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Note

High-performance liquid chromatography of the mycotoxin, sporidesmin, from *Pithomyces chartarum* (Berk. & Curt.) M.B. Ellis

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Sporidesmin, a mycotoxin produced by the fungus *Pithomyces chartarum* (Berk. & Curt.) M. B. Ellis, has been implicated as the principal toxic agent in the mycotoxicosis of sheep and cattle, commonly called facial eczema[1–3]. This disease is of major economic importance to the sheep and cattle industry in New Zealand, and a similar syndrome in sheep has been observed in the State of Texas (U.S.A.) where Ueno *et al.*[4] reported the isolation of *P. chartarum* from pastures growing in the Gulf Coast Plain of Texas but did not detect sporidesmin in cultures isolated from these pastures.

Methods that have been published for the detection and quantitation of sporidesmin include: thin-layer chromatography (TLC)[3–5], tissue culture toxicity tests[5,6], iodometric estimation[6], corneal opacity tests[6] and biological assays using guinea pigs[5,7] or rabbits[8]. In this study, a rapid, reliable, and sensitive method has been developed for the extraction and quantitation of sporidesmin from spores of *P. chartarum* using high-performance liquid chromatography (HPLC).

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of a Waters Assoc. Model M6000-A pump, U6K injector and 440 absorbance detector with a fixed wavelength of 254 nm and a Varian Model A-25 strip chart recorder. The chromatograph contained a Waters Assoc. 30 cm × 3.9 mm I.D. stainless-steel column packed with reversed-phase μBondapak C_{18} (particle size, 10 μm). The operating conditions were as follows:

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mobile phase, methanol–water (47:53); flow-rate, 2.0 ml/min; pressure, 2000 p.s.i.; ambient temperature; sensitivity, 0.01 a.u.f.s.; recorder chart speed, 5.0 in./h.

Before use, the carrier solvents were degassed for 15 min in a Bransonic 220 sonicator (Branson Instrument, Conn., U.S.A.). Extracts were evaporated using a Rotavapor-R rotary vacuum evaporator (Büchi, Switzerland) and a Temp-Blok module heater (Lab-Line Instruments, Ill., U.S.A.) under a stream of nitrogen. Samples were filtered using Sample Clarification Kits (Millipore, Bedford, Mass., U.S.A.) consisting of a 10-ml syringe, a Swinney filter holder and Millipore filters (0.5 μm).

Chemicals and reagents

A sample of sporidesmin A (benzene solvate) was donated by Dr. P. H. Mortimer, Ruakura Animal Research Station, Hamilton, New Zealand. Pure cultures of *P. chartarum*, strain C were donated by Dr. M. E. diMenna, Ruakura Soil Research Station, Hamilton, New Zealand. Scintillation-grade naphthalene (New England Nuclear, Boston, Mass., U.S.A.) was recrystallized by sublimation. Water was glass-distilled and stored in glass containers. UV-grade acetonitrile was obtained from Burdick & Jackson Labs. (Muskegon, Mich., U.S.A.). All other solvents, both HPLC-grade and ACS-grade, were obtained from Fisher Scientific (Fair Lawn, N.J., U.S.A.).

Preparation of standards

Standard solutions of sporidesmin (49 ng/μl) were prepared in UV-grade acetonitrile and the volumetric flasks were wrapped in foil to prevent degradation by light. Standard solutions of naphthalene, the internal standard, were prepared in HPLC-grade methanol (19.8 ng/μl) and the volumetric flasks were also wrapped with foil. The standard solutions were stored at room temperature, and both solutions were found to be stable for at least six months.

Culturing techniques

The culturing techniques described by DiMenna *et al.*[9] were followed and the organism was grown both on kleingrass straw (*Panicum coloratum* L.) and ryecorn (*Secale cereale* L.) as the media.

Kleingrass straw (*ca.* 10 g/flask) was added to 500-ml Erlenmeyer flasks, filled with tap water and left to soak overnight. The excess water was decanted and the flasks were autoclaved at 15 lb. (121°) for 15 min. After inoculation, the flasks were irradiated with UV light for 10 h/day between days two through eight following inoculation and then incubated in the dark. In addition, cultures can be grown in total darkness with comparable results. Both ryecorn and kleingrass cultures were incubated for a total of four weeks at ambient temperature (25–32°).

Harvesting procedure

For both types of cultures, the fungal material was wet harvested by adding distilled water to the flasks, shaking vigorously, and carefully decanting into a Buchner funnel lined with sail-cloth or coarse filter paper using suction. The fungal material was removed from the filter material and stored in foil in the freezer.

Extraction procedure

A 10-ml volume of acetonitrile (UV-grade) was added to *ca.* 0.1 g of fungal material in a 125-ml glass-stoppered Erlenmeyer flask wrapped in foil and manually shaken to disperse the spore material. A 40-ml volume of ACS-grade benzene was added to the flask and the flask contents were stirred on a magnetic stirrer for 12–17 h. The contents were then filtered through a 12.5-cm Whatman No. 52 filter paper into a 100-ml round-bottomed flask and the filtrate was evaporated to dryness on a rotary vacuum evaporator (30°). A 2-ml volume of UV-grade methanol–water (40:60) was pipetted into the evaporating flask and manually swirled for 5 min. The aqueous phase was quantitatively removed with a Pasteur pipette and transferred into a 10-ml Lur-Lok® syringe containing a Swinney filter holder and a 0.5-μm Millipore filter through which the aqueous phase was filtered into a 25-ml glass-stoppered test tube. This procedure was repeated 3 times with 2 ml of methanol–water (40:60). The aqueous phase was extracted 3 times in the test tube for 10 min each with 4 ml of ACS-grade hexanes on a mechanical shaker, and the hexane phase discarded. The aqueous phase was then extracted 3 times with 2 ml of ACS-grade benzene for 10 min each in the same test tube. The benzene phase was quantitatively collected in a tapered test tube and evaporated to dryness under a stream of nitrogen on a module block heater (40°).

Following evaporation, the residue was dissolved in 200 μl of UV-grade acetonitrile and 5–20-μl aliquot samples were transferred to separate test tubes and evaporated to dryness. The residues were dissolved in 40 μl of the standard naphthalene solution and 30 μl of the mixture were injected into the instrument with a 50-μl syringe.

RESULTS AND DISCUSSION

Chromatographic conditions

Sporidesmin exhibits a strong absorption band in the 245–260-nm region[10]. Therefore, the UV detector fixed at 254 nm is selectively sensitive to small quantities of sporidesmin. At a sensitivity of 0.005 a.u.f.s., 5.0 ng of pure sporidesmin produced peaks which were approximately four times greater in peak height than the background noise. With a 30 cm $\times$ 3.9 mm I.D. column and a solvent velocity of 2.0 ml/min, the retention time was 307 $\pm$ 18 sec for sporidesmin and 598 $\pm$ 43 sec for naphthalene based on 52 consecutive measurements. The retention time was extremely sensitive to the carrier solvent composition, *i.e.*, methanol–water (47:53), and differences in retention time between batches existed which were indicative of slight compositional changes in carrier solvent prepared daily.

Extracts of fungal material from both the ryecorn and kleingrass media give relatively clean chromatograms free of interfering peaks, but the yield of sporidesmin was considerably greater from fungal material grown on ryecorn than from that grown on kleingrass. Chromatograms are presented in Fig. 1 which represent the analysis of A, a spiked fungal sample; B, a toxic fungal sample; and C, a non-toxic fungal sample.

Extraction

During the study, it was observed that sporidesmin was rapidly degraded in the presence of ACS-grade methanol and that the rate of degradation was enhanced

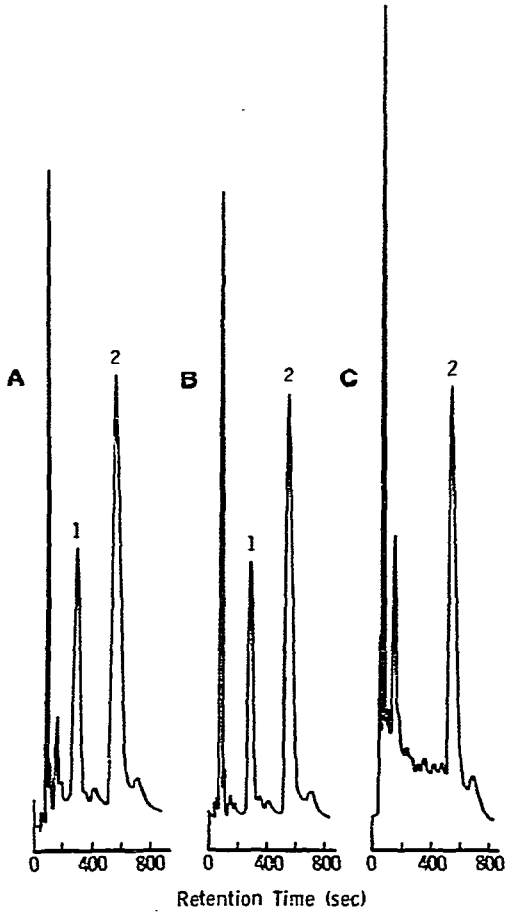

Fig. 1. Representative HPLC chromatograms showing A, a spiked fungal sample; B, a toxic fungal sample; and C, a non-toxic fungal sample. Peaks: 1 = sporidesmin, 2 = naphthalene.

by light and/or heat. Further testing revealed that sporidesmin was not chemically altered in the presence of HPLC-grade methanol, or distilled methanol. Therefore, the extraction procedure was developed to reduce the time of contact of sporidesmin with methanolic solutions. Furthermore, with HPLC-grade methanol, the analysis could be conducted under normal fluorescent lighting (excluding the benzene extraction process) and ambient temperature without noticeable loss of sporidesmin through degradation.

To ascertain the efficacy of benzene–acetonitrile (8:2) as a solvent mixture for extracting sporidesmin from fungal material, multiple extractions were made on individual toxic fungal samples ranging in weight from 100–200 mg. The initial extraction of each sample removed more than 97% of the sporidesmin content. Because of the high extraction yields and high molar extinction coefficients of sporidesmin at 254 nm, 50–100-mg samples are more than adequate for quantitative work. For highly toxic samples, serial dilutions must be made in order to bring the sporidesmin peak on scale.

TABLE I

RECOVERY OF SPORIDESMIN USING NON-TOXIC FUNGAL MATERIAL

Weight of fungal material (mg)	*Amount of sporidesmin added (μg)*	*Amount recovered* (μg)*	*Recovery (%)*
190	2.45	2.20	89.9
201	2.45	2.19	89.4
193	2.45	2.18	89.0
131	1.96	1.77	90.3
160	1.96	1.72	87.8
143	1.96	1.91	97.4
130	1.47	1.40	95.2
84	1.47	1.39	94.6
120	1.47	1.37	93.2
108	0.98	0.87	88.8
105	0.98	0.87	88.8
114	0.98	0.85	86.7

* Average of 2 samples.

Recovery study

Based on 12 parallel extractions on fungal material spiked with standard sporidesmin in the 0.98–2.45 μg range, the recoveries were found to be 91 ± 3%. To simplify calculations non-toxic fungal material was used in the study and the results of the recovery study are presented in Table I.

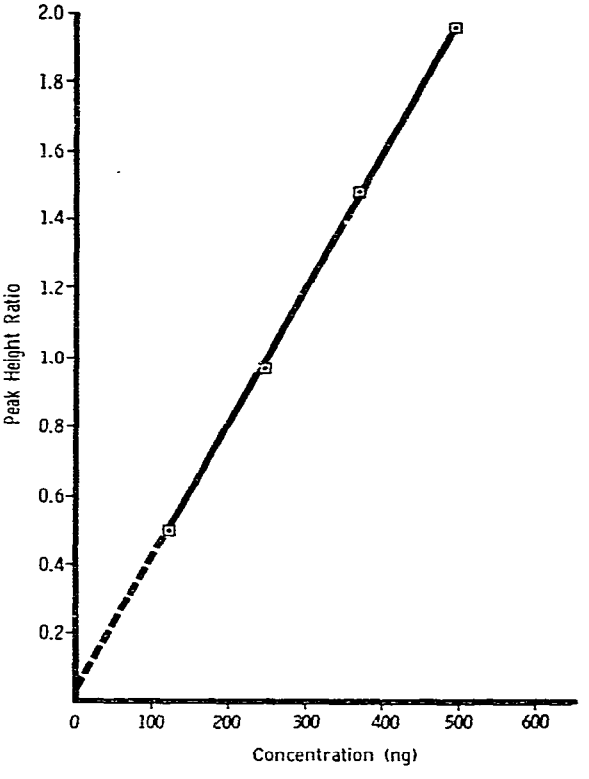

Fig. 2. A standard curve for sporidesmin with a correlation coefficient of 0.9996.

Quantitation

Unknown fungal samples were analyzed for sporidesmin content using standard curves which plot the peak height ratio (PHR) of sporidesmin over the internal standard, naphthalene, *versus* the concentration of sporidesmin as depicted in Fig. 2. Standard curves were constructed every time a new series of material was extracted using a set of standards (usually five in duplicate) covering the range from 100–500 ng. The sporidesmin content of the unknown samples was then calculated using the regression equation established from linear regression analysis of the standard curve. A plot of PHR *versus* sporidesmin concentration showed a linear relationship up to at least 500 ng, and, using linear regression analysis, a correlation coefficient ranging from 0.9949–1.0000 (av. 0.9982) was obtained.

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