QUANTIFYING DDAC IN COMMERCIAL ANTISAPSTAIN PRODUCTS AND INDUSTRIAL WORKING SOLUTIONS BY AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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Summary

An enzyme immunoassay (ELISA), based on polyclonal antibodies, was developed to measure didecyldimethylammonium chloride (DDAC). The detection limit, 50% inhibition, and effective working range were 8, 30 and 8 to 55 ppm, respectively. The immunoassay was compared to high performance liquid chromatography (HPLC) and a colorimetric titration method for the analysis of DDAC in commercial antisapstain products and industrial working solutions. The results obtained with ELISA agreed with the results from the other two methods. The immunoassay, if implemented as a simple diagnostic kit, might serve as an analytical method for quality control programs for DDAC-based antisapstain formulations.

Introduction

In Canada, softwood lumber production is valued at over five billion dollars annually. Over two billion dollars of green softwood lumber is sold to offshore markets, and approximately 90% of the exported lumber is chemically treated for protection against molds, staining and decaying fungi. Kiln-dried lumber, which can be easily rewetted in transit or storage, may also require chemical protection against fungal infection. Shortages of forest products in the near future will increase the demand to preserve and protect a wide range of wood and wood products, thereby extending the durability and the service life of these products.

Since 1987, when chlorophenates began to be removed from the lumber industry, more environmentally acceptable antisapstain formulations have been used in Canada. For example, products containing didecyldimethylammonium chloride (DDAC), a chemical registered with Agriculture Canada, account for about 95% of the sapstain control market (Byrne and Smith 1987; and personal communication). These products include NP-1 from Kop-Coat Inc., F-2 from Walker Brothers, Ecobrite III from Canfor, and Timbercote II from Napier Pacific Industries Inc.

To remain competitive and to handle environmental regulations , it is important for mills to develop effective quality control programs. However, chemicals are difficult to detect in working solutions, waste waters and on the surface of wood. Conventional detection of wood protectants (e.g. DDAC) is based on wet chemistry and colorimetric assays. However, these assays are insensitive, non-specific, and subject to interference, especially when used for wood and environmental analyses (Lonza Inc., 1988). Gas-liquid chromatography (GC), high performance liquid chromatography (HPLC) and mass spectrometry have been used with reasonable accuracy (Crowther and Fairchild, 1991; BC Research, 1991; Goetz et.al., 1985; Metcalfe, 1963, Daniels, 1992; Conboy et.al., 1990; Matthijs and de Henau, 1987; Wee and Kennedy, 1982). Often, these methods require an initial extraction step and the partial purification of the extract. These methods also demand technical expertise, they can be expensive, and they are not adequate for handling a large number of samples. Immunoassays applicable to a range of chemicals could complement traditional analytical methods. The acceptability of immunoassays is increasing for pesticides and other chemicals in food, water, soil, plants and animals (Marco et.al, 1993; Abouzied et.al., 1993; Harrison et.al., 1991a,b; Giersch and Hock, 1990; Jung et.al., 1989; Li et al., 1989).

Immunoassays are based on antibodies (Ab) raised in mammals (rabbits, mice or rats). The assay involves binding the antibody to specific chemical configurations displayed on the surface of antigen molecules, and detecting the complex formed. Almost any substance can induce an immune response and the production of antibodies in mammals. However, small chemicals like DDAC are only immunogenic when linked to large protein molecules; e.g. keyhole limpet hemocyanin, KLH. A mammal will respond to an antigen like KLH-DDAC by producing antibodies to both the foreign protein, KLH, and to the chemical (hapten) attached to the protein, DDAC. The mixture of antibodies obtained from the serum fraction of the inoculated animal's blood is referred to as polyclonal serum or polyclonal antibodies. In most immunoassays the antigen is first bound to a solid support (plate, membrane), and then the polyclonal serum is applied. If antibodies specific for the antigen are present, they will form a complex. The complex is detected by a specific secondary antibody, which is labeled with an appropriate indicator such as an enzyme, fluorochrome dye, gold or radioactive compounds. Immunoassays can be very specific and sensitive to minute quantities of chemicals, and, most importantly, can be done quickly and at low cost. This latter point can be valuable where large numbers of assays are required; for example, in quality assurance programs.

This paper describes the development of an immunoassay for detecting and quantifying DDAC, the active ingredient in antisapstain formulations. We compared the DDAC concentrations determined by ELISA, colorimetric titration and HPLC for commercial antisapstain formulations and treatment solutions as used in sawmills.

Methodology

1) Reagents and solution samples from industry

Pure DDAC (98%), F2 and NP1 were obtained from Forintek Canada Corp. (Daniels and Weigel, 1994). The different working solutions were collected by Walker Brothers (a div. of Consolidated Coatings) from various sawmills in British Columbia.

2) Indirect and competitive inhibition ELISA(s)

Microtitration plates (Immulon 4, Dynatech Laboratories, Inc., Chantilly, VA) were coated with DDAC-Hapten-BSA in $NaHCO_3$, pH 9.6, and dried overnight at 37°C. The plates were washed four times with phosphate buffered saline (PBS) and blocked with 200 µL / well of 2% milk in PBS for 1 hour at 37°C, then washed four times with PBS. Diluted DDAC polyclonal serum in 0.1% milk in PBS was added to the wells and incubated at 37°C for 2 hours. Then the plate was washed four times with PBS, and a secondary antibody-enzyme conjugate, the anti-rabbit IgG-horseradish peroxidase diluted 1:3000 into 0.1% milk in PBS, was added. Both the diluted serum and the antibody-enzyme conjugate were applied at 100 µL / well. The enzymatic reaction was carried out at room temperature, in the dark, using the SIGMA *FAST* o-phenylenediamine dihydrochloride (OPD) as substrate, 200 µL / well. The reaction was stopped after 30 min. with 50 µL of 2.5 M H_2SO_4 and the absorbance was measured at 490 nm, using a.***THERMOmax***^{TM} microplate reader (Molecular Devices Corp., Meulo Park, CA).

A competitive inhibition ELISA was used for assessing the specificity of the antibodies to free DDAC and their cross reactivity with related compounds. Stock solutions of 1000 ppm DDAC or related chemicals were prepared in methanol. Appropriate concentrations of the chemical mixed with the DDAC polyclonal antiserum diluted 2000 times into 0.1% milk in PBS, were then added to a plate which had been coated with DDAC-Hapten-BSA and blocked with 2% milk in PBS. The remaining preparation work was carried out as described in the indirect ELISA procedure.

3) DDAC analysis by chromatography

The HPLC equipment consisted of an SP8800 ternary gradient pump, Chromjet integrator, model 7950 column chiller and an ELSD III (evaporative light scattering detector). All injections were 2 µL and made with a model 728 autosampler (Alcott Chromatography). The column used in all studies was 150 mm x 3.0 mm and packed with PVA-sil OR S-5 (YMC) at 3000 psi with methanol. Mobile phase A was prepared as follows: formic acid (1 mL), water (1 mL), triethylamine (1 mL), methanol (75 mL), chloroform (250 mL) and hexane to make 1L Mobile phase B was methanol. The analysis was performed under isocratic conditions with 100% mobile phase A at a flow rate of 0.40 mL / min for 8 min (Figure 1). At 8 min the flow rate

was changed to 1.5 mL / min and 100% mobile phase B was used to clean the column for 2 min. From 10-12 min the column was equilibrated with mobile phase A at a flow rate of 1.5 mL / min and at 12 min the flow rate was changed to 0.40 mL / min allowing the system to stabilize until the next injection was made at 14 min. The air flow rate through the detector nebulizer was set at 25 mm (0.65 L / min) and the drift tube temperature was set at 95°C. Samples for quantitative analysis of DDAC were dissolved in acetonitrile containing 200 μg/mL of didodecyldimethylammonium bromide (DoDAB) as an internal standard and 500 μg/mL of tetramethylammonium chloride (TMAC).

4) Titration method

The titration method is based on the relative solubility of the material in aqueous and chloroform layers (Lonza Notebook). In a pre-titration solution, quaternary halide, chloroform, basic buffer solution (pH 10), and bromphenol blue indicator are mixed together. The indicator forms a blue colored salt, soluble in chloroform, with the quaternary ammonium cation, leaving the aqueous layer colorless. When this mixture is titrated to the endpoint with a 0.003 N solution of sodium lauryl sulfate, the anionic titrant displaces the indicator, producing a violet color in the upper aqueous layer.

Results and discussion

1) Sensitivity and specificity of the antibody raised to DDAC.

The anti-DDAC polyclonal antibodies, raised to KLH-DDAC in rabbits, recognized the free DDAC in an indirect competitive ELISA. For that, DDAC was bound to the support through a carrier protein (bovine serum albumin, BSA) which is different from the protein (KLH) used to induce the immune response. In this assay the free analyte competes for antibodies with the immobilized analyte (BSA-DDAC), as shown in figure 2. An inhibition curve with free DDAC concentrations ranging from 8 to 55 ppm is shown in figure 3. With this assay, the detection limit, 50% inhibition, and effective working range were 8, 30 and 8 to 55ppm, respectively.

It is well known that antibodies produced from a given immunogen will recognize the compound's different moieties (epitopes) to different degrees. DDAC contains four types of possible recognizable moieties (i.e., the long aliphatic chain, the methyl group, the quaternary ammonium cation, and the nitrogen center). A series of compounds with structures similar to the different parts of DDAC were tested for their cross-reactivity with the anti-DDAC polyclonal antibodies (table 1). The results from an indirect competitive ELISA indicated that the DDAC polyclonal antibodies did not react with either the quaternary ammonium cation or the nitrogen center, but only with the aliphatic chain. Also the antibodies did not react with compounds having very

long or short aliphatic chains. Only compounds with straight aliphatic chain residues containing ten to twelve carbons cross-reacted strongly with the serum. These results suggested that the antibodies produced to KLH-DDAC antigen recognized the aliphatic chain of the DDAC molecule.

2) Determination of the DDAC content of antisapstain products by ELISA

Next we determined whether the antiserum was able to measure DDAC in different commercial fungicides. Pure DDAC (98%) was used to generate a standard curve (figure 3). The curve was linear from 8 to 55 ppm, with a correlation coefficient of 0.991. Within this range, the curve was described by A = 1.014 - 0.015 [DDAC], where A is the absorbance at 490 nm, and the DDAC concentration is in ppm. This equation was used to calculate the DDAC concentration in different samples, using a competitive ELISA. The commercial product F2, containing 11.4% DDAC, was tested at assay concentrations ranging from 20 to 50 ppm DDAC. A straight line with a slope of 0.97 ± 0.01 and a correlation coefficient of 0.99 was obtained. The curve generated with the commercial product was very similar to the curve generated with pure DDAC. Similar results were obtained with another commercial product, NP-1. In general, the DDAC concentrations measured by the competitive ELISA were within 7% of the values reported by the manufacturer. This confirmed that the other chemicals present in the commercial products (e.i., 3-iodo-2-propynyl butyl carbamate (in NP-1) and borax (in F2A), did not interfere with the immunoassay.

3) Comparison with HPLC and titration methods

Table 2 shows a comparison of results obtained by three different methods: ELISA, HPLC and colorimetric titration, in the analysis of five industrial antisapstain solution samples. All three methods gave comparable results suggesting that the ELISA for DDAC may have potential as an alternative for quantifying DDAC in industrial working solutions or in yard run-off waters, where it would not require sample clean-up or extraction procedures.

Conclusions

The polyclonal antibodies raised against KLH-DDAC recognized the free chemical DDAC, and seemed to bind specifically to the two long aliphatic chains of the molecule. The antibodies could be used to measure DDAC in commercial anti-fungal products by a competitive ELISA. Similarly, DDAC was quantified in working solutions obtained from different mills. The assay was carried out directly with the original products and working solutions, neither of which required extraction or purification steps.

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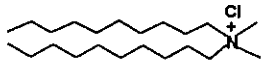
Table 1. Percentage cross-reactivity of the anti-DDAC polyclonal serum with chemicals related to DDAC

Entry	Structure	Chemicals at 30 ppm	Crossreactivity (%)
1		DDAC	100 ± 1
2		Lauric acid	100 ± 2
3	$Et_4N^+Cl^-$	Tetraethyl ammonium chloride	3.0 ± 0.1
4	Et_3N	Triethylamine	0

Coating antigen: DDAC-BSA, 100 ng / well; polyclonal serum dilution 1:2000

Table 2. Comparison of ELISA, HPLC and titration methods for the analysis of industrial samples

Sample #	ELISA (%)	HPLC (%)	Titration (%)
100-5	2.40 ± 0.1	2.20	2.42
102-5	1.60 ± 0.08	1.50	1.50
103-5	1.50 ± 0.07	1.30	1.45
104-5	1.20 ± 0.05	1.10	1.13
129-13	0.90 ± 0.04	1.00	0.93

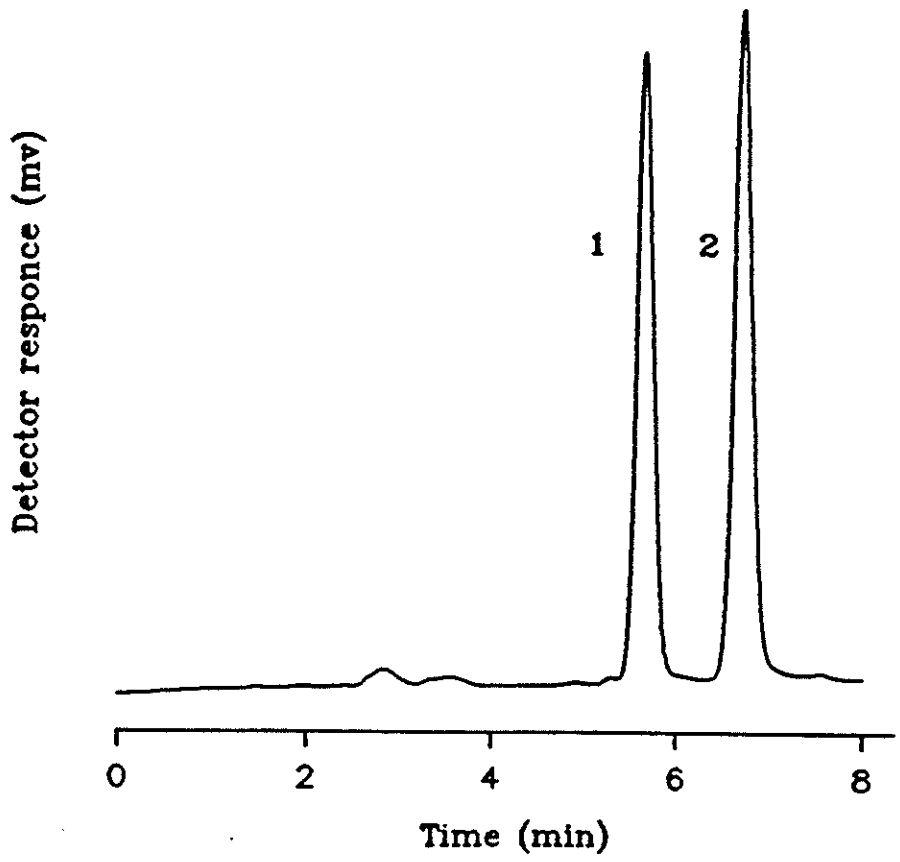


Figure 1.- Calibration standard 1-DoDAB (200 µg/ml), 2-DDAC (200 µg/ml)

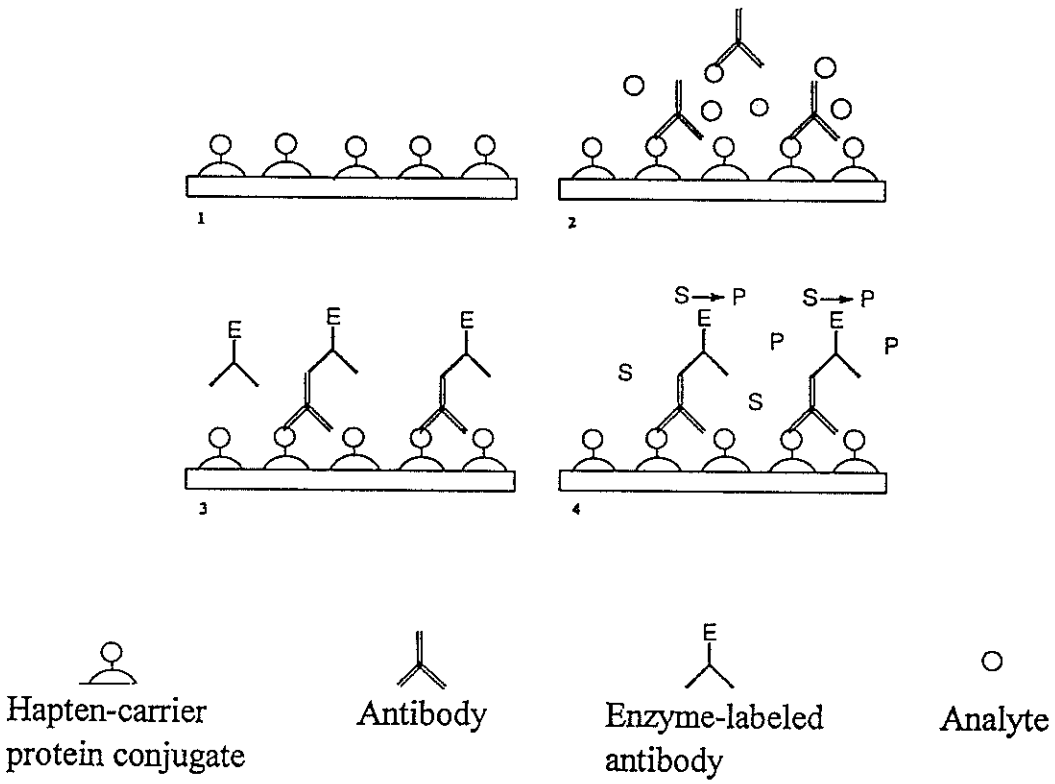


Figure 2.- Indirect Competitive ELISA

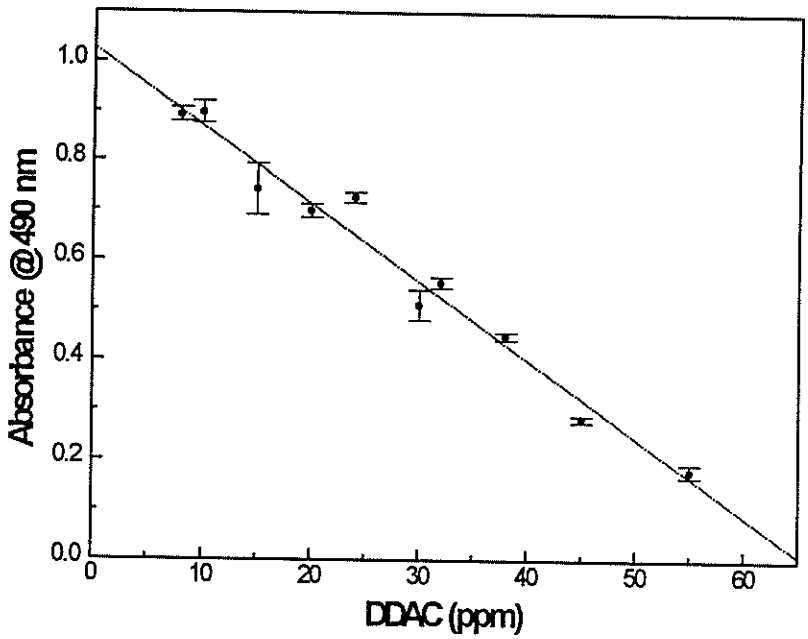


Figure 3.- Calibration curve for anti-DDAC antibodies using pure DDAC by an indirect competitive ELISA.

Serum dilution: 1:2000; coating antigen BSA-DDAC: 100 ng / well