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Development of a sensitivity enhanced multiplexed fluorescence covalent microbead immunosorbent assay (FCMIA) for the measurement of glyphosate, atrazine and metolachlor mercapturate in water and urine

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Abstract Body burdens from exposures to pesticides may be estimated from urinary analyses of pesticide parent/metabolite concentrations. Pesticide applicators and others are often exposed to numerous unrelated pesticides, either sequentially or simultaneously. Classically, body burdens of pesticides are analyzed using chemical/instrumental analysis (CIM) or enzyme immunoassays (EIAs). Both of these technologies can usually be used to quantitate one analyte (or closely related groups of analytes) per analysis. Alternatively, multiple analytes can be measured simultaneously using a multiplexed fluorescence covalent microbead immunoassay (FCMIA). We developed a multiplexed FCMIA to simultaneously measure glyphosate (Gly), atrazine (Atz), and metolachlor mercapturate (MM) in water and urine. The assay had least detectable doses (LDDs) in water/diluted urine of 0.11/0.09 ng/ml (Gly, water/urine LDD), 0.10/0.07 ng/ml (Atz), and 0.09/0.03 ng/ml (MM). The sensitivity for the measurement of Gly was enhanced by derivatization. All assays gave linear responses from the LDDs for each respective pesticide to 300 ng/ml. There was no cross-reactivity between the three analytes. Using a 96-well microplate and an autosampler, as many as 288 separate analyses can be completed in ~120 min with precision, sensitivity, and specificity equivalent to, if not better, than that found when these same analytes are measured by CIM or EIA.

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Introduction

Glyphosate (Gly; *N*[-(phosphonomethyl)glycine], atrazine (Atz; 1-chloro-3-ethylamino-5-isopropylamino-2,4,6-triazine), and metolachlor (Met; 2-chloro-6′-ethyl-N-[2-methoxy-1-methylethyl]-o-acetoluidide) are widely used herbicides in the United States (see Fig. 1for structures) in order to control broad-leaf weeds, grasses, and herbaceous plants including deep-rooted perennial weeds and brush [1, 2, 3, 4, 5]. These herbicides come from differing chemical classes, Atz being a triazine, Met being a chloroacetanilide, and Gly is a methylphosphonic acid. Some of them may also be applied as mixtures, like Met + Atz, either tank-mixed or as a commercial product. Multiple herbicides and herbicide mixtures are applied by custom applicators in the conduct of their work [2]. These applicators may apply differing herbicides and herbicide mixes in close temporal proximity to each other, sometimes within days of the week [2]. This activity can lead to multiple pesticide exposures, both from mixing and application [6] and from residue mixtures on equipment and other surfaces [7, 8]. Estimates of pesticide exposures to equipment or clothing may be performed by analytical chemical analyses of elutions [9, 10] while body burdens of pesticides are usually estimated by biological monitoring of urine samples [2, 3, 8, 11, 12, 13]. Quantitative analyses for urinary-excreted or environmentally-sampled pesticides or pesticide metabolites are usually performed by chemical/instrumental analysis (CIM) after extraction from urine or sample matrices (such as rinses of hands, patches on skin/clothing, sorbent materials, and filters) [3, 7, 9]. These procedures are costly, time consuming, labor intensive and require the acquisition of high

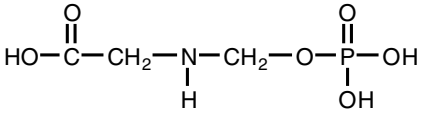
capital expenditure equipment and highly trained personnel, although they are usually highly specific. Alternatives to CIM are enzyme immunoassays (EIA), where pesticides or their metabolites can be quantified in neat or diluted urine or water using antibodies (usually polyclonal) directed against the pesticides or their metabolites [14, 15, 16]. EIAs have been used to measure numerous types of analytes in both biological and environmental matrices [2, 3, 11, 14, 15, 16, 17, 18, 19]. EIAs have the benefit of being inexpensive, relatively fast, quantitative or semi-quantitative, and can be performed simply on relatively inexpensive equipment. In many cases, EIAs have lower limits of quantitation than CIMs. Urinary pesticide/metabolite EIAs [16] and FCMIAs [3] may have the disadvantages (in some cases) of not being specific or of suffering from matrix effects from urine, limiting their sensitivity by a factor of 10–100-fold. Both CIMs and EIAs are monoplex technologies, where analytes are usually measured independently, one at a time. To measure numerous analytes, sequential single measurements are needed. Fluorescence covalent microbead immunosorbent assays (FCMIA) combine several classical methodologies: immunoassays, microspheres and flow cytometry technology [3, 17, 18, 20, 21]. In FCMIA, immunoassays are performed on solid support microspheres with characteristic internal fluorophores, which allow for the measurement of numerous analytes simultaneously (multiplexing).

The United States Environmental Protection Agency estimates that 10,000–20,000 physician-diagnosed pesticide poisonings occur each year among the ~3,380,000 US agricultural workers [22]. Exposure to low doses of pesticide mixtures is thought to be related to chronic health effects in humans [23]. Few analytical methods exist for measuring the body burden of multiple pesticides, and most deal with only one class of pesticides, such as organophosphates or pyrethroids [24]. Previously [3] our lab has shown the feasibility of using FCMIA to monitor the occupational exposure of workers to two herbicides, Atz and MM [4] in urine, simultaneously. In the present work, we extend this previous work for the simultaneous measurement of three pesticides using a sensitivity enhanced FMCIA for Gly, Atz, and MM.

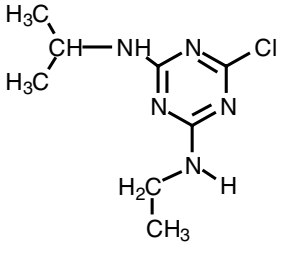
Methods and materials

Chemicals

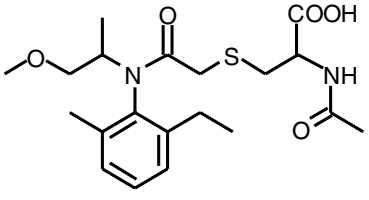
Glyphosate and Atz were obtained from ChemService, Inc. (West Chester, PA, USA). Metolachlor mercapturate (MM), MM-keyhole limpet hemocyanin (MM-KLH) (keyhole limpet hemocyanin, Pierce Biotechnology, Rockford, IL, USA) and rabbit anti-MM-bovine serum albumin (BSA, Pierce, FL, USA) were prepared as previously described [4]. MM is a major human urinary metabolite from exposure to Met [4, 25]. Atz-BSA, Gly-ovalbumin (Gly-OVA), rabbit polyclonal anti-Atz-BSA and anti-Gly-BSA were obtained from Abraxis, LLC (Warminster, PA, USA). Atz mercapturate is the major human urinary metabolite of Atz



Glyphosate
(Methylphosphonic Acid)



Atrazine
(Triazine)



Metolachlor mercapturate
(Chloroacetanilide)

Fig. 1 Structures and (chemical classes) of multiplexed herbicides

[15], although Atz parent is also excreted [26]. Polyclonal antibodies to pesticide-parent compounds have been shown to cross-react with parent-metabolites, in some cases with increased sensitivity [16]. Derivatization of Gly has been shown to increase the sensitivity of a Gly ELISA by 100-fold [19]. We derivatized our multiplexed assay with a proprietary derivatization system (Abraxis) which consisted of a derivatization agent and assay buffer. All water used was ≥18 megohm-cm (RO-Pure/E-Pure, Barnstead International, Dubuque, IA, USA). Microspheres were obtained from Luminex Corporation, Austin, TX, USA. Activation buffer (0.1 M NaH_2 PO_4, pH 6.2), coupling buffer [0.05 M 2-(*N* morpholino)ethanesulfonic acid (MES), pH 5.0], wash buffer [phosphate buffered saline (PBS), 138 mM NaCl, 2.7 mM KCl, containing 0.05% Tween 20] and storage/blocking buffer (PBS, 1% BSA, 0.05% NaN_3, pH 7.4), and HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid], were supplied or prepared with reagents supplied by Sigma Chemical Co., St. Louis, MO, USA. Biotin labeled anti-rabbit IgG, 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC), and *N*-hydroxysulfosuccinimide, sodium salt (sulfo-NHS) were obtained from Pierce Biotechnology, Inc., Rockford, IL, USA. Streptavidin R-phycoetythrin (streptavidin R-PE) was obtained from Molecular Probes, Eugene, OR, USA. UriSub synthetic urine was obtained from CST Technologies, Inc., Great Neck, NY, USA.

Preparation of microspheres

Microspheres were 5.6 μm in diameter and composed of polystyrene, divinyl benzene and methacrylic acid, which provided surface carboxylate functionality for covalent attachment of biomolecules. Internally, the microspheres were dyed with red and infrared emitting fluorochromes. By proportioning the concentrations of each fluorochrome, spectrally addressable microsphere sets were obtained. The three pesticide conjugates (Gly-OVA, ATZ-BSA and MM-KLH) were coupled to separate unique sets of carboxylate-modified (Luminex) microspheres [3, 21, 27]. After washing twice with 80 μl activation buffer, three sets of spectrally differentiable carboxylated microspheres (2.5×10^6, Luminex) were pelleted (5,000×g for 2 min) in 1.5 ml centrifuge tubes using a microcentrifuge (Eppendorf, Hamburg, Germany). The microspheres were resuspended by sonication (mini sonicator, Cole Parmer, Vernon Hills, IL, USA), and gentle vortexing (vortex Genie, VWR, Intl., West Chester, PA) in 80 μl activation buffer, to which 10 μl activation buffer containing 50 mg/ml EDC, and 10 μl of activation buffer containing 50 mg/ml sulfo-NHS were added. The mixture was allowed to incubate for 20 min at room temperature. The microspheres were then washed twice in 500 μl coupling buffer, solutions of the three pesticide conjugates (125 μg/ml, MM; 25 μg/ml, Atz and Gly) in 500 μl coupling buffer were added, and the mixture incubated for 2 h at room temperature, with gentle shaking. The coupled microspheres were then washed twice in 1 ml wash buffer, and stored in 0.5-ml storage/blocking buffer. Microsphere concentrations were determined using a microscope and hemacytometer.

Multiplexed analysis

The three pesticide conjugated microspheres (50 μl), at a working concentration of 1×10^5 microspheres/ml for each microsphere type in storage/blocking buffer, were added to wells of a 1.2 μm filter membrane microtiter plate (Millipore Corp., Part #MABVN1250, Bedford, MA, USA) and the liquid aspirated by use of a vacuum manifold filtration system (Millipore, Part #MAVM09601). Mixtures of the three pesticides (MM, Atz, Gly) were prepared at nine concentrations (300, 100, 30, 10, 3, 1, 0.3, 0.1, and 0 ng/ml) in either a mixture of water:assay buffer (3:1) (hereafter identified as "water") or pooled human volunteer urine diluted (1:10) with a mixture of UriSub:assay buffer (3:1), (hereafter identified as "diluted urine"). Derivatization agent (Abraxis) (20 μl) was added to 250 μl aliquots of the diluted pesticide mixtures in microfuge tubes (in duplicate) and allowed to incubate for 10 min at room temperature. Derivatized or derivatized and diluted mixture of pesticides (50 μl) were then added to the microspheres in the filter plate from above, and 50 μl of a mixture of the primary antibodies for each pesticide (anti-ATZ, 1:4,000–1:8,000 dilution; anti-Gly, 1:2,500–1:5,000 dilution; and anti-MM, 1:1,000–1:2,000 dilution in 0.5 M HEPES with 2% BSA, pH 6.6) were added, and then the derivatized samples, microspheres and primary antibodies were allowed to incubate at 37 °C (protected from light) for 30 min on a microplate shaker. The wells were washed three times with wash buffer. Biotin labeled anti-rabbit IgG (50 μl) in storage/blocking buffer were added to the wells and incubated at 37 °C for 30 min on a microplate shaker. The wells were again washed with wash buffer and streptavidin R-PE reporter (50 μl), at a concentration of 4 μg/ml, in storage/blocking buffer added to the assay, and the mixture was again allowed to incubate at 37 °C (protected from light) on a microplate shaker for 30 min. Finally, the wells were again washed with wash buffer, and the microspheres were resuspended in 100 μl of wash buffer. The plate was shaken vigorously for approximately 1 min to disperse the microspheres, and was placed into the autosampler platform of the LUMINEX 100 (Luminex) instrument using software, calibration microspheres, and sheath fluid supplied by the manufacturer. The instrument was programmed to collect data from 100 microspheres for each analyte (classified by their internal fluorescence ratio) and acquire the median fluorescence intensity (MFI) of the microsphere-pesticide conjugate-primary anti-pesticide conjugate IgG antibody-secondary-anti-IgG-biotin-avidin complex.

Data analyses

Standard curves were constructed from four-parameter logistic-log fits (4-PL, SigmaPlot, SPSS, Chicago, IL, USA) of $\%B/Bo$ data (where B = the MFI for each individual pesticide standard and Bo = the MFI measured for the corresponding blank) versus ng/ml of standard. The least detectable dose (LDD) of the assays was defined as $90\%B/Bo$ and was interpolated mathematically from the coefficients of the 4-PL equations. Assessment of the "goodness of fit" and the dynamic ranges of the assays were investigated by evaluating the fit of the standards data to the 4-PL model by "standards recovery" [28], calculated by evaluating interpolated results from each 4-PL fit and comparing it to the concentrations of pesticides added to the system [28] using the following relationship:

$$\%\text{recovery} = 100 \times \left(\frac{\text{observed concentration from 4 - PL fit}}{\text{expected concentration from 4 - PL fit}}\right).$$

The resultant data were analyzed for linearity from each pesticides' FCMIA LDD to 300 ng/ml by linear regression. Analyses of recoveries at each dilution were investigated by a Kruskal–Wallis one way analysis of variance (ANOVA, SigmaStat, SPSS, Chicago, IL, USA). To determine if derivatization had an effect on the response of the FCMIA, derivatized versus non-derivatized $\%B/Bo$ were investigated with a rank-sum test at each standard concentration. To test for cross-reactivity among the herbicides, increasing concentrations of the individual herbicides were added to the mixture of Gly, Atz and MM microspheres and anti-Gly, anti-Atz and anti-MM antibodies added, and the assay was run as above. The percent cross-reactivity was calculated from the following relationship:

$$\%\text{cross - reactivity} = \left(\frac{\text{ng/ml@50\% } B/Bo \text{ for heterologous analyte}}{\text{ng/ml@50\% } B/Bo \text{ for homologous analyte}}\right) \times 100.$$

Results

Four-parameter logistic fit concentration-response multiplexed curves are shown in Fig. 2 for derivatized Gly, Atz and MM, in both water (panel A) and diluted urine (panel B). The three curves had excellent fits to the 4-PL model with r^2 values of 1.000, 0.998 and 0.995 in water, and 0.999, 0.998, and 0.992 in diluted urine, respectively for Gly, Atz and MM. The assay had LDDs in water/diluted urine of 0.11/0.09 ng/ml (Gly), 0.10/0.07 ng/ml (Atz), and 0.09/0.03 ng/ml (MM). The average inter-assay coefficients of variation were 11.3% (water) and 11.7% (diluted urine) while the intra-assay coefficients of variation were 5.8% (water) and 4.1% (diluted urine)

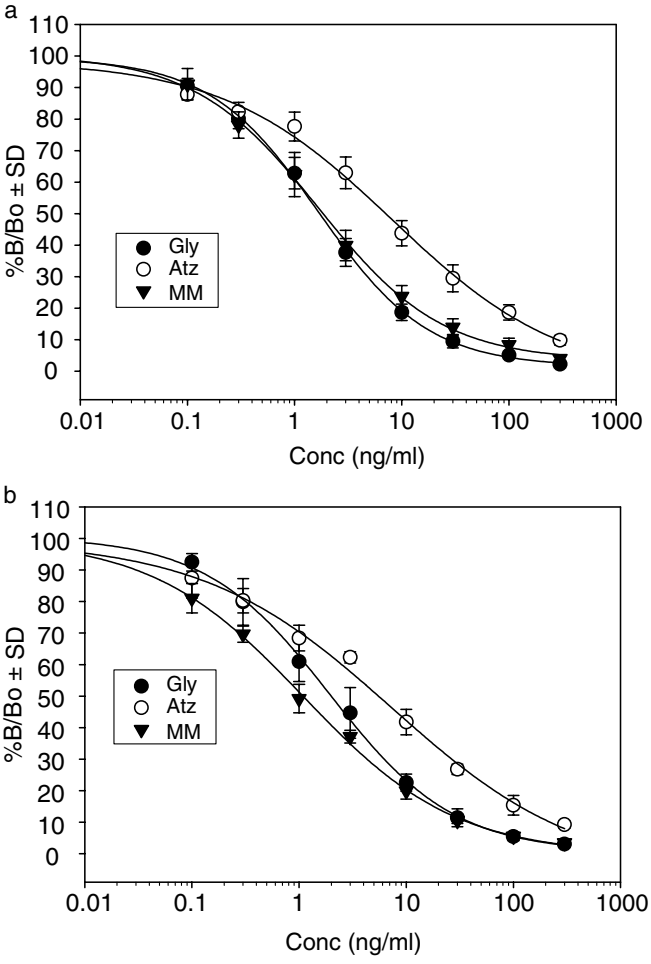


Fig. 2 Four parameter logistic regression of multiplexed FCMIA of metolachlor mercapturate (*MM*), atrazine (*Atz*) and glyphosate (*Gly*) in water (**a**) and diluted urine (**b**). Results are expressed as the mean $\%B/Bo$ of four individual runs ± SD

($n = 4$ independent assays). As the current FCMIA is a multiplexed, derivatized assay, which combines the benefits of multiplexing with the enhanced sensitivity of derivatization for the analyses of Gly, compensation for sample preparation is necessary when analyzing water or urine samples obtained from the field. Water field sample concentrations would have to be adjusted by a factor of 1.33, while urine samples would have to be adjusted by a factor of 10. Derivatization had no significant effect on concentration-effect curves for Atz ($p = 0.386$) and MM ($p = 0.862$), while dramatically increasing the sensitivity ($p < 0.001$) of Gly standard curves (data not shown).

Cross-reactivity among the herbicides was investigated by incubating increasing concentrations of single herbicides with all three antibodies and $50\%B/Bo$ was compared among homologous and heterologous analytes. Decreases in $\%B/Bo$ were observed only for homologous herbicide/antibody pairs (Table 1) with heterologous pesticides yielding essentially flat curves. Recoveries and dynamic ranges for the FCMIA assays

Table 1 Percent cross-reactivity of multiplexed FCMIA (homologous inhibitions are written in bold)

	Glyphosate (Gly)	Atrazine (Atz)	Metolachlor Mercapturate (MM)
Gly	**100**	< 0.01	< 0.01
Atz	< 0.01	**100**	< 0.01
MM	< 0.01	< 0.01	**100**

Cross-reactivity (%)

were investigated by standards recovery and regression. Significant ($p < 0.001$) regression coefficients (r^2) of > 0.984 and slopes between 0.984 and 1.000 for Gly, Atz, and MM, diluted both in water (Fig. 3a) and in diluted urine (Fig. 3b) were observed for the recovery of known additions. When the interpolated concentrations of pesticides from the 4-PL standard curves were compared to the actual concentrations of pesticide standards added, there were no significant differences in concentrations when investigated from each pesticides' LDD to 300 ng/ml ($p > 0.595$, diluted urine; $p > 0.656$, water). Mean recoveries of known added concentrations of each pesticide in diluted urine from their respective LDDs to 300 ng/ml were 100.5 ± 11.1% (range, 85.4–117.4%, Gly ± SD), 105.2 ± 11.5% (range, 86.5–121.0%, Atz) and 102.9 ± 12.1% (range, 81.4–114.4%, MM). When the pesticides were diluted in water, mean recoveries were 100.6 ± 11.2% (range, 80.6–114.2%), 105.3 ± 26.8% (range, 71.3–129.5%), and 101.4 ± 17.1% (range, 74.5–122.9%), respectively, for Gly, Atz, and MM. These recoveries are within the 70–130% range considered acceptable for these types of assays [28].

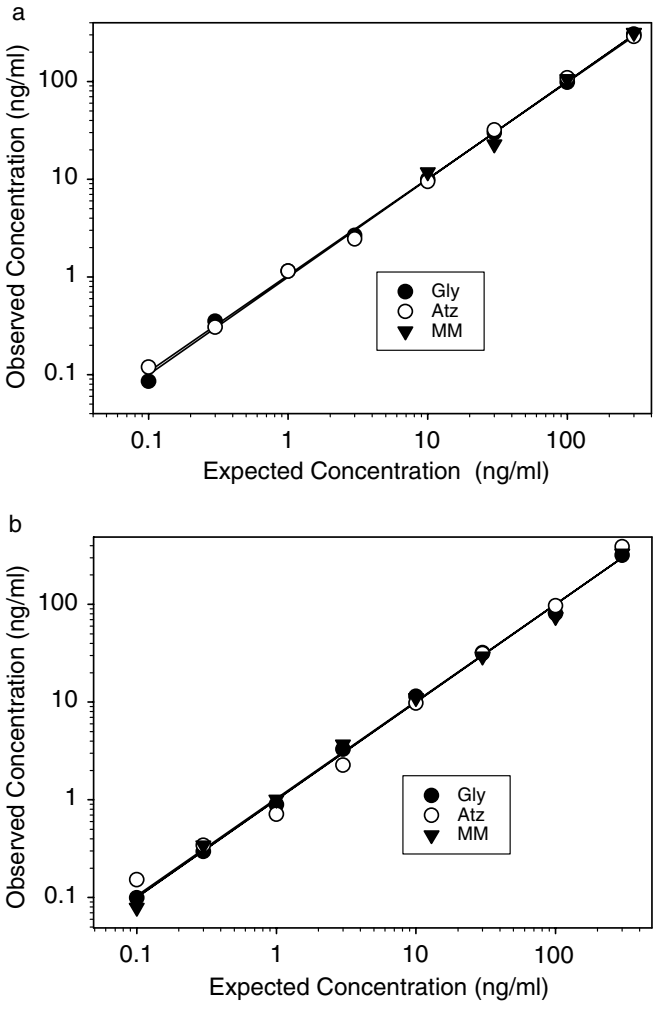


Fig. 3 Linear regression of the observed interpolated results from 4-PL fits compared to concentrations added for multiplexed FCMIA for metolachlor mercapturate (*MM*), atrazine (*Atz*), and glyphosate (*Gly*) in diluted urine (**a**) or water (**b**). All regression lines are essentially collinear

Discussion

Human exposure to pesticides is multi-media and multi-route. Agricultural workers can be exposed to numerous pesticides for variable periods of time, at variable exposure levels and by numerous routes (inhalation, dermal, ingestion). In addition, transfer exposures can occur from dermal or other contacts with contaminated equipment and surfaces. Primary and transfer exposures can be affected by weather conditions, type of applications, and work practices [2, 3]. This scenario allows for exposure to multiple pesticides, with resultant body burdens of these and their metabolites. Biological monitoring of blood or urine by CIM is the most common method used to estimate the body burden from exposure to pesticides. FCMIA can be used to measure multiple analytes without extraction or pre-concentration, has recoveries of ~100%, and usually is more sensitive for specific analytes compared to CIM. Other benefits of the multiplexed FCMIAs are smaller sample sizes, lower cost, and generally increased dynamic ranges compared to both CIM and ELISA [3, 16–18].

Using high-performance liquid chromatography-mass spectrometry (HPLC-MS), other investigators have reported limit of detections (LODs) of 0.4 ng/ml for Atz in urine [26]. Atz LDDs, measured by FCMIA in the present work, were 0.10 and 0.07 ng/ml (dilution adjusted, 0.13/0.7 ng/ml) respectively, for water and diluted urine. Instrumental methods (SPME-HPLC-MS) have been used to measured MM in spiked urine with a LOD of 3 ng/ml [25]. Using the FMCIA method reported on in the present paper, LDDs of 0.09 and 0.03 ng/ml (dilution adjusted, 0.12/0.3 ng/ml) were observed for MM in water and diluted urine, respectively. Analyses for Gly in water have been shown to have an LOD of 50 ng/ml by HPLC [19], while the FMCIA of the present work demonstrated LDDs of 0.11 and 0.09 ng/ml (dilution adjusted, 0.15/0.9 ng/ml) in water and diluted urine, respectively.

Commercially available EIA kits (all values are from vendors' documentation) for Atz (Strategic Diagnostics Inc., Newark, DE, USA) with a LDD of 0.1 ng/ml and a

range of 0.4–5.0 ng/ml (in water) are available. Kits are also available for MM in urine (EnviroLogix, Portland, ME, USA) with a LDD of 5 ng/ml and an assay range of 8–85 ng/ml and Gly [LDD of 0.10 ng/ml with a range of 0.15–5 ng/ml (Abraxis)]. Even when adjusted by a factor of 1.33 for water samples and 10 for urine, the FCMIA LDDs are essentially equivalent, if not lower, than those reported for CIMs and commercial EIAs while measuring the analytes simultaneously. In addition, the FCMIA had linear dynamic ranges, from each pesticides' LDD to 300 ng/ml, for all three pesticides in both water and diluted urine, which ranged over four orders of magnitude (~0.1–300 ng/ml).

The FCMIAs are considerably faster than EIAs or CIMs, having three 30 min incubations and a 20–40 s assay time (for each three-pesticide sample). Using the 96-well autosampler described in the present work, as many as 288 (96-wells×three pesticides) separate analyses can be performed in ~100 min with ~20 min needed to read the plates. For multi-plate runs, like three microplates, ~860 analyses can be performed in ~200 min. In FCMIA, as the time for analyses is essentially independent of the number of analytes measured, incredibly high throughput can be realized. We have described multiplexed analyses for 25 analytes [17] simultaneously, which equates to measurement of 2,400 individual analyses in under 80 min. Multi-plate runs using this assay yield measurements of ~7,000 analytes in ~160 min.

Estimates of the total body burdens of numerous pesticides/pesticide-metabolites would involve performing numerous single analyte EIAs or CIMs, either sequentially or independently. Multiple analytes can be measured using CIM after liquid–liquid extraction, liquid–solid extraction, or the use of solid phase microextraction fibers, for instance, with multi-step and time consuming extractions and sample cleanup procedures sometimes necessary before samples can be introduced to the instrumentation [24, 29, 30]. Diversity in the chemical properties of mixtures has been shown to negatively impact recoveries when measuring multiple analytes by CIM [29]. The final outcome of these efforts are methods which are either sensitive and imprecise or precise and insensitive [24, 29, 30].

Sequential and/or contemporaneous measurement of numerous analytes in separate assays from a biological sample is wrought with numerous potential sources of error. Unless the biological media (blood or urine) are stored as many independently frozen aliquots, or other contingencies are used to ensure sample integrity, sample degradation is likely in repeated freeze thaw cycles. 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 recoveries. Propagation of these errors in each individual analysis could lead to potentially large combined errors, especially for the relative ratios of concentrations of individual analytes.

Conclusion

In conclusion, we describe a method for the multiplexed analyses of Gly, Atz, and MM which is precise, accurate, sensitive, specific, has an excellent dynamic range and yields linear relationships upon dilution, both in water and diluted urine.

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