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DEVELOPMENT OF A HOMOGENEOUS PHOSPHORESCENT IMMUNOASSAY FOR THE DETECTION OF POLY-CHLORINATED DIBENZO-*p*-DIOXINS

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ABSTRACT

A homogeneous immunoassay for dioxins was developed using a phosphorescent label for detection. A dioxin derivative was conjugated to Pt-coproporphyrin. In the assay, when the antibodies against dioxin (DD3) were bound to the phosphorescent conjugate, the signal from Pt-coproporphyrin was

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quenched. The interaction between antibody and the conjugate was studied by time-resolved luminescence spectroscopy. As concentrations of dioxin standards increased from 39 pg/well to 2.5 ng/well the lifetime of the phosphorescence of the short-lived component increased from 25.6 microseconds to 58.9 microseconds. This increase in half-life was associated with a dose dependent quenching of the phosphorescence. The inhibition obtained is similar to that for a reported enzyme-based immunoassay, but the data were obtained in minutes instead of hours.

Key Words: Phosphorescence; Pt-coproporphyrin; Homogeneous immunoassay; Polychlorinated dibenzo-*p*-dioxins; Time-resolved luminescence spectroscopy

INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins (PCDDs) are a well-known group of highly toxic and widespread environmental pollutants. PCDDs have been found in many kinds of environmental matrixes including air, soil, sediment, fish and human adipose tissue, and milk (1,2). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most studied toxic congener. The LD_{50} value of this compound is 0.6–2.0 μg/kg in guinea pigs (3). The dominant analytical technique for PCDDs is a combination of high-resolution gas chromatography and high-resolution mass spectrometry (GLC-MS) with a sensitivity in the part per trillion range (4). This method is costly and time-consuming, thus alternative methods are in demand.

Ideally an alternative method would be sensitive, rapid, cost-effective, field-portable, and specific for the most toxic dioxin congeners. They should complement existing GLC-MS methods. Solid phase enzyme immunoassays (ELISA) satisfy many of these criteria. Stanker et al. generated monoclonal antibodies to dioxin and developed a monoclonal antibody-based ELISA test (5–8). The optimized assay detected 200 pg/well 2,3,7,8-TCDD as the IC_{50} (the analyte concentration giving 50% inhibition) (8). The sensitivities of the monoclonal assay as well as polyclonal antibody-based assays have been improved by the use of novel haptens (9,10).

Homogeneous immunoassays have many of the advantages attributed to ELISAs and have one additional feature: the assay is very fast, taking only 15–20 min. Homogeneous fluorescent immunoassays are widely used in clinical diagnostics, but in some cases sensitivity of these methods is limited by the background fluorescence of matrices. Phosphorescence has evoked interest

for application to immunoassay mainly because of its potential sensitivity and the possibility of offering temporal discrimination to spectral resolution to avoid background interferences (similar to time-resolved fluorometry of lanthanide chelates). Solid phase phosphorescent labels have been used for detecting clinically significant substances such as hormones (11,12).

In this paper we report a phosphorescent-labeled immunoassay for dioxins. When monoclonal antibodies against dioxin (DD3) were bound to the conjugate of a dioxin derivative with Pt-coproporphyrin, the phosphorescent signal from Pt-coproporphyrin was quenched. The interaction between these molecules was studied by time-resolved luminescence spectroscopy in order to develop a competitive homogeneous immunoassay.

EXPERIMENTAL

Materials and Reagents

The standard 2,3,7,8-TCDD was purchased from Chem Service (West Chester, PA). Pentafluorophenyl trifluoroacetate and other biochemicals were purchased from Sigma (St. Louis, MO, USA). Other chemicals and solvents used were purchased from Aldrich Chemical Co. (Milwaukee, WI). Pt-coproporphyrin I was kindly donated by Prof. G.V. Ponomarev (Institute of Biophysics, Ministry of Health of Russia). Monoclonal antibody against dioxin (DD3) was a gift from Dr. Larry Stanker (Western Regional Research Center, USDA, Albany, CA). The polystyrene microtiter strips were purchased from Eflab Oy (Finland).

Equipment

The low-resolution GLC-MS data were obtained on a Trio-2 GLC-MS system (VG Masslab, Altrincham UK) using 70 eV electron ionization. (EI). A 30 m DB1 column (0.25 mm i.d., 0.25 μm film; J & W Scientific, Folsom, CA) was used with a helium flow rate of 30 cm/s. Samples were dissolved in tetrahydrofuran (THF), and splitless injections of 1 μL were made. The column was programmed from 80°C (1 min hold) to 150°C at 20 deg/min followed by an increase to 300°C at 10 deg/min.

The solid probe mass spectrum was obtained on a Trio-2 mass spectrometer (VG Masslab, Altrichim, UK) using electron ionization upon heating of the probe from room temperature to 300°C.

Melting points were obtained on a capillary melting apparatus (Thomas Hoover, Philadelphia, PA).

Synthesis of Haptens

cis, *trans*-2,4-Dichloro-5-nitro-1-(2-(4-nitrophenyl)vinyl) benzene (I). In 10 mL of dry toluene were placed 4.3 g (10 mM) 4-nitrobenzyl triphenyl phosphonium bromide (13), 2.2 g (10 mM) 2,4-dichloro-4-nitrobenzaldehyde (14) and 10 mL (10 mM) potassium *t*-butoxide (1 M in *t*-butanol). The mixture was heated to 80–90°C for 1 h. The reaction was cooled and the triphenyl phosphine oxide was filtered off. The reaction mixture was washed with saturated sodium bisulfite. After drying over sodium sulfate, the toluene was removed to yield 1.15 g (34%) of a yellow solid, mp 133–137°C. R_f 0.4 (20% ethyl acetate : hexane, v/v). GLC-MS ($t_R = 14.75$ min) calculated for $C_{14}H_8Cl_2N_2O_4$, MW 339.13 m/z (intensity) M^+ 337.99 (100); M+2 340 (66); M^+-30, 308 (12).

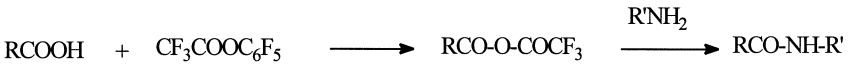
cis,*trans*-2,3,8-Trichloro-7-((4-nitrophenyl)vinyl)dibenzo[b,e]1,4-dioxin (II). In 3 mL of dry dimethylformamide under nitrogen were placed 0.42 g (2.35 mM) 4,5-dichlorocatechol (15), 0.64 g (1.89 mM) (I) and 4.0 mL (4 mM) potassium *t*-butoxide (1 M in *t*-butyl alcohol). The mixture was heated to 135°C until the *t*-butyl alcohol ceased to distill. After cooling, water was added and 1 g of a yellow solid was collected. Purification of a small amount for analysis by column chromatography (90% hexane : ethyl acetate, v/v) provided an analytically pure sample, mp 198–203°. R_f 20% hexane/ethyl acetate) 0.42. GLC-MS ($t_R = 17.67$ min) calculated for $C_{20}H_{10}C_{13}NO_4$, MW 432.97 (m/z) intensity M^+ 433 (28); 435 (27).

4-(2-(3,7,8-Trichlorobenzo[3,4-b]benzo[e]1,4-dioxnyl-2yl)vinyl)phenylamine (III). In 5 mL absolute ethanol and 2 mL ethylene dichloride were placed 0.105 g (0.24 mM) (II) and 0.6 g (2.66 mM) stannous chloride dihydrate. The reaction mixture was stirred over night and then 1 g sodium bicarbonate was added. After filtering through Celite® and drying over sodium sulfate and removal of the solvents, 0.021 g (21.6%) of a yellow, amorphous solid was obtained that discolored when in contact with air. It was used without further purification. R_f (20% hexane/ethyl acetate) 0.24. MS (solid probe) calculated for $C_{20}H_{12}C_{13}NO_2$, MW 402.99 m/z (intensity) M^+ 403 (100), 405 (88).

Conjugate Synthesis

Dioxin-ARO-NH_2 (III) was conjugated with Pt-coproporphyrin (Pt-CP$(COOH)_4$) using pentafluorophenyl trifluoroacetate to activate COOH

groups of Pt-coproporphyrin in the following reaction scheme (Figure 1):



Scheme

where RCOOH is Pt-coproporphyrin and $R'NH_2$ is dioxin-ARO-NH_2. Pt-coproporphyrin (1.7 mg, 2 μmol) was dissolved in 2 mL of dimethylformamide. Then 1.6 mg of triethylamine (16 μmol) dissolved in 10 μL of dimethylformamide, and 2.6 mg of pentafluorophenyl trifluoroacetate (9.6 μmol) dissolved in 6 μL of dimethylformamide, were added, and the mixture was stirred 20 min at room temperature. Then the mixture was added to the solution of dioxin-ARO-NH_2 (0.81 mg, or 2 μmol, in 160 μL of dimethylformamide) and stirred 2 h. Since Pt-coproporphyrin has four COOH groups, a mixture of different conjugates was obtained.

HPLC Purification of Conjugate

High-pressure liquid chromatography (HPLC) was used to separate and purify conjugates of Pt-coproporphyrin and dioxin derivatives. The fractions were monitored spectrophotometrically at 377 nm (adsorption

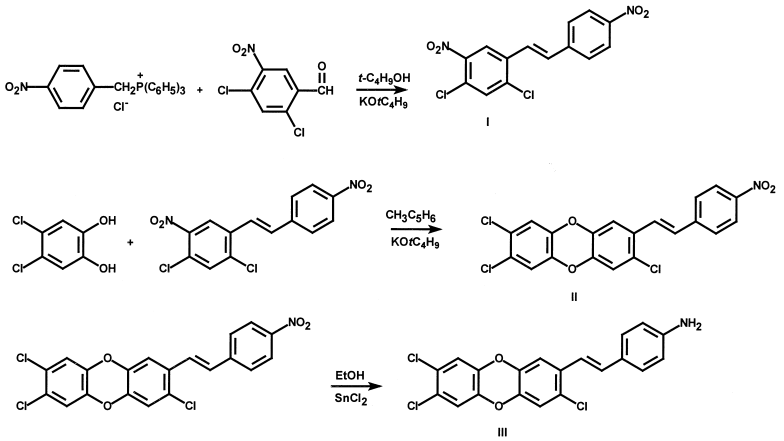


Figure 1. Synthesis scheme for the amino styryl dioxin derivative (dioxin-ARO-NH_2).

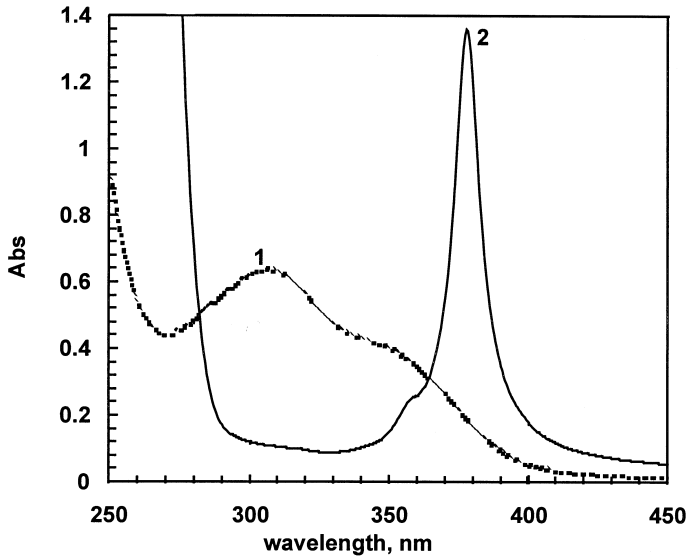


Figure 2. UV-VIS spectra of dioxin-ARO-NH_2 (1) and Pt-coproporphyrin (2).

of Pt-coproporphyrin) and 310 nm (adsorption of dioxins) (Figure 2). It was shown that the maximal difference between the retention times of Pt-coproporphyrin and dioxin derivatives was reached with a gradient from solvent A to solvent B, where A = water : acetonitrile (4 : 1) containing 0.1% NH_4OH, and B = methanol containing 0.1% NH_4OH. HPLC fractions were analyzed for specific binding with antibodies (DD3) adsorbed on the surface of a microtiter plate. Fractions that demonstrated statistically reliable phosphorescent signals were selected for sequential dilutions with the same antibodies.

Dioxin Standards

Due to the toxicity of TCDD initial screening studies were conducted using a surrogate standard, 2,3,7-trichloro-8-methyl dibenzo-*p*-dioxin (TMDD). This compound is presumed to be less toxic and more rapidly metabolized than TCDD (16). TMDD was prepared in dimethyl sulfoxide (DMSO). The stock solutions were serially diluted with DMSO–PBSB (1 : 1; PBSB, phosphate buffered saline with 0.1% bovine serum albumin). The standard curve was obtained by plotting the phosphorescence lifetime against the logarithm of the analyte concentrations.

Phosphorescent Life Time Measurement

Life time measurements were performed with an instrument for phosphorescence measurements as previously described (17). Time resolution for lifetime measurements was equal to 150 ns per channel, that was the limit for the processor ADSP-2115. Each phosphorescence decay containing 1000 points collected during 3 min and was processed with the program that detected the number of exponential components and their lifetimes (18). No deconvolution procedure was required for phosphorescence.

Assay Conditions

All measurements were performed in solution containing Na_2SO_3 (40 mg/mL) and NaH_2PO_4 (20 μg/mL) to remove oxygen in the microtiter well of 96 well plates. The final volume was 200 μL and the pH value was 7.5–7.7. Under these conditions the phosphorescence signal remained stable in an open vial or well for more than 3 h (11). Standards or samples were added to the mixture of antibody and conjugate, incubated 15 min and then measured.

RESULTS AND DISCUSSION

It was shown that the interaction of anti-dioxin antibodies and Pt-coproporphyrin-dioxin conjugate resulted in both quenching of the conjugate phosphorescence and an increase in lifetime. A homogeneous immunoassay format with competition between free TMDD (dioxin standard) and conjugate for binding with the anti-dioxin antibodies was studied. Phosphorescence of Pt-coproporphyrin in the conjugate is multi exponential, but only the short-lived component (below 50 microseconds) demonstrated clear dependence on TMDD concentration (Figure 3). At the highest concentration tested the signal was significantly lower than expected and the standard deviation of the replicates increased, indicating that at these higher concentrations quantitation would not be reliable. On all samples, both intensity of phosphorescence and lifetime of phosphorescence were measured. Both were changed in a dose dependent, reproducible manner. However, the lifetime measurements yielded more sensitive and reproducible data with a wider dynamic range and these measurements are reported here. This observation is advantageous since lifetime measurements are possible with compact and relatively inexpensive equipment.

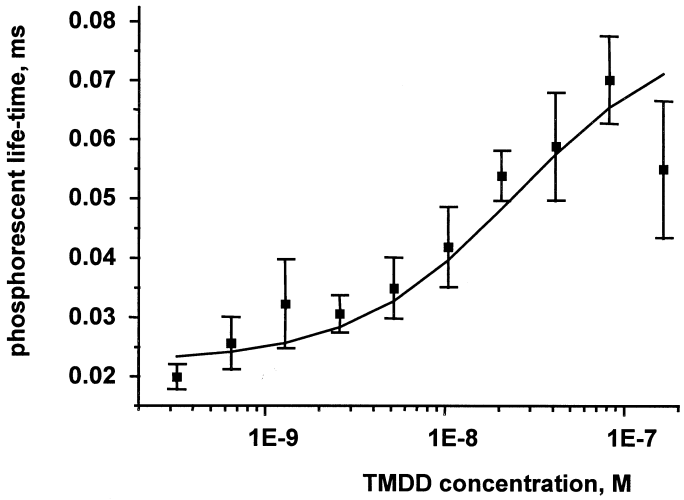


Figure 3. Standard curve for the homogeneous immunoassay of TMDD. Antibody DD3 10^{-7} M, and the conjugate of Pt-coproporphyrin with the aromatic derivative of dioxin (TCDD-ARO) were used. Assay conditions are in the Materials and Methods. Error bars correspond to 1 standard deviation for 4 replicate measurements.

When the concentration of TMDD in the presence of 10^{-7} M of antibody DD3 and conjugate was changed from 6.48×10^{-10} M (39 pg/well) to 4.14×10^{-8} M (2.5 ng/well) the lifetime of the short-lived component increased from 25.6 microseconds to 58.9 microseconds. The coefficient of variation for the same range did not exceed 20%. The detection limit was comparable with that of the ELISA test with monoclonal antibodies (8), but the time needed for detection was only 15 min instead of the 5 h needed for the ELISA. Moreover, the lifetime measurements did not need external calibration by standards during the operation.

This method is suitable for development of a lifetime phase-modulated optical biosensor for rapid detection of dioxin. The simplest scheme for this sensor may be a flow injection system taking advantage of advances in microfluidics.

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