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An Immunoassay to Evaluate Human/Environmental Exposure to the Antimicrobial Triclocarban

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Abstract

A sensitive, competitive indirect enzyme linked immunosorbent assay (ELISA) for the detection of the antimicrobial triclocarban (TCC) was developed. The haptens were synthesized by derivatizing the *para* position of a phenyl moiety of TCC. The rabbit antisera were screened and the combination of antiserum #1648 and a heterologous competitive hapten containing a piperidine was further characterized. The IC_{50} and the detection range for TCC in buffer were 0.70 and 0.13–3.60 ng/mL, respectively. The assay was selective for TCC, providing only low cross-reactivity to TCC-related compounds and its major metabolites except for the closely related antimicrobial 3-trifluoromethyl-4,4′-dichlorocarbanilide. A liquid-liquid extraction for sample preparation of human body fluids resulted in an assay that measured low part per billion levels of TCC in small volumes of the samples. The limits of quantification of TCC were 5 ng/mL in blood/serum, and 10 ng/mL in urine, respectively. TCC in human urine was largely the *N*- or *N*′-glucuronide. TCC concentrations of biosolids measured by the ELISA were similar to those determined by LC-MS/MS. This immunoassay can be used as a rapid, inexpensive and convenient tool to aid researchers monitoring human/environmental exposure to TCC to better understand the health effects.

Introduction

The chlorinated diphenylurea antimicrobial compound, triclocarban (3,4,4′-trichlorocarbanilide; TCC) is widely used as an active ingredient in personal care products, mostly added to consumer deodorant soaps. TCC is active predominantly against gram-positive bacteria.[1] A study reported that around 30% of bar soaps on the market contain this antimicrobial.[2] Exposure to TCC occurs through the use of personal care products containing TCC with the main route of exposure likely to be dermal. In the environment, TCC has been detected at μg/L levels in surface water, suggesting wide ranging contamination of aquatic ecosystems in the United States.[3,4] TCC partitions to sewage

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Supporting Information
Details of hapten synthesis, antibody screening, optimization, recovery studies and LC-MS/MS conditions are referred to in this article as SI. This information is available free of charge via the Internet at http://pubs.acs.org.

sludge and survives anaerobic digestion.[5] Biosolids from wastewater treatment are often applied to land as fertilizer, providing another potential route of entry to the aquatic environment and possibility for accumulation in soil.

The potential of this compound for bioaccumulation and exposure has raised public concern regarding its possible effects on human health due to unintended biological/physiological effects, toxicity, and microbial resistance.[6–11] Indeed, the state of California has listed triclocarban as a Designated Chemical in their Environmental Contaminant Biomonitoring Program.[12] Studies have reported that TCC enhances the activity of steroid hormones such as androgens and estrogens in *in vitro* biological tests.[13,14] TCC enhanced the production of embryos in the freshwater mudsnail.[15] TCC and its urea analogs strongly inhibited the activity of soluble epoxide hydrolase that plays an important role in metabolism of fatty acid epoxides to their corresponding 1,2-diols leading to the accumulation of epoxyeicosatrienoic acids and other epoxides in the organism.[16,17] In light of these biologies and that TCC is clearly absorbed during showering,[18,19] further investigations into the biological and toxicological effects of TCC are warranted. A routine assay for screening or monitoring TCC would be a useful research tool as epidemiological screens for this compound are of increasing interest.

Current analytical methods based on high-performance liquid chromatography (HPLC)-tandem mass spectrometry (MS) or gas liquid chromatography (GC)-MS for the detection of TCC in samples such as urine, blood, water, and biosolids include sample preparation steps such as hydrolysis of *N* or *N*′-glucuronide conjugates of TCC, liquid-liquid extraction and/or solid phase extraction, and a derivatization step of two secondary amines of TCC.[4,20–22] These instrumental methods are sensitive and precise for this target analyte, and are the method of choice when a research project investigates a delicate and varying series of actions using limited samples. However, for a large number of samples, where a single analyte is of interest, an inexpensive, simple, rapid and high throughput method is needed. Immunoassay methods were described as a rapid screening method for environmental contaminants, pesticides, and their degradation products in environmental chemistry.[23] These techniques are widely used in clinical diagnostics, environmental monitoring, food quality, agriculture, and field or on-site testing of personnel exposed to toxic chemicals and are particularly useful for laboratories that do not have LC-MS/MS capability as well as other resource limited settings. The objective of this study is the development of an enzyme-linked immunosorbent assay (ELISA) for the analysis of TCC using a polyclonal antibody as a simple monitoring tool.

Experimental

Chemicals and Instruments

The hapten coupling reagents, bovine serum albumin (BSA), conalbumin (CON), thyroglobulin (Thy), goat anti-rabbit IgG peroxidase conjugate (GAR-HRP), Tween 20, 3,3′,5,5′-tetramethylbenzidine (TMB), and carbanilide were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Triclocarban (Sigma-Aldrich) was recrystallized three times from hot methanol to yield > 99.9% pure material as measured by HPLC[13]. 3-Trifluoromethyl-4,4′-dichlorocarbanilide (TFC) was from Chembridge (San Diego, CA) while triclosan was from Fluka (St. Louis, MO). 2′-Hydroxy-triclocarban (2′-OH TCC), its sulfate conjugate, diuron, 4,4′-dichlorocarbanilide and seH inhibitor #1555 and #1709 were synthesized by condensing the appropriate isocyanate and amine according to previously published methods[24,25]. ELISA was performed on 96-well microtiter plates (Nunc MaxiSorp, Roskilde, Denmark) and read spectrophotometrically with a microplate reader (Molecular Devices, Sunnyvale, CA) in dual wavelength mode (450–650 nm). LC-MS/MS analysis was conducted by electrospray ionization in negative mode using a Quattro Ultima

tandem quadrupole mass spectrometer (Waters, Milford, MA) in multiple reaction monitoring mode interfaced with a Shimadzu LC-10A separation module (Shimadzu, Columbia, MD) utilizing a C8 Zorbax column. Details of the separation and detection conditions are described in the Supporting Information.

Hapten Synthesis

Because TCC is of small molecular weight, it requires conjugation to carrier proteins in order to be immunogenic. TCC haptens containing a reactive carboxylic acid or a primary amine group were designed and synthesized in this laboratory. Five classes of hapten were utilized (Figure 1). The main reactions to prepare haptens containing a chlorinated diphenyl urea moiety were based on methods previously described involving condensation of an isocyanate and an amine.[24,26] The synthesis of haptens is detailed in the Supporting Information.

Preparation of Immunogen and Coating Antigens

For haptens with a reactive carboxylic acid group, conjugation to proteins was made by the *N*-hydroxysuccinimide (NHS) or sulfo-NHS-carbodiimide method, and for haptens with an amine group by the diazotization method,[27] respectively. Types A, B, and C haptens (Figure 1) were conjugated to Thy for immunogen preparation. Types A, B, C, D and E haptens were conjugated to BSA or CON to prepare coating antigens.

Sulfo-N-hydroxysuccinimide (NHS) Method—Haptens type A, B, and C were coupled covalently with the lysine moieties of carrier proteins. That is, each hapten (0.02 mmol) was dissolved in 1 mL of dimethylformamide (DMF) with sulfo-NHS (0.024 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, 0.024 mmol). After the mixture was stirred overnight, the active ester was added slowly to a solution of Thy (25 mg of protein in 1 mL of 0.05 M borate buffer at pH 8) with vigorous stirring. The reaction mixture was stirred gently at 4 °C for 24 h to complete the conjugation. The conjugates were dialyzed (Spectra/Por, molecular weight cut off 6–8,000, Spectrum Laboratories, Rancho Dominguez, CA, USA) against PBS for 36 hrs with buffer changes every 12 h and stored at −20°C until use.

Diazotization Method—Hapten type D was covalently conjugated to the carrier protein. That is, 0.2 N sodium nitrite (0.5 mL) were added dropwise to a solution of hapten D1 (0.03 mmol) dissolved in a mixture of a few drops of ethanol and 0.2 N HCl (0.5 mL) until a positive starch iodide test was confirmed. The reaction vial was cooled with ice while the solution was stirred. DMF (0.3 mL) was added and stirred for 10 min, and the solution was divided into two equal aliquots. One aliquot was added to a solution of BSA, the other to a solution of CON. The BSA (25 mg) and the CON (25 mg) were dissolved in 5 mL of ice-cold borate buffer (0.2 M, pH 8.9). The reaction mixtures were cooled in an ice bath and stirred continuously for 30 min. The pH of the yellow solutions was then adjusted to 7.0 with 1 N NaOH. Each mixture was dialyzed and stored as described above.

N-Hydroxysuccinimide (NHS) Active Ester Method—The Type E haptens were coupled covalently to BSA and CON by the activated ester method. That is, each hapten (0.04 mmol) was dissolved in 0.2 mL of dry DMF with equimolar NHS and a 10% molar excess of dicyclohexylcarbodiimide. After the mixture was stirred overnight at 22 °C, the precipitated dicyclohexylurea was removed by filtration through a glass wool plugged Pasteur pipet, and about 0.2 mL of the active ester was added slowly to a solution of the protein (25 mg of protein in 1 mL of 0.05 M borate buffer at pH 8) with vigorous stirring. The reaction mixture was stirred gently at 4 °C for 24 h to complete the conjugation and then dialyzed and stored as described above.

Immunization and Antiserum Preparation

The immunization procedure followed the protocol reported previously.[28] Three female New Zealand white rabbits were immunized for each immunogen (Rabbits 1639/1640/1641, Hapten A1-Thy; Rabbits 1642/1643/1644, Hapten A2-Thy; Rabbits 1645/1646/1647, Hapten B1-Thy; Rabbits 1648/1649/1650, Hapten C1-Thy). The final bleed was collected about 5 months following the first immunization. Blood was collected in test tubes and allowed to clot. Serum was obtained by centrifugation, stored at −20°C and used without purification.

Competitive Indirect ELISA

The preparation of the buffers and the procedure for the indirect competitive ELISA was previously described.[29] The library of coating antigens was screened against the various rabbit antisera. The assay having the lowest IC_{50} with a maximum absorbance of at least 1.0 was chosen for further studies. The IC_{50} value, an expression of the sensitivity of immunoassay and the limit of detection (LOD) defined as the IC_{10} value were obtained from a four parameter logistic equation. Borosilicate glass tubes were used to prepare standard and sample solutions to minimize the surface adsorption of TCC. Each standard was analyzed in four well replicates on the same plate and each plate contained a complete standard curve.

Cross-Reactivity (CR)

The CR studies were evaluated by using standard solutions of synthesized TCC metabolites and urea analogs.[13] The compounds tested are listed in Table 1. Each concentration of each compound was tested in four well replicates. The CR was calculated as (IC_{50} of the target analyte/IC_{50} of the tested compound) × 100.

Immunological Analysis of Biological and Environmental Samples

Human Whole Blood and Sera of Calf and Mice—Methods for serum and blood sample analysis were optimized for two sample sizes (0.5 mL and 10 μL). Commercial calf (Invitrogen, Carlsbad, CA) or human serum (Sigma S-7023, St. Louis, MO) was kept at −20°C until the analysis. After thawing, 0.5 mL of the serum was transferred to a 5 mL-glass test tube. TCC (500 μg/L) dissolved in methanol was used to prepare three replicates of samples containing 3, 5, 10, and 50 ng TCC/mL serum for a recovery study. Each sample was diluted with 2 mL of sodium acetate buffer (0.2 M, pH 4.5). TCC was extracted with ethyl acetate (2 mL × 2). Centrifugation was utilized when needed. The combined organic layers were completely removed by a centrifugal vacuum evaporation overnight. The residue was redissolved with dimethyl sulfoxide (DMSO; 0.5 mL), followed by the addition of phosphate buffered saline (PBS; 2 mL) to make a 5-fold dilution prior to analysis. Each sample was analyzed in three well replicates.

For the 10 μL sample, commercial human serum and whole blood (Innovative Research, Inc. Novi. MI) were kept at −20°C and 4 °C prior to use, respectively. After thawing, 0.5 mL of the serum was transferred to a 1 mL centrifuge tube to prepare spiked samples. TCC (500 ng/mL) dissolved in methanol was used to prepare three replicates of concentrations of 5, 10, 25, 50 and 100 ng/mL of TCC in the serum. Ten-μL aliquots of each sample were added into a 1-mL microcentrifuge tube containing 50 μL of distilled water. The pipet tip was washed once with water that was added to the tube. The tube was shaken, 200 μL of ethyl acetate added and the tube was capped. The mixture was vigorously shaken for about 60 seconds on a Vortex mixer and then centrifuged at 8,160 × g at room temperature for 5 min (Eppendorf, Hamburg, Germany). The ethyl acetate layer was removed and the aqueous layer was again extracted with 200 μL of ethyl acetate. The combined ethyl acetate layers

were evaporated to dryness by a vacuum centrifugal concentrator, and dissolved in 28 μL of DMSO, followed by addition of 42 μL of PBS prior to the immunoassay. Final dilution was 7-fold in the assay buffer prior to analysis. Each sample was analyzed in three well replicates.

The same extraction method was used for blood samples collected from volunteers who used a retail solid soap containing TCC. Ten μL of whole blood was carefully collected in a pipet tip pre-rinsed with K3-EDTA using a 20-μL pipet. Samples were extracted as above and the dried extracts were dissolved in 24 μL of DMSO, followed by addition of 36 μL of PBS prior to the immunoassay. Final dilution was 6-fold in the assay buffer. Each sample was analyzed in triplicate.

Whole blood samples (10 μL) were collected from mice fed a rat chow containing 0.2% TCC (w/w). The sample was added into a 1 mL microcentrifuge tube including 50 μL distilled water. The extraction method was the same as described above. Each sample was analyzed in triplicates. Final dilution was 6-fold in the assay buffer.

Human Urine—Frozen urine samples collected from volunteers exposed to TCC were thawed at room temperature. The thawed sample was shaken vertically for 30 seconds to completely mix the sample. The sample was allowed to stand for at least two hours at room temperature. A 0.5 mL aliquot of the clear supernatant was added to a glass screw capped vials (15 mm × 4.5 mm, 1 dram, 4 mL). To hydrolyze possible TCC conjugates in urine to free TCC, in the fume hood, a 0.1 mL aliquot of 6 N HCl was added and the tube capped. The capped tube was placed in a Dri-bath heating block set a 100 °C for 20 min in the fume hood. After cooling, the acid mixture was neutralized with about 0.08 mL of 6 N NaOH. Acetic acid in water (1%, 1.5 mL) was added to the tube and mixed thoroughly. A 0.2-mL aliquot of the sample was extracted three times with 0.5 mL of ethyl acetate by shaking vigorously for 5 min at 220 rpm on an orbital shaker or for 1 min on a Vortex® mixer. After the sample had been centrifuged, the organic layers were combined in a 12 mm × 75 mm glass tube. The extract was evaporated to dryness under a gentle stream of N_2 gas. The residue was dissolved in 100 μL of DMSO followed by the addition of 150 μL of PBS for ELISA analysis. Three replicates of each sample were extracted and analyzed in triplicate. For the recovery study, 500 ng/mL TCC dissolved in methanol was spiked into 0.5 mL urine in the range of 10–100 ng/mL before hydrolysis. Three replicates were prepared at each concentration and each sample was analyzed in triplicates.

Analysis of Biosolid Samples—Three biosolid samples were collected from the outflow of a sludge holding pond at a waste water treatment plant that has a capacity of about 140 million gallons per day. The samples were dried at 70°C for 24 hrs and homogenized with a mortar and pestle. A mixture of methanol and acetone (1:1, v/v, 15 mL) was added to 1 g of dried sample. The mixture was shaken for 24 hr at 210 rpm at 60°C and centrifuged for 30 min at 10,000 × g. The supernatant was filtered with a 0.2 μm PTFE filter. Each sample was processed in triplicate. The extracts were further diluted in methanol:acetone for LC-MS/MS analysis or 50~200-fold in 40% DMSO-PBS prior to the immunoassay.

Results and Discussion

Synthesis of Haptens

The design and synthesis of immunizing haptens is critical to the development of immunoassays for small molecules in order to produce sensitive and specific antibodies to the target.[30] TCC is of small molecular weight (M.W. 316) and requires conjugation to carrier proteins in order to be immunogenic. Antibodies are generally formed to the part of

the molecule that is most distal to the point of attachment to the carrier protein. Haptens with a functional group (-COOH or $–NH_2$) for conjugation to carrier proteins were designed (Figure 1). Mainly haptens were synthesized using a reaction that condensed the appropriate isocyanate and an amine (Supporting Information Schemes 1–7).

The main idea for immunizing hapten design was to prepare a hapten that simulates the entire TCC molecule and contained a carbon linker and a carboxylic group. The numbers of carbon in the linker were 0, 3, or 4 and the linkers were attached at the 4 or 4′ position of the structure.

The immunizing haptens were divided into three different groups that exposed different parts of the TCC molecule for antibody recognition. All of the saturated hydrocarbon linkers of haptens of Type A were introduced at the 4′-carbon position. Since S is an isosteric analog of Cl, haptens of Type B contained linkers in which S was introduced in place of Cl at 4′-position, followed by the addition of a single hydrocarbon linker. For haptens of Type C, S was also introduced in place of the Cl at the 4-position in the same way as those in Type B.

For polyclonal antibody-based immunoassays for small-molecular-weight organic molecules the relative binding affinity of the target analyte TCC to the antibody is increased by utilizing relatively low affinity haptens (heterologous haptens) in the competitive assay format. An advantage of this approach is that the polyclonal antibody does not require purification from the serum because generally a dilution of 3–4 orders of magnitude is used. Because the development of heterologous immunoassays for small molecular weight targets requires synthesis of haptens, this technology is very attractive in laboratories with capability in organic synthesis.

Heterology in competitive haptens was achieved by altering the handle length (Type A), altering substitutions in the handle (Type B and D), altering placement of the handle (Type C), or modifying the parent structure (Type E). The Type D haptens utilized a different linking chemistry by coupling by diazotization. Type E haptens had a chlorinated piperidine substituted for the chlorinated phenyl moiety. Haptens of type D and E generally provided assays with the lowest IC_{50}s, lowest background and largest ratios of signal:noise (A/D Table S1).

Optimization

All of the immunizing haptens in Types A, B, and C provided reasonable affinity of the resulting antibody to the target in a competitive assay format. Rabbit antisera #1648 and #1650 generated against immunizing hapten C1 provided the best sensitivity against selected competitive haptens conjugated to BSA and CON (Supporting Information Table S1). Among the most sensitive heterologous combinations of coating antigen and antibody, a pair consisting of hapten E2-BSA and antibody #1648 was selected for TCC analysis because of the sensitivity, reasonable signal-to-noise ratio, heterologous approach, and novelty of coating hapten structure. The optimum concentrations of hapten E2-BSA and antibody #1648 were 1 μg/mL to be coated onto the plate and 1:6,000 dilution, respectively.

Since TCC is highly lipophilic and may be insoluble in the assay buffer, a high amount of co-solvent is very important for consistent assay performance and sensitivity. Varying concentrations of methanol and DMSO were evaluated and generally DMSO increased the sensitivity more than that of methanol. DMSO (40% in the assay buffer) exhibited the highest sensitivity and an A/D ratio of 10 (Supporting Information Table S2) and was selected for further experiments.

There was no significant effect of pH values ranging from 5.5 to 11 in the buffer on the IC_{50} value, but the maximum absorbance values were less variable than effects of other parameters such as ionic strength and solvents (Supporting Information Table S2). The pH of PBS was maintained at 7.5 for the assay.

Although the IC_{50} value was not significantly affected by higher ionic strength, the absorbance reduction at 2× (0.3 M), 4× PBS (0.6 M), or 6× PBS (0.9 M) shows that the binding interaction of antibody to antigen was gradually suppressed (Supporting Information Table S2). Complete screening data are shown in Tables S1 and S2 in Supporting Information.

The optimized ELISA used coating antigen hapten E2-BSA at a concentration of 1 μg/mL and polyclonal antibody #1648 produced against hapten C1-Thy at a dilution of 1/6,000 prior to adding to the microtiter plate. The coated plate was blocked with 0.5% BSA. The assay buffer was 40% DMSO in 0.15M PBS, pH 7.5. This heterologous assay had a linear range ($IC_{20–80}$) of 0.13–3.6 ng/mL of the target analyte in the buffer system and an IC_{50} value of 0.69 ng/mL. The LOD in the buffer was set as 0.03 ng/mL, the IC_{10} value (Figure 2).

Cross-Reactivity (CR)

TCC metabolites and analogs were evaluated for CR. The assay was selective for the target analyte TCC, showing low CRs (< 10%) to other tested compounds (Table 1). TFC (3-trifluoromethyl-4,4′-dichlorocarbanilide) has a $–CF_3$ group in place of a Cl, and showed relatively high CR (30%). TFC is also as an antimicrobial agent.[31]

The antibody generated against the immunizing hapten likely has more affinity to the presence of both phenyl moieties rather than a single phenyl moiety because diuron only cross reacted 0.7%. The urea bridge of the TCC molecule is also a characteristic antigenic determinant because triclosan, a biphenyl ether, cross reacted <0.1%. Surprisingly, tetrachlorocarbanilide that differs only in the addition of one symmetric chlorine only cross reacted 0.5% possibly because the antibody binding pocket generated against haptens containing three chlorines was too small. However, the symmetrical dichlorocarbanilide cross reacted significantly at 8.9% while the non-chlorinated carbanilide cross reacted <0.1% showing the importance of the Cls. The low cross reactivity of two structural analogs that are inhibitors of the enzyme soluble epoxide hydrolase (sEH) further demonstrate the importance of the presence of the chlorinated phenyl moieties.

Matrix Effects

Sample preparation is a critical step for accurate and reliable determination in many analytical methods. Liquid-liquid extraction (LLE) is an attractive alternative to solid phase extraction (SPE) because of its relatively simple process, ease of development of the method, and the removal of water compatible salts. It results in a relatively clean extract having reduced matrix interferences from blood and urine samples when analyzed by immunoassay or other instrumental methods. The LLE method described here used ethyl acetate as a simple method for crude screening of TCC in the blood/serum or urine as demonstrated by the good recoveries seen for human whole blood. Upon extraction of serum, proteins formed a gel-like precipitate between organic and aqueous layers that was easily removed by centrifugation and caused no apparent matrix effect as seen by the good overall recovery of TCC (Table 2). Recoveries were in the range of 60–120% in serum/blood and urine samples. The results of TCC measured in samples prepared from the two sized samples (10 μL and 0.5 mL) were not appreciably different. Data are shown in Supporting Information Table S3. The ability to utilize the 10 μL sample size is attractive as

samples can be obtained less invasively by finger prick and validation in whole blood eliminates the need to obtain the serum.

Validation in Various Study Samples

For the analysis of environmental biosolid samples, the ELISA was performed after a large dilution of the methanol-acetone extract in 40% DMSO-PBS. Data from the ELISA analysis indicated slightly higher levels of TCC than the data from LC/MS/MS analysis with an average ratio of 1.4 (Table 3). This method may be useful for rapid analysis in a waste water treatment plant to determine if TCC was adequately removed during digestion processes.

As seen in Table 4, there was relatively good agreement between ELISA and LC-MS/MS data for human whole blood collected from healthy volunteers, 2–3 hrs following a whole body shower that included lathering 15 min with a commercial bar soap containing 0.6% TCC. Because of the good correlation, only the ELISA was used for the follow-up study. An individual performed their normal shower habits using the commercial bar soap and whole blood samples were collected 2 hr later[19]. Taken with the previous study, these results show that TCC is clearly absorbed and shower habit does not appear to make a difference in absorption. A similar correlation between methods was obtained for serum from mice treated with TCC. These assays were performed in a blind fashion (Table 4).

TCC concentrations in urine collected over 5 days from a healthy volunteer that used a commercial TCC-containing bar soap when showering were much higher in acid hydrolyzed samples than those in non-hydrolyzed ones (Supporting Information Table S4) suggesting that more conjugates are excreted by this route than parent compound. Thus, a hydrolysis step to release free TCC was used. TCC concentrations in hydrolyzed urine correlated well to those of LC/MS/MS (Figure 3). According to LC/MS/MS analysis, oxidative metabolites of TCC such as 2′-OH-, 3′-OH and 6-OH-TCC are minor in urine. The main metabolites of TCC were found to be *N*- and *N*′-glucuronic acids of TCC.[19,32] For the urine samples, a background concentration of the analyte TCC was found in the pre-dose samples and was subtracted from the amount measured in the samples collected after TCC exposure. A control matrix (unfortified sample matrix that is used for the fortified samples) was analyzed along with the fortified matrix to detect any background concentration of analyte present in the control. A background concentration of the analyte found in the control was subtracted from the amount measured in the fortified sample for comparison of results with LC-MS/MS. As shown in Figure 3, TCC excretion reached a maximum at about 12 h and returned to pre-exposure levels by day 5. At 18 h, the TCC excretion appears to be low, but is explained by a low creatinine level. Creatinine values are in Supporting Information Table S4.

In conclusion, to develop a sensitive, selective immunoassay for TCC, the introduction of a saturated hydrocarbon spacer linked through a thioether, an isosteric analog of Cl, as a good molecular mimic of TCC aided in the development of a selective antibody as previously demonstrated for triazine herbicides and other analytes.[33,34] A heterologous coating hapten having a piperidine moiety that influenced the affinity of the antibody for the analyte in the competitive assay resulted in a sensitive heterologous assay. This immunoassay format developed for TCC was highly sensitive with IC_{50} values of 0.70 ng/mL in a competitive indirect format. Ethyl acetate extraction of aqueous samples such as serum/blood and urine was used as a common sample preparation method prior to this immunoassay. This sample preparation method facilitated quantitative measurement in serum and urine and could be applied as well to environmental water samples. This technology was demonstrated to be applicable to as little as 10 μL of urine or serum. It also can be adapted for relatively high-throughput analysis. Finally, the techniques of immunizing and competitive hapten design, screening and sample preparation can be applied to any immunoassay and should result in improved assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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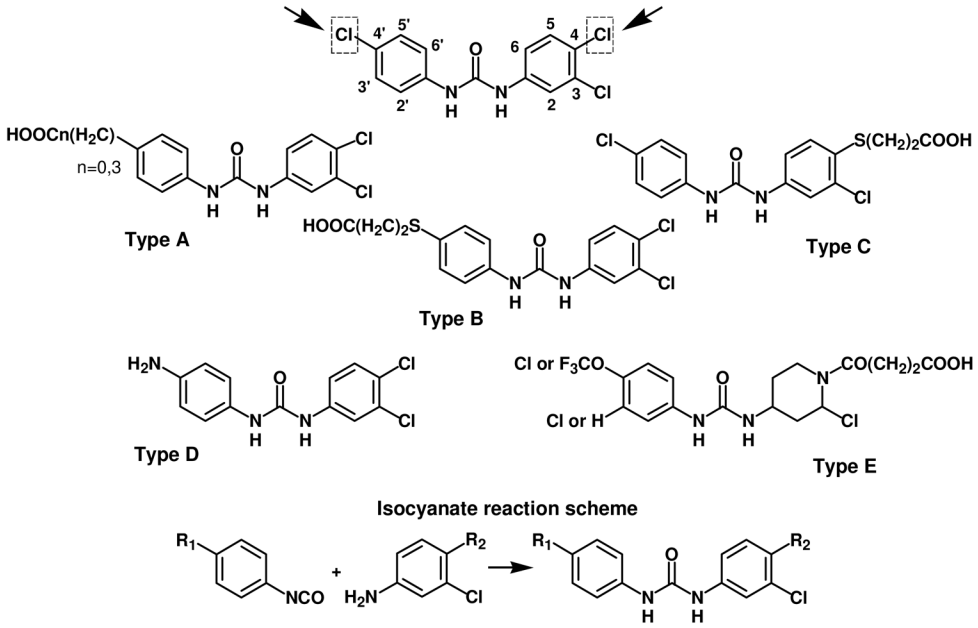

FIGURE 1.

Design of immunizing haptens and their synthetic outline. Arrows on TCC structure represent sites where linkers are introduced. The chlorines at these sites are replaced by a hydrocarbon linker containing a functional –COOH group. In the isocyanate reaction scheme, R_1 and R_2 represent a hydrocarbon linker position.

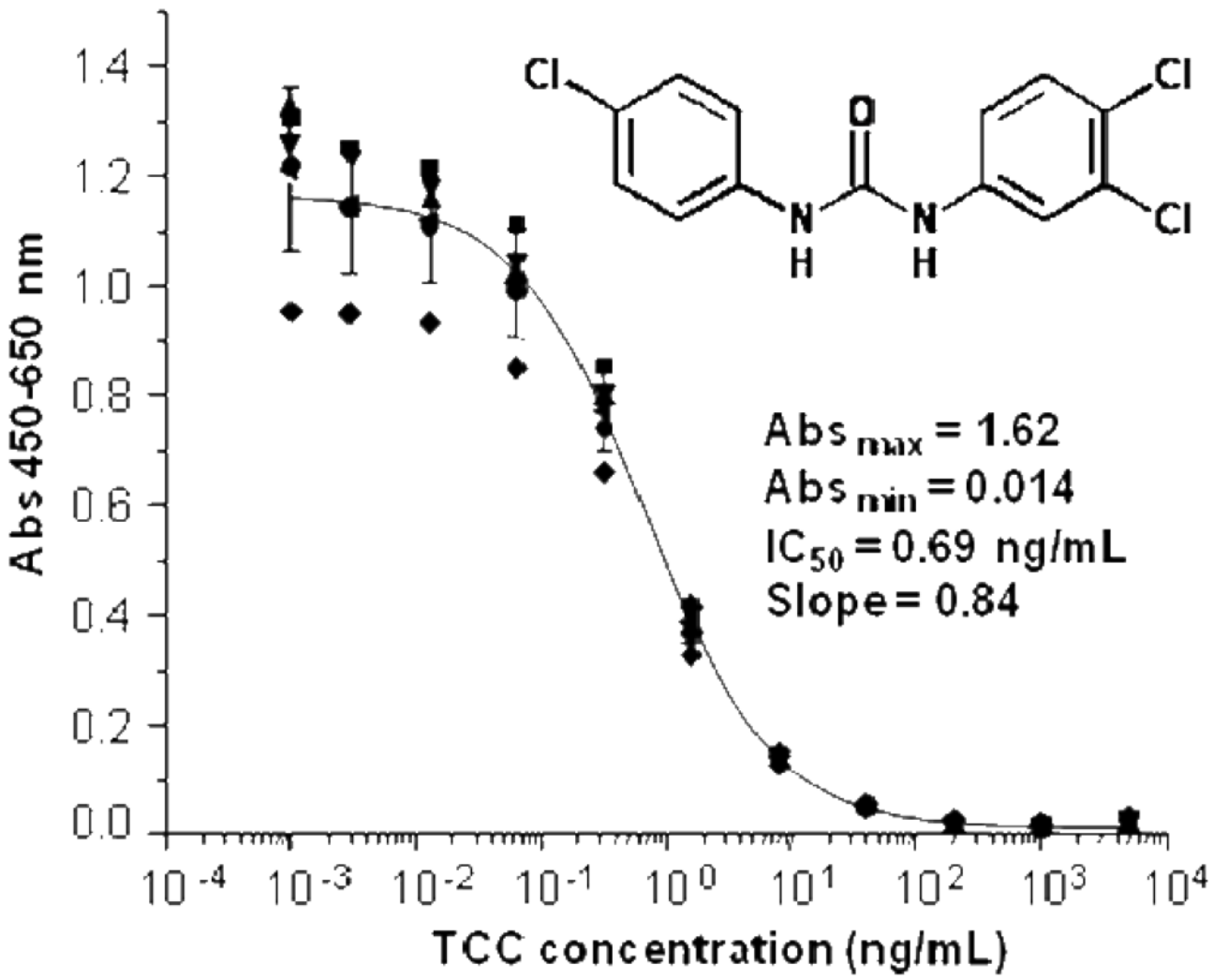

FIGURE 2.

ELISA inhibition curves for TCC determined on five different plates. Each symbol respresents the mean absorbance from 4 wells. Error bars represent the error on the mean absorbance of 5 analyses at each concentration. Some errors are smaller than the symbols. Sigmoidal fit for quadruplicate × n, n=5), $Chi^2 = 0.337$, $R^2 = 0.998$. Linear detection range = 0.10 – 3.6 ng/mL; low detection limit (IC_{10}) = 0.03 ng/mL; high detection limit (IC_{90}) = 10 ng/mL.

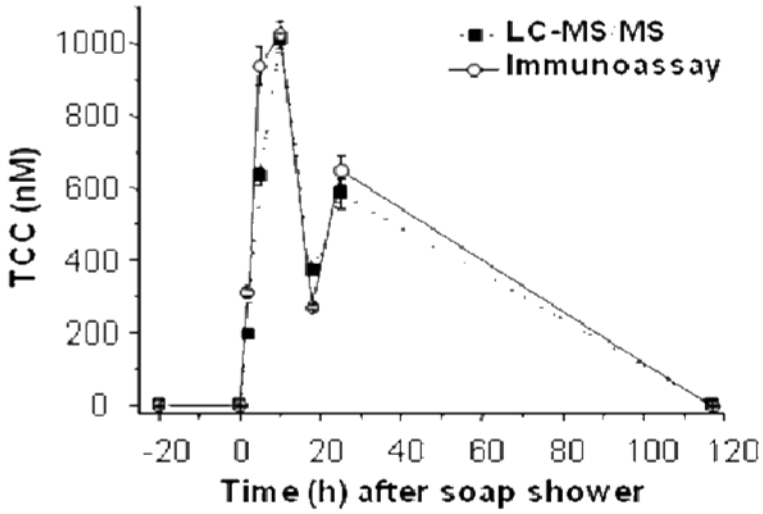

FIGURE 3.

Comparison of TCC levels in urine following hydrolysis measured by LC-MS/MS and immunoassay. Each point represents the data from a single individual at each time point. Error bars are the standard deviation on 3 replicate analyses. Participants showered for 15 min.

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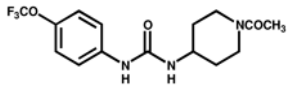
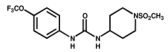
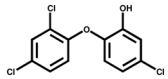
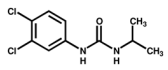
Table 1

Cross-reactivity (CR, %) of compounds structurally related to TCC

Compound	Structure					CR (%)	Remarks
	R_1	R_2	R_3	R_4	R_5		
TCC	Cl	H	H	Cl	Cl	100	Antimicrobial
2′-OH TCC	Cl	H	OH	Cl	Cl	9	Metabolite of TCC
Sulfate of 2′-OH TCC	Cl	H	OSO_3K	Cl	Cl	3	Metabolite of TCC
3-Trifluoromethyl-4,4′-dichloro-carbanilide	Cl	H	H	Cl	CF_3	27	Antimicrobial
Carbanilide	H	H	H	H	H	<0.1	Analog
4,4′-Dichlorocarbanilide	Cl	H	H	Cl	H	9	Impurity of TCC synthesis
3,3′,4,4′-Tetrachlorocarbanilide	Cl	Cl	H	Cl	Cl	0.5	Impurity of TCC synthesis
sEHi, #1555						0.3	sEH inhibitor
sEHi, #1709						<0.1	sEH inhibitor
Triclosan						<0.1	Antimicrobial
Diuron						0.6	Herbicide

A standard curve was produced for each compound tested using the optimized immunoassay conditions. Each concentration of each compound was tested in 4 well replicates. The cross reactivity was calculated by dividing the IC_{50} of TCC by the IC_{50} of the test compound and multiplying by 100.

Table 2

Recoveries of TCC in spiked samples of human serum, whole blood, and urine measured by immunoassay

Spiked concentration (ng/mL)	Serum ng/mL (% Recovered)	Whole blood ng/mL (% Recovered)	Urine ng/mL (% Recovered)
5	5.0 ± 0.3 (99 ± 6)	4.0 ± 0.7 (79 ± 13)	-
10	9.5 ± 1.7 (95 ± 17)	8.8 ± 2.8 (88 ± 28)	11 ± 0.5 (105 ± 5)
25	19 ± 3.5 (78 ± 14)	21 ± 1.8 (84 ± 7)	24 ± 1.2 (96 ± 5)
50	32 ± 2.6 (64 ± 5)	61 ± 0.2 (121 ± 1)	48 ± 2.4 (94 ± 5)
100	61 ± 3.5 (61 ± 4)	100 ± 7.8 (100 ± 8)	97 ± 11 (97 ± 11)

The sample sizes of human serum, whole blood, and urine were 10 μL, 10 μL, and 500 μL, respectively. Three samples were spiked at each concentration. Values are the mean ± standard deviation (n=3) for the absolute recovery (ng/mL) and the relative recoveries (%).

-, Not determined.

Table 3

TCC concentrations in biosolid samples determined by LC/MS/MS and immunoassay

Sample	TCC concentration determined in biosolids (ng/g dry wt)		
	Immunoassay (A)	LC-MS/MS (B)	Ratio (A/B)
BS002	12000 ± 900	8700 ± 1500	1.4
BS003	9800 ± 220	8100 ± 180	1.2
BS004	12000 ± 2700	7000 ± 60	1.8

Three replicates of each sample were extracted and analyzed in three well replicates. The values represent the mean +/− the standard deviation for n=3 sample replicates.

Table 4

Comparison of TCC concentrations in incurred samples by immunoassay and LC-MS/MS

Sample		TCC concentration determined in whole blood or serum (ng/mL)		
		Immunoassay (A)	LC-MS/MS (B)	Ratio (A/B)
Whole blood after routine shower	V0012	< 5.0	-[a]	-
	V0022	24 ± 0.6	-	-
	V0032	30 ± 0.7	-	-
	V0042	7.4 ± 0.1	-	-
	V0052	5.2 ± 0.1	-	-
	V0062	< 5.0	-	-
Whole blood after 15 min lathering using TCC-containing soap	V0072	7.2 ± 1.5	3.8 ± 0.3	1.9
	V0082	22 ± 2.3	17 ± 2.5	1.3
	V0092	16 ± 2.7	14 ± 0.1	1.1
	V0093	5.6 ± 1.0	5.6 ± 0.0	1.0
Mouse serum	MSA	5.9 ± 0.2	2.7 ± 1.3	2.2
	MSB	86 ± 0.5	49 ± 7.7	1.8
	MSE	16 ± 0.3	38 ± 2	0.4
	MSF	140 ± 0.5	127 ± 0.7	1.1

Each sample was collected at a specified time point following exposure. The values represent the mean +/− the standard deviation of the results of analysis of each sample on three separate occasions.

[a]Sufficient sample was not available for the whole blood after a routine shower analysis by LC-MS/MS.