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Identification of a Major Human Urinary Metabolite of Alachlor by LC-MS/MS

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Alachlor [2-chloro-2',6'-diethyl-N-(methoxymethyl)acetanilide] is a preemergence herbicide commonly used on soybeans, corn, and other crops. Herbicides are the most used class of pesticides accounting for more than 50% of total quantity used and more than 50% of total domestic sales. Alachlor is one of the top two herbicides with 75-100 million pounds used annually (EPA 21T-1005, 1991). Recently, we have been involved in studies of herbicide exposure that required us to develop procedures to measure alachlor and other herbicidal metabolites in urine. During our method development, we learned that the alachlor metabolism in humans was unknown. Alachlor metabolites have been identified in monkey urine and found to be mainly thioethers (Carr et al. 1986). Human alachlor exposure has commonly been estimated by quantifying the hydrolysis product, 2,6-diethylaniline (DEA), of alachlor metabolites in urine (Cowell et al. 1987). In this study we attempted to identify alachlor metabolites by using liquid chromatography coupled to mass spectrometry (LC-MS/MS) to analyze extracts of urine samples from subjects who were occupationally exposed to alachlor.

MATERIALS AND METHODS

Urine samples (25 ml) were obtained from four workers occupationally exposed to alachlor from the Centers for Disease Control and Prevention's (CDC) National Institute of Occupational Safety and Health (NIOSH).

The concentration of alachlor metabolites in these four samples were measured by a pressurized basic hydrolysis method, based on that of Cowell et al. (1987), that converts alachlor metabolites to DEA. Urine (2 mL), 0.5 mL of methanol, and 2 mL of 50% sodium hydroxide in 13-mm x 100-mm Pyrex culture tubes with teflon-lined screw caps. The Pyrex tubes were then placed in an oven at

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150° C for 1 hr. Insulated gloves were used to remove the tubes from the oven and the tubes were kept behind a safety shield until they cooled. When the tubes cooled to room temperature, 4 mL of 6 N HCl was added and the contents of the tubes were diluted to 15 ml with deionized water. The C18 solid-phase extraction cartridges were conditioned with 500 mg packing (Analytichem, Harbor City, CA) by washing them with 2 ml methanol followed by 8 ml of distilled water. After the hydrolysate was passed through the Cl8 cartridge, the cartridge was washed with 5 ml of distilled water and the eluate discarded. The analyte was eluted with 3 mL of methanol. The eluate was concentrated to 200 uL in a Savant vacuum concentrator (Farmingdale, NY); concentration to dryness was avoided because DEA is volatile. A Finnigan TSQ-7000 triple quadrupole mass spectrometer was used to measure DEA (San Jose, CA) in the atmospheric pressure chemical ionization (APCI) LC-MS/MS mode. The liquid chromatography column was a Whatman ODS-3 (4.6 mm x 25 cm) (Clifton, NJ). The mobile phase was water:methanol (3:7 v/v) with 0.1% acetic acid; at a flow rate of 1 mL/min, the elution time was about 7 min. The mass spectrometer was set in the positive ionization MS/MS mode with the collision offset at -22 V. The parent ion was the m/z 150 positive molecular ion; the daughter ion was the m/z 105, which was formed from the loss of ethylamine. We performed external standard quantitation using peak height; the standards were urine samples spiked with 1 ppb, 10 ppb, 100 ppb, and 1000 ppb of the alachlor model etabolite, 2-{(2,6-diethylphenyl)-(methoxymethyl)amino}-2-oxoethanesulfonic acid, sodium salt (Monsanto, St Louis, MO).

The following is the approach that was used to identify alachlor metabolites: We analyzed the urine samples from the occupationally exposed subjects and unexposed control subjects by LC-MS/MS with the APCI interface. The urine (2-to-5 mL volumes) was extracted twice with a 2x volume of ethylacetate and was then concentrated to 500 uL in the Savant vacuum concentrator. We used the concentrated extract for the analyses. The injection volume was 50 uL. The LC column was the same as previously described for the DEA analysis. The mobile phase was 19:l methanol:water with 0.1% acetic acid with a 1 mL/min flow rate. Our approach was to identify the MS/MS daughters of alachlor by presuming that some of the daughters would also be produced by the alachlor metabolite. Parent scanning, in which the mass of the daughter is set and the parent is scanned over a range of masses, could then be used to find the parent mass that produced an alachlor-associated daughter. Also used was daughter scanning, in which the parent mass is set and the daughter is scanned over a range of masses, to try to identify likely metabolites, such as alachlor conjugates

with glucuronic acid or cysteine.

Alachlor mercapturate was measured by the following method: The urine sample (2 mL) was passed through a Cl8 SPE cartridge, washed, eluted, and evaporated as described previously. The same LC column and conditions as described were used. The alachlor mercapturate elution time was about 9 min. We set the mass spectrometer in the positive ionization MS/MS mode with the collision offset at -18V. The parent ion was the m/z 397 positive molecular ion; the m/z 162 and m/z 130 daughter ions were scanned consecutively. The external standard measurements were done using peak height; the standards were urine samples spiked with 1 ppb, 10 ppb, 100 ppb, and 1000 ppb alachlor mercapturate that was synthesized in our laboratory.

Synthesis of alachlor mercapturate was done as follows: 35 mg alachlor and 20 mg N-acetylcysteine were added to 5 mL pyridine in a 25 ml round-bottom flask, which was sealed and let sit for 24 hr at room temperature. The pyridine was evaporated in the Savant vacuum concentrator; the oily residue was dissolved in 5 mL of a solution of acetonitrile:water:acetic acid (5:44.9:0.1 v/v) and subjected to solid phase extraction as described previously. The cartridge was rinsed with 5 mL of a solution of acetonitrile:water:acetic acid (5:44.9:0-l v/v), and the eluate discarded; the cartridge was then rinsed with 5 mL of solution of acetonitrile:water:acetic acid (27.5:22.4:0.l v/v) and the eluate discarded. Finally, the cartridge was rinsed with a solution of acetonitrile:water:acetic acid (40:9.9:0.1 v/v), the eluate was saved, and dried in the Savant centrifugal evaporator. The product was estimated to be at least 98% pure on the basis of analysis by LC-MS using a scan range from m/z 50 to 1000.

RESULTS AND DISCUSSION

The objective of this study was to identify the principal human urinary metabolite(s) of alachlor. Alachlor was analyzed in the MS/MS mode to find daughter ions that might also be produced by alachlor metabolites; two major daughter ions were present: A m/z 238 daughter ion was produced by a loss of a CH_3OH moiety, and the m/z 162 daughter ion was produced by the loss of CH_3O and $ClCH_2C{=}O$ moieties. If any alachlor metabolites were also to have one of these daughter ions, the mass of the could be determined by using the mass spectrometer in the MS/MS parent-scanning mode in which the third quad analyzes a single daughter ion such as the m/z 162 daughter ion and the first quad scans over a range of parent compounds that produced these daughter ions masses. If the

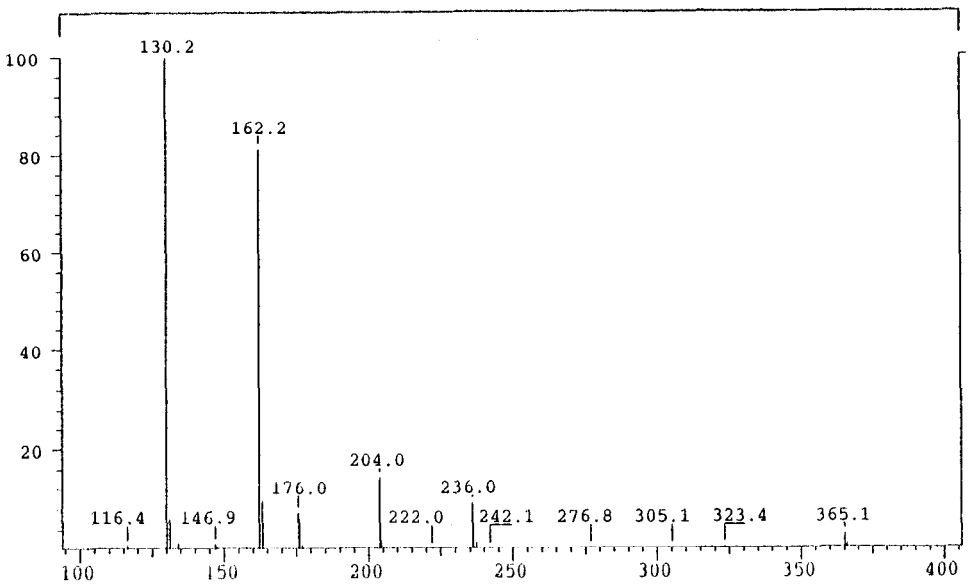
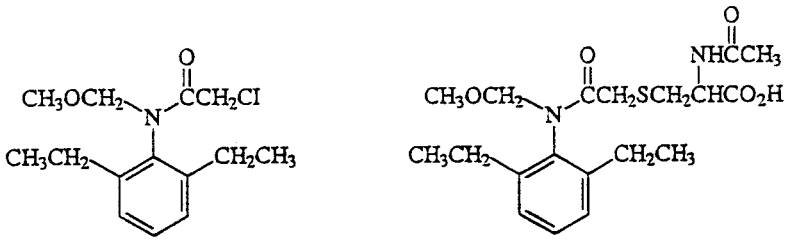


Figure 1. MS/MS spectrum (397 amu parent) of the alachlor metabolite; mass vs. relative intensity

metabolite were present at a high enough level, and if the parent mass range included the mass of its parent(s), then it is likely that the metabolite would be detected. Our samples consisted of extracts of urine samples from unexposed human subjects, extracts of each of the individual urine samples from exposed human subjects, and pooled extracts from the urine samples of exposed human subjects. We found no peak in any of the samples when the m/z 238 daughter ion was analyzed in the parent-scan mode; the parent was scanned from m/z 250 to 700 in m/z 50 increments per LC run. There was a peak with a retention time of about 5 min when the m/z 162 daughter ion was analyzed in the parent-scan mode in the samples from exposed subjects but no peak was found in samples from the control subjects. The spectrum of this peak showed three parent masses, m/z 365, m/z 383, and m/z 397. Reducing the collision offset produced no additional higher mass peak; thus, we assumed that the m/z 397 was the molecular ion, probably an MH+ ion, which is by far the most common ion in positive APCI.

We changed from parent ion scanning to daughter ion scanning to find any daughter ions other than the m/z 162 daughter ion. Figure 1 shows the daughter ion spectrum of the m/z 397 parent ion. The m/z 365 ion is a loss of methanol that is analogous to the 238 daughter ion of alachlor; this implies that the amine methoxymethyl sidechain is still intact. We postulated that the m/z



I II

Figure 2. Structure of the herbicide alachlor (I) and its human metabolite alachlor mercapturate (II)

130 daughter ion might be a product of an N-acetylcysteine moiety in which the sulfur-carbon bond is cleaved. Assuming that the m/z 397 ion is a molecular ion (MH+), then a possible structure, analogous to that of some other pesticide metabolites, is the N-acetylcysteine displacement of the chlorine of alachlor to form alachlor mercapturate. Comparing the alachlor metabolite with synthetic alachlor mercapturate, which was provided by the Monsanto Corporation, and with the alachlor mercapturate that we synthesized in our laboratory, we found the same chromatographic elution times using both acetonitrile and methanol mobile phases and the same MS/MS spectrum. On the basis of mass spectrometry and chromatographic behavior, we identified this metabolite of alachlor found in humans as alachlor mercapturate (Figure 2).

To estimate what proportion of alachlor is metabolized to alachlor mercapturate in humans, we analyzed urine samples of exposed individuals using the base hydrolysis method that quantitates DEA. We also quantitated alachlor mercapturate in the same samples (Table 1). Recovery of DEA and alachlor mercapturate from urine is about the same (about 75% recovery) in the methods used. In these four urine samples, alachlor mercapturate ranged

Table 1. Total alachlor metabolite concentration vs. alachlor mercapturate concentration

Sample	DEA (nanomolar)	Alamercapt (nanomolar)
A	930	350
B	470	120
C	4320	2750
D	1120	475

between 25% and 62% of the concentration of DEA, the hydrolysis product that probably almost all alachlor metabolites produce. Cowell et al. (1987) analyzed in urine for DEA as well as for 2,6-hydroxyethylethylaniline (HEEA), which is the base hydrolysis product that results if a metabolite contains a hydroxylated ethyl sidechain; they found no HEEA. We also analyzed the hydrolyzed urine from exposed people for the presence of HEEA and found none.

We were unable to positively identify alachlor metabolites other than alachlor mercapturate. We did detect what appeared to be alachlor cysteine in two of the urine samples from exposed persons on the basis of the MS/MS spectrum, but the signal strength was an order of magnitude lower than for alachlor mercapturate in the same samples. We did not detect alachlor-o-glucuronide.

The most complete analysis of alachlor metabolism in animals was done by following the fate of radioactively labelled alachlor that was administered intravenously in monkeys (Carr et al. 1986). Five metabolites were identified: alachlor mercapturate, alachlor cysteine, alachlor mercaptoacetate, secondary amide mercapturate, and alachlor O-glucuronide. In another study using urine from alachlor dosed monkeys (Feng et al. 1994), alachlor mercapturate was found to be the major metabolite, accounting for about 40% of the metabolite concentration. No free alachlor was detected in urine.

Thioether conjugates of pesticides, environmental chemicals, and drugs are produced by a reaction with the tripeptide glutathione (GSH = g-Glu-Cys-Gly). The products are then metabolized by the sequential loss of the glutamic acid residue, the glycine residue, and N-acetylation of the remaining S-substituted cysteine (Stanek et al. 1993). Other pesticides besides alachlor, including lindane, cyanatryn (Aizawa 1982) and atrazine (Lucas et al. 1993), and xenobiotics, including acrolein, benzene, vinyl chloride, and various allylic and benzylic halides (Stanek 1993), are metabolized by the glutathione mechanism.

Our identification of alachlor mercapturate allows development of methods to assess alachlor exposure based on measurement of levels of alachlor mercapturate in urine. This approach has advantages over the commonly used DEA method particularly when it is necessary to measure several pesticide metabolites in the same urine sample. It would be possible, in many cases, to extract all analytes together from the same urine sample, For the DEA method it is necessary to use a portion of the urine sample exclusively for alachlor exposure assessment. A method based on alachlor mercapturate like

the method described in this study would be simpler, safer, and more specific than the DEA method.

In this study, we have identified a major human metabolite of alachlor, alachlor mercapturate, developed a method to measure it in urine, and compared levels of the metabolite to levels of DEA, the hydrolysis product of alachlor metabolites, in urine samples from people exposed to alachlor. We believe exposure assessment is best accomplished by biological monitoring of human specimens, which provide an integrated sample reflecting exposure from multiple routes. Biological monitoring can provide a better understanding of exposure and can help us devise methods to evaluate effectiveness of intervention.

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