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ORIGINAL PAPER

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An immunoassay for a urinary metabolite as a biomarker of human exposure to the pyrethroid insecticide permethrin

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Abstract Permethrin is the most popular synthetic pyrethroid insecticide used in agriculture and public health. For the assessment of human exposure to permethrin, a competitive indirect enzyme-linked immunosorbent assay (ELISA) for the detection of the glycine conjugate of a major metabolite, *cis*-/*trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (DCCA), of permethrin was developed based on a polyclonal antibody. An assay based on an antibody with a high sensitivity was optimized and characterized. The IC_{50} value and the detection range for *trans*-DCCA–glycine, in the assay buffer were 1.2 and 0.2−7.0 μg/L, respectively. The antibody recognized *trans*-DCCA–glycine and the mixture of *cis*-/*trans*-DCCA–glycine with an isomer range from 30:70 to 50:50 nearly equally. Little or no cross-reactivity to permethrin and its other free metabolites or glycine conjugates was measured. The integration of the ELISA and solid-phase extraction which was used to reduce the matrix effect from human urine samples provided for analysis of total *cis*-/*trans*-DCCA–glycine at low parts per billion levels in the samples. The limit of quantitation of the target analyte was 1.0 μg/L in urine with a limit of detection of 0.1 μg/L in buffer. This assay might be a useful tool for monitoring human exposure to permethrin.

Keywords ELISA · Permethrin · Human exposure · Glycine metabolite · Polyclonal antibodies

Introduction

Permethrin is the commonest pyrethroid insecticide used around the world as well as in the USA. Its application equaled about 40% of the total pyrethroids used in the USA in 1997 [1] and about 63% in some Californian agricultural areas in 2002 [2]. It has been widely used in agriculture, forestry, homes, horticulture, and veterinary and public health programs [3–7]. Pyrethroids including this compound are neurotoxic, yet are very important insecticides because of their rapid control of insects, relatively low toxicity to mammals, and rapid degradation in the environment.

The common sources of exposure to permethrin for the general population are thought to occur via its residues in drinking water and diet and via inhalation or ingestion of contaminated household dust [8, 9]. Persons such as farmers, pesticide applicators, and manufacturers may receive occupational overexposure via inhalation and dermal contact. Military service personnel can be continuously exposed dermally to some extent to permethrin. As an example, a permethrin spray as a mosquito and fly repellent was used to treat uniforms of military personnel during the Gulf War deployment [10]. In addition, insect repellent apparel that has been treated with permethrin is currently being sold to the general public in the USA.

The US Environmental Protection Agency has classified permethrin as a potential carcinogen at high concentrations [11, 12]. It is also suspected as an endocrine disrupting compound [13], a priority pollutant [14, 15], and an environmental contaminant. Overexposure to permethrin may include acute reversible symptoms such as headache, dizziness, nausea, irritation of the skin and nose, and paraesthesia [16, 17]. In studies on chronic exposure effects, Hallenbeck and Cunningham-Burns [18] suspected that the pyrethroid fenvalerate may cause lymph node and splenic damage as well as carcinogenesis. Repetto and Baliga [19] demonstrated that pyrethroids have a suppressive effect on the immune system. Go et al. [13] suggested that pyrethroids should be considered hormone disruptors in studies on the estrogenic potential of pyrethroids in human breast carcinoma cells.

In mammals, the major metabolites of permethrin are excreted into the urine and are mainly derived from ester cleavage. The main metabolites of permethrin are the *cis* and *trans* isomers of 3-(3,3-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid (DCCA), and 3-phenoxy-

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benzoic acid (3-PBA) subsequently oxidized from 3-phenoxybenzyl alcohol (PBAlc). These can be partially excreted as polar conjugates with natural metabolites such as glycine and glucuronic acid from xenobiotic metabolism pathways [20–22]. While little is known about the conjugates of pyrethroid metabolites in humans, it has been well established that glycine is the most versatile amino acid conjugating to molecules with an available free carboxyl group to produce the glycine conjugates [23]. The glucuronide and glycine conjugates of DCCA in rat and mouse and the glucuronide, glycine, serine, glutamine, and glutamic acid conjugates of DCCA in cockroach, housefly, and cabbage looper have been found [24]. In humans, ester hydrolysis followed by conjugation is thought to be the major route of permethrin metabolism [25, 26]. Ring hydroxylation, oxidation at the gem-dimethyl group, and other oxidation pathways result in minor metabolites [27].

Urinary metabolites are one of commonest biomarkers used in human biological monitoring with agrochemicals. Compared with blood and serum samples, the amount of urine sample that can be obtained is large, and the sampling is easy. The rapid metabolism of pyrethroids allows the amount of their metabolites in urine to be remarkably higher than the amount of the parent compounds; therefore, urine is a good matrix for biomonitoring.

To choose a target analyte as a biomarker of exposure to permethrin, metabolism of this compound in humans and identification of its key metabolites are essential. No published study has specifically investigated the conjugates of pyrethroid metabolites in humans. Human metabolism studies at dose levels commensurate with potential occupational and environmental exposure are needed. However, the major permethrin metabolites, *cis*- and *trans*-DCCA, and PBA have been used as urinary biomarkers of exposed people [28]. If we consider conjugates, it is likely that the relative amount of the DCCA–glycine conjugate is higher than that of the PBA–glycine, because the PBAlc that is directly hydrolyzed from the parent compound can enter several metabolic pathways, including direct conversion to the glucuronide conjugate prior to further oxidation to PBA. The ester-type glucuronide conjugates of the free acid metabolites, PBA and DCCA, can be quite unstable for exposure studies owing to potential hydrolysis during storage. In addition, conjugates of reactive metabolites have higher molecular weights than the free metabolites. The glycine, glucuronic acid, or glutathione moiety added directly to the metabolite can serve as a good antigenic determinant for the production of a specific antibody, which is necessary for highly sensitive immunoassays [29, 30]. Therefore, DCCA–glycine should be an ideal biomarker as an indicator of exposure to this insecticide, and a good target analyte for biomonitoring in human urine.

Analytical methods for the detection of pyrethroid metabolites in samples such as urine and blood have utilized some sample preparation steps, including acid hydrolysis, liquid–liquid extraction (LLE) or solid-phase extraction (SPE) and derivatization with high-performance liquid chromatography (HPLC) or gas chromatography (GC) with mass spectrometry (MS) [25, 28, 31, 32]. Sensitive enzyme-linked immunosorbent assays (ELISAs) have been developed for the pyrethroid metabolite 3-PBA [33] and its glycine conjugate [29].

Although the instrumental methods are very sensitive for these metabolites, they can be time-consuming and expensive and are not suitable for a routine and rapid analysis. Immunoassay techniques are widely used in diagnostics, environmental monitoring, food quality, agriculture, and field or on-site testing of personnel exposed to toxic chemicals, providing rapid, sensitive, and selective analytical tools to determine trace chemicals such as agrochemicals and their metabolites as key urinary biomarkers of exposure [34].

The authors are developing and improving rapid immunoassays for possible urinary biomarkers to study human exposure to pyrethroid insecticides along with permethrin. Although the DCCA–glycine conjugate has been found as a minor conjugate in the animal studies, we think that this conjugate can also be a good target along with other glucuronide conjugates or free metabolites of pyrethroids. The objective of this study is the development of an ELISA for the analysis of a human urinary metabolite (*cis*-/*trans*-DCCA–glycine) of permethrin using a polyclonal antibody for application to a human exposure study. The preparation of haptens and the production of antibody were reported previously [26]. This study reports the characterization of antibody, assay optimization, and validation for urine analysis.

Experimental

Materials and instrumentation

Goat antirabbit immunoglobulin G (whole molecule) peroxidase conjugate as the second antibody, bovine serum albumin (BSA), Tween 20, H_2O_2, 3,3′,5,5′-tetramethylbenzidine , and dimethyl sulfoxide were purchased from Sigma-Aldrich (St. Louis, MO, USA). ELISA was performed on 96-well microtiter plates and read spectrophotometrically with a microplate reader in dual-wavelength mode (450–650 nm) described previously [26]. An Ultrawasher II plate washer (Dynatech) was used for washing the plate. The C18 column (10 mL/500 mg, Varian Sample Preparation Products, Harbor City, CA, USA) and a manifold were used for the cleanup of urine samples prior to ELISA.

Immunoreagents

The preparation of haptens and the production of antibody specific to the target analyte were described by Ahn et al. [26]. Briefly, the antibodies Ab 3703, Ab 3710, and Ab 3698, produced against *trans*-hapten-thyroglobulin (Thyr) conjugates and antibodies Ab 3701, Ab 3709, and Ab 369, produced against *cis*-hapten-Thyr conjugates, were selected for further study. A coating antigen, *cis*-hapten **5**-BSA conjugate, was used for analysis of *trans*-DCCA–glycine and the mixture of *cis*-/*trans*-DCCA–glycine in a

heterologous system. Another coating antigen, *trans*-hapten **5**-BSA conjugate, was used for analysis of *cis*-DCCA–glycine. Finally, an immunoassay with the combination of the Ab 3703 and the coating antigen, *cis*-hapten **5**-BSA conjugate, was optimized using *trans*-DCCA–glycine as the standard and was used for the analysis of the mixture of *cis*-/*trans*-DCCA–glycine conjugate in urine. The structures of the haptens used for the production of the antibodies and the coating antigens are shown in Fig. 1.

Enzyme-linked immunosorbent assay and assay optimization

The preparation of the buffers and the procedure for the ELISA has been previously described [26]. An indirect competitive ELISA was performed according to the method of Voller et al. [35]. An IC_{50} value expressed as the sensitivity of immunoassay and a limit of detection (LOD) defined as the IC_{10} value were obtained from a four-parameter logistic equation.

The ELISA was optimized with some factors in the assay buffer. The effect of blocking agents was tested with BSA, nonfat dry milk, and gelatin in normal-strength phosphate-buffered saline (PBS). To investigate the effect of solvent amounts, the target analyte standards were dissolved in PBS buffer containing 0, 3, 6, 10, 20, 40, 60, or 80% (v/v) methanol. The effects of pH (4.5, 6.5, 7.5, 8.5, and 9.5) and ion strength (1× and 2×) in PBS buffer were also tested.

Cross-reactivity

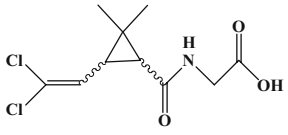
The optimized assay was used to run cross-reactivity (CR) studies by using a standard solution of the permethrin metabolites and other structurally related compounds in methanol. The test compounds are listed in Tables 2 and 3. The CR was calculated as (IC_{50} of the target analyte/IC_{50} of the tested compound)×100 to get the percentage figure.

Analysis of urine samples

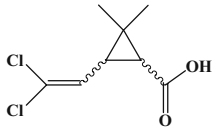
The urine samples were stored at −20°C prior to analysis. The samples were thawed at room temperature, and then about 1 mL of the urine sample was centrifuged for 5 min at 1,500 g (5,000 rpm) to remove precipitates. When 10 μg of the target analyte was spiked into 0.5 mL of urine sample that had not been centrifuged, the amount of the compound detected in the precipitates was negligible (less than 0.1%) (data not shown).

Sample cleanup methods: LLE

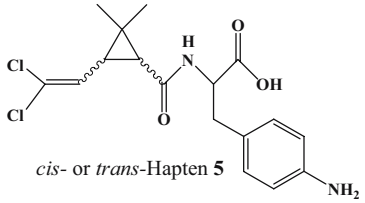
The LLE was slightly modified from that in the previous report [25]. That is, the 0.5-mL aliquot of the sample was acidified with one drop of 6 N hydrochloric acid. The acidic sample was twice extracted with 0.5 mL of ethyl acetate by shaking vigorously for 2 min. After the sample had been centrifuged, the organic layers were combined in a 2-mL centrifuge vial. For further cleanup, 1 mL of 0.5 N NaOH was added to the organic phase and the target analyte was reextracted into the aqueous phase by shaking vigorously for 2 min. After centrifugation, the organic phase was discarded. The remaining aqueous phase was acidified by adding ten drops of 6 N hydrochloric acid and was twice extracted with 1 mL of ethyl acetate. Following centrifugation, the organic phase was combined in a 4-mL glass vial. The extract was evaporated to dryness under a gentle stream of N_2 gas. The residue was dissolved in 0.5 mL of 1×PBS and then diluted up to 50 times prior to the ELISA.



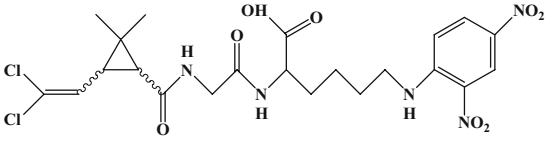
Target analyte: *cis*- or *trans*-DCCA-glycine (Hapten **3**)



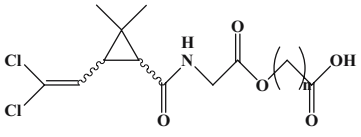
cis- or *trans*-Hapten **1**



cis- or *trans*-Hapten **5**



cis- or *trans*-Hapten **9**



cis- or *trans*-Hapten **24** (n=3)
cis- or *trans*-Hapten **26** (n=1)

Fig. 1 Target analyte and haptens used for this study. *trans*-Hapten **3** was used as an immunogen for the production of Ab 3703, *trans*-hapten **9** for Ab 3710, *trans*-hapten **24** for Ab 3698, *cis*-hapten **3** for Ab 3701, *cis*-hapten **9** for Ab 3709, and *cis*-hapten-**24** for Ab 369, respectively. *cis*- or *trans*-Hapten **5** was used as a coating antigen. *DCCA* 3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid

SPE under acidic conditions (SPE method A)

The SPE method was based on a previously reported method for the extraction of PBA–glycine [29] from human urine using a C18 column. The C18 column was preconditioned with 3 mL of methanol, deionized water (DW), and 0.1 M phosphate buffer (pH 2). The supernatant from centrifuged urine samples (0.5 mL) was mixed with 19.5 mL of the phosphate buffer and loaded onto the column, and eluted with flows of 2–4 mL/min . The columns were subsequently washed with 10 mL of the buffer, DW, and 20% methanol in DW, sequentially. After being dried under high vacuum for 15 min, the column was eluted with 1.5 mL of 100% methanol. The methanol was evaporated to dryness under a gentle stream of N_2 gas, dissolved in 0.5 mL PBS, and then diluted up to 50 times prior to the ELISA.

Improved SPE under acidic and ion-pairing conditions using trifluoroacetic acid (SPE method B)

The C18 column was preconditioned with 3 mL of methanol, 3 mL of 0.1% TFA in 50% methanol in DW, and 3 mL of 0.1% trifluoroacetic acid (TFA) in DW. The centrifuged urine samples (0.5 mL) were loaded on the column, and eluted with flows of 2–4 mL/min. The columns were subsequently washed with 10 mL of 0.1% TFA in DW, and 2 mL of 40% methanol in 0.1% TFA in DW. After drying under high vacuum for 15 min, the column was finally eluted with 1.5 mL of ethyl acetate, methanol, or a mixture of isopropyl alcohol and methylene chloride (20:80, v/v). The organic solvent fraction was evaporated to dryness under a gentle stream of N_2 gas, dissolved in 0.5 mL PBS, and then diluted up to 5 times prior to the ELISA.

Instrument for chromatographic analysis of the urine samples

Chromatographic separation was performed using a Shimazu LC-10A separation module (Shimazu, Japan) equipped with a 150 mm×2.1 mm Waters 3.5-μm C18 Xterra column (Waters, MA, USA) held at 20°C. A solvent system consisting of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B) was used. The target analyte was separated using a gradient program (0.3 mL/min) starting with a solvent composition of 15% solvent B, ramped using a linear gradient for 6 min to 45% solvent B, ramped again for 2 min to 100% solvent B, and then held for 5 min. The injection volume was 20 μL. The samples were kept at 15°C in the autosampler. The target analyte was detected by electrospray ionization in negative mode and tandem quadrupole MS in multiple reaction monitoring mode (MRM) using a Quattro Premier tandem quadrupole mass spectrometer (Micromass, UK). Nitrogen gas flow rates were fixed with a cone gas flow of 50 L/h and a desolvation gas flow of 650 L/h. Electrospray ionization was performed in negative mode with a capillary voltage fixed at 1.5 kV and a cone voltage fixed at 35 V using a source temperature of 125°C and a desolvation temperature of 300°C. Argon was used as the collision gas (2.3×10^{-3} Torr). The optimum collision voltage was 15 V experimentally by acquisition of the product ion spectrum. This spectrum was used to select a dominant product ion ($[M+H]^+$ 264.30) to set up the transition monitored in the MRM (264.30→192.40). Urine samples were purified with SPE method B. The ethyl acetate fraction that was eluted from the SPE tube was evaporated to dryness under a gentle stream of N_2 gas, and was dissolved in 100 μL of methanol prior to the instrumental analysis.

Results and discussion

Optimization of the ELISA

The previous report described several suitable combinations of antibody and coating antigen for the analysis of DCCA–glycine [26]. A combination of Ab 3703/*cis*-hapten **5**-BSA as a coating antigen was selected for the assay optimization and for sample analysis. The antiserum specific to *trans*-DCCA–glycine cross-reacted about 30% with the *cis* isomer of target analyte. To determine the target analyte in samples, it is essential to develop an ELISA with optimum sensitivity. For that purpose, the immunoassay was optimized for the main factors of the assay buffer, including the blocking agents on the coated plate, detergent content, solvent content, pH, and ionic strength in the assay buffer, in order to obtain the best sensitivity and reproducibility.

Effect of blocking agents

To minimize the background and to maximize the signal-to-noise ratios in the assay, blocking agents were evaluated to hinder nonspecific sorption of an antibody onto the coated plate. Because there is no universal blocking buffer for all immunoassays, various blocking agents for each immunoassay were evaluated. As shown in Table 1, all of the blockers tested enhanced the assay sensitivity compared with no blocking. BSA showed a lower background signal and a higher sensitivity (based on IC_{50} value and slope) for the ELISA than no blocking, and the highest ratio of signal to noise among blockers. BSA as a blocker of the coated plate was selected for further study.

Effect of detergent and organic solvent

Tween 20 is a nonionic detergent and has been used in the assay buffer of immunoassays to reduce nonspecific binding and improve sensitivity [36], whereas, in some cases, the assay sensitivity was enhanced in the buffer without detergent [37, 38]. In this study, the addition of Tween 20 at the commonest concentration of 0.05% to the

Table 1 Effect on blocking reagent and Tween 20 on the sensitivity of the enzyme-linked immunosorbent assay (*ELISA*)

	A_{max}	Slope	IC_{50} (μg/L)	A_{min}	A_{max}/A_{min}
Blocking reagent on the coated plate					
No blocking	0.851±0.015	0.817±0.071	2.76±0.308	0.107±0.014	8
0.5% BSA	0.629±0.013	0.642±0.042	1.39±0.140	0.036±0.008	18
3% Ovalbumin	0.271±0.006	0.861±0.082	1.17±0.141	0.035±0.004	8
3% Nonfat dry milk	0.369±0.007	0.785±0.059	1.22±0.123	0.037±0.005	10
Tween 20 in the assay buffer					
0%	0.897±0.008	0.703±0.037	2.48±0.153	0.029±0.007	31
0.05%	0.778±0.015	0.749±0.044	2.06±0.248	0.022±0.013	35

Assay conditions: coating antigen *cis*-hapten **5**-bovine serum albumin (*BSA*) (1 μg/mL); antiserum 3703 finally diluted by 1:32,000 in 1× phosphate-buffered saline (*PBS*) in wells; goat antirabbit immunoglobulin G (*IgG*)–horseradish peroxidase (*HRP*) (1:5,000). The data are the means of quadruplicates

PBS buffer slightly decreased the absorbance owing to the inhibition of nonspecific binding and thus increased slightly the signal to noise ratio and the sensitivity of this assay (Table 1). PBS with 0.05% Tween 20 was used for further study. Meanwhile, the effect of different methanol content in the buffer on the ELISA was evaluated. Methanol is the most employed solvent miscible in buffer for the immunoassay. As shown in Fig. 2a,b, with increasing

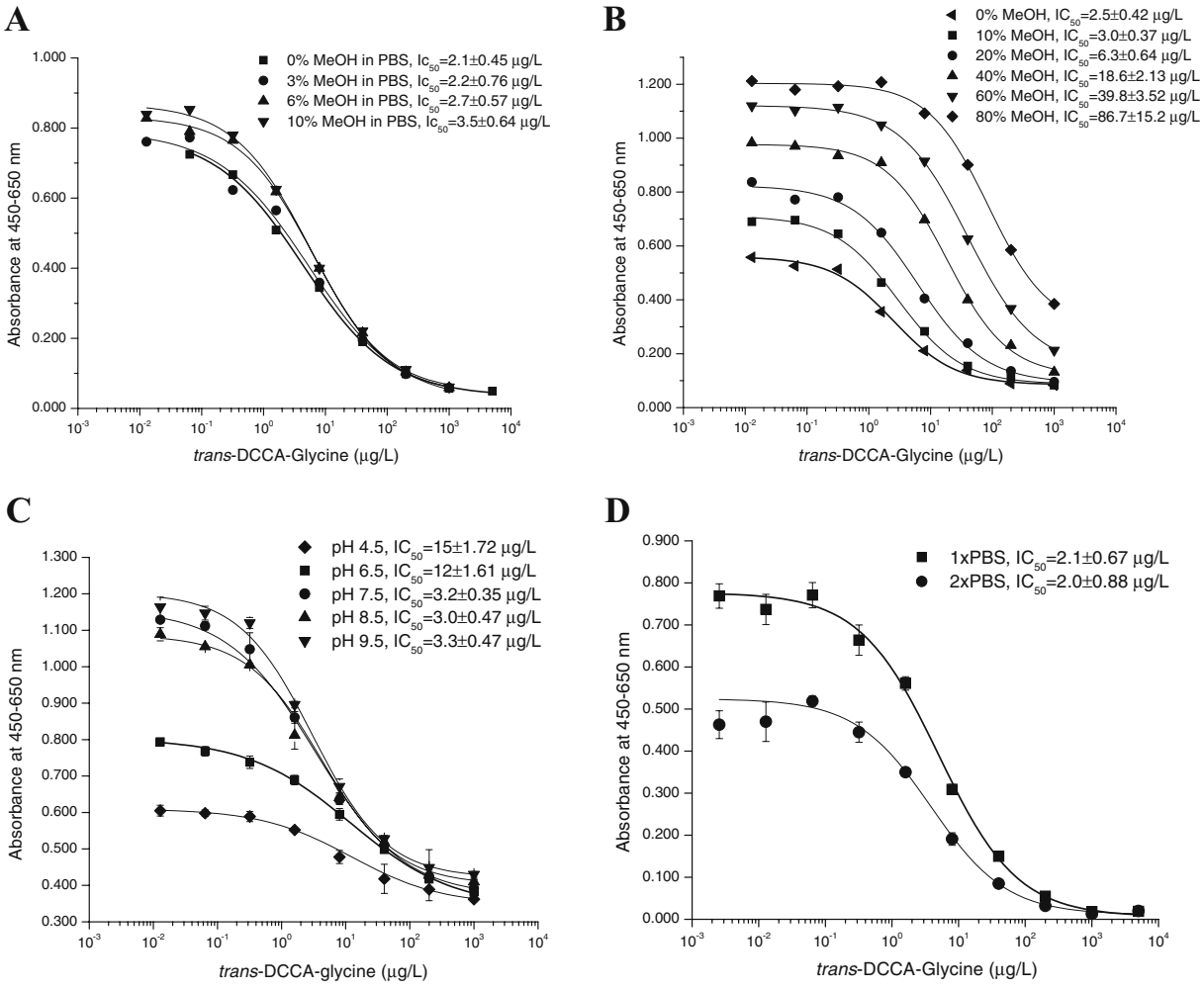


Fig. 2 Effect on various factors in assay buffer on the sensitivity of the enzyme-linked immunosorbent assay (*ELISA*). **a**, **b** Different solvent content (%) of methanol, **c** different pH, and **d** different ionic strength. Assay conditions: coating antigen *cis*-hapten **5**-bovine serum albumin (*BSA*) (1 μg/mL); antiserum 3703 diluted by 1:32,000 (final dilution in wells); goat antirabbit immunoglobulin G –horseradish peroxidase (1:5,000). The data are the means of quadruplicates

amount of methanol in the PBS buffer, both the IC_{50} value of the target analyte and the absorbance increased. The assay sensitivity was higher in the buffer containing no or a small amount (3%) of the organic solvent. The IC_{50} value in the assay buffer containing 20% methanol was about twice as high as that without the organic solvent, indicating that an increase in the amount of organic solvent decreases the assay sensitivity in the ELISA. Organic solvent concentration below 3% in the assay buffer had little effect on the sensitivity of the ELISA. On the basis that the glycine conjugates of metabolites excreted in urine by the phase II reactions are quite hydrophilic, the use of organic solvent will not likely be necessary for this assay, but up to 3% methanol could be utilized if needed.

pH effect

As seen in the Fig. 2c, there was no significant effect of pH values ranging from 7.5 to 9.5 in the buffer on the IC_{50} value, whereas the acidic buffers decreased the absorbance and thus increased the value. The signals of maximum absorbance were different as the pH was changed. The assay was highly suppressed under the acidic conditions. This result was coincident with the results with assays for esfenvalerate metabolites, the glycine conjugates of PBA and fenvalerate acid [29]. The glycine moiety ($–NHCH_2$-COOH) on the target analyte structure has both acidic (carboxylic acid) and basic (amine) functional groups. The relative amounts of RCOOH and $R–NHH^+–$ functional groups on the target under acidic conditions might be increased with a corresponding decrease in the concentration of the base form ($RCOO^-$ and R–NH–). At the increased pH, the opposite trend could be found. On the basis of this chemistry at different pH values, relatively higher pHs than pH 6.5 provided the interaction of totally negatively charged target analyte to antibody to be more attractive and the assay to be more sensitive. Although the assay is applicable in PBS with pH 7.5−9.5, the pH of PBS was kept at 7.5 for the assay.

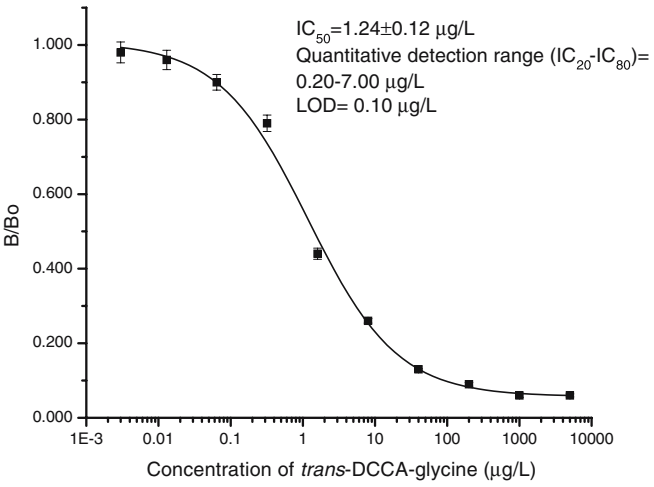


Fig. 3 ELISA inhibition curves for DCCA–glycine. Reagent concentration: antiserum 3703, 1/32,000 (final dilution in wells); coating antigen *cis*-hapten **5**-BSA, 1 µg/mL. Calibration standards were prepared in PBS. The standard curve represents the average of ten curves (± standard deviation). *PBS* phosphate-buffered saline, *LOD* limit of detection

Ionic strength

Although the IC_{50} value was not affected by higher ionic strength, the absorbance reduction at 2× PBS (0.3 M) shows that the binding interaction of antibody to antigen was strongly suppressed (Fig. 2d). This result suggests that the assay may be sensitive to the ionic strength changes in various urine samples. The immunoassay requires a dilution or a desalting method of urine samples with various physiological salt concentrations to reduce the salt

Table 2 Cross-reactivities (*CRs*) (%) of permethrin metabolites and other structurally related compounds to the selected antibodies for *trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane-1-carboxylic acid (*DCCA*)–glycine

Compound	*cis*-Hapten **5**-BSA/3703 IC_{50} (CR)	*cis*-Hapten **5**-BSA/3710 IC_{50} (CR)	*cis*-Hapten **5**-BSA/3698 IC_{50} (CR)
trans-DCCA–glycine	1.9 (100)	2.60 (100)	0.82 (100)
cis-DCCA–glycine	6.7 (28)	18 (15)	37 (2)
Mixture of DCCA–glycine isomers (*cis and trans*)			
20:80	1.4 (133)	2.0 (133)	–[a]
30:70	1.8 (107)	2.1 (125)	–
40:60	2.0 (102)	3.5 (75)	1.8 (46)
50:50	1.9 (99)	4.1 (63)	–
60:40	3.1 (62)	4.3 (60)	–
trans-DCCA	430 (0.44)	808 (0.32)	>8,200 (<0.01)
Permethrin	4,475 (0.04)	2,357 (0.11)	2,357 (0.04)
Glycine	>19,000 (<0.01)	>26,000 (<0.01)	>8,200 (<0.01)
3-PBA–glycine	>19,000 (<0.01)	>26,000 (<0.01)	>8,200 (<0.01v)

3-PBA 3-phenoxybenzoic acid
[a]Not tested

Table 3 CRs (%) of permethrin metabolites and other structurally related compounds to the selected antibodies for *cis*-DCCA–glycine

Compound	*trans*-Hapten **5**-BSA/3701 IC_{50} (CR)	*trans*-Hapten **5**-BSA/3709 IC_{50} (CR)	*trans*-Hapten **5**-BSA/369 IC_{50} (CR)
cis-DCCA–glycine	3.1 (100)	0.7 (100)	6.2 (100)
trans-DCCA–glycine	194 (1.61)	179 (0.38)	322 (1.93)
Mixture of DCCA-glycine isomers (*cis*/*trans* 40:60)	11 (28.04)	1.7 (38.69)	16 (37.83)
trans-DCCA	590 (0.53)	22 (3.00)	2947 (0.21)
cis-/*trans*-DCCA (*cis*/*trans* 40:60)	–[a]	40 (1.75)	–
trans-DCCA	–	1,194 (0.06)	–
Permethrin	>31,400 (<0.01)	>6,700 (<0.01)	>61,900 (<0.01)
Glycine	>31,400 (<0.01)	>6,700 (<0.01)	>61,900 (<0.01)
3-PBA–glycine	>31,400 (<0.01)	>6,700 (<0.01)	>61,900 (<0.01)

[a]Not tested

effect prior to ELISA. The adjustment of pH and ionic strength of urine sample is required to minimize errors due to the effect on absorbance.

The optimized ELISA used a coating antigen of *cis*-hapten **5**-BSA at a concentration of 1 μg/mL and Ab 3703 (against *trans*-hapten **3**-Thyr) at a dilution of 1/32,000 in wells. The plate coated with the coating antigen was blocked with 0.5% BSA. The assay PBS buffer was 0.15 M PBS, pH 7.5 plus 0.05% Tween 20. This heterologous assay had a linear range ($IC_{20\text{-}80}$) of 0.2–7.0 μg/L of the target analyte in the buffer and an IC_{50} value of 1.2 μg/L. The LOD in the buffer was set as 0.1 μg/L, the IC_{10} value (Fig. 3).

Cross-reactivity

The CRs were described with the previously reported data [26]. Briefly, the antibodies generated against the immunizing haptens of the *trans* configuration bound more strongly to the *trans* configuration of target analyte than the *cis* configuration, whereas the antibodies generated against the *cis* configuration more strongly bound to the *cis*-target analyte. Ab 3703 (against a *trans*-hapten) showed relatively higher CR to *cis*-DCCA–glycine than that of other tested antibodies, indicating the mixture of *cis*-/*trans*-DCCA–glycine can be analyzed using this antibody.

Interestingly, the CRs of Ab 3703 to the mixture of *cis*-/*trans*-DCCA–glycine (50:50–30:70) were close to 100% (Table 2). A shorter linker presents the hapten relatively close to the protein carrier surface; thus, the antibody produced against the hapten can provide broader selectivity to the structurally related compounds [39]. This theory was applicable to Ab 3703. Antibodies produced against the *trans* isomer of haptens **9** and **24** (with longer linkers) showed lower CR to the *cis*-target analyte. The antibody (Ab 3703) raised against *trans*-hapten **3** (*trans*-DCCA–glycine with no spacer) displayed better recognition to the *cis* isomer of the target analyte than that of other antibodies (Ab 3710 and Ab 3698). The ELISA combination of Ab 3703 and coating antigen, *cis*-hapten **5**-BSA, may be good for the detection of total *cis*-/*trans*-DCCA–glycine. All of the antibodies showed no or very little CR to the free permethrin metabolite (DCCA), permethrin, PBA–glycine conjugate, and glycine, suggesting that the antibodies are very selective for the target structure.

Unlike the aforementioned antibodies produced against the *trans* immunogens, the antibodies, Ab 3701, Ab 3709, and Ab 369, generated against the *cis* configuration cross-reacted poorly with the *trans*-configuration analogs (Table 3), and were highly specific to *cis*-target analyte. These antibodies also showed very low CRs to other structurally related compounds. On the basis of these results, it is thought that the antigenic determinants of the target analyte for the antibody production are the glycine moiety (–$NHCH_2COO$–) in the structure, the cyclopropane moiety as a hydrophobic position, and the *cis* or *trans* isomeric

Table 4 The comparison of recovery of *trans*-DCCA–glycine from the spiked urine sample by different sample preparation methods

Spiked concentration (μg/L)	Theoretical concentration in the ELISA (μg/L)[a]	ELISA	
		Liquid–liquid extraction (μg/L)	C18 SPE (method A) detected (μg/L)
0	0	0.27	0.19
3	0.06	0.40	0.16
5	0.10	0.50	0.22
10	0.20	1.91	0.19
20	0.40	3.07	0.41
40	0.80	5.02	0.78
80	1.60	5.25	1.31
160	3.20	–[b]	3.13

SPE solid-phase extraction
[a]Assay conditions: coating antigen *cis*-hapten **5**-BSA (1 μg/mL); antiserum 3703 finally diluted by 1:32,000 in 1× PBS in wells; goat antirabbit IgG-HRP (1:5,000). The data are the means of quadruplicates
[b]Not tested

Table 5 Recovery of a mixture of *cis*-/*trans*-DCCA–glycine (40:60) from spiked urine samples, prepared by C18-SPE-trifluoroacetic acid (*TFA*)

Spiked concentration (μg/L)	Theoretical concentration in the ELISA (μg/L)	Detected by SPE-TFA-ELISA (μg/L)	
		Sample 1	Sample 2
0	0	0.03±0.01	ND
1.0	0.2	0.19±0.05 (80±0.3%)[a]	0.13±0.02 (65±10%)
2.0	0.4	0.26±0.03 (65±4.0%)	0.34±0.18 (85±14%)
3.0	0.6	0.74±0.03 (123±9.4%)	0.67±0.17 (112±17%)
5.0	1.0	0.94±0.06 (94±3.3%)	0.92±0.11 (92±11%)
10	2.0	1.35±0.01 (68±0.7%)	1.62±0.06 (81±3.3%)

ND not detected
[a]Mean ± standard deviation

configuration of the structure as well as two Cl atoms as an electronegativity position. Permethrin is mostly produced in *cis*/*trans* isomeric mixtures of 40:60−25:75. The amount of *trans*-DCCA metabolite not conjugated and/or after acid hydrolysis of conjugates of permethrin in human urine ranged from 65 to 87% [8, 40]. The ratios of conjugates of *cis*- and *trans*-DCCA might be similar to that of free *trans*-DCCA. If each isomer of *cis*- and *trans*-target analyte could easily be separated for the assay, simultaneous assay with the specific antibody to *cis*- and *trans*-DCCA–glycine might be used. Alternatively, on the basis of the CR of the mixture of *cis*/*trans* isomers, the ELISA with Ab 3703 selective for both *trans* and *cis* isomers could be used for the analysis of total *cis*-/*trans*-DCCA–glycine in the urine sample. Interestingly, Ab 3709 generated against immunizing *cis*-hapten **9** combined with a coating hapten *trans*-hapten **5** recognized to *cis*-DCCA (3%) and *cis*-/*trans*-DCCA (1.71%), suggesting a potent antibody for the *cis*-/*trans*-DCCA analysis (Table 3). The recognition of antibodies except Ab 3709 to the parent compound and others including DCCA or the glycine moiety was negligible.

Sample preparation of urine

The LLE method of the target analyte where the pH is adjusted to acidic, basic, and then acidic conditions was used for sample cleanup. Because the target analyte possessing a carboxylic acid group is acidic, the aqueous urine sample was adjusted to pH 2 with 1 N HCl and partitioned with ethyl acetate. The target analyte moved to the organic phase from the aqueous phase under acidic conditions. After the aqueous phase had been discarded, 1 N NaOH solution was added and extracted with ethyl acetate, where the analyte moved back to the aqueous phase from the organic phase under alkaline conditions. This LLE cleanup technique was not satisfactory to eliminate the matrix effect of urine samples. As shown in Table 4, the ELISA results coupled to LLE showed high recovery values (above 300%), indicating that the normal constituents [41] of urine such as uric acid and hippuric acid may move with the target analyte and thus the urine matrix interference was removed incompletely by LLE.

SPE under acidic conditions (SPE method A)

A reversed (C18) SPE was used for urine sample extraction. The retention strength of the target compound with a carboxylic acid on reversed (C18) silica absorbent is quite low without acidification of the aqueous sample [42]. Therefore, the 0.1 M sodium phosphate buffer adjusted to pH 2 with phosphoric acid was used as a diluent for the urine sample before loading onto the top of the adsorbent. The ELISA results coupled to the SPE showed good recoveries (Table 4) at a 1:50 dilution (calculated on the basis of the original urine volume loaded onto SPE) of eluate evaporated. Chromatography under acidic conditions removed more acidic components in urine than the LLE method, but the sensitivity was not enough to quantify at the concentrations below 10 μg/L in the urine samples.

Improved SPE under acidic and ion-pairing conditions with TFA (SPE method B)

TFA both reduced the pH and acted as an ion-pairing reagent that masked the positive charge of the target improving the hydrophobicity of the target [43]. Reversed-phase retention of the target analyte was enhanced on the reversed-phase silica column. The retention strength of the target analyte increased on the C18 silica absorbent compared with that for the SPE technique. The ELISA results coupled to the SPE-TFA cleanup method showed good recovery (Table 5) at 1:5 dilution. This method completely removed the urine matrix and allowed the ELISA to detect much lower concentrations of target analyte.

As shown in Table 6, all of the final eluting solvents showed good recovery on the SPE column. Ethyl acetate was selected as the best eluting solvent for the target compound because the color of the ethyl acetate extract of urine was clearer than that of the extracts of methanol or a

Table 6 Different eluting solvents for SPE to reduce the matrix effect of urine samples in the ELISA

Eluting solvent (1.5 mL)	Recovery of *trans*-DCCA–glycine (%)[a]	Color of the urine extract after SPE-TFA (%)[b]
Methanol	>95	60
Ethyl acetate	>95	48
Isopropyl alcohol/ methylene chloride (20:80, v/v)	>95	66

[a]Recovery at 10 μg of target analyte in 0.5 mL of the buffer based on high-performance liquid chromatography–UV analysis
[b]Color was determined as the optical density of 100 μL of the urine extract at 405 nm

mixture of isopropyl alcohol and methylene chloride. In addition, the ethyl acetate evaporated more rapidly to dryness. This result suggests that the use of ethyl acetate as an eluting solvent for the target analyte can reduce the urine matrix effect in the ELISA.

Analysis of control urine samples

Several different urine samples from individuals with no known exposure to permethrin were obtained, as matrix effects from urine can vary from person to person. The urine samples were randomly collected from young and old, and male and female individuals. The collected urine samples were extracted by the optimized SPE sample preparation method and analyzed by HPLC/MS. DCCA–glycine was determined to be below the limit of quantitation (LOQ) of 0.38 μg/L (LOD, 0.038 ng/20 μL), and so identified no or negligible exposure to pyrethroid insecticides permethrin or cypermethrin. As shown in Fig. 4, the apparent DCCA–glycine concentration resulting from the individual urine samples ranged from 0.04 to 0.83 μg/L by the ELISA. Thus, the LOQ in urine was set at 1 μg/L. The theoretical LOQ value in the buffer was calculated as 0.79 μg/L. It was estimated as the concentration that corresponded to the absorbance of the control (zero concentration of analyte minus 3 times the standard deviation of the control [44]). The practical LOQ value was set slightly higher than the theoretical value.

Assay validation

As seen in Fig. 5, the assay validation was achieved by an SPE-TFA method to eliminate the matrix effect of the urine samples at concentrations of 0–10 μg/L, which were spiked with a mixture of *cis*-/*trans*-DCCA–glycine (40:60). The linear regression of the results showed a good correlation (r^2=0.98) with a slope of 0.75. The result demonstrates that the assay is appropriate for the detection of a glycine conjugate of *cis*-/*trans*-DCCA at low levels in urine samples.

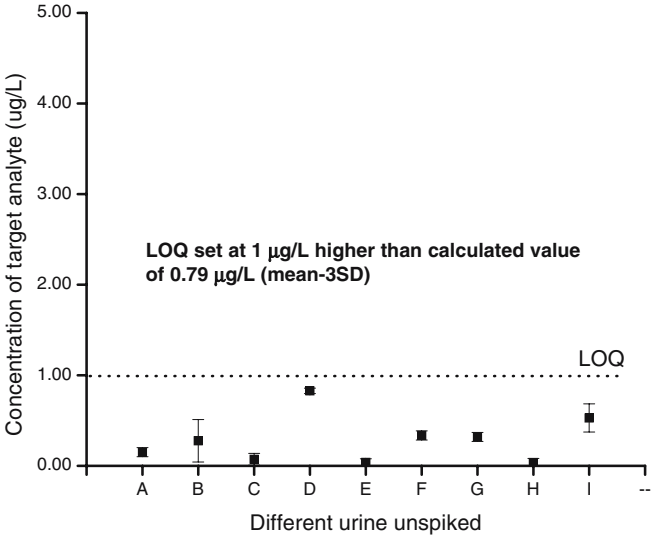


Fig. 4 The apparent concentrations of the target analyte resulted from matrices of individual unspiked urine samples. Urine samples were obtained from individuals with no known exposure to permethrin. The limit of quantitation (*LOQ*) is higher than the calculated value (zero concentration of analyte minus 3 times the standard deviation of the control equals 0.79)

Conclusions

A sensitive and specific ELISA was developed and characterized for the detection of a glycine conjugate of *cis*-/*trans*-DCCA as a biomarker of exposure to permethrin. The linear range of the extent of inhibition of the target analyte in buffer is approximately 0.20−7.0 μg/L, and the IC_{50} value is 1.24 μg/L. The ELISA coupled to an SPE-TFA method for the sample preparation was successfully applied to the detection of trace amounts of the target in

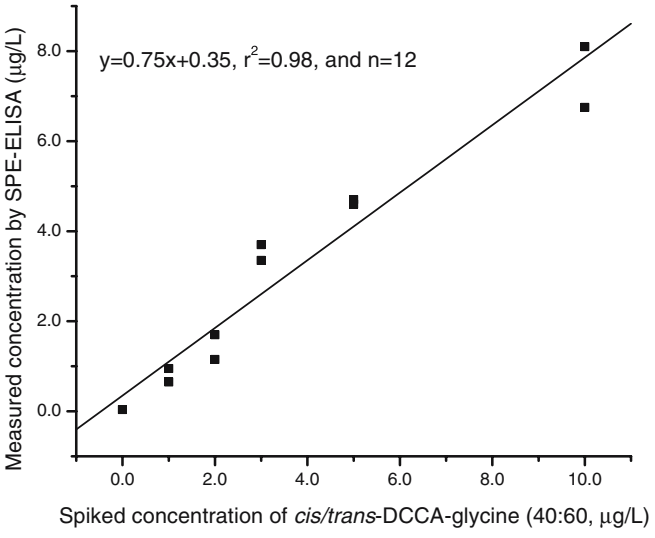


Fig. 5 Relationship between different concentrations of target analyte spiked into urine and determined by solid-phase extraction (*SPE*) trifluoroacetic acid ELISA. Urine samples were obtained from individuals with no known exposure to permethrin. y=0.75x+0.35, r^2=0.98, and n=12

human urine samples with an LOQ of 1.0 μg/L. Since the *cis*-/*trans*-DCCA metabolite is a common structural group in some pyrethroids, including permethrin, cypermethrin, and cyfluthrin, the assay is class-specific for the metabolism study of pyrethroids containing DCCA. This assay may be a useful and significant tool together with the immunoassays for a PBA–glycine conjugate [29] and free PBA metabolite [33] as biomarkers of exposure to pyrethroids, including permethrin.

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