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Analysis of Pyrrolizidine Alkaloids: A Competitive Enzyme-Linked Immunoassay (ELISA) for the Quantitative Determination of Some Toxic Pyrrolizidine Alkaloids

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ABSTRACT A highly sensitive and specific competitive enzyme-linked immunoassay (ELISA) for the determination of retrorsine, senecionine, and integerrimine in the nanogram range has been developed. Pyrrolizidine alkaloids were detected in *Senecio rupestris*. No cross-reactivity was seen with either retrorsine N-oxide, otonecine alkaloids, or other macrocyclic pyrrolizidine alkaloids.

Key Words: Enzyme immuno assay, Determination of toxic pyrrolizidine alkaloids, Retrorsine, Senecionine, Integerrimine

INTRODUCTION

Pyrrolizidine alkaloids (PAs) are often toxic compounds which occur naturally in some plant species belonging to Boraginaceae, Compositae and Leguminosae [Bull et al., 1968; Mattocks, 1986; Rizk, 1991]. Pyrrolizidine alkaloid poisoning is a public health problem in many areas of the world due to wide geographical distribution of PA containing plants [Roitman, 1983].

Poisoning by PAs causes severe acute hepatotoxicity in humans in the form of venoocclusive disease [McLean, 1970]. Especially in countries of the third world, many people fall ill and die by consumption of cereals contaminated by PA containing plants, medicaments, spices, or food.

Due to the risk of plants containing PAs, specific and sensitive methods are of considerable importance. In the literature, spectrophotometric [Mattocks, 1967; Roeder et al., 1992a], spectroscopic [Pieters et al., 1989] and chromatographic [Roeder and Neuberger, 1988] methods are described.

In this article, a competitive enzyme-linked immunosorbent assay (ELISA) is described, which allows the specific and precise measurement of the macrocyclic 12-membered unsaturated PAs of the senecionan-type most present in nature.

One of these alkaloids, retrorsine (Fig. 1.1) has been used to produce antibodies in rabbits by immunization with retrorsine hemisuccinate-bovine serum albumine conjugate (Fig. 1.4). Horseradish-peroxidase coupled to retrorsine hemisuccinate was used as tracer with ABTS® and hydroxyperoxide as a chromogenic system [Gallati, 1979; Porstmann et al., 1981; Tijssen, 1985]. This assay allows the quantitative determination of retrorsine, senecionine, and integerrimine in the nanogram range in plants and other biological material.

RESULTS AND DISCUSSION

The measuring range of the retrorsine-ELISA extends from 2–150 ng/ml. The sensitivity of this system is between 1 and 2 ng/ml and the precision of the retrorsine standard curve between 7.1 and 12.1%, expressed by the coefficient of variation. The cross reactions of the antibodies against the PAs **2–11** were investigated by comparing the concentrations which displace 50% of the enzyme marked retrorsine from the antibody. Figure 2 shows the standard curve of the enzyme immunoassay.

The data show that the competitive ELISA is likewise specific for the alkaloids retrorsine **(1)**, senecionine **(2)**, and integerrimine **(3)**. Molecular modifications concerning the structure of the macrocycle cause a loss of specificity. This is shown by the alkaloid seneciphylline **(4)**, and senecivernine **(5)**. Retrorsine N-oxide **(6)** gives a cross reaction of 1% only, obviously a free nitrogen in the PA base is necessary for the antibody adhesion. The behaviours of the tested compounds senkirkine **(7)** and otosenine **(8)**, both 12-membered macrocyclic otonecine-alkaloids support this supposition. The methylated nitrogen and the "quasi ketonfunction" at the position C-8 gives a cross reaction of only 0.7 (senkirkine) and 0.5 (otosenine), respectively, and is thus in the same order as the cross reaction of retrorsine N-oxide.

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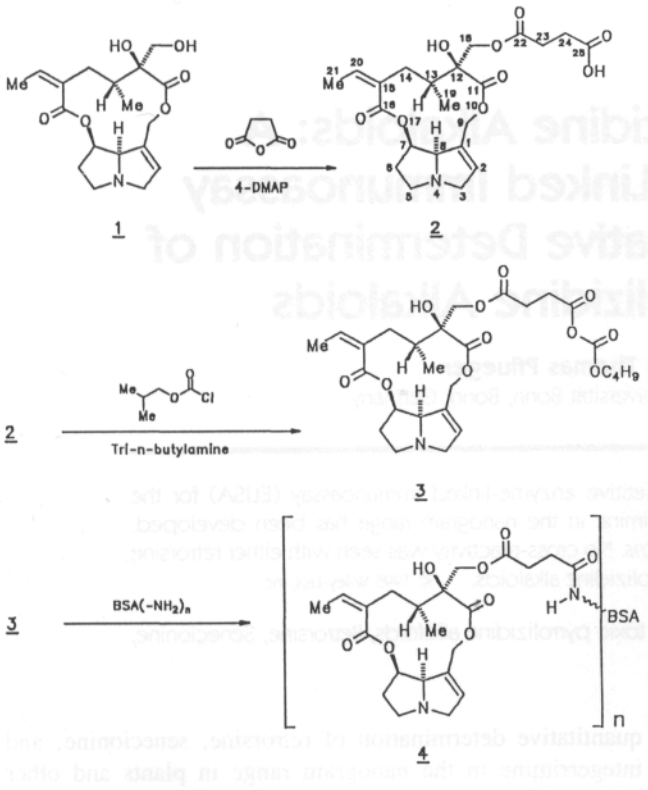

Fig. 1. Synthesis of retrorsine hemisuccinate-BSA-conjugate (DMAP = dimethylaminopyridine; BSA = bovine serum albumin).

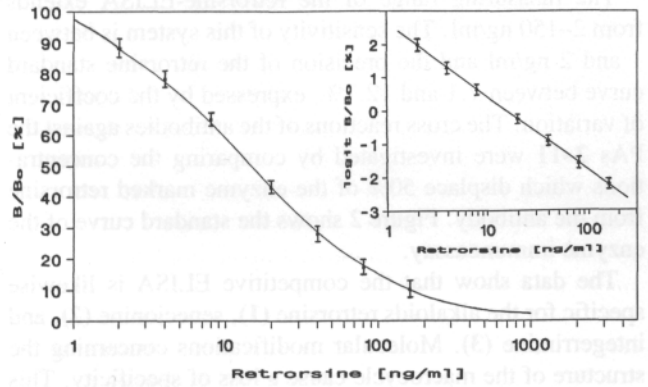

Fig. 2. Standard curve enzyme immunoassay obtained by showing standard deviation and linearization after logit transformation (inset).

Monocrotaline **(10)**, an alkaloid of the 11-membered macrocyclic alkaloids, crosemperine **(11)**, a 11-membered macrocyclic alkaloid of the otonecan-type, and doronenine **(9)**, an alkaloid of the 13-membered macrocyclic alkaloids give no reaction. A mixture of alkaloids from *Symphytum officinale*, comfrey, was also tested, but no cross-reaction takes place. All standard curves of the tested PAs are shown in the log/lin representation in Figure 3.

Analysis of Biological Samples

A linear dose-response relationship was obtained when aliquotes of dilute, methanolic extracts from dry leaves of *Senecio rupestris* were analyzed either by ELISA or by colour spectrophotometric determination (Table I) [Mattocks, 1967; Roeder et al., 1992]. *Senecio rupestris* contains senecionine **(2)** and integerrimine **(3)** in different amounts (Roeder and Wiedenfeld, 1983; Unpublished results).

EXPERIMENTAL METHODS

Chemicals

Bovine serum albumin (BSA) was obtained from Serva (Heidelberg, FRG), isobutylchloroformate from Sigma-Aldrich (Deisenhofen, FRG). Horseradish peroxidase (POD) and ABTS® were provided by Boehringer (Mannheim, FRG). Freund's complete and incomplete adjuvans were purchased from Difco (Detroit, MI). Nunc Maxisorp-tubes, 70 × 11 mm, Renner (Karlsruhe, FRG) were used in the assay. All other chemicals used were standard products of analytical grade. The tested alkaloids **1–11** were isolated from *Senecio vernalis, S. doronicum, S. inaequidens, S. rupestris, S. leucanthemifolius*, and *S. rodriguezii* [Roeder et al., 1979, 1980, 1981; *Emilia sonchifolia* [Cheng et al., 1986], *Crotalaria aegyptiaca* [Roeder et al., 1993], and *Werneria nubigena* [Roeder et al., 1992b]. The structures of the PAs, designated with numbers from **1–11**, are shown in Figure 4. A mixture of *Symphytum* alkaloids was isolated from *Symphytum officinale* [Roeder and Neuberger, 1988].

Preparation of Retrorsine Hemisuccinate (Fig. 1.2)

Succinic anhydride (12 mg, 0.12 mmol) and dimethylaminopyridine (7.3 mg, 0.06 mmol) were dissolved in 2 ml CH_2Cl_2. After cooling the solution, retrorsine (35.1 mg, 0.1 mmol), dissolved in 1.5 ml CH_2Cl_2, was added. The reaction mixture was then stirred for 2 days at room temperature. After evaporation of the CH_2Cl_2, the residue was recrystallized twice from acetone; yield 40.2 mg (89%). M.p. 201–203°; IR ν KBr_{max}; 3,500–3,200, 1,730, 1,590 cm^{-1}; MS $(M)^+$ 451.1829 (calc. 451.1834) $C_{22}H_{29}NO_6$.

^{1}H-NMR (300 MHz, DMSO, int. standard: TMS); δ = 6.06 (1H, m, H-2); 5.73 (1H, qd, J = 7.0,1.5 Hz, H-20); 5.28 (1H, d, J = 11.5 Hz, H-9); 4.93 (1H, tm, J = 3.5 Hz, H-7); 4.60 (1H, s, HO-12); 4.25 (1H, m, H-8); 4.19 (1H, d, J = 11.5 Hz, H-18); 4.07 (1H, dm, J = 11.5 Hz, H-9); 3.96 (1H, d, J = 11.5 Hz, H-18); 3.78 (1H, dm, J = 16.0, H-3); 3.28 (1H, ddd, J = 16.0, 6.0, 1.5 Hz, H-3); 3.11 (1H, t, J = 8.0 Hz, H-5); 2.45 (4H, t, J = 3.0 Hz, H-23,24); 2.42 (1H, m, H-5); 2.19 (2H, m, H-6); 2.09 (1H, m, H-14); 1.74 (3H, dd, J = 7.0, 1.5 Hz, H-21); 1.71 (1H, m, H-14); 1.67 (1H, m, H-13); 0.78 (3H, d, J = 6.5 Hz, H-19).

^{13}C-NMR (75 MHz, DMSO, int. standard: TMS); δ = 173.0, C-11; 172.7, C-25; 171.2, C-22; 166.5, C-16; 134.9, C-2; 133.1, C-20; 132.5, C-1; 131.8, C-15; 78.2, C-12; 76.7, C-8; 74.6, C-7; 67.6, C-18; 62.2, C-3; 59.3,

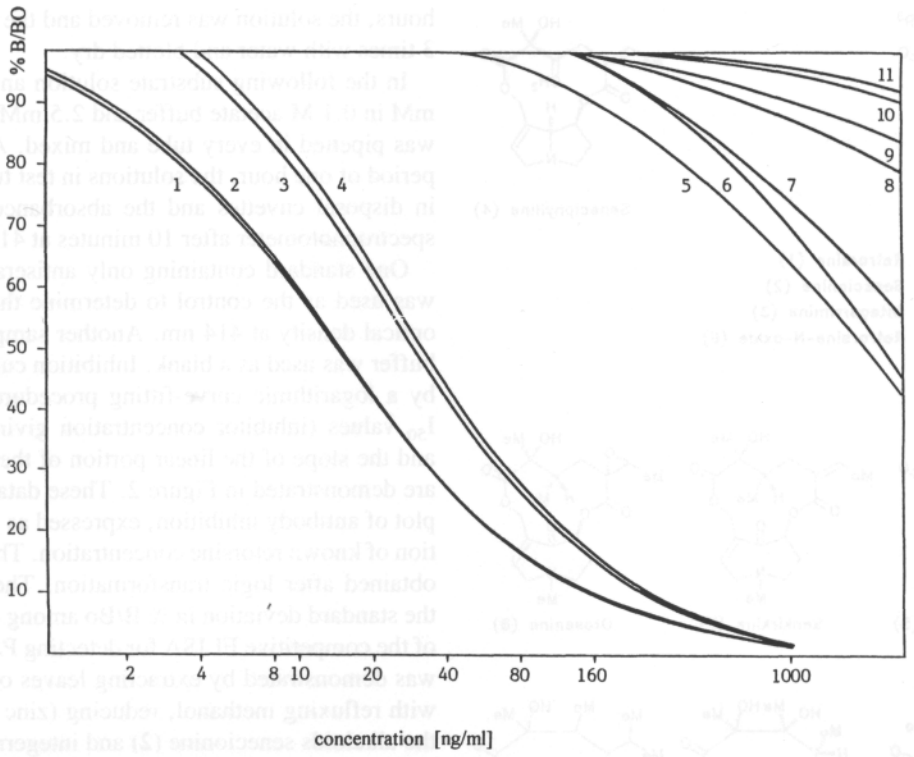

Fig. 3. Standard curves obtained by enzyme immunoassay for the tested pyrrolizidine alkaloids 1–11.

TABLE I. Quantitative Determination of the Complete Amounts of the Alkaloids From *Senecio rupestris

Sample no.	ELISA (%)	Colour reaction (5)
1	0.17 ± 0.05	0.18 ± 0.01
2	0.14 ± 0.03	0.15 ± 0.01
3	0.21 ± 0.07	0.22 ± 0.02

*The two methods showed nearly identical results.

C-9; 52.3, C-5; 37.4, C-14; 35.6, C-13; 34.2, C-6; 28.8, C-24; 28.6, C-23; 14.6, C-21; 11.1, C-19.

Preparation of Retrorsine Hemisuccinate-BSA-Conjugate (Fig. 1.4)

Solution A: BSA (45 mg, 6.7×10^{-4} mmol) was dissolved in H_2O (3 ml). The pH was adjusted to 9 by addition of 1 N NaOH. Dioxan (1 ml) was added and the solution was stirred at 10–12°C.

Solution B: To a suspension of retrorsine hemisuccinate (31 mg, 6.85×10^{-2} mmol) in anhydrous dioxan (1 ml) was added under stirring at 10–20°C, tri-n-butylamine (0.2 ml) and isobutylchloroformate (20 µl).

Solution B was slowly and dropwise added to solution A. The mixture was stirred at 4°C overnight and intensively dialyzed against deionized H_2O (2,000 ml) at 4°C overnight to remove any uncoupled hapten. The dialysate was lyophilized and stored at −20°C until required for immunochemical studies. The retrorsine hemisuccinate to BSA ratio was determined by UV-spectroscopy and found to be about 20:1 (mol/mol).

Preparation of Retrorsine-POD-Conjugate

Solution A: POD (22 mg), was dissolved in H_2O (2 ml) and cooled to 10–12°C.

Solution B: Retrorsine hemisuccinate (4.51 mg, 0.01 mmol), was suspended in dry dioxan (0.3 ml). After cooling to 10–12°C, tri-n-butylamine (0.1 m) and isobutylchloroformate (5 µl) was added.

After 30 min, solution B was added in one portion to the well-stirred solution A. Stirring and cooling was continued for a total of 4 hours. This mixture was chromatographed on a Sephadex G-25 column (internal diameter = 10 mm, vol. = 10 ml) using H_2O as eluent. The first fraction (5 ml) was lyophilized and stored until required for immunochemical studies at −20°C.

Immunization Procedure and Purification of Antibody (AB)

The retrorsine hemisuccinate-BSA-conjugate (3 mg) was dissolved in 0.9% NaCl solution (1.5 ml) and emulsified with an equal volume of Freund's complete adjuvant. Three approximately 6 month-old New Zealand female rabbits were injected subcutaneously for four weeks with the above mentioned solution. After this, 1.5 mg of the immunogen was emulsified in Freund's incomplete adjuvant and injected at

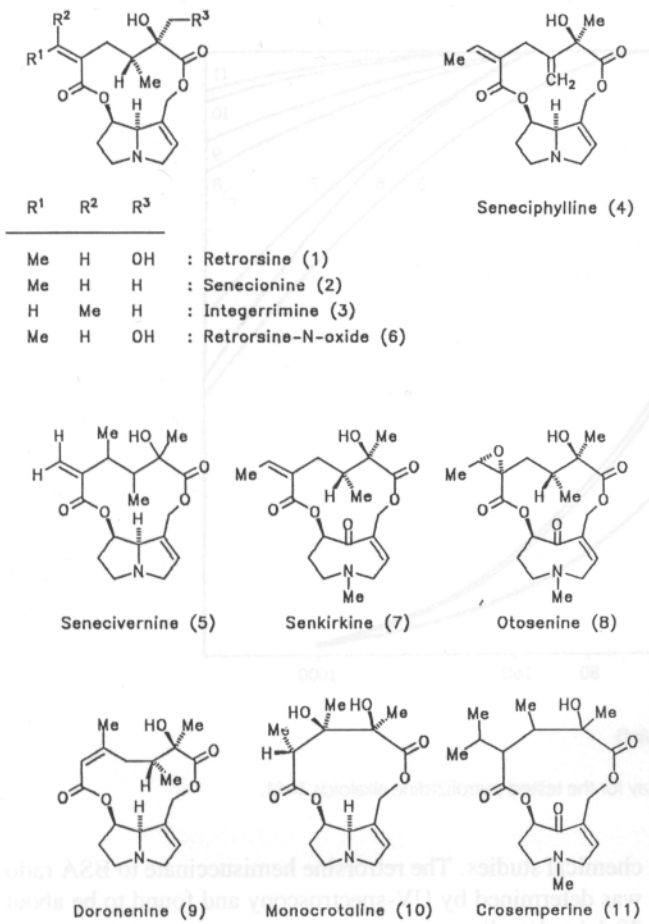

Fig. 4. Structure of the tested pyrrolizidine alkaloids.

monthly intervals. After 6 months, the blood samples were collected and allowed to clot overnight. Ammonium sulfate was used for the precipitation of gamma globulin (Ig) by a salting-out procedure. After a gel filtration and lyophilization the Ig fraction (AB) was stored until required for immunochemical studies at −20°C.

Competitive ELISA With Retrorsine Standards

For standard curves, a primary standard of 10.00 mg/ml of retrosine was used to make a series of diluted standards in phosphate buffer (0.1 M; pH 7.4) with concentrations ranging from 2 ng/ml to 150 ng/ml.

The AB (4.0 mg) was diluted in carbonate buffer solution (pH 9.6) and was immobilized by passive adsorption upon the inside of polystyrene test tubes. The tubes were then incubated with a solution of 1% caseine in order to prevent nonspecific adsorption of the tracer. After washing and carefully drying, the tubes were ready for use. To each tube, 1 ml of incubation solution (retrorsine hemisuccinate-POD-conjugate, 250 μg/l in 0.1 M phosphate buffer; pH 7.4) followed by alkaloid standard solution or sample dilution (0.1 ml) were pipetted. After an incubation period of 4 hours, the solution was removed and the tubes were washed 3 times with water and blotted dry.

In the following substrate solution and ABTS® (1 ml, 2 mM in 0.1 M acetate buffer and 2.5 mM sodium perborate) was pipetted in every tube and mixed. After an incubation period of one hour, the solutions in test tubes were decanted in disposal cuvettes and the absorbance measured with a spectrophotometer after 10 minutes at 414 nm.

One standard containing only antisera and buffer (1:10) was used as the control to determine the maximum of the optical density at 414 nm. Another sample containing only buffer was used as a blank. Inhibition curves were analyzed by a logarithmic curve-fitting procedure which calculated I_{50} values (inhibitor concentration giving 50% inhibition) and the slope of the linear portion of the curve. The results are demonstrated in Figure 2. These data are converted to a plot of antibody inhibition, expressed as % B/Bo, as a function of known retorsine concentration. The linearization was obtained after logic transformation. The error bars reflect the standard deviation in % B/Bo among eight runs. The use of the competitive ELISA for detecting PAs in plant samples was demonstrated by extracting leaves of *Senecio rupestris* with refluxing methanol, reducing (zinc dust) and assaying the alkaloids senecionine **(2)** and integerrimine **(3)**.

It is important to know, that the PAs readily undergo oxidation to the corresponding N-oxides. Extreme care has to be taken to exclude oxygen from the PA samples (compare standard curve of retrorsine N-oxide).

Cross-reactivity studies with some 11-, 12-, 13-membered PAs and 11-, 12-membered otonecine PAs have shown, that ELISA is very sensible for retrorsine **(1)** and senecionine **(2)**, less sensible for integerrimine **(3)** and even less than seneciphylline **(4)**. There was no response to senecivernine **(5)**, retrorsine N-oxide **(6)**, and the alkaloids **(7–11)**. No reaction took place with the *Symphytum* alkaloids that are retronecine mono- and diesters of monocarboxylic acids [Roeder and Neuberger, 1988].

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