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# Development and evaluation of an enzyme-linked immunosorbent assay (ELISA) method for the measurement of 2,4-dichlorophenoxyacetic acid in human urine

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**Abstract**

An enzyme-linked immunosorbent assay (ELISA) method was developed to quantitatively measure 2,4-dichlorophenoxyacetic acid (2,4-D) in human urine. Samples were diluted (1:5) with phosphate-buffered saline containing 0.05% Tween and 0.02% sodium azide, with analysis by a 96-microwell plate immunoassay format. No clean up was required as dilution step minimized sample interferences. Fifty urine samples were received without identifiers from a subset of pesticide applicators and their spouses in an EPA pesticide exposure study (PES) and analyzed by the ELISA method and a conventional gas chromatography/mass spectrometry (GC/MS) procedure. For the GC/MS analysis, urine samples were extracted with acidic dichloromethane (DCM); methylated by diazomethane and fractionated by a Florisil solid phase extraction (SPE) column prior to GC/MS detection. The percent relative standard deviation (%R.S.D.) of the 96-microwell plate triplicate assays ranged from 1.2 to 22% for the urine samples. Day-to-day variation of the assay results was within ±20%. Quantitative recoveries (>70%) of 2,4-D were obtained for the spiked urine samples by the ELISA method. Quantitative recoveries (>80%) of 2,4-D were also obtained for these samples by the GC/MS procedure. The overall method precision of these samples was within ±20% for both the ELISA and GC/MS methods. The estimated quantification limit for 2,4-D in urine was 30 ng/mL by ELISA and 0.2 ng/mL by GC/MS. A higher quantification limit for the ELISA method is partly due to the requirement of a 1:5 dilution to remove the urine sample matrix effect. The GC/MS method can accommodate a 10:1 concentration factor (10 mL of urine converted into 1 mL organic solvent for analysis) but requires extraction, methylation and clean up on a solid phase column. The immunoassay and GC/MS data were highly correlated, with a correlation coefficient of 0.94 and a slope of 1.00. Favorable results between the two methods were achieved despite the vast differences in sample preparation. Results indicated that the ELISA method could be used as a high throughput, quantitative monitoring tool for human urine samples to identify individuals with exposure to 2,4-D above the typical background levels.


*Keywords:* 2,4-Dichlorophenoxyacetic acid (2,4-D); Urine; Enzyme-linked immunosorbent assay (ELISA); Immunoassay; 96-Microwell; Gas chromatography/mass spectrometry

## 1. Introduction

The herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) is one of the most widely-used herbicides in the United States (U.S.) for control of weed growth. 2,4-D belongs to the group of synthetic compounds called chlorophenoxy herbicides. The chemical structure of 2,4-D resembles indoleacetic acid, a naturally occurring hormone produced by plants to regulate their own growth. This resemblance allows 2,4-D to artificially regulate plant growth on a controlled basis. Herbicides containing 2,4-D are typically formulated as either free acids, amine salts, or as esters and are used in agriculture, forestry, and residential lawn care. A few of the common

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trade names of 2,4-D products sold in U.S. are Chloroxone, Salvo, Weed-no-more and Aqua-Kleen.

2,4-D has been identified in multiple environmental media such as air, dust, and soil [1–6]. Non-occupational routes of exposure include inhalation of contaminated air, dietary and non-dietary ingestion of contaminated food and non-food items, and dermal contact with contaminated surfaces. Although 2,4-D has not been classified as a human carcinogen, an association between exposure to herbicides containing 2,4-D and an increased incidence of tumor formation has been reported in several studies [7–12]. Acute exposure to 2,4-D via dermal contact has resulted in nervous system damage; ingestion of high-dose 2,4-D formulations has led to death; and low-dose 2,4-D ingestion has led to neuromuscular problems [13,14]. Most of the 2,4-D is excreted in urine within days after exposure with elimination rates differing slightly among 2,4-D formulations (acids, esters, or salts) [15]. Once in the body, the ester and amine salts of 2,4-D are converted to the acid for excretion in the urine. The urinary concentrations of 2,4-D in adult and children subjects without recent occupational 2,4-D exposures are typically less than 10 ng/mL (ppb) while applicators who used hand-held, backpack sprayers had a reported average urinary 2,4-D concentration of 454 ppb [1,2,5,16]. Thus, urinary 2,4-D concentrations could be used as a primary indicator of human exposure.

Instrumental analytical methods have been developed for determining 2,4-D in multiple sample media including urine at low- or sub-ppb levels [4,6]. However, extraction, derivatization, and clean up procedures are necessary prior to gas chromatography/mass spectrometry (GC/MS) or GC/electron capture detection (ECD). The procedures employed in these instrumental methods are labor-intensive, time-consuming, and costly. Enzyme-linked immunosorbent assay (ELISA) methods are generally sensitive, selective, and cost effective. They can facilitate a high sample throughput and can be used as qualitative or quantitative tools. Several ELISA methods have been developed for the detection of environmental pollutants including pesticides, metabolites of pesticides, polycyclic aromatic hydrocarbons, and polychlorinated biphenyls with performance data reported for real-world samples such as soil, sediment, food, and urine [17–25].

This paper describes the development of a 96-microwell high sample capacity ELISA method for measuring 2,4-D in urine; the analysis of 2,4-D in real-world urine samples by both ELISA and GC/MS methods; and compares the ELISA and GC/MS results in several key areas: accuracy, precision, sample throughput and detection limits. The 2,4-D ELISA method employed a monoclonal antibody [26] and a coating antigen in a 96-micowell format. The ELISA utilized a streamline sample preparation for a simple, high throughput and cost effective analysis. The method was then challenged with human urine samples collected as part of the EPA Pesticide Exposure Study (PES) [27]. The EPA PES is a sub-study in the Agricultural Health Study, which is co-sponsored by the National Cancer Institute and the National Institute of Environmental Health Sciences.

## 2. Experimental

### 2.1. Chemicals and instruments

The monoclonal antibody for 2,4-D (clone E2/G2) and the 2,4-D ovalbumin coating antigen was purchased from Dr. Milan Franek [25] and Joint Forum for Environmental Health, which is now owned by Diagenode, Belgium, respectively. Phosphate-buffered saline with 0.05% Tween and 0.02% sodium azide (PBST), pH 7.4, goat anti-rabbit IgG alkaline phosphatase conjugate, *p*-nitrophenol phosphate tablets, carbonate-bicarbonate buffer, sodium azide; diethanolamine, 2,4-D, Diazald, carbitol, potassium hydroxide, anhydrous sodium sulfate, sodium chloride, ethyl ether, and Florisil SPE columns were purchased from Sigma (St. Louis, MO). Labeled 2,4-D-($^{13}C_6$) and phenanthrene-$d_{10}$ were purchased from Cambridge Isotope Laboratories (Andover, MA). Drug-free urine (DFU) was purchased from American Biological Technologies Inc. (Sequin, TX). Solvents including hexane, chlorobutane, dichloromethane (DCM), and methanol for preparing standard solutions and samples, were distilled-in-glass grade and obtained from Burdick and Jackson (Indianapolis, IN). ELISA experiments were performed in 96-microwell plates (Nunc, MaxiSorp$^{TM}$, Sigma). Absorbances were read with a SpectraMax Plus microplate spectrophotometer with SoftMax Pro version 4.3E software (Molecular Devices, Sunnyvale, CA). A Hewlett-Packard (HP) GC/MS instrument with a ChemStation data system was used for the GC/MS analysis.

### 2.2. Urine sample preparation

Spiked samples were prepared for GC/MS by placing a known amount (25–50 ng) of 2,4-D into the urine samples (5–10 mL). A known amount (25–50 ng) of the surrogate recovery standard (SRS) 2,4-D-($^{13}C_6$) was added to both the spiked and neat samples. An aliquot of 5–10 mL of each urine sample was placed in a vial with 1 mL of chlorobutane and concentrated hydrochloric acid (0.5 mL). The sample was heated to $80 \pm 5\,^{\circ}C$ in a water bath for 1 h. The resulting solution was extracted with DCM ($2 \times 5$ mL) and a 20% sodium chloride solution ($2 \times 1$ mL) in a separatory funnel and dried over $Na_2SO_4$. The resulting concentrated urine DCM extract was methylated with diazomethane in ethyl ether generated in situ from Diazald, carbitol, and 37% aqueous KOH. The methylated sample extract was solvent-exchanged into hexane and processed through a conditioned Florisil solid-phase extraction (SPE) column. The SPE column was eluted with 18 mL of 50% ethyl ether in hexane, and the collected fraction was concentrated to 1 mL. A known amount of the internal standard (IS), phenanthrene-$d_{10}$ was added for subsequent GC/MS analysis.

For initial ELISA development, urine samples were prepared using the same extraction procedures as described above except that the SRS and IS were not added to the urine samples and the solid phase clean up was not performed. The concentrated DCM extracts were solvent exchanged into methanol and diluted with PBST (1:10) for subsequent ELISA analysis.

To investigate a urine matrix effect, and to determine the concentration of urine tolerated in the ELISA without a clean up step, a 25-fold urine concentrate was prepared using the above procedures. Five aliquots ($5 \times 10$ mL) of DFU were hydrolysed, extracted into $5 \times 1$ mL of DCM, and solvent exchanged into 2 mL of methanol. This 25-fold urine concentrate was further diluted to a 2.5-fold urine concentrate in 10% methanol in PBST (10% methanol/PBST). Standard solutions were prepared in 10% methanol/PBST containing varying amounts of urine concentrate for subsequent ELISA. Based on these results, a more streamlined sample preparation approach consisting of diluting the urine sample prior to ELISA analysis was developed.

### 2.3. ELISA procedure

The ELISA analyses for urine samples were performed using a laboratory based 96-microwell format [28]. A 96-microwell plate (Nunc MaxiSorp$^{TM}$) was coated with 100 μL of coating antigen (250 ng/mL) and incubated overnight at 4 °C. Each plate was washed three times with PBST in a plate washer (Skatron model 300) programmed for a three-cycle wash. The plates were rotated 180° between wash cycles to effectively remove unbound antigen. The plates were further dried by tapping on absorbent paper. Each plate was then sealed with an acetate cover and stored in a refrigerator until needed. In the initial ELISA analyses for the evaluation of possible urine matrix effects, the standard solutions and hydrolyzed urine sample extracts were prepared in 10% methanol/PBST.

For the final ELISA format, the 2,4-D calibration standard solutions and quality control (QC) spiked samples were prepared in 80% PBST/20% DFU, and analyzed in triplicate for each assay. A 10-point (3.9–2000 ng/mL) calibration curve (in triplicate microwells) was generated for each plate analysis. The highest standard solution was prepared at 2000 ng/mL in DFU. The other standard solutions were prepared in DFU from the highest standard solution to concentrations of 1000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8 and 3.9 ng/mL. Note that the concentrations designated on the calibration curve (Fig. 3) referred to the actual standard concentrations of 2,4-D in DFU, which were then diluted with PBST (1:5) for assay. Thus the standards, urine samples and QC samples were in the same buffer composition (80% PBST/20% DFU or 80% PBST/20% urine sample) for quantitation. A 0.00 ng/mL standard solution containing no 2,4-D in 80% PBST/20% DFU was employed as a negative control and routinely prepared fresh for each assay. The dilution of the human urine samples with PBST (20 μL of urine and 80 μL of PBST) prior to the ELISA analysis minimized the urine matrix effect such that a cleanup step was not required. If the assay results were above the calibration curve, the extract was further diluted with 80% PBST/20% DFU and reassayed.

Individual aliquots (100 μL) of the standard solutions, diluted urine extracts, QC samples, and blanks were added to appropriate microwells in triplicate. An aliquot (100 μL) of the 2,4-D monoclonal antibody E2/G2 (1:32,000) in PBST was added to all microwells except those used as instrument blanks. The plates were incubated for 2 h at room temperature on an orbital shaker at 160 rpm. Excess reagent not bound to the plate was removed by washing six times with PBST as described above. The plate was further dried by tapping on absorbent paper. An aliquot (100 μL) of goat anti-mouse IgG alkaline phosphatase conjugate (Sigma Chemical) at a 1:3000 dilution in PBST was added to each microwell. Plates were again incubated for 2 h at room temperature on the orbital shaker (160 rpm). Excess conjugate was removed by washing with PBST. A 100 μL aliquot of *p*-nitrophenyl phosphate (Sigma Chemical) at 1 ng/mL in diethanolamine buffer was added to each microwell. Following a 30 min room-temperature incubation, each microwell was read using a Molecular Device SpectraMax Plus microplate spectrophotometer (Sunnyvale, CA) interfaced to a personal computer. The absorbance of the microwells was determined at 405 nm and normalized to a 1 cm pathlength. Data processing was performed with SoftmaxPro software using a four-parameter curve fit.

### 2.4. GC/MS procedure

The sample extracts and standard solutions were analyzed by 70 eV electron impact (EI) GC/MS. A Hewlett-Packard (HP) GC/MS was operated in the selected ion monitoring (SIM) mode. Data acquisition and processing were performed with a ChemStation data system. The GC column was a DB-5 fused-silica capillary (60 m × 0.32 mm, 0.25 μm film thickness). Helium was used as the GC carrier gas. Following injection, the GC column was set at 90 °C, temperature programmed to 290 °C at 8 °C/min, and held at 290 °C for 5 min. Peaks monitored were the molecular ion peaks and their associated characteristic fragment ion peaks (234, 236, 238 for 2,4-D; 240 and 242 for the SRS 2,4-D-($^{13}C_6$); and 94 and 188 for the IS phenanthrene-$d_{10}$). Identification of the target analytes (2,4-D and the SRS) was based on their GC retention times relative to the IS and the relative abundances of the monitored ions. Quantification was performed by comparing the integrated ion current response of the target ions to those of the IS. The average response factors of the target ions were generated from the standard calibrations [5]. In brief, the Rf value was obtained using $Rf = (A_S/A_{IS}) \times (C_{IS}/C_S)$ from the analyses of standard solutions used for generating the calibration curve. Note that $A_S$ and $A_{IS}$ refer to the area counts of the quantification ions of target analytes and the IS, respectively, and $C_S$ and $C_{IS}$ are the concentration

values of target analytes and the IS, respectively. The concentration of target analytes ($C_S$) in the samples was obtained from $(A_S/A_{IS}) \times (C_{IS}/Rf_{avg})$, where the $Rf_{avg}$ value was the average Rf value generated from the analyses of standard solutions.

## 3. Results and discussion

### 3.1. ELISA method evaluation

Results of the initial 2,4-D ELISA evaluation experiments showed that the estimated detection limit was 12.5–25 ng/mL of 2,4-D in 100% PBST. Tolerance to methanol in the 2,4-D ELISA was investigated with results indicating that 10% methanol in PBST did not significantly affect the calibration curve. To analyze the hydrolyzed urine extracts by both GC/MS and ELISA, the DCM sample extract was solvent-exchanged into 10% methanol/PBST for the ELISA. The potential sample loss from the nitrogen evaporation during the solvent exchange step was determined. Standard solutions (1 mL each) were evaporated to dryness under a stream of nitrogen and resuspended in 1 mL of 10% methanol/PBST. Acceptable recoveries were obtained from both urine samples and standard solutions with recoveries ranging from 68 to 147%.

A group of low-level background urine samples collected from a children's exposure study [1] was analyzed by both ELISA and GC/MS, following the procedures described in Section 2.2. Acceptable recoveries (60–126%) of 2,4-D were obtained for the ELISA from control spiked buffer solutions. However, acceptable recoveries from the spiked urine samples were not obtained. The ELISA results did not correlate well with the GC/MS results, yielding higher concentrations of 2,4-D in all the urine samples tested. The correlation coefficient between the ELISA and GC/MS data was 0.6. The ratio of the ELISA and GC/MS data ranged from 2 to 55. The higher bias of 2,4-D levels in the ELISA was attributed to interferences from the 10-fold concentrated urine matrix, indicating either a clean up step or a reduction in the urine concentration would be necessary.

The sample matrix effects in the ELISA were further investigated to determine the optimum urine concentration for analysis and to develop an overall streamlined method. A 2,4-D standard curve (0.78–400 ng/mL) was prepared in triplicate in a 2.5-fold urine concentrate prepared in 10% methanol/PBST (Section 2.2) using doubling dilutions. A second dilution series of 2,4-D in 10% methanol/PBST (without the urine matrix) was assayed on the same plate. Both sets of data were fit to four-parameter curves. Fig. 1 shows the mean response of the standards prepared with (squares) and without (circles) the urine matrix. The urine matrix depressed the response of the assay from a maximum absorbance of approximately 1.1 optical density (OD) units in the 10% methanol/PBST buffer to approximately 0.4 OD units in a dose dependent manner. These data suggested that

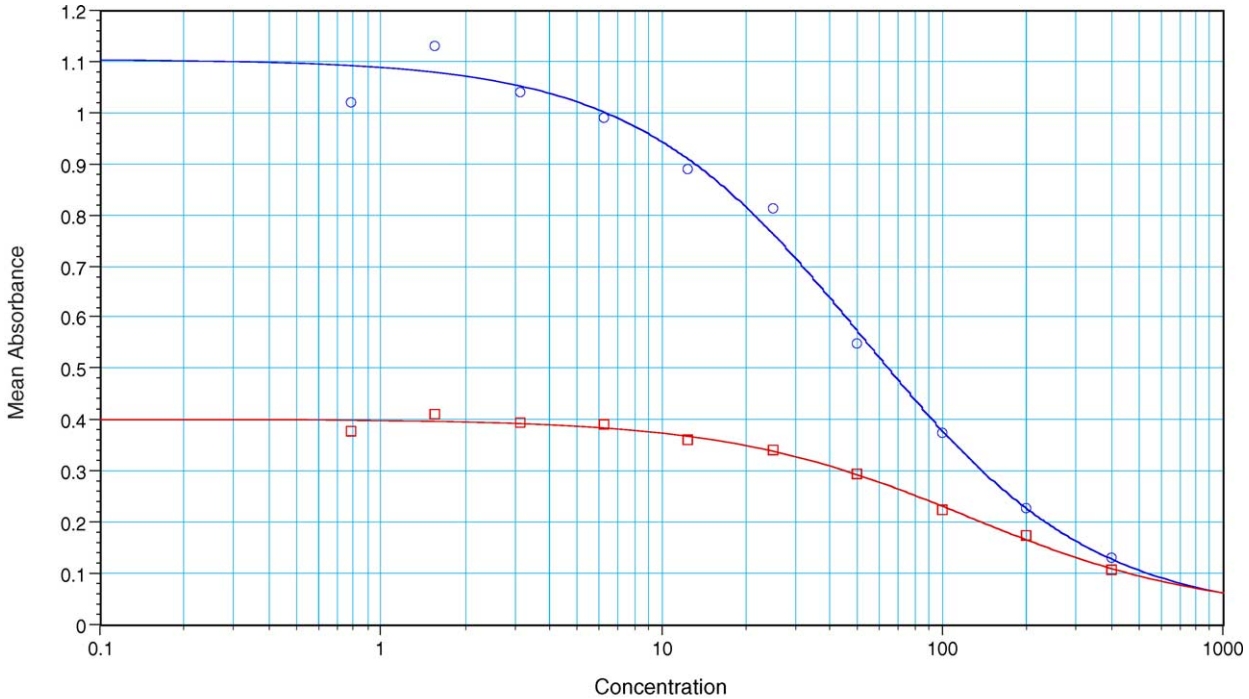


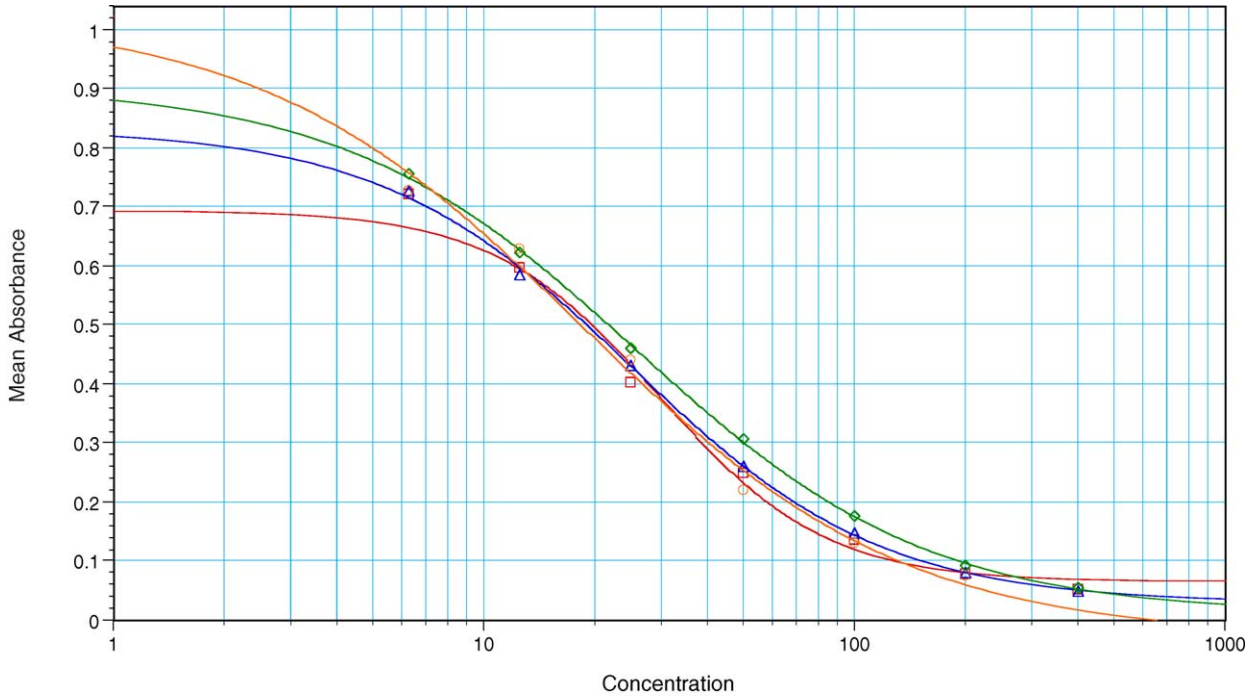

Fig. 1. ELISA calibration curves for 2,4-D without (circles), with (squares) hydrolyzed 2.5-fold urine concentrate in 10% methanol/PBST.

unknown components in the urine matrix inhibited the ability of the antibody to bind to the reference antigen immobilized on the assay plate. The maximum amount of urine matrix that could be tolerated in the assay without a clean up step was determined by analyzing a constant amount (100 ng/mL) of 2,4-D in several dilutions of the 2.5-fold urine concentrate. Results indicated that a 1:8 dilution of the 2.5-fold urine concentrate (yielding 31.2% urine) minimized the adverse effect on the ELISA performance. Three levels (31.2, 15.6, and 7.8%) were chosen for further testing. Note that the 31.2, 15.6, and 7.8% urine in 10% methanol PBST were prepared from dilutions (1:8, 1:16, and 1:32) of the 2.5-fold urine concentrate (Section 2.2).

Standard curves of 2,4-D (6.25–400 ng/mL) were constructed using 31.2, 15.6, 7.8 and 0% of urine in 10% methanol/PBST, and assayed on the same ELISA plate. The concentrations of 2,4-D in these standard solutions were 400, 200, 100, 50, 25, 12.5, and 6.25 ng/mL. The calibration curves with different urine concentrations are displayed in Fig. 2. Curves, one through three (squares, triangles, and diamonds) in Fig. 2, are plots of the 2,4-D standard solutions prepared in 31.2, 15.6, and 7.8% of urine in 10% methanol/PBST, respectively. The fourth curve (circles) in Fig. 2 shows the plot for the standard solutions prepared in 10% methanol in PBST (containing 0% urine). The curves are superimposable, indicating that at these concentrations, the diluted urine ranging from 7.8 to 31.2% does not interfere with the assay. The average and standard deviation of the % recovery for the calculated values of each standard solution in triplicate are summarized in Table 1. With few exceptions, the percent relative standard deviation (%R.S.D.) was within ±10% for standard solutions prepared with and without the urine matrix. The calculated concentrations derived from the calibration curve were above 90% of that expected for most standards. In summary, the analyte can be accurately quantified in diluted urine (7.8–31.2%). A dilution factor of 1:5 (20% urine) was selected for the finalized ELISA method described in Section 3.2.

### *3.2. Streamlined ELISA method evaluation for 2,4-D in urine*

A simple ELISA procedure was established for the determination of 2,4-D in urine samples as described in Section 2.3. Note that the cross-reactivity of the antibody with 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) was 13.8 and 9.5%, respectively, as previously reported [26]. Cross-reactivity with structural analogs of other chlorophenoxyacetic acids was less than 3% [26]. As expected, the 2,4-D antibody had a high cross-reactivity for 2,4-D methyl ester (104.8%). To negate any sample matrix effect, the standard solutions




| y = ( (A - D)/(1 + (x/C)^B ) ) + D: | A | B | C | D | R^2 |
|---|---|---|---|---|---|
| 31.2% Urine Matrix (10% Methanol/90% PBST) | 0.694 | 1.935 | 29.672 | 0.065 | 0.986 |
| 15.6% Urine Matrix (10% Methanol/90% PBST) | 0.832 | 1.286 | 24.87 | 0.029 | 0.999 |
| 7.8% Urine Matrix (10% Methanol/90% PBST) | 0.905 | 1.1 | 25.751 | 0.011 | 1 |
| 0% Urine Matrix (10% Methanol/90% PBST) | 1.025 | 1.002 | 18.391 | -0.029 | 0.995 |

Fig. 2. Calibration curves for 2,4-D with 31.2% (squares), 15.6% (triangles), and 7.8% (diamonds), and 0% (circles) of urine sample matrix in 10% methanol/PBST (urine prepared from dilutions of a 2.5-fold urine concentrate).

Table 1
Average recovery for calculated concentrations of standards in PBST/10% methanol with various urine concentrations

| Standard 2,4-D (ng/mL) | Recovery (%) | | | |
|---|---|---|---|---|
| | 10% Methanol in PBST without urine matrix | 10% Methanol in PBST with 7.8% urine matrix | 10% Methanol in PBST with 15.6% urine matrix | 10% Methanol in PBST with 31.2% urine matrix |
| 400 | NA | 97 ± 7.6 | 109 ± 16 | NC |
| 200 | 85 ± 3.8 | 106 ± 4.9 | 102 ± 3.2 | 107 ± 5.4 |
| 100 | 104 ± 1.0 | 99 ± 7.0 | 97 ± 5.0 | 86 ± 7.0 |
| 50 | 119 ± 9.8 | 98 ± 9.5 | 100 ± 4.2 | 94 ± 2.6 |
| 25 | 92 ± 6.8 | 102 ± 7.8 | 99 ± 7.0 | 110 ± 11 |
| 12 | 89 ± 1.0 | 102 ± 6.0 | 106 ± 3.6 | 98 ± 25 |
| 6 | 116 ± 16 | 96 ± 20 | 91 ± 21 | NC |

NA, not analysed; and NC, not calculated because the value was outside the calibration range.

and QC spikes were prepared in 80% PBST/20% DFU. As shown in Fig. 3, a typical standard curve consisted of 10 doubling dilutions of 2,4-D ranging from 3.9 to 2000 ng 2,4-D/mL of DFU (or 0.78–400 ng 2,4-D/mL of 80% PBST/20% DFU). Based on GC/MS analysis, only a trace amount (2.63 ± 0.06 ng/mL) of 2,4-D was present in the DFU. The 20% concentration of DFU did not contribute a significant amount of 2,4-D in the standard solutions. The shape of the curve was consistent from day to day. The ELISA had a 50% inhibition ($IC_{50}$) value of approximately 80 ng/mL. The QC spikes prepared in 80% PBST/20% DFU were analyzed in three separate assays conducted on different days to determine the maximum quantitation limit. Recoveries of the highest QC spike sample (1000 ng 2,4-D/mL DFU) were 130 and 80%, respectively, in assays 1 and 3 but were greater than 130% in assay 2 (out of range). Thus, 1000 ng 2,4-D/mL-urine was considered beyond the maximum quantification limit of the assay. The 250 ng/mL QC spike was accurately measured in all three assays with an average recovery of 103 ± 1%. The 31 and 15.6 ng/mL QC spike samples were tested in assays 2 and 3. The measured concentration for the 31 ng/mL QC spike was 93 and 107% of the expected values in assays 2 and 3, respectively. Day-to-day variation for these two assays was within ±10%. Recoveries of the 15.6 ng/mL QC spike were 54% in assay 2 and 120% in assay 3, indicating that this level was below the quantification limit of the method. In summary, the working range of the ELISA was 30–800 ng 2,4-D in 1 mL of urine. Three human urine samples were also tested in these three assays concurrently with the QC spike samples (Table 2). Day-to-day assay variation for the human urine samples was within ±18%.

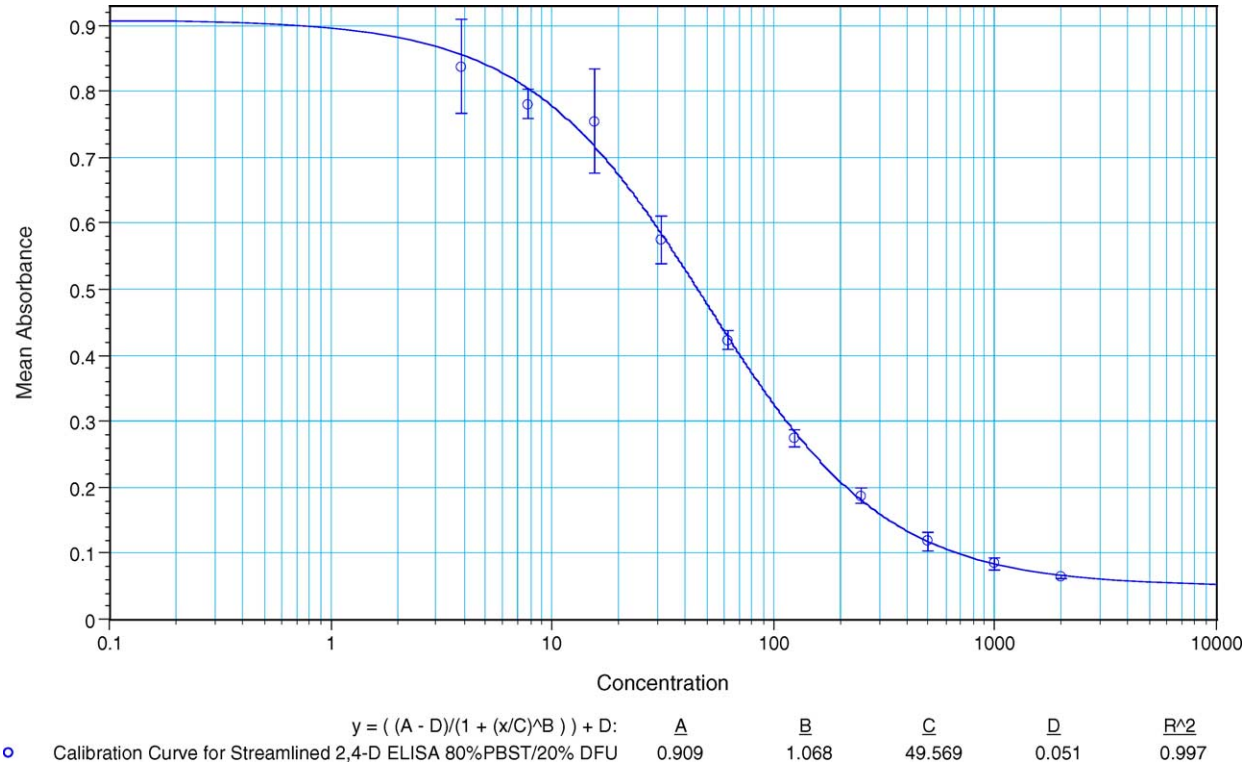



Fig. 3. Calibration curve of streamlined ELISA method for measuring 2,4-D in DFU standard, diluted 1:5 in 80%PBST/20%DFU.

Table 2
Day-to-day variations of urine samples analyzed by streamlined ELISA method[a]

| Sample code | Dilution factor[b] | Day-1 (ng/mL) | Day-2 (ng/mL) | Day-3 (ng/mL) | Average (ng/mL) | R.S.D. (%) |
|---|---|---|---|---|---|---|
| 204-A | 1 | 109 | 152 | 138 | 133 | 16 |
| 212-B | 1 | 440 | 307 | 490 | 420 | 18 |
| | 5 | | 472 | 370 | | |
| 212-C | 5 | – | 793 | 893 | 826 | 10 |
| | 10 | | 725 | | | |

[a] Each reported concentration is the average concentrations of triplicate assays.
[b] Dilution factor 1 denotes 20 μL of the urine sample and 80 μL of PBST was placed in the well; dilution factors 5 and 10 denotes 1:5 and 1:10 dilutions of the above diluted urine sample using 80% PBST/20% DFU as diluent.

### 3.3. Streamlined 2,4-D ELISA for human urine samples

Fifty urine samples received without identifiers from a subset of pesticide applicators and their spouses in the EPA PES [27] were analyzed by the streamlined ELISA method as described in Section 2.3. Each set of urine samples was analyzed using a calibration series of 10 concentrations of 2,4-D standard solutions (triplicate analyses). Data acceptance criteria for the 96-microwell-plate assay were established and used as guidance for the urine analysis. The four-parameter curve-fit values of: (a) upper asymptote, (b) slope, (c) $IC_{50}$, and (d) lower asymptote were generated for each calibration curve. The %R.S.D. values of the triplicate analyses were less than 30% for all samples and standard solutions. The % differences of the QC spike samples were within 30% of the expected values. If the QC spike samples were out of this acceptance range the samples were reassayed.

For each assay, triplicate values were obtained for all urine samples. The means of the triplicate values were used to calculate the final concentration of each sample. Sample concentrations of 2,4-D ranged from <30 to 2480 ng/mL. Of the 50 urine samples analyzed by ELISA, two samples had no detectable concentration of 2,4-D. Six samples had detectable levels of 2,4-D, with levels below the quantification limit of the assay (∼30 ng/mL). The remainder of the samples contained between 40 and 2480 ng/mL 2,4-D as measured by ELISA. Eighteen urine samples were reanalyzed over different days to obtain ELISA performance data. The %R.S.D. values of these replicate analyses (day-to-day variation) were between 1.2 and 20%, indicating good inter-assay reproducibility. Quantitative recoveries were obtained for the spiked urine samples ranging from 70 to 124%. In summary, the overall accuracy for the streamlined ELISA method was greater than 70% and the overall assay precision was within ±20% for urine samples.

### 3.4. Comparison of ELISA and GC/MS data

Table 3 summarizes the 2,4-D concentrations (ng/mL) of the 50 urine samples using the streamlined 2,4-D ELISA and GC/MS methods. The summary statistical data for the measured 2,4-D in the urine samples ranged from non-detectable to 2480 ng/mL with a mean of 365 ± 517 ng/mL for the ELISA and from non-detectable to 2500 ng/mL with a mean of 448 ± 551 ng/mL for GC/MS. In general, similar 2,4-D concentrations were observed in these urine samples between the two detection techniques.

Fig. 4 displays the relationship between the data from the 2,4-D ELISA and the GC/MS methods. The ELISA method has a much simpler sample preparation step consisting of only a dilution. The GC/MS method involves an acid extraction, concentration, methylation, and clean up by solid phase extraction. Overall, there was good correlation between the ELISA and GC/MS methods for this set ($N = 50$) of urine samples (correlation coefficient, $r = 0.94$, $R^2 = 0.89$, and slope = 1.00). The ELISA method as applied to diluted urine samples provided quantitative data comparable to the GC/MS results, with a quantification limit of approximately 30 ng/mL. This quantification limit would enable the use of the ELISA method as a screening tool to identify highly exposed individuals or as a quantitative measuring tool in occupation health studies.

Table 4 summarizes the method precision, accuracy, quantification limit, and sample throughput between the ELISA and GC/MS methods for monitoring 2,4-D in human urine samples. The overall method accuracy and precision are similar between the two methods. The estimated quantification limit is lower for the GC/MS method. This is primarily because a 10-fold concentration was coupled to a solid phase cleanup in the GC/MS method while a 5-fold sample dilution without any clean up was used in the ELISA method. It is possible to lower the quantification limit of the ELISA method if a clean up step (SPE or immunoaffinity chromatography) is implemented to remove the urine matrix effects prior to detection. However, for this study, the streamline ELISA approach using diluted urine samples provided satisfactory results. The estimated sample throughput by the streamlined ELISA method is about twice of the GC/MS method due to

Table 3
Summary statistics for 2,4-D concentrations in urine samples

| Summary statistics | Streamlined ELISA (ng/mL) | GC/MS (ng/mL) |
|---|---|---|
| Sample size | 50 | 50 |
| Mean | 365 | 448 |
| Standard deviation | 517 | 551 |
| Minimum | <30 | <0.1 |
| Maximum | 2480 | 2500 |

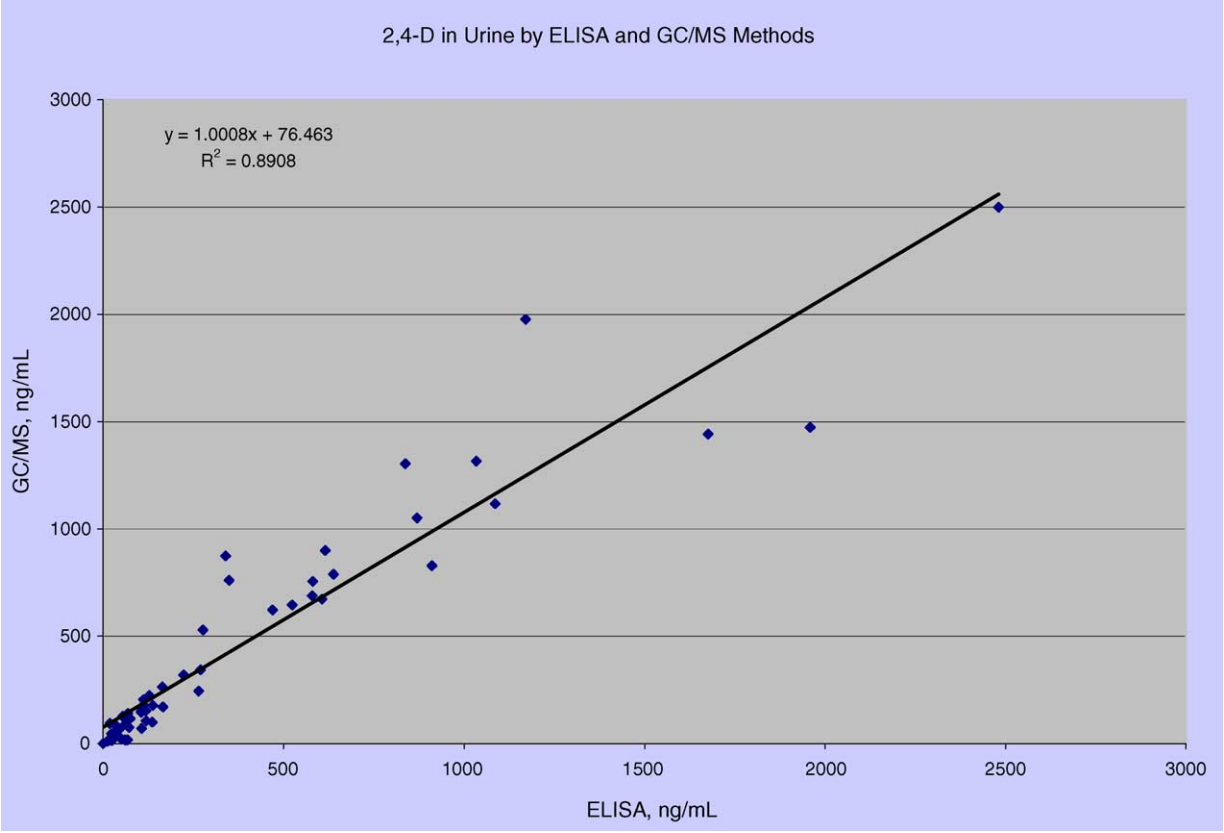



Fig. 4. Comparison of streamlined ELISA and GC/MS data for 50 human urine samples. ELISA concentrations were the mean values of triplicate measurements.

Table 4
Comparative analyses of accuracy, precision, detection limit, and sample throughput for the ELISA and GC/MS methods

| | ELISA | GC/MS |
|---|---|---|
| Accuracy[a] | 70–130% | 70–130% |
| Precision | ±30% | ±30% |
| Quantification limit | ~30 ng/mL of urine | ~0.2 ng/mL of urine[b] |
| Sample throughput[c] | ~40 samples/3 days | ~20 samples/3 days |

[a] Accuracy was based on the recovery of the spiked urine samples and precision was based on the % relative standard deviation of triplicate urine samples.

[b] The estimated quantification limit for GC/MS method was based on 10 mL of a urine sample processed into a final volume of 1 mL.

[c] The estimated sample throughput for the ELISA method is based on the assumption that the samples outside the calibration range will be diluted and reassayed. The estimated sample throughput includes sample preparation steps and triplicate sample analyses by ELISA.

the simpler sample preparations, which also lowers analytical costs. Note that the estimated costs do not include the purchase of a GC/MS system, ELISA photometer, or the specific immunologic reagents.

## 4. Conclusion

A streamlined and high sample throughput ELISA method was developed and compared with GC/MS for the determination of 2,4-D in urine. The data from the two methods were highly correlated for spiked and real-world samples. Linear relationships were observed between the ELISA and the GC/MS data for 50 human urine samples. In conclusion, the ELISA method could be used for quantitatively measuring 2,4-D in urine samples in a cost-effective manner for large-scale measurement studies. If lower detection levels are required, the ELISA could be coupled with a clean up procedure such as SPE or immunoaffinity chromatography.

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