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## Note

# Separation and purification of metabolites of the mycotoxin, sporidesmin, by high-performance liquid chromatography

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Sporidesmin, the fungal toxin produced by *Pithomyces chartarum* (Berk & Curt) M. B. Ellis, is the principal agent responsible for facial eczema, a disease affecting ruminants in many areas of the North Island of New Zealand. A similar syndrome in sheep and cattle has been observed in Texas (U.S.A.)[1,2]. Some animals are more susceptible to the toxic effect of sporidesmin than others, and differences in the rate of sporidesmin metabolism by the liver seems to be one factor determining resistance or susceptibility in sheep[3]. In order to study this possibility, some knowledge of the mode of metabolism of sporidesmin by the liver is required.

In this study, the ethyl acetate soluble metabolites produced from hepatic microsomal incubations, have, after preliminary fractionation on Lipidex 5000 columns, been further examined using high-performance liquid chromatography (HPLC). Some metabolites were then purified on a cyano-bonded phase (CN) column for use in structural determinations.

## EXPERIMENTAL

### *Apparatus*

The liquid chromatograph was a modular system consisting of two Spectra Physics Model 740B pumps, a Spectra Physics Model 744 solvent programmer and a Model 714 pressure monitor, a Valco 7000 p.s.i. sample injection valve fitted with either a 10-$\mu$l or 100-$\mu$l sample loop, and a Tracor 970A variable-wavelength detector with scanning option. The recorder was a Linear Instruments Model.

### *Solvents*

All solvents were analytical grade and were further purified before use. Methanol, isopropanol, ethyl acetate and dichloromethane were distilled through a 1.5-m Hempel column. Hexane was washed sequentially with concentrated sulphuric acid and water, then dried ($Na_2SO_4$) and distilled as above. Chloroform was distilled as above and stabilised with approximately 2% redistilled ethanol. Water was from a Millipore Milli-Q water purification system.

### *Chromatographic conditions*

The columns used were $\mu$Bondapak CN (30 cm × 3.9 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.) and Zorbax $C_{18}$ (25 cm × 4.6 mm I.D.) (Dupont,

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Wilmington, DE, U.S.A.). The analyses were performed at room temperature. The detection wavelength was 225 nm when using hexane–isopropanol or methanol–water as solvents, or 254 nm when the eluting solvent contained chloroform, ethyl acetate or dichloromethane. For micro-preparative separation, the fractions were simply collected from the detector exit line as indicated by the detector response.

*Lipidex fractionation of microsomal incubation*

Sporidesmin was incubated with liver microsomes (2 mg/ml) in the presence of an NADPH generating system as described previously[3]. Labelled sporidesmin ($^3H$ and $^{35}S$) was included to aid the detection of metabolites. After incubation the solution was extracted with ethyl acetate and the dried organic phase was then transferred to a Lipidex 5000 column (10 × 2 cm) and eluted with a stepwise hexane–chloroform gradient as illustrated in Fig. 1. Fractions (1 ml) of the eluate were collected and a small sub-fraction counted for both $^3H$ and $^{35}S$ activity. The resulting radioactivity pattern indicated the 1 ml fractions could be pooled into ten larger fractions containing 1–3 mg of material. These fractions were evaporated and stored at 0°C before HPLC analysis.

## RESULTS AND DISCUSSION

The Lipidex 5000 fractionation of the metabolites is shown in Fig. 1. Some fractions (D, E, F and H[1]) were shown to be mixtures by thin-layer chromatography, and these were separated by HPLC. Analysis of sporidesmin on a $C_{18}$ column with methanol–water has been reported[2], and we have routinely used similar conditions to check the purity of large scale isolations of the toxin. However micro-preparative separation of trace quantities of metabolites from a mixture has requirements different from those needed for the analytical separation of one component. After preliminary

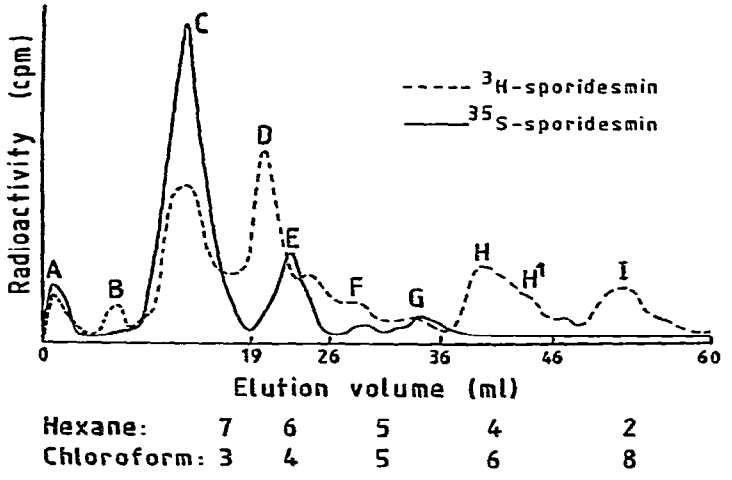


Fig. 1. Lipidex 5000 chromatography of metabolites produced by incubating sporidesmin with hepatic microsomes from sheep. The various compounds are labelled A to I, with sporidesmin corresponding to C.

investigation on both the $C_{18}$ column (with water–methanol) and the CN column (with ethyl acetate–dichloromethane, hexane–isopropanol, and hexane–chloroform–methanol), the CN column with the latter two solvent systems was chosen as most suitable for the present work. Peaks were generally broader and less resolved on the $C_{18}$ column. Added advantages of the CN column were that easily evaporated solvents could be used, and that a variety of chromatographic conditions could be obtained by simple solvent manipulation without relying on the addition of nonvolatile modifiers.

Fig. 2 shows the complexity of the four crude metabolite fractions. The advantage of combining the preliminary lipophilic gel column fractionation with HPLC is obvious. The four fractions, some well separated on Lipidex, gave components with a range of overlapping retention times on the CN column.

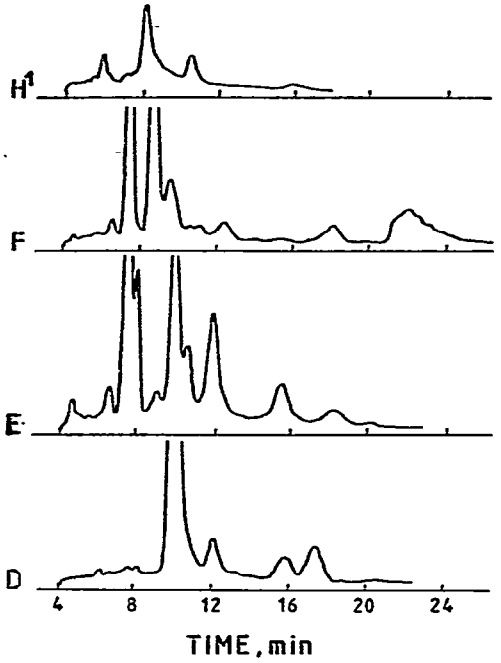


Fig. 2. HPLC of the four Lipidex 5000 fractions, D, E, F and H¹. Conditions: column, μBondapak CN: eluent, hexane–isopropanol (83:17); flow-rate, 1 ml/min; pressure, 230 p.s.i.; detector wavelength, 225 nm; detector sensitivity, 0.08 a.u.f.s.; sample conditions equivalent to the total fraction (1–3 mg) dissolved in methanol–chloroform (1:1, 10–20 ml); sample size, 10 μl.

Fraction D could be well separated analytically using either hexane–isopropanol (90:10) or hexane–chloroform–methanol (85:13.5:1.5). The former solvent system gave greater selectivity and shorter analysis time, but was unsuitable for use with larger samples since overloading occurred relatively easily. This showed up as the appearance of a leading shoulder on the major component peak (D), which developed into a double peak with larger injections (Fig. 3). A similar effect has been reported[4] in systems where the sample solvent is of much stronger eluting power than the eluant, however, in our system, the effect could not be associated directly with any one factor such as sample size, sample solvent or eluting solvent, but seemed likely to be a local concentration effect associated only with compound D. The same compound, present as a secondary component of fraction E, also gave double

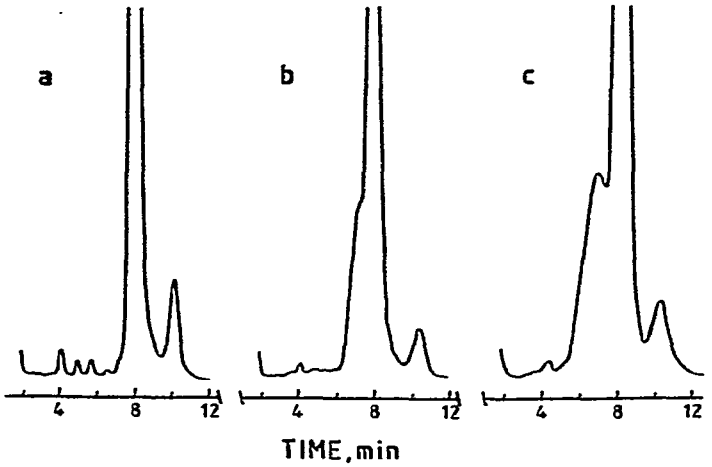


Fig. 3. Overloading effects with Lipidex fraction D. Conditions as in Fig. 2 except for eluent, hexane–isopropanol (90:10); flow rate, 2 ml/min; pressure, 480 p.s.i.; detector sensitivity, a, 0.32 a.u.f.s.; b and c, 1.28 a.u.f.s.; sample conditions, 3 mg/ml in $CHCl_3$; sample size, a, 10 $\mu$l; b, 30 $\mu$l; c, 50 $\mu$l.

peaking during purification of fraction E. The shoulder, while still present, was not so apparent in the ternary solvent system.

Table I shows the value of ternary solvent systems for HPLC when nonvolatile modifiers cannot be used. Rather than relying simply on increased retention for increased separation, selectivity can be manipulated independantly to retention. For example, comparison of the fourth and sixth lines of Table I shows greater separation of the major component was obtained with a shorter retention time. In addition the generally sharper peak shapes in the sixth eluent option meant that a well defined baseline resolution was achieved, compared to broader fused peaks in the fourth option. Similar solvent selectivity effects are now being reported for $C_8$ and $C_{18}$ reversed-phase chromatography[5]. Fraction D was purified using hexane–chloroform–methanol (85:13.5:1.5) at 1 ml/min.

The same chromatographic conditions were used for the purification of fraction E, and gave the major component at 41 min. Comparison with the separation

TABLE I

RETENTION TIMES AND SEPARATION OF THE MAJOR COMPONENT OF LIPIDEX FRACTION D ON ELUTION WITH DIFFERENT TERNARY SOLVENT MIXTURES

$\mu$Bondapak CN column with eluent flow of 1 ml/min. Detector wavelength was 254 nm at 0.16 a.u.f.s. sensitivity. Sample conditions as for Fig. 2. Pump A, hexane; pump B either 2:1, 3:1 or 9:1 chloroform–methanol.

| *Hexane–chloroform–methanol (%)* | $t_R$* *(min)* | $\Delta t$** *(min)* |
|---|---|---|
| 80:13.3:6.7 | 8.6 | 0.9 |
| 85:10:5 | 12.8 | 1.8 |
| 80:15:5 | 11.1 | 1.5 |
| 85:11.25:3.75 | 17.1 | 3.0 |
| 80:18:2 | 9.8 | 1.5 |
| 85:13.5:1.5 | 16.2 | 3.2 |

* Total retention time of major component; $t_0 = 3.2$ min.

** Difference in retention time of major component and the nearest other significant component in the particular eluents; this was the peak of next higher $t_R$ in all cases.

of fraction E using hexane–isopropanol (83:17) (Fig. 2) showed a reversal of the elution order of the three major components from one system to the other. In addition, with hexane–isopropanol mixtures, lowering the solvent strength did not simply space out the peaks; some moved faster than others, leading to overlapping peaks and changing elution patterns (Fig. 4).

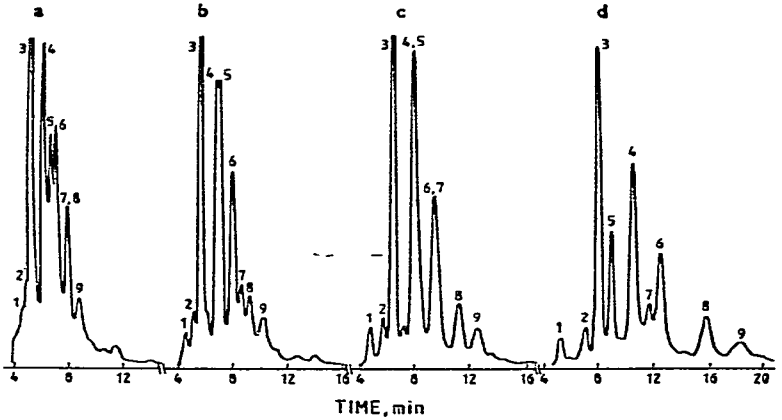


Fig. 4. Elution patterns of Lipidex fraction E with different solvent strengths. Conditions as in Fig. 2 except for: eluent, a, 70:30; b, 73:27; c, 78:22; d, 83:17 hexane–isopropanol; pressure, a, 300 p.s.i.; b, 290 p.s.i.; c, 240 p.s.i.; d, 230 p.s.i. Peaks numbered relative to elution order with eluent condition b. Peaks: 3 = molecular weight 505, tentatively sporidesmin E; 4 = compound D, molecular weight 409; all others uncharacterized.

Fraction F was purified using hexane–isopropanol (96:4) at 1.0 ml/min, to give 2 main components at 28 and 37 min. Fraction $H^1$ gave one main peak ($H^1$) at 16 min with hexane–isopropanol (92:8) at 1.0 ml/min.

The purity of each accumulated HPLC fraction was checked on both CN and $C_{18}$ columns. The major components from Lipidex fractions D, E, and F showed one peak on both columns, but $H^1$ showed two roughly equal components on the $C_{18}$ column. These were separated and collected using methanol–water (64:36).

A CN column has proved useful for the rapid separation and collection of metabolite mixtures. The variety of solvents suitable for use with this column packing offers the chromatographer a great range of possible chromatographic conditions. It has been suggested[6] that $C_{18}$ columns from different manufacturers be used to give changes in selectivity between different compounds, and a more recent article[7] has illustrated considerable differences from column to column. However, this paper has presented some data which suggest that an increase in selectivity may be more easily enhanced using a cyano column and a variety of solvent systems. Exploitation of the full potential of this type of column will be simpler on instruments now coming available with ternary solvent mixing capability.

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