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Determination of Dihydroergosine in Sorghum Ergot Using an Immunoassay

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Dihydroergosine (DHES) is the principal toxic alkaloid produced by sorghum ergot (*Claviceps africana*). It has recently been shown that DHES levels as low as 1 mg/kg in animal feed can cause significant production losses. Quantitative immunoassays for detecting the related rye ergot alkaloid, ergotamine, are described in the literature, but those assays are relatively insensitive for DHES. This paper describes competitive enzyme-linked immunosorbent assays (ELISA) for measuring the DHES concentration in grains and mixed animal feed. The assays were developed using a DHES specific mouse monoclonal antibody and rabbit polyclonal antibodies raised against DHES conjugated to bovine serum albumin. Recoveries of between 77 and 103% were obtained from spiked grain using a simple, one step extraction with 70% methanol. Both the monoclonal and the polyclonal assays are capable of detecting DHES concentrations above 0.01 mg/kg, but quantification is most reliable at concentrations of 0.1 mg/kg or higher.

KEYWORDS: Dihydroergosine; alkaloid; sorghum ergot; *Claviceps africana*; immunoassay; ELISA; mycotoxin

INTRODUCTION

Sorghum ergot (*Claviceps africana)* is widespread in Africa and Asia and has recently been introduced into Australia (*1*). The fungus infects the ovary of flowering plants and eventually produces hard sclerotia (ergots), which contain toxic alkaloids. The major alkaloid produced by sorghum ergot is the ergo peptide, dihydroergosine (DHES), which usually represents at least 80% of the total alkaloids present (*2*). Minor alkaloids include festuclavine and dihydroelymoclavine, which lack a peptide moiety. These three alkaloids are saturated at the 9,-10-position, in which respect they differ from ergotamine and the various other ergo peptides produced by rye ergot (*Claviceps purpurea*) (*3*).

Until recently, sorghum ergot had not been associated with disease in livestock, but cases of poisoning in pigs and dairy cattle occurred in 1997 in Queensland (*4*). Subsequent research has shown marked depression of milk production in sows fed diets containing 3−6 mg DHES/kg for only a few days prior to farrowing and reduced growth rates in beef cattle fed diets containing as little as 1 mg DHES/kg (*5*). These effects were associated with a profound depression in plasma prolactin.

Quantitative high-performance liquid chromatography (HPLC) and semiquantitative thin-layer chromatography (TLC) methods have been developed for detecting DHES in extracts from affected grain (*6*). TLC is simple and has a high throughput but is relatively insensitive. HPLC is sensitive and specific but requires expensive equipment and trained staff and is therefore most suited to reference laboratories. HPLC methods also typically involve lengthy extraction and cleanup procedures, thus limiting throughput. As compared to HPLC, immunoassays usually have lower precision but greater sensitivity and much higher throughput and are consequently cheaper and more practical for screening large numbers of samples. Immunoassays have been developed for rye ergot alkaloids and shown to be very sensitive (*7*, *8*), but the assays are relatively insensitive to DHES (Shelby, R. A. Personal communication).

This paper describes the development and validation of immunoassays for DHES using polyclonal antibodies raised in rabbits and a mouse monoclonal antibody. The assays employ a simple one step extraction with 70% methanol and are reliable for quantifying DHES concentrations of 0.1 mg/kg or higher in grain or mixed feed.

MATERIALS AND METHODS

Alkaloid Standards. Alkaloids were purchased or acquired from other laboratories, generally as methanesulfonate derivatives. They produced single peaks or spots in HPLC and TLC, respectively, and

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were assumed to be pure. In quantitative calculations, allowance was made for the mass of the methanesulfonate group, so that results are quoted in terms of the concentration of the base alkaloids.

Alkaloid standards ranging from 0.1 to 10 000 ng/mL were prepared in 70% methanol. DHES standards were also prepared by spiking the 70% methanol extracts of ergot-free sorghum grain or a typical mixed feed formulated with ergot-free sorghum.

Conjugation of Hapten. DHES was conjugated to bovine serum albumin (BSA) and ovalbumin (OVA) using a Mannich reaction as described previously (*9*). Protein concentrations of the BSA−DHES and OVA−DHES conjugate preparations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA) based on Coomassie brilliant blue G250, according to the manufacturer's instructions.

Animal Inoculation and Antibody Production. Three rabbits each received three subcutaneous injections containing approximately 500 μg of BSA−DHES conjugate dialyzed into sterile phosphate-buffered saline (PBS), at approximately 2 week intervals. The first injection only included 100 μg of saponin as adjuvant. After the third injection, blood was collected from the ear vein and tested for antibodies to DHES in an enzyme-linked immunosorbent assay (ELISA) using the OVA−DHES conjugate as antigen. All rabbits were then exsanguinated by heart puncture under general anaesthetic and euthanized by lethal injection before recovery.

Mice received three intraperitoneal injections each containing approximately 125 μg of the BSA−DHES conjugate, at approximately 2 week intervals. The first injection only included 10 μg of saponin as adjuvant. After the third injection, blood was collected from the tail and tested for antibodies to DHES as described above. The mice were left for at least 1 month and then given a final intraperitoneal booster injection with OVA−DHES (125 μg) 3 days before harvesting the spleen for fusion. Monoclonal antibodies were produced by conventional hybridoma technology (*10*). Hybridomas were screened for DHES specific antibody production in an ELISA using OVA−DHES as test antigen and OVA as control antigen. For use in ELISAs, monoclonal antibodies were affinity-purified from cell culture supernatant using prepacked Protein G Sepharose columns (Pharmacia Biotech, Upsala, Sweden) according to the manufacturer's instructions.

ELISA. Grain and feed samples were hammer-milled to pass a 1 mm screen. A 4 g sample was extracted in 40 mL of 70% methanol for 30 min in a sealed plastic tube with constant agitation and then allowed to settle briefly before sampling the supernatant for ELISA.

Competitive ELISAs were developed using both the rabbit polyclonal antibodies and the mouse monoclonal antibody. For ELISAs using the monoclonal antibody, 96 well microtiter plates (MaxiSorp, Nunc, Naperville, IL.) were coated with 100 μL/well of OVA−DHES diluted to a concentration of 40 μg/mL in 0.1 M sodium carbonate buffer (pH 9.6). Blank wells were included on each plate and were coated with a similar amount of OVA in 0.1 M sodium carbonate buffer (pH 9.6). After an overnight incubation at 4 °C, excess antigen was removed and 200 μL of a 2% solution of low fat skim milk powder (SMP) in PBS containing 0.1% Tween 20 (PBST) was added to each well. The plate was then incubated for 1 h at room temperature after which the wells were washed with PBST (5 × 200 μL rinses followed by 1 × 200 μL soak for 5 min with agitation). Next, 25 μL of 2% SMP in PBST was added to each well, followed by 25 μL of either undiluted sample extract, DHES standard (0, 0.1, 1, 10, 100, 1000, or 10 000 ng/mL in 70% methanol) or 70% methanol (blank wells), and 50 μL of monoclonal antibody diluted 1/4000 in 2% SMP in PBST. The plate was then incubated for 30 min at room temperature with gentle agitation. Wells were then washed as described previously: 100 μL of peroxidase-labeled goat anti-mouse IgG conjugate (KPL, Gaithersburg, MD) diluted 1/10 000 in 2% SMP in PBST was added to each well, and the plate again was incubated for 30 min at room temperature with gentle agitation. Wells were again washed as described previously, and 100 μL of peroxidase substrate 3′,3′,5′,5′-tetramethylbenzidine (TMB) (KPL) was added to each well. Color was allowed to develop until the absorbance of the wells containing the 0 ng/mL DHES standard was between one and two absorbance units, at which time the reaction was stopped by addition of 50 μL of 2 M phosphoric acid. Absorbance was read at 450 nm. Sample extracts were tested in duplicate. On each plate, the 10, 100, and 1000 ng/mL DHES standards were included in quadruplicate and the remaining standards were included in duplicate. DHES concentrations were quantified using SOFTmax Pro software (Molecular Devices Corporation, Menlo Park, CA) with standards fitted to a four parameter curve and expressed as mg/kg. Sample extracts with concentrations of DHES in excess of 200 ng/mL were diluted with 70% methanol so that they fell within the optimum range for quantification in the ELISA (10−100 ng/mL).

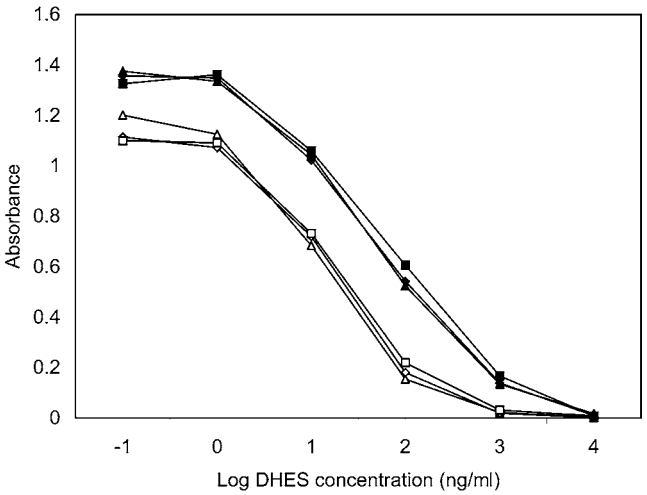

Figure 1. Sensitivity of ELISAs using polyclonal rabbit antibodies (filled symbols) and mouse monoclonal antibody (open symbols) for detecting varying concentrations of DHES standard diluted in 70% methanol (△), 70% methanol extract of ergot-free sorghum (◇), and 70% methanol extract of an ergot-free sorghum-based mixed feed (□).

ELISAs using polyclonal antibodies were conducted in the same manner except that the rabbit antiserum was used at a dilution of 1/3000 and peroxidase-labeled goat anti-rabbit IgG conjugate (KPL) was diluted 1/5000.

HPLC. Grain samples were analyzed for ergot alkaloids in duplicate or triplicate using HPLC (*6*). Recoveries for the HPLC method are quoted at about 75% (*6*), but for purpose of the comparison between HPLC and ELISA shown here, uncorrected assay figures were used.

RESULTS

Sensitivity. Sensitivity was estimated by assaying DHES standards prepared in 70% methanol and in the 70% methanol extracts of ergot-free sorghum and mixed feed (**Figure 1**). The assays using the monoclonal and polyclonal antibodies were both capable of detecting DHES concentrations above 1 ng/mL and could reliably be used to quantify concentrations above 10 ng/mL. Accounting for a 10-fold dilution during the extraction process, these figures equate to 0.01 and 0.1 mg/kg in a grain or mixed feed sample. The assays were unaffected by pigments and other compounds present in 70% methanol extracts from ergot-free grain or mixed feed.

Specificity. The monoclonal and polyclonal antibodies were tested for cross-reactivity with the minor sorghum ergot alkaloids, festuclavine and dihyroelymoclavine, the rye ergot alkaloid ergotamine, and synthetic dihydroergotamine (**Figure 2**). Neither showed any evidence of cross-reaction with festuclavine or dihyroelymoclavine. The polyclonal antibodies cross-reacted with both ergotamine and dihydroergotamine, but the sensitivity of the assay for these alkaloids was at least 10-fold lower than for DHES. The monoclonal antibody showed some evidence of cross-reaction with ergotamine and dihydroergotamine but only at concentrations of 10 000 ng/mL or higher.

Recovery of DHES from Spiked Grain. DHES in 70% methanol was added to milled ergot-free sorghum grain at levels of 0.1, 1, and 10 mg/kg and allowed to absorb into the milled

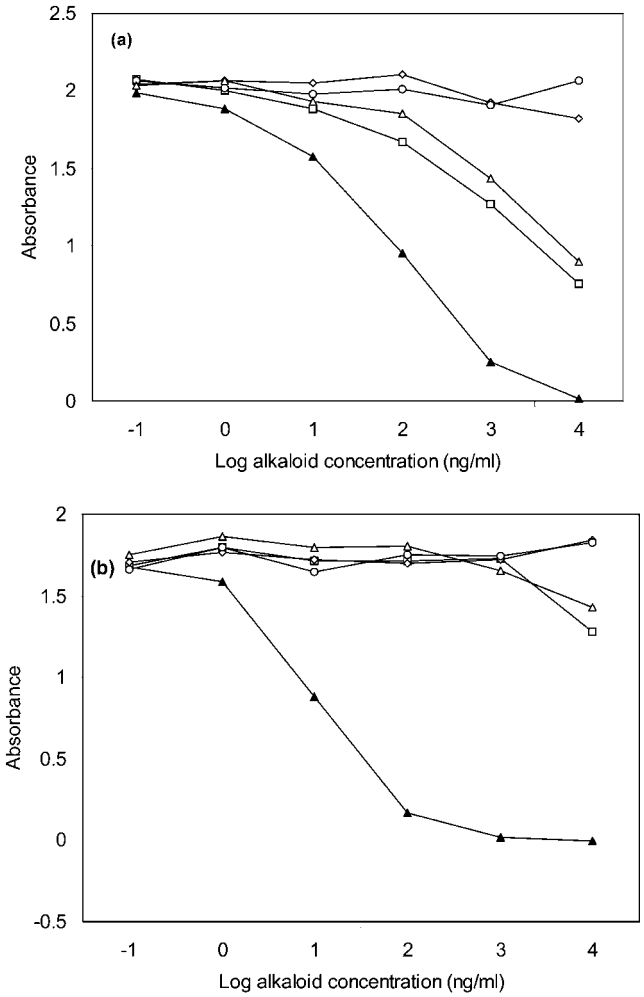

Figure 2. Specificity of ELISAs using (a) polyclonal rabbit antibodies and (b) mouse monoclonal antibody. Graphs show inhibition of antibodies by varying concentrations of DHES (▲), dihydroelymoclavine (◇), festuclavine (○), dihydroergotamine (□), and ergotamine (△).

grain for at least 2 h. The spiked grain was then extracted and assayed in quadruplicate using the monoclonal antibody ELISA. Percentage recoveries were 77 (±3.5), 103 (±1.3), and 88 (±3.3)%, respectively.

Extraction Efficiency. Two samples of grain with moderate and high levels of ergot infestation were each extracted with 70% methanol in five replicate tubes and 200 μL aliquots of the 70% methanol extract were removed from each tube for later testing in the monoclonal antibody ELISA after 5, 15, and 30 min, 1 h, and overnight for approximately 20 h (**Figure 3**). Extraction was complete after 30−60 min, but at least 75% of the alkaloid was extracted within the first 5 min. An extraction time of 30 min was selected for routine use.

Comparison with HPLC. Forty-four samples of sorghum grain previously assayed for DHES content by the HPLC method were analyzed by the monoclonal antibody ELISA, and the results were compared (**Figure 4**). DHES concentrations determined by HPLC ranged between 0 and 246 mg/kg. Overall ELISA and HPLC results were comparable although the ELISA did tend to give slightly higher results for most samples. However, that difference would be entirely accounted for if the ELISA results were corrected for a recovery of 90% and the HPLC results for a recovery of 75% (*6*).

Repeatability. Estimates of ELISA repeatability were based on results obtained for a grain sample containing approximately 1 mg/kg DHES that was included on each plate. The coefficient of variation over eight plates run over 5 days was 15.5% (mean = 1.27 mg/kg, SD = 0.20).

DISCUSSION

Detrimental effects of sorghum ergot on livestock have been reported at DHES concentrations as low as 1 mg/kg in feed (*4*, *5*). A sensitive test is therefore required for screening grain and mixed feed. The ELISAs that we have developed are capable of detecting DHES at concentrations above 0.01 mg/kg and are reliable for quantifying concentrations of 0.1 ng/mL or higher. The monoclonal antibody assay is specific for DHES with measurable cross-reactivity with the most closely related naturally occurring alkaloid (ergotamine) and synthetic dihydroergotamine occurring only at concentrations above 1000 mg/kg. In contrast, when rabbit polyclonal antibodies were used, the assay detected the rye ergot alkaloid, ergotamine, at

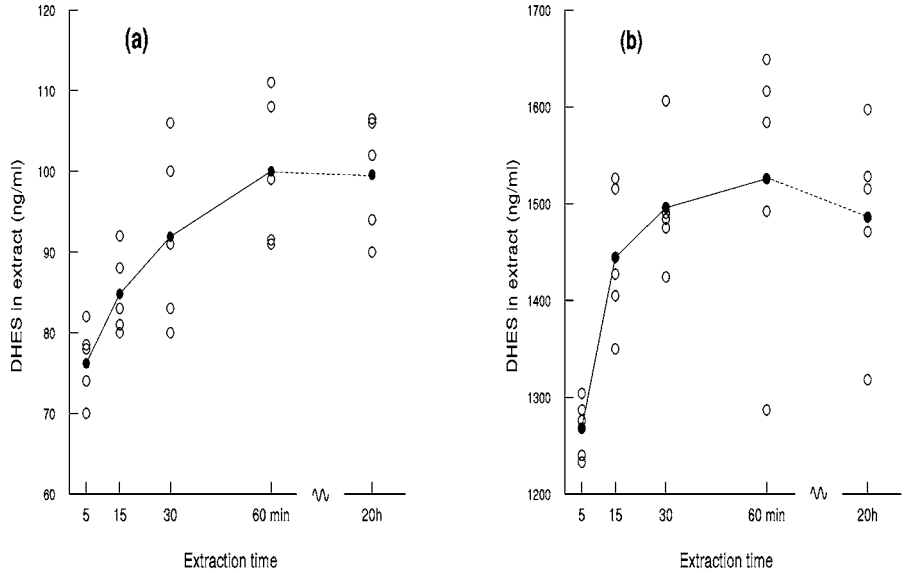

Figure 3. Efficiency of extraction of DHES with 70% methanol from sorghum grains with (a) moderate and (b) high levels of ergot infestation. Grains were extracted in five replicate tubes, and the extraction supernatant was sampled after 5, 15, 30, and 60 min and 20 h. Data points for individual tubes are shown as open circles (○), and the averages for each set of five tubes are shown as filled circles (●).

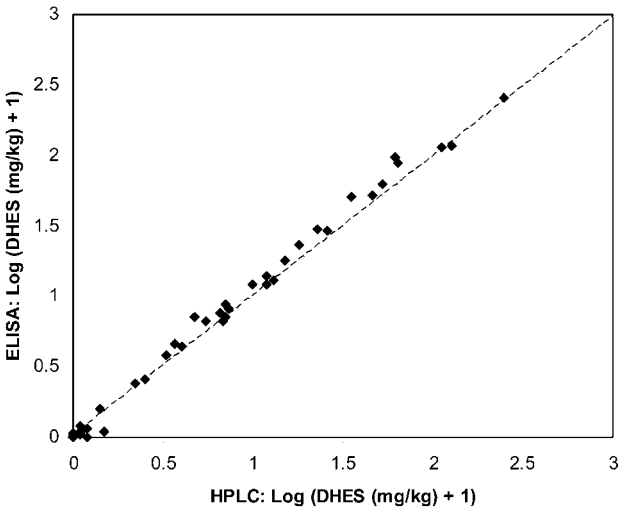

Figure 4. Comparison of DHES concentrations in 44 samples of sorghum grain with varying levels of ergot infestation as measured by HPLC and ELISA. Log_{10} (DHES concentration + 1) was plotted to better represent values over the full range assayed (one was added to each value to avoid complications arising from zero values). The diagonal line represents perfect agreement between the two assay methods.

concentrations of 1 mg/kg or higher and could therefore be useful for screening for ergot infestation in both sorghum and rye. The specificity of the assays suggests that the monoclonal antibody and the majority of the polyclonal antibodies recognize an epitope on the unique peptide domain of the molecule.

Extraction of DHES with 70% methanol appears to be remarkably efficient with the majority of alkaloid being extracted within 5 min. The 9,10-dihydro alkaloids are known to be more stable than their unsaturated equivalents, and we have found DHES standards to be stable in methanol solution for at least 6 months at −20 °C (*6*). We have also found no evidence that isomerization of DHES in methanol solution affected epitope recognition by either our monoclonal or polyclonal antibodies. The repeatability of the ELISA between plates, measured as the coefficient of variation, was 15.5%. Variability of that order is almost inevitable when using a log scale for quantification and is similar to that recently reported for a competitive ELISA for ergot alkaloids in tall fescue (*11*). Recovery of DHES from spiked grain was about 90%, as far as can be estimated given the precision of the assay. We recognize however that this may not reflect the true recovery from naturally contaminated grain and we have therefore relied mainly on comparison with the established HPLC method to validate our results. Once allowance was made for the different recoveries of the two methods (75% for HPLC and 90% for ELISA), there was excellent agreement between the two methods over a wide range of DHES concentrations (0.01−250 mg/kg).

The ELISAs reported here are suitable for screening sorghum grain and mixed feeds for the presence of DHES. The specificity of the monoclonal antibody should make it the reagent of choice when testing for DHES alone, but substitution with the polyclonal antibodies would be advantageous when screening for a range of ergot alkaloids, including ergotamine. The assays are more robust and more sensitive than HPLC and require only a rapid one step extraction. The precision of ELISA is inferior to HPLC, but recoveries are apparently higher. For regulatory work, ELISA and HPLC methods should complement one another.

ACKNOWLEDGMENT

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