Practical Immunochemical Method for Determination of 3,5,6-Trichloro-2-pyridinol in Human Urine: Applications and Considerations for Exposure Assessment

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An analytical method is described for the quantitative determination of 3,5,6-trichloro-2-pyridinol (3,5,6-TCP) in human urine. This is the primary analyte found in urine as a result of exposure to chlorpyrifos, chlorpyrifos-methyl, triclopyr, or 3,5,6-TCP. Conjugates of 3,5,6-TCP are released from urine by acid hydrolysis. The free 3,5,6-TCP is purified using C_{18} solid-phase extraction, eluting the analyte with 1-chlorobutane. An aliquot of 1-chlorobutane is placed in a vial containing Trichloropyridinol Sample Diluent and evaporated, leaving the 3,5,6-TCP in the aqueous sample diluent. The samples are assayed using the Trichloropyridinol RaPID Assay immunoassay test kit. Final results are calculated using a standard curve constructed by linear regression after a ln/Logit data transformation is performed of the concentration and the absorbance readings, respectively. The calculated lower limit of quantitation for 3,5,6-TCP in fortified control urine samples is 2.96 ng/mL (2.96 ppb). Residues of 3,5,6-TCP determined using both immunochemical and gas chromatography with mass spectrometric detection correlate well.

Keywords: *3,5,6-Trichloro-2-pyridinol; chlorpyrifos; triclopyr; immunoassay; ELISA; urine; exposure assessment*

INTRODUCTION

Chlorpyrifos is a broad-spectrum, nonsystemic insecticide used to control a variety of insect pests with both crop and noncrop applications. It is the active ingredient of Dursban and Lorsban. Chlorpyrifos is one of the most widely used insecticides in the United States, with 4500−7000 t (10−15 million pounds) applied per year for crop protection and 4000−5500 t (9−12 million pounds) applied per year for nonagricultural or urban uses (U.S. EPA, 1994). Chlorpyrifos has been used for the control of insect infestations in crops and in urban settings for ∼30 years. Because of this diverse use profile, it is anticipated that low-level baseline exposures to chlorpyrifos and its primary metabolite, 3,5,6-trichloro-2-pyridinol, occur within the U.S. population (Hill et al., 1995; Shurdut et al., 1998).

The major urinary metabolite of chlorpyrifos (as well as chlorpyrifos-methyl and triclopyr) is 3,5,6-TCP (3,5,6-trichloro-2-pyridinol). The 3,5,6-TCP is rapidly excreted in human urine after exposure to chlorpyrifos, chlorpyrifos-methyl, or triclopyr (Nolan et al., 1984; Bartels and Kastl, 1992; Ormand et al., 1998) (Figure 1). The determination of 3,5,6-TCP is performed in human urine using negative-ion chemical ionization gas chromatography/mass spectrometry (GC/MS) (Bartels and Kastl, 1992; Ormand et al., 1998).

Immunochemical detection of analytes in urine is widely practiced in clinical chemistry. Only recently have investigators begun to describe urinary immunoassay as a screen for potential exposures to a variety of compounds, including pesticides. By correlating analytes in the urine with knowledge of product pharmacokinetics, absorbed doses from all potential routes of exposure may be estimated accurately (Nolan et al., 1984). Although assays are generally sensitive, specific, and accurate, unique and varying degrees of sensitivity and cross-reactivity can occur, which can lead to misinterpretation of data by overestimation of apparent analyte present (Feng et al., 1990; Biagini et al., 1995). A carefully validated method is necessary to avoid potential interference with performance from conjugates or other products excreted in the urine which may lead to possible overestimation of exposures. A new immunochemical analytical method is described for the rapid quantitative determination of 3,5,6-TCP in human urine resulting from chlorpyrifos, chlorpyrifos-methyl, or triclopyr exposure. The method employs the release of conjugated 3,5,6-TCP by acid hydrolysis and sample purification using a C_{18} solid-phase extraction (SPE) cartridge prior to assay using a magnetic particle-based immunoassay (ELISA) kit.

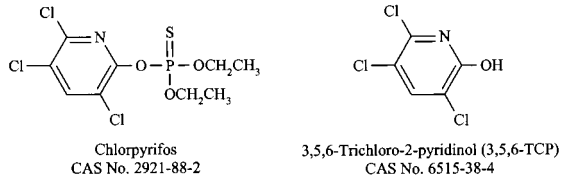

Figure 1. Structures of chlorpyrifos and 3,5,6-trichloro-2-pyridinol.

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An immunochemical method validation study is conducted in which untreated control urine samples are fortified with 3,5,6-TCP over a concentration range of 2.0−200.0 ng/mL. Data from this study are used to generate method recoveries. In addition, a study was performed to compare the immunochemical method with a gas chromatography with mass spectrometric detection (GC/MSD) technique. Twenty-four human urine samples are collected over an 8-day period from four different individuals participating in an occupational exposure study. Samples are analyzed using immunochemical detection and GC/MSD, and the data generated by the two different detection systems were correlated.

EXPERIMENTAL PROCEDURES

Apparatus. *(a) Trichloropyridinol RaPID Assay Kit.* Samples were assayed using the Trichloropyridinol RaPID Assay kit (Strategic Diagnostics Inc., Newark, DE).

(b) SPE Cartridges. C_{18}, 1-g packing, SPE cartridges (Whatman Inc., Clifton, NJ) were used for sample purification. Elution profiles were generated with standards on cartridges to ensure adequate recoveries.

(c) Reacti-Vap Evaporator. A Reacti-Vap (Pierce Chemical Co., Rockford, IL) was used for small-scale sample evaporation.

(d) Vortex Mixer. A Fisher Vortex Genie 2 (Scientific Industries, Inc., Bohemia, NY) was used for sample mixing.

(e) Pipets. A precision pipet capable of delivering 250 and 500 μL was used, along with a repeating pipet capable of delivering 1.0 mL. Eppendorf pipets were used throughout sample analysis (Brinkmann Instruments, Inc., Westbury, NY).

(f) Magnetic separation rack was from Strategic Diagnostics Inc.

(g) Fixed-Wavelength Spectrophotometer (RPA-1 RaPID Analyzer). The RPA-1 RaPID Analyzer, fixed-wavelength spectrophotometer (Strategic Diagnostics Inc.) or equivalent photometer capable of making absorbance readings at 450 nm was used.

(h) 3-Dram (11.1 mL) Glass Vials. Screw-cap, glass vials, 12-mL capacity, with PTFE-lined caps (Fisher Scientific, Pittsburgh, PA) were used throughout sample purification.

Reagents. *(a) Solvents* (acetonitrile, 0.1 N hydrochloric acid, 1.0 N hydrochloric acid, concentrated hydrochloric acid, 1-chlorobutane, HPLC grade water) were all of HPLC grade or better and were purchased from EM Science, Gibbstown, NJ; Fisher Scientific, Fair Lawn, NJ; or Mallinckrodt Baker, Inc., Paris, KY. The trichloropyridinol aqueous diluent was obtained with the trichloropyridinol RaPID Assay kit (Strategic Diagnostics Inc.). The analytical standard of 3,5,6-TCP was obtained from Dow AgroSciences LLC, Indianapolis, IN.

Safety Precautions. Each analyst should be acquainted with the potential biological and chemical hazards of the biological matrix (human urine), reagents, products, and solvents used in this method before commencing laboratory work. Safety information on the reagents and chemicals listed should be obtained from the suppliers in the form of material safety data sheets, literature, and other related data. Disposal of potentially biohazardous matrix materials, reagents, reactants, and solvents must be made in compliance with local, state, and federal laws and regulations. All solvent evaporation steps should be performed in a well-ventilated fume hood away from ignition sources. Protective gloves, proper eye protection, and protective clothing should be worn when working with potentially biohazardous materials and with chemicals.

Fortification Solution Preparation. A stock solution of 3,5,6-TCP was prepared at 1.0 mg/mL in HPLC grade water. An aliquot (1.0 mL) of the stock solution was quantitatively transferred to a 100-mL volumetric flask and diluted to volume with HPLC grade water to obtain a 10 μg/mL solution. An aliquot (10.0 mL) of the 10 μg/mL solution was quantitatively transferred to a 100-mL volumetric flask and diluted to volume with HPLC grade water to obtain a 1.0 μg/mL solution. A 0.1 μg/mL solution was made by taking an aliquot (1.0 mL) of the 10.0 μg/mL solution, quantitatively transferring it to a 100-mL volumetric flask, and diluting it to volume with HPLC grade water.

Calibration Standards. Calibration standards incorporated in the Trichloropyridinol RaPID Assay kit were used for direct comparison with both fortified and unknown samples. Three concentrations (0.5, 2.5, and 6.0 ng/mL) of 3,5,6-TCP standards in buffered saline with preservative and stabilizers are supplied with each kit.

Sample Preparation and Fortification. Previously frozen urine samples were prepared for analysis by thawing at ambient temperature. Thawed or refrigerated samples were warmed to ∼35 °C to dissolve any suspended solids that may be present. (This step was omitted for freshly collected samples.) A 1.0-mL aliquot of urine was taken from each well-mixed sample, and aliquots were placed in a series of 12-mL (3-dram) vials having PFTE-lined caps. For generation of recovery data, untreated control urine samples were fortified with 20.0-μL aliquots of the appropriate 3,5,6-TCP fortification solution. Fortification solutions were dispensed directly into the 1.0-mL urine aliquots to obtain concentrations ranging from 0.002 to 0.2 μg/mL (2.0−200.0 ng/mL). A portion of each sample of the urine used for the preparation of recovery samples was retained to be used as unfortified control samples. A reagent blank (consisting of only HPLC grade or deionized water), containing no sample matrix, was carried through the method with each sample set.

Acid Hydrolysis. To free any conjugated 3,5,6-TCP by acid hydrolysis, samples received 100 μL of concentrated hydrochloric acid (12 N) and were vortex mixed, capped, and placed in an 80 °C water bath for 1 h. Following hydrolysis, samples were cooled in a refrigerator at ∼4 °C for ∼10 min prior to uncapping. After cooling, each sample was diluted with 9 mL of HPLC grade water.

Sample Purification. Samples were purified using reversed phase SPE cartridges that were rinsed with 5 mL of acetonitrile and then conditioned with 5 mL of 0.1 N hydrochloric acid. Columns were not allowed to go to dryness. The diluted urine samples were added to the tops of the SPE cartridges. With the aid of vacuum, the samples were slowly passed through the SPE cartridges at a flow rate of 1−2 mL/min, discarding the eluate. Each sample vial was rinsed with 4.0 mL of a 40% acetonitrile/59% HPLC grade water/1% 1.0 N hydrochloric acid solution, which was quantitatively transferred to the top of the corresponding SPE cartridge. The solution was slowly passed through the cartridge, again with the flow through the cartridge maintained at ∼1−2 mL/min. The rinse was discarded. The SPE cartridges were briefly dried by leaving them attached to the vacuum manifold and drawing air through them for 1−2 min at 20 in. of Hg. The 3,5,6-TCP was then eluted from the SPE cartridges with 5.0 mL of 1-chlorobutane, and the eluate was collected in a 5-mL volumetric flask. Samples were brought to volume (5.0 mL) with 1-chlorobutane, sealed with ground glass stoppers, and mixed well by inverting several times. Elution profiles for 3,5,6-TCP were obtained on each new lot of SPE cartridges prior to purification to ensure optimum recovery and efficiency.

Preparation of Samples for Assay Using the Trichloropyridinol RaPID Assay Kit. New 12-mL (3-dram) vials received 1.0-mL aliquots of the Trichloropyridinol Sample Diluent (Strategic Diagnostics Inc.) found in the Trichloropyridinol RaPID Assay kit. These were weighed, and the weights were recorded. Aliquots of the 1-chlorobutane eluate (1.0 mL) were transferred to each 11-mL vial containing 1 mL of the Trichloropyridinol Sample Diluent. The Trichloropyridinol Sample Diluent and 1-chlorobutane layers were not vortex mixed. The 1-chlorobutane layer of each sample was evaporated completely under a stream of nitrogen using an evaporator at ambient temperature, leaving the 3,5,6-TCP in the Trichloropyridinol Sample Diluent. The volume of the Trichloropyridinol Sample Diluent of each sample was adjusted to the original 1-mL volume by weight by adding aqueous diluent in a dropwise manner to each sample, if

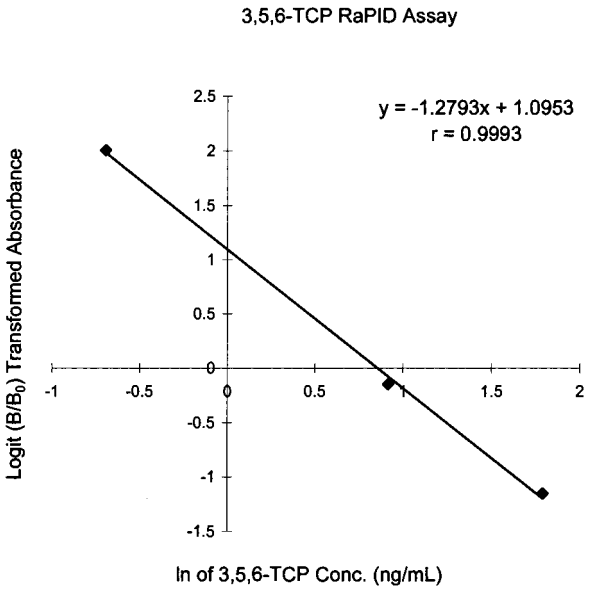


Figure 2. Typical calibration curve for the determination of 3,5,6-TCP in urine.

necessary. A 250-μL aliquot of each standard, the quality control solution, and each sample were analyzed following the instructions found in the Trichloropyridinol RaPID Assay kit insert. Samples known or found to contain between 30.0 and 200.0 ng/mL of 3,5,6-TCP were diluted 10-fold with the trichloropyridinol aqueous diluent prior to analysis to keep them within the working range of the standard curve.

Calculation of Standard Curve Results. The RPA-1 RaPID Analyzer (Strategic Diagnostics Inc.) contained preprogrammed data reduction capabilities that calculated a standard curve for each analytical set using the absorbance readings obtained from the standards supplied with the kit. The calibration curve was constructed by linear regression after a ln/Logit data transformation of the concentration and the absorbance values, respectively, had been performed.

The regression equation (Freund et al., 1991) was as follows (eqs 1 and 2):

$$\text{Logit } B/B_0 = [\text{slope} \times \ln(\text{concn})] + Y - \text{intercept} \quad (1)$$

In eq 1

$$\text{Logit } B/B_0 = \ln (B/B_0)/[1 - (B/B_0)] \quad (2)$$

B = the mean absorbance value measured at each standard 3,5,6-TCP concentration, B_0 = the mean absorbance measured for the 0.00 ng/mL standard, and concn = the 3,5,6-TCP concentration of the standard.

An example of a calibration curve is shown in Figure 2.

The average correlation coefficient (r) for the linear regression analysis describing the detector response as a function of the standard calibration curve over the concentration range of 0.5−6.00 ng/mL was >0.995 for each validation study set.

Calculation of Sample Results. The RPA-1 RaPID Analyzer was used to calculate the concentration of 3,5,6-TCP in each sample using the preprogrammed data reduction parameters. Each sample was assayed in duplicate tubes. Reported were the absorbance value and calculated 3,5,6-TCP concentration for each sample tube as well as the mean absorbance, the mean 3,5,6-TCP concentration, and the percent coefficient of variation (%CV) of the duplicate concentration measurements for each sample. The mean results were reported as the final results for each sample.

The following equations (eqs 3 and 4) were used to calculate the concentration of 3,5,6-TCP in a sample:

$$\text{measured concentration} = e^a \quad (3)$$

where

$$a = [\text{Logit } (B/B_0) - Y \text{ intercept}]/\text{slope} \quad (4)$$

Calculation of Method Factor (eq 5):

$$\text{method factor} = \frac{\text{vol of extraction solvent (mL)}}{\text{vol of sample (mL)}} \times \text{final dilution factor} \quad (5)$$

Thus, for a sample receiving no additional dilution (eq 6)

$$\text{method factor} = (5 \text{ mL}/1 \text{ mL}) \times 1$$
$$\text{method factor} = 5 \quad (6)$$

Quality Control. A quality control solution containing 3 ng/mL of trichloropyridinol (supplied with the Trichloropyridinol RaPID Assay kit) was assayed as part of every batch of samples. Study samples were assayed in duplicate. If the concentration of a sample exceeded the range of the calibration curve, the sample was diluted with the aqueous sample diluent supplied with the RaPID Assay kit (typically a 10-fold dilution is performed), and a diluted sample aliquot was reassayed. The results were multiplied by the appropriate method factor to obtain the final result.

Criteria for Acceptance of an Analytical Batch. The correlation coefficient (r) for the linear regression of each calibration curve was >0.995. The replicate %CV for absorbance was ≤10% for each duplicate pair of calibration standards, and the replicate %CV for the calculated concentration for the quality control sample, the samples fortified for recoveries, and the analytical study samples was ≤20%. The recovery value for the quality control sample should be within ±20% of the expected concentration.

Specificity. Neither chlorpyrifos nor triclopyr, parent compounds of the 3,5,6-TCP urinary metabolite, exhibited any cross-reactivity in the trichloropyridinol assay. Additionally, 37 pesticides, 17 organic/inorganic compounds, and 4 solvents were tested for the potential to interfere with conjugate binding in the trichloropyridinol assay. There was low-level cross-reactivity to fluroxypyr-2-pyridinol, which is a fluroxypyr metabolite; however, there was no cross-reactivity to fluroxypyr itself. None of the pesticides exhibited an IC_{50} concentration below 10.0 μg/mL with the exception of chlorpyrifos-methyl, which had an IC_{50} concentration of 1.85 μg/mL (Figure 3). A variety of commonly used and/or structurally similar pesticides and metabolites demonstrated no reactivity in the Trichloropyridinol RaPID Assay at concentrations up to 10 μg/mL: alachlor, aldicarb, ametryn, atrazine, azinphos-methyl, benomyl, carbaryl, carbendazim, carbofuran, clopyralid, 2,4-D, diazinon, dinoseb, fenitrothion, fluroxypyr, glyphosate, lindane, malathion, MCPA, methamidophos, methomyl, 2-methoxy-3,5,6-trichloropyridine, oxamyl, parathion, parathion-methyl, phosmet, picloram, pirimcarb, pirimphos-ethyl, pirimphos-methyl, propachlor, terbufos, and thiophanate-methyl.

Background Levels of 3,5,6-TCP in Unfortified Control Samples. Control samples were collected from Dow AgroSciences employees. Low background levels of 3,5,6-TCP were found in all unfortified control urine samples that were assayed. These background levels of 3,5,6-TCP typically ranged from 3.0 to 15.0 ng/mL, consistent with ranges found in the general U.S. population (Hill et al., 1995). Unfortified control samples were analyzed with each analytical set to determine the background levels of 3,5,6-TCP for use in background subtraction from samples fortified for determination of method recoveries of 3,5,6-TCP. No background subtraction was performed for samples assayed for comparison of the immunochemical and GC/MSD detection methods.

Confirmation of Residue Identity. Duplicate samples were analyzed by GC/MSD for confirmation of analyte identity. Confirmation ratios were used to determine whether peaks detected at the expected retention time of the analyte are in fact 3,5,6-TCP. If the ion ratio was not within the established

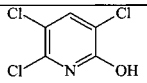
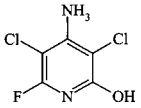
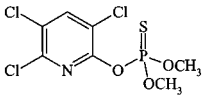
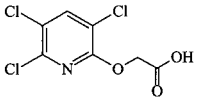
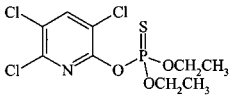
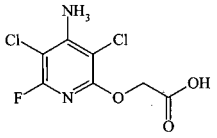
Compound	IC_{50}, ng/mL	% Cross Reactivity	Structure
3,5,6-Trichloro-2-pyridinol	2.3	100.	
Fluroxypyr-2-pyridinol	531.	0.44	
Chlorpyrifos-methyl	1850.	0.40	
Triclopyr	>10,000.	<0.01	
Chlorpyrifos	>10,000.	<0.01	
Fluroxypyr	>10,000.	<0.01	

Figure 3. Cross-reactivity of the Trichloropyridinol RaPID Assay for various pesticides, metabolites, and structurally related compounds.

limits of ±15% of the average found for the calibration standards, the identity of a detected peak was not considered confirmed as 3,5,6-TCP. The peak area ratios of ion *m*/*z* 256 divided by *m*/*z* 254 were calculated for each calibration standard injected (Olberding, 1997).

Using the 3,5,6-TCP Analyte as a Biomarker of Pesticide Exposure. Measurement of 3,5,6-TCP in urine may be observed either following uptake and metabolism of chlorpyrifos, chlorpyrifos-methyl, or triclopyr pesticides or following exposure to the 3,5,6-TCP hydrolysis product of these pesticides. Chlorpyrifos is rapidly hydrolyzed to 3,5,6-TCP following application to either agricultural commodities or residential surfaces such as turfgrass. Hence, urinary 3,5,6-TCP measurements can represent potential exposures to chlorpyrifos, its moieties, and similar pesticides with common metabolites.

Assuming that the urinary concentration of 3,5,6-TCP reflected exposure to chlorpyrifos only, such measurements may be transformed into an estimated chlorpyrifos-equivalent absorbed dose. Concentrations of 3,5,6-TCP may be used to calculate chlorpyrifos equivalent doses using pharmacokinetic data developed by Nolan et al. (1984) and accounting for the molecular weight difference between chlorpyrifos and its primary metabolite, 3,5,6-TCP. The concentration of 3,5,6-TCP should first be normalized for creatinine content. Urinary creatinine can be measured using a modification of the method described by Fabiny and Ertingshausen (1971), which is based on the Jaffe reaction. Endogenous creatinine is a muscular breakdown product and released into bodily fluids at a relatively constant rate. Hence, it is often used as an indicator of urinary clearance for normalization of chemicals cleared in the urine. Following normalization of the 3,5,6-TCP concentrations for creatinine content, the normalized concentration (micrograms of 3,5,6-TCP per gram of creatinine) is multiplied by the standard daily creatinine elimination rate relevant to a given gender and age group (Diem et al., 1970; Tietz, 1987; Snyder et al., 1981). Creatinine excretion varies most dramatically as a function of age and muscle mass, with younger children exhibiting a lower excretion rate than adults. Consequently, the amount of chlorpyrifos absorbed (μg/kg/day) by a subject using urinary 3,5,6-TCP concentrations may be estimated using the following equation:

$$\text{estimated absorbed chlorpyrifos dose } (\mu\text{g/kg/day}) = \frac{A\ (\text{g of creatinine/day}) \times B\ (\mu\text{g of TCP/g of creatinine}) \times C}{D \times E\ (\text{kg})} \quad (7)$$

where A = age-specific daily creatinine excretion rate, B = 3,5,6-TCP concentration normalized for creatinine content, C = molecular weight ratio of chlorpyrifos to 3,5,6-TCP (350.6/198) (Diem et al., 1970; Tietz, 1987; Snyder et al., 1981), D = fraction of absorbed chlorpyrifos dose eliminated in urine as 3,5,6-TCP (0.7151) (Nolan et al., 1984), and E = individual body weight.

Hence, transformation of 3,5,6-TCP results to pesticide equivalent exposure is necessary to ensure complete and accurate interpretation of data. An understanding of the relationship between the analyte and the parent molecule for which it is being used as a as biomarker of exposure is critical to the overall evaluation.

RESULTS AND DISCUSSION

Data generated were summarized from the analysis of urine samples collected from four adult study subjects potentially exposed to chlorpyrifos during occupational exposure. Unfortified control urine samples were analyzed with each analytical set to determine the background levels of 3,5,6-TCP likely attributed to an individual's potential exposures to chlorpyrifos, chlorpyrifos-methyl, triclopyr, and/or the hydrolysis product 3,5,6-TCP. Background concentrations found were subtracted from the control samples fortified with a known amount of 3,5,6-TCP to determine method recovery levels. Actual test samples resulting from the occupational exposure study received no background subtraction and were reported without correction for recovery. Urine samples analyzed for comparison of the immunoassay and the GC/MSD methodology contained levels of 3,5,6-TCP ranging from approximately 2.6 to 85.5 ng/mL. Random samples from the exposure study were selected for analysis by immunoassay to avoid bias. Determination of 3,5,6-TCP in urine samples was performed following the sample preparation and purification procedures described herein, and samples were assayed following the Trichloropyridinol RaPID Assay kit procedure. Comparative data for this method were generated, and the levels of 3,5,6-TCP were quantified and confirmed by GC/MSD. Comparative data are shown in (Figure 4).

Recovery of 3,5,6-TCP was achieved by hydrolysis of samples for release of conjugated 3,5,6-TCP, followed by purification by chemical separations. Validation of the method involved two separate studies: a method validation study for the immunochemical method and additionally a correlation study between the immunochemical method with immunochemical detection and the methodology employing GC/MSD. The immunochemical method validation study was performed to demonstrate acceptable recovery of 3,5,6-TCP from fortified samples fortified over a concentration range of 2.0−200.0 ng/mL above background levels of 3,5,6-TCP found in the corresponding control samples. The average recovery at 2.0 ng/mL, the limit of quantitation (LOQ) at which the method validated, was 90 ± 15%. Average recoveries at 20.0 and 200.0 ng/mL were 92 ± 9 and 96 ± 6%, respectively. Recovery over the entire validation

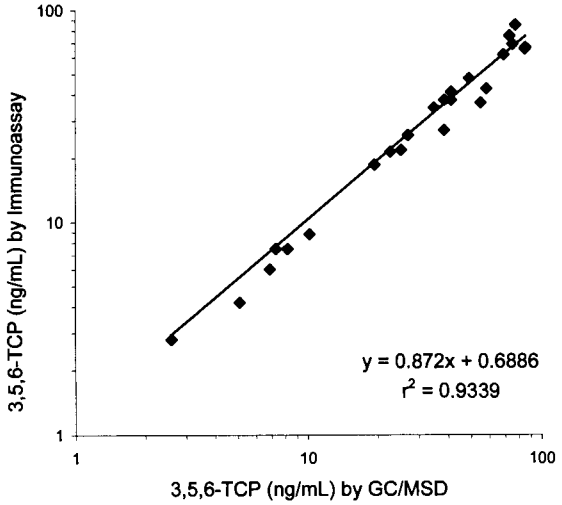


Figure 4. Determination of 3,5,6-TCP in urine by immunoassay versus GC/MSD.

Table 1. Summary of Recovery of 3,5,6-TCP from Urine during Method Validation Study

matrix	fortification level, ng/mL	*n*	% recovery mean	SD	% RSD
control urine	2.0	18	90	15	16.6
	20.0	10	92	9	9.4
	200.0	11	96	6	5.9
	overall	39	92	11	

range of the method was 92 ± 11% (Table 1). Selectivity and sensitivity were sufficient to reliably measure 3,5,6-TCP over the concentration range of 2.0−200.0 ng/mL in human urine samples. Results obtained during the correlation study using the immunochemical methodology and detection demonstrate good agreement with the results obtained by GC/MSD for the quantitative determination of 3,5,6-TCP in human urine samples obtained from an occupational exposure study.

The time required to prepare samples, perform the analysis, and generate the final data on a typical immunochemical analytical set consisting of 25 samples, 4 standards, and the quality control sample, in duplicate, is ∼7−8 h. Sample preparation time for GC/MSD analysis was similar to that required for immunochemical analysis; however, GC/MSD analysis requires overnight injection of samples followed by integration of chromatographic peaks and spreadsheet summarization.

Background levels of 3,5,6-TCP typically ranged from 3.0 to 15.0 ng/mL in control samples, which were consistent with low background levels previously measured in the general U.S. population as reported by Hill et al. (1995). Control urine samples were fortified at 2.0, 20.0, and 200.0 ng/mL above background for the purpose of the determination of percent recovery of 3,5,6-TCP using the method. Control samples fortified at 2.0 ng/mL above the background concentration of 3,5,6-TCP were used for calculating the limit of detection (LOD) and the LOQ of the method. Calculations were performed on the samples fortified at 2.0 ng/mL above background with background subtraction of the concentration levels of 3,5,6-TCP found in the control samples. Calculation of the LOD and LOQ was made following a published technique (Keith et al., 1983), using the standard deviation of the 3,5,6-TCP concentrations found in samples fortified at the lowest fortification level of 2.0 ng/mL (with background subtraction) for the validation study. By this technique, the LOD was calculated as three times (3σ) the standard deviation of the concentrations found, and the calculated LOQ for the validation study was calculated as 10 times (10σ) the standard deviation of the concentrations found at the lowest fortification level of 2.0 ng/mL (Table 1).

During the immunochemical method validation study, 18 samples were fortified at 2.0 ng/mL above background levels of 3,5,6-TCP and were analyzed on several different days. The calculated LOQ for the method obtained during the validation study was 2.96 ng/mL. The calculated LOD of the method generated during the validation study was 0.89 ng/mL.

Preliminary method development began by taking urine samples, simply diluting them 1:10 with water, and analyzing the samples directly by immunoassay in hope that any conjugated 3,5,6-TCP would react similarly to free 3,5,6-TCP during immunoassay. The result was underestimation of recovery values as compared to the GC/MSD assay, thus indicating that hydrolysis of the urine to release conjugated 3,5,6-TCP was a necessary step prior to immunoassay. The use of a liquid−liquid partition between 1-chlorobutane and hydrolyzed urine matrix, saturated with sodium chloride, but which did not incorporate the SPE purification step in sample preparation procedure, led to overestimations of 3,5,6-TCP in urine samples in the range of 2−20 times the results expected on the basis of the GC/MSD assay. The cause for these overestimations was unknown but believed to be due to several factors including the antibody sensitivity relative to 3,5,6-TCP and conjugated metabolites as well as matrix interferences liberated during the hydrolysis step. Once the SPE purification step was incorporated into the sample preparation, residues of 3,5,6-TCP, determined following the immunochemical methodology, correlated very well with results obtained by GC/MSD. A correlation coefficient (r^2) of 0.9339 was obtained from the comparison of data collected by the immunochemical methodology as compared to the GC/MSD methodology (Figure 4). These results clearly demonstrate that it is very important to carefully validate an immunochemical method for determination of urinary metabolites to avoid any under- or overestimation of final results. It is equally important that the results achieved by immunochemical methodology be compared to an alternate analytical technique during validation. A properly validated immunochemical method can be an effective tool for measuring 3,5,6-TCP as a surrogate for a chlorpyrifos absorbed dose. Knowledge of pharmacokinetic dynamics as well as temporal patterns of potential exposure are absolutely necessary for proper interpretation of results.

It is important to recognize that the immunoassay kit was to be treated as a reagent that is incorporated into an analytical procedure—the immunochemical method. The methodology described here was validated using reagents manufactured by Strategic Diagnostics Inc., following the Trichloropyridinol RaPID Assay kit procedure. Modifications to this method are not recommended without prior validation. The data presented in this paper demonstrate the suitability of this immunochemical method for the determination of residues of 3,5,6-TCP in human urine samples.

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