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Development of an Enzyme-Linked Immunosorbent Assay for the Quantification of DDA (2,2-bis(p-chlorophenyl) acetic Acid) in Urine

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The widespread use of 1,1,1-trichloro-2,2-bis (p-chlorophenyl) ethane (DDT) over a number of years, coupled with its extreme stability and slow metabolism, has lead to environmental contamination and the ultimate carry-over from the food chain into humans (Ramachandran et al. 1984a). A large number of metabolites are formed by biotransformation during partial detoxication of DDT in the mammalian system. During the course of a study of the body burden of DDT and its metabolites in humans the assay of the metabolite 2,2,bis (p-chlorophenyl) acetic acid (DDA) was investigated as it was the chief urinary metabolite of DDT (WHO 1979; Watts 1981; Ramachandran et al. 1984b). Urinary excretion of DDA has been reported as a sensitive index of DDT exposure in humans (WHO 1979; Watts 1981; Zaidi et al. 1984; Ramachandran et al. 1984b).

The usual procedure for analysis of DDA in urine is gas chromatography or colorimetry but these methods require extensive sample preparation and clean up (Watts 1981; Zaidi et al. 1984; Ramachandran et al. 1984b). Moreover, these methods are often time consuming, tedious and expensive. Therefore, it is necessary to have an efficient and rapid assay system to determine DDA concentration in urine. This paper describes the development of enzyme-linked immunosorbent assay (ELISA), a rapid and efficient method that can detect nanogram quantities of DDA in crude urine extracts with high sensitivity and specificity. This was used to examine 24-hr urinary excretion of the DDA in human subjects, and the values were compared with gas chromatographic and colorimetric procedures.

MATERIALS AND METHODS

p,p'-DDT (99%) was obtained from Aldrich Chemical Co., Milwaukee, WI. Bovine serum albumin (BSA), rabbit serum albumin (RSA), goat anti-rabbit IgG-horseradish peroxidase conjugate, o-phenylenediamine and Tween-20 were purchassed from Sigma Chemical Co., St. Louis, MO. Freund's complete adjuvant (FCA) was obtained from Difco Laboratories, Detroit, MI. Reference

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standards of p,p'-DDT, DDA, 1,1-dichloro-2,2-bis (p-chlorophenyl) ethane (DDE) and 1,1-dichloro-2,2-bis (p-chlorophenyl) ethane (DDD) were received through the courtesy of the Environmental Protection Agency, Research Triangle Park, NC.

DDA was prepared from p,p'-DDT by the method of Grummitt et al. (1946). The analysis of DDA by gas chromatography indicated a purity of greater than 99%. DDA was conjugated to BSA or RSA by anhydride reaction according to the method of Centeno et al. (1970). Briefly, the requisite protein (120 mg) was dissolved in 20 mL of phosphate-buffered saline (0.15 M, pH 7.4). To this was added DDA anhydride (75 mg) dissolved in 7.5 mL of absolute ethanol and the mixture stirred overnight at 4^{o}C. The solution was then dialysed against several changes of 0.05 N NaOH, and the finally extensively against distilled water, all at 4^{o}C. The protein conjugates were then lyophilized and stored under dessication at -20^{o}C.

Two female albino rabbits (1.5 kg average weight) were immunized intramuscularly thrice with 5 mg of DDA-BSA conjugate emulsified in Freund's complete adjuvant in the hind limb at 15-day intervals. The rabbits were bled one week after the last immunization at weekly intervals, and sera were collected for testing antibodies for another 8 weeks. Sera were stored at -20^{o}C. The DDA-antibody activity in the antisera was assayed by immunoelectrophoresis (IEP). DDA-RSA or DDA-BSA conjugate (10-15 μL) was electrophoresed (7.5 mA current per microslide) in 0.9% agarose-A (Pharmacia Fine Chemicals, Uppsala, Sweden) by using 0.05 M Veronal buffer, pH 8.6 in LKB-multiphor system for 90 min. The application pattern in the gel consisted of two sample holes (1-mm diameter) and one antibody trench with a distance of 5 mm between the trench and the holes. Rabbit antiserum was added to the trench after electrophoresis and kept for diffusion in a humid chamber at room temperature for 18-24 hr. The slides were later washed for 48 hr, dried and stained with 0.5% coomassie brilliant blue.

The enzyme immunoassay was performed according to Voller et al. (1976) with modifications as follows. The assays were performed in 96-well polystyrene microtiter plates (Titertek, Flow Laboratories, Irvine, Scotland). The wells were coated with 10 μg of the DDA-RSA conjugate dissolved in 100 μL of 0.1 M sodium carbonate buffer (pH 9.6) containing 0.02% NaN_3 at 4^{o}C for 12 hr. The coated plates were washed 3 times with phosphate-buffered saline (0.15 M, pH 7.2) containing 0.05% Tween-20 (PBS-Tw). The wells were incubated with 100 μL of 1% RSA in sodium carbonate buffer at 37^{o}C for 1 hr and washed 3 times with PBS-Tw. Serial dilutions of antisera in PBS-Tw were prepared, and 100 μL was incubated with the coated wells for 1 hr at 37^{o}C. After three further washes the PBS-Tw, the 100 μL of goat anti-rabbit IgG-horseradish peroxidase conjugate diluted in PBS-Tw (1:1000) was added, incubated at 37^{o}C for 1 hr and washed with PBS-Tw. The activity of the horseradish peroxidase was determined by introducing 100 μL of the enzyme substrate o-phenylenediamine (400 μg/mL) dissolved in 0.1 M sodium citrate buffer, pH 4.9 containing 1.5 μL of 30%

H_2O_2/mL into the wells. The plates were incubated at room temperature for 30 min. The reaction was stopped by the addition of 8 N H_2SO_4 (50 µL) and absorbance was measured in a microcell read in a spectrophotometer (LKB Ultrospec 4050) at 490 nm.

The preparation of inhibition curve was as follows. One milliliter of appropriately diluted anti-DDA rabbit serum in PBS-Tw was incubated with different levels of DDA dissolved in 50 µL of ethanol for 2 hr at 25°C. Aliquots (100 µL) of the inhibited antiserum were added to the DDA-RSA coated plates. The enzyme immunoassays were then performed as described above. Results are expressed as the inhibition of absorbance of color produced due to enzymatic activity in ELISA when a fixed concentration of antibodies was plotted against a variable concentration of DDA. Maximum antibody binding and background absorbance were determined by adding solvent only or no antiserum respectively to the system. All assays were run in triplicate. Further, in the inhibition experiments DDA was replaced with known concentrations (10-100 ng/mL) of structurally related compounds to study effects of hapten structure on antibody specificity in DDA immunoassay development. The haptens used for inhibition studies were DDT, DDE and DDD.

The 24-hr urine samples were collected over 10 mL benzene as preservative from healthy male staff members of the centre and, DDA was extracted into benzene as described earlier (Zaidi et al. 1984; Ramachandran et al. 1984b). An aliquot of 200 mL of urine (pH 6.3) was ascidified to pH 2 by the addition of 1.8 mL of 5 N HCl and extracted twice with 80 mL and 40 mL benzene and used without any cleanup. The organic extracts were evaporated, and the residues dissolved in 1 mL ethanol for the assay and compared with standard inhibition curve for the quantitative estimation of DDA. All samples were run in triplicate. In order to counter check values obtained with enzyme immunoassay, aliquots of the benzene extract were used for the colorimetric assay of DDA and for the gas chromatographic determination according to the procedures described earlier (Zaidi et al. 1984; Ramachandran et al. 1984b). A Packard gas chromatograph (GC) equipped with an electron-capture detector was used in the estimation. Known amounts of DDA were added to urine samples prior to extraction, and the recoveries were determined by the immunoassay and gas chromatographic procedures.

RESULTS AND DISCUSSION

The presence of specific anti-DDA antibodies was demonstrated by IEP and ELISA as early as the first bleeding. Antibodies against both the hapten (DDA) and its carrier molecule (BSA) were observed in the antiserum as evidenced by the formation of two precipitin bands with DDA-BSA conjugate in IEP. The DDA-RSA conjugate produced a single precipitin band with antiserum against DDA-BSA conjugate indicating further anti-hapten specificity. Subsequently in ELISA, the DDA-RSA conjugate was used as a coating antigen in order to prevent cross-reactivities between carrier.

The anti-DDA antibody titers were in the range of 128-512 when analyzed by ELISA. The antiserum samples were pooled, and a dilution of 1:10 was used for the residue analysis of DDA. The rabbit serum collected prior to immunization had no detectable antibody titres by using DDA-RSA conjugate as the coating antigen in the ELISA procedure.

The optimal concentration of the immobilized antigen (DDA-RSA conjugate) was determined after checkerboard titration of both the antigen and antisera. The rabbit sera (1:10 diluted) exhibited reactivity with the concentration of coating antigen as low as 1 μg/mL and the plateau was observed at concentration higher than 80 μg/mL. Therefore, for the further development of the immunoassay, 100 μg/mL (10 μg/well) of DDA-RSA conjugate was used as the coating antigen.

Inhibition studies were performed to determine the ability of various concentrations of competitive antigen (DDA) to inhibit binding of DDA antibodies to the DDA-RSA coated plates in an indirect ELISA. Immune serum was incubated with free DDA molecules. The relative binding capacity of anti-DDA antibodies was inhibited on incubation of these antisera with the DDA when subsequently analyzed by ELISA (Fig.1). A dose-response inhibition curve was observed. This inhibition curve is linear over a range of 10 to 100 ng/mL DDA concentration. DDA exhibited a 50% inhibition of binding of antibodies to the hapten-coated wells at a concentration of 72 ng/mL. To confirm the antibody specificity further, the cross-reactivities of structurally related haptens (DDT, DDE and DDD) were determined. These haptens in inhibition studies exhibited less than 5% cross-reactivities indicating a fine specificity of anti-DDA antibodies which discriminates between minor differences in the structure among DDA and related compounds.

The enzyme immunoassay described in this report allows the quantitative estimation of DDA in urine samples at nanogram level without the need for a time consuming cleanup or sample preparation prior to analysis. There was no significant interference with sensitivity or reproducibility of the assay. The values of urinary DDA as determined by the ELISA are given in Table 1. The urinary excretion of DDA for eleven healthy adult males with no known occupational or other special exposure to DDT ranged from 0.025 to 0.120 μg/mL with a mean of 0.066 μg/mL and falls in lines with those reported earlier (WHO 1979; Zaidi et al. 1984; Ramachandran 1984b). For reproducibility of the method, five different aliquots of the same urine sample were analysed and yielded mean and standard deviation for the DDA content of 0.058 ± 0.0025 μg/mL.

The gas chromatographic and colorimetric assays were used as reference methods. The values of DDA as determined by ELISA were compared with colorimetric and gas chromatographic methods, and they were in close agreement which showed the sensitivity and specificity of the method (Table 1). The comparative study

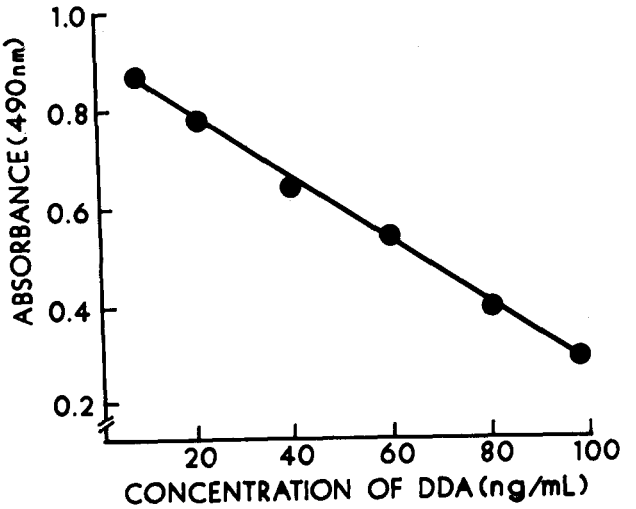

Figure 1. Standard inhibition curve for the determination of DDA concentration by ELISA.

Table 1. Comparative ELISA, colorimetric and GC values of DDA in human urine samples*

Sl. No.	ELISA values	GC values	Colorimetric values
1	0.102	0.094	0.090
2	0.120	0.130	0.125
3	0.090	0.096	0.093
4	0.040	0.038	0.025
5	0.051	0.056	0.043
6	0.058	0.046	0.054
7	0.055	0.062	0.050
8	0.025	0.031	0.020
9	0.084	0.072	0.097
10	0.039	0.030	0.034
11	0.065	0.078	0.056
Mean ± SD	0.066± 0.028	0.067± 0.030	0.062± 0.032

* Values are expressed in μg/mL. Each determination was performed in triplicate.

shows that the immunoassay is rapid, efficient, relatively inexpensive and suitable for the routine analysis of DDA residues in urine. The recovery of DDA, added to urine samples prior to extraction as determined by ELISA was compared with those measured by gas chromatographic procedure (Table 2). The recoveries

Table 2. Recoveries of DDA added to urine samples prior to extraction by ELISA and GC*

Concentration of DDA (ug/mL)	Recovery (%) ELISA	GC
0.025	96.1 (6)	99.4 (4)
0.050	96.0 (3)	100.6 (3)
0.075	98.7 (4)	93.4 (4)
0.100	97.0 (4)	97.8 (5)
0.125	98.2 (3)	97.0 (3)
Mean ±	97.1 ± (20)	96.5 ± (19)
SD	3.2	7.6

* Each determination was performed in triplicate. The value of recovery gives the mean value of number of determination given in parentheses.

obtained by ELISA by using lowest and highest values of DDA estimated in urine samples were more than 96% and correlated well with those of the gas chromatography.

In view of its simplicity, low cost and high specificity, sensitivity and reproducibility, the ELISA described in this report would appear to be a suitable alternative method for DDA assay.

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REFERENCES

Centeno ER, Johnson WJ, Sehon AH (1970) Antibodies to two common pesticides; DDT and malathion. Int Arch Allergy 37: 1-13

Grummitt O, Buck A, Egan R (1946) Di-(p-chlorophenyl) acetic acid (DDA). Organic Synthesis 26: 21-22

Ramachandran M, Banerjee BD, Gulati M, Grover A, Zaidi SSA, Hussain QZ, (1984a) DDT and HCH residues in the body fat and blood samples from Delhi Hospitals. Indian J Med Res 80: 590-593

Ramachandran M, Zaidi SSA, Banerjee BD, Hussain QZ (1984b) Urinary excretion of DDA: 2,2-bis (4-chlorophenyl) acetic acid as an index of DDT exposure in men. Indian J Med Res 80: 483-486

Voller A, Bidwell DE, Bartlett A (1976) Enzyme immunoassay in diagnostic medicine. Theory and practice. Bull WHO 53: 55-65

Zaidi SSA, Banerjee, BD, Ramachandran M, Hussain QZ (1984) Colorimetric assay of 2,2-bis (4-chlorophenyl) acetic acid (DDA) in urine. Anal Biochem 136: 185-186

Watts RR (1981) Manual of Analytical Quality Control of Pesticides and Related Compounds in Humans and Environmental Samples. U.S. Environmental Protection Agency, Research Triangle Park NC, p.319

WHO (1979) DDT and its Derivatives. Environmental Health Criteria 9. World Health Organization, Geneva

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