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Saliva biomonitoring of atrazine exposure among herbicide applicators

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Abstract A field study was conducted in which saliva samples were collected from a cohort of herbicide applicators during the pre-emergent spray season in Ohio in 1996. Atrazine concentrations were detected in human saliva samples using an enzyme-linked immunosorbent assay (ELISA) method. Trend due to atrazine exposure and subsequent elimination in the body were evidenced by the temporal pattern of decreasing atrazine concentrations in saliva over time. Median salivary concentrations of atrazine on non-spray days were significantly lower than on spray days for each sampling time (Mann-Whitney *U*–Wilcoxon rank sum test, $P < 0.01$). Within spray days, median salivary atrazine concentrations were significantly higher on days atrazine was sprayed than on days herbicides other than atrazine were sprayed for each sampling time (Mann-Whitney *U*–Wilcoxon rank sum test, $P = 0.02$ for 4–6 p.m. samples, $P = 0.04$ for bedtime samples, $P = 0.03$ for next-morning samples). Median salivary atrazine concentrations on days atrazine was sprayed were higher than the median concentration for the corresponding sampling time on non-spray days and on days when other herbicides were sprayed. Salivary concentration of atrazine is a plausible indicator of those days in which atrazine spraying was likely to have occurred. Salivary concentrations of atrazine not only reflect exposures resulting from spraying atrazine, but also exposures from other field activities where applicators may come in contact with atrazine. The results of this study confirmed data from animal experiments that atrazine is able to cross the cell membranes of salivary glands, and can be measured in human saliva with high sensitivity. The sampling method itself is convenient and easy to use in the field, with a high compliance rate, and analytical procedures are rapid and inexpensive. It is, therefore, concluded that saliva sampling of atrazine exposure among herbicide applicators is a feasible biomonitoring method.

Key words Saliva · Biomonitoring · Atrazine · Herbicide · Pesticide exposure · Biological monitoring

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Introduction

Pesticide applicators are exposed to pesticides through inhalation, ingestion and dermal contact. Dermal absorption generally contributes a large portion of the total pesticide dose (Fenske 1993, Woollen 1993). Since human skin varies greatly in its ability to absorb chemicals, it is difficult to quantify the relationship between dermal exposure and absorbed pesticide dose. For this reason, biological monitoring has become the preferred method for determining workers' exposure (Henderson et al. 1993, Woollen 1993). Biological monitoring for pesticide exposures has relied on blood or urine collection, and both methods have demonstrated their value in certain cases. However, other practical issues, such as analytical matrix difficulties, the invasive nature of blood sampling, and the inconvenience of urine sampling, have made the use of these methods problematic in the field (Nigg and Stamper 1989, Chester 1993).

Saliva is a biological fluid that is relatively simple to obtain from workers, and does not require invasive procedures or collection by medical personnel. It also appears that the watery matrix which constitutes a typical saliva sample, is relatively simple to work with analytically, when compared to matrices such as blood or urine. Saliva has been employed quantitatively to monitor a variety of therapeutic drugs (Drobitsch and

Svensson 1992) and hormones (Vining and McGinley 1987) in human clinical studies. Recently, saliva sampling has been explored as a practical method for examining occupational and environmental exposure to chemicals, including pesticides (Jarvis et al. 1991, White et al. 1992, Nigg et al. 1993, Parazzini et al. 1996). Measurement of pesticides in saliva has great potential due to the convenience of sampling and analysis, and the accuracy of saliva concentration as an indicator of tissue availability (Lu et al. 1998). Saliva biomonitoring for pesticide exposure may prove to be useful in studies to evaluate absorption, metabolism and excretion, to establish re-entry periods, and to assess the effectiveness of engineering controls and protective clothing.

Lu et al. (1997a, b) investigated the feasibility of saliva biomonitoring for atrazine exposure in an animal model and found that atrazine concentrations in saliva and in plasma were parallel over time after administering atrazine by gavage or i.v. infusion. This parallel relationship was not affected by saliva flow rate or atrazine dose. In a later study, Lu et al. (1998) demonstrated that, with the consideration of protein binding of atrazine in plasma, salivary concentrations of atrazine reflect the free atrazine (non-protein bound) levels in plasma. These animal studies led us to conduct this field study, which was designed to investigate the feasibility of monitoring applicator atrazine exposure using saliva sampling.

This saliva biomonitoring study was part of a larger herbicide exposure assessment study conducted by the National Institute for Occupational Safety and Health (NIOSH) which involved both environmental and biological sampling of custom herbicide applicators during the pre-emergent spray season. Urine, dermal patch, hand wash, and air samples were also collected, together with chemical use and spray activity data. One of the objectives of the main study was to estimate the distribution of applicator herbicide exposure across the season by collecting samples on both spray and non-spray days. This paper reports only the saliva results.

Methods

Subject recruitment

Applicators were recruited based on the following criteria: (1) full-time employment status, (2) anticipated exposure to target herbicides including atrazine, (3) willingness to participate (informed consent was obtained), (4) employing company located within a four-county area in Ohio. In April 1996, a NIOSH investigator visited the companies, explained the study and recruited the applicators. The NIOSH Human Subjects Review Board has approved the use of human subjects in this project.

Study design

Fifteen applicators participated in the saliva-sampling portion of the study. All participants were male, and ranged in age from early 20s to late 50s. They were sampled at systematic intervals over the course of six consecutive weeks during the spring application season. Each participating applicator was sampled approximately every fourth day, even when no spraying was conducted, since applicators were likely to be exposed to herbicides while performing other work activities. Every sampling day was called an event and three saliva samples were collected at each event. The first saliva sample was taken between 4:00 and 6:00 in the evening. The second, or bedtime sample, was done just before the subject went to bed and the third or next-morning sample, was completed just after the participant woke up the next morning. This sampling schedule was designed to capture the applicator's highest exposure, and subsequent excretion of atrazine by the next morning. Since applicators may work late into the evening, in some cases, the post-shift measurement is best represented by the 4–6 p.m. samples, and in other cases, the bedtime sample most closely represents the post-shift measurement. Each applicator's herbicide spraying activity was recorded for each sampling event. Records included chemicals sprayed, time spraying began and ended, and amount sprayed.

Pre-season samples were taken during the recruiting period, approximately one month before the spraying season started. The pre-season sampling schedule followed the design for season samples except that the first pre-season sample was taken at mid-day instead of during the evening period (4–6 p.m.). Three samples from each applicator were collected, for a total of 45 pre-season samples.

Sample collection

A commercial device, called the Salivette (Sarstadt) was used for collecting saliva samples in this study. It is convenient and easy to use by both the participant and the researcher, and is not readily contaminated. The Salivette is a cotton roll with its own centrifuge tube and cap. The saliva sample is easily confined within the sampling device, which is used for transportation and storage. The Salivette collects approximately 2 ml of saliva in less than 2 min.

Applicators were asked, at each sampling period, to rinse their mouths thoroughly with water twice, and then put the cotton roll into their mouths. They were asked to avoid touching the lip of the plastic tube or the cotton roll with their hands or lips in order to minimize exogenous contamination. The applicators moved the cotton roll around inside their mouths for 1 min attempting to fill it with as much saliva as possible. They were told to hold the saturated cotton roll with their teeth, guide it back into the tube and recap the tube tightly.

Upon collection of saliva, the Salivette was labeled by applicators themselves and stored in a cooler with a blue ice pack. After being returned to the laboratory, the sample was then centrifuged at 3,000 rpm (2,600 *g*) for 5 min. The cotton roll and inner plastic tube were then removed and discarded, leaving the ultrafiltrate remaining in the outer centrifuge tube. The tube was recapped and stored at −20 °C until sample analysis.

Sample analysis

An enzyme-linked immunosorbent assay (ELISA) method (Karu et al. 1991, Lucas et al. 1991) was used to determine concentrations of atrazine in saliva. Ninety-six-well microtiter plates (Nunc-Immuno Maxisorp Plates) were first coated with goat anti-mouse IgG (Boehringer-Mannheim, Indianapolis, Ind., USA) as the trapping antibody, and then with the monoclonal triazine-specific antibody (AM7B2.1). A hapten-enzyme conjugate (SIM-N(C2)-AP), a small molecule similar in structure to atrazine which has an alkaline phosphatase enzyme attached, was added with the saliva sample to each well. If atrazine is present, it will compete with the hapten-enzyme conjugate for binding sites, which results in less yellow color-development with the addition of enzyme substrate. Plates were read in an automated Microplate Reader EL311SX (Bio-Tek Instruments) at 405 nm. Because of the matrix effect observed in the previous study (Lu et al. 1997a), saliva samples were cleaned up in a solid phase extraction method (SPE) using C18 cartridges (Accu-Bond, J&W Scientific) prior to ELISA analysis. This ELISA assay has a limit of detection (LOD) of 0.22 μg/l for atrazine in saliva.

The ELISA assay was validated in a laboratory study in which the Salivette cotton rolls were immersed in a beaker containing blank saliva spiked with 8.64 μg/l of atrazine. Blank saliva samples obtained from laboratory staff showed no atrazine level. Samples were centrifuged and cleaned up as described above. The average recoveries for those samples, analyzed immediately and stored for a month at −20 °C, were 77% ($n = 5$, CV = 3.5%) and 72% ($n = 5$, CV = 14.4%), respectively. Mean recoveries of atrazine for the two time-periods were not significantly different (*t*-test, $P = 0.09$), and therefore data from both experiments were pooled to produce a final recovery efficiency of 75% (CV = 10.2%, $n = 10$). Final atrazine concentrations in the saliva samples were adjusted by the recovery efficiency of 75%.

Data analysis

Median values were lower than mean values in all cases, suggesting a skewed distribution of atrazine concentrations in saliva samples. Non-parametric tests were used rather than the assumption of a log-normal approximation. Statistical analyses were performed using SPSS (SPSS, 6.1.1, Chicago, Ill. USA) with the level of significance set at 0.05.

Results

Atrazine applications

Fifteen applicators participated in a total of 89 seasonal sampling events, and 15 pre-seasonal events. They were unable to spray on many of the days, because of the unusually wet weather during the study period, however, sampling was conducted whether they were able to spray or not. The collection of saliva samples, therefore, was done either on non-spray days, or on spray days where any herbicide was sprayed, but not necessarily atrazine. Table 1 lists the sampling events for each applicator and the amount of atrazine sprayed during each event. Participants were sampled for five to seven events each, and sprayed atrazine in three or fewer events. Five of the 15 applicators did not spray atrazine on any of the sampling days.

Table 1 Amount of atrazine sprayed by worker during each sampling event (kg of active ingredient)

Worker	Sampling event[a]								No. of sampling events[b]
	1	2	3	4	5	6	7	8	
1	+[c]	13	+	*[d]	*	31	+	9	6
2	+	17	+	*	*	15	0[e]	149	6
3	0	+	+	*	*	0	+	0	6
4	+	+	+	*	*	+	*	0	5
5	0	+	+	*	*	+	+	0	6
6	*	*	+	+	32	+	*	+	5
7	+	*	+	0	50	+	*	+	6
8	+	*	+	+	169	36	*	+	6
9	+	*	+	+	275	+	*	*	5
10	+	*	+	+	87	54	*	+	6
11	+	*	+	+	*	+	*	+	5
12	+	*	+	109	0	+	*	+	6
13	+	+	51	464	+	66	+	*	7
14	*	+	104	220	+	15	+	+	7
15	+	+	+	+	0	+	+	*	7

[a] Does not include pre-season samples
[b] The total number of events for which the worker was sampled
[c] Worker was sampled but did not spray any chemicals in the field
[d] Worker was not sampled
[e] Worker sprayed herbicides other than atrazine

Pre-season samples

Descriptive statistics of the pre-season saliva samples are shown in Table 2. All 45 of the pre-season samples were completed, and had measurable atrazine concentration, except for one. Median salivary concentrations of atrazine in mid-day, bedtime and next-morning pre-season samples did not clearly suggest a decreasing trend across sampling time. None of the pre-season saliva samples contained atrazine concentrations exceeding 10 μg/l, therefore, this level was considered to be a cut-off concentration for pre-seasonal exposure.

Season samples

Descriptive statistics of saliva samples collected during the spraying season are also shown in Table 2. During the season, nine samples (one 4–6 p.m., six bedtime and three next-morning) were not completed because of non-compliance. One 4–6 p.m. sample, which had an extremely high atrazine level (> 800 μg/l) was determined to be an outlier (box plot and extreme studentized deviate methods) and was not included in the statistical analysis. This 4–6 p.m. sample was collected on a non-spray day, and the following samples at bedtime and the next-morning had atrazine concentrations of 5.7 and 4.0 μg/l. It is likely that such a high concentration was due to exogenous contamination.

The box plots in Fig. 1 show the distribution of salivary concentrations of atrazine for samples collected on non-spray days and spray days across sampling times. The spray day samples are further separated into two groups: spray with atrazine and spray without atrazine. Each type of sample (non-spray days, spray days, spray with atrazine, and spray without atrazine) exhibited a distinct temporal pattern of salivary concentration of atrazine across three sampling times, with medians decreasing from 4–6 p.m., to bedtime, to next-morning samples, except for non-spray days. Median salivary concentrations of atrazine on non-spray days were significantly lower than on spray days for each sampling time (Mann-Whitney *U*–Wilcoxon rank sum test, $P < 0.01$). Within spray days, median salivary atrazine concentrations were significantly higher on days atrazine was sprayed than on days herbicides other than atrazine were sprayed, for each sampling time (Mann-Whitney *U*–Wilcoxon rank sum test, $P = 0.02$ for 4–6 p.m. samples, $P = 0.04$ for bedtime samples, $P = 0.03$ for next-morning samples). Median salivary atrazine concentrations were not significantly different for each

Table 2 Descriptive statistics of salivary concentrations of atrazine by sampling times (μg/l)

	N	Mean	Median	Standard deviation	Min.	Max.
Pre-season samples						
Mid-day	15	2.4	1.5	2.3	0.5	8.3
Bedtime	15	1.3	0.9	1.1	< LOD[g]	4.4
Morning	15	1.6	0.8	2.4	0.3	10.0
Non-spray days						
4–6 p.m.	58	10.4	3.9[a]	17.2	0.6	86.9
Bedtime	56	1.9	1.0[b]	2.9	< LOD[g]	19.9
Morning	57	1.8	1.2[c]	1.9	< LOD[g]	9.8
Spray days						
4–6 p.m.	30	27.6	16.0[a]	33.6	0.6	148.7
Bedtime	27	10.5	4.3[b]	11.8	0.9	39.7
Morning	29	4.3	2.1[c]	4.8	0.5	19.1
Spray without atrazine						
4–6 p.m.	10	7.7	3.1[d]	9.0	0.6	25.6
Bedtime	9	7.3	2.0[e]	12.5	0.9	39.7
Morning	10	2.2	1.1[f]	2.5	0.5	8.0
Spray with atrazine						
4–6 p.m.	20	37.6	21.5[d]	37.0	6.7	148.7
Bedtime	18	12.1	6.9[e]	11.4	1.2	37.1
Morning	19	5.4	3.1[f]	5.3	1.0	19.1

[a] Significantly different (Mann-Whitney *U*–Wilcoxon rank sum test, $P < 0.01$)
[b] Significantly different (Mann-Whitney *U*–Wilcoxon rank sum test, $P < 0.01$)
[c] Significantly different (Mann-Whitney *U*–Wilcoxon rank sum test, $P < 0.01$)
[d] Significantly different (Mann-Whitney *U*–Wilcoxon rank sum test, $P = 0.02$)
[e] Significantly different (Mann-Whitney *U*–Wilcoxon rank sum test, $P = 0.04$)
[f] Significantly different (Mann-Whitney *U*–Wilcoxon rank sum test, $P = 0.03$)
[g] Limit of detection (LOD) = 0.29 μg/l, adjusted by the recovery of 75%. Values below LOD were determined from extension of four-parameter standard curve below the lowest calibration concentration

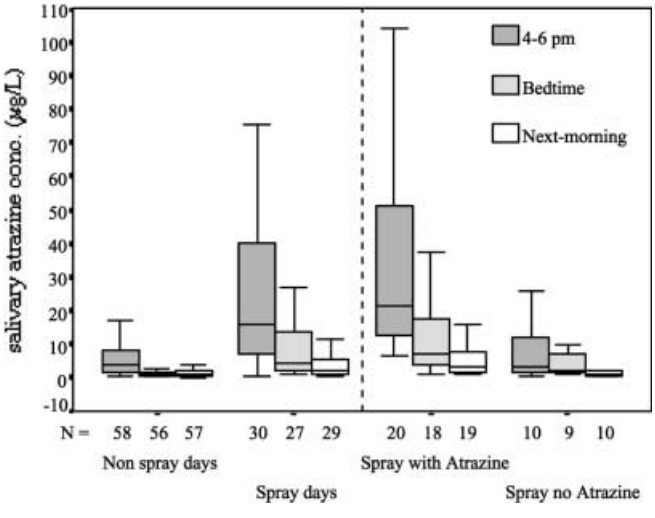

Fig. 1 Box plots of salivary concentrations of atrazine for non-spray days, spray days, spray days with atrazine and spray days without atrazine samples. From the bottom to the top, the *lines* in the figure represent 10th, 25th, 50th, 75th, and 90th percentiles, respectively. Sample size (*n*) for each box plot is provided on the *x*-axis

sampling time between non-spray days and days that herbicides other than atrazine were sprayed.

Table 3 shows the distribution of salivary concentrations of atrazine in 4–6 p.m. season samples for atrazine-sprayed vs. no atrazine-sprayed events, categorized according to the 10 μg/l cut-off concentration derived from the pre-season sampling. The no atrazine-sprayed events include 58 non-spray days, and 10 spray days where herbicides other than atrazine were used. For 17 of 20 events in which atrazine were sprayed (85%), salivary concentrations of atrazine were greater than 10 μg/l. For 54 of the 68 events in which no atrazine was sprayed or herbicides other than atrazine were used (79%), salivary concentrations of atrazine were less than or equal to 10 μg/l. The hypothesis of independence between atrazine concentration in saliva using the cut-off pre-season concentration of 10 μg/l and spraying atrazine during an event was rejected by a Chi-square test (Pearson, $P < 0.001$), indicating that salivary a concentration of atrazine greater than 10 μg/l is a good indicator of atrazine spraying.

Table 3 Distribution of salivary atrazine concentrations of 4–6 p.m. samples for atrazine sprayed vs. no atrazine sprayed events [significantly different (chi-square Pearson test, $P < 0.001$)]

Sample concentration	Number of seasonal events	
	Atrazine sprayed	No atrazine sprayed[a]
> 10 μg/l	17 (85%)	14 (21%)
< 10 μg/l	3 (15%)	54[b] (79%)
Totals	20	68

[a] Includes non-spray day samples ($n = 58$) and spray day samples where herbicides other than atrazine were sprayed ($n = 10$)
[b] One outlying data point was excluded

Discussion

Before evaluating the findings, it is important to note several study limitations. First, the ELISA used in this study was developed primarily for routine analysis of atrazine in groundwater samples, and can cross-react with other triazines, such as simazine and cyanazine (Karu et al. 1991, Lucas et al. 1991). Even though atrazine was reported as the chemical measured in the saliva samples, it is possible that the applicators were exposed to simazine or cyanazine in the same event. Further analysis of the entire NIOSH data set should include comparisons similar to those performed in this study on the days in which other triazines were sprayed. Second, in the application of saliva biomonitoring, oral contamination may be a potential confounding factor (Nigg and Stamper 1989). The mouth-rinsing procedure prior to saliva sample collection was incorporated in the sampling protocol to minimize contamination. However, the effectiveness of this procedure is unknown. A controlled laboratory study is needed to determine the efficacy of mouth-rinsing decontamination. Finally, blood samples were not taken from the applicators. Therefore, the concentration ratio of atrazine in saliva and plasma, demonstrated in animal studies (Lu et al. 1997a, b), could not be validated in humans.

Despite these limitations, this study has several valuable findings. Even though saliva sampling was relatively novel to the applicators, they acclimated well to this technique, as demonstrated by the very high sampling compliance rate of 97%. The Salivette seems to be a convenient sampling device for use in the field. It is quick, easy to use, and is not easily contaminated. Some applicators, however, did not like the taste or the texture of the cotton roll. Other saliva sampling devices are available, such as the SalivaSac (BioQuant) which is a semi-permeable bag about 20 mm in diameter, which contains 2 g of sucrose. Through osmosis, the SalivaSac collects molecules smaller than 12,000 Da. The SalivaSac was not chosen for this study because it takes approximately 8 to 15 min to fill the 2 ml membrane sac, while it takes only 1 min to fill the Salivette.

Atrazine was detected in all pre-season samples even though they were collected approximately one month prior to the beginning of the spray season. Several explanations for these pre-season levels of atrazine are possible. First, atrazine measured in the pre-season samples could represent background exposure during the non-spray season. Objects and surfaces in work areas may be contaminated with atrazine. Second, since atrazine can be retained in the fatty tissue due to its lipid solubility, pre-season levels may reflect elimination of stored atrazine. And third, during the time period in which the pre-season samples were collected, applicators were likely to have been preparing for the spray season, and may have been exposed to atrazine while working on spray rigs and other equipment.

Saliva sampling can capture the trends of atrazine exposure and subsequent elimination in the body, as evidenced by the temporal pattern of decreasing atrazine concentrations in 4–6 p.m., bedtime, and next-morning samples. However, 12 bedtime samples (six from spray days and six from non-spray days) had higher salivary atrazine levels than the corresponding 4–6 p.m. samples. Of these 12 bedtime samples, only three collected on spray days showed salivary concentrations of atrazine greater than 10 μg/l. On one of these three spray days, worker continued to spray atrazine after giving the 4–6 p.m. samples. Therefore, the highest atrazine level in this event was expected to be in the bedtime samples. On the other two spray days, one applicator finished spraying atrazine less than 2 h before the 4–6 p.m. sample, and the other applicator sprayed herbicides other than atrazine during the day, including after giving the 4–6 p.m. sample. In both cases, the highest salivary concentration of atrazine did not show up in saliva until the bedtime sample. In the former case, the time between exposure and sample collection may not have been sufficient to capture the peak elimination in the 4–6 p.m. sample, or the applicator may have become exposed to atrazine after giving the 4–6 p.m. sample, by some means other than spraying. In the latter case, it is not clear why the highest salivary concentration of atrazine was in the bedtime sample; however, the applicator sprayed non-atrazine herbicides after giving the 4–6 p.m. samples. Atrazine exposure may have occurred from contact with equipment contaminated with atrazine from previous spray jobs.

Salivary concentrations of atrazine greater than 10 μg/l seem to be a plausible predictor of those days in which atrazine spraying was likely to have occurred (Table 3). For samples taken during events in which atrazine was sprayed, 85% of the 4–6 p.m. samples had salivary atrazine concentrations higher than this level (10 μg/l). When atrazine was not sprayed in the field, 79% of 4–6 p.m. samples had salivary atrazine concentrations of less than or equal to 10 μg/l. Information on whether an applicator sprayed atrazine or not during a sampling event, in this case, may not be the only predictor of exposure. Because of the abnormal weather conditions during the study period, it was not uncommon for scheduled spraying to be postponed, even in some cases where atrazine or other herbicides had already been mixed and loaded into the spray rigs. In these cases, exposure related to mixing and loading may have already occurred. In addition, contact with contaminated surfaces may have been a source of exposure. Spraying equipment, glove use, and maintenance activities varied among applicators, and these factors may have also influenced atrazine exposures.

It is concluded that saliva biomonitoring of atrazine exposure among herbicide applicators is feasible. The sampling method itself is convenient and easy to use in the field, with a low non-compliance rate. The results of this study confirmed the animal experiment data that atrazine is able to cross the cell membranes of salivary

glands, and can be measured in human saliva with high sensitivity (4 of 302 total samples were non-detects) using the ELISA method. These results also demonstrated an expected elimination of atrazine from the body with time, away from the workplace. Salivary concentrations of atrazine not only reflect exposures resulting from spraying atrazine, but also exposures from other field activities during which applicators may come in contact with atrazine. An in-depth data analysis incorporating other data from the main study when it becomes available, may be helpful in identifying sources and determinants of exposure among these applicators.

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