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Development and Application of an Indirect Competitive Enzyme-Linked Immunosorbent Assay for the Detection of *p,p′*-DDE in Human Milk and Comparison of the Results against GC-ECD

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ABSTRACT: 1,1-Dichloro-2,2-bis(*p*-chlorophenyl) ethylene (*p,p′*-DDE) is the major metabolite of insecticide 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (*p,p′*-DDT) and a persistent organic pollutant (POPs) with concerns regarding its bioaccumulation and persistence in the environment and food chain. In the present study, an indirect competitive enzyme-linked immunosorbant assay (ic-ELISA) specific for the detection of *p,p′*-DDE is described. In hapten synthesis, 2,2′-bis(4-chlorophenyl)ethanol and glutaric anhydride were used as precursor and spacer arm, respectively. The hapten was then conjugated to bovine serum albumin (BSA) as immunogen for mouse immunization and also conjugated to ovalbumin as coating antigen for ELISA. The developed ic-ELISA was used for detecting *p,p′*-DDE in human milk samples and validated against the results from conventional gas chromatography−electron capture detection (GC-ECD). Coefficients of variation (%CV) of ELISA were 5.7−10.4% for intra-assay and 10.6−19.6% for interassay variations. The Pearson correlation coefficient of *p,p′*-DDE concentrations between ic-ELISA and GC-ECD was $r = 0.766$, which was in an acceptable range. The results indicate that the developed assay could be an alternative analytical tool for monitoring *p,p′*-DDE in lipimic matrices such as human milk.

KEYWORDS: *DDT, DDE, ELISA, GC-ECD, human milk*

INTRODUCTION

1,1-Dichloro-2,2-bis(*p*-chlorophenyl) ethylene (*p,p′*-DDE), the major metabolite of the insecticide 2,2-bis(*p*-chlorophenyl)-1,1,1-trichloroethane (*p,p′*-DDT or DDT as most common use), is a persistent organic pollutant (POP) with concerns over its bioaccumulation and persistence in the environment and food chain. The structure of *p,p′*-DDE and *p,p′*-DDT is shown in Figure 1.[1−3] *p,p′*-DDE is most frequently detected at high

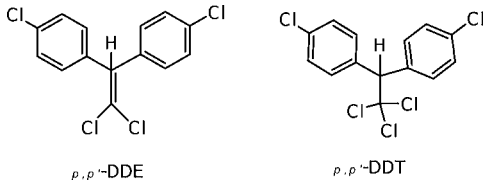

Figure 1. Structures of *p,p′*-DDE and *p,p′*-DDT.

levels in human biological fluids from former malaria endemic areas.[4−9] Recently, DDT has been reportedly involved in endocrine disruption activity,[8−10] resulting in adverse human and wildlife developmental defects and potentially causing cancer.[11−13]

Since DDT was banned in vector-control by the Thai government in 1999,[7] the human population has been exposed to DDT and its metabolites mainly from the environment and food chain.[14] From their chemical characteristics of nonpolar compounds, nonmetabolized DDT and *p,p′*-DDE will be mainly stored in fatty tissues.[14,15] Highest concentrations are found in body fat tissues, and these are higher in milk than in serum. *p,p′*-DDE in human tissues can remain for several decades as its half-life is approximately 7−11 years.[16,17] Several studies reportedly found high *p,p′*-DDE concentrations in the breast milk of mothers living in malaria endemic area where DDT had been used.[4,18,19]

Methods for detecting DDT and its metabolites mostly rely on conventional techniques such as gas chromatography equipped with electron capture detection (GC-ECD)[19,20] and mass spectrometry (GC-MS).[21,22] However, these techniques have some disadvantages such as requirements for thorough sample cleanup, expensive equipment, and a skilled operator. Therefore, a method with rapid and effective sample preparation for the analysis of massive samples is a quest for development. Accordingly, the present study demonstrates that the developed indirect competitive enzyme-linked immunosorbent assay (ic-ELISA) is applicable for analyzing *p,p′*-DDE in human milk, which is a fatty matrix. The ic-ELISA result obtained was compared against results from GC-ECD.

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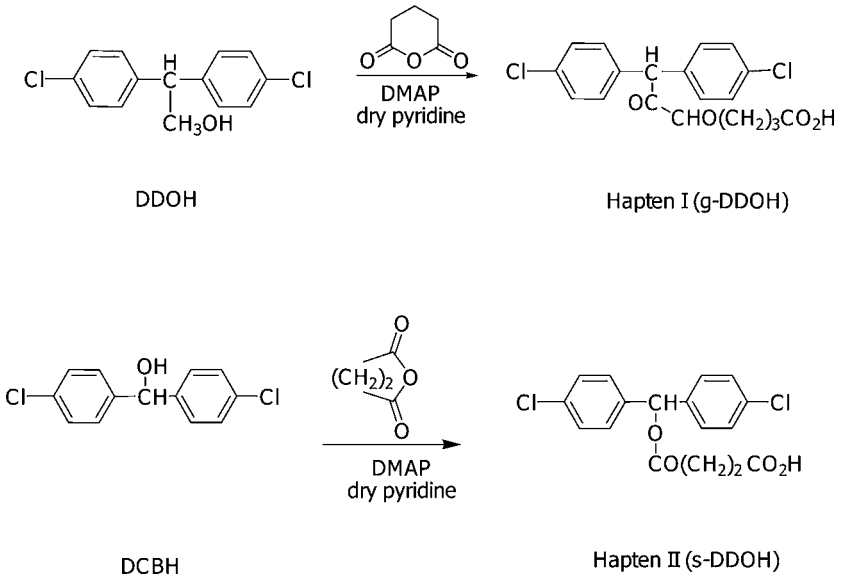

Figure 2. Diagram of the synthesis routes for targeted haptens.

MATERIALS AND METHODS

Chemicals and Reagents. Analytical stock solutions were prepared in dimethyl sulfoxide (DMSO; Sigma) and stored at −20 °C. Protein concentrations were measured by Bradford assay using reagents from Bio-Rad Laboratories. Thin-layer chromatography (TLC) was performed on silica gel 60F254 20 × 20 cm aluminum sheets (Merck). Solvent systems used for hapten elution were hexane/ethyl acetate (40:60 v/v) and others as indicated in the individual syntheses. Substances resulting from synthesis reaction were detected by viewing plates under ultraviolet (UV) light at 254 nm. Column chromatography was carried out on 40 μm silica gel (Merck). Dialysis was performed using 6000−8000 MW cutoff, Spectra/Por membrane tubing (Spectrum Laboratory, Inc.).

Hapten Synthesis. *Pentanedioic Acid Mono[2,2-bis(4-chlorophenyl)ethyl] Ester (Hapten I, g-DDOH).* Hapten I was synthesized following the method of Beasly et al.[29] by using 2,2′-bis(4-chlorophenyl)ethanol (DDOH, 100 mg, 0.37 mmol) as reactant and reacted with glutaric anhydride (375 mg, 3.74 mmol) in 5 mL of dry pyridine with 5 mg of dimethyl aminopyridine overnight at room temperature (25 °C). A diagram of the reaction is shown in Figure 2. Twenty milliliters of water was added to the mixture and evaporated to dryness to remove pyridine. The crude compound was rinsed with toluene and solvent removed by evaporation and then dissolved in ethyl acetate. The mixture was washed with 1 M HCl, water, and salt water before evaporation and drying over $MgSO_4$. The crude product was purified by column chromatography on silica gel using ethyl acetate/petroleum ether (40:60 v/v) with 0.01% acetic acid as mobile solvent. A single-compound fraction was collected and evaporated to dryness. The structure of the compound was confirmed by ^{1}H nuclear magnetic resonance (NMR) spectroscopy, and the molecular weight was confirmed by GC (7890A)-MS (5975C) (Agilent Technology). The hapten obtained was a white powder compound: R_f 0.50 (ethyl acetate/hexane, 40:60); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.268 (dd, J = 2.4, 8H), 7.172 (dd, J = 2.8 Hz, 4H), 4.587 (d, J = 7.6 Hz, 2H), 4.341 (t, J = 7.6 Hz, 1H), 2.479 (t, J = 7.2 Hz, 1H), 2.321 (q, J = 7.2 Hz, 4H). MS was used to confirm the homogeneous peak, t_R =11.31 min. MSD 5975 (EI) Hewlett-Packard calculated for $C_{19}H_{18}Cl_2O_4$ (M + H^+) 381.2 was 381.0.

Succinic Acid Mono[bis(4-chlorophenyl)methyl] Ester (Hapten II, s-DCBH). Hapten II was synthesized following a method from Honsibsong et al.[23] In brief, a mixture of DCBH (200 mg, 0.78 mmol), succinic anhydride (750 mg, 7.5 mmol), and dimethylamino-pyridine (DMAP; 10 mg, 0.08 mmol) in 10 mL of dry pyridine was stirred overnight. Twenty milliliters of water was then added, and the mixture was evaporated to dryness. Crude was rinsed twice with 10 mL of toluene and dissolved in 10 mL of ethyl acetate. Crude solution was washed once with 10 mL of cool HCl, twice with 10 mL of water, and twice with 10 mL of salt water, respectively. The product was dried over magnesium sulfate ($MgSO_4$). The purity of the product was confirmed by TLC. Crude product was purified by column chromatography on silica gel using ethyl acetate/hexane (40:60 v/v) as solvent. A single-compound fraction was collected and evaporated to dryness. The structure of the compound was confirmed by ^{1}H NMR and GC-MS.

Preparation of Immunogens. Haptens I and II were conjugated to proteins: bovine serum albumin (BSA, from Sigma-Aldrich) for preparing immunogens, and ovalbumin (OVA, from Fluka, Sigma-Aldrich) for preparing capture antigens. In all cases, an active *N*-hydroxysuccinimide (NHS) ester was used to couple the carboxylic acid moieties of the haptens to proteins.[24] Immunogens and capture antigens were prepared from 25 μM of the haptens and incubated overnight at 30 °C with stoichiometric amounts of NHS and dicyclohexylcarbodiimide (DCC) in 0.5 mL of dimethylformamide (DMF). The mixture was then centrifuged at 7000 RCF for 10 min. Four hundred microliters of supernatant containing the active NHS ester of hapten was collected and then slowly added to 2 mL of 15 mg/mL BSA or OVA in 50 mM carbonate buffer, pH 9.6. The mixture was allowed to react at 25 °C for 4 h with stirring. The mixture was dialyzed overnight in PBS, pH 7.2, at 25 °C. Protein concentration in the dialysates was determined according to the Bradford protein assay.

Production of Polyclonal Antibody (pAb). *Immunization and Antibody Preparation.* The first aim of the present study was to generate pAb, and mice instead of rabbits were employed for immunization. The animal experiment was performed in accordance with the guidelines for the care and use of animals for experimental procedures and approved by the Animal Ethics Committee, Faculty of Medicine, Chiang Mai University, Protocol 29/2550. Female BALB/c mice were injected subcutaneously (sc) with 30 μg of an immunogen emulsified in complete Freund's adjuvant. Mice were given the subsequent injections sc with the immunogen emulsified in incomplete Freund's adjuvant at 2 week interval. Blood samples were collected from the tail veins at 7 day interval ,and heart puncture blood sample was collected 24 weeks after the first immunization. Blood was allowed to clot, and sera were separated and stored at −20 °C in the freezer. The sera pooled from the mice having good antibody response either to hapten I or hapten II were used as pAb for ELISA development.

Determination of Sensitivity and Specificity of pAb by ic-ELISA. The sensitivity and specificity of pAb were tested following our standard protocol as described previously.[23] In brief, 96-well maxisorp immunoplates were coated with 2 μg/mL DCBH-S-OVA in 50 mM

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carbonate−bicarbonate buffer, pH 9.6, at 4 °C overnight. The wells were washed four times with 0.05% Tween 20/PBS and then blocked by incubation with 200 μL/well of 1% gelatin in PBS. One hundred microliters of a mixture containing *p,p′*-DDT and *p,p′*-DDE at different concentrations and antibodies was diluted in PBS, pH 7.2, with 0.05% Tween 20 (final dilution 1:5000) dispensed onto each well, and the wells were then incubated at room temperature (25 °C) for 90 min. The wells were washed with PBS, pH 7.2, with 0.05% Tween 20 as washing buffer (four times), and 100 μL of 1:5000 HRP-conjugated goat anti-mouse IgG in washing buffer was added to each well. After incubation at 37 °C for 1 h, the plates were washed and 100 μL of *o*-phenylenediamine (OPD) solution was added to the wells. The reaction was allowed to continue for 30 min and stopped by adding 50 μL of 2 N H_2SO_4. The color developed (yellow) was read for absorbance at 492 nm using an ELISA plate reader (Sunrise, TECAN).

Optimization of ELISA. *Effect of Coating Antigen.* Two types of coating antigens, s-DCBH-OVA from hapten I and g-DDOH-OVA from hapten II, were compared by ic-ELISA. *p,p′*-DDE (Laboratory of Dr. Ehrenstorfer, Augburg, Germany) was used as a competitor.

Effect of Diluents. DMSO is often used as an organic modifier of the buffer formulation in the extraction of less hydrophilic analytes from samples. To determine optimal concentrations of DMSO in the buffer, noncompetitive indirect ELISA was carried out using various solutions of DMSO (10, 20, 30, 40, and 50%) with washing buffer (v/v). It was found that 10−20% DMSO solution had no effect on antibody reaction (Figure 3) and was used as diluent's composition.

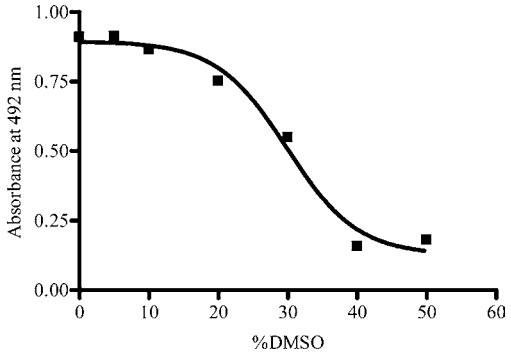

Figure 3. Effect of DMSO on antibody at different concentrations.

The standard *p,p′*-DDE was diluted at the optimal concentration of DMSO in washing buffer (v/v) by ic-ELISA. The assays were done in duplicate wells.

Human Milk Samples. These human milk samples were part of samples collected from health care clinics of the Shoklo Malaria Research Unit (SMRU) in Maela camp, 50 km north of Mae Sot district (at the Thai−Myanmar border), Tak province, northern Thailand, from 2004 to 2008. The study was approved by the Ethics Committee of Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand, and the Oxford Tropical Research Ethics Committee, University of Oxford, United Kingdom. In brief, the purpose and methods of the survey were explained to all participants in their own language, mostly Karen. Breast milk samples were collected by manual expression into glass tubes. The samples were mixed and aliquoted into Eppendorf tubes and then kept frozen at −20 °C in Maela camp and transported to the SMRU office in Mae Sot district. The collected samples were stored at −80 °C. Samples were shipped frozen on dry ice to the toxicology laboratory of the Research Institute for Health Sciences, Chiang Mai University, Chiang Mai, for the analysis of DDT and its metabolites using GC-ECD (HP 5890 series II).

GC-ECD Analysis. Human milk samples were extracted using the method described by Prapamontol and Stevenson[25] with slight modification. In brief, 2 mL of human milk was extracted with 10 mL (ethyl acetate/methanol/acetone = 1:2:2) and cleaned up with C_{18} solid phase extraction (BondElute, Varian). Lipid residue in the eluate was treated with 300 μL of concentrated sulfuric acid. One microliter of clean eluate was injected for analysis on GC-ECD-^{63}Ni (HP 5890 A series II) equipped with an automatic sampler (HP 7673) and a fused silica capillary column (Ultra 2, Agilent Technologies; 25 m × 0.32 mm i.d., with 0.52 μm film thickness) for separation. Spiked skim cow's milk was used for standard calibration curve construction. The calibration curve for *p,p′*-DDE was constructed at 3, 6, 9, 12, 15, and 30 μg/L. The recoveries of *p,p′*- DDE at concentrations 6 and 21 μg/L were 98.2 ± 0.2 and 89.1 ± 0.4%, respectively. The limit of detection (LOD) as described by a signal-to-noise ratio of 10:1 was 0.04 μg/L. As part of the quality assurance of analysis, besides internal quality control, we participated in the German External Quality control (G-EQUAS, 40 in 2007), University of Erlangen-Nuremberg, Erlangen, Germany, and obtained qualified results.

RESULTS AND DISCUSSION

Synthesis of Haptens. DDE, as well as DDT and other small molecules, is not immunogenic.[26] DDE has two aromatic

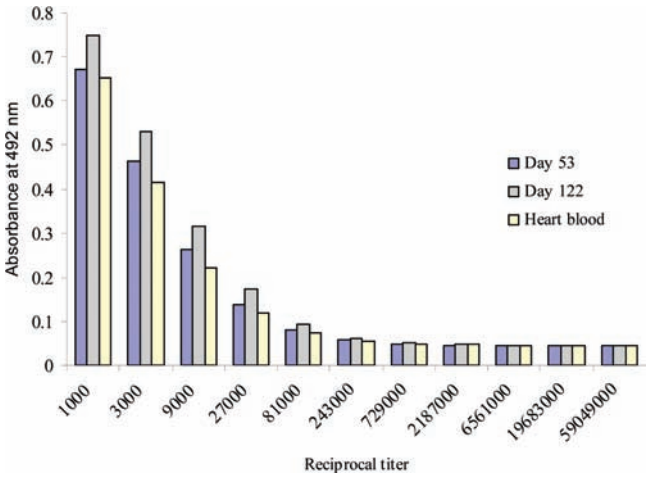

Figure 4. Antibody titer from mouse P3 at different days and heart blood.

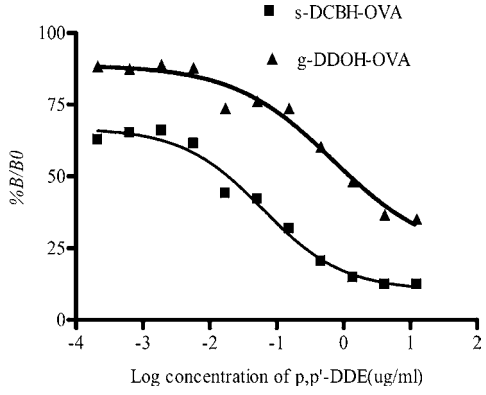

Figure 5. Dose−response curve between $\%B/B_0$ and log concentration of *p,p′*-DDE in different coating antigens determined by the developed ic-ELISA.

rings, which have chlorine atom substitution and are connected with a carbon atom in the center (Figure 1). To synthesize an immunogen, we employed a hapten having a structure similar to that of DDE. DDOH has a similar structure to DDE and provides a −OH group for substituting with glutaric anhydride (5 carbon atom length) as spacer arm for hapten I. DCBH was also used for hapten synthesis in our previous study, and succinic acid anhydride (4 carbon atoms) was used as spacer

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Table 1. Sensitivity and Cross-Reactivity of Pooled Mouse Sera with DDT Metabolites

compound	IC_{50} (ng/mL)	% cross-reactivity
p,p′-DDE	22.5	100
p,p′-DDD	20.0	112.5
p,p′-DDM	37.7	59.7
DCBH	243.3	9.25
p,p′-DDT	340	6.62
o,p′-DDD	450	5.00
DDOH	785	2.87
o,p′-DDE	3500	0.64
p,p′-DDA	8025	0.28
p,p′-DBP	8357	0.27
o,p′-DDT	>20000	
dicofol	>22500	

Table 2. Recovery, Accuracy, and Reproducibility of the Developed ic-ELISA

analytical parameter	developed ic-ELISA
LOD (ng/mL)	3.5
LOQ (ng/mL)	10.5
IC_{50} (ng/mL)	20.1
IC_{15} (ng/mL)	9.6
CV, intra-assay (%, N = 3)	5.7−10.4
CV, interassay (%, N = 3)	10.6−19.6
recovery (%)	88.8−116.8

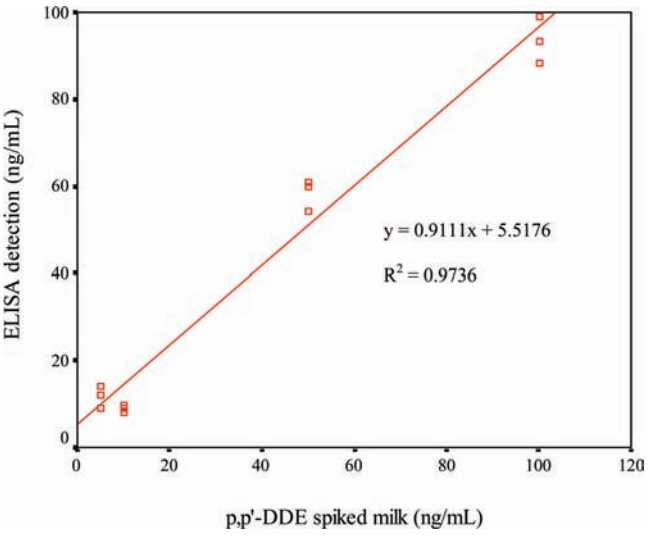

Figure 6. Correlation between concentration of *p,p′*-DDE-spiked milk and concentration detected by the developed ic-ELISA against standard curve of *p,p′*-DDE.

arm for hapten II.[23] The present study results show that the hapten with the 5 carbon atom spacer arm provided better antibody production than the shorter spacer arm hapten (4 carbon atoms). Meanwhile, hapten with the shorter spacer arm (4 carbon atoms) performed better as a coating antigen than the hapten with the longer spacer arm (5 carbon atoms).

Antibody Response to DDE. To evaluate the ability of the synthesized haptens to induce anti-DDE antibody response, mice were immunized with g-DDOH-BSA. After three injections, the antibody response and titer of each mouse was determined by noncompetitive indirect ELISA using g-DDOH-OVA as coating antigen. Sera from all mice showed antihapten

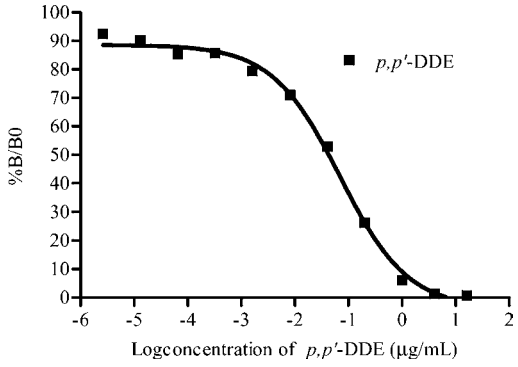

Figure 7. Developed indirect competitive ELISA standard curve of *p,p′*-DDE.

reactivity after the first immunization. Mouse P3 gave the best antibody response with a titer of >162,000 after the third injection, because nonimmune serum control showed low absorbance at 0.045. Sera from mice at day 80 were used to determine sensitivity and specificity to *p,p′*-DDT and *p,p′*-DDE by ic-ELISA. The results show that the binding activity of antibodies in sera of mouse P3 was inhibited by *p,p′*-DDT and *p,p′*-DDE at high concentration with IC_{50} values of about 2.85 and 0.08 μg/mL, respectively, before the ELISA was optimized. Sera from mouse P3 collected at different time points of postimmunization, which gave similar antibody titers to sera collected by cardiac puncture, were then pooled for further study (Figure 4).

Assay Optimization. *Effect of Coating Antigens.* To determine a suitable coating antigen, s-DCBH-OVA and g-DDOH-OVA were verified by ic-ELISA. *p,p′*-DDE was used as competitor. A dose−response curve was generated to select a matching pair of antibody and coating ligand. Two matching pairs of antibody and coating ligand were tested for sensitivity to *p,p′*- DDE. Figure 5 shows the results for dose responses of *p,p′*-DDE using a pair of antibody and coating ligand. The results show that the IC_{50} when s-DCBH-OVA was used as coating antigen (0.06 μg/mL) was 10 times better than the IC_{50} (0.69 μg/mL) of g-DDOH-OVA. Hence, there is a high binding between antibody and the hapten used for immunization. The pair of antibody and capture antigen showed high binding response, but the bound complex of antibody and the coating antigen could not be displaced by *p,p′*-DDE. It was also reported that increased sensitivity of antibody was achieved by reducing an antibody affinity of the tracer.[27] These results confirmed our study that a longer spacer arm used for coupling of a carrier protein enhances immunogenicity of the hapten for antibody production. However, a short spacer arm showed a better binding efficiency than a longer spacer arm for dose response as determined by ic-ELISA (Figure 5). Therefore, s-DCBH-OVA will be used as coating antigen in ic-ELISA in the subsequent assays.

Effect of Diluent. To investigate the effect of DMSO, different concentrations of DMSO at 50, 40, 30, 20, 10, and 5% were tested by noncompetitive indirect ELISA. The results show that buffer containing 10 and 20% DMSO gave similar absorbance to buffer containing no DMSO. Then diluents containing 10 and 20% of DMSO were used for further assays to examine the optimal condition for ic-ELISA when *p,p′*-DDE was used as a competitor. The diluent 20% DMSO in washing buffer (v/v) gave the most satisfactory result with an IC_{50} at 1.0

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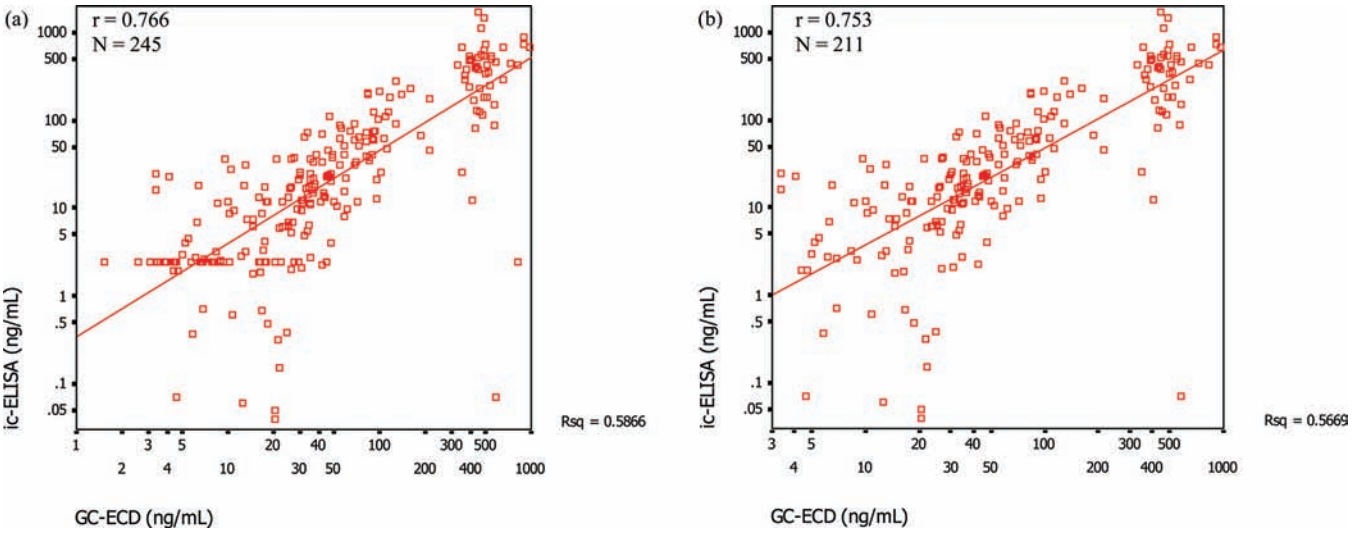

Figure 8. (a) Correlation of *p,p′*-DDE data between imputed data of ic-ELISA and data of GC-ECD. (b) Correlation of *p,p′*-DDE between cutoff nondetectable data of ic-ELISA and data from GC-ECD.

μg/L. Therefore, 20% DMSO in washing buffer (v/v) was used as the diluent for ic-ELISA.

Sensitivity and Specificity of Polyclonal Antibody. The sensitivity and specificity of pooled antibody were assessed by ic-ELISA using *p,p′*-DDE standard and other compounds structurally related to *p,p′*-DDE, including *o,p′*-DDT, *p,p′*-DDT, *o,p′*-DDD, *p,p′*-DDD, *p,p′*-DDA, dicofol, DDOH, DCBH, and DDM, as inhibitors. The absorbance was transformed to % B/B_0, where B is the value of absorbance for each standard and B_0 is the value of absorbance for no standard. Inhibition concentrations at 50% (IC_{50}) were fit to a four-parameter logistic equation. The LOD of the developed ELISA was 0.02 ng/mL at 80% B/B_0 and 0.05 pg/mL at 90% B/B_0. Cross-reactivity was calculated as follows. The concentration ratio of test chemical that caused 50% inhibition ($IC_{50\ p,p'\text{-DDE}}/IC_{50\ \text{chemicals}}$) × 100. $IC_{50\ \text{chemicals}}$ was 50% inhibition of test chemical or inhibitors including *o,p′*-DDT, *p,p′*-DDT, *o,p′*-DDD, *p,p′*-DDD, *p,p′*-DDA, dicofol, DDOH, DCBH, and DDM. The cross-reactivity patterns against various DDT metabolites and DDE-related compounds are shown in Table 1. Using the developed ELISA, it was determined that anti-*p,p′*-DDE antibody had high cross-reactivity with *p,p′*-DDD (112.5%) and mild cross-reactivity with *p,p′*-DDT, *o,p′*-DDE, and *p,p′*-DDA at 6.62, 0.64, and 0.28%, respectively. Dicofol, *o,p′*-DDT, DDOH, and DCBH showed almost no cross-reactivity with the obtained antibody. In addition, characterization of the pAb indicated that antiserum possesses a high specificity for *p,p′* isomers.[28] A study reported[29] that pAb produced in rabbits could detect *p,p′*-DDE at concentrations ranging between 3.5 and 300 ng/mL. However, from this study, we report the production of antibody highly specific to *p,p′*-DDE and some metabolites of *p,p′*-DDT. The obtained antibody from the present study was from mouse and gave high sensitivity with the LOD of *p,p′*-DDE in the ELISA system at 3.5 ng/mL, IC_{15} = 9.6 ± 24.4 ng/mL, and IC_{50}= 20.1 ± 31.3 ng/mL.

Analysis of Human Milk Samples. *Analysis of p,p′-DDE.* The *p,p′*-DDE in milk samples was determined using a GC-ECD method as described earlier.[25] The extract, left over in dry and kept in the freezer, was redissolved with DMSO for determining *p,p′*-DDE with the developed ic-ELISA. The results show no interference from the redissolved DMSO in ELISA.

Recovery and Precision of Developed ic-ELISA. Recovery of the assay was carried out using three different concentrations (low, medium, and high concentrations, respectively) of spiked milk samples with *p,p′*-DDE (10, 50, and 100 ng/mL). The results in Table 2 show that the recovery of *p,p′*-DDE ranged from 88.8 to 116.8%. The coefficient of variation (CV; the ratio of the standard deviation (SD) to the mean of detected *p,p′*-DDE) reflects the assay precision. The CVs of the intrabatch assay, which was performed in three different concentrations, varied from 5.7 to 10.4%, and those of interbatch assay for 3 days of analysis were 10.6−19.6% (Table 2). The correlation of spiked milk samples detected by ELISA gave good correlation between developed ELISA (y) and spiked standard (x) with a linear regression equation of $y = 0.9111x + 5.5176$ (R^2 = 0.9736) as shown in Figure 6. The LOD of the ic-ELISA was interpreted from interaction of the equation when the standard deviation (SD) was plotted against three concentrations of spiked milk. The obtained LOD was 3.5 ng/mL, whereas the limit of quantitation (LOQ) was 10.5 ng/mL (3-fold of LOD). Then, the results suggest that the developed ic-ELISA could be used for determining *p,p′*-DDE in biological samples.

Validation of Developed ic-ELISA with GC-ECD. Human milk samples (N = 245) were determined for *p,p′*-DDE using the developed ic-ELISA. The concentrations of *p,p′*-DDE in samples were computed against the standard curve and ranged from 0.047 ng/mL to 12.5 μg/mL *p,p′*-DDE (Figure 7). Concentrations of *p,p′*-DDE detected by the developed ELISA were compared with those results detected by GC-ECD. From the result of cross-reactivity, although the obtained pAb shows high specificity to *p,p′*-DDD, this compound was the minor metabolite of DDT in the environment and biological fluids.[9] Of 245 human milk samples, the developed ic-ELISA detected *p,p′*-DDE in 211 samples (86.1%). Concentrations of *p,p′*-DDE detected by the developed ELISA and GC-ECD were 108.8 ± 224.1 and 133.4 ± 204.0 ng/mL, respectively. The nondetectable data by ELISA were imputed with the value of its LOD by dividing by the square root of 2, which was 1.414 for further calculation.[30] The geometric mean concentrations of *p,p′*-DDE detected by ic-ELISA and GC-ECD were 19.5 and

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45.5 ng/mL, respectively. The concentration of *p,p′*-DDE at high concentration (75th and 95th percentiles) gave results close to GC-ECD. Please note that one of the limitations of the present study might be the storage of milk sample extracts in iso-octane, which was left over from GC-ECD analysis and kept at −20 °C in the freezer for about 2 years. This is might be the reason for the lower number of sample detection by the developed ELISA. However, the results transformed to natural log (ln) concentration detectable by both ic-ELISA and GC-ECD show good correlation at Pearson correlation coefficient $r = 0.766$ with imputed data ($N = 245$) and $r = 0.753$ with data from detectable samples ($N = 211$) (Figure 8). The present study has demonstrated that the developed ic-ELISA using our own developed antibody could be used for determining *p,p′*-DDE at low levels reported in epidemiology study[31,32] and used in biological samples and environmental samples of *p,p′*-DDE analysis at levels comparable with the GC-ECD standard method. The current commercial kits available for DDE/DDT detection are from EnviroGard and Abraxis. Enviroguard's method relies on polyclonal antibodies coated on the plate, whereas the method in the present study is based on the antigens. Although it is similar to Abraxis's format, our method can be applied to lipimic biological samples, such as milk. Therefore, we believe that we are presenting the innovation of using mouse polyclonal antibodies in an indirect competitive ELISA to detect *p,p′*-DDE in human milk samples. In conclusion, the present study has demonstrated that the obtained antibody gave a good sensitivity for detecting *p,p′*-DDE and the developed ic-ELISA could be employed for analyzing *p,p′*-DDE in lipimic matrices such as human milk. Furthermore, the obtained ic-ELISA can be performed rapidly, is inexpensive, and allows high sample throughput when compared with the conventional GC method. Moreover, this assay can also be employed to analyze *p,p′*-DDE in other biological samples such as serum because of its high sensitivity characteristic to detect very low levels.

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