Determination of Fumagillin by High-Performance Liquid Chromatography

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A stability-indicating assay for the antibiotic fumagillin in technical materials has been developed. Measurement is by reversed-phase HPLC with detection at 351 nm. The assay is rapid, specific, and precise within the fumagillin concentration range of 0.000–0.035 mg/mL. It was also found that protection of fumagillin from room fluorescent lights is required to prevent photodegradation.

Fumagillin is an antibiotic produced by the fermentation of certain strains of *Aspergillus fumigatus*. Fumagillin, first isolated in 1951 (Eble and Hanson, 1951), has been demonstrated to be effective against *Nosema apis*, a universally occurring protozoan parasite found in the gut of the adult honey been (Girardeau, 1972). Additionally, it has been found to control diseases caused by microorganisms in fish (Takeda Chemical Industries, 1983).

Fumagillin has been assayed by thin-layer chromatography (Issaq et al., 1977), spectrophotometry (Garrett and Eble, 1954), and a microbial assay (Girolami, 1963). Each of these techniques has disadvantages limiting their utility in the analysis of fumagillin. Thin-layer chromatography yields only a limited amount of quantitative information and is not ideally suited for the assay of fumagillin powders. The spectrophotometric assay is subject to error due to interferences from UV-absorbing impurities, especially in complex samples (Girolami, 1963). Finally, the microbial assay requires approximately 16–18 h to complete an analysis.

Described in this paper is an HPLC assay for fumagillin that is rapid and precise and separates fumagillin from possible impurities and degradation products.

EXPERIMENTAL SECTION

Glassware. Nonactinic glassware was used whenever possible. Standard glassware was wrapped in aluminum foil when its use was necessary.

Reagents. The acetonitrile used in this study was HPLC grade. All other reagents were analytical grade.

Fumagillin Standard and Samples. The fumagillin standard used was an Abbott Laboratories house reference standard. The standard had been previously characterized and assigned a purity of 98.5% (w/w). All samples of fumagillin powder were actual production lots supplied by Abbott Laboratories.

Fumagillin Standard Solution Preparation. The fumagillin stock standard solution was prepared by accurately weighing 50 mg of fumagillin standard into a 100-mL volumetric flask. The fumagillin standard was dissolved in and diluted to volume with acetonitrile. The working standard solution was prepared by diluting the stock solution 1:25 with acetonitrile.

Fumagillin Sample Preparation. About 100 mg of the fumagillin sample was accurately weighed into a 100-mL volumetric flask and dissolved in and diluted to volume with acetonitrile. This solution was diluted 1:50 with acetonitrile for the assay preparation.

Liquid Chromatography. The separation and assay of fumagillin was accomplished isocratically on an IBM C_{18} (5 μm, 15 cm × 4 mm (i.d.), P/N 8635329) at ambient temperatures. The mobile phase was acetonitrile/water/glacial acetic acid (500:500:1.5, v/v/v). Detection was with a continuously variable wavelength UV/vis detector set at 351 nm. A suitable computing integrator was used to calculate peak area and/or peak height, both of which were used to quantitate the fumagillin in the sample.

The samples were assayed by two analysts using two different HPLC systems. A summary of the equipment used is given in Table I.

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Table I. HPLC Equipment Used for the Separation and Quantitation of Fumagillin

	analyst 1	analyst 2
pump	Water's Model M45	Water's Model M6000
autosampler	Beckman Model 501	Water's Wisp Model 710 B
detector	Schoeffel Model SF770	Kratos Model SF769Z
recorder/integrator	Perkin-Elmer LCI 100	Water's Data Module

Table II. Precision of the Fumagillin HPLC Assay

	fumagillin, μg/mg			
	analyst 1		analyst 2	
replicate	peak ht	peak area	peak ht	peak area
1	659	661	650	651
2	659	667	671	671
3	660	668	647	645
4	658	659	655	652
5	658	652	654	650
6	650	646	649	646
7	659	673	650	648
8	667	673	653	649
mean	659	662	654	652
std dev	4.6	9.8	7.5	8.2
rel std dev, %	0.7	1.5	1.1	1.3

RESULTS AND DISCUSSION

A typical HPLC chromatogram of fumagillin is shown in Figure 1. The detector response was found to be linear over a concentration range of 0.000–0.035 mg/mL fumagillin. The correlation coefficients were 0.9999 for both peak area and peak height measurements, and the intercepts were found to be less than 1% of the x and y values for the nominal concentration of 0.02 mg/mL fumagillin.

The precision of the assay was determined by two analysts assaying eight replicate sample preparations. The assay results are presented in Table II. The relative standard deviation of the assay was found to be 1.4% or less for peak height and peak area quantitation by both analysts.

The reproducibility of the assay was studied by two analysts assaying eight different samples of fumagillin powder. The results in Table III show good agreement

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Table III. Analysis of Various Lots of Fumagillin

	fumagillin, μg/mg			
	analyst 1		analyst 2	
sample no.	peak ht	peak area	peak ht	peak area
1	572	566	548	552
2	496	495	488	487
3	607	602	593	593
4	844	837	834	838
5	296	292	306	316
6	639	630	628	627
7	880	878	873	874
8	659	662	657	655

Table IV. Effect of Acetonitrile Content in Mobile Phase upon the Fumagillin Assay

% CH_3CN: % buffer	fumagillin k'	fumagillin, μg/mg	
		peak ht	peak area
40/60	24.0	870	875
50/50	6.2	880	878
60/40	2.7	967	975

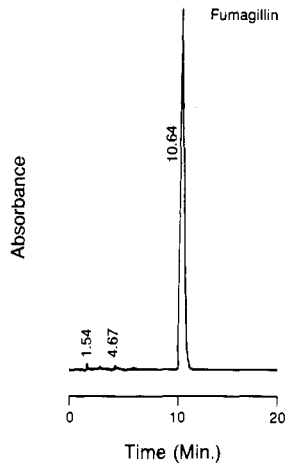

Figure 1. Typical HPLC chromatogram of fumagillin powder.

between both analysts using both peak height and peak area measurements.

The ruggedness of the assay was studied by varying the mobile phase and column temperature. A variation of acetic acid concentration in the mobile phase sufficient to cause a ±1 pH unit change from the nominal pH of 3.5 was found to have little or no effect on peak retention time or quantitation by peak height or area. However, it was found that changing the percentage of acetonitrile had a significant effect on the assay of fumagillin as shown in Table IV. Increasing the acetonitrile content of the mobile phase to 60% (v/v) caused the fumagillin peak to coelute with impurities that normally elute early in the chromatogram (See Figure 1). Therefore, the k' of fumagillin should be approximately 6 or greater to ensure complete resolution of fumagillin.

A change of ±10 °C in the column temperature was found to have little or no effect upon the assay results of fumagillin.

A sample of fumagillin was subjected to various stress conditions and assayed. These stress conditions and assay results are presented in Table V. It is interesting to note that 254-nm UV light was found not to degrade fumagillin. This latter observation is thought to be due to the fact that the molecular absorption band of fumagillin peaks at 351 and 336 nm with very little molecular absorption at 254 nm (Garrett and Eble, 1954).

Photodegradation of fumagillin has been reported in the literature (Garret and Eble, 1954) and was further studied

Table V. Analysis of Fumagillin Powder Exposed to Acid, Base, Heat, and UV Light

conditions	fumagillin, μg/mg
1 N HCl, 3-h reflux	58
1 N NaOH, 3-h reflux	nd[a]
distilled water, 3-h reflux	439
105 °C dry heat, 1 week	0.4
UV light (254 nm), 3 h	521
UV light (366 nm), 3 h	458
no treatment	517

[a] Not detected.

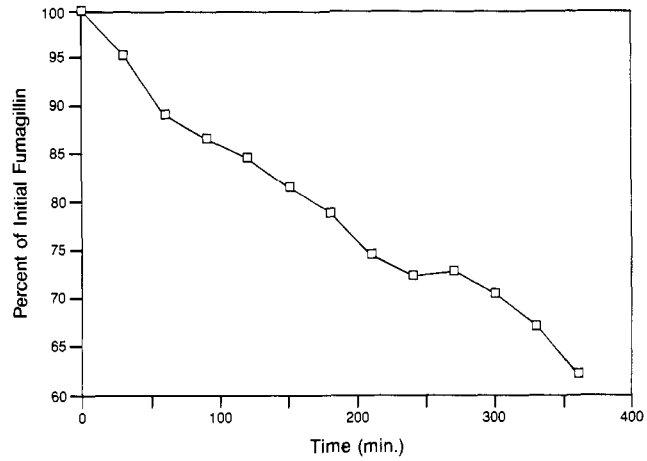

Figure 2. Degradation of fumagillin by fluorescent room lights. Calculated as the percent of fumagillin initially present.

by exposing a sample solution of fumagillin to fluorescent room lights for a period of 6 h. The sample solution was assayed every 30 min. Figure 2 shows the degradation of fumagillin due fluorescent room lights over the period studied.

Two other C_{18} columns were tried: a Water's μBondapak C_{18} and an Alltech Associates C_{18}. A discrepancy of about 5% between peak height and peak area assay results for fumagillin was observed on both of the above columns. Further investigation with thin-layer chromatography revealed that an unknown impurity was coeluting with fumagillin. This unknown impurity was observed to have a retention time of 3.3 min ($k' = 1.2$), with the IBM column using the nominal chromatography conditions.

CONCLUSIONS

An accurate, precise HPLC assay for fumagillin is described. The method has a precision of about 1.5% (RSD) and has been shown to be stability indicating. The assay procedure is relatively easy; however, two points must be observed to obtain accurate results. First, the k' of fumagillin must be about 6 (or greater) or the fumagillin peak will not be completely resolved from the impurities present in the powder samples. Second, the sample solutions must be protected from light by using nonactinic glassware or aluminum foil. Protection from light is especially important when a number of samples are loaded on an autosampler for an extended period of time.

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Determination of Fluvalinate Metabolite Residues in Cottonseed, Apples, Tomatoes, and Soil

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Fluvalinate is the active ingredient in Mavrik and Spur insecticides used to control insects on numerous field crops. Radiolabel experiments have shown that the residue of concern in most crops is the parent molecule. However, an analytical method was needed to determine the major hydrolytic metabolites of fluvalinate, 3-phenoxybenzoic acid (PBA) and 2-[2-chloro-4-(trifluoromethyl)anilino]-3-methylbutanoic acid (CAA), in selected crops. The method entails standard extraction and partition steps, basic hydrolysis to free conjugated metabolites, conversion to methyl esters, and determination by capillary gas chromatography/mass spectrometry. The methods were validated for the title crops with detection limits of 0.02 and 0.05 ppm, respectively, for CAA and PBA.

Fluvalinate [(*RS*)-α-cyano-3-phenoxybenzyl (*R*)-2-[chloro-4-(trifluoromethyl)anilino]-3-methylbutanoate; **1** in Figure 1] is the ingredient of Mavrik and Spur insecticides being developed by Sandoz Crop Protection Corp. for use on a variety of field crops (Henrick et al., 1980). Previous studies have demonstrated (Quistad et al., 1982) that the primary metabolic pathway of fluvalinate in plants is via ester hydrolysis and oxidation to yield 3-phenoxybenzoic acid (PBA, **2**) and 2-[2-chloro-4-(trifluoromethyl)anilino]-3-methylbutanoic acid (CAA, **3**). These metabolites exist in the plants in both free and conjugated forms.

In conjunction with field studies on the environmental fate of fluvalinate, analytical methods were needed for residues of PBA and CAA. Although PBA is a known metabolite from many synthetic pyrethroids, scant literature is available on determination of its residues. An early report on the high-pressure liquid chromatography behavior of PBA (Ram and Grushka, 1977) has not been expanded upon. The only method for PBA in the *Pesticide Analytical Manual* (FDA, 1985) was published as part of a method for the determination of permethrin and its metabolites. The method described herein is similar to the published permethrin method but is adapted to allow determination of the fluvalinate-specific acid CAA.

EXPERIMENTAL SECTION

Reagents. Fluvalinate and CAA were synthesized as previously described (Henrick et al., 1980). [*trifluoromethyl*-^{14}C]Fluvalinate was prepared as previously described (Quistad et al., 1982). PBA was purchased from Aldrich Chemical Co. The ethyl ester of CAA was synthesized from CAA by treatment with diazoethane. All solvents were high purity from Burdick and Jackson. Florisil (PR Grade, J. T. Baker) was heated at 150 °C overnight and deactivated with 6% water prior to use. Sodium sulfate (anhydrous, reagent grade) was slurried with anhydrous ethyl ether, treated with 0.5% by weight concentrated H_2SO_4, filtered, and dried at 100 °C prior to use.

Sample Preparation. Apples were chopped finely, and 20 g was treated with 5 mL of 0.5 N HCl. The sample was then Soxhlet extracted for 10 h with 200 mL of methanol containing 2.5% 0.5 N HCl. Tomatoes were chopped, and 20 g was blended with 50 mL of methanol and 5 mL of 0.5 N HCl. The extract was vacuum filtered and the residue reextracted as before. The final filter cake was washed with two additional 25-mL portions of methanol, and the filtrates were combined. Soil samples were ground and sifted through a No. 6 sieve. The soil was then sprinkled with water and allowed to equilibrate for 1 h. A 20-g portion of soil was then treated with acid and Soxhlet extracted. A separate 5-g sample of the moistened soil was dried to constant weight at 100 °C. Residues were corrected to a dry-weight soil basis. Cottonseed samples were ground in a coffee grinder. A 20-g cottonseed sample was then treated with acid and Soxhlet extracted.

Partitioning of Extract. For each substrate, the extract was diluted to 200 mL and a 4-g equivalent portion (40 mL) transferred for further processing to a 500-mL separatory funnel. To the funnel were added 200 mL of 0.1 M $NaHCO_3$, 5 mL of saturated NaCl, and 50 mL of isooctane that had been saturated with methanol. After 1 min of extraction, the upper isooctane layer was discarded. The isooctane partitioning was repeated three times to ensure complete removal of all fluvalinate.

Hydrolysis. The aqueous layer from the partitioning was treated with 5 mL of 5 N KOH and heated at reflux for 1 h. The basic solution was then cooled in ice and

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