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Note

High-performance liquid chromatographic procedure for determining the profiles of aflatoxin precursors in wildtype and mutant strains of *Aspergillus parasiticus*

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Aspergillus flavus and *A. parasiticus* produce the carcinogenic aflatoxins. As part of an investigation of anthraquinone and xanthone metabolites of *A. parasiticus* as putative precursors of aflatoxins, individual pigments have been isolated by thin-layer chromatography (TLC) and column chromatography[1]. The most recently proposed scheme for aflatoxin biosynthesis is: Norsolorinic acid (NOR) → averantin (AVN) → averufanin (AVNN) → averufin (AVF) → versiconal hemiacetal acetate (VHA) → versicolorin A (VERA) → sterigmatocystin (ST) → O-methylsterigmatocystin (OMST) → Aflatoxin B_1 (AFB_1)[1,2]. Since several of the known aflatoxin precursors and other related metabolites, such as NOR, VERA, versicolorin C (VERC), AVF, AVNN and AVN, have very similar R_F values on TLC with several solvent systems, quantitation of precursors using this method is difficult. For this reason, an high-performance liquid chromatographic (HPLC) system, with the improved resolution inherent in that technique, which can rapidly separate and quantitate the major anthraquinone metabolites from *A. parasiticus* cultures, is desirable.

Several HPLC systems have been developed to separate aflatoxins, but only a few have been described to separate their anthraquinone precursors. Berry *et al.*[3] used a reversed-phase HPLC system to separate metabolites in cell-free extracts of *A. flavus*. They were able to separate aflatoxins, VHA, NOR, VERA, and AVF using a gradient of methanol, tetrahydrofuran (THF) and water. Their procedure, however, could neither effectively resolve VERC from VERA, nor afford a complete separation of a mixture of all the components. Ito *et al.*[4], using a Zorbax HPLC column and a solvent system of hexane, methylene chloride, chloroform and acetic acid, separated ST and a number of related metabolites from *A. versicolor*. The HPLC system reported by Kingston *et al.*[5] to separate anthraquinones and aflatoxins used a solvent system of hexane and acetic acid with ethyl acetate, chloroform or *n*-propanol using a Porasil column. The chloroform system gave the best separation but had the disadvantage of having a total running time of nearly 85 min. The *n*-propanol and chloroform system used by these authors gave adequate separation of the aflatoxins and ST but yielded very poor resolution of the anthraquinones, particularly AVNN and AVF.

In this paper we report an HPLC system which uses a C_{18} μBondapak column

and a solvent system of methanol–THF acidified with a small amount of acetic acid for the separation of anthraquinone metabolites from *Aspergillus* spp. This system offers the advantage of a good separation of all the anthraquinones of interest and a running time of under 40 min. We have also demonstrated the use of this system as a means of screening fungal strains and mutants for the metabolites they accumulate, as well as its use for rapid quantitation of enzymatic and non-enzymatic interconversions of the metabolites.

EXPERIMENTAL

Fungal strains and growth conditions

A highly pigmented wild-type aflatoxigenic strain of *A. parasiticus* (SRRC 143)[1] was used to isolate AVNN and VERC. The blocked mutant strains of *A. parasiticus* used in the feeding study, for metabolite profile determinations, and to isolate standards were SRRC 163 (AVN), SRRC 162 (NOR), SRRC 164 (VERA), and SRRC 165 (AVF). Growth medium (GM) and low-sugar replacement medium (LSRM) were prepared by the methods of Adye and Mateles[6].

Extraction of secondary metabolites

Strains of *A. parasiticus* were grown for 5–7 days on GM in static or shake cultures (150 rpm, Lab-Line Instruments) before extraction of pigments[1]. For feeding studies, cultures were grown for 2 days in shake culture on GM; the mycelia were harvested, washed thoroughly with distilled water and transferred (1 g) to 10 ml LSRM. The appropriate anthraquinone metabolite such as NOR, was added to the incubation medium in 10 μl of acetone. After the desired incubation, mycelial mats or pellets were extracted with aqueous acetone and partitioned with dichloromethane according to established procedures[7]. The extracts were dried and reconstituted in an appropriate volume of mobile phase solvent.

Purification and analysis of metabolites

Individual compounds were purified as described earlier[1] using Sephadex column chromatography and repeated thin-layer silica gel chromatography with toluene–ethyl acetate–acetic acid (50:30:4, v/v/v) as the developing solution. The identities of these metabolites were confirmed by mass spectrometry (MS)[8,9]. Aflatoxins, ST and OMST standards were obtained from Sigma (St. Louis, MO, U.S.A.).

Apparatus

A Waters Model 6000A solvent delivery system equipped with a Waters U6K injector, a Waters 660 solvent programmer and a Waters Model 440 absorbance detector were utilized for the separations. A Waters C_{18} μBondapak (30 cm $\times$ 3.9 mm I.D.) column was used at 25°C for these studies. The solvent system utilized was a binary step gradient consisting of A (methanol–THF, 2:1) and B (0.1 *M* acetic acid), going from 67 to 79% A at 30 min. The total flow-rate for the solvent system was 1 ml/min. Peaks were detected at 313 nm and 0.1 a.u.f.s. The lower limit of detection for each metabolite was 1 μg.

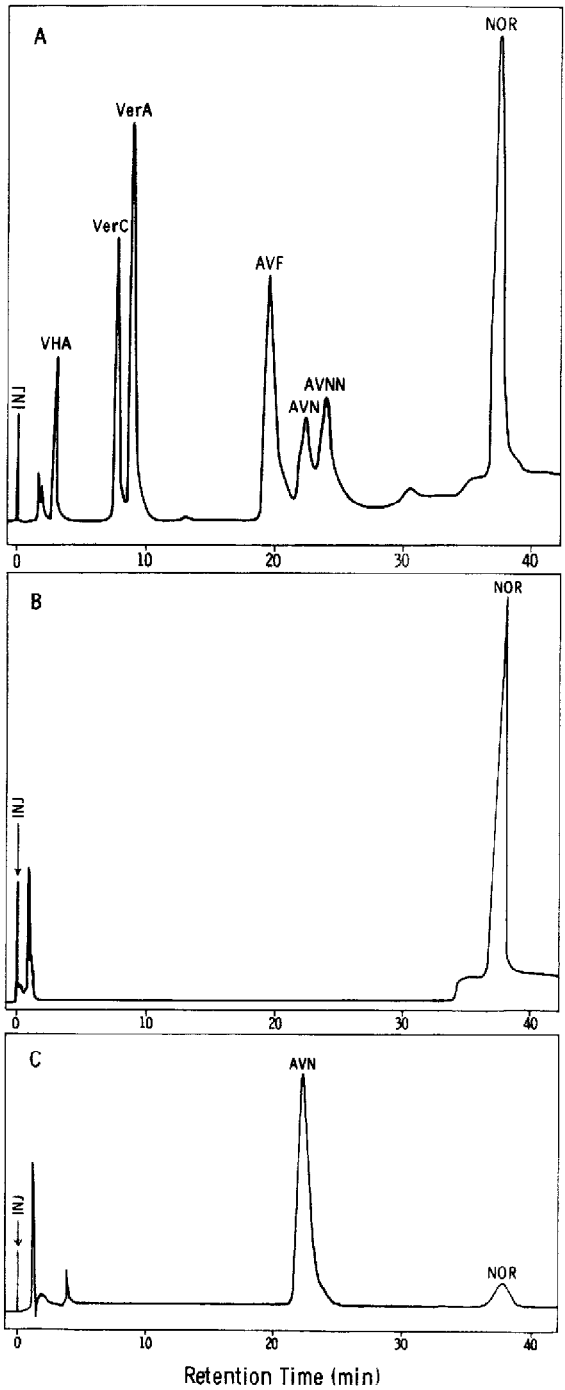

Fig. 1. HPLC traces of anthraquinone precursors of aflatoxin utilizing the solvent system described in the Experimental section. (A) Major anthraquinone metabolites isolated from *A. parasiticus* SRRC 143; versiconal hemiacetal acetate (VHA), versicolorin C (VerC) versicolorin A (VerA), averufin (AVF), averantin (AVN), averufanin (AVNN), norsolorinic acid (NOR). (B) NOR accumulated by SRRC 162 cultures. (C) AVN and a small amount of NOR accumulated by SRRC 163 cultures.

RESULTS AND DICUSSION

A chromatogram showing the separation of the major anthraquinone pigments found in the mycelial extracts of the wildtype strain of *A. parasiticus*, SRRC 143 is given in Fig. 1A. The methanol–THF–acetic acid binary step gradient system was effective in distinctly separating all the major anthraquinone metabolites in *A. parasiticus* as shown in Fig. 1A: VHA (3.1 min), VERC (7.8 min), VERA (9.0 min), AVF (19.7 min), AVN (22.4 min), AVNN (24.0 min), and NOR (37.5 min). The xanthones in the aflatoxin biosynthetic pathway, OMST and ST, and the coumarin, AFB_1, were also separated from the anthraquinones, being eluted early at 1.53, 1.8 and 2.3 min, respectively. The major aflatoxins, B_1 and G_1, could not be separated with this solvent system.

Several applications were made of this HPLC procedure. The mycelial extracts from two known mutant strains of *A. parasiticus*, SRRC 162 and SRRC 163, were examined by HPLC to determine the anthraquinone metabolite accumulated by these strains. As can be seen in Fig. 1, SRRC 162 accumulates only NOR (Fig. 1B), whereas SRRC 163 accumulates AVN as well as small amounts of NOR (Fig. 1C). Other mutants developed could be similarly screened for the metabolites they accumulate.

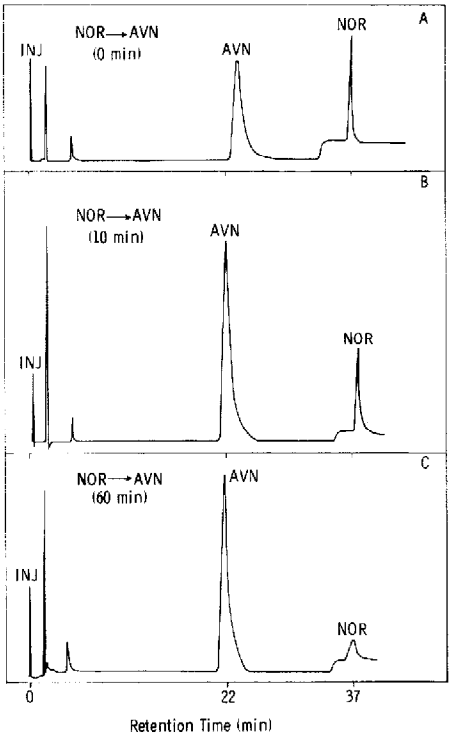

Fig. 2. HPLC traces of a time course study for the conversion of NOR to AVN by submerged cultures of SRRC 163 as described under Experimental. NOR and AVN present in the culture extract at the start of the incubation, after 10 min of incubation, and after 60 min of incubation are shown in panels (A), (B) and (C), respectively. NOR was converted to averantin at a rate of 1.15 μmol/h/g wet mycelial weight.

TABLE I

CONVERSION OF AVN BY METHANOL AT ROOM TEMPERATURE TO A PRODUCT WITH SIMILAR CHROMATOGRAPHIC PROPORTIONS TO AVF OR AVNN

	AVN	*AVN product*	*AVF*	*AVNN*
R_F value*	0.71	0.77	0.77	0.77
Color (NA)**	Purple	Pink	Pink	Pink
Color (VIS)***	Yellow	Yellow	Yellow	Yellow
HPLC retention time (min)	22.4	30.5	19.7	24.0

* As observed on TLC after developing in toluene–ethyl acetate–acetic acid (50:30:4, v/v/v).

** NA = Diphenylaminoborinate spray reagent (Sigma, St. Louis, MO, U.S.A.). The TLC plates were sprayed with NA, air-dried and visualized under normal light.

*** VIS = Visible under normal light.

The HPLC system was also used for a time course study (0 to 6 h) for the conversion of NOR to other metabolites by the mycelia of *A. parasiticus* mutants. NOR fed to a culture of SRRC 163 (Fig. 2) was converted to AVN at a rate of 1.15 μmol/h/g mycelial wet weight.

This HPLC method was used to study the apparent decomposition or interconversion on storage of the metabolite averantin. In order to investigate the nature of this problem, standards of AVN were purified and stored as solutions in methanol,

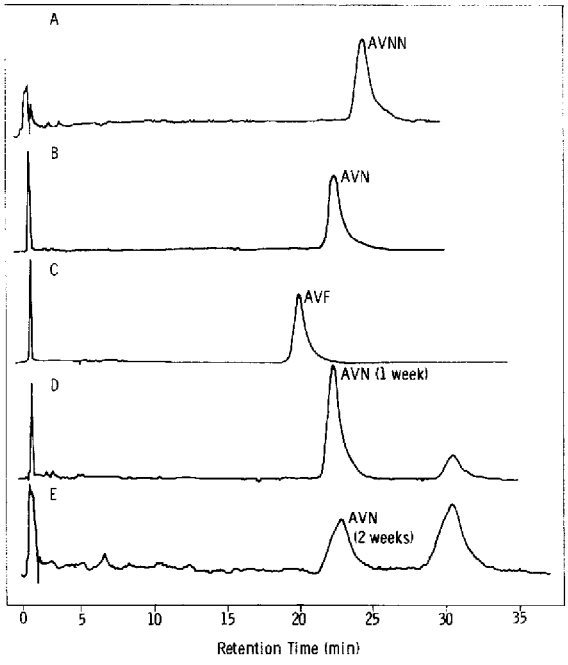

Fig. 3. HPLC traces showing elution of (A) AVNN, (B) AVN, (C) AVF, (D) and (E) the degradation product produced on room temperature storage of AVN in methanol for 1 week and 2 weeks, respectively. The AVN product did not co-elute with either AVF or AVNN.

acetone or dichloromethane in a freezer (−4°C) or at room temperature (27°C). At weekly intervals the composition of these standards was checked by TLC and by HPLC. Some of the standards were found to decompose significantly within a few weeks. One example is that of chromatographically pure AVN stored in methanol at room temperature. AVN converted to a compound with the same R_F behavior and color on TLC as that of AVF or AVNN (Table I). The step-wise gradient HPLC procedure (Fig. 3) provided experimental evidence of a new product (30.5 min) that did not co-elute with AVF (19.7 min) or AVNN (24 min). On MS analysis, the decomposition product of AVN was found to be averantin-2'-methyl ether (Fig. 4). The product exhibited a molecular ion at m/z 386 and a base peak at 315 ($M^+ - C_5H_{11}$) as opposed to 372 for AVN[10]. This fragment, and the appearance of the anthraquinone skeletal ion at m/z 272 indicated that the additional methyl group was on the 2'-position of the side chain of averantin.

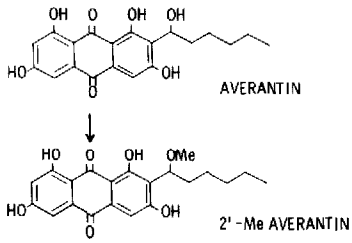

Fig. 4. Conversion of AVN to averantin-2'-methyl (Me) ether (AVN product) during storage of AVN at room temperature.

In conclusion, the above applications show the versatility of the HPLC technique described here. The technique could also be used to study the effect of chemical blocking agents on the aflatoxin biosynthetic pathway based on the anthraquinone precursor accumulation, thereby establishing the position of the block in the aflatoxin pathway due to the mutation or chemical block. The binary step-wise gradient solvent system used in this study overcame the disadvantages of previously described HPLC procedures which do not allow for the complete separation of all the anthraquinone pigments of interest in one run. The addition of acetic acid to the solvent system was observed to be responsible for the improvement of the anthraquinone metabolites.

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