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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.
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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 (2×5 mL) and a 20% sodium chloride solution (2×1 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.

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