Journal of Chromatography B, 878 (2010) 1045–1050

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Quantification of monofluoroacetate and monochloroacetate in human urine by isotope dilution liquid chromatography tandem mass spectrometry

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ARTICLE INFO

Article history:
Received 7 October 2009
Accepted 8 March 2010
Available online 15 March 2010

Keywords:
Monofluoroacetate
Monochloroacetate
Tandem mass spectrometry
Compound
Urine excretion
LC/MS/MS
Solid-phase extraction
Chloroacetic acid

ABSTRACT

The rodenticide monofluoroacetate (MFA) and monochloroacetate (MCA), a chemical intermediate from several chemical syntheses, have been identified as potential agents of chemical terrorism due to their high toxicity. In preparation for response to poisonings and mass exposures, we have developed a quantification method using isotopic dilution to determine MFA and MCA in urine from 50 to 5000 ng/mL. Both analytes were extracted from urine using solid-phase extraction; extraction recoveries were 62% (MFA) and 76% (MCA). The extracts were then separated with isocratic high-performance liquid chromatography and identified using electrospray ionization tandem mass spectrometry, with detection limits of 0.9 and 7.0 ng/mL for MFA and MCA, respectively. Selectivity was established for both analytes with unique chromatographic retention times which were correlated with isotopically labeled internal standards and the use of two mass spectral transitions for each compound. The intra-day variability was less than 5% for both analytes and the inter-day variability was 7% for MFA and 6% for MCA.

1. Introduction

Monofluoroacetate (MFA), identified as a potential chemical terrorist agent [1], and monochloroacetate (MCA) are both highly toxic compounds. MFA has been used in suicides [2] and has caused accidental poisonings of animals and humans alike [3,4]. Monochloroacetate along with monochloroacetic acid have been the cause of accidental human deaths from both ingestion and dermal exposure [5–7].

Sodium monofluoroacetate, also known as Compound 1080, is a widely used rodenticide, as well as a naturally occurring compound in many botanical species worldwide [8,9]. Once in the body, monofluoroacetate acts as a poison by disrupting the citric acid cycle, causing an accumulation of citrate and ultimately depriving cells of necessary energy which results in cellular death. The human lethal dose of MFA is estimated to be 2–10 mg/kg [2]. Exposure to MFA results in approximately 20% excretion of the intact compound along with fluorocitrate and other metabolites [10]. Therefore, it is reasonable to evaluate MFA as a urinary biomarker to assess exposure to this poison. Previous studies, all of which prescribed derivatization of MFA, have examined MFA in urine and other biological matrices using gas chromatography (GC) [11] and high-performance liquid chromatography (HPLC) using fluorescence and UV detectors [12,13]. Methods without derivatization have also been developed for both HPLC and GC with mass spectrometric detection [14,15].

MCA and the acidic form, monochloroacetic acid, are common intermediates for the production of many chemicals, including thioglycolic acid, phenoxy acetic acid, carboxymethylcellulose and indigoid dyes. MCA is also used as a post-emergent herbicide and defoliant. Monochloroacetic acid is quickly absorbed through the skin, acting as a systemic metabolic poison, so much that only 5–15% of body surface exposure to an 80% monochloroacetic acid solution may be toxic [7]. The metabolism of monochloroacetate is not well understood [16], but up to 70% of the monochloroacetate dose has been detected unchanged in urine post-exposure urine [17]. Methods for the identification of MCA in water and biological matrices using liquid–liquid microextraction, derivatization and subsequent identification using solid-phase microextraction coupled with GC with electron capture detection have also been developed [18].

In order to respond quickly to analyze the large number of samples that could arise from a major poisoning event, it is important to have a methodology that is rapid and simple while still meeting the requirements for sensitivity, accuracy and reproducibility. In order to meet these requirements, we developed a method for

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doi:10.1016/j.jchromb.2010.03.008

determining the quantity of MFA and MCA in human urine utilizing isotopic dilution liquid chromatography tandem mass spectrometry (LC–MS/MS) without derivatization. This unique use of isotope dilution LC–MS/MS for these analytes will provide more accurate quantitative determinations in urine and an improved means to identify people who may have been exposed to these compounds and the relative extent of exposure.

2. Experimental

2.1. Materials

Calibration standards were prepared in urine from sodium monofluoroacetate (Sigma–Aldrich, St. Louis, MO), and chloroacetic acid (Supelco, Bellefonte, PA). To prepare the internal standard mixture, isotopically labeled monofluoroacetate ($^{13}C_2$, D_2), was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA) and labeled monochloroacetate ($^{13}C_2$), was purchased from Sigma (St. Louis, MO). Additional solvents used included HPLC-grade methanol, HPLC-grade acetonitrile, hydrochloric acid and ammonium hydroxide purchased from Fisher Scientific (Fairlawn, NJ), and formic acid (98%) from EM Science (Gibbstown, NJ). Organic-free 18.2 MΩ Type I water was obtained from a purifier purchased from Aqua Solutions, Inc. (Jasper, GA) for these studies. Individual human urine samples for reference range studies were obtained from Tennessee Blood Services Corporation (Memphis, TN). Oasis® HLB solid-phase extraction 96-well plates were obtained from Waters Corporation (Milford, MA). Standard laboratory glassware used for the preparation of solutions was purchased through Fisher Scientific (Fairlawn, NJ), pipettes and pipette tips from Rainin (Oakland, CA), and 96-well plates from Nunc (Rochester, NY).

MFA and MCA internal standard stock solutions were prepared at a concentration of 500 μg/mL each of labeled MFA and labeled MCA in HPLC-grade methanol. The stock solutions were diluted to yield a mixed internal standard containing MFA and MCA both at a concentration of 500 ng/mL in deionized water. Dilute hydrochloric acid solution was prepared by diluting 1 mL of 35% HCl to 100 mL with 18.2 MΩ water resulting in a 0.35% HCl solution.

For the calibration standard and quality control sample (QC) matrix, urine was collected anonymously from six persons with no known exposure to MFA or MCA and mixed together to create a homogeneous urine pool. Standards were prepared in human urine from stock solutions of MFA and MCA at concentrations of 100 μg/mL and 200 μg/mL, respectively. The urine pool was spiked with the MFA and MCA stock solutions to produce standards with the following concentrations: 50, 100, 250, 500, 1000, 2500 and 5000 ng/mL of both MFA and MCA. Two QC sample concentrations were prepared by diluting the MFA and MCA stock solutions in pooled urine, resulting in final MFA and MCA concentrations of 200 and 2000 ng/mL; a blank urine sample was also prepared. All solutions were stored at approximately −70 °C.

2.2. Sample preparation

In preparation for LC–MS/MS analysis 200 μL urine or standard samples were spiked with 20 μL of the internal standard mixture and diluted with 200 μL of dilute HCl in a 2-mL 96-well plate to obtain a pH∼1. The 96-well plate was covered and vortexed for 5 min. Oasis® HLB solid-phase extraction plates (30-mg/30-μm particles) were pretreated with 300 μL of acetonitrile followed by 300 μL of water. A volume of 200 μL of the sample mixture was loaded onto the cartridge, followed by a 130-μL rinse with 18-MΩ water. The analytes were eluted using 200 μL of acetonitrile into a clean 2-mL 96-well plate and were ready for analysis by LC–MS/MS. This method was automated with the Zephyr® SPE Workstation (Caliper, Hopkinton, MA).

2.3. Instrumental analysis

HPLC separation was performed on an Agilent 1100 HPLC with a well plate autosampler, HPLC pump, degasser and column oven (Santa Clara, CA). The LC column used was a 2.1 mm × 50 mm Primesep B2 by SIELC (Prospect Heights, IL) containing 5-μm particles and maintained at 30 °C. The mobile phase used for the isocratic separation was 60% acetonitrile and 40% water; formic acid was added to the acetonitrile/water solution to yield a total concentration of 0.05% formic acid. The analytes were eluted during the initial chromatographic flow rate of 500 μL/min. After 2 min, the flow rate was increased to 1000 μL/min and subsequently decreased to 500 μL/min after 5 min to equilibrate the system pressure prior to the next injection. The HPLC was programmed to inject 15 μL of sample for analysis resulting in on column masses ranging from 0.35 to 36 ng across the calibration curve.

Using the parameters defined in Table 1, tandem mass spectrometry was performed on an Applied Biosystems API 4000™ (Foster City, CA), to detect and quantify the analytes. Negative electrospray ionization was used for both MFA and MCA; two transitions were selected for each analyte as quantification and confirmation ions with a dwell time of 100 ms for each transition. Internal standards for both MFA and MCA were monitored using one transition each.

Data analysis was performed using the Analyst® 1.4.2 software. Calibration curves were determined by linear regression analysis of the standard concentration versus the ratio of the quantification ion area to the internal standard ion area; a $1/x$ weighting was applied.

Method characterization included replicate analysis of 20 calibration curves and 20 replicates of low QC samples (200 ng/mL) and high QC samples (2000 ng/mL). SAS® (Cary, NC) software was used to determine the statistical limits; method precision and accuracy were determined over 47 days. Fifty individual urine samples were also extracted and analyzed using this procedure to determine the reference range concentration of MFA and MCA in an anonymous population with no known exposure.

Concentration is a commonly used means to increase sensitivity following solid-phase extraction of samples. To reduce the amount of analyte loss, the stability of both MFA and MCA with respect to pH and temperature was assessed. Urine, spiked with 1 mg/mL of both MFA and MCA, was adjusted to the following pH levels: 1, 2, 3, 4, 5, and 6. The urine at a pH of 1 was prepared by the addition of hydrochloric acid; the pH 2 sample was prepared by the addition of formic acid. Acetic acid was added to create a urine sample at a pH of 3; the urine at pH 4 was prepared by adding 10% acetic acid.

Table 1
Mass spectrometer parameters for the analysis of monofluoroacetate and monochloroacetate.

Analyte	Precursor ion (m/z)	Product ion (m/z)	Cell exit potential (V)	Collision energy (V)	Declustering potential (V)
Monofluoroacetate—quantitation	77	57	−7	−16	−35
Monofluoroacetate—confirmation	77	33	−3	−18	−35
Monofluoroacetate—internal standard	81	60	−11	−16	−35
Monochloroacetate—quantitation	93	35	−5	−10	−30
Monochloroacetate—confirmation	93	49	−5	−20	−30
Monochloroacetate—internal standard	97	37	−5	−16	−30

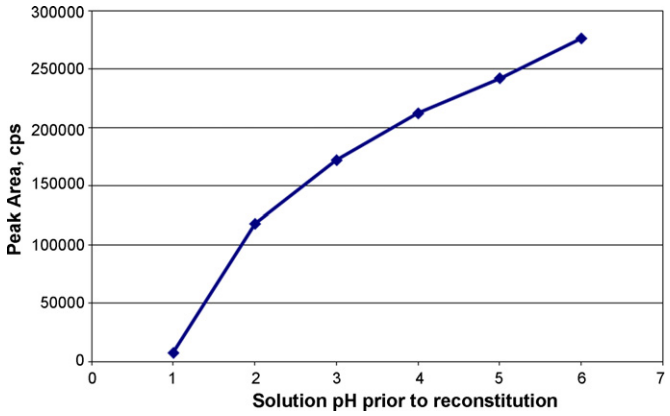

Fig. 1. The effect of solution pH on monofluoroacetate signal intensity following a heated concentration and reconstitution step.

Finally, the pH 5 urine sample was created with the addition of ammonium formate and the unadjusted urine was determined to be at a pH of 6. These samples were taken to dryness at 70 °C with a nitrogen purge of 11 psi. The samples were then reconstituted with acetonitrile containing both labeled internal standards and analyzed according to the LC/MS/MS method.

Recovery and matrix effects were measured using alterations of the standard procedure. To determine recovery, 12 replicates of a blank urine sample and 12 replicates of a urine sample spiked to 250 ng/mL of MFA and MCA were extracted according to the method protocol. Following extraction, MFA and MCA were added to the extracted blank urine to match the theoretical concentration of the originally spiked samples. All samples were analyzed using LC–MS/MS and the instrument responses were compared. Matrix effects were determined by comparing the instrument response from extracted blank urine samples spiked to a concentration of 119 ng/mL to the instrument response of a standard prepared in water at the same concentration.

Urine samples taken from people exposed to MFA and MCA were not available to evaluate this method. To determine accuracy and precision across the calibration range, five urine samples were spiked with MFA and MCA to achieve the following concentrations: 75, 175, 750, 1750, and 3750 ng/mL. These samples were extracted and analyzed five times each. In addition, the 50 reference range samples described above were spiked at a low-level concentration (150 ng/mL) of MFA and MCA. These 50 spiked samples were analyzed to determine the accuracy and precision of this method as well as elucidate if matrix effects from individual urine samples affected analyte recovery.

3. Results/discussion

Monofluoroacetate and monochloroacetate were assessed for pH and heat stability to determine the most appropriate means of sample preparation. Both analytes were spiked into pH adjusted deionized water, taken to dryness with a nitrogen purge at 70 °C, reconstituted with internal standard and analyzed by LC–MS/MS. The recovery of MFA and MCA was assessed over a pH range of 1–6 compared to the known internal standard concentration. The results of this study (Fig. 1) indicated that MFA was not stable when heated at a low pH; the intensity of the MFA transition for the samples eluted at pH 2 were approximately 50% of the intensity of the MFA transition for samples eluted at pH 5. With this information in hand, subsequent studies determined that the use of Oasis HLB (hydrophilic–lipophilic balance) solid-phase extraction provided optimal recovery for both MFA and MCA, which required only acetonitrile for adequate elution of MFA and MCA and avoided any dry down step for sample concentration.

The chromatographic column chosen for this method, Primesep B2, was a weak basic column, designed to accommodate low pH ranges. Primesep B2 has both reverse-phase and anion-exchange interactions with the analytes at the pH of the mobile phase which was used. This column demonstrated less tailing than the other columns which were tested and resulted in a stable retention time for more than 1000 injections. The retention time of MFA and MCA could be adjusted by the concentration of formic acid in the mobile phase; 0.05% formic acid eluted the analytes with k' values of 2.5 and 2.8 for MCA and MFA respectively.

A contaminant was observed in the chromatogram for both the monochloroacetate quantification and internal standard ions signals. To determine the source of the contaminant, a deionized water sample was extracted using the SPE method and resulted in the presence of the contamination peaks; the solvents were also independently evaluated and did not contain this contaminant. This indicated the source of the contaminant to be the SPE cartridges and not the solvents or the urine matrix. Neither additional conditioning of the SPE cartridge, washing of the SPE cartridge, nor modification of the elution solution eliminated the contaminant. A longer chromatographic run time was required to separate this potential interference from the analytes of interest. To minimize the impact on run time, the flow rate was increased following the elution of MCA to elute the contaminant within 5 min (Fig. 2), resulting in a final chromatographic run time of 6 min.

The mass transitions chosen were the most abundant ions as determined by infusion of a solution containing MFA and MCA. The quantification transition for MCA occurs through the loss of $C_2H_2O_2$ to form chloride at m/z 35, and the confirmation ion is formed by the loss of CO_2 from $(M-H)^-$ (m/z 93 to m/z 49). MFA loses HF from $(M-H)^-$ (m/z 77 to m/z 57) to produce the quantification ion. The confirmation ion for MFA is created through the loss of CO_2 forming CH_2F^- with an m/z of 33 Da. The product ion spectra of MFA and MCA are shown in Fig. 3, with the tranisition employed for the SRM method indicated. The mass spectra have been smoothed using a Savitsky–Golay filter, provided with the Analyst software, and three points.

This method used isotopic dilution to compensate for the variable recovery encountered with the extraction and reconstitution steps, as well as to account for ion suppression differences in individual urine extracts. The internal standard solution contained $^{13}C_2$, D_2-MFA and $^{13}C_2$-MCA. The MFA internal standard was monitored with the transition from m/z 81 to m/z 60; the same loss as the transition used for the MFA quantification ion taking into account

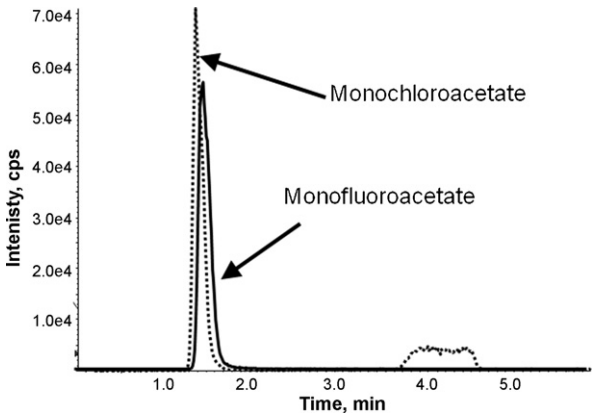

Fig. 2. Overlaid chromatograms of monofluoroacetate (solid line) and monochloroacetate (dashed line) using the quantitation ions. The chromatograms were smoothed using a Savitsky–Golay filter and three points.

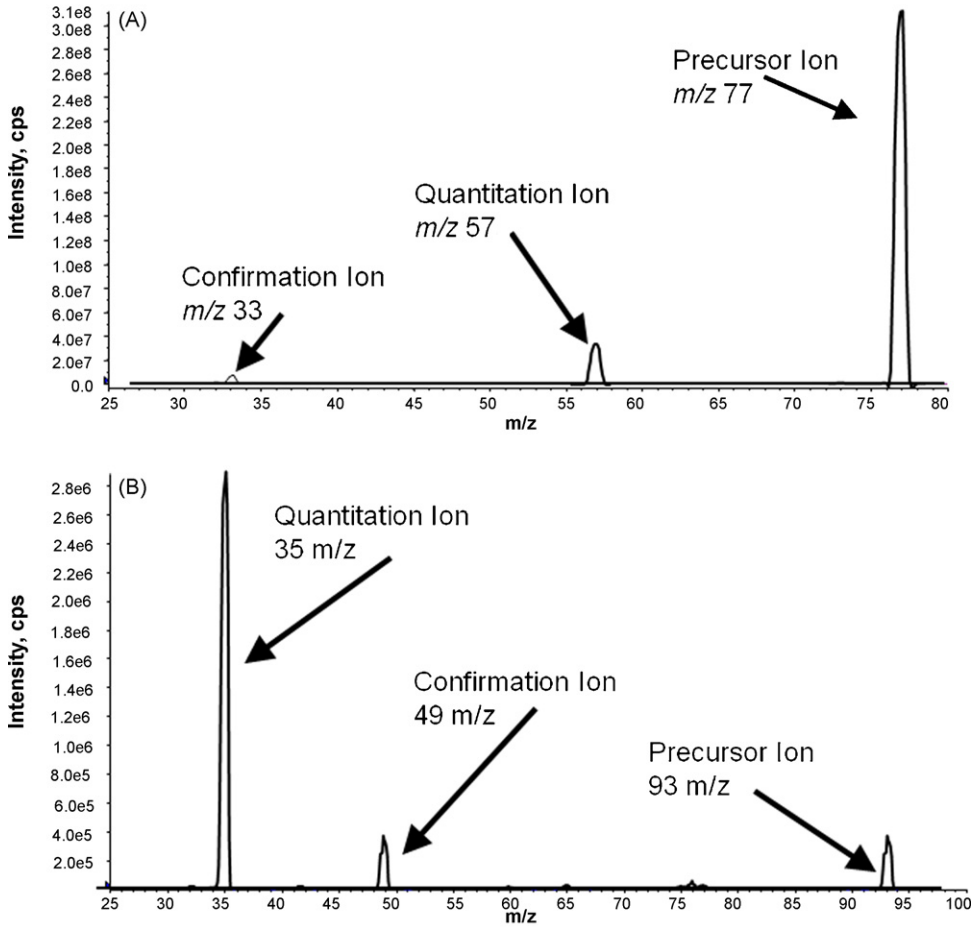

Fig. 3. Product ion spectra of monofluoroacetate (A) and monochloroacetate (B). The spectra were smoothed using a Savitsky–Golay filter and three points.

the isotopic mass difference. Since chlorine has a significant contribution from both ^{35}Cl and ^{37}Cl isotopes, the precursor ion for the MCA internal standard was chosen to be 97 instead of 95 *m*/*z*. The selection of 97 *m*/*z* for the internal standard eliminated any interference from the contribution of naturally occurring ^{37}Cl from the native MCA (95 *m*/*z*) to the internal standard ion transition.

3.1. Method validation

Twenty standard sets with QC samples were extracted and analyzed by LC–MS/MS to determine the precision of this method. Multiple analysts prepared no more than two standard sets with QC samples per day over a period of 54 days; the use of multiple analysts and preparation of samples over a 54-day period was intended to better characterize the long-term variability and reproducibility of the method. Quality control characterization data for the low and high quality control samples are presented in Table 2. The means for the low QC samples, spiked at 200 ng/mL, were 185 and 195 ng/mL for MFA and MCA, respectively, and the means for the high QC samples, spiked at 2000 ng/mL, were 2016 ng/mL for MFA and 2009 ng/mL for MCA. The relative standard deviation of the quality control samples indicates the inter-day consistency of this method across the range of the calibration curve as well as over the 54-day period in which this data was collected. The lower limit of quantitation (LLOQ) for this method is equivalent to the lowest calibrator of 50 ng/mL.

The limits of detection (LOD) for MFA and MCA were determined using the approach specified by Taylor [20]. This approach determines the LOD by calculating the standard deviation of the four lowest standards in urine; these standard deviations are then plotted versus concentration of the respective standards. A least-squares fit of this line is generated and the intercept is described as S_0; the LOD is defined as $3 \times S_0$. The LOD for this method was calculated to be 0.9 ng/mL for MFA and 7 ng/mL for MCA. To support the calculated LOD experimentally, both MFA and MCA were evaluated near the calculated LOD. The results, presented in Fig. 4, demonstrate the detection of these analytes near the calculated LOD as compared with blank urine samples. This data supports the

Table 2
Quality control sample characterization data for monofluoroacetate and monochloroacetate.

QC Pool	Analyte	Prepared concentration (ng/mL)	Mean (ng/mL)	Standard deviation (ng/mL)	Relative standard deviation (%)	Accuracy of mean (%)
Low	Monofluoroacetate	200	185	10	6.5	92.5
High	Monofluoroacetate	2000	2016	134	5.2	100.9
Low	Monochloroacetate	200	195	12	5.2	89.8
High	Monochloroacetate	2000	2009	121	5.5	100.5

Table 3
Evaluation of spiked urine samples, $n = 5$.

	75 ng/mL	175 ng/mL	750 ng/mL	1750 ng/mL	3750 ng/mL
Monofluoroacetate					
Mean (ng/mL)	66.9	170	722	1764	3484
Standard deviation (ng/mL)	3.05	2.83	20.51	32.9	90
Accuracy of mean	89.2%	97.1%	96.2%	100.8%	92.9%
Monochloroacetate					
Mean (ng/mL)	70.4	181.2	769	1878	3574
Standard deviation (ng/mL)	2.4	7.0	32	26	49
Accuracy of mean	93.9%	103.5%	102.6%	107.3%	95.4%

Taylor method as a viable means of calculating the LOD for this method.

A documented case of fatal ingestion of 465 mg of sodium fluoroacetate resulted in 368,000 ng/mL of fluoroacetate in urine [19]. In this study it was reported that a calculated lethal dose of 2–10 mg/kg resulted in the excretion of 19,000–93,000 ng/mL of MFA in urine, using an estimated average urine output of 1.5 l per day and an average human mass of 70 kg. Similarly, two separate exposures to MCA resulted in a urinary concentration of 100,000 ng/mL [7] and plasma levels ranging from 220 to 33,000 ng/mL [5]; both of these exposures resulted in death. Given this information, the method presented here could easily detect MFA and MCA in urine at concentrations equivalent to sub-lethal doses. Other methods in biological matrices have reported limits of detection from 3.9 to 30 ng/mL for MFA [11,12] and 16 ng/mL for MCA [20].

The average recoveries from the SPE extraction for urine samples were determined to be 62% for MFA and 76% for MCA using the final sample extraction protocol. Matrix effects resulting from the extracted urine were also determined; 93% of the sample response for MFA and 86% of the sample response for MCA was detected in a urine matrix as compared to water. The relative intensities of the analytical response indicated that suppression of the signal from the urine matrix was small, since the signal from the sample in urine is almost as intense as the sample in water.

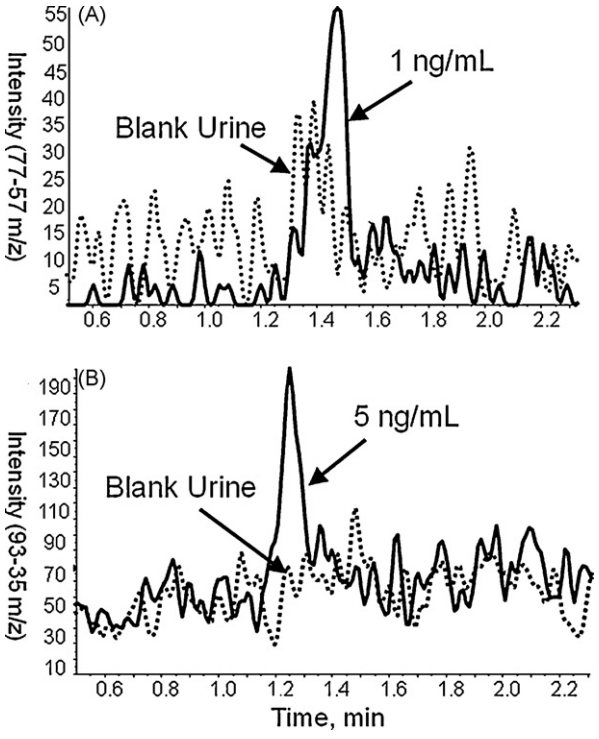

Fig. 4. Chromatograms of monofluoroacetate (A) and monochloroacetate (B) spiked in urine (solid line) near the limit of detection, at concentrations of 1 and 5 ng/mL, respectively, overlaid with blank urine samples (dashed line). The chromatograms were smoothed using a Savitsky–Golay filter and three points.

Fifty blank individual urine samples were extracted and analyzed to evaluate the presence of MFA and MCA in the urine of persons with no known exposure to these compounds. No peaks were observed in either SRM transition corresponding to MFA in the individual blank urines. The MCA quantitation transition displayed a measurable peak in 77% of the urine samples, but only 7 of those also displayed a peak in the confirmation transition. Those that had peaks in both transitions were below the LLOQ and the ratio of the confirmation ion to the quantitation ion was not within the acceptable ratio range to confirm the presence of MCA. In addition, 50 individual urine samples spiked at a concentration of 150 ng/mL were extracted and analyzed. These 50 spiked samples were evaluated to determine if the variability of matrix effects from individual urine samples might interfere or cause a bias in the results. All of the spiked urine samples were successfully identified and quantified with the expected confirmation ratios. The average measured concentrations were determined to be 144 ng/mL for MFA and 151 ng/mL for MCA with standard deviations of 9.6 and 9.0 ng/mL, respectively. The range was 121–164 ng/mL for MFA and 130–169 ng/mL for MCA; there were no significant outliers within these 50 samples at a 0.01 confidence level. These results indicate that variability in the matrix composition of individual urine samples does not affect the quantification of MFA and MCA in urine.

To determine intra-day accuracy and precision across the calibration range, five urine samples were prepared with MFA and MCA at the following concentrations: 75, 175, 750, 1750, and 3750 ng/mL. These samples were extracted and analyzed five times to generate the statistical data presented in Table 3. The relative standard deviations for all five levels were less than 5% indicating an acceptable intra-day precision for this analysis. The means for all five levels resulted in accuracy of between 89% and 107%.

4. Conclusions

An isotopic dilution method for the determination of MFA and MCA in urine has been developed. This method combined solid-phase extraction sample preparation with LC–MS/MS analysis resulting in detection limits of 0.9 and 7.0 ng/mL for MFA and MCA, respectively. A linear response was maintained from 50 to 5000 ng/mL, with a correlation coefficient of greater than 0.990 and quality control sample variability of less than 10% at 200 and 2000 ng/mL. This method successfully determined the concentrations of MFA and MCA in spiked human urine samples. To further this work, urine samples from actual MFA and MCA exposures are needed to verify the applicability of this method for exposure assessment. Evaluation of exposed urine samples would also provide insight into the appropriateness of the calibration range for the application of this method to assess poisonings and exposures to these compounds.

Acknowledgements

Rayman Stanelle and Mike Trinidad for their assistance with extraction and evaluation of the data included in the method characterization data.

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