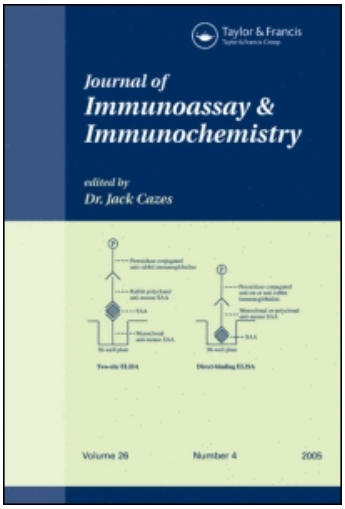

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

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To cite this Article Ragupathi, G. , Prabhasankar, P. , Sekharan, P. Chandra , Annapoorani, K. S. and Damodaran, C.(1992) 'Enzyme-Linked Immunosorbent Assay for the Phytotoxin Cleistanthin A', Journal of Immunoassay and Immunochemistry, 13: 3, 321 — 338
To link to this Article: DOI: 10.1080/15321819208021236
URL: http://dx.doi.org/10.1080/15321819208021236

JOURNAL OF IMMUNOASSAY, 13(3), 321-338 (1992)

ENZYME-LINKED IMMUNOSORBENT ASSAY FOR THE PHYTOTOXIN CLEISTANTHIN A

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ABSTRACT

Enzyme - linked immunosorbent assay is reported for the estimation of cleistanthin A, a major constituent of the toxic plant Cleistanthus collinus. Rabbit antibodies were obtained by immunisation with cleistanthin A hemisuccinate-BSA conjugate and the ELISA developed thereupon could detect cleistanthin A at as low a concentration as 3 ng/ml. Cross-reactivity studies with structural analogs as well as with other phytotoxins and drugs of common occurrence established the suitability of the ELISA to specifically monitor the C. collinus marker molecules in emergency clinical and forensic cases. The simplicity and specificity make the ELISA superior to the other available techniques.

(KEY WORDS: Cleistanthin, C.collinus, ELISA, Penicillinase, Toxicology).

INTRODUCTION

Cleistanthin A (clei A), a lignan glycoside is a toxic principle of the plant Cleistanthus collinus (1). This plant belongs to the Euphorbiaceae family and

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is abundant in India. The poisonous nature of the plant especially its leaves has been well documented (2). In addition to cleistanthin A, other lignan lactones namely cleistanthin B, diphyllin, and collinusin are also present (1,3); spectral and chromatographic methods of quantifying them in biospecimens have been reported (4-6). However no immunoassay has yet been developed. The objectives of this investigation were therefore to couple the haptenic clei A with carrier protein using peptide bond forming agents in order to produce antibodies, characterise their specificity for clei A and develop an ELISA for qualitative and quantitative application in cases of C.collinus poinoning.

MATERIALS AND METHODS

Cleistanthin A was isolated from the leaves of C.collinus by a preparative chromatographic procedure and its purity checked through melting point analysis, thin-layer chromatography and spectral data by comparison with an authentic sample obtained from CIBA-GEIGY (India).

Bovine serum albumin (BSA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), soluble starch, 2,4,6,- trinitrobenzene sulfonic acid (TNBS) and Tween 20 were obtained from Sigma; penicillinase and penicillin V were from Hindustan

Antibiotics Ltd (India); and Freund's adjuvants (complete and incomplete) were the products of Difco Laboratories, USA. Buffer salts of reagent grade quality were used.

Preparation of cleistanthin A-protein (BSA) immunogen

Preparation of cleistanthin A - hemisuccinate (clei A-HS) and its conjugation to BSA were carried out according to the method developed earlier by us (7).

Enzyme labelling of cleistanthin A

Cleistanthin A - HS derivative was labelled with the marker enzyme penicillinase in the following way. The conjugation was initiated by adding 1ml of an aqueous solution of EDC (15 mg/ml; 80 μmoles) to a solution of clei A-HS (4.5 mg/ml; 8.2 μmoles) in sodium phosphate buffer (pH 6.0; 0.01M). The mixture was incubated at room temperature for 30 min with occasional shaking and then added to a solution of penicillinase (1 ml ; 5mg/ml; 0.16 μmole) in sodium phosphate buffer (pH 8.0; 0.2M). After incubation at 2-8^{o}C for 16 to 18h, the reaction mixture was dialyzed extensively against sodium phosphate buffer (pH 7.4; 0.01M). To the dialyzed enzyme conjugate, BSA and sodium azide were added to a concentration of 1% and 0.2% and stored refrigerated in aliquots.

Evaluation of molar participation of hapten in conjugates

Determination of the number of haptenic clei A residues per protein or enzyme molecule was carried out

by spectrometric analysis (8) and TNBS method (9). The protein content before and after conjugation was assayed by the method of Lowry et al (10) and the enzyme activity of penicillinase was measured as described by Joshi (11).

Preparation of antiserum to cleistanthin A

New Zealand white rabbits were injected intradermally at multiple sites with 1 ml of clei A-HS-BSA conjugate (corresponding to a concentration of 100 μg of bound clei A-HS) dissolved in phosphate-buffered saline (PBS; 0.1 M; pH 7.4) and emulsified in Freund's complete adjuvant (1ml). After four weeks a booster injection of the immunogen (40 μg) in Freund's incomplete adjuvant was given. The antiserum obtained after seven days of the booster dose was tested for the presence of clei A antibodies by direct ELISA and purified by ammonium sulfate precipitation and dialysis after which aliquots were stored at - 60°C until use.

Development of competitive ELISA for clei A

Optimal combination of the antibody and enzyme labelled analyte for use in ELISA was ascertained as described in 'Results'. Accordingly 200 μl of anti-clei A antibody diluted with coating buffer (bicarbonate buffer; 0.05M; pH 9.6) (1:1600) was added to the wells of microtiter plates and incubated for 3h at 37°C or overnight in refrigerator. After washing the

plates (three times) with wash buffer (PBS-containing 0.05% Tween 20) the empty sites were blocked with 1% solution of BSA in PBS. The plates were then incubated for 1h at room temperature and washed again with wash buffer. This was followed by the addition of known amounts of clei A (100 μl) or analyte sample (100μl) like clei A - spiked urine and a specified aliquot of the predetermined dilution of the clei A - HS - penicillinase conjugate (100 μl; 1:100). The plates were incubated at 37^{o}C for 2h. Subsequently the plates were washed with wash buffer and 200 μl of the substrate solution was added to each well. (The substrate solution was starch-iodine-penicillin (SIP) reagent obtained by mixing 0.2ml of iodine reagent which in turn was a preparation of potassium iodide (5.32g) and iodine (200 mg) in 10 ml of distilled water, and 20 ml of 2% starch solution with 100 ml of penicillin V (15.2mg) solution in phosphate buffer; SIP reagent was prepared just before use). The plates were then left at ambient temperature for 20 min immediately following which the absorbance measurements were made at 620 nm with an ELISA Reader (Biotek, EL 310 model).

Evaluation of ELISA for specificity to clei A

The competitive ELISA was conducted with different "analytes" such as the clei A structural analogs present in C.collinus, other phytotoxins and drugs of common occurrence in clinical and forensic

toxicology. The cross-reactivity was calculated as below according to the method described by Joshi (personal communication):

$$\%\text{binding at known concentration} = \frac{(\text{O.D of clei A excess}) - (\text{O.D of known concentration of clei A})}{(\text{O.D of clei A excess}) - (\text{O.D without clei A})} \times 100$$

The percent binding for various concentrations of clei A were calculated and plotted as percent binding vs corresponding concentration on a semi-logarithmic graph.

Similarly the percent binding with various concentrations of the cross-reacting compounds (CRC) were calculated and the graph constructed.

Let T_{50} be the concentration of clei A,and CRC_{50} that of the CRC; with respect to 50% binding, then

$$\%\text{ Cross-reactivity of the CRC} = \frac{T_{50}}{CRC_{50}} \times 100$$

RESULTS

A hemisuccinate derivative of the toxin cleistanthin A (clei A-HS) was prepared and conjugated with carrier protein/enzyme for obtaining the immunogen and label. The BSA-coupled conjugate contained 26 molecules of clei A-HS per protein while the enzyme (penicillinase) - tagged product retained on average 4 haptens on a molar basis. Determination of

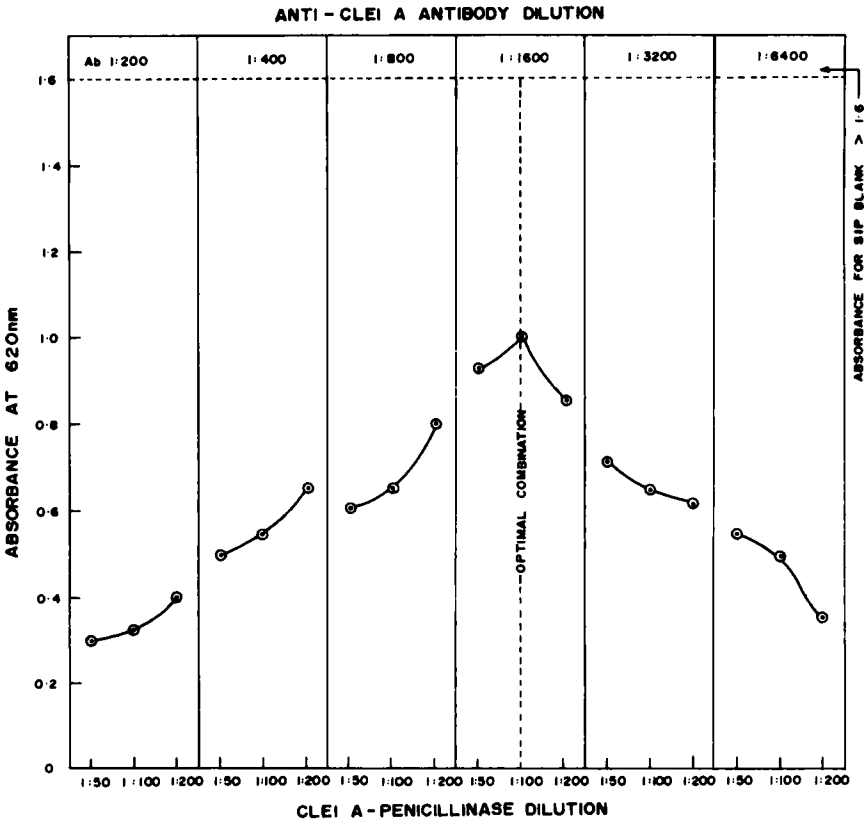


FIGURE 1 Optimum combination of anti-clei A antibody and clei A-penicillinase label.

penicillinase activity in the conjugate showed retention of more than 85% of the original activity.

Optimal dilutions of the anti-clei A antibody and the clei A-HS- penicillinase conjugate as required for ELISA were determined by constructing a graph from the difference in absorbance values of the reagent

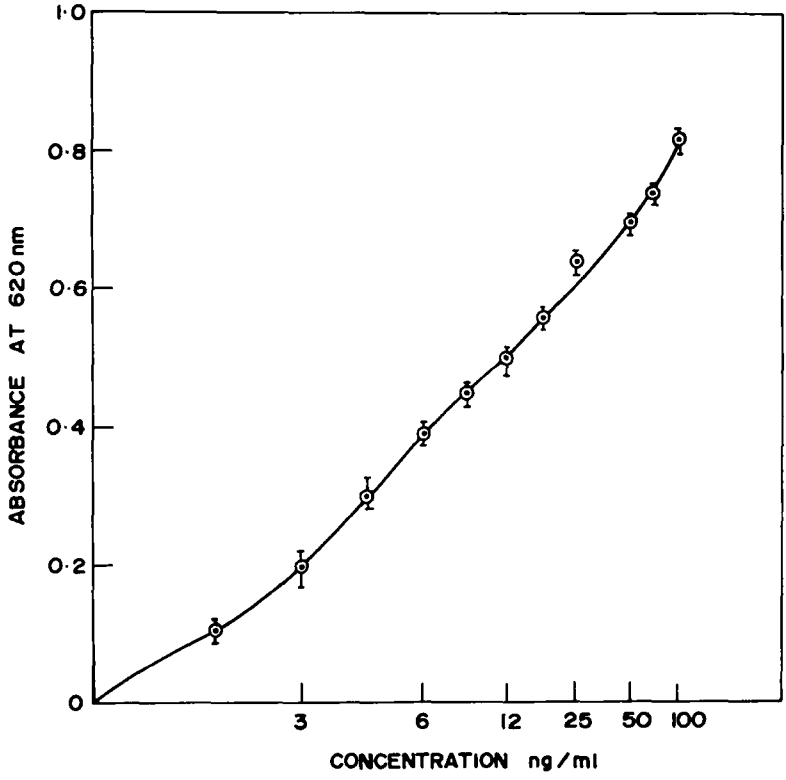


FIGURE 2 Calibration curve for cleistanthin A.

blank and analyte wells for the various combinations of anti-clei A- antibody and clei A-HS - penicillinase. The dilution that gave an absorbance-difference close to 1.0 was considered as optimum dilution. The corresponding data are shown in Figure 1. The dilution factors were found to be 1600 and 100 for the antibody and the enzyme conjugate respectively.

TABLE 1

Reproducibility of ELISA for the estimation of cleistanthin A

Added (ng/ml)	Estimated recovery*							
	Intra-assay				Inter-assay			
	ng/ml	SD	%	CV,%	ng/ml	SD	%	CV,%
5	4.90	0.187	98.00	3.82	5.12	0.377	102.40	7.36
10	10.00	0.412	100.00	4.12	9.57	0.714	95.70	7.46
25	23.90	0.689	95.60	2.88	26.82	2.295	107.28	8.55
50	48.66	3.850	97.32	7.91	49.31	3.245	98.62	6.58
75	67.28	5.554	89.70	8.25	72.64	1.887	96.85	2.59
100	91.00	4.920	91.00	5.40	94.78	4.037	94.78	4.25

*Each sample (spiked urine) was assayed in quadruplicate

TABLE 2

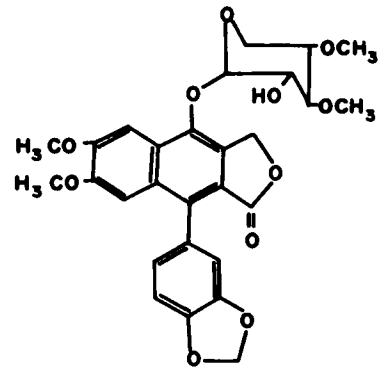
Cross-reactivity of anti-cleistanthin A antibody during ELISA as tested with cleistanthin A structural analogs

Compounds tested	% Cross-reactivity*
Cleistanthin B	84.4
Diphyllin	98.0
Collinusin	96.6

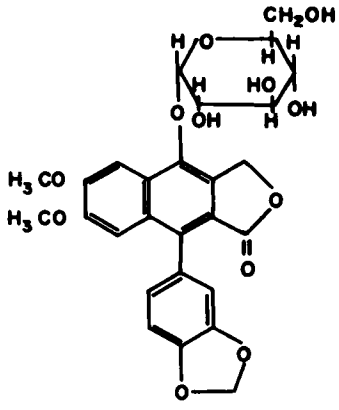
*Cleistanthin A-HS was assigned a value of 100

A calibration graph constructed with clei A standard by plotting the semilog of concentration against absorbance at 620 nm showed linearity in the range 3-100 ng/ml (Figure 2). The reproducibility of the ELISA was verified by estimating the amount of clei A in spiked urine samples. The figures on percent recovery are presented in Table 1.

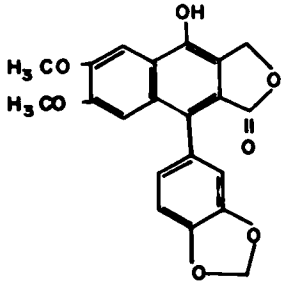
The possible cross-reactivity of anti-clei A antibody with certain other C.collinus lignan lactones that are structurally similar to clei A is shown in Table 2 and their structures in Figure 3. Table 3 lists the non-C.collinus phytotoxins and drugs of common occurrence in clinical and forensic toxicology that were tested in the ELISA for cross-reactivity and ruled out therefor.



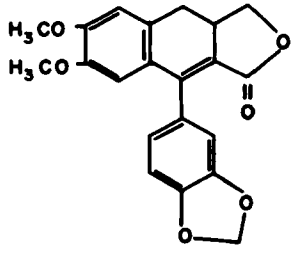
CLEISTANTHIN - A



CLEISTANTHIN - B



DIPHYLLIN



COLLINUSIN

FIGURE 3 Major lignan lactones of C.collinus.

TABLE 3

Drugs and plant toxins tested and found negative for cross-reactivity with anti-clei A antibody during ELISA

Drugs	Plant toxins
Amylobarbitone	Brucine
Butobarbiton	Cerberin
Chlordiazepoxide	Digitoxin
Chlorpromazine hydrochloride	Digoxin
Chlorpromazine sulphoxide	Ellagic acid
Cyclobarbitone calcium	Gitoxin
Dextropropoxyphene hydrochloride	Neriifolin
Diazepam	Oleandrigenin
Dicoumarol	Oleandrin
Diphenhydramine hydrochloride	Ouabain
Heptabarbitone	Peruvoside
Imipramine	Strychnine
Indomethacin	Thevetigenin
Lorazepam	Thevetin
Nitrazepam	
Nortriptyline	
Oxazepam	
Oxyphenbutazone	
Pentobarbitone sodium	
Phenacetin	
Phenobarbitone sodium	
Promethazine hydrochloride	
Quinalbarbitone sodium	
Temazepam	
Thiopentane sodium	
Trimipramine maleate	

DISCUSSION

Identification and determination of xenobiotics in biospecimens are essential for therapeutic drug monitoring and to establish the cause of poisoning in clinical and forensic problems. For this many

investigators have been turning to enzyme immunoassays. The increasing incidence of C.collinus poisoning calls for the development of such simple, sensitive and rapid assays.

Cleistanthin A, as shown in Figure 3, is not an immunogen and hence a conjugate with a carrier protein like BSA needs to be prepared. Various methods are available for the preparation of hapten-protein conjugate (12). Direct coupling of clei A to protein is however not feasible due to the absence of relevant reactive groups in clei A. The hemisuccinate derivative of clei A (clei A-HS) was prepared which was then conjugated with BSA by the mixed anhydride method (7). This method of conjugation was preferred for the preparation of good immunogen since the resulting conjugate had a higher number of clei A-HS residues per molecule of protein than the one obtained by the carbodiimide method. On the other hand, for labeling the analyte with enzyme marker, the carbodiimide method of conjugation was preferred since less analyte molecules are required in the resulting analyte- enzyme conjugate in order that the enzyme retain its activity (13). The BSA conjugate had 26 clei A molecules per BSA molecule whereas the penicillinase conjugate contained only 4 clei A residues per enzyme molecule.

Among the haptens, excepting steroids (14), the arylnaphthalene lignan lactone glycoside used in the present study is the first compound to be labelled with penicillinase by the carbodiimide method. Determination of the enzyme activity in clei A-HS penicillinase conjugate showed the enzyme to retain more than 85% of its activity. Besides the presence of fewer clei A-HS molecules in the conjugate the other factors that contribute to retention of enzyme-activity are the pH of the reaction medium during conjugation and the concentrations of the other reactants. The initial reaction of clei A-HS with EDC at pH 6 presumably permits the formation of O-acylisourea (15), which then reacts with the nucleophilic amino group in the enzymic protein to form peptide bonds at pH 8.0. Self polymerisation of clei A-HS is not possible under these conditions since it is devoid of an amino group. This apart, the effect of carbodiimide on penicillinase in initiating self coupling is further prevented by raising the pH of the reaction medium from 6 to 8 during the addition of penicillinase and by increasing the concentration of phosphate buffer from 0.01 to 0.2 M (16). Penicillinase was chosen as the marker enzyme since it is known to be stable at ambient temperature over the pH range 5 to 9 (17).

The sensitivity of the ELISA developed here for clei A was 3ng/ml. The reproducibility of the method

was verified by assaying clei A from spiked urine samples. The coefficients of variation in the intra- and inter-assays were less than 9% (Table 1).

The specificity study conducted by testing other structurally similar arylnaphthalene lignan lactones of C.collinus clearly indicated the ability of anti-clei A-HS antibody to react with cleistanthin B, diphyllin and collinusin (Table 2). This is because for preparing the immunogen the conjugation of clei A-HS with BSA was carried out through the carboxyl group introduced in the 2-position of the 3,4-di-o-methyl-xylose (carbohydrate moiety) of clei A (7) thereby permitting the aromatic nucleus to be the major haptenic determinant site. Since cleistanthin B, diphyllin and collinusin also have the same aryl nucleus, the anti-clei A-HS antibody exhibited cross-reactivity. However the degree of cross-reactivity varied, with the glycoside cleistanthin B showing less affinity (Table 2) which might be due to its carbohydrate moiety, namely D-glucose as shown in Figure 3.

The cross-reactivity of anti-clei A-HS antibody with the other three major lignan lactones of C.collinus is in fact an added advantage in the end-user context of the developed ELISA since in cases of C.collinus poisoning there is the presence of all of

these four marker compounds in urine, blood and other tissues. In addition the major metabolite after C.collinus poisoning has been reported to be diphyllin (18). Thus the present method can be applied for the specific detection of C.collinus by monitoring the characteristic active glycosidic principle as well as the metabolite. The operational promise and specificity of the proposed ELISA are further enhanced by the fact that other phytotoxins and drugs frequently encountered in clinical and forensic toxicology exhibit no cross-reactivity with anti-clei A-HS antibody (Table 3).

ACKNOWLEDGEMENTS

The authors thank Dr. Gowri Chandrakasan (CLRI, Madras) and Dr.U.M. Joshi (IRR, Bombay) for their help/suggestions. The financial assitance provided by the Bureau of Police Research and Development, New Delhi and Tamil Nadu State Council for Science and Technology, Madras is acknowledged.

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