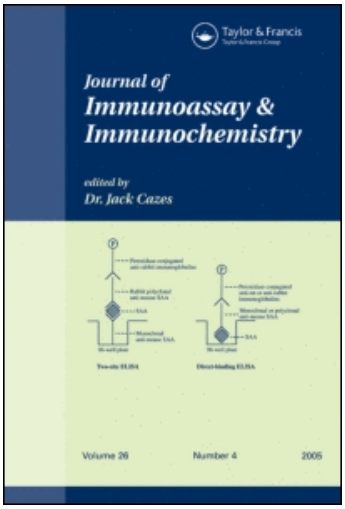

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PRODUCTION OF ANTIBODIES AND DEVELOPMENT OF AN IMMUNOASSAY FOR THE ANTICOAGULANT, DIPHACINONE

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ABSTRACT

Diphacinone, a commonly used anticoagulant rodenticide, was coupled to protein via an (O-carboxymethyl) oxime bridge. Immunization in rabbits produced antibodies with good ability to recognize the hapten as demonstrated by indirect EIA and affinity column adsorption. A competitive EIA was developed which clearly measured 10 µg/L diphacinone concentrations showing the sensitivity of the assay. Cross-reactivity study with chlorophacinone showed that the antisera possessed a high degree of diphacinone specificity.

INTRODUCTION

Diphacinone (Fig. 1; I; 2-(Diphenylacetyl)-1H-indene-1,3(2H)-dione) a commonly used rodenticide, is representative of the indandione anticoagulants (1). It has been used therapeutically as a antithrombotic agent in man (2). This anticoagulant prevents recycling of vitamin K from the inactive epoxide metabolite back to the functional quinone structure (3). Vitamin K is biochemically responsible for carboxylation of glutamate residues to 4-carboxyglutamate located on the vitamin K dependent coagulation factors (4). This carboxylation results in functionalizing coagulation factors II, VII, IX, and X so they can participate as zymogens in the enzymatic cascade resulting in thrombin generation which is vital in providing proper hemostasis (5).

Chromatographic methods using TLC (6), GLC (7), and HPLC (8) have been applied for diphacinone determination but no immunoassay has been described. Chlorophacinone (also an anticoagulant rodenticide) differs only by a single chlorine on one of the bisbenzene rings of diphacinone. It would be of great interest to compare chlorophacinone and diphacinone with respect to antibody specificity and cross-reactivity since both are commonly used rodenticides.

Objectives of this investigation were to couple diphacinone to protein using peptide bond forming agents (9) in order to produce antibodies in rabbits, to characterize their affinity for diphacinone, and to develop an enzyme immunoassay (EIA) (10).

MATERIALS AND METHODS

Reagents and related materials

Diphacinone analytical standard (99%) was provided by Velsicol Chemical Corporation, Rosement, IL. N-hydroxysuccinimide (NHS), N,N'-dicyclohexylcarbodiimide (DCC), bovine serum albumin (BSA), and keyhole limpet hemocyanin (KLH) were obtained from Sigma Chemical Company, St. Louis, MO. Carboxymethoxylamine hemihydrochloride (CMA) was obtained from Aldrich Chemical Company, Inc., Milwaukee, WI. The proteins were twice dialyzed in 1 L distilled water prior to usage. Beads of aminoalkyl agarose (AFFI-GEL 102) were obtained from Bio-Rad, Richmond, CA. Spectrapor membrane tubing (Spectrum Medical Industries, Inc., Los Angeles, CA) utilized in dialysis had a molecular cutoff at 12-14,000 and 0.002 inch dry thickness. Buffer salts and tetrahydrofuran (THF) and other reagent-grade solvents were obtained commercially. Pyridine was freshly distilled and peroxide-free. Ultraviolet measurements were determined on a scanning UV/VIS spectrophotometer (Hewlett Packard, Model 8450, Palo Alto, CA).

Antigen preparation

Diphacinone-carboxymethyloxime, D-CMO, (Fig. 1; II) was synthesized by reacting diphacinone with CMA in pyridine for 24 h at 25°C (11). Following

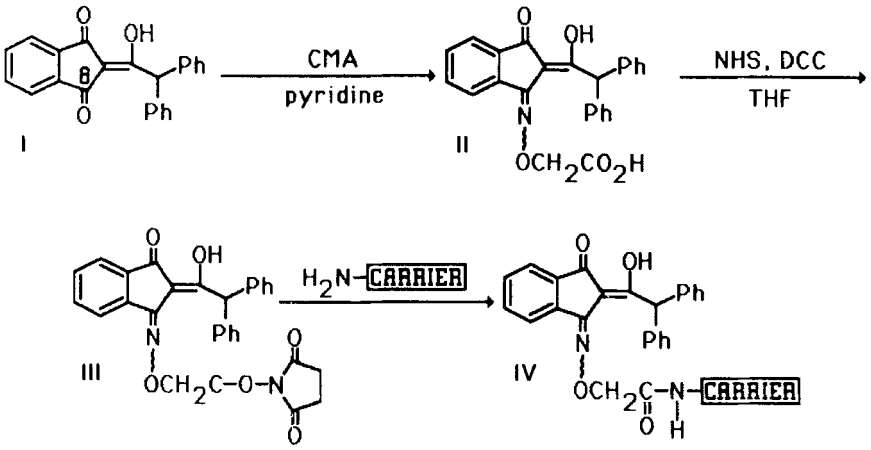

Figure 1: Synthetic pathway used to couple diphacinone to primary amines.

purification of D-CMO by extraction into aqueous 4% sodium bicarbonate and partitioning into dichloromethane after 10% HCl addition, the active succinimide ester (Fig. 1, III) was synthesized by mixing with NHS and DCC in THF at 4°C for 12 h (12). The resulting product was a near 1:1 stereoisomer at the C8 position as determined by proton- and ^{13}C-NMR (13).

Diphacinone succinimide active ester (10 mg; 0.02 mmol) dissolved in THF (4 mL) at 4°C was added directly to the protein (60 mg) dissolved in lithium borate buffer (4 mL; 0.4 M; pH 9.3) at 4°C and stirred 12 h. The resulting solution containing D-CMO proteins [IV] was dialyzed in 10 mmol/L sodium tetraborate containing 25% lauric acid (2 times) and a solution of 144 mmol/L sodium chloride and 10 mmol/L sodium carbonate (3 times). The epitopic density of BSA following coupling was 14 D-CMO molecules/protein molecule. The coating antigen (KLH-antigen) had 16% of the available lysine residues coupled to D-CMO. An extinction coefficient at 327 nm was determined as 1.622 x 10^4 for D-CMO in phosphate buffered saline pH 7.4.

Antibody production and collection

New Zealand White rabbits were immunized subcutaneously with 1 mg/immunization of BSA-antigen as previously described (14). Blood was collected 7-10 days post immunization and the titer determined by indirect EIA. A portion of the antisera was stored as received at -40°C for titering while the remainder was purified by ammonium sulfate precipitation (15).

Evaluation of titer and antibody affinity

Ability of the KLH-antigen (coating antigen) to recognize antibodies produced against BSA-antigen (immunogen) was tested by a modified indirect EIA procedure of Engvall and Perlmann (16). The molecular weight of KLH was assumed to be 814,000 (17) and to contain 7% of amino acid composition as available lysine residues (18). Optimization of coating antigen and conjugate (goat antirabbit IgG coupled to HRP; Antibodies Inc., Davis, CA) concentrations were determined to establish an indirect EIA. Coating antigen (5 µg/mL) was added (100 µL/well) in carbonate buffer (50 mmol/L; pH=9.6) was added to 96 well microtiter plates (Linbro/Titertek^{R}, Flow Laboratories, McLean, KA) and incubated overnight at 4°C. Plates were washed 5 times with 150 mmol/L (0.9%) NaCl plus 0.1% Tween 20 (wash solution) on a Dynawasher II (Dynatech Laboratories, Inc., Alexandria, VA). Antisera dilutions were made in PBST [phosphate buffered saline (150 mmol/L NaCl and 10 mmol/L Na/phosphate) plus Tween 20 (0.05%)] and were added (100 µL/well) and incubated for 1 h at 37°C. Plates were washed (5 times). The conjugate (1:4000) was added (100 µL/well) in PBST and incubated 1 h at 37°C followed with wash (5 times). The substrate 2,2'-azino-di-(3 ethyl benzothiazolin sulfonic acid) [ABTS; 200 µL of 40 mmol/L added to 19.8 mL of buffer] in citrate buffer (50 mmol/L, pH=4) was added (100 µL/well) with hydrogen peroxide (60 µL of 500 mmol/L added to 19.8 mL of buffer) and incubated at 25°C for 30 min. The reaction was halted by addition (100 µL/well) of stop solution. The stop solution was prepared by

making a solution of 3.75 mol/L HF in 375 mmol/L NaOH and diluting this 1:10 in 1 mmol/L disodium EDTA.

Comparisons with plates coated with KLH were measured to evaluate the affinity of antibody for the coupled hapten. Absorbance ratio (410/450 nm) of the resulting color development was determined on a Dynatech Microplate Reader. The end point titer was determined as the values greater than or equal to 0.1 absorbance and possessing a 5 times higher margin of absorbance over the corresponding presera values. The titer was expressed as the reciprocal of the end point serum dilution.

An affinity column made by coupling D-CMO was used to measure antibodies affinity for the hapten

Diphacinone succinimide active ester (Fig. 1; III) in THF was added (1:1 v/v) to aminoalkyl agarose beads in lithium borate buffer (400 mmol/L, pH=9.3) at a 2 molar excess of the amine groups. The available free primary amines were determined as described by Failla and Santi (19). The mixture was gently shaken overnight at 25°C. The beads were extensively washed with phosphate buffered saline. The amount of D-CMO coupled to the beads [IV] was determined by UV measurement (327 nm). Beads coupled to D-CMO were added to rabbit antisera and incubated 10 h at 25°C. Uncoupled beads were in like manner incubated with rabbit antisera to measure nonspecific binding. The antisera was recovered followed centrifugation (3000 rpm for 20 minutes) and titered against the original antisera. Significant removal of antibody by D-CMO coupled beads would indicate antibody affinity toward the D-CMO hapten (Fig. 1; IV).

Development of a competitive EIA and evaluation of sensitivity

Horseradish peroxidase (HRP; MW=40,000; 30 mg; previously dialyzed in 1 L water 2X) was added to lithium borate buffer (200 mmol/L; pH=9.3; 5 mL) at 4°C. Diphacinone succinimide active ester [III] (MW=513; 7.7 mg) in 5 mL THF

was added in one portion to the HRP solution and stirred overnight at 4°C. The resulting conjugate was dialyzed as described for D-CMO coupled proteins. The HRP coupled to D-CMO (HRP-HAPTEN) was diluted to 1 mg/mL and then frozen (-40°C) until needed. Binding of the D-CMO to HRP was determined by UV absorbance (327 nm).

Antisera (rabbit No. 136) was purified by ammonium sulfate precipitation (15) and reconstituted to a final concentration of 15 mg/mL. Optimization of the concentrations of HRP-HAPTEN (0.01 mg/mL) and antibody (1:500) was performed. The antibody solution was added (100 µg/well) in the coating buffer and incubated overnight at 4°C. The HRP-HAPTEN and diphacinone standards (each 2 times the desired concentration) were mixed (1:1 v/v) and added (100 µl/well) in PBST/dog serum (4 to 1) to the plates following washing and incubated at 37°C for 1 h. After washing, the ABTS substrate solution was added (100 µL/well) and stopped after 1 h incubation at 25°C in the dark. Measurements were made as described earlier.

Evaluation of specificity

The procedure for the competitive immunoassay was repeated using chlorophacinone to measure cross-reactivity. Serial dilutions of equal concentrations to the diphacinone were compared. The standard solutions were mixed equal volume with HRP-HAPTEN on the plate and incubated for 1 h at 37°C followed by substrate addition.

RESULTS

Evaluation of titer and antibody affinity

Figure 2 shows the increasing titer following successive immunizations using indirect EIA. Comparison of pooled presera (line **a**) to post immunization sera (lines **b-e**) clearly demonstrates increasing antibody concentrations. The pooled sera samples taken after the initial immunization showed titers of

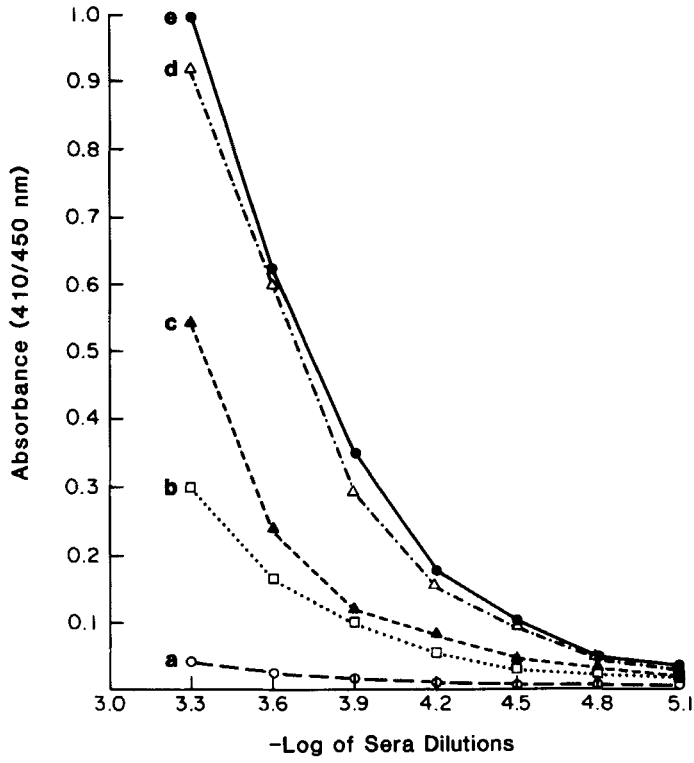

Figure 2: Titering of pooled rabbit sera immunized with BSA-antigen using indirect EIA. Presera (**a**), 1 month (**b**), 3 month (**c**), 4 month (**d**), and 6 month (**e**) post initial immunization antisera are illustrated. Log = Logarithm to the base 10.

8,000 at 1 month (line **b**), 10,000 at 3 months (line **c**), and 32,000 at both 4 (line **d**) and 6 (terminal, line **e**) months. The increasing titers demonstrated elevation in antibody concentrations following repeated exposure to the immunogen.

Figure 3 demonstrates the specificity of the antisera toward the D-CMO hapten. The antibody titer was 1500 when KLH was the coating antigen compared to 50,000 when the coating antigen was KLH-antigen.

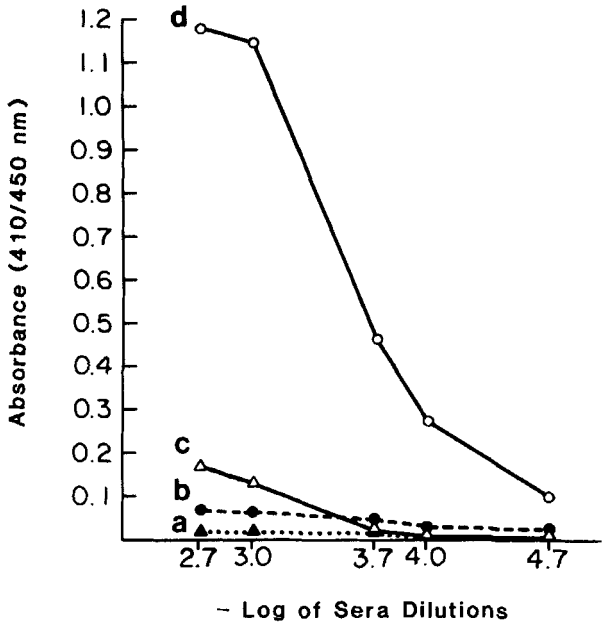

Figure 3: Preserum (broken lines) [a,b] and antiserum (solid lines [c,d] of Rabbit No. 136 measured using indirect EIA on a 96-well microtiter plate coated with KLH-antigen (circle) [b,d] or KLH (triangle) [a,c]. Log = Logarithm to the base 10.

Measurement of free amine groups on the uncoupled beads was 32 µmol/mL of beads. The concentration of D-CMO coupled to the beads was 6 µmol/mL of beads. The D-CMO coupled beads removed 59% of the antisera titer compared to only 37% removal by the beads alone.

Competitive EIA and Sensitivity

The epitopic density of HRP-HAPTEN was 3. The standard curve obtained from the competitive immunoassay for diphacinone standards is shown in Fig. 4. The curve was linear over the concentrations 10 to 320 µg/L. The 10 µg/L concentration was clearly detected.

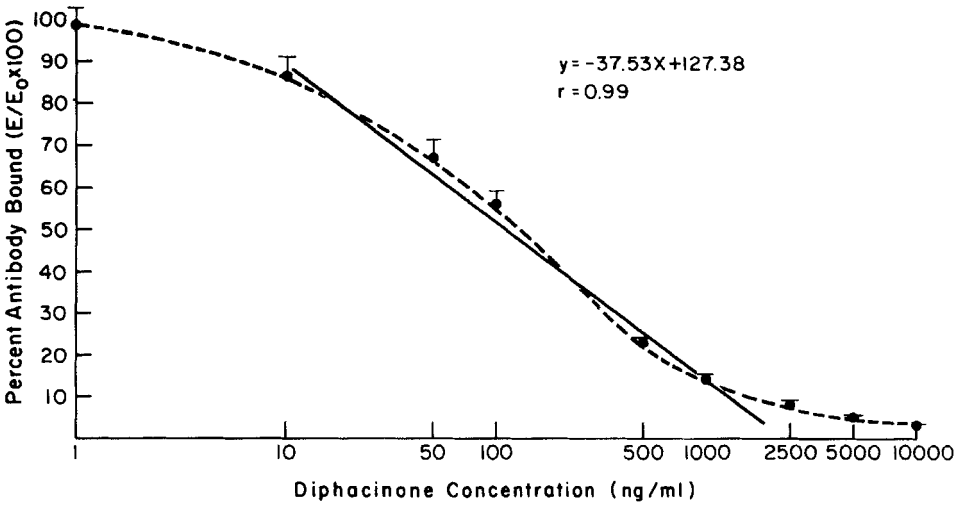

Figure 4: Competitive EIA standard curve of diphacinone standards constructed from predicted points (solid line) based on regression equation. Data points (N=3) shown with standard deviation (brackets) are illustrated. Diphacinone concentration on a logarithm scale.

Chlorophacinone cross-reactivity is shown in Fig. 5 using this competitive EIA. It requires 8 fold as much chlorophacinone to produce the same degree of inhibition caused by diphacinone.

DISCUSSION

Diphacinone was successfully coupled to antigenic proteins via (O-carboxymethyl)oxime bridge (Fig. 1). Antibodies raised in rabbits against this hapten-coupled protein demonstrated high specificity for the anticoagulant, diphacinone (Fig. 2,3,5). A highly sensitive method for detecting diphacinone was developed using a competitive EIA (Fig. 4).

When the KLH-antigen is used to blanket the microtiter plates, the indirect EIA measures those antibodies having affinity to the D-CMO hapten and/or those antibodies cross-reacting with KLH. Antibodies which cross-react

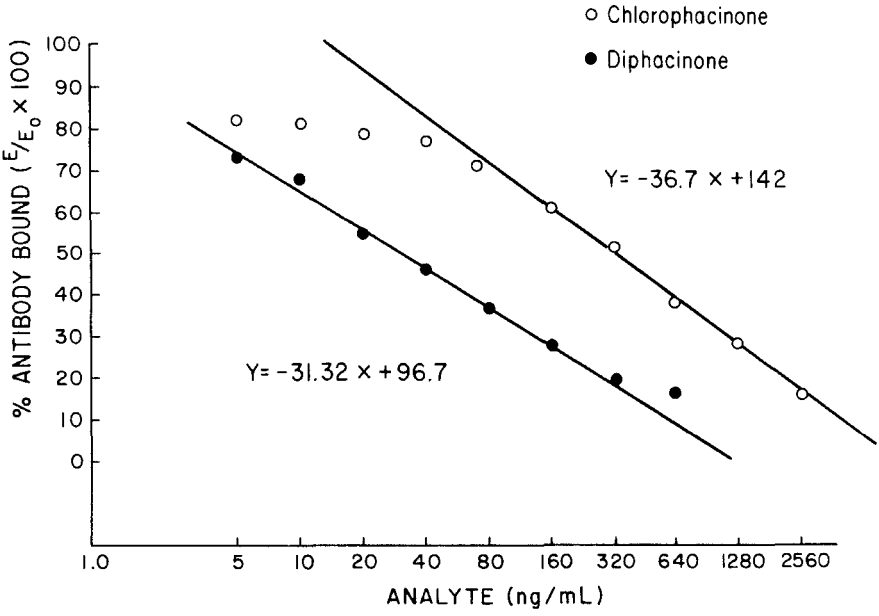

Figure 5: Cross-reactivity of chlorophacinone with the diphacinone competitive EIA is illustrated.

with KLH would falsely indicate immunoglobulins having specificity to the hapten. Therefore, to appreciate the degree of cross-reactivity, coating of plates with KLH would establish the baseline for measuring D-CMO affinity. Minimal cross-reactivity of the antisera (titer = 1500; Fig. 3, line **c**) was determined when tested with KLH. In contrast, a much greater affinity (titer = 50,000; Fig. 3, line **d**) was measured when tested with KLH-antigen. These experiments demonstrate that the increasing titer observed following successive immunizations is due predominately to antibody affinity toward the D-CMO hapten.

The affinity bead experiment clearly demonstrated antisera affinity toward the D-CMO (Fig. 1; II). Approximately 59% of the antibody was removed by the D-CMO coupled beads when compared to the antisera prior to bead

adsorption. In comparison, uncoupled beads removed 37% of the antibody. This nonspecific adsorption is due to free amine groups (32 μmol/mL). The uncoupled beads could function as an ion exchange resin causing nonspecific protein adsorption. The pKa of ϵ-amino groups of lysine range between 9.5 to 10.6 (20). At physiological pH (7.4), virtually 100% would be positively charged. The D-CMO coupled beads have less available free amines (6 μmol/mL are bound to D-CMO) resulting in less nonspecific binding. Thus the difference (59%-37%) between the D-CMO coupled beads and the uncoupled beads verifies removal of antibody having specific affinity toward the D-CMO hapten [IV].

Successful development of the competitive EIA verifies the antibodies' affinity for diphacinone (Fig. 1; I). Free diphacinone successfully competes with HRP-HAPTEN for diphacinone-specific antibody binding sites. The effect of this competition results in a decrease in absorbance which is correlated to the concentration of free diphacinone. This is illustrated in Fig. 4 by the standard curve of diphacinone concentrations. The assay clearly detected 10 μg/L concentration of diphacinone which both demonstrates the sensitivity and specificity of the assay. The cross-reactivity of antibody with chlorophacinone (Fig. 5) required approximately 10 fold the concentration to produce equivalent inhibition. This clearly demonstrates the high specificity of antibody for diphacinone. Chlorophacinone is identical structurally to diphacinone except for a single Cl^- on one of the benzene rings.

Application of this assay to anticoagulant overdose or rodenticide poisonings can now be performed.

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