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Journal of Chromatography B, 830 (2006) 185–195
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Quantification of six herbicide metabolites in human urine

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Received 31 May 2005; accepted 25 October 2005
Available online 16 November 2005

Abstract

We developed a sensitive, selective and precise method for measuring herbicide metabolites in human urine. Our method uses automated liquid delivery of internal standards and acetate buffer and a mixed polarity polymeric phase solid phase extraction of a 2 mL urine sample. The concentrated eluate is analyzed using high-performance liquid chromatography–tandem mass spectrometry. Isotope dilution calibration is used for quantification of all analytes. The limits of detection of our method range from 0.036 to 0.075 ng/mL. The within- and between-day variation in pooled quality control samples range from 2.5 to 9.0% and from 3.2 to 16%, respectively, for all analytes at concentrations ranging from 0.6 to 12 ng/mL. Precision was similar with samples fortified with 0.1 and 0.25 ng/mL that were analyzed in each run. We validated our selective method against a less selective method used previously in our laboratory by analyzing human specimens using both methods. The methods produced results that were in agreement, with no significant bias observed.
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Keywords: Herbicide; Urine; Biomonitoring; HPLC–MS/MS; Chloroacetanilide; Alachlor; Acetochlor; Metolachlor; Triazine; Atrazine; 2,4-D

1. Introduction

Herbicides composed 44% of the total pesticide use in 2001 in the United States [1]. About 430 million pounds of herbicides and plant growth regulators are used annually in U.S. agriculture, with another 80 million pounds used in home and garden applications [1]. An additional 40 million pounds are used in industrial, commercial, and government applications. Four of the five most abundantly used pesticides in agricultural and residential setting in the United States are herbicides [1]. Similarly, four of the five most abundantly used residential pesticides are herbicides [1].

Three common herbicide classes include the triazines, chloroacetanilides and phenoxyacetic acid herbicides. Triazine herbicides, the most common of which is atrazine, are preemergent herbicides used to kill annual broadleaf weeds and grasses by inhibiting photosynthesis [2,3]. Over 70 million pounds of atrazine were applied in the United States in 2001, making it the second most abundantly applied pesticide [1]. Chloroacetanilide herbicides, such as alachlor, acetochlor and metolachlor also are preemergent herbicides targeting annual grasses and some broadleaf weeds by inhibiting protein synthesis [2,3]. Over 75 million pounds of acetochlor, metolachlor, and alachlor were applied in the United States in 2001, ranking them as the 4th, 9th, and 16th, respectively, most abundantly applied pesticides [1]. Both chloroacetanilide and triazine herbicides are used widely on corn crops in the Midwest. 2,4-Dichlorophenoxyacetic acid (2,4-D) is a postemergent herbicide used for control of annual and perennial broadleaf weeds. It is applied abundantly in both agricultural and residential uses and ranks first in residential applications [1].

Biomonitoring has been a useful tool for assessing herbicide and other pesticide exposures [4–7]. The primary reported human metabolites of atrazine and the chloroacetanilde herbicides are their mercapturates [8–10]. The phenoxy acid herbicides are largely excreted as the parent compounds [11]. In the past, measurement of polar pesticide metabolites was complex and time-consuming, if possible, because their analysis was not conducive to gas chromatography–mass spectrometry without first protecting the polar groups (derivatization). With the advent of effective interfaces to couple high performance liquid chromatography (HPLC) with mass spectrometry in the 1990s, easy

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1570-0232/$ – see front matter. Published by Elsevier B.V.
doi:10.1016/j.jchromb.2005.10.041

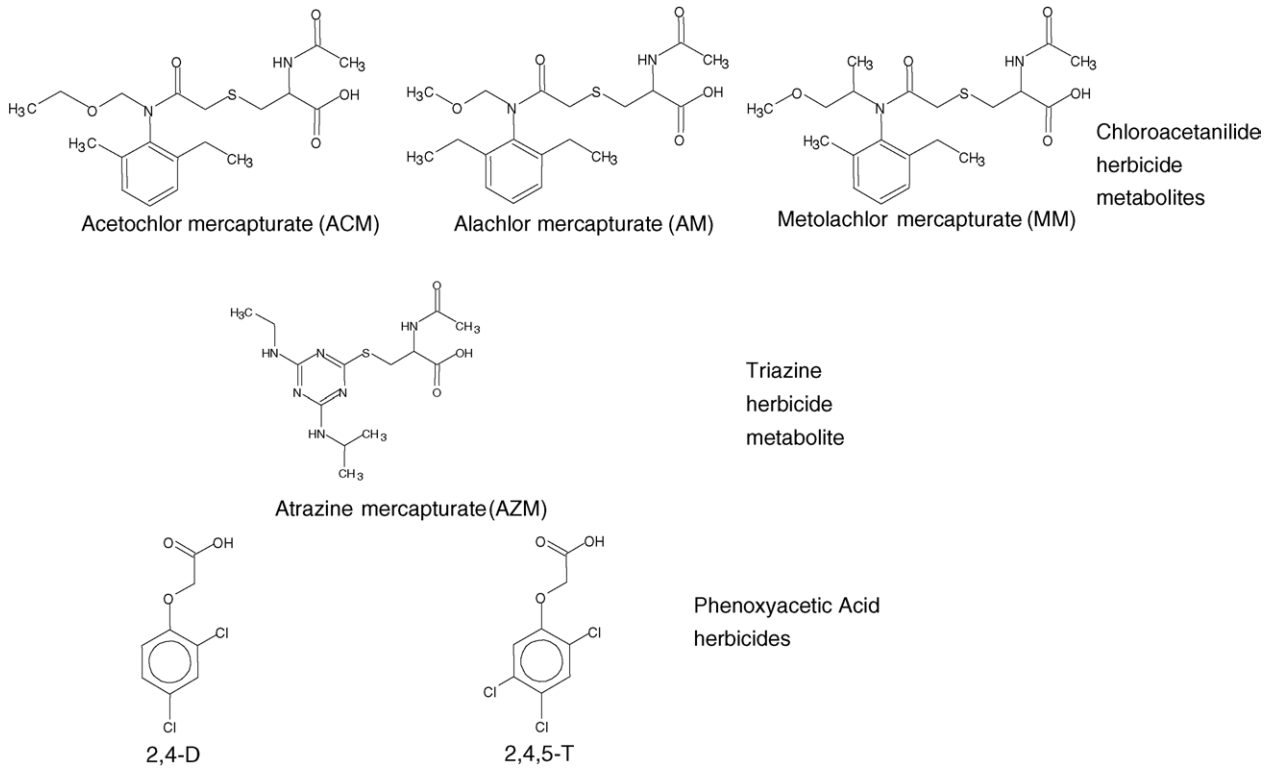

Fig. 1. Structures of the target analytes.

measurement of polar metabolites has become possible. Furthermore, with the widespread availability of selective tandem mass spectrometers (MS/MS), sensitive and selective applications for measuring pesticide metabolites have been reported [12–18] and the overall potential of HPLC–MS/MS in biomonitoring has been explored [19].

Because of the potential for widespread exposure to herbicides in both occupational and environmental settings, health effects associated with herbicide exposures or determinants of herbicide exposures have been the focus of several studies, primarily evaluating occupational exposures [4,5,20–26]. Thus, to further accommodate such exposure and health effects assessment studies, we have modified an existing method [15] to provide a more sensitive, accurate, precise, and selective method for measuring the herbicides 2,4-D and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), and the mercapturic acid metabolites of the herbicides alachlor, acetochlor, metolachlor, and atrazine in human urine (Fig. 1). These chemicals, their herbicide class, and metabolite types are listed in Table 1. Our new method has a wide dynamic range, making it suitable for measuring in urine the parent herbicide or its metabolite resulting from high-level occupational exposures, but also with the sensitivity and selectivity to measure them in urine from people with lower level environmental exposures.

2. Materials and methods

2.1. Chemicals

All solvents used were analytical grade. Acetonitrile was obtained from Burdick & Jackson Inc. (Muskegon, MI, USA) and methanol from Caledon Laboratories Ltd. (Georgetown, Ont., Canada). Glacial acetic acid and sodium acetate were purchased from JT Baker (Phillipsburg, NJ, USA). Deionized water was organically and biologically purified with a NANOpure® Infinity UF from Barnstead International (Dubuque, IA, USA). Nitrogen was purchased from Airgas Inc. (Radnor, PA, USA) and had a minimum purity of 99.99%. The OASIS HLB® 3cc

Table 1
The target analytes, their abbreviations, their metabolic status, and herbicide class

Analyte name	Abbreviation	Type of marker	Indicator of exposure to (pesticide class)
Atrazine mercapturate	AZM	GSH-derived conjugate	Atrazine (triazine)
Acetochlor mercapturate	ACM	GSH-derived conjugate	Acetochlor (chloroacetanilide)
Metolachlor mercapturate	MM	GSH-derived conjugate	Metolachlor (chloroacetanilide)
Alachlor mercapturate	AM	GSH-derived conjugate	Alachlor (chloroacetanilide)
2,4,5-Trichlorophenoxyacetic acid	2,4,5-T	Parent	2,4,5-T (phenoxyacetic acid)
2,4-Dichlorophenoxyacetic acid	2,4-D	Parent	2,4-D (phenoxyacetic acid)

GSH: glutathione.

(60 mg) mixed-bed polymeric cartridges used for solid phase extraction (SPE) were purchased from Waters Corporation (Milford, MA).

The native standards of acetochlor mercapturate (ACM; 98% purity; *N*-acetyl-*S*-[2-(2-ethyl-6-methylphenyl)(ethoxymethyl)amino]-2-oxoethyl-L-cysteine); alachlor mercapturate (AM; 99% purity; *N*-acetyl-*S*-[2-(2,6-diethylphenyl)(methoxymethyl)amino]-2-oxoethyl-L-cysteine); metolachlor mercapturate (MM; 98% purity; *N*-acetyl-*S*-[2-(2-ethyl-6-methylphenyl)(2-methoxy-1-methylethyl)amino]-2-oxoethyl-L-cysteine); and atrazine mercapturate (AZM; 98% purity; *N*-acetyl-*S*-[4-(ethylamino)-6-[(1-methylethyl)amino]-1,3,5-triazin-2-yl]-L-cysteine) were custom synthesized by Cambridge Isotope Laboratories (Andover, MA, USA). 2,4-D (98% purity) and 2,4,5-T (97% purity) were obtained from Aldrich Chemical Company (Milwaukee, WI, USA). All isotopically labeled standards were $^{13}C_6$-ring labeled, except AZM, which was $^{13}C_3$-ring labeled, and were custom synthesized by Cambridge Isotope Laboratories (Andover, MA, USA) and had chemical and isotopic purities >99%.

2.2. Standard preparation

Individual stock solutions of each labeled internal standard were prepared in acetonitrile to give concentrations ranging from 53 to 142 μg/mL. Appropriate amounts of each stock solution were combined and diluted with acetonitrile to produce a working internal combined standard solution (ISTD) of 0.96 ng/μL for each labeled standard. This solution (15 μL) was used to deliver the ISTD into each 2 mL urine sample to yield an ISTD concentration of about 7 ng/mL.

Individual stock solutions (~200 ng/μL) of the unlabeled analytes were prepared from their solid standards. Appropriate volumes were pipetted from the stock solutions to prepare nine multianalyte calibration standard spiking solutions with the following concentrations of each individual analyte: 0.018, 0.036, 0.072, 0.180, 0.360, 0.720, 1.8, 3.6, and 7.2 ng/μL. To prepare a calibration standard set, 13 μL of each standard solution was added to 2 mL of the matrix material, which was prepared according to the method detailed below. All concentrations were corrected for each analyte's chemical purity.

2.3. Quality control materials

Urine was collected from multiple anonymous donors, combined, and mixed overnight at 20 °C. The urine was divided into four pools, three of which were used to prepare quality control (QC) materials. The first QC pool (low concentration; QCL) was spiked with the native materials to yield an approximate analyte concentration of 0.6 μg/L. The second QC pool (medium concentration; QCM) was spiked with the native materials to yield an approximate analyte concentration of 6 μg/L. The third QC pool (high concentration; QCH) was spiked with the native materials to yield an approximate analyte concentration of 12 μg/L. The final pool was not spiked and was used as matrix material for calibration standards and urine blank samples.

2.4. Instruments

Samples were concentrated to dryness using a TurboVap LV Evaporator (Zymark, Farmingham, MA, USA) where the water temperature was set to 40 °C and nitrogen (10 psi pressure) was used as the evaporating gas. The high-performance liquid chromatography (HPLC) was performed on an Agilent 1100 system (Agilent Technologies, Waldbronn, Germany) consisting of a binary pump, a degasser, an auto sampler, and a temperature-stable column compartment. A TSQ Quantum Ultra® triple quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA, USA) with an atmospheric pressure chemical ionization (APCI) interface was used for analysis.

2.5. Sample preparation

Samples were thawed and vortex mixed to ensure sample homogeneity. Two milliliters of each urine sample was pipetted into a 16 × 100 round-bottomed tube. The urine tubes were loaded onto a Gilson 215 Liquid Handler with dual syringes (Gilson, Middleton, WI) where 15 μL ISTD was added automatically to each sample tube, including calibration samples, blanks, and QC materials. Each sample was buffered with 1.5 mL of a 0.2 M acetate buffer (pH 5) to allow for a more repeatable analyte recovery, which was delivered automatically, and mixed thoroughly. The SPE cartridges were preconditioned with 1 mL of methanol followed by 1 mL of 1% acetic acid. The samples were added and passed through the cartridges. To reduce the chemical noise observed during analysis, the cartridges were washed with 1 mL 5% methanol in 1% acetic acid. The cartridges were dried for approximately 30 s using vacuum. Methanol (1.5 mL) was eluted through the cartridges and collected. Two milliliters of acetonitrile was added to the methanol fraction to help facilitate the evaporation of residual water eluted from the cartridge because acetonitrile forms a lower boiling azeotrope with water. The extracts were evaporated to dryness and reconstituted in 100 μL acetonitrile:water (1:1), resulting in an overall 20-fold concentration of the original urine samples. The samples were transferred to autosampler vials and capped for analysis using HPLC–MS/MS.

2.6. HPLC operating conditions

Chromatographic separation was performed on a Betasil® Hexylphenyl column (3 μm particle size, 100 Å pore size, and 4.6 mm I.D. × 100 mm length; Keystone Scientific Inc., Bellefonte, PA, USA). The flow rate was 0.5 mL/min, and the injection volume was 5 μL. The column temperature was kept at 25 °C during the analysis. Mobile phase A was 0.1% acetic acid in water, and mobile phase B was 0.1% acetic acid in acetonitrile. To accommodate two injections for each sample to analyze ions in both the positive and negative ion modes, two gradient elution programs were employed using the same mobile phases. Although the mass spectrometer is capable of switching between positive and negative ion modes within a single injection, more reliable results were obtained if two separate injections were used. For positive ions, the mobile phase compo-

sition was initially 50% A and 50% B which was held for 1 min. The composition was changed linearly over 1 min to 40% A and 60% B. Over the next min, the composition changed linearly to 30% A and 70% B and changed to 15% A and 85% B over the following min. Finally, the mobile phase changed to 100% B linearly over 1 min and was held for 2 min before reverting to initial conditions for 3 min to reequilibrate the column. The total run time for positive ion analysis and reequilibration was 10 min. For negative ions, the initial mobile phase composition was 47% A and 53% B, which was held over the next 5 min. The composition was changed linearly over the next min to 15% A and 85% B. Finally, the composition was changed linearly over the next min to 100% B and held for 2 min before reverting to initial conditions for 3 min to reequilibrate the column. The total run time for negative ion analysis and reequilibration was 12 min.

2.7. Tandem mass spectrometry operating conditions

The TSQ Quantum Ultra® was operated in the multiple reaction monitoring (MRM) mode. The precursor-product ion pairs, collision offset energies, and scan times for the analysis of each target analyte are summarized in Table 2. The scan width was ±0.05 *m/z* in both the first and last quadrupoles, and the chrom filter was set to 5 s. Data were collected as centroid peaks. In the APCI source, the heated capillary was set at 450 °C, the corona discharge was set at 4.0 kV, and the capillary temperature was 250 °C. The sheath and auxiliary gas (N_2) pressures were set to 25 and 5 psi, respectively, and the collision gas (Ar) pressure was set to 1.5 mTorr. All samples were injected twice. For the first injection, data were acquired in positive ionization mode, and the total run time was 10 min. The positive ion run was divided into four distinct timed segments (Table 2). For the second injection, data were acquired in the negative ionization mode, and the total run time was 12 min. The negative ion run was divided into three distinct timed segments (Table 2). The mobile phase for the first 2 min timed segment (dead volume time) for both positive and negative ion injections was diverted to waste to maintain cleanliness of the ion source, thus prolonging optimum system performance.

2.8. Quantification and quality control of analytical runs

A nine-point matrix-based calibration plot for quantification was generated during each analytical run. For each calibration standard, 2 mL urine was spiked automatically with 13 μL of the appropriate standard using the Gilson 215 Liquid Handler to produce a full calibration set. The concentrations of the nine calibration points ranged from 0.1 to 50 ng/mL urine for all analytes. A linear regression analysis of the area of the native analyte/area of the ISTD plotted against the analyte concentration in urine produced a linear equation from which unknown concentrations could be calculated. This equation was appropriately corrected for any contribution that the ISTD provided to the native ion channel and vice versa using standard isotopte dilution calculations [27]. Each analytical run consisted of a full nine-standard calibration set, one blank urine sample, two to five reagent blank samples, one of each QC material concentration level, and up to 36 unknown samples. All samples were prepared concurrently using the sample preparation method outlined above. In addition during the validation process, two samples spiked at 0.1 ng/mL and two samples spiked at 0.2 ng/mL were included in the runs to ensure the method could easily detect levels at or near our previous limits of detection (LODs) [15].

Several QC criteria were used to evaluate the validity of a given analytical run. All three QC pools were characterized to determine the mean and 95th and 99th control limits by consecutive analysis of at least 20 samples from each QC pool. After establishing the control limits of the pools, all QC samples contained within each analytical run were evaluated for validity using the Westgard multirules [28].

Table 2
Optimized precursor-product ion pairs and other instrumentation parameters for the target analytes

Target analyte	Ion type	Precursor ion (*m/z*)	Product ion (*m/z*)	CE (V)	Ion mode	Scan time (s)	Timed segment (min)
AZM	Q	343	214	27	+	0.35	2–3.82
AZM	C	343	172	37	+	0.35	2–3.82
$^{13}C_3$-AZM	Q	346	217	27	+	0.35	2–3.82
ACM	Q	351	130	10	+	0.17	3.83–7.01
ACM	C	351	148	28	+	0.17	3.83–7.01
$^{13}C_6$-ACM	Q	357	130	10	+	0.17	3.83–7.01
AM	Q	365	162	27	+	0.17	3.83–7.01
AM	C	365	130	10	+	0.17	3.83–7.01
$^{13}C_6$-AM	Q	371	168	27	+	0.17	3.83–7.01
MM	Q	409	280	22	–	0.30	2–4.50
MM	C	409	150	32	–	0.30	2–4.50
$^{13}C_6$-MM	Q	415	286	22	–	0.30	2–4.50
2,4-D	Q	219	161	19	–	0.35	4.51–5.61
2,4-D	C	221	161	19	–	0.35	4.51–5.61
$^{13}C_6$-2,4-D	Q	225	167	19	–	0.35	4.51–5.61
2,4,5-T	Q	255	197	21	–	0.40	5.62–9.02
2,4,5-T	C	257	197	21	–	0.40	5.62–9.02
$^{13}C_6$-2,4,5-T	Q	261	203	21	–	0.40	5.62–9.02

CE: collision energy; Q: quantification ion; C: confirmation ion; AZM: atrazine mercapturate; ACM: acetochlor mercapturate; MM: metolachlor mercapturate; AM: alachlor mercapturate.

Table 3
Descriptive measurements of the method

Analyte	Mean extraction recovery ± S.D.		Relative recovery (%), $N = 72$	LOD (ng/mL)	Q/C ion ratio ± S.D.[a]
	5 ng/mL ($N = 19$)	50 ng/mL ($N = 10$)			
AZM	96 ± 3.8	94 ± 1.8	101	0.060	3.4 ± 0.25
ACM	98 ± 5.1	94 ± 3.1	101	0.048	1.9 ± 0.1
AM	96 ± 4.6	95 ± 5.3	102	0.036	1.0 ± 0.1
MM	91 ± 8.6	90 ± 7.2	102	0.039	2.7 ± 0.5
2,4-D	96 ± 8.6	87 ± 3.8	99	0.054	1.5 ± 0.23[a]
2,4,5-T	97 ± 5.1	90 ± 2.4	101	0.075	1.0 ± 0.15[a]

N: number; S.D.: standard deviation; LOD: limit of detection; Q/C: ratio of quantification ion area to confirmation ion area; AZM: atrazine mercapturate; ACM: acetochlor mercapturate; MM: metolachlor mercapturate; AM: alachlor mercapturate.

[a] All ion ratios quantified from calibration standards and quality control materials, except for 2,4-D and 2,4,5-T where the theoretical ratio of ^{35}Cl to $^{37}Cl \pm 15\%$ was used.

Additional QC criteria were used to judge the validity of an analytical run or a measured concentration. The reagent blank could not contain any measurable concentration of the analyte over the method LOD. Furthermore, for an individual analyte chromatographic peak to be identified as the target chemical and for a valid concentration to be calculated, the peak was required to [1] coelute with its respective labeled internal standard (±3 s); [2] have the confirmation ion; and [3] have a ratio of the area of the quantification ion to the confirmation ion that fell within the predetermined range shown in Table 3.

2.9. Method validation

2.9.1. Limits of detection

The LODs were calculated as three times the standard deviation of the noise at zero concentration [29]. The estimate of the noise is based on the variation in precision at concentrations close to the LOD. This was calculated using the confirmation ion of the four lowest calibration standards from available validation and analytical runs. This gives an integrated and conservative LOD value over several ($n > 10$) runs. By using the confirmation ion (typically the less abundant ion) for LOD calculations, we ensured that a confirmation ion would always be detectable at or near the LOD. Furthermore, the LODs were compared with the results of the calibration standard samples and low-level spiked samples to ensure that the calculated values agreed with the peaks observed and that a minimum signal-to-noise ratio of 3 was present at these low levels.

2.9.2. Extraction efficiency

The extraction recovery of the method was determined at two concentrations (5 and 50 μg/L) by spiking five "blank" urine samples with the appropriate standard concentration and extracting according to the method. Five additional "blank" urine samples (unspiked) were extracted concurrently. Before the evaporation steps, all the extracts were spiked with a known amount of labeled internal standard to correct for instrument variation resulting in a more accurate extraction recovery calculation. The samples that were not spiked before preparation were then spiked with the appropriate native standard to serve as control samples representative of 100% recovery. After evaporation and reconstitution, the samples were analyzed. The recovery was calculated by comparing the responses of the blank urine samples spiked before extraction with the responses of the blank urine samples spiked after the extraction.

2.9.3. Precision

The precision of the method was determined by calculating the relative standard deviation (RSD) of repeat measurements of samples from the three QC pools. Five new samples from each of the QCL, QCM, and QCH pools were prepared and analyzed every day during a 7-day period, and the results were used to determine the within-day precision. The between-day precision was determined by analyzing each QC pool in a minimum of 15 analytical runs over a 1-month period.

2.9.4. Cross-method validation

The urine samples used for cross-validation of the present method with our previous, less selective multianalyte method [15] consisted of split samples collected from 14 men in Missouri who were part of the study of Swan et al. [30] and were properly archived at −70 °C immediately after collection and processing. These samples were chosen for cross-validation because many of these herbicide metabolites were measured previously at low concentrations in these samples and because AM and AZM concentrations were associated with sperm quality [30]. The archived samples used in this evaluation never had been subjected to a thaw–refreeze cycle. We compared the results obtained using this method with the results obtained using the previous method, both analyzing the archived sample. In addition, we compared the results on the archived samples with the results we previously obtained on the same person's split sample, which previously was reported in Swan et al. [30]. Also, QC materials were analyzed using both methods, and the results were compared. All protocols, including those for collecting anonymous urine for pooled QC and matrix materials, were reviewed and approved by a human subjects review committee and complied with all institutional guidelines for the protection of human subjects.

3. Results

The optimized precursor-product ion pairs for the target analytes are summarized in Table 2. The ionization polarity that

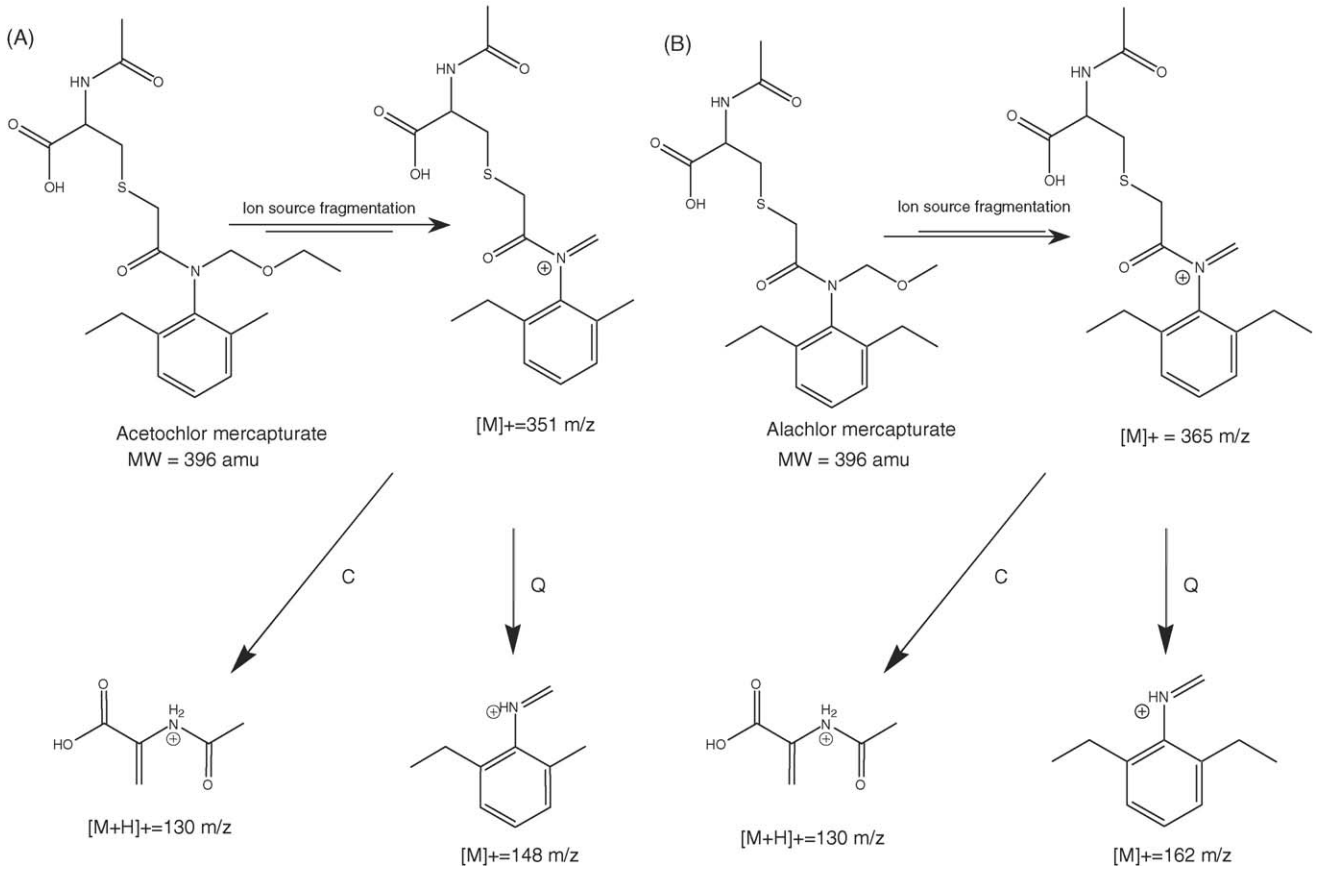

Fig. 2. MS/MS fragmenting schemes for acetochlor mercapturate and alachlor mercapturate.

produced the cleanest fragmentation and produced the most abundant signal was chosen for each analyte. All transitions were based on the $[M + H]^+$ or $[M - H]^-$ precursor ions except for ACM and AM, which were based on a source-fragmented ions representing the loss of ethanol and methanol, respectively. The precursor-product ion fragmenting scheme for both ACM and AM are shown in Fig. 2.

Mass chromatograms of AZM, ACM, and AM, which were monitored in the positive ion mode, are shown in Fig. 3. AZM is chromatographically separated from ACM and AM; however, ACM and AM coelute. Mass chromatograms of MM, 2,4-D, and 2,4,5-T, which were monitored in the negative ion mode, are shown in Fig. 4. All analytes that were analyzed in the negative ion mode were chromatographically resolved.

The method validation data for all analytes are summarized in Tables 3 and 4. The extraction recoveries ranged from 87 to 98%, and the relative recoveries ranged from 99 to 102%. A regression plot showing the spiked concentrations plotted against the quantified concentrations for AM and ACM is shown in Fig. 5. The slopes of 1.02 and 1.01 for AM and ACM, respectively, indicate relative recoveries of 102 and 101%.

The LODs for all analytes were <0.1 ng/mL. The within-day variation for the analytes was between 2.5 and 9%, at con-

Table 4a
Method precision calculated from quality control materials at three concentrations

Target analyte	Within-day R.S.D. (%)			Between-day R.S.D. (%)			Total R.S.D. (%)		
	0.6 ng/mL $N = 5$	6 ng/mL $N = 5$	12 ng/mL $N = 5$	0.6 ng/mL $N = 7$	6 ng/mL $N = 8$	12 ng/mL $N = 7$	0.6 ng/mL $N = 30$	6 ng/mL $N = 32$	12 ng/mL $N = 30$
AZM	3.6	3.1	2.5	16	7.4	9.1	15	6.9	7.4
ACM	3.8	4.3	3.0	15	8.8	8.8	14	11	8.7
AM	6.7	5.2	2.7	13	8.8	8.9	13	11	8.8
MM	4.7	6.6	2.9	4.7	7.4	2.9	16	16	7.2
2,4-D	9.0	8.2	6.3	8.8	8.2	6.3	20	10	6.4
2,4,5-T	6.8	4.3	3.2	7.1	4.4	3.2	15	10	5.3

RSD: relative standard deviation; AZM: atrazine mercapturate; ACM: acetochlor mercapturate; MM: metolachlor mercapturate; AM: alachlor mercapturate.

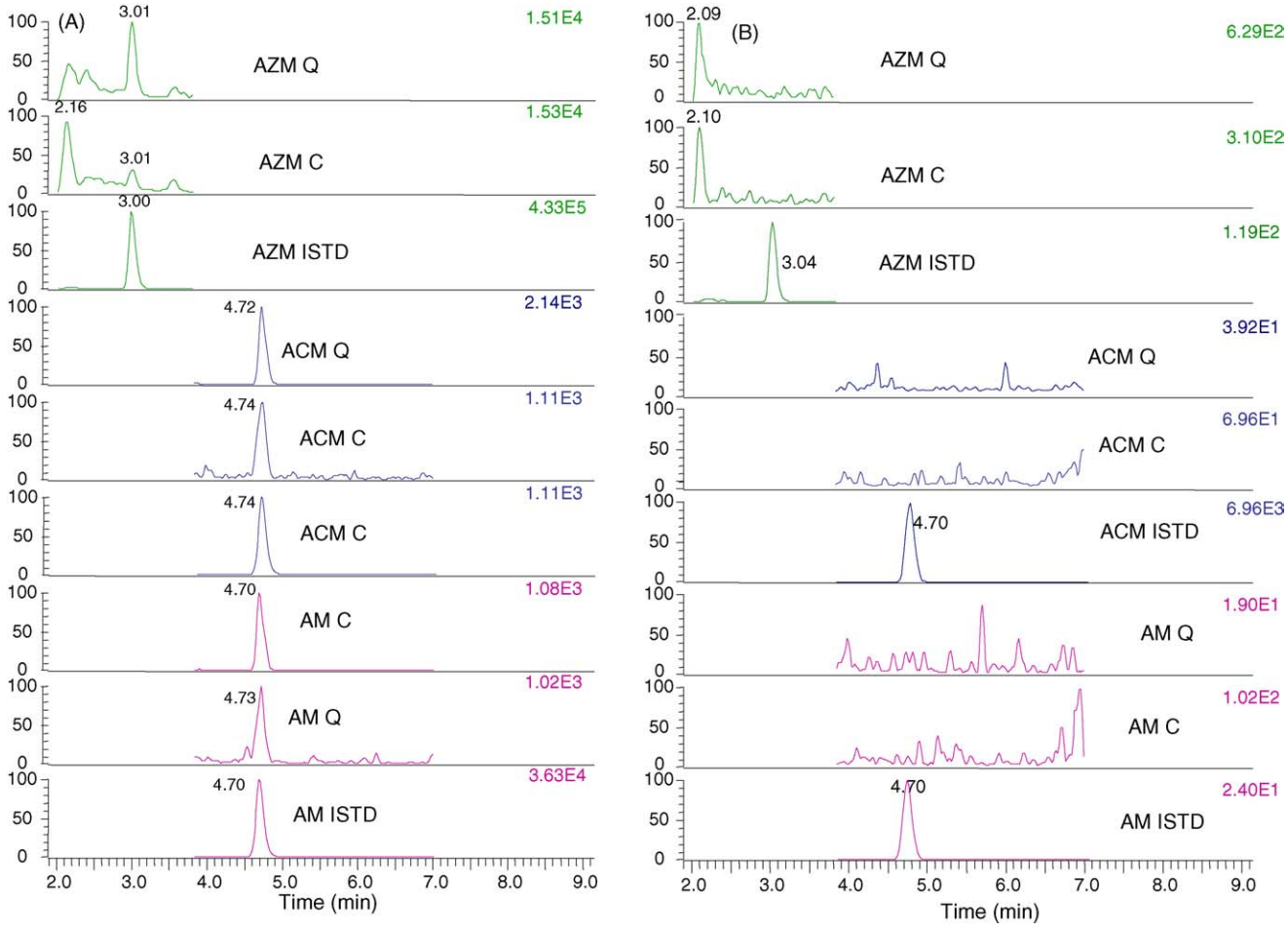

Fig. 3. Mass chromatograms showing atrazine mercapturate (AZM), acetochlor mercapturate (ACM), and alachlor mercapturate (AM), the analytes measured in positive ion mode, in a urine sample spiked at 0.1 ng/mL (A) and in an unspiked blank sample (B).

centrations ranging from 0.6 to 12 ng/mL. The between-day R.S.D.s ranged from 3.2 to 16%, whereas the total R.S.D.s ranged from 5.3 to 20%. Similarly, the within-day variation at 0.1 and 0.25 ng/mL was 1.2–22% and the between-day variation was 6.5–22%. If only the mercapturic acid metabolites were considered, the within-day variation was <10% and the between-day variation was <13% at 0.1 and 0.2 ng/mL. The highest R.S.D.s were observed for 2,4-D. A typical Shewart plot for QC is shown in Fig. 6.

Table 4b
Method precision calculated from fortified samples near the method limit of detection.

Target analyte	Within-day R.S.D. (%)		Between-day R.S.D. (%)	
	0.1 ng/mL $N = 4$	0.25 ng/mL $N = 4$	0.1 ng/mL $N = 8$	0.25 ng/mL $N = 8$
AZM	3.2	5.5	7.1	9.1
ACM	10	1.9	13	11
AM	4.6	9.9	8.9	9.1
MM	3.1	8.1	6.5	11
2,4-D	22	17	21	15
2,4,5-T	16	8.3	22	8.9

R.S.D.: relative standard deviation; AZM: atrazine mercapturate; ACM: acetochlor mercapturate; MM: metolachlor mercapturate; AM: alachlor mercapturate.

Data from the cross-method validation are shown in Table 5. Pearson correlation coefficients comparing the previous measurements reported in Swan et al. [30] and measurements made on archived split samples from the same persons using the herbicide method reported here ranged from 0.8545 to 0.9914 with $p < 0.0001$. Pearson correlation coefficients of the new measurements using the herbicide method with measurements on the same new sample using the previous multianalyte method [15] ranged from 0.9354 to 0.9992 with $p < 0.0001$. Similarly, both samples measured using the previous multi-analyte method [15] also agreed well ($r = 0.9206$–0.9904; $p < 0.0001$). Individual paired measurements agreed well as shown for AM and ACM in Table 6. Chromatograms of AM in the same sample measured using both methods along with the previous measurement reported in Swan et al. [30] are shown in Fig. 7. Measurements made on QC materials and spiked samples using both the multianalyte method [15] and the herbicide method also agreed well (Fig. 8). These data confirm our previous results reported on these samples.

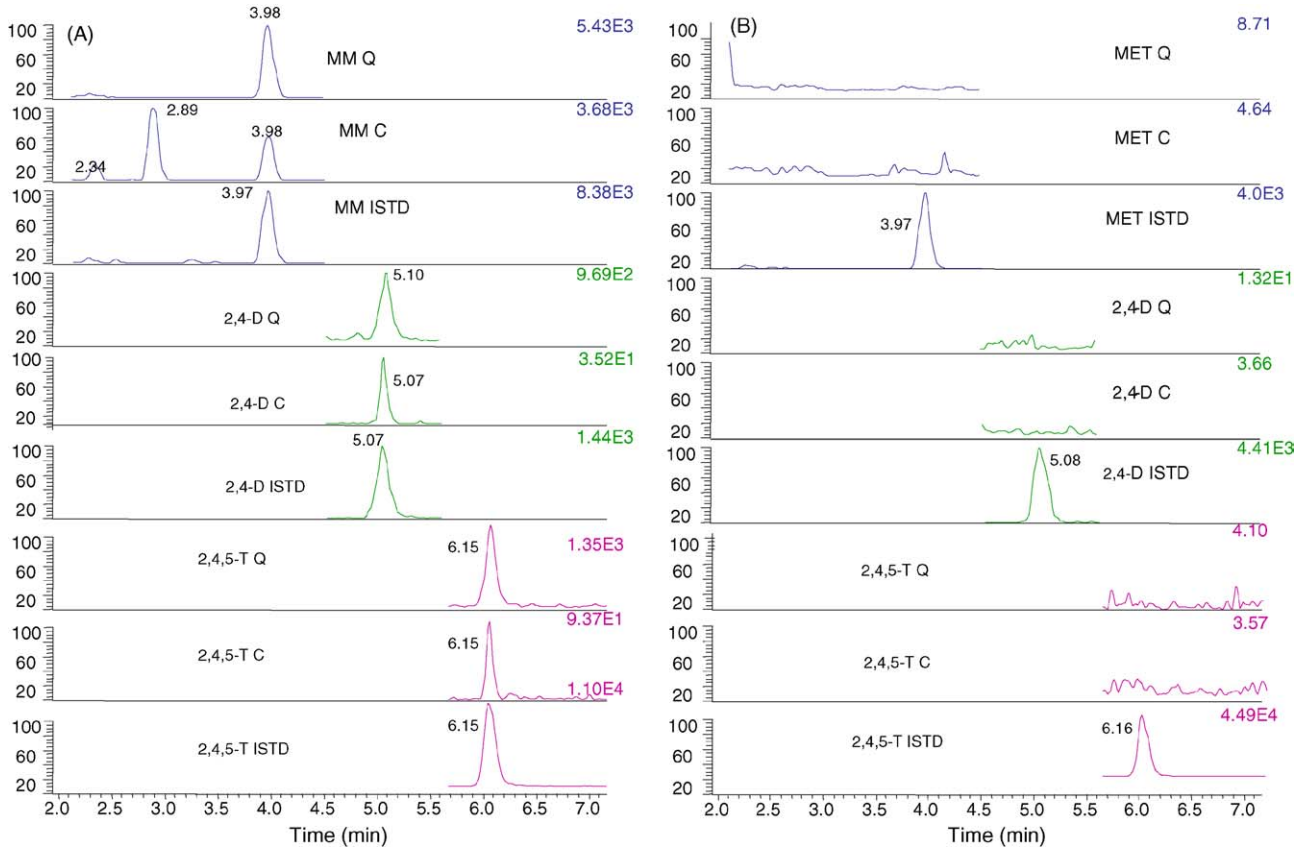

Fig. 4. Mass chromatograms showing metolachlor mercapturate (MM), 2,4-D and 2,4,5-T, the analytes measured in negative ion mode, in a urine sample spiked at 0.1 ng/mL (A) and in an unspiked blank sample (B).

Table 5
Pearson correlation coefficients of urinary results previously reported in Swan et al. [30] using our multi-analyte method [15] with new results obtained on archived split specimens using an the multi-analyte method [15] and the herbicide method presented in this paper

Analyte	Previous measurements using old method versus new measurements using new method	Previous measurements using old method versus new measurements using old method	Newly measured values using the old method versus the new method
AZM	0.8545[a]	0.9852	0.9354
ACM	0.9435	0.9206	0.9434
AM	0.9914	0.9904	0.9992
MM	0.9256	0.9795	0.9725
2,4-D	0.9610	0.9621	0.9700
2,4,5-T	[b]	[b]	[b]

All correlation coefficients were significant at $p < 0.0001$.
[a] Correlation becomes >0.92 when the same LOD is imposed on both data sets.
[b] Detected too infrequently in sample sets to calculate.

4. Discussion

The use of newer instrumentation which allowed for lower LODs and inclusion of a confirmation ion allowed us to make dramatic improvements in measuring herbicide metabolites in human urine; thus, this method has improved specificity and sensitivity. In addition, the newer instrumentation and pared list of target analytes allowed us to measure fewer ions during each MRM time segment, allowing more scan time per ion and ultimately increasing the overall sensitivity.

The use of confirmation ions adds selectivity to analytical methods providing more reliability in the detection of a given analyte, especially at lower levels. In general, confirmation ions which are less abundant than the quantification ions pose problems in positively identifying or confirming the identity of a given analyte at or near the LOD. To circumvent this problem, we calculated our LODs based upon the response and precision of the confirmation ions rather than the quantification ions. The ratio of the quantification ion to the confirmation ion is usually less precise at lower concentrations; thus, using criteria similar to

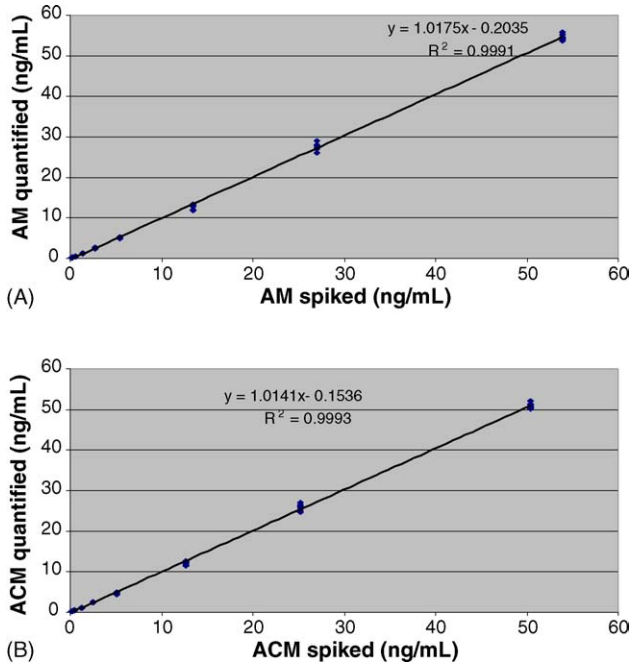

Fig. 5. Linear regression plots showing the amount spiked in urine samples plotted against the amount quantified in urine samples for alachlor mercapturate (AM) and acetochlor mercapturate (ACM). A slope of 1.00 indicates perfect agreement.

the QC requirements (e.g., 95th and 99th confidence intervals) mentioned above allow a reasonable degree of confidence in detection of a given analyte. Certainly the use of multiple quantification ions would further enhance the reliability of a positive detection, but may provide false negative detections. Thus, one confirmation ion with strict ratio evaluation criteria should be an acceptable practice for positively detecting analytes at low concentrations.

Because AM and ACM have the same molecular weight and coelute chromatographically, they were challenging to measure. To obtain mass resolution of AM and ACM, we selected a source fragment ion for each of these analytes representing the loss of methanol and ethanol, respectively, from the same *N*-alkyl side chain, thus allowing different precursor ions to be selected. Similarly, the quantification product ion for AM and ACM represented the same loss (i.e., loss of the mercapturate moiety and the other *N*-alkyl side chain); however, because their aromatic rings are substituted differently, the ions could be differentiated on the basis of their mass. The confirmation ions for AM and ACM were identical and were derived from a portion of the mercapturate moiety; however, because the precursor ions differed for AM and ACM, no cointerference was observed. In fact, in all testing performed, we found no interferences in the ion channels of AM that could be attributable to ACM and vice versa.

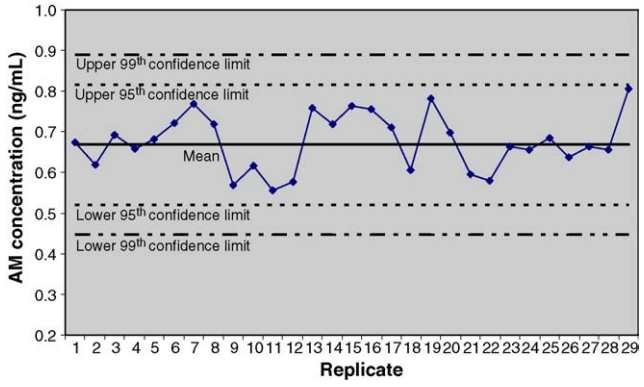

Fig. 6. A Shewart quality control plot for alachlor mercapturate (AM).

Few methods reported in the literature have focused solely on the measurement of herbicides or their metabolites in human urine. Of the herbicides targeted in this method, 2,4-D and atrazine have been the most widely studied. Aprea et al. [31] reported a method for measuring 2,4-D and another similar herbicide in human urine with and LOD of 15 ng/mL. Lyubimov et al. [32] reported a novel immunoassay for measuring 2,4-D exposure with an LOD of 19 ng/mL. These methods are suitable for measuring occupational exposures but probably would miss any environmental exposures to 2,4-D because the population levels are low [33]. Our laboratory has reported several multianalyte methods [12,15,16] in which 2,4-D was measured with LODs ranging from 0.2 to 1 ng/mL; all of these method have been used to measure 2,4-D in general population samples.

Table 6
Comparison of measured values (ng/mL) for alachlor mercapturate and acetochlor mercapturate using a multianalyte method [15] and the present method

Analyte	ID	Old value with multianalyte method	New value with new method	New value with multianalyte method
AM	1	<0.1	<0.036	<0.1
	2	0.41	1.444	1.545
	3	0.34	0.397	0.324
	4	0.40	0.493	0.522
	5	<0.1	<0.036	<0.1
	6	0.35	0.388	0.295
	7	0.39	0.393	0.269
	8	0.37	0.468	0.359
	9	0.42	0.687	0.496
	10	0.34	0.444	0.398
	11	0.78	0.857	0.639
	12	0.66	0.726	0.762
	13	0.44	0.454	0.429
	14	8.91	8.107	8.795
ACM	1	0.23	0.340	0.330
	2	<0.1	<0.048	<0.1
	3	<0.1	0.048	<0.1
	4	<0.1	<0.048	<0.1
	5	<0.1	<0.048	<0.1
	6	<0.1	<0.048	<0.1
	7	<0.1	<0.048	<0.1
	8	<0.1	<0.048	<0.1
	9	0.11	0.090	0.100
	10	0.33	0.302	0.220
	11	<0.1	0.048	<0.1
	12	<0.1	0.049	<0.1
	13	<0.1	<0.048	<0.1
	14	<0.1	0.055	<0.1

Measurements previously determined using a separate aliquot and the multianalyte method. Current measurements determined using an archived split sample from the same individual.

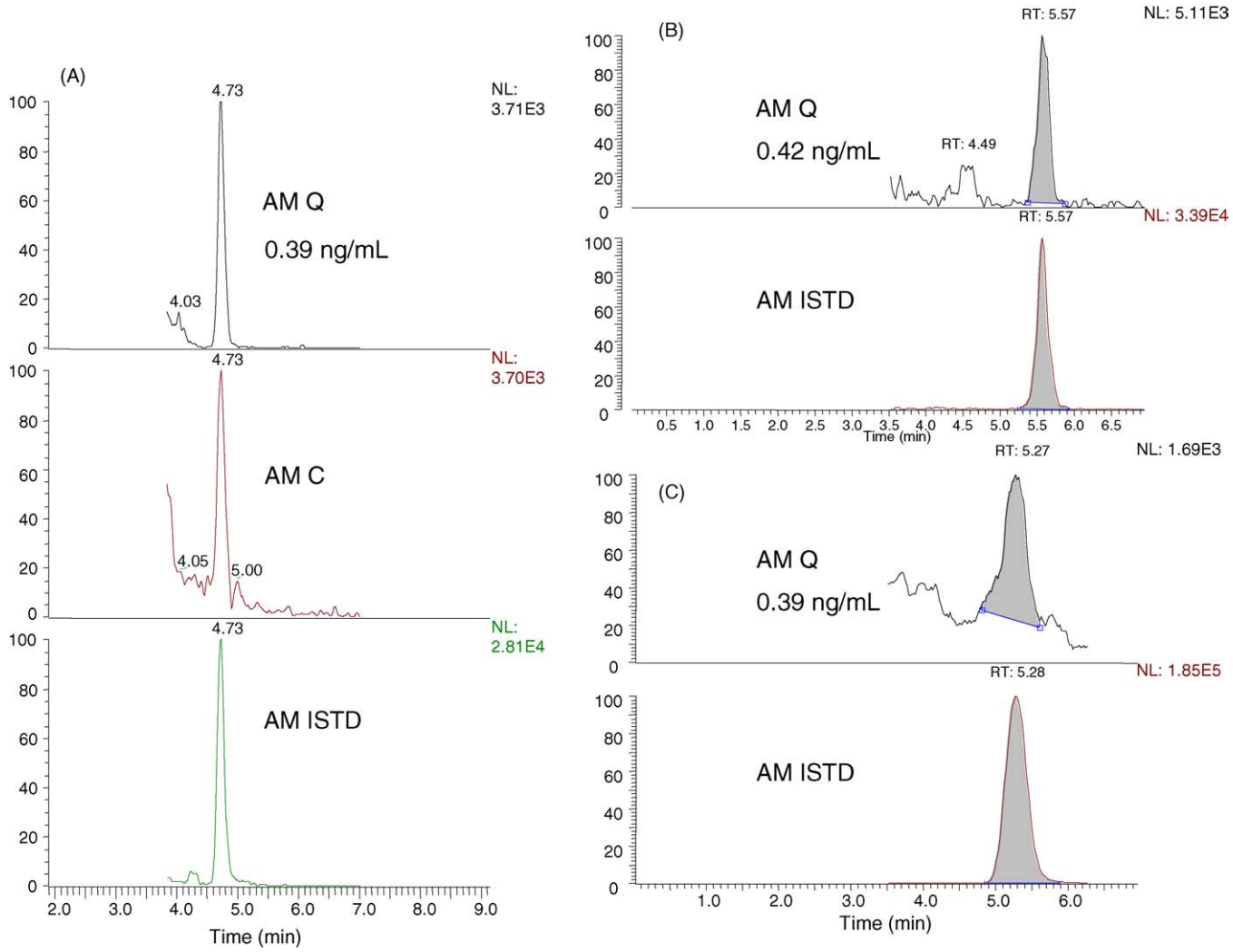

Fig. 7. Chromatograms showing alachlor mercapturate (AM) in an archived split sample from the same individual. (A) Shows the measurement using the method presented here on the archived sample that was newly prepared. (B) Shows the measurement using our previous multianalyte method [15] on the archived sample that was newly prepared. (C) Shows the measurement using our previous multianalyte method [15] on the split sample previously analyzed and reported in Swan et al. [30].

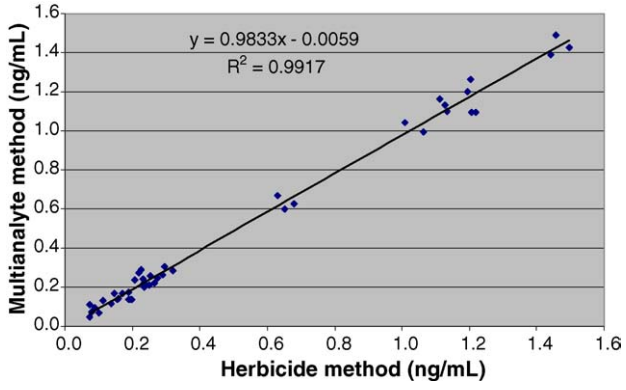

Fig. 8. Agreement among quality control materials and spiked samples using the method presented here and our previous multianalyte method [15].

Several methods measuring AZM have been reported using both mass spectrometry [12,15,16] and immunoassay [34]. These methods are sensitive and selective for quantifying AZM with LODs ranging from 0.3 to 0.5 ng/mL. However, the present method has improved the sensitivity of AZM measurements over three-fold.

Methods to measure human exposure to chloroacetanilide herbicides have been rarely reported. To date, the only methods reported in the literature to measure human exposure to alachlor, acetochlor, and/or metolachlor have been developed in our laboratory [6,15]. In fact, our laboratory first identified the primary metabolites of alachlor [10] and metolachlor [35], and soon will do the same for acetochlor (unpublished data). The present method has allowed us to increase both our sensitivity and specificity for measuring the mercapturic acid metabolites of these herbicides in urine. In fact, in 14 archived urine samples, we improved our frequency of detection for ACM from 21 to 50%. The excellent agreement with our previous multianalyte method [15] also will allow us to easily compare data

obtained in studies conducted before the development of this method.

5. Conclusions

We have developed an HPLC–MS/MS method for assessing human exposure to six herbicides. This method is sensitive, selective, accurate and precise. Isotope dilution calibration is used for quantification of all analytes. The LODs ranged from 0.036 to 0.075 ng/mL for 2 mL urine. The within- and between-day variation ranged from 2.5 to 9.0% and 3.2 to 16%, respectively, for all analytes at concentrations ranging from 0.6 to 12 ng/mL. We validated our method against a less selective method used previously in our laboratory by analyzing human specimens using both methods. The methods produced results that were in agreement with no significant bias observed, thus allowing comparison of results from this study with those produced using our previous method. Additionally, the results of our new method confirm our previous results.

Acknowledgments

We thank Rebekah Emrich for her technical assistance during the method validation process. We thank Dr. Eric Sampson, Dr. Jim Pirkle, Dr. John Osterloh and Ms. Dorothy Sussman for their input into the method development and validation process. In addition, we thank Dr. Shanna Swan, Dr. Robin Kruse, and the Study for Future Families research team for providing the samples for the cross-method validation process. This work was supported in part by appointments (JN and PR) to the Research Participation Program at the Centers for Disease Control and Prevention (CDC), National Center for Environmental Health, Division of Laboratory Sciences, administered by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and CDC.

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