High-Pressure Liquid Chromatographic Method for Detection and Resolution of Rubratoxin, Aflatoxin, and Other Mycotoxins

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A high-pressure liquid chromatography system (HPLC) was developed to resolve a mixture of seven mycotoxins (aflatoxin B_1, aflatoxin G_1, ochratoxin A, zearalenone, rubratoxin B, patulin, and penicillic acid). This HPLC system includes a new UV detector, a reverse phase μ Bondapak/C_{18} column and a solvent system of acetonitrile–water–glacial acetic acid (55:45:2 or 45:55:2, v/v/v). Detection limits were as low as 5 ng of rubratoxin B, 1 ng of zearalenone, 0.04 ng of ochratoxin A (254 nm), and 1 ng of aflatoxin B_1 or G_1 (365 nm). This system also resolved trichothecin from the other six mycotoxins in the absence of ochratoxin A, roseotoxin B from the other six mycotoxins in the absence of rubratoxin B, and sterigmatocystin from the other six mycotoxins in the absence of zearalenone.

Interest in the use of high-pressure liquid chromatography (HPLC) for the detection, identification, and quantitation of aflatoxin and other toxic fungal metabolites is evidenced by several reports of its use (Seiber and Hsieh, 1973; Rao and Anders, 1973; Ware et al., 1974; Ware, 1975; Garner, 1975; Seitz, 1975; Kovacs et al., 1975; Hsieh et al., 1976; Pons, 1976; Kmieciak, 1976; Stack et al., 1976).

Aflatoxin B_1, the most toxic of a series of metabolites produced by the mold *Aspergillus flavus*, is one of the most powerful hepatotoxins (Patterson, 1973) and potent chemical carcinogens (Wogan and Newberne, 1967) known. Sterigmatocystin and penicillic acid are also demonstrated carcinogens (Dickens and Jones, 1965; Dickens et al., 1966). Ochratoxin A is a nephrotoxin and hepatotoxin but is not carcinogenic (Purchase and Theron, 1968; Krogh, 1974). Zearalenone causes hyperestrogenism or "estrogenic syndrome" (Mirocha and Christensen, 1974) and patulin inhibits DNA, RNA, and protein synthesis and some sulfhydryl enzymes (Wilson, 1974). The aflatoxins and mycotoxins produced by other molds (Bamberg et al., 1969; Smalley et al., 1970) constitute a potential hazard as contaminants of animal feed and could cause major economic losses.

Determination of the extent of this hazard requires analytical methods for detection, identification, and quantitation of all these important mycotoxins. High-pressure liquid chromatography might become the method of choice for mycotoxin analyses due to the advantages of good resolution, high degree of precision, reproducibility, and sensitivity (Hsieh et al., 1976; Pons, 1976). Improvements in column packings and detectors have enhanced the performance capability of HPLC methods.

In 1957 Burnside et al. reported that of 13 fungi isolated from toxic corn, *Aspergillus flavus*, which produces aflatoxins, and *Penicillium rubrum*, which produces rubratoxin, were the only two that caused illness and death when fed to swine, horses, and mice. This observation is important because those fungi are frequently isolated from the same foodstuff, and a synergistic relationship between rubratoxin B and aflatoxin B_1 was demonstrated when the two mycotoxins were given simultaneously to rats (Wogan et al., 1971). The lethal action of rubratoxin B increased but the carcinogenic action of aflatoxin B remained unchanged. Other investigators have confirmed the toxicity of diets contaminated by *Penicillium rubrum* (Forgacs et al., 1958; Newberne et al., 1955; Blevins et al., 1969;

Madhavikutti and Shanmugasundaram, 1968). Hood et al. (1973) reported that rubratoxin B is a potent embryocide, a teratogen, and a growth retardant. The LD_{50} values for rubratoxin B in several species were reported by Townsend et al. (1966) and Wogan et al. (1971). The values ranged from 0.2 mg/kg of body weight for cats given a single ip dose in dimethyl sulfoxide to 400–450 mg/kg for rats given a single dose by stomach tube. Richard et al. (1974) reported that rubratoxin B given orally at a dose level of 6 mg/day suppressed complement activity in guinea pigs and also caused a significant increase in prothrombin time of serum.

Hayes and McCain (1975) reported a method for detection of rubratoxin B to the 0.5 μg level. In this method the developed thin-layer chromatography (TLC) plates were heated to 200 °C to produce a fluorescent derivative that is visible under long wave UV light. The detection level was higher than the levels for several of the other mycotoxins. The potential importance of rubratoxin B as a contaminant of feedstuffs provided impetus for developing a more sensitive detection system.

We now describe a HPLC system that resolves and detects nanogram amounts of seven mycotoxins either as single standards or in a standard mixture.

MATERIALS AND METHODS

Materials. Mycotoxins and sources were: crystalline ochratoxin A, aflatoxins B_1, B_2, G_1, and G_2 (Calbiochem, LaJolla, Calif.), patulin, penicillic acid (Alex Ciegler, Northern Regional Research Center, Peoria, Ill.), sterigmatocystin (Sigma Chemical Co., St. Louis, Mo.), zearalenone (C. J. Mirocha, University of Minnesota, St. Paul, Minn.), and trichothecin (W. Sorenson, University of Oklahoma, Norman, Okla.). Crude rubratoxin B was extracted from the liquid of the surface cultures of *Penicillium rubrum* Stoll, purified by the method of Hayes and Wilson (1968), and recrystallized from ethyl acetate–diethyl ether. Crude roseotoxin B was extracted from fungal rice cultures of *Trichothecium roseum*, purified by the method of Engstrom et al. (1975) and recrystallized from diethyl ether–petroleum ether. Standard samples were dissolved in ethyl acetate or acetonitrile to give concentrations of 100 ng/μL, 10 ng/μL, 5 ng/μL, and 1 ng/μL. A 40-pg/μL standard solution of ochratoxin A was also prepared. Aqueous solutions prepared for use with the HPLC systems contained 0.02% sodium azide as a bacteriostatic agent.

Method. High-pressure liquid chromatographic separations were conducted with a Model ALC 502/401 Liquid Chromatograph (Waters Associates Inc., Milford, Mass.) equipped with a Model 440 UV detector, M6000

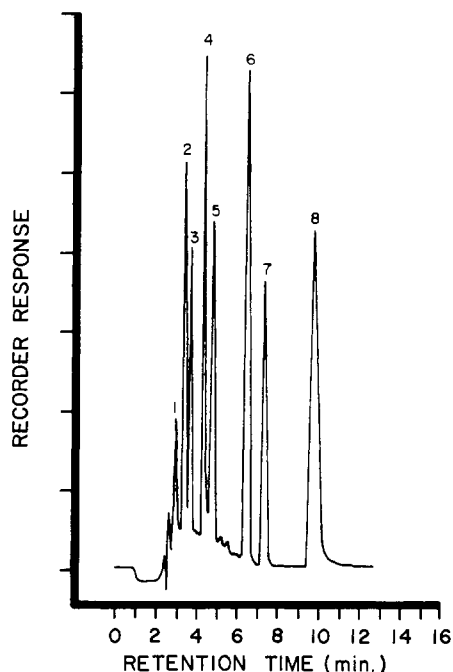The National Animal Disease Center, Veterinary Research Service, U.S. Department of Agriculture, Ames, Iowa 50010.

Figure 1. High-pressure liquid chromatography of a mixture of seven mycotoxins eluted from one μ Bondapak/C₁₈ column with acetonitrile–water–glacial acetic acid (55:45:2, v/v/v/) solvent system (pH 3.85) at a flow rate of 1.0 mL/min with a 254-nm filter: peak 1, solvent peak (acetic acid); peak 2, 0.25 μg of patulin; peak 3, 1.9 μg of penicillic acid; peak 4, 0.85 μg of aflatoxin G₁; peak 5, 0.65 μg of aflatoxin B₁; peak 6, 2.3 μg of rubratoxin B; peak 7, 0.025 μg of ochratoxin A; peak 8, 0.25 μg of zearalenone. UV detector sensitivity setting = 0.1 AUFS.

pump, U6K septumless injector, and a Model 25 UV-vis recording spectrophotometer (Beckman Instruments Inc., Fullerton, Calif.) with a 80 μL volume, 1 cm path length flow-through cell. The Model 440 UV detector was used at sensitivities of from 0.2 to 0.005 absorbance units full scale (AUFS). Chromatograms were recorded on a Linear Instruments (Costa Mesa, Calif.) Model 281 10-in. two-pen recorder with a chart speed of ¹/₄ in./min. The reverse phase μ Bondapak/C₁₈ column was from Waters Associates, Inc., Milford, Mass. It is important to use column care procedures recommended by the manufacturer.

RESULTS AND DISCUSSION

The chromatogram shown in Figure 1 illustrates the resolution of a mixture of seven mycotoxins with our HPLC system. This system includes one μ Bondapak/C₁₈ column, the acetonitrile–water–glacial acetic acid (55:45:2, v/v/v) solvent system and the Model 440 UV detector. The seven mycotoxin chromatogram was completed in 12 min at a flow rate of 1 mL/min. Retention times of the mycotoxins are listed in Table I. Samples of the respective mycotoxins obtained from sources cited in the Materials and Methods section were used as standards without additional purification. There were minor contaminants in the standard samples which were observed by HPLC of the individual mycotoxins. These contaminants accounted for the baseline noise. The small peak between peaks 3 and 4 in Figure 2 was due to a minor breakdown product of rubratoxin B. Peak 1 was due to acetic acid which was added to the mixture to provide a so-called solvent peak or marker. Baseline resolution was obtained between all but two pair of mycotoxins (Figure 1). Resolution between patulin (peak 2) and penicillic acid (peak 3) was satisfactory but could be improved either by reducing the flow rate or by increasing the percentage of water in the solvent mixture (45:55:2, v/v/v) (Figure 2).

Table I. Retention Times of Mycotoxins on μ Bondapak/C₁₈ Column[a,b]

Mycotoxin	1.0 mL/min flow rate, min	0.6 mL/min flow rate, min
Patulin	3.6–3.7	5.9–6.0
Penicillic acid	3.9–4.0	6.4–6.5
Rubratoxin B (artifact)	4.6–4.7	7.4–7.5
Aflatoxin G₁	4.7–4.8	7.5–7.6
Aflatoxin B₁	5.2–5.3	8.3–8.4
Rubratoxin B	6.7–6.8	11.1–11.2
Ochratoxin A	7.5–7.6	12.3–12.4
Zearalenone	10.1–10.2	16.7–16.8
Roseotoxin B	7.2–7.3	11.8–12.0
Trichothecin	7.8–7.9	13.1–13.3

[a] Solvent system was (acetonitrile–water–acetic acid, 55:45:2,v/v/v). [b] Retention time measured from time of sample injection to point of maximum peak height.
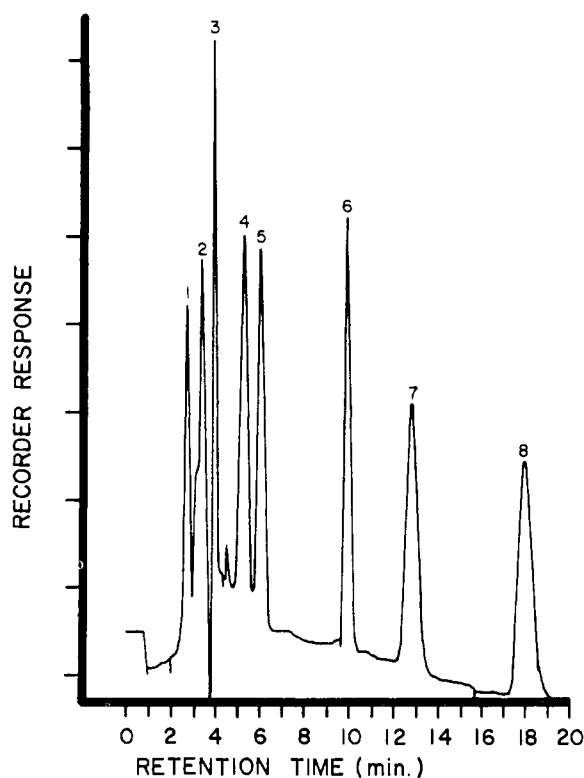

Figure 2. High-pressure liquid chromatography of a mixture of seven mycotoxins eluted from one μ Bondapak/C₁₈ column with acetonitrile–water–glacial acetic acid (45:55:2, v/v/v) solvent system (pH 3.50) at a flow rate of 1.0 mL/min with a 254-nm filter: peak 1, solvent peak (acetic acid); peak 2, 0.20 μg of patulin; peak 3, 3.0 μg of penicillic acid; peak 4, 0.65 μg of aflatoxin G₁; peak 5, 0.70 μg of aflatoxin B₁; peak 6, 0.022 μg of ochratoxin A; peak 7, 1.6 μg of rubratoxin B; peak 8, 0.25 μg of zearalenone. UV detector sensitivity setting = 0.1 AUFS.

Resolution of the most important aflatoxins, G₁ (peak 4) and B₁ (peak 5), was good but not quite baseline. Aflatoxins G₂ and B₂ were not included in the mixture because they were not resolved from aflatoxins G₁ and B₁. Good resolution was achieved when roseotoxin B (Engstrom et al., 1975) and trichothecin (Freeman, 1955; Godtfredsen and Vangedal, 1965) were substituted for rubratoxin B and ochratoxin A in the mycotoxin mixture. Rosetoxin B and trichothecin were not included in the mycotoxin mixture because they were not resolved from rubratoxin B and ochratoxin A, respectively (Table I). Retention times for sterigmatocystin and zearalenone were essentially the same (10.0 and 10.1 min). Only zearalenone was included in the standard mixture. Citrinin gave an excessively broad peak
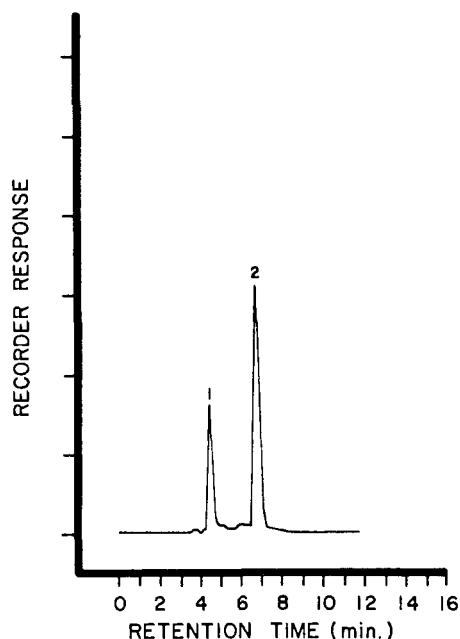
Figure 3. High-pressure liquid chromatography of rubratoxin B eluted from one μ Bondapak/C_{18} column with acetonitrile–water–glacial acetic acid (55:45:2, v/v/v) solvent system at a flow rate of 1.0 mL/min with a 254-nm filter: peak 1, rubratoxin B artifact; peak 2, 0.6 μg of rubratoxin B. UV detector sensitivity setting = 0.05 AUFS.

Table II. Detection Limit of Mycotoxins by HPLC[a,b]

Mycotoxin	Detection limit, ng	
	254 nm	365 nm
Patulin	1	
Penicillic acid	10	
Aflatoxin G_1	25	1
Aflatoxin B_1	25	1
Rubratoxin B	5	
Ochratoxin A	0.04	
Zearalenone	1	
Roseotoxin B	250	
Trichothecin	100	

[a] HPLC system includes one μ Bondapak/C_{18} column and the solvent system of acetonitrile–water–acetic acid (55:45:2, v/v/v). [b] The sensitivity setting used in all cases was 0.005 AUFS. Detection limit was lowest amount of mycotoxin which gave a recorder response of at least 2% of full scale when detector was set at its highest sensitivity setting.

and was not included in the mycotoxin mixture. Only ochratoxin A had a retention time that was dependent on pH of the elution solvent. The solvent system used to obtain the chromatogram shown in Figure 1 (acetonitrile–water–glacial acetic acid, 55:45:2, v/v/v) had a pH of 3.85 and ochratoxin A (peak 7) a retention time of 7.6 min. The solvent system used to obtain the chromatogram shown in Figure 2 (acetonitrile–water–glacial acetic acid, 45:55:2, v/v/v) had a pH of 3.5 and ochratoxin A (peak 6) a retention time of 10.3 min. The difference of 0.35 of a pH unit between the solvent systems was sufficient to cause ochratoxin A to be eluted from the column after rubratoxin B in Figure 1 (peak 7) and before rubratoxin B in Figure 2 (peak 6). The seven mycotoxin chromatogram in Figure 2 was completed in 20 min at a flow rate of 1 mL/min.

Questions about the identity of an unknown peak could be answered in part by use of the stop-flow capability of the equipment to scan the UV–vis spectrum of the unknown compound and comparison of the spectrum with that of a known standard run under the same conditions.

Instability of rubratoxin B to conditions involved in thin-layer chromatography has been a problem. Hayes and Wilson (1968) obtained a single spot from rubratoxin B on silica gel HF_{254} chromatoplates if the plates were developed immediately in an unlined tank with glacial acetic acid–methanol–chloroform (2:20:80, v/v/v). However, if the plates were exposed to the atmosphere for more than 2 to 3 min, between sample application and development, an artifact developed rapidly at a lower R_f value. Moss and Hill (1970) reported that the pure rubratoxin A and B always gave two spots each in the acetic acid–methanol–chloroform (2:20:80, v/v/v) solvent system and that the low R_f value artifact spots were found to increase in relative intensity with the time between sample application and development. They suggested that this phenomenon could be explained by hydrolysis of the anhydride groups. In a review on rubratoxin, Moss (1971) reported that the chromatography of such polar materials

is very sensitive to a number of factors such as temperature and relative humidity. Hayes and McCain (1975) reported that the low R_f value artifact could be eliminated by applying the rubratoxin B sample to TLC plates under nitrogen and developing the plates in unlined tanks with an ascending methanol–chloroform–glacial acetic acid–water (20:80:1:1, v/v/v/v) solvent system. We have determined that there is an artifact peak (retention time = 4.6 min) formed with the reverse phase μ Bondapak/C_{18} column and acetonitrile–water–glacial acetic acid (55:45:2, v/v/v) solvent system which appears in the chromatogram before the main rubratoxin B peak (Figure 3). In Figure 1, the rubratoxin artifact peak is superimposed with aflatoxin G_1, since their retention times are almost identical (Table I). Despite the fact that there is some significant conversion of rubratoxin B to another form in this HPLC system, it was still possible to detect two orders of magnitude less rubratoxin B (5 ng) than is possible with the best published TLC system reported to date (0.5 μg, Hayes and McCain, 1975).

Detection limits for the mycotoxins are listed in Table II. The criterion for determining the detection limits was simply the lowest concentration at which a mycotoxin would produce a small peak (2% of full scale) on the recorder when the detector was set at its highest sensitivity (0.005 AUFS = absorbance units full scale). In order to obtain maximum sensitivity for detection of aflatoxins, it was necessary to change to the 365-nm filter (Table II). Of the seven mycotoxins, only aflatoxins B_1 and G_1 gave a peak at 365 nm.

We believe that this HPLC solvent system could be very useful in conjunction with one of several published extraction procedures: Stoloff et al., 1971; Romer, 1973; Roberts and Patterson, 1975; AOAC, 1975; Wilson et al., 1976. A modification of the extraction procedure would need to be made to include rubratoxin, since it is not soluble in chloroform or benzene but is soluble in ethyl acetate.

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Synthesis of Radiolabeled T-2 Toxin

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Tritium-labeled T-2 toxin [4β,15-diacetoxy,8α-(3-methylbutyryloxy)-3α-hydroxy-12,13-epoxytrichothec-9-ene] was synthesized using a two-step process. First, the C-3 hydroxyl was oxidized to a ketone using either chromium trioxide–pyridine or dimethyl sulfide–N-chlorosuccinimide complex. In step two, the ketone was reduced with either tritiated sodium cyanoborohydride or tritiated sodium borohydride to a mixture of epimers which were separable by thin-layer chromatography. The α epimer (naturally occurring T-2 toxin) was formed predominantly, i.e., α to β ratio was 4 to 1. Final reduction with tritiated sodium borohydride yielded [3-^{3}H]T-2 toxin in 23% yield with a specific activity of 790 mCi/mmol. The labeled product, when equilibrated with 0.01 N HCl, did not lose the tritium, indicating that no labile tritium was incorporated into T-2 toxin. Further, the oxidation of [3-^{3}H]T-2 toxin resulted in total loss of activity in the corresponding ketone.

The metabolites of species of *Fusarium* are commonly found in foodstuff and are prominent because of their association with cases of mycotoxicoses of humans and animals. One such metabolite, T-2 toxin (1) [4β,15-diacetoxy,8α-(3-methylbutyryloxy)-3α-hydroxy-12,13-epoxytrichothec-9-ene] has been implicated as a mycotoxin responsible for the hemorrhagic syndrome and death of dairy cattle (Hsu et al., 1972). T-2 toxin causes an extreme dermal necrosis in most animals and is also one of the most

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potent inhibitors of protein synthesis in eucaryotic cells (Wei et al., 1974).

Our primary interest was to prepare radiolabeled T-2 toxin with sufficient specific activity to enable us to study its metabolism in various animal species. We considered two basic methods of incorporating radioactive elements into T-2. One route was biosynthesis of 1 by feeding labeled precursors such as mevalonic acid (Jones and Lowe, 1960; Achilladelis and Hanson, 1972), farnasyl pyrophosphate (Evans et al., 1973), and trichodiene (Machida and Nozoe, 1972) to actively metabolizing cultures of *Fusarium*. Although the biosynthesis of radiolabeled T-2 toxin appears simple, it is time consuming, expensive, and results in a product with a relatively low specific activity.