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Quantitative Detection of Trichloroacetic Acid in Human Urine Using Isotope Dilution High-Performance Liquid Chromatography−Electrospray Ionization Tandem Mass Spectrometry

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The chemical disinfection of drinking water to control microbial contaminants results in the formation of disinfection byproducts (DBPs). The volatile trihalomethanes and the nonvolatile haloacetic acids (HAAs) are the most prevalent DBPs. It is important to monitor human exposure to HAAs because of their potential adverse health effects, such as cancer. Among the HAAs, urinary trichloroacetic acid (TCAA) is a potential valid biomarker for assessing chronic ingestion exposure to HAAs from drinking water. We have developed a rugged, high-throughput, sensitive, accurate, and precise assay for the measurement of trace levels of TCAA in human urine using a simple solid-phase extraction (SPE) cleanup followed by isotope dilution high-performance liquid chromatography tandem mass spectrometry (HPLC−MS/MS). TCAA is extracted from the urine using SPE, separated from other extract components by reversed-phase HPLC, and analyzed by negative ion electrospray ionization-isotope dilution-MS/MS using a multiple reaction monitoring experiment. The method is simple and fast and is not labor intensive (sample preparation and analysis can be performed in ∼15 min) with a limit of detection of 0.5 ng/mL in 1 mL of urine.

Disinfection byproducts (DBPs) are formed as a result of the chemical disinfection of drinking water to control microbial contaminants.[1] When chlorine is used as the disinfecting agent, the volatile trihalomethanes (THMs) and the nonvolatile haloacetic acids (HAAs) are of the highest concentration DBPs generated.[2] Toxicologic studies on laboratory animals have found that some DBPs are carcinogenic and may have adverse reproductive outcomes.[3,4] Several epidemiologic studies have reported associations between consumption of chlorinated water and both cancer and reproductive effects in humans.[4,5] These epidemiologic investigations evaluated long-term exposure to THMs only. DBPs other than THMs, such as the HAAs, may pose different risks and can confound observed associations.

Because of their potential harmful effects on human health, THMs and HAAs have received increasing attention in recent years, and the U. S. Environmental Protection Agency introduced regulations for the maximum contaminant levels of these compounds in drinking water.[3] The Stage 1 Disinfectant and Disinfection Byproducts Rule sets a maximum level of 60 ng/mL for the sum of five HAAs (monobromo-, monochloro-, dibromo-, and dichloroacetic acid (DCAA) and trichloroacetic acid (TCAA)) in drinking water; stage 2 rules are in preparation. Conformity to these regulations requires a significant investment, so as these rules go into effect or change, it is important to evaluate their effectiveness. In addition, it is important to establish which interventions are the most useful in reducing exposure.

Although to date, a clear association between human exposure to HAAs in drinking water and adverse health effects has not been established, further studies to investigate the human health relevance of HAAs exposure are warranted. An important aspect of this research is the selection of a reliable biomarker of exposure to HAAs. Urinary TCAA has been proposed as a biomarker of chronic ingestion exposure to HAAs from chlorinated drinking water.[6−9] Weisel et al. showed that DCAA and TCAA, the two major HAAs in water, are readily excreted in the urine after ingestion of chlorinated water.[9] However, only TCAA showed a relationship between ingestion exposure and urinary levels, which was explained by the longer elimination half-life of TCAA vs

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DCAA. Recently, Froese et al. calculated an elimination half-life of 3.6 days for TCAA after controlled exposure to chlorinated water by analyzing first morning urine samples.[6] This value agrees with the 70−120 h range found by measuring TCAA in plasma after oral administration of chloral hydrate, a sedative drug typically used in the elderly or the young, which is metabolized in vivo to TCAA.[10] TCAA is also a urinary metabolite of 1,1,1,-trichloroethane, and tri- and tetrachloroethylene, three widely used solvents and common groundwater contaminants.[11−13]

TCAA has been previously measured in urine by using gas chromatography-electron capture detection (GC-ECD) after liquid−liquid extraction and derivatization to a methyl ester.[7−9,14] The limit of detection (LOD) was 0.15 ng/mL in 5 mL of urine.[14] Recently, a modified version of this GC-ECD method was used to determine TCAA in 40 mL of urine by combination of solid-phase microextraction and liquid−liquid extraction.[6] These methods, similar to the EPA methods 552 and 552.2 used for the simultaneous determination of all HAAs in water,[15] have adequate sensitivity; however, they are labor-intensive and time-consuming.

We have developed a high-throughput (∼15 min/sample), sensitive, and selective method for measuring TCAA in 1 mL of urine using a simple solid-phase extraction (SPE) cleanup followed by high-performance liquid chromatography-negative ion electrospray ionization-tandem mass spectrometry (HPLC/ES-MS/MS). We have enhanced the reproducibility of the method by using an isotope-labeled analogue (i.e., TCAA-2-^{13}C) as a reference compound.

EXPERIMENTAL SECTION

Preparation of Standard Solutions and Quality Control (QC) Materials. We prepared a 1 g/mL solution of TCAA by dissolving 5 g of TCAA (99+%, Sigma-Aldrich, Milwaukee, WI) in 5 mL of methyl-*tert*-butyl ether (MTBE, Sigma-Aldrich, Milwaukee, WI). This solution was diluted in three steps with HPLC-grade water (J. T. Baker, Phillipsburg, NJ) to afford a 10 μg/mL stock solution, which was stored at −4 °C. This TCAA stock solution was used to prepare 8 standard solutions whose concentrations encompassed the entire linear range of the method. The concentration of each standard was adjusted such that a 50-μL aliquot in 1 mL of urine gave the desired TCAA amount (i.e., from 0.1 to 25 ng). An isotopically labeled reference stock solution was prepared by dissolving 500 mg of TCAA-2-^{13}C (Cambridge Isotope Laboratories, Inc., Andover, MA) in 2 mL of MTBE. To prepare the working labeled isotope solution, the stock solution in MTBE was diluted with HPLC-grade water in three steps such that a 25-μL aliquot onto 1 mL of urine provided an approximate final amount of 5 ng. Both the native and the isotopically labeled standard solutions were divided into 100−300 μL aliquots, flamed-sealed in ampules and stored at −20 °C; TCAA native and labeled standards were stable for at least 6 months at −20 °C.

The QC materials were prepared from a base urine pool obtained from two donors who drank only bottled water containing nondetectable levels of TCAA. The urine pool was filtered using a sterile apparatus and divided into three subpools. One subpool was used for blank QC, and the other two were enriched with TCAA as needed to afford low (∼3 ng/mL) and high (∼16 ng/mL) level pools. The subpools were uniformly mixed, dispensed into small aliquots (∼2−7 mL) into prerinsed glass vials and stored at −20 °C until used. Long-term storage of QC materials at −20 °C for two months showed no decrease in TCAA levels. The QC materials were characterized to define the mean and the 95th and 99th confidence intervals of TCAA concentration by the analysis of at least 20 repeat measurements.

Sample Preparation. Oasis-HLB columns, 60 mg/3 mL, (Waters Corporation, Milford, MA) were conditioned with 1 mL of HPLC-grade methanol (EM Science, Gibbstown, NJ) and 1 mL of 10 mM formic acid, which was prepared from concentrated formic acid (98% min, GR, EM Science, Gibbstown, NJ). A 1-mL portion of urine was dispensed into test tubes, diluted with 2 mL of 10 mM formic acid, and spiked with 25 μL of the isotopically labeled working solution. For the standards, 50 μL of the appropriate standard was added to the urine. The diluted urine was loaded onto the conditioned SPE cartridge and allowed to pass through it by gravity. Next, residual salts were removed by washing the SPE column with 150 μL of 10 mM formic acid and by drying the cartridge for 20 s under vacuum (10 mmHg). TCAA was eluted with 1 mL of 20% methanol/water under vacuum (2 mmHg) into a 1.5-mL autosampler vial; residual TCAA was eluted by drying the cartridge (5 s, 5 mmHg). Total preparation time per sample was ∼8 min. Prepared samples could be stored at 4 °C for up to one month and at room temperature for up to one week before analysis without degradation.

Instrumental Analysis. A 20-μL portion of the SPE extract were injected into an Agilent 1100 liquid chromatograph (Agilent Technologies, Wilmington, DE) coupled with an API 3000 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). The TCAA was separated from the rest of the urine extract components on a Prism RP (2 × 100 mm, 3 μm) HPLC column (Keystone Scientific, Inc., Bellefonte, PA), which was preceded in-line by a 2-mm Prism RP (3 μm) guard column (Keystone Scientific, Inc., Bellefonte, PA) to extend the lifetime of the analytical column. The HPLC operating conditions were isocratic separation using a 75% methanol/25% 5 mM ammonium acetate (pH 5.2) eluent at a 200 μL/min flow rate and ambient temperature. The 5mM ammonium acetate HPLC buffer was prepared daily (vide infra) from concentrated acetic acid (99.7% min, GR, EM Science, Gibbstown, NJ) and HPLC-grade water. The pH of the buffer was adjusted with 1 M ammonium hydroxide (GR, EM Science,Gibbstown, NJ) using a ThermoOrion pH meter (Orion Research, Inc., Beverly, MA). The HPLC analysis run was 8 min long and included a 3-min equilibration time. During the first 3 min following the injection, a switching valve in the 1100 HPLC system directed the postcolumn flow to waste, then the automatic switching valve directed the postcolumn flow to the mass

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Table 1. Multiple Reaction Monitoring Analysis of Tricholoacetic Acid (TCAA) and TCAA-2-^{13}C[a]

	precursor ions $(M - H)^-$ (m/z)	product ions $(M - CO_2H)^-$ (m/z)
TCAA	162*, 164§, 166, 168	117*, 119§, 121, 123
TCAA-2-^{13}C	163, 165*, 167, 169	118, 120*, 122, 124

[a] The 3:1 isotopic ratio of $^{35}Cl/^{37}Cl$ yields four peaks at interval of two mass units in the ratio 3:3:1:0.1 for ion species containing three chlorine atoms. The native and labeled TCAA product ion transitions, due to CO_2 loss, used for quantitation (*) and confirmation (§) are indicated. The ratio of quantitation to confirmation ion was used as an interference quality control index.

spectrometer to detect the TCAA and TCAA-2-^{13}C signals at ~5.5 min.

Negative ion TurboIonSpray was used to form negatively charged ions. The TurboIonSpray ionization source is a variant of the electrospray source and is used to convert liquid-phase ions into gas-phase ions. The TurboIonSpray source incorporates a heated probe that blows hot dry gas at the spray produced by the electrospray; the electrospray effluent and the heated gas intersect at an angle of approximately 90° near the orifice. The heated gas increases the efficiency of ion evaporation, thus resulting in increased sensitivities and the ability to handle higher liquid sample flow rates than regular electrospray. The TurboIonSpray source allowed us to use the 2-mm-i.d. HPLC column operating at 200 μL/min without postcolumn splitting. The TurboIonSpray settings were curtain gas (N_2), 12 arbitrary units (au); auxiliary gas (zero air), 6000 cm^3/min; heated gas (zero air), 11 au; heated gas temperature, 400 °C; and ion spray voltage, −4000 V. The declustering potential (−20 V) and focusing potential (−60 V) were optimized for TCAA and TCAA-2-^{13}C. For MS/MS collision-induced dissociation, the collision energy was −10 V, with nitrogen as the collision gas (8 au); the collision cell exit potential was −5 V; the channel electron multiplier and deflector voltage were 2200 V and −400 V, respectively. The TCAA and TCAA-2-^{13}C $[M - H]^-$ precursor ions and their transitions (because of CO_2 loss) used for quantitation and confirmation, measured in multiple reaction monitoring mode at unit resolution, are given in Table 1. The dwell time for each transition was 200 ms/cycle.

We also used the above HPLC/MS/MS method, without SPE separation, for a quick (8 min) and sensitive (LOD = 0.1 ng/mL) measurement of TCAA in drinking water samples.

Data Analysis. All of the samples, blanks, standards, and QC material were processed identically using the Analyst software of the API 3000. Each ion of interest in the chromatogram was automatically selected and integrated. The peak integrations were checked for errors and corrected manually if necessary. We used the peak area ratio of TCAA to TCAA-2-^{13}C (i.e., response factor, RF) for quantification. Eight standard TCAA concentrations (0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 15.0, and 25.0 ng/mL), which encompassed the entire linear range of the method, were used to construct a calibration curve of RF versus standard amount. The calibration curve weighted by the reciprocal of the standard amount was used for quantification. Samples with values below the LOD were designated as nondetectable.

The integrated peak areas for each analyte and retention times were saved in a report file. An excel macro, created in-house, converted the report file into a report text file, which was imported into an R:BASE (Microrim, Inc., Redmond, WA) database specifically created for this analysis using an automated, custom-written routine. In the R:BASE database, RF values were calculated and corrected for natural isotope distribution contribution and standard purity. Statistical analysis of the data was performed using Statistical Analysis System (SAS) software (SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Traditionally, TCAA has been extracted from urine using liquid−liquid extraction.[7−9] Although the extraction efficiency is adequate (~85%[9,14]), the extraction is labor-intensive. Instead, we chose to extract TCAA from urine using SPE for several reasons, such as the wide variety of SPE sorbent types, the advances in cartridge design, and the fact that SPE is more readily automated than traditional liquid−liquid extraction. Urine is a highly polar matrix with variable and high concentration of ionic species, such as phosphates, urates and metal ions, sugars, proteins, creatinine, urinary pigments, and many metabolic products.[16] To extract TCAA from the urine, we selected the Oasis-HLB cartridges (Waters Corp., MA), which contain a synthetic sorbent with high absorbency for a wide array of hydrophilic compounds. We optimized the SPE separation to afford an SPE extract with a minimum amount of ionic compounds, which might have interfered with the HPLC separation and the ES-MS/MS detection, and with a TCAA concentration high enough to avoid time-consuming evaporation and reconstitution steps. The SPE recovery of TCAA from urine was calculated as RF_a/RF_b, where RF_a and RF_b are the TCAA response factor obtained from spiking the urine sample with the isotopically labeled standard after and before the SPE separation, respectively.

We found that the addition of an acidic solution to the urine was essential for the retention of TCAA to the HLB sorbent; we chose formic acid for its strength and volatile nature. We tested several urine/formic acid dilution ratios (i.e., 2:1, 1:1, 1:2, 2:3, and 1:4), and formic acid concentrations (i.e., 5, 10, and 25 mM) to achieve an optimal recovery using urine samples of low, medium, and high concentrations, tentatively set by the color of the urine.[16] We found that a 1:2 urine:formic acid dilution ratio provided the best recovery of TCAA using 1 mL of urine diluted to 3 mL, the volume of the SPE cartridge (data not shown). We also observed that the retention of TCAA by the SPE sorbent was dependent on the concentration of both the urine and the formic acid. The recovery of TCAA from the concentrated urine samples diluted with 25 mM formic acid was 65%; the less concentrated urine specimens diluted with 25 mM formic acid afforded nonreproducible HPLC separations. The recoveries of TCAA from urine samples of medium and high concentration diluted with 5 mM formic acid were 84% and 42%, respectively. The 10 mM formic acid provided excellent recoveries of TCAA for the diluted, moderately, and highly concentrated urine specimens (>95%, >75%, 60%, respectively). We chose 10 mM formic acid, because it provided the best average recovery (~80%) for all urine samples; this value compares well to the recovery of urinary TCAA achieved by traditional liquid−liquid extraction methods (~85%).[9,14]

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The diluted urine−formic acid mixture had a pH ~6, at which TCAA is in its anionic form ($pK_a = 0.65$).[17] We speculate that the interactions that facilitate the retention of TCAA by the sorbent are the result of some competition between formic acid, TCAA, and other urine components for the interaction sites in the sorbent. In addition, we cannot rule out the fact that the formic acid may also disrupt the interaction of TCAA with other urine components that may not be easily retained by the sorbent.[18] We observed that washing the cartridge with a volume of water as small as 0.5 mL resulted in poor recoveries of TCAA, which suggested that the retention of TCAA by the sorbent was relatively weak. Therefore, we washed the SPE column with a very small volume of 10 mM formic acid (150 μL), and dried the cartridge before eluting TCAA with a 20% methanol/water solution. In contrast, we observed that TCAA in 1 mL of water diluted 1:2 with 10 mM formic acid was strongly retained by the OASIS HLB sorbent; TCAA was eluted only with 100% methanol. We believe that the weaker retention in urine was due to the presence of salt and other compounds in urine that effectively compete with TCAA for the sorbent sites.

To optimize the HPLC separation and achieve both short retention times and adequate resolution, we evaluated four short microbore columns, namely Xterra RP_{18} (2.1 × 150 mm, 3.5 μm, Waters), Aquasil C18 (2 × 100 mm, 3 μm, 100 Å, Keystone Scientific), BetaMax Acid (2 × 100 mm, 5 μm, 60 Å, Keystone Scientific), and Prism RP (2 × 100 mm, 3 μm, 100 Å, Keystone Scientific). The major difference among them is that the BetaMax Acid, Prism RP, and the Xterra RP_{18} columns contain embedded polar groups in a long alkyl chain attached to the silica surface, but the Aquasil C18 column does not. Columns with embedded polar groups are developed to enhance retention and selectivity for polar compounds, such as those with carboxylic acid groups. We found that the retention time of TCAA on the Xterra RP_{18} and the Aquasil C18 columns using a methanol/ammonium acetate buffer was between 3 and 4 min, even with a low 35% methanol/buffer mobile phase, but TCAA coeluted with other interfering compounds. In contrast, the retention time of TCAA on the BetaMax Acid column was 17.5 min, even with 50−95% MeOH gradient elution, a much longer time than desired for high sample throughput. The retention time for TCAA on the Prism RP column was between 5 and 10 min, depending on the pH of the buffer and methanol content of the mobile phase; TCAA eluted with no detectable interfering compounds. On the basis of these results, we chose the Prism RP column.

Despite the low pK_a of TCAA in water,[17] we found that the retention of TCAA was highly pH-dependent at pHs 4.5−5.75 using mobile phases with 70−90% methanol (Figure 1). An increase in pH from 4.5 to 5.75 caused the TCAA retention time to shift between 5 and 10 min. We believe that this change in TCAA retention time is related to the higher pK_a of TCAA in high-percentage methanol solvent mixtures, as compared with that in water.[17] Further analyses indicated that the retention time shift plateaued at pH values greater than 5.4 (Figure 1), but the peak shape began to distort, probably because of the interaction of TCAA with the deprotonated silanol groups. Therefore, we

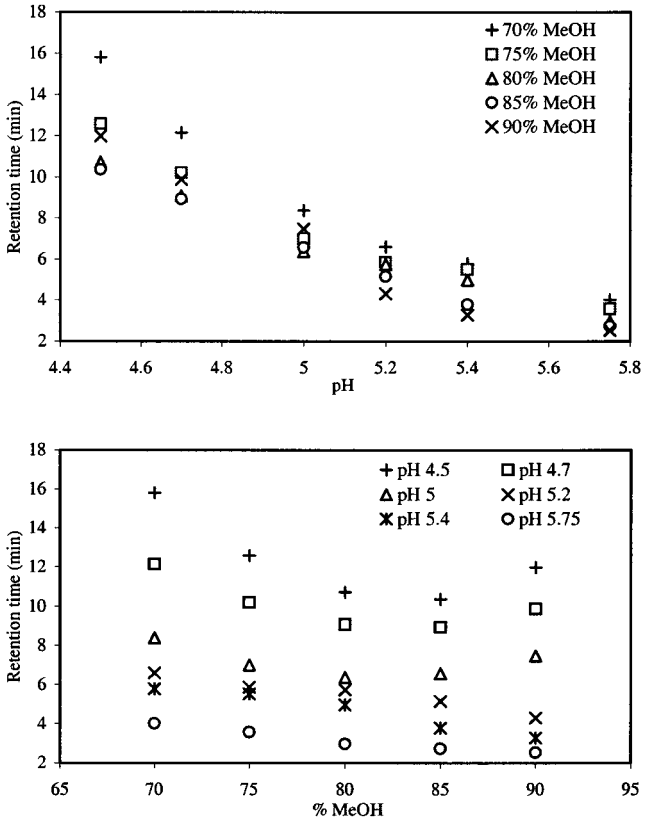

Figure 1. Changes in the trichloroacetic acid (TCAA) retention time as a function of pH (up) and percentage of MeOH (down).

selected 5.2 as the optimal pH where the TCAA retention was adequate for high sample throughput and was also less dependent on the percentage methanol of the HPLC mobile phase (Figure 1). Even with these carefully chosen conditions, a ~0.05-unit change in pH could cause a 0.1−0.3-min change in the retention time (estimated from Figure 1). To minimize these variations, we prepared the 5 mM ammonium acetate buffer daily and set the pH with a ±0.01 accuracy. During the course of the analytical runs, the standby time was minimized, so the buffer did not spend extended periods of time in the degasser compartment of the HPLC system, which seemed to slightly affect both buffer composition and pH and, ultimately, retention. A typical chomatogram of a urine extract collected with these optimized conditions is shown in Figure 2.

Because urine samples vary in both their composition and ion strength, we expected significant variability in the TCAA recovery during the extraction procedure and in the extent of ionization of TCAA. To minimize this confounder, we used the isotope dilution technique, which allows for automatic recovery correction for each sample and improves the assay precision. The native and isotopically labeled TCAA, being chemically identical, elute at the same time during the SPE and HPLC separations, but they can be differentiated by mass spectrometry.

In the mass spectra of the native analyte and its labeled analogue, the deprotonated molecular ions $[M - H]^-$ at m/z 162 and 164 (TCAA), and m/z 163 and 165 (TCAA-2-^{13}C) were the most intense peaks; the TCAA m/z 162 and 164 ions and the TCAA-2-^{13}C m/z 165 ion were selected as the precursor ions for collision-induced dissociation. The collision-induced dissociation conditions for TCAA and TCAA-2-^{13}C were selected so that

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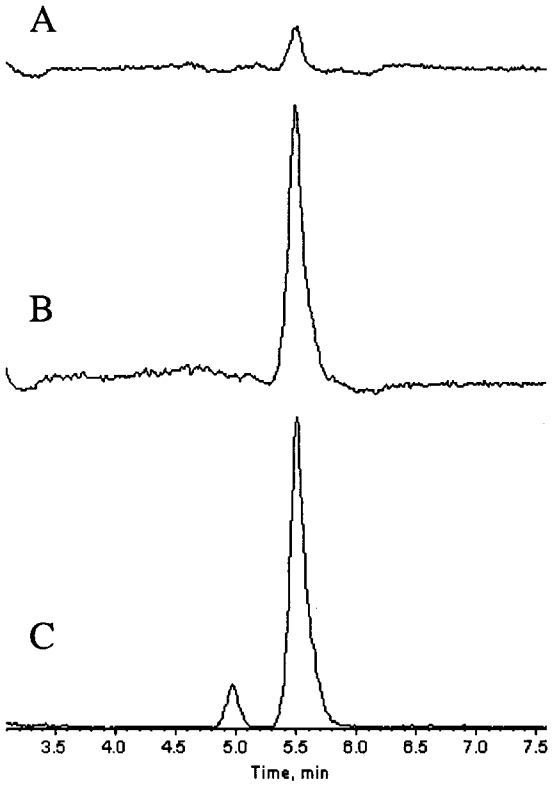

Figure 2. Typical multiple reaction monitoring chromatogram of two urine extracts: (A) native trichloroacetic acid (TCAA) (*m*/*z* 162/117) in a blank, and (B) a urine spiked with 5 ng/mL TCAA and 5 ng/mL TCAA-2-^{13}C (*m*/*z* 165/120), shown in (C).

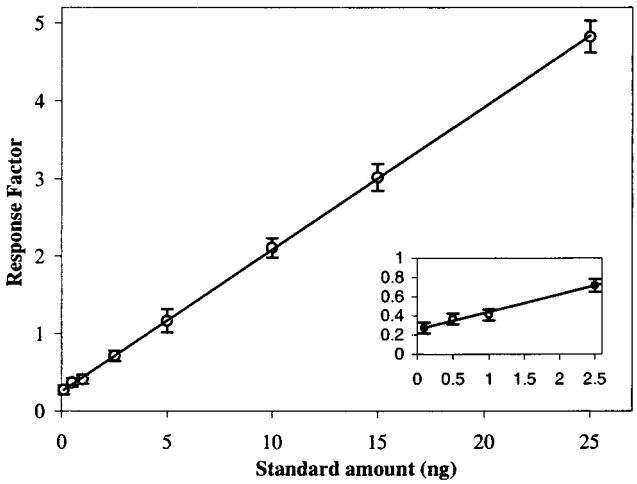

Figure 3. Average calibration curve for trichloroacetic acid based on the analysis of eight standard solutions across the linear range (± standard deviation). The values are weighted (1/*x*) by the SAS software and produced curves with excellent linear correlation coefficients (typically ~0.98). The calibration curve spanned 1 order of magnitude and was linear across the entire range, including the lowest levels, as shown in the figure inset.

Table 2. Quality Control (QC) Statistics for Measuring TCAA in Urine QC Materials over 8 Weeks

	N[a]	mean	median	std dev[b]
QC low	21	3.16	3.25	0.36
QC high	21	16.27	16.00	0.94

[a] Number of samples analyzed. [b] Standard deviation.

the precursor ions and the highest-mass product ions, represented by the neutral loss of CO_2H $[M - 45]^-$ (Table 1), were the most intense ions. Maximum sensitivity was obtained by using these product ions for quantitation of both TCAA and TCAA-2-^{13}C.

TCAA-spiked urine was repeatedly analyzed to determine the method recovery, reproducibility, and LOD. The calibration curves showed good linearity, with linear correlation coefficients generally ~0.98. A typical average calibration curve of TCAA over a 2-month period is shown in Figure 3. The interday variability of calibration curves was small; over 2 months, the standard deviation of the slope of 8 calibration curves was 3%. The LOD and limit of quantitation (LOQ) were calculated as $3S_0$ and $10S_0$, respectively, where S_0 is the standard deviation value as the concentration approaches 0.[19] S_0 was determined from the replicate analysis of low-level standards. The calculated method LOD was 0.5 ng/mL, and the LOQ was 1.7 ng/mL. These values compare well with the detection limits achieved by GC-ECD in urine.[9,14]

The accuracy was established by determining the recovery of spiked urine samples. To examine the consistency of the recovery over a range of TCAA levels, the measurements were taken by quintuplicate at three different concentrations (i.e., 1.0, 5.0, and 10.0 ng/mL). The mean recoveries of TCAA in urine, expressed as a percentage of the expected value, were excellent at spiking levels of 5 (97%) and 10 ng/mL (98%); the recovery at 1 ng/mL was higher (124%). This elevated recovery at the lowest concentration is likely due to variations associated with making measurements near the LOQ. The slope of a linear regression analysis of the calculated versus the expected concentration was 0.97, which confirmed the excellent accuracy of the method.

The precision of the method was determined by calculating the average coefficient of variation (CV) of 21 repeated measurements of the QC materials (i.e., ~3 and ~16 ng/mL) over an 8-week period (Table 2). The average CV was 8.5%. This value, which reflects both the intraday and interday variability of the assay, indicates the excellent reproducibility of this trace analysis method.

We evaluated the applicability of the method to detect TCAA in human urine by analyzing 402 samples from the Third National Health and Nutrition Examination Survey (NHANES III) that were collected during 1991−1994 and stored at −20 °C until analysis.[20] Participants in our study were selected to include persons 20 to 59 years of age, both sexes (50% male), and urban and rural residences (70% urban, >100 000 inhabitants). Further details of this reference range are discussed elsewhere.[21] TCAA was found at detectable concentrations in 75.6% of the samples. The high

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frequency of TCAA detection reflects the high frequency of exposure of the U. S. population to disinfection byproducts, the high sensitivity of our analytical method, and its value for future studies assessing environmental exposure to TCAA in the general human population.

In summary, we have developed an analytical method based on an SPE separation coupled with HPLC/ES-MS/MS for the quantitative determination of urinary TCAA, a HAA exposure biomarker. This rapid, sensitive, and simple high-throughput method (∼50 samples per day) is suitable for large epidemiologic studies to assess the relevance of human exposure to HAAs.

DISCLAIMER

The use of trade names is for identification purposes only and does not constitute endorsement by the U.S. Department of Health and Human Services or the Centers for Disease Control and Prevention. Use of the NHANES III samples was approved by the National Center for Health Statistics (NCHS) Institutional Review Board (Protocol #2001-05).

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