Bull. Environ. Contam. Toxicol. (1995) 54:245–250

Bulletin of Environmental Contamination and Toxicology

Urinary Biomonitoring for Alachlor Exposure in Commercial Pesticide Applicators by Immunoassay

R. E. Biagini,[1] W. Tolos,[1] W. T. Sanderson,[2] G. M. Henningsen,[1] B. MacKenzie[1]

Department of Health and Human Services, Public Health Service, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, [1]Division of Biomedical and Behavioral Science, Applied Biology Branch, Immunochemistry Research Section; [2]Division of Surveillance Hazards Evaluation and Field Studies, Industry Wide Studies Branch and Hazards Evaluation and Technical Assistance Branch, Cincinnati, Ohio, USA

Received: 12 January 1994/Accepted: 10 July 1994

Numerous investigators have described urinary immunoassay as a screen for occupational exposure to a variety of compounds including pesticides (Feng et al. 1994; Lucas et al. 1993). Screening assays are generally quite sensitive, specific and accurate; however, unique patterns of sensitivity and cross-reactivity appear to be assay dependent and a detailed knowledge of assay performance characteristics is necessary for accurate interpretation of urine testing data (Cone et al. 1992). Despite these shortcomings, screening immunoassays have been shown to have high concordance with instrumental methods for the analysis of clinical specimens (Cone et al. 1990). Immunoassays have also been proposed for use in the screening of large numbers of water samples for the analysis of pesticide residues, as they are cost effective and highly efficient (Feng et al. 1990). Alachlor (2-chloro-2',6'-diethyl-N-[methoxymethyl] acetanilide; Figure 1) is one of the most commonly used chloroacetanilide herbicides in the United States, being the active ingredient of Lasso® and several other herbicide products (Feng et al. 1990). The Environmental Protection Agency (EPA) has restricted the use of alachlor or alachlor containing products to use by certified applicators or persons under their direct supervision (Federal Register 1987), because alachlor met or exceeded EPA's oncogenicity risk criteria (the existence of evidence alachlor resulted in increased incidence of tumors at multiple sites in mice and rats). However, epidemiological evidence for the carcinogenic potential of alachlor in humans is lacking. In the present work, we describe an immunoassay for the detection of alachlor or putative alachlor metabolites, in human urine obtained from alachlor-exposed pesticide applicators and compare these results with measurements obtained by high-performance liquid chromatography (HPLC) analysis of urine hydrolysate for diethylaniline (DEA).

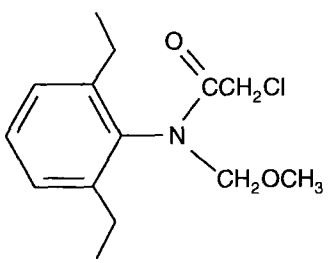

Figure 1. Structure of Alachlor

MATERIALS AND METHODS

The present investigation was part of a field study of workers who commercially apply agrichemicals. Twenty applicators and seven hauler/mixers participated in the study. Also, eight employees of the application companies, who were thought to have limited exposure to pesticides, submitted urine samples for estimates of alachlor dose. All study participants were male. Participants in the study were asked to provide three urine voids over a 24-hr period: one on the morning of the exposure survey before they began work; one at the end of the application period; one as the first-void sample the morning following the exposure survey. Each void was collected separately in a wide-mouthed 500-mL polyethylene bottle, and the time and volume of the void were noted. Two 25- to 50-mL aliquots of each void were transferred to a 60-mL high density polyethylene bottle and immediately frozen on dry ice. To estimate possible contamination of urine samples during voiding, a second 500 mL high density polyethylene bottle containing 50 mL of distilled water was taped to the side of the urine collection bottle (N=4).

Correspondence to: R. E. Biagini

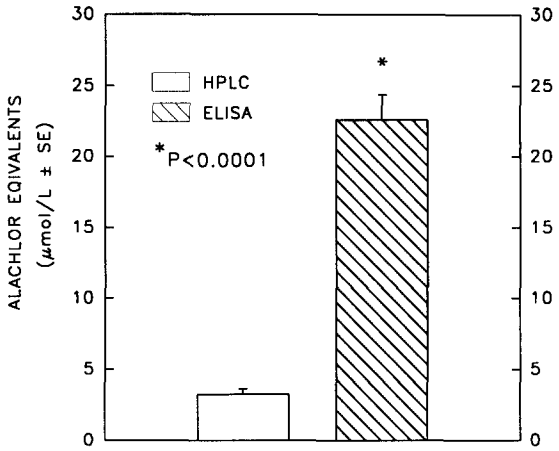

Figure 2. Results for alachlor equivalents determination by HPLC (μmole/L DEA) and ELISA (μmole/L as alachlor equivalents), (N=82).

After voiding, the participants were asked to recap the water bottle and return it along with the urine sample. The distilled water was analyzed for the presence of alachlor using the same technique as used to analyze urine samples. Samples were stored at -80^{o}C until analyzed. The HPLC method used is a modification of a GC method previously described (Cowell et al. 1987). Briefly, putative alachlor metabolites present in the urine are alkaline-hydrolysed at 150^{o}C, and the resultant DEA produced measured by HPLC. Methanol (0.125 mL) and sodium hydroxide [0.5 mL of 50% (w/w)] were added to a urine specimen (0.5 mL) in a screw-cap tube. The cap was sealed tightly using Teflon® and plastic tape and hydrolysis was performed for 1 hr in a bath consisting of sand and aluminum oxide which was fluidized by compressed air and maintained at 150^{o}C. After the hydrolysis, the sample was cooled, and 6 N HCl (1.2 mL) and deionized water (3 mL) were added. The treated, hydrolysed urine sample was passed through a MP-2 solid phase extraction cartridge (Interaction, Mountain View, CA), which had been pre-conditioned (washed with 1 mL methanol, then with 5 mL deionized water) and was connected to a twelve-port vacuum manifold (Baxter S/P,Obetz, OH). After the hydrolysate had passed through the cartridge, the cartridge was rinsed with 1 mL of deionized water. DEA was eluted from the cartridge bed with 1.5 mL methanol. The effluent was collected in a 2-mL volumetric flask brought to volume with 0.041 M sodium acetate buffer (pH 5), and mixed well. A 100 μL sample of the processed urine sample was introduced into a HPLC for DEA determination (Model 715 Ultra WISP sample processor; Model 590 solvent delivery system, and column heater with associated temperature control module; Waters, Division of Millipore, Milford, MA). The stationary phase was a 15 cm X 4.6 mm (i.d.) stainless-steel column packed with 5 μm Zorbax^{TM} C_8 (Mac-Mod Analytical, Chadds Ford, PA), maintained at 30 ^{o}C, and protected by a pre-column cartridge, 4 cm X 6 mm (i.d.), packed with 5-μm Zorbax^{TM} C_8. The mobile phase was 60:40 (v/v) methanol, 0.041 *M* sodium acetate in deionized water, adjusted to pH 4.8 with glacial acetic acid. The solvent flow rate was maintained at 1 mL/min. DEA was quantified using a Coulochem Model 5100A detector (ESA, Bedford, MA) and the data collected by a Dionex Advanced Computer Interface System (Dionex Corp., Sunnyvale, CA). In order to control for hydrolyses and systematic losses, normal volunteer control urines were spiked with a DEA-yielding pseudo-metabolite of alachlor [(2-[2,6-diethylphenyl)-(methoxymethyl)-amino]-2-oxo-ethane acid, pseudo-DEA] and analyzed bysulfonic the HPLC method outlined above.

A commercially available immunoassay kit (EnviroGuard^{TM}, ImmunoSytems (a subsidiary of Millipore Corp, Scarborough, ME), designed for the analysis of alachlor in water, was modified for the urinary analyses. The exact structure of the alachlor-immunogen used to produce the polyclonal antibodies used in the kit is propietary, however, it is similar in structure to

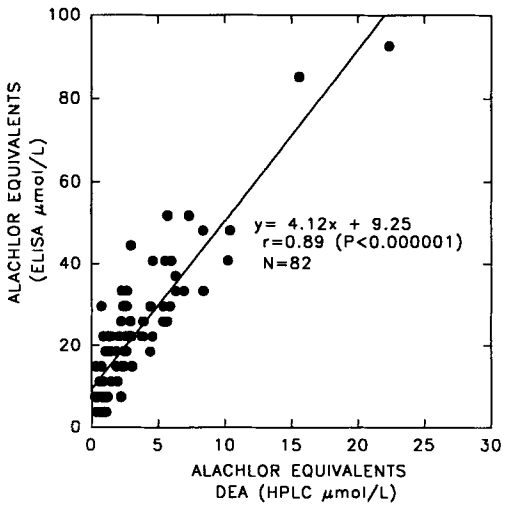

Figure 3. Results of linear regression of alachlor urinary equivalent concentrations by HPLC and ELISA.

immunogens used by other investigators performing ELISA quantitation of alachlor residues (Feng et al. 1994). The immunoassay format is a competitive solid phase ELISA method which is based on the inhibition of the reaction of enzyme-labelled (horseradish peroxidase) alachlor with immobilized polyclonal anti-alachlor antibodies by any free alachlor present in the standard or test sample. Briefly, 80 µL of standardized sample or diluted experimental urine sample was added (in triplicate) to each of the wells of the pre-coated 96-well microtiter plates. Eighty (80) µL of alachlor-enzyme-conjugate was then added to each well and the plates were covered and mixed on an orbital shaker (200 rpm) for 2 hr at room temperature. Plates were then thoroughly washed with a phosphate buffered saline (PBS)-Tween solution (0.02 M phosphate buffer, pH 7.4, containing 0.9% NaCl and 0.05% Tween). Eighty (80) µL of substrate (hydrogen peroxide) followed by 80 µL of chromogen (tetramethylbenzidine) were then added to each well, and the plates were incubated at room temperature for 1 hr, again, with shaking. Forty (40) µL of stop-solution (H_2SO_4) was then added and the plate solutions were agitated again (200 rpm). After 20 min of shaking, the absorbance of the solutions in the wells were read on an automatic microplate reader (Dynatech MR700) at 450 nm against a deionized water blank. The output of the plate reader was transferred to a microcomputer for data analysis. Preliminary analyses had shown urine from these alachlor exposed workers yielded results 100-1000 times higher than the dynamic range of the kit's standard curve, using parent alachlor in urine as standard. To rectify this, worker urines were diluted either 1:100 or 1:1000 with deionized water to bring their results into the range of the ELISA standard curve. In order to control for dilution effects on the urine matrix, alachlor parent standard curves were prepared using diluted pooled urine obtained from a minimum of three volunteers. Alachlor standard was obtained (ImmunoSystems, Scarborough, ME) as a 10 mg/mL solution in methanol. This solution was used to prepare alachlor standards from 0.0004 to 0.1 µmoles/L in diluted urine (1 ppb=0.004 µmoles/L). Diluted urine from the volunteers served as the negative control. Alachlor standards at 0.1, 0.05, 0.025, 0.0125, 0.004 and 0.0004 µmoles/L were freshly prepared on the day of analysis. Levels of alachlor equivalents in the urine samples were estimated by interpolation from a fitted four parameter logistic transformation of results from the diluted urine standard curves. Results from ELISA and HPLC analyses of co-analyzed urines were investigated for correlation using simple regression techniques and a microcomputer based statistical analysis package (Stagraphics, STSC, Rockville, MD). Alachlor equivalent results obtained from the ELISA method and DEA concentrations obtained from HPLC were analyzed for homogeneity using a one-way ANOVA. The analytical LOD of the ELISA assay system (calculat-

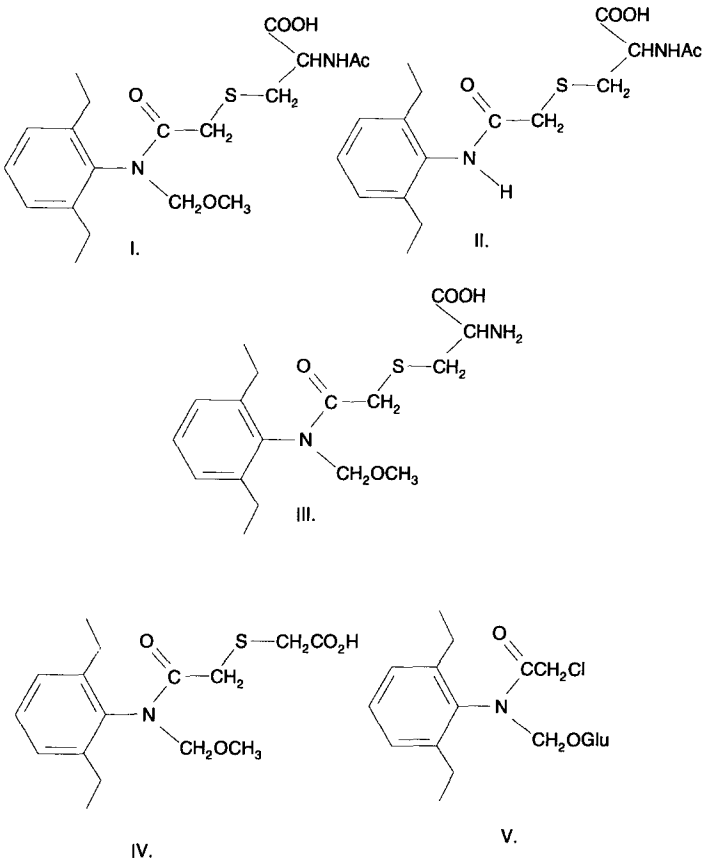

Figure 4. Structure of alachlor Rhesus monkey metabolites. Glu=glucuronidyl (modified from Sharp, 1988).

ed by the kit manufacturers protocol) is about 0.004 µmole/L alachlor equivalents for parent alachlor in diluted (1:1000) urine. Standard curves for parent alachlor in diluted normal volunteer urine provided data whose day to day and intra-plate variation were generally in the range of ≤ 15%. The accuracy and precision of the HPLC method were determined by the analysis of quality control samples and a duplicate test sample. The average recovery for quality control samples (pseudo-DEA, n=18) was 92 ± 10% (standard deviation), with a range of 65% to 122%. The HPLC's method analytical LOD was 0.19 µmole/L DEA.

RESULTS AND DISCUSSION

The ELISA and HPLC analytical methods gave statistically significant ($P<0.0001$) different results when applied to the 82 specimens that were above the analytical LOD for both methods. The mean result for the samples, analyzed by ELISA (N=82) with results above the analytical LOD was 22.6 ± 1.79 µmole/L as alachlor equivalents (± standard error, [SE]), while the HPLC method gave a mean result of 3.23 ± 0.38 µmole/L DEA (Figure 2). When correlation between the two methods was investigated using simple orthogonal regression techniques, a highly significant ($P<0.000001$; $r=0.89$) linear association was observed. The relationship between the two methods was ELISA results (as alachlor equivalents [µmole/L]) = 4.12 HPLC (as DEA, [µmole/L]) + 9.25 (Figure 3). Because the metabolism and excretion of alachlor in humans is not well defined, discussion of the bio-monitoring significance of the findings in the present study are hampered. Metabolism studies

in Rhesus monkeys with [^{14}C] alachlor have shown the production of five major urinary metabolites from alachlor exposure (two mercapturates [Figure 4, I. and II.], a cysteinyl conjugate [Figure 4, III.], a thioacetic acid conjugate [Figure 4, IV.] and a glucuronide [Figure 4, V.] (Sharp, 1988). Eighty-eight percent of administered [^{14}C] alachlor was excreted in the urine of monkeys with 80% yielding DEA forming analytes and 20% yielding 2,1-(hydroxyethyl)-aniline (HEEA) moieties (Dubelman and Cowell 1987). Although it may appear intuitive that monkey metabolism and excretion studies based on exposure to [^{14}C] alachlor could be used to model human metabolism and excretion, this has never been rigorously verified. The results of the present study which demonstrate a highly significant linear relationship ($r=0.89$; $P<0.000001$; slope=4.12) between methods for measuring putative alachlor metabolites in vitro strongly suggests that the ELISA method is positively biased. The basis of this systematic bias is unknown, but probably is related to similarities in structure between putative human alachlor metabolites excreted in the urine and the primary immunogen used to produce the polyclonal antibodies used as the basis of commercial kits. Alachlor, MW 270, is too small to be immunogenic in its own right. To overcome this, most antibodies for alachlor and other chloroacetanilide herbicides are raised against a derivatized chloroacetanilide that is coupled to a carrier macromolecule (usually a protein) forming a thioether linkage (Rittenburg et al. 1991; Feng et al. 1990). Polyclonal antisera to these alachlor-protein-thioethers would be expected to contain antibodies to numerous antigenic determinants on the immunogen molecule, including the thioether region, probably with differing affinities and avidities for each antigenic determinant. As can be seen from Figure 4, four of the 5 monkey urinary metabolites of alachlor have a thioether bond. Other investigations have shown that cross-reactivity can be as high as 188% in alachlor polyclonal antibody ELISAs using individual metabolites containing sulfur or thioether linkages (Feng et al. 1990). It is intriguing to speculate that combined cross-reactivity of the putative thiolated human urinary metabolites of alachlor present in the operators' urine in the current study are the reason for the discrepancy between our observed HPLC and ELISA results. Alternatively, metabolites of other acetanilide herbicides such as metolachlor and/or propachlor could have been present in the applicators urines. These acetanilide metabolites would be expected to potentially cross-react with anti-alachlor antibodies; however, upon base hydrolysis for HPLC they would not form DEA. This discrepancy could also account for the apparent "over estimation" of alachlor equivalents by ELISA when compared to HPLC results. Finally, the ELISA kits used for the urinary analyses are designed for use in a water matrix. The standard curves generated using dilute urine as a matrix have higher LODs than water standard curves (about 1 ppb vs. 0.1 ppb for water). This matrix effect, if present to a greater or lesser extent for the urinary metabolites of alachlor, could conceivably had an effect on the correlation between hydrolysis derived DEA alachlor equivalents and ELISA quantitated alachlor equivalents. In general, the use of urinary immunoassay as a primary analytical method, although shown to have high concordance with instrumental methods (as in the present work), is precarious, due to unique patterns of sensitivity, cross-reactivity and matrix effects. Even after rigorous investigation of assay performance characteristics, prudence dictates confirmation by independent assay. ELISA methods have benefits in comparison to instrumental methods with regard to differences in sample size required by the two methods (20-30 mL for HPLC vs. 80 µL for the ELISA method), and the ELISA's benefits in time of analysis, cost, sample throughput and portability. These factors strongly suggest that the ELISA method is a viable adjunct to classical instrumental methods of analysis, especially in screening for occupational exposure.

Acknowledgements.

The authors gratefully acknowledge Bruce Ferguson of ImmunoSystems (Millipore) for suggestions concerning the content of this manuscript. Mention of a product or company name does not constitute endorsement by NIOSH.

REFERENCES

Cone EJ, Dickerson S, Paul BD, Mitchell JM (1992) Forensic drug testing for opiates. IV.

Analytical sensitivity, specificity, and accuracy of commercial urine opiate immunoassays. J Anal Toxicol 16: 72-78

Cone EJ, Yousefnejad D, Dickerson SL (1990) Validity testing of commercial urine cocaine metabolite assays: IV. Evaluation of the EMIT d.a.u. cocaine metabolite assay in a quantitative mode for detection of cocaine metabolite. J Forensic Sci 4: 786-91

Cowell J, Danhaus R, Kuntsman J, et. al. (1987) Operator exposure from closed system loading and application of alachlor herbicide. Arch Environ Contam Toxicol16: 327-332

Dubelman S, Cowell J (1987) Biological monitoring for pesticide exposure. In: Wang, Franklin, Honeycutt, Reinhart (ed) Am Chem Soc Symposium Series 382, Division of Agrichemicals, 194th Meeting, New Orleans, LA.

Federal Register (1987). "Alachlor; Notice of Intent to Cancel Registrations; Conclusion of Special Review", Vol. 52. No. 251, Thursday, December 31, pp. 49480-49583

Feng, PCC, Sharp, CR, Horton, Sr (1994) Quantitation of alachlor residues by ELISA. J Agric Food Chem 42: 316-319

Feng PCC, Wratten SJ, Horton SR, Sharp CR, Logusch EW (1990) Development of an enzyme-linked immunosorbent assay for alachlor and its application to the analysis of environmental water samples. J Agric Food Chem 38: 159-163

Lucas AD, Jones AD, Goodrow MH, Saiz SG, Blewett C, Seiber JN, Hammock BD (1993) Determination of atrazine metabolites in human urine: development of a biomarker of exposure. Chem Res Toxicol 6:107-16

Sharp, DB (1988) Alachlor. In: Kearny PC and Kaufman DD (ed) Herbicides: Chemis try, Degradation and Mode of Action. Vol. 3, Marcel Dekker, NY, pp 301-333.

Rittenberg, JH, Grothaus, GD, Fitzpatrick, DA, Lankow, RK (1991) Rapid on-site Immunoassay System, Agricultural and Environmental Applications. In Vanderlann M, Stanker LH, Watkins BE, Roberts, DW (ed) Immunoassays for Trace Chemical Analysis, American Chemical Society Symposium Series 451, American Chemical Society, Washingtion, DC pp 28-39.