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Biological Monitoring for Selected Herbicide Biomarkers in the Urine of Exposed Custom Applicators: Application of Mixed-effect Models

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Metabolites and/or parent compounds of the herbicides atrazine, alachlor, metolachlor, cyanazine and the 2-ethylhexyl ester of 2,4-dichlorophenoxyacetic acid (2,4-D) were measured in the urine of 15 custom applicators who each provided from five to seven 24 h urine samples during a 6 week period (n = 87). Each applicator provided a pre-season urine sample and a reference population (n = 46) provided first-morning urine samples. Urinary biomarkers were measured by either immunoassay or gas chromatography. During the spraying season, the geometric mean amount of alachlor mercapturate equivalents (eq.), atrazine eq., 2,4-D and metolachlor mercapturate eq. excreted in 24 h was 17, 19, 110 and 22 nmol, respectively. Mixed-effect models were used to determine predictors of the amount of atrazine eq. and 2,4-D excreted in 24 h. The specific days of herbicide spraying associated with increased biomarker excretion varied for the two analytes, and included one or more days prior to urine collection. This confirms the importance of collecting covariate information on day(s) most relevant to the biomarker of interest. The within-worker variance component, expressed as a geometric standard deviation ($_W$GSD range: 2.5–2.9), was substantially larger than the between-worker component ($_B$GSD range: 1.3–1.5) for the modeled biomarkers. Alachlor mercapturate eq. and metolachlor mercapturate eq. were detected in more than half of the applicator pre-season urine samples. All biomarkers were detected infrequently in the reference population. Evaluation of non-spray exposure determinants was limited by inclusion of prior day spraying, adjustment for time and the small sample size.

Keywords: atrazine; alachlor; metolachlor; 2,4-D; cyanazine; biological monitoring; mixed-effect models; variance components; immunoassays

INTRODUCTION

In the spring of 1996, the National Institute for Occupational Safety and Health (NIOSH, Cincinnati, OH) conducted an exposure assessment study of custom (or commercial) applicators who applied pre-emergent herbicides to corn and soybean fields using ground boom application equipment. This study included multiple measures of exposure to the herbicides alachlor, atrazine, cyanazine, metolachlor and the 2-ethylhexyl ester of 2,4-dichlorophenoxy acetic acid (2,4-D EH). A description of the herbicide application process and the results of air, handwash, dermal patch and saliva sampling have been reported elsewhere (Denovan *et al.*, 2000; Hines *et al.*, 2001). The results of biological monitoring for selected urinary biomarkers of these five herbicides are reported here.

Biological monitoring can provide an accurate measure of the absorbed dose. Ideally, the biomarker should not be present in the urine at the start of the monitoring period and the applied dose, absorption, metabolism, distribution and elimination parameters of the parent compound should be known. In addition, all urine should be collected until the biomarker is nearly completely eliminated and no

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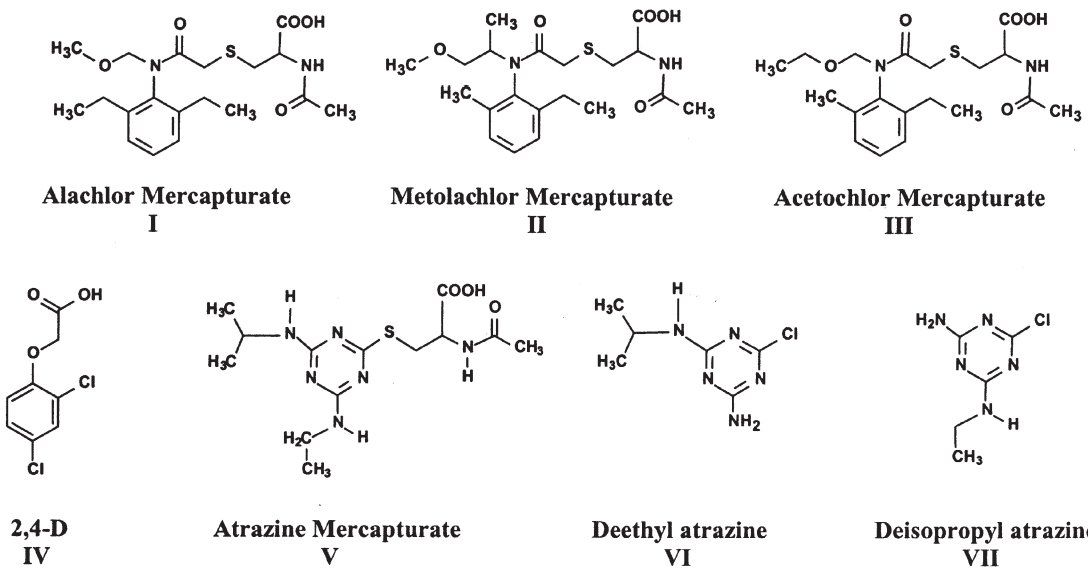

Fig. 1. Mercapturic acid metabolites of alachor, metolachor and acetochlor (I–III), 2,4-D metabolite of 2-ethylhexyl ester (IV) and three atrazine metabolites (V–VII).

additional exposure should occur during the collection period. These conditions are difficult to achieve in many work environments, including the one described here. In these cases, it is often more feasible to estimate the urinary body burden of a substance over a specified time period.

In this study, the body burden of selected herbicide urinary biomarkers was characterized among 15 custom applicators during a 6 week pre-emergent spraying season. The influence of recent herbicide spraying on biomarker levels was evaluated using mixed-effect regression models. Since investigators could not control the timing of an applicator's exposure and the biomarkers had varying elimination half-lives, a sampling strategy that was optimal for all herbicides was not possible. Instead, a systematic sampling scheme that provided information about body burden at multiple points in time over the 6 weeks was used (Hines *et al.*, 2001). Applicator urine sampling was conducted concurrently with patch, air, hand wash and saliva sampling. Urine samples were also collected from a reference population for comparison to applicator samples.

BACKGROUND

Biomarkers that might serve as exposure markers were identified for each of the five herbicides using information from human studies whenever possible (Fig. 1). The acetanilide herbicides, alachlor and metolachlor, are primarily metabolized in humans to their respective mercapturic acid conjugates and excreted in the urine (Driskell *et al.*, 1996; Driskell and Hill, 1997). In rhesus monkeys, 92–94% of an intravenous (i.v.) alachlor dose was excreted in the first 24 h, and 88% of a dermal dose within 48 h, with the major metabolite being alachlor mercapturate (Feng *et al.*, 1994). In a second study, radiolabeled alachlor administered i.v. or dermally to rhesus monkeys was eliminated primarily in the urine within 48 h, with an estimated elimination half-life ($_{e}t_{1/2}$) of 3.5–6.5 h by the i.v. route (Kronenberg *et al.*, 1988).

Atrazine is primarily metabolized in humans to its mercapturic acid conjugate (Lucas *et al.*, 1993; Brady *et al.*, 1998; Buchholz *et al.*, 1999). The mono-*N*-dealkylated metabolites of atrazine (deethylatrazine and deisopropylatrazine) and its di-*N*-dealkylated metabolite (2-chloro-4,6-diamino-1,3,5-triazine) have also been detected in varying proportions in human urine (Catenacci *et al.*, 1993; Lucas *et al.*, 1993; Buchholz *et al.*, 1999; Mendaš *et al.*, 2000). The bi-dealkylated metabolite degrades rapidly when urine is stored for an extended time or thawed and refrozen (Bradway and Moseman, 1982), and is less suitable for biomonitoring. In rhesus monkeys dosed with i.v. [^{14}C]atrazine, atrazine mercapturate elimination in urine peaked at 12 h after dosing and decreased to nearly baseline levels by 24 h (Brady *et al.*, 1998). In dermally dosed human volunteers, atrazine, measured as ^{14}C-labeled urinary metabolites, had a $_{e}t_{1/2}$ of 24–28 h (Gilman *et al.*, 1998). Human or primate data on cyanazine metabolism were not found.

Esters of 2,4-D are primarily hydrolyzed to the free acid in humans (Sauerhoff *et al.*, 1977). Reported urine elimination rates for 2,4-D vary across studies. In six human volunteers administered 2,4-D by i.v., 100% of the dose was excreted in the urine within 120 h ($_{e}t_{1/2}$ =13 h), whereas following dermal admin-

istration, only 5.8% of the dose was recovered after 120 h ($_{e}t_{1/2}$ not reported) (Feldmann and Maibach, 1974). In one oral ingestion study of 2,4-D in a human volunteer (Kohli *et al.*, 1974), 75% of the dose was excreted unchanged in the urine after 96 h, while in a similar study of five human volunteers (Sauerhoff *et al.*, 1977), an average of 95.1% of the dose was excreted in the urine, primarily as the free acid (82.3%), and to a lesser extent as a conjugate (12.8%), with an average urine $_{e}t_{1/2}$ of 17.7 h. In 26 occupationally exposed mixers, loaders and applicators applying predominantly an amine formulation of 2,4-D by ground application, a urine $_{e}t_{1/2}$ of 35–48 h was estimated after a single exposure event (Nash *et al.*, 1982). An average of 4.5% of a 2,4-D dose applied to the hands of five human volunteers was recovered in the urine after 144 h, with an estimated $_{e}t_{1/2}$ of 39.5 h (Harris and Solomon, 1992). Thus, depending on the route of exposure and possibly formulation, the urine elimination half-life of 2,4-D may range from as low as 13 h to >40 h.

In summary, the above evidence suggests that biomarkers of the target herbicides are eliminated in the urine within several hours to several days of exposure and that for some biomarkers, accumulation in the body is possible depending on the frequency and intensity of exposure.

MATERIALS AND METHODS

Sampling strategy

Fifteen custom applicators in Ohio, all males, were sampled at ~4 day intervals, including weekends, over six consecutive weeks (6 May–13 June 1996) as described in Hines *et al.* (2001). Samples were collected on the applicator's scheduled day whether the applicator sprayed or not, unless the applicator was given the day off. For each sampled and unsampled day in the 6 week period, information on the type and amount of all herbicides applied was obtained for each applicator by interview and from records. Other covariate information, such as glove use, was recorded on only the sampled day. For each applicator, at least three males residing in the same township who did not use pesticides at work were recruited as a reference population. In this paper, the term 'herbicide' refers to the active ingredient. Participation in the study was voluntary and informed consent was obtained. This study was approved by the NIOSH Human Subjects Review Board.

Sample collection

A 'pre-season' urine sample was collected from each applicator in early April before herbicide spraying began. On each sampling day during the spray season, 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). Urine was collected in five time periods starting on day 0: wake-up, wake-up to 12 p.m., 12 p.m. to 6 p.m., 6 p.m. to 12 a.m., and 12 a.m. to wake-up on day 1. A portable urine collection kit consisting of a 9.5 l cooler containing five 500 ml high-density polyethylene (HDPE) bottles labeled by time period, a refrigerant pack, pen, brown paper bags, time-piece and instructions were provided. Applicators were instructed to void into the appropriate bottle depending on the time of day and to write all void times on the label. Bottles were removed from the cooler as each collection period elapsed. Urine volume was measured with a clean 250 ml graduated cylinder, and aliquots were poured into 30 ml HDPE bottles. Aliquots were frozen and transported to the laboratory on dry ice, then stored at –80°C. A duplicate aliquot was collected from one of the applicator's bottles each day for quality control (QC). A first-morning urine void was also collected from each person in the reference population. Applicator and reference population participants were compensated $200 and $10 each, respectively, for full participation.

Immunoassays: alachlor mercapturate, atrazine and cyanazine

RaPID Assay® enzyme-linked immunosorbent assay (ELISA) kits (Strategic Diagnostics, Newtown, PA) were used to determine metabolites of alachlor, atrazine and cyanazine in urine. Kits were used according to the manufacturer's instructions. Calibration standards (0, 0.1, 0.5, 1.0 and 5.0 µg/l) were prepared by fortifying diluted pooled urine from anonymous volunteers with alachlor mercapturate (prepared in-house; Striley *et al.*, 1999), atrazine or cyanazine (Chem Service, West Chester, PA). The alachlor response was greater for alachlor mercapturate than for alachlor parent (Biagini *et al.*, 1995). Since alachlor mercapturate is a major human metabolite of alachlor, it was used as the calibration standard. Pooled urine used as calibration standard diluent was diluted with water 1:10 or 1:100 to match the dilution of the samples. Laboratory QC samples were prepared in neat urine so that when diluted 1:10 or 1:100 the concentration would be 1.0 µg/l.

The RAPID Assay® for metolachlor had little cross-reactivity to metolachlor mercapturate. Since metolachlor is excreted as the conjugated form in humans, an ELISA for metolachlor mercapturate was developed. Analyses were performed according to a previously published method (Striley *et al.*, 1999). Calibration standards (0, 0.15, 0.25, 0.5, 1.0, 2.0 and 5.0 µg/l) were prepared by fortifying diluted pooled urine with metolachlor mercapturate (prepared in-house; Striley *et al.*, 1999). Pooled urine used as calibration standard diluent was diluted with Superblock™, a buffer (Pierce Chemical, Rockford, IL) 1:10 or 1:100 to match the dilution of the samples.

Laboratory QC samples were prepared in neat urine so that when diluted 1:10 or 1:100 the concentration would be 1.0 and 2.5 µg/l.

Calibration, QC and field samples were run in duplicate for all ELISA analyses. Field samples with an absorbance higher than the highest calibration standard were diluted 1:10 or 1:100 in water or Superblock™, and additional calibration standards in diluted pooled urine were similarly prepared. Since enzyme-labeled pesticide was in competition with unlabeled pesticide, the developed color was inversely proportional to the concentration of the unlabeled pesticide in the sample. The % B/B_o (bound/unbound) was computed by dividing each standard or sample absorbance by the absorbance of the blank. The standard curve was plotted as a logit % B/B_o versus the log concentration of the standard. Simple linear regression was performed and the regression parameters, the sample absorbance and the dilution factor were used to compute the final concentration.

Cross-reactivities to compounds structurally related to the metabolites were examined for each immunoassay (Table 1). When *in vivo* metabolism adds characteristics to a metabolite that make it more similar to the hapten–protein immunogen used to immunize animals, increases in affinity with resultant increases in sensitivity can be observed (Biagini *et al.*, 1995). This appears to be the case for the anti-alachlor and anti-atrazine antibodies, which strongly cross-react with their respective mercapturate metabolites. Because of cross-reactivity and affinity issues, immunoassay results are expressed as analyte equivalents (eq.).

For all immunoassays, except metolachlor mercapturate, the least detectable dose (LDD) was based on the manufacturer's reported LDD, taking into account sample dilution. The LDD for metolachlor mercapturate was defined as 90% $B/B_o \times 10$ (the urine dilution) and interpolated mathematically from the coefficients of a four-parameter logistic equation. The immunoassay LDDs were 1.3, 2.0, 1.7 and 4.9 nmol/l, for alachlor mercapturate eq., atrazine eq., cyanazine eq. and metolachlor mercapturate eq., respectively. The LDD is used as the method limit of detection (LOD).

Table 1. Herbicide immuoassay cross-reactivities for selected compounds

ELISA	% Cross-reactivity at 50% B/B_o[a]
Antibody: alachlor	
Standard: alachlor mercapturate	
Alachlor	100
Alachlor mercapturate	130
Metolachlor	0.87
Metolachlor mercapturate	2.04
Acetotochlor	0.28
Acetochlor mercapturate	0.092
Antibody: atrazine	
Standard: atrazine	
Atrazine	100
Atrazine mercapturate	79
Cyanazine	<0.01[b]
Deethylatrazine	*
Deisopropylatrazine	*
Simazine	22[b]
Antibody: cyanazine	
Standard: cyanazine	
Cyanazine	100
Atrazine	<0.004[b]
Atrazine mercapturate	Not tested[c]
Deethylatrazine	Not tested
Deisopropylatrazine	Not tested
Simazine	0.21[b]
Antibody: metolachlor mercapturate	
Standard: metolachlor mercapturate	
Metolachlor	39
Metolachlor mercapturate	100
Acetochlor	Not tested
Acetochlor mercapturate	23
Alachlor	*
Alachlor mercapturate	*

[a]Absorbance of the sample (B) divided by the absorbance of the blank (B_o), expressed as a percentage.
[b]Kit insert value—confirmed in laboratory.
[c]Parent compound had very low cross-reactivity.
*50% inhibition not observed at concentrations tested (0.1–10 p.p.b.).
Note: ELISA = enzyme-linked immunosorbent assay.

Gas chromatography: 2,4-D, deethylatrazine, deisopropylatrazine, parent triazines

A gas chromatography (GC) method with electron capture detection was used to determine 2,4-D in urine. Concentrated phosphoric acid (1.000 ml) and an internal standard solution (150 µl, 4676 ng/ml) of 2,4-dichlorophenylacetic acid in methanol were added to 6.0 ml of urine in a test tube and vortexed for 60 s. A 4.0 ml aliquot of the urine-internal standard mixture was loaded onto a C_{18} solid phase extraction (SPE) cartridge conditioned with propanol (5 ml) and water (5 ml). The loaded cartridge was rinsed with 25% acetic acid (5 ml) and water (3.0 ml). The cartridge was then dried under a flow of nitrogen for 10 min and eluted with acetone (5 ml). The acetone extract was collected in a tube containing pentafluorobenzyl (PFB) bromide (100 µl) and potassium carbonate (63 mg). The reaction mixture was vortexed for 15 min at room temperature and trans-

ferred to a tared test tube containing 120 mg of the quenching agent, hordenine (prepared from hordenine hemisulfate). The quenched solution was vortexed for 30 s and weighed. For each gram of solution, 0.4 M sulfuric acid (0.7 ml) was added and the solution vortexed for 15 s. This aqueous solution was loaded onto a second C_{18} SPE cartridge conditioned as above. The loaded cartridge was rinsed with 5 ml each of 0.4 M sulfuric acid and water, then dried under a flow of nitrogen for 10 min. After drying, the cartridge was eluted with toluene (4 ml) and the extract collected in a test tube. The extract contained the PFB derivatives of both 2,4-D and the internal standard. An aliquot of the toluene eluate (1.0 ml) was transferred to a GC vial. An internal instrument performance standard (0.1 ml of dinoseb methyl ether in toluene, 15000 ng/ml) was added to the vial.

The final extract was analyzed using a Hewlett-Packard (HP) 6890 gas chromatograph with a ^{63}Ni electron capture detector and an HP-5 fused silica capillary column (60 m × 0.325 mm i.d., 1.05 μm film thickness). The carrier gas was hydrogen at 2.0 ml/min. The injection volume was 2 μl and the injection and detector temperatures were 300°C and 325°C, respectively. The initial temperature was held at 80°C for 1.5 min, then ramped at 20°C/min to 220°C where it was held for 41.8 min, then ramped at 70°C/min to 325°C, where it was held for 15 min. Eight calibration standards (nominal range 15–200 μg/l) and two laboratory QC samples (30 and 200 μg/l) were made by fortifying blank pooled urine with 2,4-D standard. A water blank was also run with each batch. If a field sample did not have 6 ml, then the available sample volume was diluted to 6 ml with water and the dilution factor used in the final calculations. If the 2,4-D concentration exceeded the highest standard, the sample was diluted and rerun. Sample concentrations of 2,4-D were determined from the regression parameters of a linear curve of the calibration standards. The method LOD was 86 nmol/l (19 μg/l).

A GC method with mass selective detection (MSD) was used to determine atrazine, deethylatrazine, deisopropylatrazine, simazine, propazine and cyanazine (NIOSH, 1994). Urine (5 ml) was added to a test tube containing sodium chloride (0.7 g) and sodium bicarbonate (0.5 g), and then mixed thoroughly. The urine was extracted consecutively with ethyl ether and ethyl acetate (5 ml each). Samples were mixed for 15 min after solvent addition and centrifuged for 5 min at 3000 r.p.m. The organic layers were removed, combined and transferred to an anhydrous sodium sulfate filtration tube. The filtrate was evaporated to dryness under a flow of nitrogen. An internal standard (IS), phenanthrene-d_{10} (10 μl of a 100 μg/ml solution in methanol), and ethyl acetate (90 μl) were added to the extract. Salts and solvents were from Fisher Scientific (Pittsburgh, PA) while the IS and parent triazines were from ChemService (West Chester, PA).

The extract was analyzed using an HP5971A GC/MSD (Agilent, Santa Clara, CA) in the selected ion monitoring mode. An SPB-5 (Supelco, Bellefonte, PA) fused silica capillary column (30 m, 0.20 mm i.d., 0.20 μm film thickness), with a 1.5 m guard column of the same material was used. Helium was used as the carrier gas (1.5 ml/min). The injection volume was 1 μl, the injector and detector temperatures were 280°C and 285°C, respectively. The initial temperature (50°C) was held for 1 min, then ramped at 50°C/min to 160°C, ramped at 3.5°C/min to 230°C, and finally ramped at 50°C/min to 280°C where the temperature was held for 2 min. Ions at *m/z* 172 (deethylatrazine) and *m/z* 158 (deisopropylatrazine) as well as ions for all the parent triazines were monitored. Seven calibration standards (nominal range 9–4200 nmol/l) were prepared in ethyl acetate. Two laboratory QC samples (deethylatrazine: 78 and 253 nmol/l; deisopropylatrazine: 86 and 282 nmol/l) were made by fortifying blank pooled urine with the appropriate standards. A water blank was also run with each batch. If a field sample did not have 5 ml, then the available sample volume was used and noted in the final calculations. Sample concentrations of the six analytes were determined from the regression parameters of a linear curve of the calibration standards. The method LODs for deethylatrazine and deisopropylatrazine were 27 and 49 nmol/l, respectively. The method LODs for simazine, atrazine, propazine and cyanazine were 20, 28, 24 and 47 nmol/l, respectively.

Creatinine analysis

Creatinine in urine was determined using a BM/Hitachi 717 Analyzer (Boehringer Mannheim, Indianapolis, IN) and a reagent based on the Jaffé method. The method LOD was 30 mg/l. Creatinine was used to evaluate completeness of urine collection.

Statistical analysis

Arithmetic and/or geometric means and standard deviations were computed for individual measurements of urinary creatinine, volume and herbicide biomarkers. The LOD divided by two was imputed for samples below the detection limit, except where the percentage of samples below the detection limit was >50%, in which case only the range of detectable values was reported (Hornung and Reed, 1990). For 2,4-D, the laboratory also provided estimated values for samples below the LOD when an instrumental response was present at the retention time of 2,4-D. Therefore, 2,4-D data could be examined as being censored at either the LOD or the minimum instrumental response. In the latter case, the lowest instrumental response divided by two was imputed for

samples below the minimum instrumental response for 2,4-D. Use of estimates below the LOD is considered appropriate if the intended use of the data is for statistical analysis (Analytical Methods Commitee, Royal Society of Chemistry, 1987). For comparison purposes, statistical estimates for 2,4-D are reported separately for these two censoring points. All dependent variables were highly skewed to the right and a natural log transformation was applied.

Mixed-effects linear regression modeling was used to examine the influence of spraying on the amount of herbicide biomarker excreted in 24 h in the applicator population. This 24 h total was computed by summing the number of nanomoles of biomarker in each urine sample collected after the first morning urine sample on day 0 through to the first-morning urine sample on day 1. This *summed* 24 h biomarker total was used as the dependent variable. For each summed 24 h dependent variable, the number of cases where all four samples were below the LOD was evaluated as an indicator of censoring in the summed dependent variable. Two variations of the dependent variable, computed biomarker concentration in 24 h (nmol/l) and the amount of biomarker excreted in 24 h divided by the applicator's body weight (nmol/kg body wt), were also evaluated in the models.

PROC MIXED in SAS v. 8.12 (SAS Institute Inc., Cary, NC) was used to build linear models. Subject was treated as a random effect. Three covariance structures—compound symmetry, first-order autoregressive and spatial power—were evaluated for each dependent variable to address the correlation of measurements within subjects (Symanski *et al.*, 2001). A spatial power structure was included as measurements were not equally spaced with respect to date. Structures were compared using maximum likelihood estimation in a model consisting of terms for spraying the target herbicide (yes/no) on day 0 and the 2 days immediately before day 0 (day –1 and day –2) and kg of target herbicide sprayed on days 0, –1 and –2. Akaike's Information Criterion was used to compare the model fit.

Plots of the data revealed that levels on non-spray days had a fairly consistent pattern during the spraying season [either gradually increasing (e.g. 2,4-D) or increasing initially then decreasing (e.g. atrazine)]. In order to adjust for these temporal patterns, restricted cubic splines (cubic curves between knots, smooth at the knots, and linear at the ends) were used to examine the effect of time (i.e. number of days since the start of the study) for each dependent variable as described by Harrell (Harrell *et al.*, 1988; Harrell, 2001).

Once the optimal covariance structure and time effect had been determined, a backward selection procedure using restricted maximum likelihood estimation was performed on a model containing the covariates spraying the target herbicide on days 0, –1 and –2, kg of target herbicide sprayed on days 0, –1 and –2, acres sprayed with target herbicide on days 0, –1 and –2, and duration (min) of spraying target herbicide on day 0 only (duration data were not available for days –1 and –2). These covariates were examined together because they were possibly related predictors of biomarker levels. A 0.05 level of significance was used to remove terms from the models. All other covariates and possible confounders were tested one at a time after the best base model had been determined. Continuous covariates included age, years worked as an applicator, years worked for the current company, duration of work day, number of spray jobs during the day (all herbicides combined), number of nozzle changes during the day (all herbicides combined), and number of spray jobs with a cab window open (all herbicides combined). Categorical covariates included certified applicator status (yes/no), smoking tobacco (yes/no), chewing tobacco (yes/no), glove use (yes/no), charcoal filter (yes/no), dust filter (yes/no), air conditioning in cab (yes/no) and boom location (front/back).

Total variance was computed by fitting a model containing subject only (Burstyn *et al.*, 2000). Within- and between-worker variance components were estimated from the random effects portion of the models. In order to obtain within- and between worker variance estimates under a first-order autoregressive or a spatial power structure, both the REPEATED and RANDOM statements in PROC MIXED were used (Littell *et al.*, 2000).

RESULTS

Applicators

All 15 applicators provided a pre-season urine sample. Alachlor mercapturate eq. and metolachlor mercapturate eq. were detected in more than half (60% each) of these samples (Table 2). Atrazine eq. and 2,4-D were detected to a lesser extent (13 and 20%, respectively). Cyanazine eq., deethylatrazine and deisopropylatrazine were not found in pre-season samples.

During the 6 week study season, 15 applicators from seven field stations provided urine samples for 5–7 days each, for a total of 89 applicator-days and 408 urine samples (Table 3). Applicator demographics and herbicide use are reported in Hines *et al.* (2001). On two applicator-days, a day 1 first-morning urine sample was not provided. These two applicator-days were excluded from analyses that involved 24 h totals. For nine of the remaining 87 applicator-days, the 24 h urine volume was <600 ml (range: 353–597 ml), and on one of these applicator-days, 24 h creatinine was <0.5 g/day (0.429 g/day). The normal range for 24 h urine volume and 24 h creatinine in men is

Table 2. Frequency and concentrations of herbicide biomarkers at or above the limit of detection in applicator pre-season and reference population urine samples

Sample type/analyte	*n*	Frequency (no. samples ≥ LOD[a])	%	Concentration of samples ≥ LOD (nmol/l)[b]
Applicator pre-season				
Alachlor mercapturate eq.	15	9	60	5.0, 5.2, 5.5, 5.9 ,6.2, 7.1, 8.0, 8.2, 23
Atrazine eq.	15	2	13	12, 19
Deethylatrazine	15	0	0	NA
Deisopropylatrazine	15	0	0	NA
Cyanazine eq.	15	0	0	NA
2,4-D	15	3	20	86, 110, 150
Metolachlor mercapturate eq.	15	9	60	5.1, 5.5, 6.3, 8.0, 12, 19, 21, 22, 24
Reference population				
Alachlor mercapturate eq.	46	1	2.2	17
Atrazine eq.	46	1	2.2	5.0
Deethylatrazine	46	1	2.2	32
Deisopropylatrazine	46	0	0	NA
Cyanazine eq.	46	2	4.4	1.8, 5.1
2,4-D	46	5	10.9	86, 86, 90, 150, 170
Metolachlor mercapturate eq.	46	1	2.2	5.0

[a]The LOD was the mass of analyte that gave a mean signal three standard deviations above the mean blank signal and was based on low-level calibration standards that covered the range from less than the expected LOD to no greater than 10 times the expected LOD. For biomarkers determined by immunoassay, the LDD or least detectable dose was used as the LOD. The LODs (or LDDs) were in nmol/l: alachlor mercapturate eq., 1.3; atrazine eq., 2.0; deethylatrazine, 27; deisopropylatrazine, 49; cyanazine eq., 1.7; 2,4-D, 86 ; metolachlor mercapturate eq., 4.9.
[b]Molecular weights: alachlor mercapturate, 396.5; atrazine, 215.68; deethylatrazine, 187.6; deisopropylatrazine, 173.6; cyanazine, 240.69; 2,4-D, 221.04; metolachlor mercapturate, 410.53.
Note: LOD = limit of detection. NA = not applicable.

600–2500 ml and 0.5–3.5 g/day, respectively (Que Hee, 1993). Since we could not be certain that lower than normal 24 h urine volume or creatinine actually indicated incomplete urine collection, these data were left in the analysis. Insufficient hydration may explain low urinary volumes.

Biomarker results were reported on all 408 urine samples, except for 2,4-D and the dealkylated atrazine metabolites where the number of samples with results was 406 and 405, respectively, due to insufficient sample volumes (Table 4). For cyanazine eq., deethylatrazine and deisopropylatrazine, where >50% of the samples were below the LOD, only the range of concentrations above the LOD is reported (Table 4). Parent atrazine was found in only two of 405 (0.5%) spray season samples at levels of 53 and 29 nmol/l. Parent cyanazine, simazine and propazine were not detected in any urine samples. Total biomarker excreted in 24 h adjusted and unadjusted for body weight, biomarker concentration in the composite 24 h urine sample and biomarker excretion rates by collection time period were also computed for alachlor mercapturate eq., atrazine eq., 2,4-D and metolachlor mercapturate eq. (Tables 5 and 6). Excretion rates peaked in the afternoon and were lowest in the morning and overnight samples. The sample size was not sufficient to determine the effect of spraying on excretion rates by time period.

Exposure modeling

Regression model parameters for atrazine eq. and 2,4-D are given in Tables 7 and 8. Two models are presented for atrazine eq.: model A, which includes kg of atrazine sprayed (all other terms dropped out), and model B, in which kg of atrazine sprayed was excluded *a priori*. The latter model was constructed because information on the amount of herbicide active ingredient sprayed may not be available to the investigator. A compound symmetric covariance structure was used for atrazine eq. and a spatial power covariance structure for 2,4-D. Time (i.e. days since the study started) was significant in the atrazine eq. model and best modeled as a three-knot restricted cubic spline function. The percentage of applicator-days where all samples comprising the summed 24 h biomarker total were below the LOD for atrazine eq. and 2,4-D was 10 and 19, respectively. For 2,4-D, the percentage of applicator-days where all samples in the 24 h total were below the minimum instrumental response was 3.

In both atrazine eq. models, either kg of atrazine sprayed or spraying atrazine (yes/no) on day –1 had a greater impact on the amount of atrazine eq. excreted

Table 3. Summary statistics for volume and creatinine concentrations of applicator spray season urine samples by collection time period

Parameter/time period[a]	*n*	AM	SD	Range
Urine volume (ml)				
All samples	408	308	172	20–1286
Wake-up, day 0	85	275	128	20–522
Morning	73	324	224	42–1286
Afternoon	87	307	155	70–1010
Evening	76	317	218	28–978
Overnight, including wake-up, day 1	87	322	121	23–493
24 h volume (ml/24 h)	87	1165	505	353–2546
Creatinine (g/l)				
All samples	408	1.44	0.697	0.1–4.69
Wake-up, day 0	85	1.61	0.544	0.43–3.41
Morning	73	1.15	0.743	0.1–3.03
Afternoon	87	1.57	0.839	0.29–4.69
Evening	76	1.41	0.720	0.1–3.4
Overnight, including wake-up, day 1	87	1.39	0.520	0.37–2.78
24 h creatinine (g/24 h)	87	1.37	0.467	0.317–2.59

[a]Time periods: Morning = after day 0 morning urine void to 12 p.m.; afternoon = 12 p.m. to 6 p.m.; evening = 6 p.m. to12 a.m.; overnight = 12 a.m. to day 1 first morning urine void.
Note: AM = arithmetic mean, SD = standard deviation.

Table 4. Concentration of herbicide biomarkers in individual urine samples corrected and uncorrected for creatinine

Analyte	n	% < LOD[a]	AM	SD	GM	GSD	Range
Individual samples (nmol/l[b])							
Alachlor mercapturate eq.	408	17	83	220	14	7.1	<1.3–2000
Atrazine eq.	408	25	58	140	14	6.4	<2.0–1100
2,4-D[c]	406	24[d]	220	330	58	7.7	<2.5–2600
Metolachlor mercapturate eq.	408	26	49	94	18	4.5	<4.9–1300
Individual samples (nmol/g creatinine)							
Alachlor mercapturate eq.	408	17	47	110	11	5.5	0.34–1400
Atrazine eq.	408	25	34	66	11	5.2	0.33–540
2,4-D[c]	406	24[d]	160	240	47	7.4	0.71–2000
Metolachlor mercapturate eq.	408	26	31	44	15	3.7	0.81–500

[a]Refers to herbicide biomarkers. All creatinine concentrations were above the creatinine LOD.
[b]Summary statistics were not computed for cyanazine, deethylatrazine and deisopropylatrazine as 54, 59 and 92% of the data were below the LOD, respectively. The range of concentrations for cyanazine, deethylatrazine and deisopropylatrazine were <1.7–160 (n = 408), <27–230 (n = 405) and <49–430 (n = 405) nmol/l, respectively.
[c]2,4-D data included estimates for some samples below the LOD. The lowest estimated value for 2,4-D was 5 nmol/l. 5nmol/l was used as the censoring point to compute 2,4-D summary statistics; samples <5 nmol/l were assigned one-half this value. By comparison, the percentage of samples below the LOD for 2,4-D was 45.1%. If the LOD was used as the censoring point and one-half the LOD imputed for samples below the LOD, the AM, SD, GM and GSD for 2,4-D uncorrected for creatinine would be 230, 330, 120 nmol/l, and 3.0, respectively. The AM, SD, GM, and GSD for 2,4-D corrected for creatinine would be 170, 240, 96 nmol/g, and 2.9, respectively.
[d]Percentage of samples <5 nmol/l (minimum value estimated for 2,4-D).
Note: AM = arithmetic mean. SD = standard deviation. GM = geometric mean. GSD = geometric standard deviation.

in 24 h compared with day 0 (factors of 2.32 versus 1.75 and 2.86 versus 1.95, respectively). Day –2 was not significant in either model A or B. Spraying 2,4-D one and two days before urine collection was highly predictive of the amount of 2,4-D excreted in the 24 h urine sample (factors of 4.25 and 3.90,

Table 5. Estimated amount of herbicide biomarker excreted in 24-h adjusted and unadjusted for body weight, and estimated herbicide biomarker concentration in 24-h composite sample

Analyte	*n*	AM	SD	GM	GSD	Range
Amount excreted in 24 h (nmol/24 h)						
Alachlor mercapturate eq.	87	63	130	17	4.6	0.85–600
Atrazine eq.	87	42	65	19	3.9	1.0–360
2,4-D[a]	86	220	330	110	3.7	2.4–2400
Metolachlor mercapturate eq.	87	38	43	22	3.0	2.5–240
Amount excreted in 24 h adjusted for body weight (nmol/kg body wt/24 h)						
Alachlor mercapturate eq.	87	0.68	1.3	0.20	4.6	0.008–6.5
Atrazine eq.	87	0.52	0.89	0.22	4.0	0.01–5.0
2,4-D[a]	86	2.6	3.8	1.2	3.8	0.026–27
Metolachlor mercapturate eq.	87	0.46	0.53	0.26	3.1	0.025–3.1
Concentration in 24 h sample[b] (nmol/l)						
Alachlor mercapturate eq.	87	67	140	16	5.5	0.65–620
Atrazine eq.	87	53	110	18	4.7	1.0–690
2,4-D[a]	86	210	280	100	3.7	2.5–1400
Metolachlor mercapturate eq.	87	43	62	21	3.4	2.4–390

[a]2,4-D data included estimates for some samples below the computed LOD. The lowest estimated value for 2,4-D was 5 nmol/l. 5 nmol/l was used as the censoring point to compute 2,4-D summary statistics; samples below 5 nmol/l were assigned one-half this value. If the LOD was used as the censoring point and one-half the LOD imputed for samples below the LOD, the AM, SD, GM and GSD for total 2,4-D excreted in 24 h uncorrected for body weight would be 240, 330, 140 nmol and 2.6, respectively. The AM, SD, GM and GSD for total 2,4-D excreted in 24 h corrected for body weight would be 2.8, 3.7, 1.6 nmol/kg and 2.7, respectively. The AM, SD, GM and GSD for he estimated 2,4-D concentration in the 24 h composite urine would be 220, 270, 130 nmol/l and 2.6, respectively.

[b]Computed by summing for each applicator-day, the number of nanomoles in each collection period of the 24-h sample; then dividing total nmol by the 24 h urine volume in liters.

Note: AM = arithmetic mean. SD = standard deviation. GM = geometric mean. GSD = geometric standard deviation.

respectively), while spraying 2,4-D on day 0 was not significant. The kg of 2,4-D sprayed on any of the 3 days did not remain in the final model. Spraying the target herbicide 3 days before urine collection (day –3) and all other covariates were not significant ($P > 0.05$) in any of the models. Using computed biomarker concentration in 24 h or the amount of biomarker excreted in 24 h adjusted for body weight as dependent variables had negligible effect on the atrazine eq. and 2,4-D models.

Variance components were computed (Table 9) for the atrazine eq. and 2,4-D models. The within-worker variance component, expressed as a geometric standard deviation ($_{W}$GSD range: 2.5–2.9) was substantially larger than the between-worker component ($_{B}$GSD range: 1.3–1.5) for both analytes. The within-worker component explained 47–62% of the total variance. Fixed effects explained 28–49% of the total variance.

Alachlor mercapturate eq. and metolachlor mercapturate eq. were not modeled because the number of spray days on day 0, day –1 and day –2 were not sufficient (range 1–2 days for alachlor mercapturate eq.; 6–9 days for metolachlor mercapturate eq.). In addition, the metolachlor mercapturate immunoassay moderately cross-reacted with acetochlor mercapturate (Table 1). Acetochlor was commonly substituted for alachlor during the study. The atrazine antibody also cross-reacted moderately with simazine (Table 1); however, simazine was not detected in any urine samples.

Reference population

Forty-six men were recruited as a reference population. Their median age (40 yr; range 19–63 yr) compared favorably with the applicator population (median age 40 yr; range 23–58 yr). The frequency of detection of the seven herbicide biomarkers in the reference population was low (range 0% for deisopropylatrazine to 10.9% for 2,4-D) (Table 2).

Quality control

Mean recoveries in laboratory QC samples ranged from 77 to 122%, while mean percent relative standard deviations for laboratory QC samples and for field duplicates ranged from 10 to 27 and from 12 to 24, respectively (Table 10). Field samples have not been corrected for recovery.

DISCUSSION

Custom applicators in this study were exposed, on average, to 13 (± 2.8, range 9–17) different herbi-

Table 6. Estimated geometric means and standard deviations for herbicide biomarker excretion rates (nmol/h) by collection time period

Analyte/time period[a]	*n*	% < LOD[b]	GM (nmol/h)	GSD
Alachlor mercapturate eq.				
Morning	69	32	0.44	5.6
Afternoon	87	9.2	0.86	5.1
Evening	76	18	0.70	6.0
Overnight	87	18	0.40	6.7
Atrazine eq.				
Morning	69	43	0.40	4.9
Afternoon	87	17	0.94	5.3
Evening	76	24	0.70	5.0
Overnight	87	22	0.48	5.2
2,4-D				
Morning	69	26	2.5	7.4
Afternoon	87	23	3.3	7.8
Evening	75	23	2.8	7.5
Overnight	87	16	2.6	7.3
Metolachlor mercapturate eq.				
Morning	69	43	0.61	3.7
Afternoon	87	20	1.1	3.8
Evening	76	24	0.80	3.9
Overnight	87	23	0.64	4.2

[a]Collection time periods: morning = after day 0 first morning urine void to 12 p.m.; afternoon = 12 p.m. to 6 p.m.; evening = 6 p.m. to12 a.m.; overnight = 12 a.m. to day 1 first morning urine void.
[b]2,4-D data included estimates for some samples below the LOD. The lowest estimated value for 2,4-D was 5 nmol/l. 5nmol/l was used as the censoring point to compute 2,4-D summary statistics; samples < 5 nmol/l were assigned one-half this value. If the LOD was used as the censoring point and one-half the LOD imputed for samples below the LOD, the percentage of samples below the LOD for 2,4-D for morning, afternoon, evening and overnight were 59, 38, 43, and 39, respectively, while the estimated GMs (GSDs) by time period would be (i) morning: not estimated (censoring >50%); (ii) afternoon: 6.2 nmol/h (3.1); (iii) evening: 5.5 nmol/h (3.1); (iv) overnight: 4.3 nmol/h (3.6).
Note: GM = geometric mean, GSD = geometric standard deviation, AM = arithmetic mean, SD=standard deviation.

cides. The group was exposed to >30 different herbicides. All monitored biomarkers were detected to varying degrees of frequency and intensity in the urine of participating applicators. In pre-season samples, alachlor mercapturate eq. and metolachlor mercapturate eq. were detected at substantially higher frequencies (60% each) than other biomarkers (range 0–20%). The concentration range for these two biomarkers was low compared with samples collected during the spray season. Because applicators had not sprayed the target herbicides before the pre-season samples were collected, the high frequency of detection suggests applicator exposure to unidentified herbicide sources prior to sample collection.

During the spray season, 2,4-D was found in applicator urine samples at GM levels 3–4 times higher than the other biomarkers (Table 4). Surprisingly, alachlor mercapturate eq. was detected in nearly 83% of the spray season urine samples and at GM levels comparable to those found for metabolites of two heavily used herbicides, atrazine and metolachlor, even though most farmers switched from alachlor to acetochlor during the study year. Alachlor was sprayed on only two of the 87 applicator-days. Since alachlor mercapturate levels were generally higher in the spray season than in the pre-season, and very little alachlor spraying was done, this increase might be due to alachlor residues on equipment from previous seasons or to antibody cross-reactivity with an unidentified compound. The high percentage of hand wash samples with detectable levels of alachlor (~70%) suggests the former may be the case (Hines *et al.*, 2001). The presence of alachlor mercapturate in a subset of the urine samples has been confirmed by GC/MS/MS (unpublished data) The excretion rate peak seen in the afternoon for all analytes, may reflect that samples came from an occupationally exposed population where exposure typically starts early in the morning and ends late in the day.

Atrazine eq. measured by ELISA was detected more frequently in spray season samples than either of the two mono-dealkylated atrazine metabolites measured by GC. These findings are consistent with literature reports that atrazine mercapturate, which cross-reacts with the atrazine ELISA, is the major human metabolite of atrazine. Forty-six percent of the samples tested with the cyanazine immunoassay gave a response above the assay's LOD. Since parent cyanazine was not found in any sample analyzed by GC, we hypothesize that a putative cyanazine metabolite or congener was possibly present in the urine which reacted with the cyanazine antibody.

The monitored biomarkers were found infrequently in the urine of the reference population and, when detected, levels were low compared with exposed applicators. 2,4-D was found most often in the reference population (10.9%), even though these participants did not report application of 2,4-D to their property, either by themselves or by others. 2,4-D is a widely used herbicide and participants were possibly exposed to 2,4-D at or away from home without their knowledge. Hill *et al.* (1995) reported a similar frequency of 2,4-D detection (12%) in a much larger set of 983 urine samples collected from adults participating in the National Health and Nutrition Examination Survey III (NHANES III). MacIntosh *et al.* (1999) found atrazine mercapturate in only 0.3% of 348 urine samples

Table 7. Herbicide urinary biomarkers: regression model parameters

Model	*n*	β	SE	*P*-value[a]	Factor[b]
Atrazine					
Model A: with kg atrazine sprayed					
ln(atrazine eq., nmoles/24 h)[c]					
Intercept	87	1.177	0.283	<0.001	3.24
kg of atrazine sprayed, day 0[d]		0.00560	0.00163	0.001	1.75[e]
kg of atrazine sprayed, day –1		0.00841	0.00298	0.006	2.32[e]
Time (days since start of study)		0.115	0.0261	<0.001	NA
Time (spline function), $T_{32}(t)$[f]		–0.00011	0.00004	0.009	NA
Model B: without kg atrazine sprayed					
ln(atrazine eq., nmol/24 h)[c]					
Intercept	87	1.142	0.286	<0.001	3.13
Spray atrazine, day 0		0.666	0.272	0.016	1.95
Spray atrazine, day –1		1.050	0.328	0.002	2.86
Time (days since start of study), *t*		0.116	0.0268	<0.001	NA
Time (spline function), $T_{32}(t)$[f]		–0.00011	0.00004	0.006	NA
2,4-D					
ln(2,4-D, nmol/24 h)[c,g]					
Intercept	86[h]	4.319	0.179	<0.001	75.1
Spray 2,4-D EH, day –1		1.446	0.334	<0.001	4.25
Spray 2,4-D EH, day –2		1.361	0.398	<0.001	3.90

[a]*P*-values < 0.05 were retained in the models.
[b]exp(β). Factor by which levels are increased or decreased from baseline condition, $\exp(\beta_{intercept})$.
[c]Covariance error structure: compound symmetry for atrazine eq. and power for 2,4-D. Restricted maximum likelihood estimation used in all models.
[d]Day 0 = start of 24 h urine collection; day –1 = the first day before day 0; day –2 = the second day before day 0.
[e]Per 100 kg of atrazine sprayed.
[f]Three-knot restricted cubic spline function applied. $T_{32}(t) = (x-6)_+^3 - 1.933(x-20)_+^3 + 0.933(x-35)_+^3$, where $(x-c)_+ = x-c$ if $x-c>0$, else $(x-c)_+ = 0$.
[g]2,4-D data included estimates for some samples below the LOD. The lowest estimated value for 2,4-D was 5 nmol/l. 5 nmol/l was used as the censoring point for the 2,4-D model; samples <5 nmol/l were assigned one-half this value. Table 8 shows the 2,4-D model if the LOD was used as the censoring point and one-half the LOD imputed for samples below the LOD.
[h]Urine quantity not sufficient to determine 2,4-D in one sample on one applicator-day.
Note: β = regression coefficient. SE = standard error. NA = not applicable.

collected from 80 individuals living in five Maryland counties. This percentage is slightly less than the 2.2% reported here for atrazine eq., where the major metabolite measured was most likely atrazine mercapturate. General population reference data are not available for the other biomarkers monitored. Other published applicator studies have measured exposure only on spray days, whereas our study included both spray and non-spray days.

Regression modeling was used to determine significant predictors of the amount of atrazine eq. and 2,4-D excreted in the 24-h sample. For both analytes, spraying information on days *prior* to urine collection (day –1 for atrazine eq., and days –1 and –2 for 2,4-D) was significant (Table 7). Unlike atrazine, spraying 2,-4 D (yes/no) on day 0 was not an important predictor of total 2,4-D excreted in the 24 h sample (Table 7). Also unlike atrazine, the amount of 2,4-D sprayed in kg was less significant than whether 2,4-D was sprayed or not and did not remain in the final model. The reason for the difference in the importance of the kg covariate between the atrazine eq. and 2,4-D models is not readily apparent. The application method was the same for both herbicides; however, the range of 2,4-D EH sprayed on days 0, –1 and –2 (3–24, 2–84 and 0.3–87 kg, respectively) was much narrower than for atrazine on the corresponding days (9–464, 5–236 and 10–402 kg, respectively). The 2,4-D findings are consistent with urinary half-life estimates for 2,4-D in the range of 13 to >40 h (Sauerhoff *et al.*, 1977; Nash *et al.*, 1982; Harris and Solomon, 1992). The small sample size and adjustment for more than one day of spraying in the models probably reduced the power to detect other significant covariates. This contrasts with the analysis of hand and dermal exposures for this population where a significant glove effect was found for

Table 8. Herbicide urinary biomarkers: 2,4-D model with one-half the LOD imputed for samples below the LOD

Model	*n*	β	SE	*P*-value[a]	Factor[b]
ln(2,4-D, nmol/24 h)	86				
Intercept		4.368	0.190	<0.001	78.9
Spray 2,4-D EH, day –1		1.127	0.208	<0.001	3.09
Spray 2,4-D EH, day –2		1.100	0.254	<0.001	3.0
Time (days since start of sampling), *t*[a]		0.0155	0.00701	0.037	NA

The LOD was used as the censoring point and one-half the LOD imputed for samples below the LOD. The lowest estimated value for 2,4-D was 5 nmol/l. 5 nmol/l was used as the censoring point for the 2,4-D model; samples <5 nmol/l were assigned one-half this value.
[a]Additional spline terms not significant.

Table 9. Herbicide urinary biomarkers: variance component estimates for regression models

Model[a]	${}_{T}S^2_y$[b]	${}_{W}S^2_y$[c] (%)[d]	${}_{W}GSD$[e]	${}_{B}S^2_y$[f] (%)[d]	${}_{B}GSD$[g]	%Fixed[h]	$\hat{\rho}$[i]
Atrazine eq.							
Model A: with kg	1.84	0.86 (47)	2.5	0.08 (4)	1.3	49	NA
Model B: without kg	1.84	0.91 (49)	2.6	0.06 (3)	1.3	48	NA
2,4-D[j]	1.80	1.11 (62)	2.9	0.18 (10)	1.5	28	0.71

[a]Includes worker, fixed effects and intercept.
[b]Estimated total variance from a model containing only worker as a random effect.
[c]${}_{W}S^2_y$ = Estimated variance of the within-worker distribution.
[d]Percentage of the estimated total variance.
[e]${}_{W}GSD$ = Estimated geometric standard deviation of the within-worker distribution.
[f]${}_{B}S^2_y$ = Estimated variance of the between-worker distribution.
[g]${}_{B}GSD$ = Estimated geometric standard deviation of the between-worker distribution.
[h]% Fixed = Percentage of the variance explained by the fixed effects.
[i]Estimated one-day lag autocorrelation coefficient.
[j]2,4-D data included estimates for some samples below the LOD. The lowest estimated value for 2,4-D was 5 nmol/l. 5 nmol/l was used as the censoring point for the variance component estimates in the above table; samples below 5 nmol/l were assigned one-half this value. If the LOD was used as the censoring point and one-half the LOD imputed for samples below the LOD, the total, within-worker, and between-worker variances would be: total, 0.96; within-worker, 0.48 (50%); between-worker, 0.11 (11%), percentage of the variance explained by fixed effects, 39; and $\hat{\rho}$ = 0.81.
NA = Not applicable.

alachlor, atrazine, 2,4-D EH, and metolachlor (Hines *et al.*, 2001).

The time or number of days into the study when the sample was collected was a significant predictor of the amount of atrazine eq. excreted independent of the amount of target herbicide sprayed (Table 7). Therefore, in order to clearly test the effect of spraying the target herbicides on the amount of biomarker excreted, it was necessary to adjust for time in the atrazine regression models. Our approach was to use the restricted cubic spline function that most closely modeled the observed atrazine time profile and gave the best model fit. Time trends, such as that found here, may be present in other longitudinal exposure data, and should be examined and adjusted for, when necessary, as a part of exposure determinant modeling.

This study also allowed an empirical evaluation of the statistical impact of using observed estimates for 2,4-D below the LOD compared with imputing a constant value. Not surprisingly, use of a single imputed value in regression models reduced total variability (Table 9) and decreased standard errors (Tables 7 and 8). When an imputed value was used (compared with using all observed estimates), reduction in the within-worker variability was more pronounced than in the between-worker variability (Table 9). The effect of using an imputed value versus observed estimates is seen even more clearly in estimates of the geometric mean (GM) and standard deviation (GSD) for 2,4-D levels in individual samples (Table 4) where estimates using a single imputed value resulted in ~2-fold higher GMs and ~2-fold lower GSDs as compared to using observed estimates.

Traditionally, chemists have been largely concerned with the reporting of *individual* measurements rather than the use of data to make statistical inferences and estimates (Lambert *et al.*, 1991; Porter *et al.*, 1988). The Analytical Methods Committee of the Royal Society of Chemistry (1986) noted, however, that 'At low levels of analyte, many observations of concentration will fall below the c_L [detection limit] given an unbiased analytical method. Such observations are not devoid of meaning: they are estimates of true concentrations' and '…the preferred method of reporting such values depends on the *intended use of*

Table 10. Results of laboratory and field quality control urine samples

Analyte	n[a]	Level[b] (nmol/l)	Mean recovery (%)	Mean % RSD[c]
Alachlor mercapturate eq.				
Laboratory	20	2.5	122	12
Field duplicates	77	3.5–2000	NA	17
Atrazine eq.				
Laboratory	15	4.6	115	19
Field duplicates	70	2.8–1000	NA	16
Cyanazine eq.				
Laboratory	9	4.2	121	23
Field duplicates	44	3.7–160	NA	12
Metolachlor mercapturate eq.				
Laboratory—low	22	0.6	97	17
Laboratory—high	22	4.9	91	11
Field duplicates	66	5.9–440	NA	24
2,4-D				
Laboratory—low	42	140	83	23
Laboratory—high	45	900	94	10
Field duplicates	40	90–1800	NA	21
Deethylatrazine				
Laboratory—low	38	78	96	24
Laboratory— high	39	250	77	19
Field duplicates	30	30–180	NA	21
Deisopropylatrazine				
Laboratory—low	38	86	118	27
Laboratory— high	39	280	81	19
Field duplicates	5	52–140	NA	19
Creatinine				
Field duplicates—batch 1	102	0.21–2.82 g/l	NA	3.5
Field duplicates—batch 2	38	0.26–3.43 g/l	NA	1.6

[a]QC samples were run in duplicate. For field duplicates, n is the number of pairs where both values were greater than or equal to the LOD or LDD.
[b]Fortification level for laboratory samples. Measured levels for field duplicates.
[c]The mean %RSD is the average relative standard deviation of the n pairs.
Note: RSD = relative standard deviation. NA = not applicable.

the data [emphasis added]', Moreover, when a value is part of a data set subjected to statistical analysis 'various substitute values ranging from 0 to c_L have been suggested but are not recommended' and 'Generally the observed value should be used.' The use of observed estimates below the LOD, although controversial, preserves information, and in particular, approximates the true variability in the data set better than using substitute values. Clearly, the level of censoring in the data should still be considered before statistical analyses are performed, regardless of the method used for observations below the detection limit; however, when statistical analyses are performed more consideration should be given to using all observed data to ensure more reliable estimates and inferences.

Few studies have reported within- and between-worker variability for pesticide biomarkers in urine. The high within-worker variability observed in this study ($_W$GSD range 2.5–2.9) indicates that repeated measurements on workers in this job are necessary in order to estimate individual biomarker levels more

accurately, if a similar sampling frequency (i.e. ~1–2 times per week) is used. Within-worker variability of dimethylphosphate, a metabolite of azinphosmethyl, was also greater than between-worker variability ($_W$GSD range 2.2–2.6; $_B$GSD range 1.1–1.4) in spot urine samples collected one to several days apart from apples thinners (Simcox *et al.*, 1999). On the other hand, between-worker variability of creatinine-corrected urinary 3,5,6-trichloro-2-pyridinol was greater than within-worker variability ($_W$GSD = 1.4, $_B$GSD = 2.4) when spot urine samples were collected every day for a week from termiticide applicators using chlorpyrifos (Hines and Deddens, 2001). Variability in the environmental exposure, the elimination half-life of a substance, and the frequency of sample collection are all factors that may influence within- and between-worker variability.

Some study limitations should be noted. The concurrent collection of dermal and urine samples, and any incomplete urine collection may have produced underestimates of biomarker levels. Herbicide captured or removed by dermal sampling would not have been available for uptake. In the 2,4-D analysis, samples did not undergo acid hydrolysis and any 2,4-D present as a conjugate would not have been measured. The study year was unusually wet and applicators sprayed on only one-third of the applicator-days compared with two-thirds of the days in a more typical year. Therefore, biomarker levels in this study may not be representative of levels found in years with more spraying.

CONCLUSION

The specific days of herbicide spraying, measured as either a dichotomous variable (yes/no) or as kg of herbicide sprayed, that were associated with an increased amount of atrazine eq. and 2,4-D excreted in the 24-h urine samples, varied for the two biomarkers and included information from one or more days prior to the urine sample collection. This inclusion of day(s) prior to urine collection in the models confirms the importance of collecting covariate information on the day(s) most relevant to the biomarker of interest. In addition, time trends in longitudinal data that may be independent of the effects of interest should be evaluated and adjusted for in statistical models. Larger sample sizes may be needed to evaluate exposure determinants for biological measures of exposure compared with non-biological measures if the biomarker of interest is not excreted within 24 h and exposures on days prior to biomarker collection are significant.

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