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Identification of human urinary metabolites of acetochlor in exposed herbicide applicators by high-performance liquid chromatography-tandem mass spectrometry

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Acetochlor is a preemergent chloroacetanilide herbicide used to control annual grasses and small-seeded broadleaf weeds. It is the second most abundantly applied herbicide on corn crops in the United States; however, human metabolites associated with known exposure to acetochlor have not been positively identified and confirmed. We positively identified acetochlor mercapturate (ACM) as a metabolite of acetochlor in urine samples collected during a 24-h period from custom (commercial) applicators who had applied acetochlor on either the day of or the day before urine collection. Concentrations in applicator urine samples ranged from 0.5 to 449 μg/l (0.3–121 μg/g creatinine). We found that ACM accounted for as much as 42% of the total acetochlor-derived metabolites; however, as the exposure level decreased (based on total acetochlor metabolite level), ACM became a less abundant metabolite of acetochlor ($<17\%$). Unmetabolized acetochlor was also measured in the urine samples analyzed. At high exposures (classified as $>100\,\mu$g/l), acetochlor accounted for about 0.8% of the total excreted acetochlor metabolites ($\sim 2\%$ of the ACM concentrations). At lower exposures (classified as ACM $<10\,\mu$g/l), ACM and acetochlor concentrations were similar. Additionally, we tentatively identified another acetochlor metabolite that appeared to be important at low levels of exposure.
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Introduction

Acetochlor, (2-chloro-*N*-(ethoxymethyl)-*N*-(2-ethyl-6-methyl-phenyl)-acetamide), is a preemergent chloroacetanilide herbicide used to control annual grasses and small-seeded broadleaf weeds (Monaco *et al*., 2002; Weed Science Society of America: Herbicide Handbook 2002). Acetochlor was registered for use on corn in the United States in 1994 (US EPA, 1994), largely replacing the use of the herbicide alachlor on corn crops. After atrazine, acetochlor is the second most abundantly applied herbicide on corn in the United States (USDA, 2004). A 2003 survey of 92% of the US corn acreage indicated that 36.1 million pounds of acetochlor had been applied to corn, whereas only 2.6 million pounds of alachlor were used (USDA, 2004). By contrast, a 1993 survey of 90% of the US corn acreage (USDA, 1994) indicated that the amount of alachlor used in 1993 (32.1 million pounds) was comparable to the amount of acetochlor used in 2003. Mean application rates for acetochlor and alachlor from 1994 to 2003 were similar (range 1.69–2.00 lbs/acre and 1.61–2.16 lbs/acre, respectively) (USDA, 1994, 1995, 1996, 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004).

Acetochlor induces nasal tumors in rats at the maximum-tolerated dose of 1000 parts per million and is classified as "likely to be carcinogenic to humans" by the US EPA (Ashby *et al*., 1996; Dearfield *et al*., 1999). In human and rat liver microsomes, acetochlor is bioactivated via a multistep pathway to the putative DNA-reactive metabolite, 2-methyl-6-ethylbenzoquinoneimine (Jefferies *et al*., 1998; Coleman *et al*., 2000; Green *et al*., 2000). Exposure to acetochlor has been reported to significantly increase estrogen-binding activity in rat uteri (Rollerova *et al*., 2000).

Acetochlor is structurally similar to the herbicides alachlor and metolachlor (Figure 1). Glutathione-derived mercapturate metabolites of alachlor and metolachlor have been identified as important human urinary metabolites after occupational exposures (Driskell *et al*., 1996; Driskell and Hill Jr., 1997). By analogy, acetochlor mercapturate (ACM) has been hypothesized to be a human urinary metabolite of

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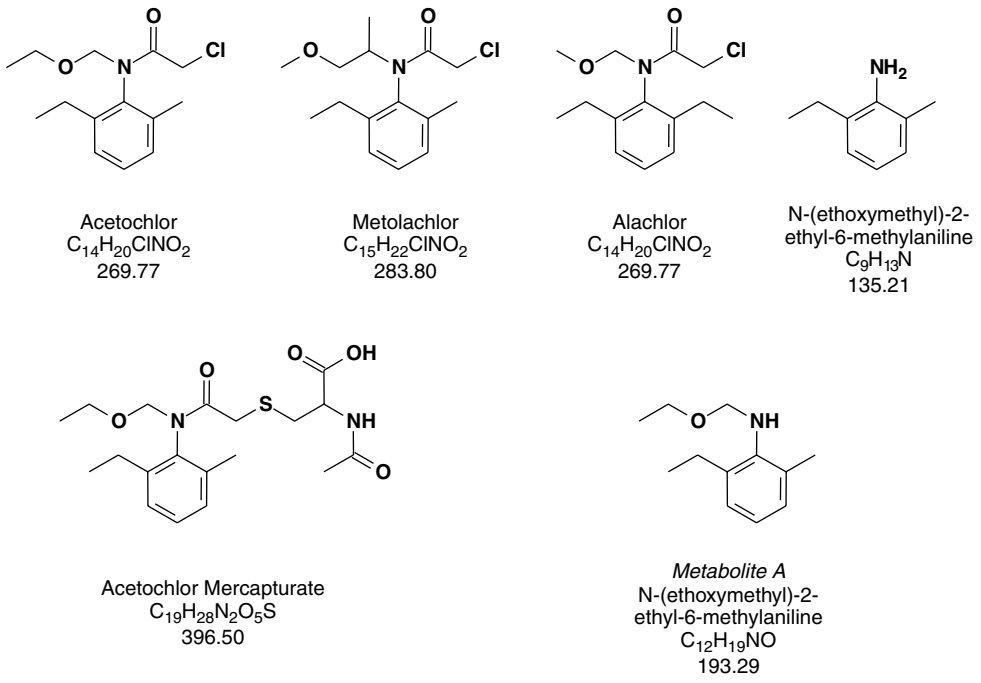

Figure 1. Structures of acetochlor, related herbicides and acetochlor metabolites or degradates.

acetochlor (Swan *et al*., 2003; Olsson *et al*., 2004; Curwin *et al*., 2005). Human exposure to alachlor has also been estimated by quantifying the hydrolysis product of alachlor-related metabolites, 2,6-diethylaniline, in urine (Driskell *et al*., 1996). The major portion of acetochlor metabolites in the urine may be similarly estimated by quantifying the hydrolysis product, 2-ethyl-6-methylaniline (EMA). EMA will not represent an estimate of the total acetochlor metabolite concentration because hydroxylation of the ethyl chain on the phenyl ring has been reported (Chloroacetanilides, 1998). These metabolites will not be accounted for by EMA, but in this paper we will still consider EMA an estimate of the total metabolite concentration.

The aim of our study was to identify metabolites resulting from occupational exposures to acetochlor and to quantify these metabolites in 24-h applicator urine collections. We positively identified one acetochlor metabolite in most of the urine samples analyzed and tentatively identified an additional metabolite. In addition, we estimated total acetochlor metabolite concentrations by measuring EMA after base hydrolysis.

Materials and methods

Study Population

In 1996, as part of a herbicide exposure assessment study, the National Institute for Occupational Safety and Health (NIOSH) at the Centers for Disease Control and Prevention (CDC) collected urine, air, dermal patch, hand rinse and saliva samples from 15 male custom (or commercial) applicators in Ohio who applied herbicides in the spring to corn and soybean fields (Hines *et al*., 2001, 2003). Samples were collected from each applicator at approximately 4-day intervals over six consecutive weeks for a total of 89 applicator days, with 5–7 sampled days per applicator. Each custom applicator sprayed, on average, 13 ± 2.8 (range 9–17) different herbicides, and as a group, sprayed more than 30 different herbicides (Hines *et al*., 2003). Applicators worked full-time (mean 12.3 ± 2.9 h per day) and up to 7 days per week. Participation was voluntary and informed consent was obtained. This study complied with all national and international guidelines for the protection of human research subjects and was approved by the NIOSH Human Subjects Review Board.

Sample Collection

On each sampling day, applicators collected all urine starting with that day's first-morning void (day 0) through to the first-morning void of the next day (day 1). The volume of each sample was recorded. Occasionally, applicators either did not have any urine or forgot to collect their urine during one of the time periods. Because of the repeated measures design of the study, multiple urine samples were collected on the same person, both within a day and across days. Samples were frozen in the field on dry ice and stored at $-70^{\circ}C$ until analysis. Details of the sampling and field procedures have been reported previously (Hines *et al*., 2003). Acetochlor was

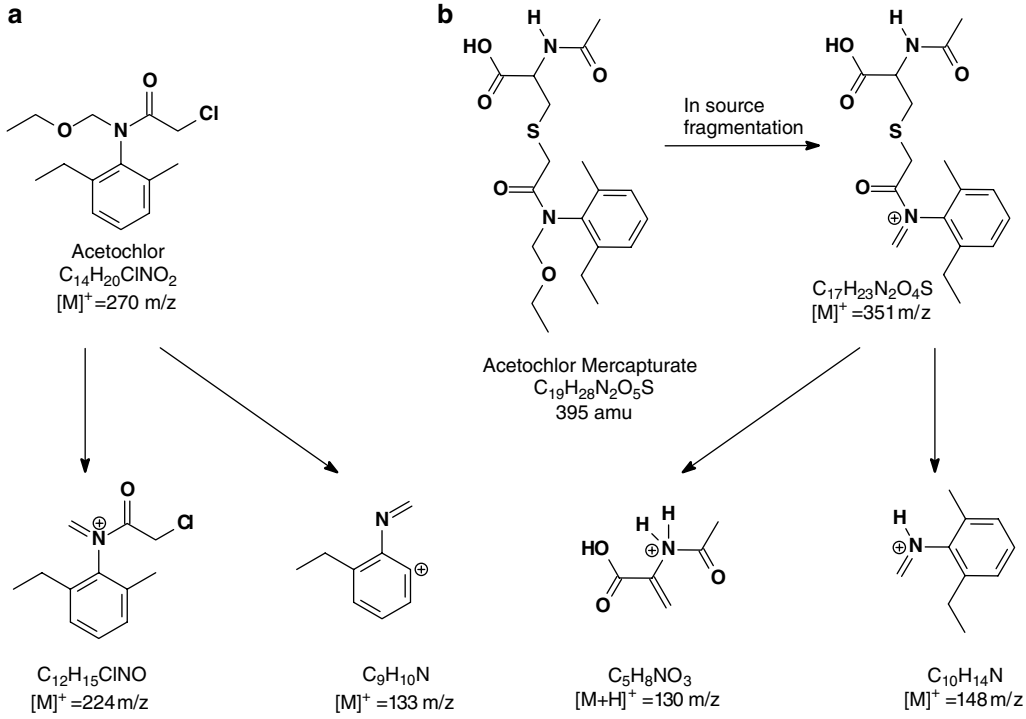

Figure 2. MS/MS analysis scheme for acetochlor (a) and its mercapturate metabolite (b). Q = quantification ion; C = confirmation ion; amu = atomic mass unit

commonly substituted for alachlor during the study period. Metabolites of alachlor and metolachlor were found in many of the applicator urine samples (Hines *et al.*, 2003); thus, we also expected to find acetochlor metabolites. The number of pounds of acetochlor sprayed on day 0 and on the day before day 0 (day −1) was also recorded for each applicator day.

Laboratory Analysis

All urine samples collected on days where the applicator sprayed acetochlor on either day 0, day −1 or both days were analyzed for acetochlor metabolites ($n = 59$ of 408 samples collected) at the National Center for Environmental Health (NCEH) at the CDC. Six duplicate (samples split directly after application) samples were included among the samples shipped. These samples were blinded to all analysts at NCEH and were analyzed concurrently with other applicator samples. For quantification of ACM, the samples were analyzed by a previously validated positive ion atmospheric pressure chemical ionization (+APCI) high-performance liquid chromatography-tandem mass spectrometry method (HPLC-MS/MS) (Olsson *et al.*, 2004). Blank samples (unspiked pooled urine samples) and positive quality control materials (pooled urine samples spiked at 1, 8 and 25 ng/ml) were analyzed in concert with unknown urine samples. Quantification was achieved using isotope dilution calibration plots generated for each individual analytical run. Concentrations of ACM in all samples were later confirmed by a second analysis including confirmatory ions (Figure 2). The method limit of detection (LOD) in the repeat analyses was 0.09 μg/l.

A set of four urine samples representing higher level exposures, three urine samples representing lower level exposure, and four "blank" urine samples were analyzed to estimate total metabolite concentration and for metabolite identification. The classification of exposure level was determined based on their urinary ACM concentrations (high >100 ng/ml; low <1.5 ng/ml; and "blank" <0.09 μg/l). Furthermore, these samples had little or no metolachlor mercapturate that could potentially lead to overestimation of total acetochlor metabolites because EMA can be derived from metolachlor metabolites as well as acetochlor metabolites. Total acetochlor metabolite concentrations in urine were estimated after a base hydrolysis under pressure to produce EMA using the preparation method of Cowell *et al.* (1987). The +APCI HPLC-MS/MS conditions used were those of Olsson *et al.* (2004).

To determine unknown metabolite structures, samples were extracted according to Olsson *et al.* (2004) analyzed using positive ion full-scan low-resolution mass spectrometry (MS) and MS/MS in both the precursor and product ion modes. Ions potentially retaining the 2-ethyl-6-methyl-aniline (*m/z* 136), the dealkylated aniline (i.e., ethyl aniline (*m/z* 122), methyl aniline (*m/z* 108) or aniline (m/z 94)), or 1-ethyl-5-methyl benzene (*m/z* 119) moieties (Figure 3) were

investigated using precursor ion scans. Precursor ion scans allow the determination of ions in the extract that can potentially produce the specified fragment ions. Our rationale was that most acetochlor-related metabolites would retain one of these moieties after fragmentation, allowing us to determine the source ion. In addition, dominant MS ions found in the full-scan MS mode were further analyzed using full-scan product ion analyses. The chemical structures were reconstructed from the fragment ion patterns.

Urinary creatinine concentrations were determined using a BM/Hitachi 717 Analyzer (Boehringer Mannheim, Indianapolis, IN, USA) and a reagent based on the method by Jaffe (1886). The LOD was 30 mg/dl. Creatinine concentrations were used to adjust for variable urine volume.

Data Analysis

Summary statistics were computed for ACM concentrations, both adjusted for creatinine content (μg/g) and unadjusted on a urine volume basis (μg/l). Creatinine unadjusted ACM concentrations in μg/l units were converted into molar unit concentrations (nmol/l) for comparison with the EMA molar concentrations. The unadjusted ACM concentration was multiplied by the total volume of each urine void to get total amount (nmol) contained within each urine sample. Total amount of ACM excreted in 24 h was computed by summing the amount of ACM in each urine sample collected after the first-morning urine sample on day 0 through to the first-morning urine sample on day 1. Descriptive statistics were calculated in S-PLUS v 6.0 (Insightful Corp, Seattle, WA, USA) and SAS, v. 9.1, (SAS Institute, Cary, NC, USA).

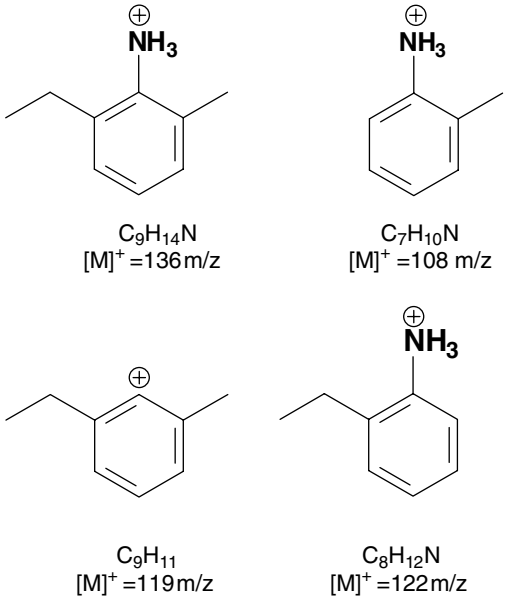

Figure 3. Potential fragment ions monitored using precursor ion MS/MS.

Results

ACM was successfully quantified in 55 out of 59 urine samples. An unknown compound interfering with the labeled internal standard ($^{13}C_6$-labeled ACM) precluded quantification in the remaining four samples. The mean relative standard deviation for the concentration of ACM in the six blind duplicates was 1.6% (range 0.1–4.6%) (Table 1). The Pearson correlation between the first and repeat analysis (repeat analysis contained confirmation ions as well as quantification ions) was 0.998 ($P<0.0001$).

The 55 samples were collected from eight individuals who were sampled 1–3 days each with a range of 1–13 samples per person for a total of 14 applicator days. ACM concentrations in the 55 samples ranged from 0.5 to 449 μg/l. The mean, standard deviation and range of ACM concentrations by applicator day are shown in Table 2. Mean ACM concentrations by applicator day ranged from 1.80 to 332 μg/l (1.41–135 μg/g creatinine). Total amount of ACM excreted in 24 h ranged from 7.71 to 350 nmol/24 h for the 10 applicator days with complete 24-h urine collection (Table 2). Total amount of ACM excreted in 24 h could not be computed for four applicator days because of incomplete urine collection. Boxplots of ACM concentrations by applicator day are shown in Figure 4.

Applicators sprayed acetochlor on day 0 on 11 out of the 14 applicator days (79%), on day -1 on 4 out of 14 applicator days (29%), and on both day 0 and day -1 on 1 applicator day (7%). The mean number of pounds of acetochlor sprayed on day 0 and on day -1 for the 14 applicator days was 157 ± 185 (range 0–642) and 92 ± 211 (range 0–743) pounds, respectively. The highest observed ACM level (449 μg/l) was found in the person who had applied the most acetochlor over the 2-day period (total of 1385 lbs).

Concentrations of EMA and ACM in a subset of samples are shown in Table 3. EMA (75 ± 8%) was recovered less efficiently than ACM (95 ± 4%), thus, the final concentrations for EMA were recovery corrected. Because our analysis

Table 1. ACM results for duplicate samples submitted blind to all analysts and analyzed concurrently with applicator samples.

Duplicate pair	
ACM No.1 (μg/l)	ACM No.2 (μg/l)
144	154
147	148
92.9	93.1
4.36	4.51
49.0	49.2
6.84	7.04
Mean % RSD = 1.6	

ACM = acetochlor mercapturate; RSD = relative standard deviation.

Table 2. Summary statistics for ACM by applicator day ($N = 55$).

Applicator-Day		ACM (μg/l)		ACM μg/g creatinine		ACM (nmol/24 h)	AC sprayed day 0 (lbs)	AC sprayed day −1 (lbs)
Worker no.	*n*	AM (SD)	Range	AM (SD)	Range			
1	3	3.72 (1.16)	2.87–5.04	2.48 (0.12)	2.40–2.61	NA	103	0
2	3	37.1 (31.8)	14.0–73.4	49.0 (22.5)	27.4–72.3	NA	21	0
2	5	128 (76.2)	35.3–228	68.2 (41.2)	18.8–121	234	351	0
6	4	11.5 (11.3)	1.57–23.5	6.69 (6.23)	1.26–13.8	33.8	105	0
8	4	22.6 (21.8)	9.81–55.3	11.5 (8.03))	4.79–23.1	29.0	0	65
8	1	NE	2.42	NE	1.06	NA	16	0
9	4	2.40 (1.41)	1.38–4.36	2.28 (1.20)	1.30–3.82	NA	0	138
9	4	1.80 (1.58)	0.50–3.93	1.41 (1.21)	0.30–2.77	7.71	254	0
12	5	8.44 (6.01)	1.29–16.5	8.09 (1.79)	6.16–10.7	29.9	334	0
13	5	14.6 (20.6)	0.90–49.0	8.85 (8.06)	1.18–20.8	34.6	170	0
13	4	114 (22.0)	82.3–133	48.7 (15.7)	29.8–68.3	158	45	0
14	4	332 (140)	144–449	135 (39.3)	86.5–181	350	642	743
14	4	111 (27.8)	81.5–147	62.2 (16.6)	43.5–83.7	118	161	0
14	5	148 (92.1)	47.4–281	87.0 (31.9)	55.0–133	205	0	350

AC = acetochlor; ACM = acetochlor mercapturate; *n* = number of urine samples for specified applicator day; NA = not available. Missing one or more samples needed for 24 h total; NE = not estimated, only one sample.

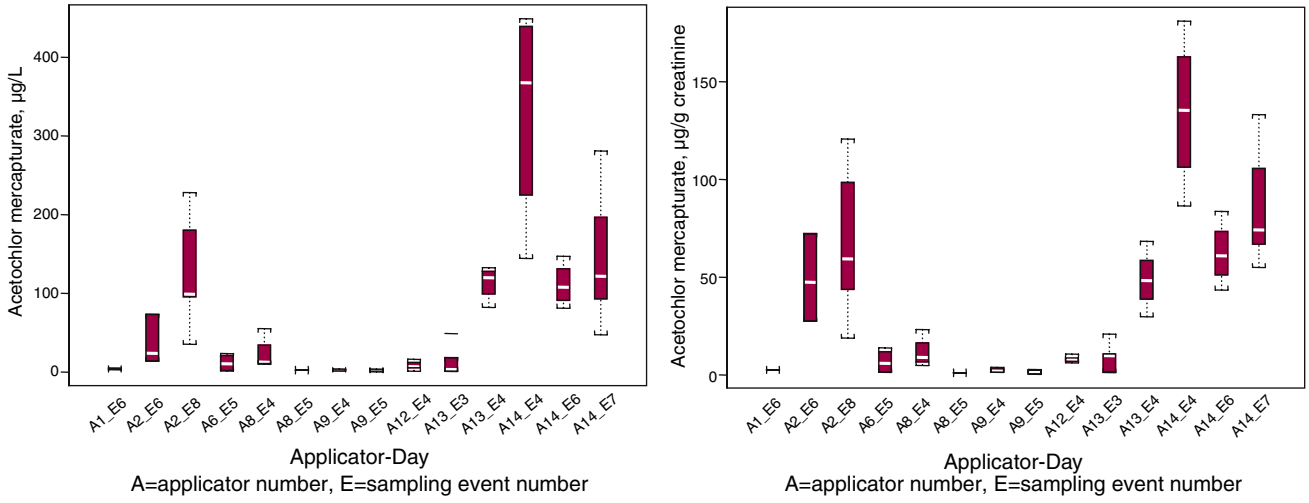

Figure 4. Boxplots showing urinary ACM concentrations for each applicator by sampling event. The horizontal line inside the box represents the median. The box boundaries are the twenty-fifth and seventy-fifth percentiles. Whiskers above and below the box are 1.5 times the interquartile range.

of ACM included a labeled internal standard, concentrations were automatically corrected for recovery. ACM represented on average about 27% of the total recovery-corrected EMA. However, when the ACM concentrations were higher, its percentage of the total EMA was higher in a concentration-dependent fashion (Figure 5).

We tentatively identified an additional acetochlor metabolite in the subset of urine samples analyzed. The $[M + H]^+$ ions of these metabolites were identified with precursor ion scan with m/z 119 as set mass. Additional fragment ions were determined after performing a product ion scan on the found precursor ion. The MS analysis did not determine any further metabolites. The metabolite (Figure 1) was tentatively identified as the N-dealkylation product *N*-(ethoxymethyl)-2-ethyl-6-methylaniline (metabolite A). Ion characteristics of this compound are m/z 119, 148, 164 and 178 with m/z 119 predominating (Figure 6). Unmetabolized acetochlor was also identified in the subset of urine samples, but at low concentrations (range < 0.20–$6.6\ \mu$g/l).

ACM and acetochlor were quantified using the scheme shown in Figure 2. Additionally, the tentatively identified metabolite was semiquantified (based upon ACM signal) using four precursor–product ion transitions (Table 4). The relative abundance of the metabolites is shown in Table 5. We

observed metabolite A in both higher and lower exposure samples; the relative abundance appeared to be associated with exposure level (i.e., increased as exposure level increased). At higher exposures, metabolite A was present at concentrations <1% of ACM concentrations (assuming equivalent MS response and recoveries were equivalent for the metabolites). Similarly, acetochlor was also found in the higher exposure samples but only at concentrations approximately 2% of the ACM concentrations. Unexpectedly, at the lower exposure levels, metabolite A concentrations were similar to ACM concentrations, and where ACM could not be detected, acetochlor and metabolite A were present at concentrations 1–4 times that of the ACM LOD. Because we have no authentic standards, we cannot verify this observation but it is consistent across ions and multiple ions were analyzed per analyte. Furthermore, these data seem to correspond with the changing percentage of ACM/EMA presented earlier.

Discussion

Although ACM has been hypothesized to be a human metabolite of acetochlor based on its structural similarity to alachlor and metolachlor, this is the first peer-reviewed report to unequivocally establish this identification. Most urine samples collected within 24–48 h of acetochlor application contained ACM at concentrations that varied over 900-fold among individual samples. The 14 applicator-day mean ACM concentrations adjusted and unadjusted for creatinine varied 100- and 180-fold, respectively.

Measurements of ACM have been reported in only a few studies. Curwin *et al.* (2005) recently reported the detection of ACM in farmer applicators who may or may not have applied acetochlor. Custom applicators in this study had mean ACM concentrations unadjusted for creatinine up to 40-fold higher than those reported for farmer applicators by Curwin *et al.* (2005). Two reports have indicated that ACM is rarely detected in the general US population. Swan *et al.* (2003) detected ACM in only 21% and 8% of urine samples from men in Missouri and Minnesota, respectively, with LODs similar to this study. Further, in CDC's Third National Report on Human Exposure to Environmental Chemicals (CDC, 2005), ACM was detected in fewer than 5% of the total samples analyzed. These studies suggest that acetochlor exposure in the general population is low and that ACM is a predominant metabolite in individuals exposed to large amounts (e.g., occupational levels) of acetochlor.

Interestingly, we observed a changing pattern in metabolite concentrations at changing exposure level (as determined by ACM concentrations). At lower exposure levels, ACM was a

Table 3. Concentrations of EMA and ACM in urine samples selected from among acetochlor-exposed applicators.

Sample	EMA (nmol/l)	Recovery-corrected EMA (nmol/l)[a]	ACM (nmol/l)	% ACM
A	2015	2687	1136	42.3
B	1758	2344	711	30.3
C	1880	2506	772	30.8
D	671	895	253	28.3
E	14.1	18.8	3.5	18.6
F	11.3	15	2.5	16.7
G	7.2	9.6	2.2	22.9

EMA = 2-ethyl-6-methyl aniline; ACM = acetochlor mercapturate.
The percentage of the total urinary metabolite concentrations (as measured by EMA) attributable to ACM are also shown. The samples are sorted by descending ACM concentrations.
[a]EMA concentrations corrected for the 75% recovery established previously.

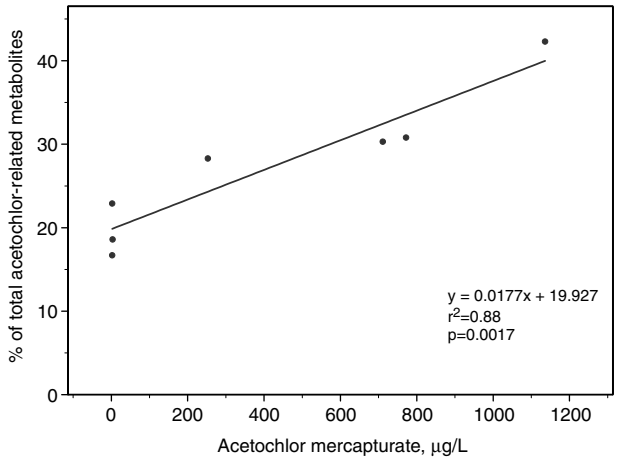

Figure 5. Concentration-dependent excretion of acetochlor mercapturate in urine.

Table 4. Precursor to product ion transitions measured for quantification of each proposed metabolite.

Quantification level	Analyte (or proposed analyte)	Precursor → product #1 (*m/z*)	Precursor → product #2 (*m/z*)	Precursor → product #3 (*m/z*)	Precursor → product #4 (*m/z*)
Fully quantitative	ACM	351 → 130	351 → 148	NA	NA
Semiquantitative	Metabolite A	194 → 119	194 → 148	194 → 164	194 → 178
Fully quantitative	Acetochlor	270 → 224	270 → 133	NA	NA

ACM = acetochlor mercapturate; metabolite A = *N*-ethoxymethyl-2-ethyl-6-methyl aniline; NA = not applicable.

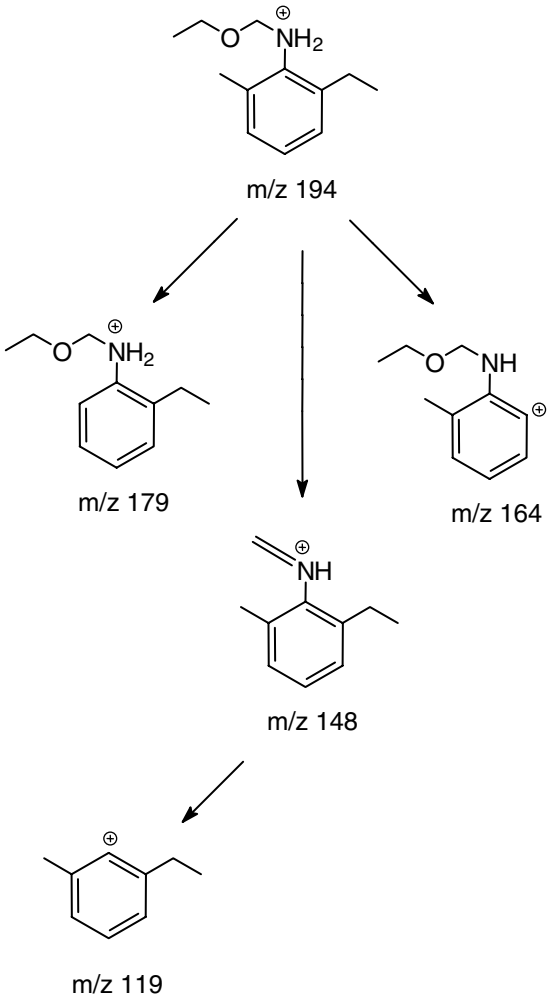

Figure 6. Analysis scheme for a tentatively identified acetochlor metabolite.

Table 5. Concentrations of acetochlor and its metabolites in urine samples of acetochlor applicators.

Exposure Category[a]	N	Mean levels		
		ACM (μg/l)	Metabolite A	Acetochlor (μg/l)
<LOD of ACM	4	<0.09	0.35	<0.02
Low ACM	3	1.1	1.42	1.47
High ACM	4	284	1.83	5.27

Concentrations of metabolite A was calculated semiquantitatively based on acetochlor mercapturate concentrations.
ACM = acetochlor mercapturate; metabolite A = *N*-(ethoxymethyl)-2-ethyl-6-methyl aniline; LOD = limit of detection; N = number of samples.
[a]Exposure category defined by ACM concentrations.

lower percentage of the acetochlor-related metabolite concentration as determined by EMA concentrations. This suggests a change in the metabolic pathway depending on the degree of exposure. Thus, at low background exposure ACM might not be the best indicator of exposure. Actually according to our results even unmetabolized acetochlor is a better indicator than ACM at lower exposure levels. However, because acetochlor and ACM only make up a small part of the EMA concentrations at low exposure, we attempted to determine other potential indicators of low-level acetochlor exposure.

Although ACM was identified as a metabolite of acetochlor, our identification and semiquantification of metabolite A are tentative and subject to several limitations. First, the intensity of each metabolite was low, thus, our mass spectra were subject to a great deal of noise. Consequently, only four fragment ions could be identified that would be derived from the proposed metabolite. Secondly, we did not have authentic standards for the tentative metabolite to provide an unequivocally positive identification. However, we verified that the proposed metabolite was not a fragment ion derived from acetochlor or ACM in the samples. Lastly, the relative magnitude of ACM to its proposed metabolite A could have varied also by different extraction recoveries or mass spectrometer response, thus changing their relative contribution in the urine samples. The obvious future direction in our research includes the synthesis of the proposed metabolite A and authenticating it as a potential metabolite of acetochlor.

Conclusions

We measured ACM in urine samples collected from custom applicators within 24–48 h of applying acetochlor. We positively identified ACM as a primary metabolite resulting from occupational exposures to acetochlor. We also measured small amounts of unmetabolized acetochlor in the urine samples and tentatively identified an additional metabolite. Our next steps for the biomonitoring of acetochlor include the acquisition of authentic standards for proposed metabolites A. This metabolite may be an important marker of exposure in the general population because it tends to predominate when exposure levels are low.

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Disclaimer

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Center for Environmental Health or the National Institute for Occupational Safety and Health.

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