as an effective screening method for detecting adulteration of milk with small nitrogen-rich molecules and sucrose. *J Dairy Sci.* 2016; 99:2520–2536. [PubMed: 26874427]
61. Rodriguez Mondal AM, Desmarchelier A, Konings E, Acheson-Shalom R, Delatour T. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) method extension to quantify simultaneously melamine and cyanuric acid in egg powder and soy protein in addition to milk products. *J Agric Food Chem.* 2010; 58:11574–11579. [PubMed: 21038852]
62. Sivashanmugan K, Liao JD, Liu BH, Yao CK. Focused-ion-beam-fabricated Au nanorods coupled with Ag nanoparticles used as surface-enhanced Raman scattering-active substrate for analyzing trace melamine constituents in solution. *Anal Chim Acta.* 2013; 800:56–64. [PubMed: 24120168]
63. Tyan YC, Yang MH, Jong SB, Wang CK, Shiea J. Melamine contamination. *Anal Bioanal Chem.* 2009; 395:729–735. [PubMed: 19669733]
64. Yang L, Huo D, Jiang Y, Hou C, Zhang S. Monitoring the adulteration of milk with melamine: a visualised sensor array approach, *Food Addit. Contam. Part A. Chem analysis, control, Expo risk Assess.* 2013; 30:786–795.
65. Sun F, Ma W, Xu L, Zhu Y, Liu L, Peng C, Wang L, Kuang H, Xu C. Analytical methods and recent developments in the detection of melamine. *Trac-Trends Anal Chem.* 2010; 29:1239–1249.
66. Ivesic M, Babic S, Krivohlavek A, Smit Z. Determination of cyanuric acid in whey powder using HILIC-MS/MS. *Anal Methods.* 2013; 5:5188–5194.

Highlights

- Cyanuric acid in urine is utilized as a quantitative biomarker of pool water ingestion.
- The novel method presented integrates preservation and sample cleanup with analysis.
- Holding time studies indicate suitable preservation of cyanuric acid in urine.
- Integrated method results in quality data for large scale studies of ingestion.

Fig. 1.

Comparison of isocratic and gradient HPLC analyses of cyanuric acid in urine. The concentration of cyanuric acid in the chromatogram is 2.15 mg/L. Sample pre-treatment included acid precipitation of proteins and, for illustrative purposes, only the C₁₈ cartridge was used (section 2.2, except the elution solvent was de-ionized water instead of 0.05 M hydrochloric acid). A volume of 20 µL of solution was injected into the HPLC with UV detection at 213 nm. A 10 × 0.3 cm PGC column was used. For the isocratic separation, the mobile phase was 95:5 (% v/v) 50 mM K₂HPO₄ buffer (pH 9.1): methanol at 0.6 mL/min flow rate. For the gradient elution, conditions were the same except that the methanol content was ramped to 100% after 5 min and held for 40 min.

Fig. 2.

Analysis of cyanuric acid in urine, after preservation and sample clean-up (section 2.2). The concentration of cyanuric acid represented in the HPLC-UV chromatogram is 1.16 mg/L (concentration uncorrected for dilution effects). The corrected concentration is 4.85 mg/L representing a recovery of 97%. Table 1 summarizes the HPLC conditions.

Table 1
HPLC method of analysis of cyanuric acid in human urine

Stationary Phase	Porous Graphitic Carbon (hypercarb) (5 µm particle size)
Column Dimensions	10 cm (length) × 0.30 cm (width)
Guard Column Dimensions	1 cm
Column Compartment Temperature	45° C ^a
Sample Loop	25 µL
Eluent	94.5% Phosphate buffer: 5 %methanol: 0.5% acetonitrile (v/v/v)
Buffer Composition	0.013 M K HPO
Buffer pH	9.1
Flow Rates	0.8 mL/min
UV Detector	213 nm
Detection Limit	0.02 mg/L
Analysis Time	1 h

^aTemperature selected to minimize chromatographic interferences for cyanuric acid.

Table 2
Results from holding time experiments for HPLC method of analysis for cyanuric acid in human urine

Holding time	Mean recovery (mg/L)	Standard deviation	Percent of initial recovery		
			Mean	95% confidence level ^a	
			Lower limit	Upper limit	
Acid-preserved samples^{b,c}					
1 day (cleaned day 1)	4.85	0.043			
22 days					
- cleaned day 1	4.83	0.040	96.7	94.8	104.5
- cleaned day 22	4.80	0.192	99.1	95.7	102.5
5 months (cleaned day 1)	4.91	0.436	101.3	92.6	106.7
Unpreserved samples^d					
0 h	4.75	0.268			
6 h	4.70	0.087	99.0	90.3	105.5
21 h	4.79	0.360	100.7	92.7	106.4
30 h	4.67	0.187	98.2	89.5	104.7
45 h	4.29	0.105	90.2	81.6	96.7
69 h	4.06	0.133	85.5	76.8	92.0

^a 95% confidence level limits based on pooled variance among preserved or unpreserved samples, respectively (with exception of 5-month samples).

^b Mean recoveries for preserved samples based on n = 5.

^c At refrigerated storage conditions of 4 °C.

^d Mean recoveries for preserved samples based on n = 3, except at 0 h and 21 h (n = 4).