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Novel solid-state fluorodensitometric method for the determination of haptens in protein–hapten conjugates

Demonstration with a toxic glycoside of *Cleistanthus collinus*

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ABSTRACT

A method is reported for the solid-state fluorodensitometric determination of haptens in hapten–protein conjugates. The applicability of the method is shown using the bovine serum albumin conjugate of cleistanthin B, a toxic glycoside of the plant *Cleistanthus collinus*. This non-destructive technique, which is relatively simple, sensitive, rapid and versatile, can be used for the determination of protein conjugates of fluorogenic haptens.

INTRODUCTION

Immunoassay-based diagnostic kits, owing to their specificity, sensitivity, simplicity and direct applicability to unprocessed biological specimens, are of immense help to clinicians and toxicologists, especially when traditional chemical methods fail to detect low concentrations of analytes in the presence of impurities [1]. The prerequisite of any immunoassay is the production of specific antibodies against the analyte. Many xenobiotics, such as drugs and toxins, are low-molecular-mass compounds and hence not by themselves antigenic. Such compounds, known as haptens, can, however, be made immunogenic by conjugation with certain carrier protein molecules such as serum albumin, egg albumin and globulin. These hapten–protein conjugates, or neoantigens, generate antibodies in the challenged host with specificity to the hapten.

The procedure for the preparation of hapten–protein conjugates varies depending on the reactive functional group(s) of the hapten and as a consequence there is no method which can be used for all compounds. Nevertheless, the efficiency of the different methods can be evaluated by determining the number of haptenic molecules conjugated to the protein molecule [2]. This is important as the quality of the antibody produced depends on the extent of hapten participation in the conjugate. Too many or too few haptens act as poor antigens [3]. With serum albumin as the carrier molecule, which is generally preferred because of the relative stability and solubility of its conjugates, 10 to 25 covalently coupled haptenic groups seems to be the optimum number. In this context the formation of the desired conjugate can be controlled by varying the ratio of hapten to protein [4,5].

The number of haptenic molecules in the conjugate can be determined from the UV spectral data of the hapten, protein and hapten–protein

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conjugate [6]. An estimation of the number of free amino groups in the protein molecule before and after conjugation is another common method [7]. The incorporation of radioactive haptens in the conjugation procedure has also been reported [8]. However, these methods have limitations, which prompted this work based on fluorodensitometry for haptens with an intrinsic fluorescence property. The applicability of the developed procedure is shown with an arylnaphthalene lignan lactone, cleistanthin B, the glycosidic structure of which is shown in Fig. 1.

Cleistanthin B (clei B) is an active principle of the highly toxic plant *Cleistanthus collinus* [9], for which a fluorodensitometric technique has been developed [10]. Work was begun on a dipstick enzyme immunoassay method for bedside poison monitoring in emergency clinical cases. For this purpose, to raise antibodies to clei B, the haptenic clei B (Fig. 1) needs to be conjugated to a carrier protein molecule. This was made possible by the periodate method of conjugation as clei B has vicinyl hydroxyl groups in the carbohydrate moiety of its structure (Fig. 1).

Clei B, as it is an aromatic compound with a lactonic ring and hydroxyl groups, shows a strong fluorescence which is not affected by conjugation with proteins. In contrast, the carrier molecule used, bovine serum albumin (BSA), like many other proteins does not fluoresce, thereby facilitating the interference-free determination of clei B. Based on this observation this fluorodensitometric method could be developed for the determination of the clei B (hapten) molecules in the clei B–BSA (hapten–protein) conjugate.

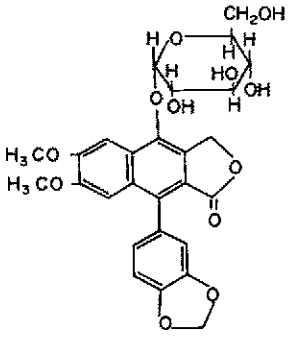

Fig. 1. Structure of cleistanthin B.

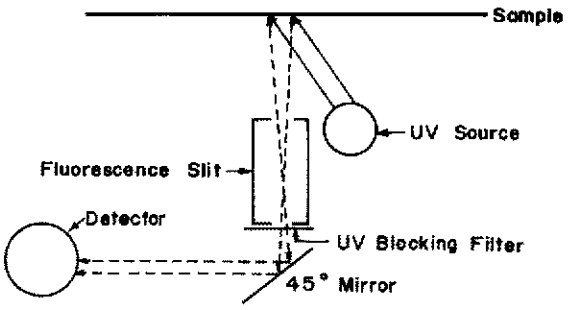

Fig. 2. Schematic diagram of the fluorodensitometry system.

EXPERIMENTAL

Instrumentation and chemicals

All densitometric measurements were made with a soft laser (SL-TRFF) scaning densitometer (Biomed, CA, USA). The layout of the fluorodensitometry system is shown in Fig. 2.

The UV spectra were recorded using a UV–visible Specord spectrophotometer (Carl Zeiss, Jena, Germany).

Precoated thin-layer chromatography (TLC) plates (Kieselgel 60 F_{254}, E. Merck, Darmstadt, Germany; 0.2 mm) were used throughout the study.

Authentic clei B was obtained from Osmania University (Hyderabad, India) and checked for purity by chromatographic and spectral analyses. A standard solution of clei B was prepared in ethanol (500 μg/ml) and stored at 4°C until use. Suitable working solutions were prepared as required.

Chemicals and solvents were of analytical-reagent or spectral grade. BSA (A 4503), sodium metaperiodate (S 1878), sodium borohydride (S 9125) and 2,4,6-trinitrobenzene sulphonic acid (TNBS) (P 5878) were from Sigma (St. Louis, MO, USA).

Conjugation of clei B with BSA

The clei B–BSA conjugate was prepared according to the method of Ragupathi *et al.* [11], as described below. To 32.5 mg (60 μmol) of clei B, 2 ml of distilled water were added and the solution warmed slightly until clei B completely dissolved. To this solution, 1 ml of sodium metaperiodate (12.8 mg/ml in distilled water; 60 μmol)

was added dropwise and the reaction mixture stored in the dark with occasional shaking for 45 min at room temperature. At the end of this period, ethylene glycol (135 μmol) was added to neutralise the unreacted sodium metaperiodate and allowed to stand at room temperature with occasional stirring for 20 min. To the reaction mixture was then added 66 mg of BSA (1 μmol) dissolved in hydrogencarbonate buffer (pH 9.5). The pH of the reaction mixture was maintained between 9 and 9.5 by the addition of hydrogencarbonate buffer. The reaction mixture was stirred for 2 h at room temperature. After this, 1 ml of sodium borohydride solution (2.6 mg/ml in distilled water; 60 μmol) was added and the reaction allowed to proceed at 37°C for 3 h or at 4°C for 24 h. At the end of the specified period, the solution was dialysed against distilled water at 4°C for 24 h with repeated changes. The conjugate was also prepared in different molar ratios of protein to hapten.

Determination of protein in the conjugate

The protein content in the conjugate after dialysis was determined by Lowry's method using BSA as the standard [12]. The possible interference of clei B with the protein (BSA) in Lowry's method was checked and found to be negligible, even at three times, the concentration of the clei B to BSA.

TLC of clei B, BSA and clei B–BSA conjugate

The TLC was carried out on silica gel with a known amount of standard clei B, BSA and the conjugate with an estimated protein content. Chloroform–methanol (9:1, v/v) was used as the developing solvent system and detection was achieved by viewing the plate under short-wavelength UV light.

Determinations of clei B in clei B–BSA conjugate using TLC–fluorodensitometry

To obtain the best results, the densitometer was warmed up for 15 min. The TLC plate was mounted upright on the stage with suitable spacers on the top and bottom of the plate. The sample was excited with a low pressure mercury lamp with an emission peak at 366 nm. The emissions above 400 nm were blocked by a cobalt blue filter, which is an integral part of the lamp. The source was placed on the same side of the sample as the detector (Fig. 2). The emitted light from the detector passed through a collimating slit. A filter that blocks energy below 410 nm was placed between the slit and the detector to block the UV light of the exciting source reaching the detector. The secondary filter allows only the light emitted from the sample into the detection and measuring system. The photomultiplier tube voltage, the analogue gain and the zero position control knobs were adjusted to give 85–90% full-scale deflection and the scan length was adjusted by the thumb screw.

For the determination of clei B in the conjugate, a standard graph was constructed for various amounts of clei B by plotting the percentage peak area against concentration. A known amount of the clei B–BSA conjugate was also chromatographed with standard clei B. As the amount of protein in the conjugate is known, the determination of clei B in the conjugate allows the determination of the number of clei B molecules conjugated to each protein molecule.

UV method

The number of clei B molecules in the conjugate was calculated from the UV spectral data obtained for clei B, BSA and the conjugate [6]. The UV spectra of clei B, BSA and clei B–BSA in water were recorded. Clei B showed an absorption maximum at 262 nm with a molar absorptivity of 60 000. The absorbance of the clei B moiety in clei B–BSA at 262 nm was determined by subtracting the absorbance value of BSA (262 nm) from that of the clei B–BSA conjugate (262 nm), which, similar to free clei B showed a maximum peak at 262 nm. The hapten was then determined by comparing these values with a calibration graph.

TNBS method [7]

This was carried out by determining the number of free amino groups in the protein (BSA) before and after conjugation with clei B. A standard graph was constructed using known concentrations of free BSA reacted with TNBS and the absorbance was subsequently monitored at 420 nm. The clei B–BSA conjugate with the estimated

TABLE I

NUMBER OF CLEI B MOLECULES PRESENT FOR EACH BSA MOLECULE (MOLAR PARTICIPATION) IN CLEI B–BSA CONJUGATE AS ESTIMATED BY DIFFERENT METHODS

Results are averages of three experiments.

BSA to clei B molar ratio	Solid-state fluorodensitometry (this work)	UV spectrophotometric method (ref. 6)	TNBS method (ref. 7)
1:70	26	30	25
1:60	26	29	26
1:50	25	34	26
1:40	22	30	24
1:30	17	24	18

protein content was then treated with TNBS and the absorbance (420 nm) measured for calculating the free amino groups as defined from the standard graph.

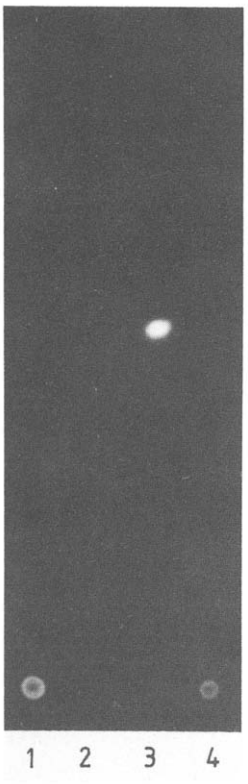

Fig. 3. UV photograph of the TLC plate for clei B–BSA, BSA and clei B after development with chloroform–methanol (9:1, v/v) as the solvent system. Lanes: 1 and 4 = clei B–BSA conjugate; 2 = BSA; and 3 = clei B.

RESULTS AND DISCUSSION

Table I shows the molar participation of the haptenic clei B with BSA in the clei B–BSA con-

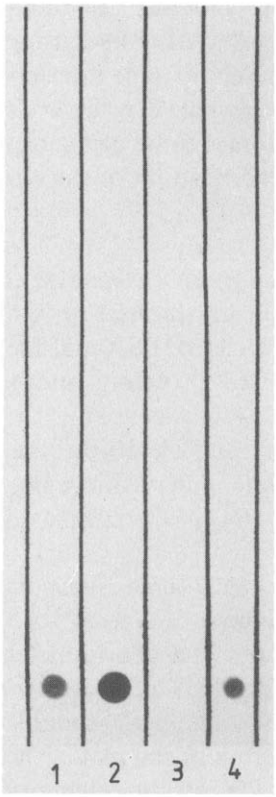

Fig. 4. Photograph of the TLC plate shown in Fig. 3 after spraying with ninhydrin.

jugate as calculated by this fluorodensitometric and other methods. Also shown are the results obtained for conjugates prepared beginning with different molar ratios of protein to hapten.

As is evident from this table, the number of haptenic molecules calculated by fluorodensitometry and by the TNBS method are similar. However, the results obtained by the UV method are higher, which might be due to the possible presence of free hapten in the conjugate even after extensive dialysis [13] for 24 h against distilled water at low temperatures, with repeated changes. As clei B has a high molar absorptivity (60 000), even a trace amount of excess compound can introduce errors. This problem is, however, circumvented in the TLC–fluorodensitometric method as the free hapten is well separated (R_F 0.45) from the origin-bound conjugate (Fig. 3). Thus this method precludes the time-consuming, diluting dialysis after conjugation.

The behaviour of the free hapten, protein and the conjugate are shown in Fig. 3. As illustrated, the clei B–BSA conjugate showed the characteristic fluorescence of its hapten-participant clei B, whereas the free protein molecule (BSA), even at a concentration three to four times higher than clei B, showed no fluorescence, thereby ruling out any interference by BSA in the fluorodensitometric determination of clei B. In addition, as the free BSA and the conjugate remained in the origin itself, the presence of the protein in and accounting for the conjugate after TLC was confirmed by reaction with ninhydrin (Fig. 4).

CONCLUSIONS

The following points highlight the merits of the relatively versatile and non-destructive TLC–fluorodensitometric method for the determination of fluorogenic haptens in protein–hapten conjugates:

(a) Unlike the UV method [6], interference due to the free hapten is eliminated.
(b) Extensive dialysis of the conjugate is not required, in contrast to the UV method [6].
(c) The TNBS method [7] of determining the number of free amino groups in the protein before and after conjugation is applicable only in instances where conjugation takes place through the amino group of the protein; this method does not have this limitation.
(d) Fluorodensitometry is more sensitive than other methods as fluorescence can be monitored even at nanogram levels of the hapten, thus allowing both qualitative and quantitative determinations *in situ* on the TLC plate.
(e) The inherent problems in handling and disposing of radioactive materials [8] are avoided.
(f) The progress of the conjugation reaction can also be followed by adapting the TLC–fluorodensitometry method.

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