Quantitation of Alachlor Residues in Monkey Urine by ELISA

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An ELISA was used to quantitate the total residues of alachlor in monkey urine samples. Rhesus monkeys (six of each gender) were administered intravenously with [^{14}C]alachlor, and urine samples were collected from 0 to 120 h. Urinary residues from different time points were quantitated by immune response in an ELISA and by radioactivity. The immune response was based on antibody cross-reactivity toward three of the five major urinary metabolites of alachlor. The urinary concentrations of residues by both quantitation methods were expressed as parts per million equivalents of alachlor mercapturate, which was previously identified as the most abundant metabolite of alachlor in monkey urine. Analysis of 135 urine samples by radioactivity and by ELISA produced a correlation coefficient of 0.89. Time after dosing, gender, and dosing levels had no effect on the performance of the ELISA. The results suggest potential application of an immunoassay as a biomonitoring tool for alachlor exposure.

INTRODUCTION

The development of immunoassays for small molecules is a rapidly expanding field of research. Some of the advantages of immunoassays include speed, sensitivity, selectivity, and specificity (Hammock et al., 1990). An immunoassay-based biomonitoring method could permit a quick assessment of potential exposure to xenobiotics. Immunoassays are commonly applied as a single-residue method. However, many of the xenobiotics undergo rapid degradation, with the majority of residues being accounted for by metabolites rather than the parent compound. For those xenobiotics that undergo rapid metabolism, an immunoassay-based biomonitoring method would have to detect a mixture of metabolites. Using monkeys as a model, we examined the application of an immunoassay to measure total urinary residues of alachlor.

The disposition and metabolism of alachlor in monkeys were studied previously (Carr et al., 1986). Alachlor administered intravenously (iv) was shown to be rapidly eliminated from the monkeys, with 92–94% of the dose being excreted in the urine within the first 24 h after dosing. Alachlor itself was not detected in urine, and the five major metabolites were identified as alachlor mercapturate (ala-merc), alachlor cysteine (ala-cys), alachlor mercaptoacetate (ala-MA), secondary amide mercapturate (sec-merc), and alachlor *O*-glucuronide (ala-glc) (Figure 1). HPLC fractionation of pooled urine samples (0–96 h postadministration) from the 7 mg/kg dose group showed ala-merc as the most abundant metabolite, accounting for an average of 18% of the administered dose. The average concentrations of other metabolites were 10% for sec-merc, 8% for ala-MA, 2.5% for ala-cys, and 2.5% for ala-glc (Carr et al., 1986). Alachlor administered dermally to monkeys produced a similar disposition, with 88% of the absorbed dose being excreted in the urine within 48 h (Kronenberg et al., 1988). These results demonstrated two important features of alachlor metabolism in monkeys: the urine contains almost all of the residues, and most of the residues are thioether metabolites.

The development of immunoassays for alachlor and other chloroacetanilide herbicides has been a subject of our research for several years (Feng et al., 1990, 1992; Sharp et al., 1991). Rabbit polyclonal antibodies against alachlor were generated by use of an alachlor–protein conjugate. We conjugated alachlor to protein through the chlorine-bearing carbon via a thioether linkage. Antibodies generated to that hapten–protein conjugate showed good selectivity for alachlor from other chloroacetanilide herbicides; however, the antibodies also showed strong cross-reactivity to thioether metabolites of alachlor. Taking advantage of the antibody cross-reactivity for thioether metabolites, we now report the use of an ELISA to quantitate total residue levels of alachlor in monkey urine. Our objective is to examine if the ELISA immune response from a mixture of urinary metabolites would correlate with total residue levels determined by radioactivity.

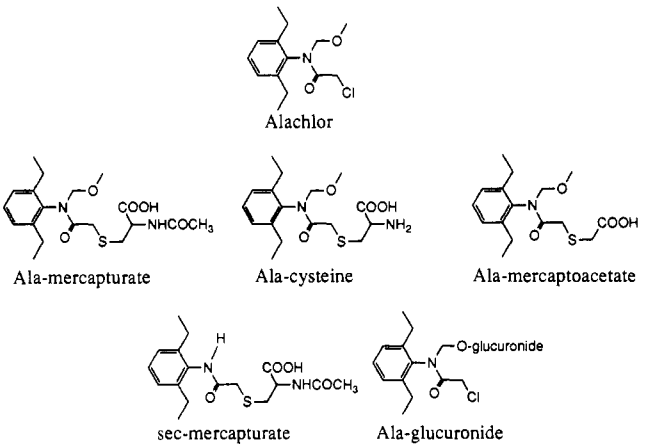

Figure 1. Major urinary metabolites of alachlor in monkeys following intravenous administration.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA), horseradish peroxidase (HRP), protein A–agarose, and Freund's complete and incomplete adjuvants were purchased from Sigma Chemical Co. Dicyclohexylcarbodiimide (DCC) and 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from Fluka Chemical Co. *N*-Hydroxysuccinimide (NHS) was purchased from Kodak. *N*-Acetylhomocysteine thiolactone (AHT) was purchased from Aldrich Chemical Co. Immulon 2 96-well microtiter plates were obtained from Dynatech. Nonfat dry milk powder (NFDM, Food Club brand) was obtained locally. Atomlight cocktail was obtained from New England Nuclear.

Intravenous Administration of Alachlor to Monkeys. Alachlor (^{14}C-phenyl UL, 10.1 mCi/mmol) in 1,2-propylene glycol was administered by injection into the saphenous vein. Two groups of six rhesus monkeys (three males and three females)

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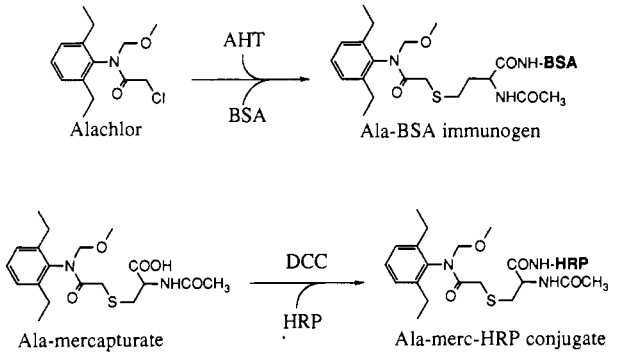

Figure 2. Synthesis of alachlor–BSA immunogen and alachlor mercapturate–HRP conjugate.

were administered at 7.0 or 0.7 mg/kg of body weight. Urine samples were collected in vessels maintained at 0–4 °C at 4, 8, and 12 h and every 12 h thereafter through 120 h. Urine samples were stored at −20 °C until analysis. The in-life portion of this study was conducted at International Research and Development Corp. (Mattawan, MI).

Synthesis of Metabolites. Synthetic standards of thioether metabolites were prepared by reacting alachlor with the appropriate thiols (cysteine, *N*-acetylcysteine, and mercaptoacetic acid) in a basic methanolic solution (Feng and Patanella, 1988). The secondary amide mercapturate was synthesized by reacting the secondary chloroamide analog of alachlor (Monsanto Sample Retention Center) with *N*-acetylcysteine. The alachlor glucuronide standard was previously synthesized and provided by Dr. G. Yalamanchili (Carr et al., 1986).

Synthesis of Immunogen (Alachlor–BSA Conjugate). Alachlor was covalently attached to BSA via AHT, a protein thiolating agent. The reaction was carried out at basic pH, and the alachlor–BSA conjugate was purified through dialysis (Feng et al., 1990). The proposed structure for the resulting hapten–protein conjugate is shown in Figure 2.

Synthesis of Alachlor–Horseradish Peroxidase Conjugate. Conjugation of alachlor mercapturate to horseradish peroxidase was accomplished by a modification of the method of Bauminger and Wilchek (1980). Alachlor mercapturate, dicyclohexylcarbodiimide, and *N*-hydroxysuccinimide (0.15 mmol each) were dissolved in 1 mL of *N*,*N*-dimethylformamide and stirred at room temperature for 30 min. After removal of the precipitated dicyclohexylurea by centrifugation, 100 μL of the supernatant was added to a solution of 100 mg of horseradish peroxidase in 5 mL of 0.1 N sodium bicarbonate. After stirring at 4 °C for 2 h, the conjugate was dialyzed against 0.005% thimerosal in phosphate-buffered saline (PBS, pH 7.4, 0.01 M phosphate and 0.15 M NaCl), aliquoted, and stored at −20 °C. The proposed structure of ala-merc–HRP conjugate is shown in Figure 2.

Antibody Generation and Purification. Ala–BSA conjugate (1 mg) was dissolved in 0.3 mL of PBS, emulsified with Freund's complete adjuvant (1 mL), and injected subcutaneously into female New Zealand white rabbits. Animals were subsequently boosted at 3-week intervals with 0.1–0.5 mg of the same immunogen in Freund's incomplete adjuvant. Whole blood, obtained 10–14 days after each boost by bleeding from the ear vein, was allowed to coagulate overnight at 4 °C and centrifuged to generate the serum. The IgG fraction in the serum was isolated by protein A affinity chromatography. A 5-mL aliquot of serum was added to a 5-mL column of protein A–agarose which was then washed with approximately 75 mL of PBS to remove the unbound serum proteins. The IgG fraction was then eluted with 10 mL of 0.58% acetic acid in 0.15 M NaCl and dialyzed against PBS. Aliquots of the purified IgG fraction were stored frozen at −80 °C.

ELISA for Alachlor Mercapturate. The purified IgG fraction (100 μL of 1:400 dilution in 0.1 M carbonate buffer, pH 9.6) was bound to the wells of Immunlon 2 microtiter plates by incubation overnight at 4 °C. Following three washes with water, wells were blocked with 8% NFDM in PBS for 1 h at room temperature. Wells were then washed three times with deionized water, and the plates were either used in assays or stored at 4 °C in PBS containing 1% BSA and 0.01% sodium azide for up to 1 week.

A stock solution of alachlor mercapturate in methanol was spiked into a sample of pooled human control urine to generate the standards at 0, 0.25, 0.5, 1, 2, 4, and 16 ppb. The standards were aliquoted, frozen (−20 °C), and freshly thawed for each assay. Standards or monkey urine samples (50 μL each) were dispensed into six replicate wells, followed by the addition of ala-merc–HPR (50 μL/well, 1:10000 dilution in PBS–2% NFDM). The plate was incubated at room temperature for 1 h, and wells were washed three times with deionized water. TMB substrate solution (100 μL/well) was prepared by mixing 100 μL of the stock solution (10 mg/mL in DMSO), 30 μL of hydrogen peroxide, and 10 mL of 0.1 M sodium acetate buffer, pH 6.0. Color was developed for 30 min at room temperature and quenched with 50 μL/well of 3.6 N sulfuric acid. Absorbances were measured at 450 nm, and the median absorbance of the six replicate wells was interpolated against the standard curve to calculate the concentration in parts per billion equivalents of ala-merc. Urinary samples with residue levels that exceeded the 16 ppb standard were serially diluted with human control urine until the concentration was measured within the standard curve range. The resulting concentration was then multiplied by the dilution factor to obtain the actual urinary concentration in parts per million equivalents of ala-merc.

Antibody Cross-Reactivity. The antibody cross-reactivity was examined against alachlor and the major urinary metabolites. The IC_{50} was the concentration of analyte required to generate a 50% reduction in absorbance. The percentages of cross-reactivities for the metabolites were calculated on the basis of alachlor which was arbitrarily set at 100%. The metabolite IC_{50} in molar concentrations was divided by the corresponding IC_{50} for alachlor and expressed as a percent (Feng et al., 1990).

Determination of Metabolite Concentration by Liquid Scintillation Counting (LSC). Crude monkey urine was dispensed into Atomlight cocktail (10 mL) and analyzed in a Tracor liquid scintillation counter, and disintegrations per minute were calculated on the basis of instrument efficiency. The dpm radioactivity was then divided by the specific activity of ala-merc (56 300 dpm/μg or 10.1 mCi/mmol) to obtain the concentration of urinary residues in ppm equivalents of alachlor mercapturate.

Instrumentation. Spectrophotometric absorbances of 96-well microtiter plates were recorded on a BioTek EL 310 reader equipped with a 450-nm filter. Plates were washed using a Dynatech Dynawasher II. A 12-channel Titertek pipet (50–200 μL) from Flow Laboratories was used for dispensing liquids.

RESULTS AND DISCUSSION

Animal metabolism of alachlor has been shown to differ greatly among species. Alachlor is rapidly metabolized to numerous products by rats and mice with biliary excretion as the major route of excretion (Sharp, 1988). In comparison, monkeys metabolized alachlor rapidly but to a few structurally similar metabolites that are excreted almost exclusively in urine (Carr et al., 1986). In none of the species was alachlor excreted intact in urine. On the basis of its characteristic metabolism and disposition, we chose monkeys as a model to evaluate the use of an immunoassay to quantitate total residues of alachlor in urine.

Specificity of the Antibodies against Alachlor Metabolites. Figure 1 shows the five major urinary metabolites of monkeys following iv administration (Carr et al., 1986). Ala-merc was the most abundant metabolite, accounting for about 18% of the administered dose. With the exception of ala-glc, the rest of the metabolites all share a common thioether linkage. All of the thioether metabolites were produced from an initial displacement of the chlorine atom in alachlor by glutathione (Feng and Patanella, 1988). The glutathione conjugate was subsequently metabolized through the mercapturate pathway

Table 1. Percentages of Antibody Cross-Reactivity for Alachlor and the Major Monkey Urinary Metabolites

analyte	% cross-reactivity
alachlor	100
alachlor mercapturate	34
alachlor cysteine	38
alachlor mercaptoacetate	75
secondary mercapturate	0
alachlor *O*-glucuronide	0

to the cysteine and *N*-acetylcysteine conjugates. The alachlor glucuronide was formed via a combination of O-demethylation and glucuronidation reactions (Feng and Patanella, 1989).

Table 1 lists the percentages of antibody cross-reactivity toward the five major urinary metabolites of alachlor. On the basis of a 100% reactivity for alachlor, three of the major monkey metabolites (ala-merc, ala-cys, and ala-MA) showed significant reactivities, ranging from 34 to 75%. The sec-merc showed no cross-reactivity, which was in agreement with our earlier observation that the *N*-methoxymethyl side chain of alachlor was critical for antibody reactivity (Feng et al., 1990). Not surprisingly, ala-glc also showed no cross-reactivity. The alachlor–BSA conjugate used in generating rabbit polyclonal antibodies is also based on the thioether chemistry (Figure 2). Because of the structural similarities of the alachlor–BSA conjugate and the monkey metabolites, it was not surprising to find extensive cross-reactivities of the antibodies toward these metabolites.

Quantitation of Residues by ELISA and LSC. The ELISA that was developed for this study is a simplification from our earlier ELISA (Feng et al., 1990). By conjugating the hapten (ala-merc) directly to HRP, we eliminated the need for the second antibody and achieved significant savings in time. We used ELISA to analyze crude urines from monkeys that were administered radiolabeled alachlor. The only sample preparation conducted was dilution, to bring the concentration within the standard curve range of the ELISA (0.25–16 ppb). LSC analysis was conducted with crude urine in scintillation cocktail.

A total of 135 urine samples from 12 rhesus monkeys were analyzed by ELISA and by LSC. Samples were obtained from both male and female monkeys administered at high (7.0 mg/kg) or low (0.7 mg/kg) doses of [^{14}C]-alachlor, and urines were collected between 0 and 120 h. The ELISA employed as standards seven concentrations of alac-merc (0, 0.25, 0.5, 1, 2, 4, and 16 ppb). The concentrations of residues as determined by ELISA were corrected for appropriate dilution factors and reported as ppm equivalents of ala-merc. The same urine samples were also analyzed by LSC for radioactivity and converted to ppm equivalents of ala-merc based on radio-specific activity. A comparison of urinary residue levels as determined by ELISA and LSC is shown in Figure 3. A linear correlation was observed between the two methods with an overall correlation coefficient of 0.89, a slope of 0.5, and a *Y*-intercept of −1.0. The slope of the line and its *Y*-intercept are explained by the inability of the ELISA to account for all of the residues in urine. This was expected, since the antibodies cross-reacted with three of the five major metabolites. In contrast, radioactivity analysis provided a comprehensive quantitation of all the residues. The data clearly demonstrate a linear correlation between the immune response by ELISA and total residues by radioactivity. The data were also analyzed in subgroups based on dose level or gender. With respect to dose level, the correlation between ELISA and LSC was 0.88 for both

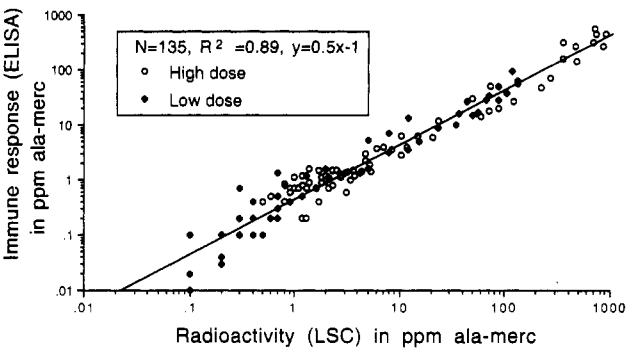

Figure 3. Comparison of monkey urinary residue levels in ppm equivalents of alachlor mercapturate as measured by the immune response (ELISA) or radioactivity (LSC).

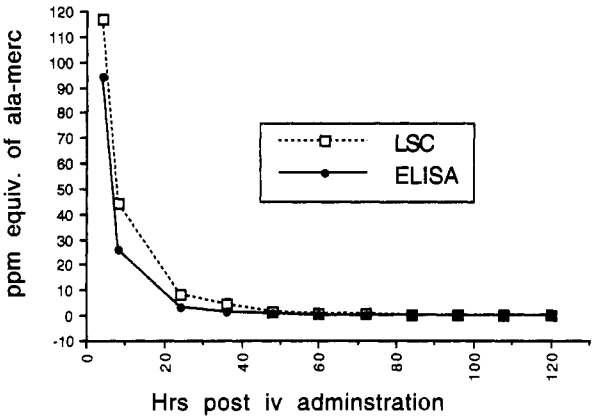

Figure 4. Time course of urinary excretion of alachlor residues in a male low-dose monkey (no. 1641) as measured by ELISA or LSC.

the high- and low-dose groups and produced superimposable lines on the graph. Figure 3 is plotted using different symbols for the high- and low-dose groups to further illustrate the correlations of ELISA with LSC. With respect to gender, we observed correlations of 0.85 and 0.95 for the males and females, respectively. The results suggest that gender or dosing level had no apparent effect on the performance of the ELISA.

Figure 4 compares the urinary residue levels of a male low-dose monkey (no. 1641) at various times after dosing as quantitated by ELISA or LSC. Correlations between the ELISA and LSC results were very good and suggest that time after dosing also has no apparent effect on the performance of ELISA. It is apparent that monkeys metabolized alachlor very rapidly, excreting almost all of the residues in urine within the first 2 days after administration. Although similar disposition patterns were observed with all of the animals, the actual concentrations of residues varied from animal to animal and from high- to low-dose levels.

Our data clearly showed that an immunoassay can be used to monitor total residue levels of alachlor in monkey urine. The immune response generated from a mixture of metabolites did correlate linearly to total residues and suggests potential applications of immunoassays as a biomonitoring method for exposure. In contrast to the monkeys, we do not believe an immunoassay could successfully monitor alachlor residues in rats and mice. Rodents metabolize alachlor to a myriad of metabolites with drastically different structures (Sharp, 1988), and developing antibodies that would recognize the majority of the residues would be difficult. Furthermore, rodents excrete most of the residues in feces, which is a more complex matrix requiring sample preparation prior

to analysis. There is obviously a lot of interest in developing an ELISA as a biomonitoring tool to assess human exposure to alachlor. Our studies indicate that the development of a reliable ELISA for human biomonitoring needs to be based on a thorough understanding of alachlor metabolism and disposition in humans and the design of appropriate antibodies.

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