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Development of Multiplexed Fluorescence Microbead Covalent Assays (FMCAs) for Pesticide Biomonitoring

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Atrazine (6-chloro-N-2-ethyl-N-4-isopropyl-1,3,5-triazine-2,4-diamine; ATR), and metolachlor (2-Chloro-N-(2-ethyl-6-methylphenyl)-N-[2-methoxy-1-methyl-ethyl] acetamid) are widely used herbicides in the United States (see Figure 1 for structures) for pre-emergence control of broad-leaf weeds. Metolachlor and ATR are frequently applied together. Biological monitoring of urine for these chemicals or their metabolites effectively provides a non-invasive estimate of occupational exposure. Quantitative analyses for urinary excreted pesticides or pesticide metabolites are classically performed by instrumental analysis after separation from a urine matrix. This procedure is costly, time consuming, labor intensive and requires the acquisition of high capital expenditure equipment and highly trained personnel, although it is usually highly specific. Alternatives to instrumental analyses are enzyme immunoassays (EIA), where pesticides or their metabolites can be quantitated in neat or diluted urine by using antibodies (usually polyclonal) directed against pesticides or their metabolites. EIAs have the benefit of being inexpensive, relatively fast, quantitative or semi-quantitative, can be performed simply on relatively inexpensive equipment and in most cases have lower limits of quantitation than classical chemical methods. EIAs may have the disadvantage (in some cases) of not being specific and that the separation of unreacted ligands, antibodies and addition of detector systems (enzymes) has to be performed in sequential steps (either washes, separation of magnetic beads, etc.), with added loss of sensitivity. Urinary pesticide/metabolite EIAs may also suffer from matrix effects (interference from substances other than the targeted analyte), limiting their sensitivity by a factor of 10-100 fold. FMCAs combine several classical methodologies: immunoassays, microspheres and flow cytometry technology (Vignali 2000; McHugh et al. 1994; Fulton et al. 1997; McHugh et al. 1997). In FMCA, immunoassays are performed on solid support microspheres with a characteristic internal fluorophore on which antigen or antibody are covalently linked. The microspheres are impregnated with combinations of proprietary dyes to produce 100 distinct microsphere sets. Flow analyzers using flow cytometry methods/technology have the ability to discriminate different particles on the basis of size or color (McHugh et al. 1994; Oliver et al. 1998; Horan et al. 1979). A dual laser instrumentation system allows both the

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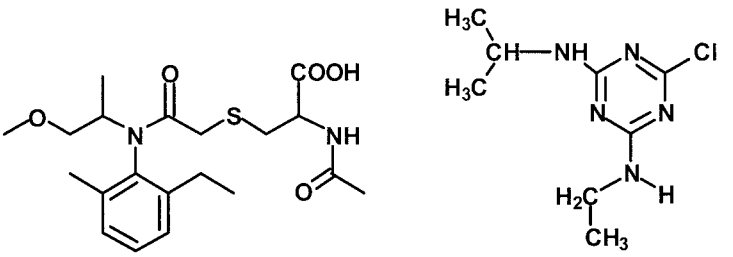

Metolachlor mercapturate Atrazine

Figure 1. Structures of metolachlor mercapturate and atrazine.

identification of the antigen-coupled microsphere by its characteristic color and the quantization of the fluorescent dye corresponding to the reporter molecule, which is bound to reagents at the surface of the microsphere. Multiplexing, the ability to perform multiple discrete assays in a single tube with the same sample simultaneously, is possible because each analyte can be bound to a discrete microsphere population characterized by a unique internal fluorophore. In addition, a no-wash assay format is feasible because the reporter molecule is read only at the surface of the microsphere. Any residual (e.g.unreacted) reporter molecule will remain in solution and thus not contribute to the assay value, simplifying the assay protocol relative to that of a typical ELISA. The sensitivity and
specificity of multiplexed assays have been reported to be comparable to conventional immunoassays (Fulton et al. 1997). The advantages of multiplexing include increased throughput and decreased cost. Our lab is currently investigating the feasibility of using FMCA to monitor the occupational exposure of workers to ATR and metolachlor by measuring ATR (the primary human metabolite of ATR, ATR- mercapturate, is detected by anti-ATR-antibodies, Striley et al. 1996) and metolachlor mercapturate (MM, the primary metabolite of metolachlor) in urine, simultaneously.

MATERIALS AND METHODS

The herbicide ATR was obtained from Chem Service, Inc. (West Chester, PA). Metolachlor mercapturate, MM-bovine serum albumin (MM-BSA) and anti-MM-BSA were synthesized and purified as previously described (Striley et al. 1999). Atrazine-BSA (ATR-BSA) and anti-ATR-BSA were obtained from Fitzgerald Industries International Inc. (Concord, MA). Carboxylate microspheres were obtained from Luminex Corporation (Austin, TX).
1-ethyl-3-(3-dimethylaminopropyl)-carbo-dimide hydrochloride (EDC), N-hydroxysulfosuccinimide sodium salt (sulfo-NHS), sodium bicarbonate, polyethylene sorbitan monolaurate (Tween 20), sulfo-NHS-LC-Biotin, and HABA (2,-[4'-hydroxyazobenzene]benzoic acid) were obtained from Pierce Chemical Company (Rockford, IL). Sodium azide and BSA were obtained from Sigma Chemical Company (St. Louis, MO). Streptavidin

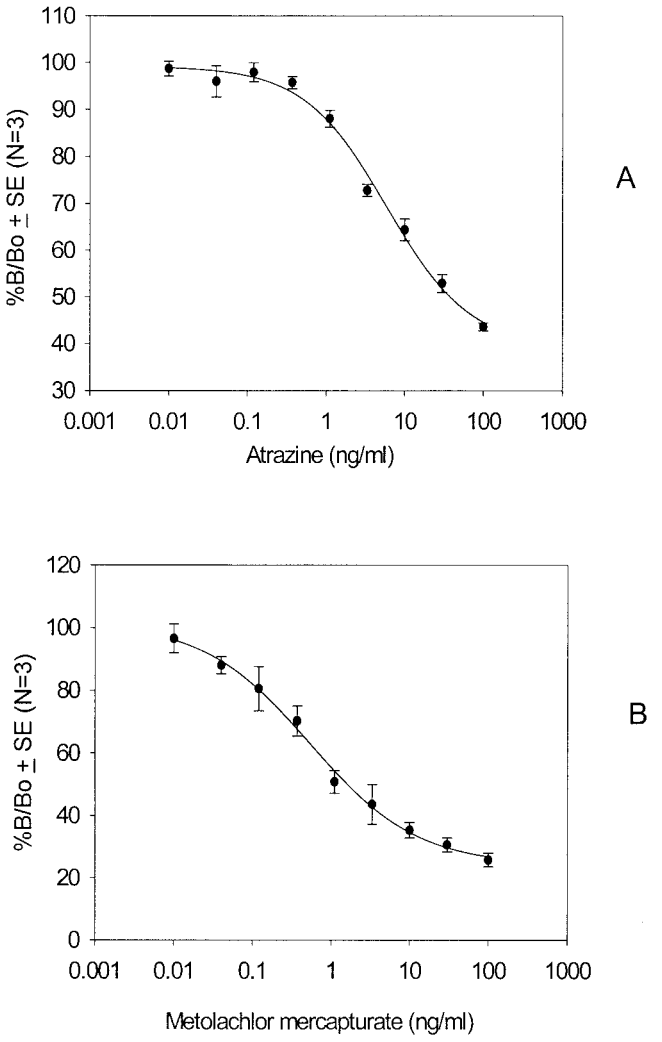

Figure 2. Four parameter logistic regression of monoplexed flow microbead immunoassays of atrazine (panel A) and metolachlor mercapturate (panel B). Results are expressed as %B/Bo of three individual runs performed in triplicate ± SE.

R-phycoetythrin (PE) conjugate was obtained from Molecular Probes (Eugene, OR). Analyses were performed on a Luminex 100 flow analyzer, coupled with a 96-well plate autosampler (XY Platform, Luminex, Austin, TX) using software, calibration microspheres, and sheath fluid supplied by the manufacturer. Two protein-coupled pesticides, MM-BSA and ATR-BSA were coupled to separate unique sets of carboxylated-modified polystyrene (Luminex, Austin, TX) microspheres (Fulton et al. 1997; Oliver et al. 1998). In triplicate, 200 µl of each

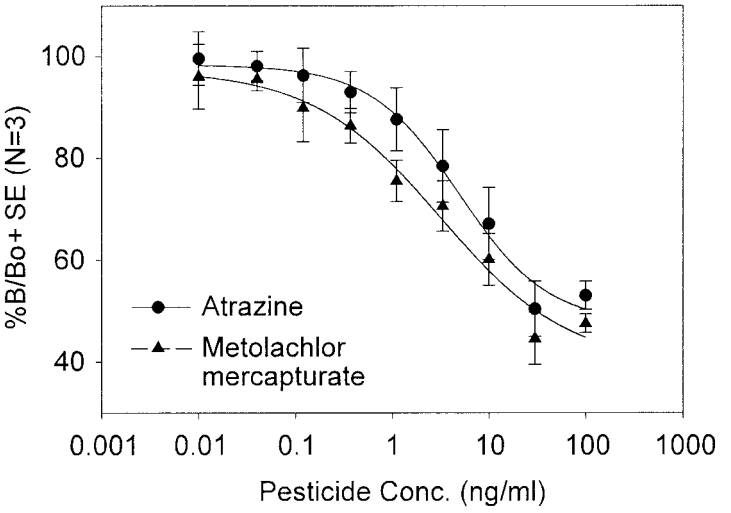

Figure 3. Four parameter logistic regression of multiplexed flow microbead immunoassays of metolachlor mercapturate and atrazine. Results are expressed as %B/Bo of three individual runs performed in triplicate ± SE.

of the unique sets of carboxylate microspheres, corresponding to 2.5 x 10^6 microspheres, were separately suspended in 80 µl of activation buffer (0.1 M sodium phosphate, pH 6.2). Ten µl each of 50 mg/ml sulfo-NHS and 50 mg/ml EDC were added, and the microspheres incubated at room temperature for 20 minutes, in the dark. The suspensions were then centrifuged, the supernatant aspirated, and the activated microspheres washed with 500 µl of PBS (0.02 M phosphate buffer, pH 7.3, containing 0.9% NaCl). The discrete sets of microspheres were coupled to ATR-BSA or MM-BSA at concentrations of 25 µg/ml, 125 µg/ml and 250 µg/ml, prepared in PBS, after being vortexed and sonicated thoroughly to reduce aggregates. Coupling of the beads to antigen-BSA was achieved by incubating activated beads in 500 µl of the appropriate antigen-BSA solution in the dark at room temperature for two hours with gentle shaking. The beads were then washed with 1 ml of PBS (0.05% Tween) enumerated with a hemocytometer, and stored in PBS (0.1% BSA, 0.05% azide) buffer at 4° C, protected from light. The antibodies (anti-ATR and anti-MM) were conjugated to biotin using sulfo-NHS-LC-Biotin reagent (Pierce Chemical Company, Rockford, IL). The sulfo-NHS-LC-biotin (556 Da) was prepared to give a 20-fold molar excess of reagent to antibody. The antibody and biotin reagent were allowed to incubate at room temperature for 30 minutes. Excess biotin was removed via dialysis using 10,000 MWCO dialysis cassettes (Pierce) against PBS buffer for four days at 4° C, with six buffer exchanges. The extent of antibody biotinlylation was determined by measuring the decrease in absorbance at 500nm in the presence of HABA (2,-[4'-hydroxyazobenzene] benzoic acid). The extent of biotin

incorporation was determined to be 17 moles biotin per mole of anti-MM and 13 moles per mole of anti-ATR. The assay reporter, streptavidin R-PE, was prepared at a concentration of 16 μg/ml in PBS containing 0.1% BSA (PBS-BSA). Each antigen-bead coupling concentration (e.g. 25 μg/ml, 125 μg/ml and 250 μg/ml) was tested over a broad range of antibody dilutions, and titration curves were prepared (data not shown) to determine both the optimum antigen-bead coupling concentration and antibody concentration to yield high median fluorescence intensities (MFIs), good dynamic range and assay sensitivity.

Monoplexed FMCAs, using a single antibody-analyte system, were developed for ATR and MM in a 96 well plate (Corning, Rochester, NY) format. ATR-BSA or MM-BSA conjugated microspheres (50 μl), at a working concentration of 10^5 beads/ml in PBS-BSA, were added to each well. To the microspheres, 50 μl of a 1:500 dilution of anti-ATR- (3.2 μg/ml,) or 1:100 (8 μg/ml,) of anti-MM-biotinylated antibody and 50 μl of the competitive inhibitor (e.g. ATR or MM) prepared at nine concentrations (100, 30, 10, 3.33, 1.11, 0.37, 0.12, 0.04 and 0.01 ng/ml in urine diluted 1:10 in PBS-BSA) were added. Pooled urine, diluted 1:10 in PBS-BSA served as the blank. The microspheres, antibody and competitive inhibitor were allowed to incubate at room temperature (protected from light) for 30 minutes on a microplate shaker. Streptavidin R-PE reporter (50 μl), at a concentration of 16 μg/ml, was added to the assay, and the mixture again allowed to incubate at room temperature (protected from light) on a microplate shaker for 30 minutes. The total assay volume was 200 μl. The plate was placed on the Luminex XY plate-sampler platform, and approximately 150 μl were read in the Luminex 100.

A multiplexed FMCA was developed to simultaneously detect the presence of ATR and MM. To perform the multiplexed assay, a mixture of ATR-BSA and MM-BSA conjugated microspheres (50 μl), at a working concentration of 10^5 beads/ml in PBS-BSA for each unique bead set, was added to each well. To the mixture of ATR and MM beads, 50 μl of an antibody cocktail containing a 1:250 working dilution (6.4 μg/ml) of biotinylated anti-ATR antibody and a 1:100 working dilution (8 μg/ml) of the biotinylated anti-MM antibody were added. ATR and MM (50 μl), at concentrations of 100, 30, 10, 3.33, 1.11, 0.37, 0.12, 0.04 and 0.01 ng/ml in urine diluted 1:10 were added to the microspheres and antibody cocktail. The microspheres, antibodies, and competitive inhibitors were protected from light and placed on a microplate shaker for 30 minutes. Streptavidin R-PE (50 μl), at a working solution of 32 μg/ml in PBS-BSA, was added to the assay, and allowed to incubate at room temperature (protected from light) for 30 minutes. The total assay volume of the multiplexed assay was 200 μl. Approximately 150 μl were read on the Luminex 100. Data were reported as median fluorescent intensity (MFI). To evaluate cross reactivity between anti-ATR and anti-MM, multiplexed beads mixtures and either 30 ng/ml of ATR or MM and either anti-ATR or anti-MM were evaluated. Standard curves were constructed from four parameter logistic regressions (SigmaPlot, SPSS, Chicago , IL) of %B/B0 transformed data, where B = the median fluorescence intensity

(MFI) for each standard and Bo = the MFI measured for the corresponding diluted urine blank. Median fluorescence intensities were adjusted for background by subtracting the MFI with no additions. The least detectable dose (LDD) of the assays, was defined as 90%B/Bo and was interpolated mathematically from the coefficients of the four parameter logistic equation. All assays were independently run 3 times in triplicate (9 individual assays).

RESULTS AND DISCUSSION

The mean intra-assay coefficient of variation (CV) for the triplicate monoplexed ATR standards was 3.45; for MM, the mean monoplexed intra-assay CV was 5.46. The LDD for the monoplexed atrazine assay was 0.73 ng/ml, while for MM it was 0.04 ng/ml (1:10 diluted urine). R values from the logistic fit were 0.996 for ATR and 0.998 for MM. Monoplexed curves are shown in Figure 2 for both ATR (panel A) and MM (panel B). The mean intra-assay coefficient of variation (CV) for the triplicate multiplexed ATR standards was 9.61; for MM the mean multiplexed intra-assay CV was 7.17. The LDD for the multiplexed ATR assay was 0.88 ng/ml, while for MM it was 0.17 ng/ml (1:10 diluted urine). R values from the logistic fit were 0.993 for ATR and 0.990 for ATR. Multiplexed curves for both MM and ATR are shown in Figure 3. Analyses of cross-reactivity between anti-ATR or anti-MM yielded 7.8% cross-reactivity for the anti-ATR antibody and MM and 1.7% cross-reactivity for the anti-MM antibody and ATR.

Agricultural workers are usually exposed to numerous pesticides for variable periods of time and exposure levels are extremely variable according to weather conditions, type of applications and work practice. Subsequent body burdens of pesticide parent(s) or parent-metabolite(s) are affected by an individual's general health status, diet, nutrition, degree of hydration, time after chemical exposure, route of exposure, etc. In addition, it is common in the formulation of commercial pesticides to have more than one active ingredient as well as numerous co-formulants. This scenario allows for body burdens of multiple native pesticides, pesticide metabolites and other agents. Biological monitoring of blood or urine by chemical analysis is the most common method used to estimate the body burden from exposure to pesticides. Commonly (depending on the chemical/physical similarities of the parent compounds), only one (or related classes) of parent(s) or pesticide metabolite(s) can be analyzed contemporaneously as sequential elution events. In most cases instrumental analytical procedures require meticulous clean-up and extractions. Urinary immunoassays have been used as quantitative analytical methods for occupational exposure to a variety of compounds, including pesticides (Biagini et al. 1995; Striley et al. 1999; Feng et al. 1990; Mastin et al. 1998). Immunoassays are generally quite sensitive, specific and accurate and have benefits in comparison to instrumental methods with regard to differences in sample size, time of analysis, cost, sample throughput and portability. However, in the classic immunoassay format, analysis is limited to the measurement of one analyte. Estimates of the total body burdens of numerous pesticides/pesticide-metabolites would involve performing numerous

immunoassays (or chemical analyses) independently. Sequential and/or contemporaneous measurement of numerous analytes from a biological sample is wrought with numerous sources of error. Unless the biological media (blood or urine) is stored as many independently frozen aliquots, or other contingencies are used to ensure sample integrity, sample degradation is likely. Errors in the estimates of the concentrations of individual analytes are likely as the individual methods most probably have their own unique inter- and intra assay coefficients of variation and recovery. Propagation of these errors in each individual analysis could lead to potential large combined errors, especially for the relative ratios of concentrations of individual analytes.

In the present work we describe FMCAs for ATR and MM, both in the monoplexed and multiplexed format. Atrazine and its metabolites have been measured by high-performance liquid chromatography (HPLC) coupled with tandem mass spectrometry (HPLC-MS/MS) using atmospheric pressure chemical ionization. This method has limits of detection ranging from 20 to 500 ng/l (parts per trillion) and relative standard deviations of less than 11% (Baker, et al., 2000). Commercially available kits for ATR are available (The Atrazine RaPID Assay, Strategic Diagnostics Inc., Newark, DE) with a LDD of 0.046 ppb (in water). Commercially available kits for MM analysis are also available (EnviroLogix Metolachlor Mercapturate in Urine Plate Kit, EnviroLogix, Portland, ME) with a LDD of 5 ppb and assay range of 8 - 85 ppb. The LDDs for the FMCAs described in the present work are in the same range with published and commercial LDD ELISA values. The multiplexed FMCAs presented herein have substantial benefits over classical chemical and traditional ELISA methods. The FMCAs are "no-wash" in that sequential reagents are added to the assay without removing the previous reagent(s) by repeated washings. Repeatedly washing of ELISA plates has the possibility of removing analyte as well as affecting the binding of adsorbed antibodies/antigens. In FMCA, the antibodies/antigens are covalently bound to the solid support The FMCAs are considerably faster than ELISAs, having two 30 minute incubations and a 20-40 second assay time (per sample). This speed enhancement may be explained by considerably improved reaction kinetics, the assays being performed on the surface of the mobile microspheres vs. immunosorbed/adsorbed reagents in immunosorbent assays. The adaptation of the Luminex Flow Metrix system from single tube assays to 96-well plate assays (using the instrument's XY platform) adds automated capability to the instrument in that as many as 96 assays can be performed in one microwell plate.

The ability to multiplex, the savings in time, minimization of systematic error, the "no wash" format and improved reaction kinetics with sensitivity and precision comparable to ELISA methods suggest that multiplexed FMCA for pesticides/pesticide metabolites is a viable adjunct to classical instrumental methods and ELISA methods for urinary biological monitoring.

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