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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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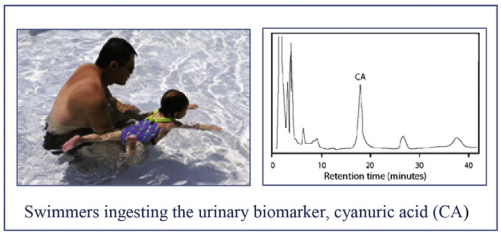
HIGHLIGHTS

- 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.

GRAPHICAL ABSTRACT



Swimmers ingesting the urinary biomarker, cyanuric acid (CA)

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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

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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.

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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 occurring 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. Briggle 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%), K_2HPO_4 (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 K_2HPO_4 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 (C_{18}, SCX, Polymer as described below) connected in series, after each conditioned separately. During