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CHROMATOGRAPHIC AND SPECTROSCOPIC PROPERTIES OF HEMIACETALS OF AFLATOXIN AND STERIGMATOCYSTIN METABOLITES

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SUMMARY

Improved fluorescence detection of aflatoxin B_1 by chromatographic analysis is accomplished by conversion to the corresponding hemiacetal, aflatoxin B_{2a}. Because the metabolites aflatoxin M_1, aflatoxin P_1, aflatoxin Q_1, sterigmatocystin, and O-methylsterigmatocystin have the same molecular conversion site, we investigated the chromatographic and spectroscopic properties of hemiacetals of these compounds to assist in confirming aflatoxins and sterigmatocystins in human urine. Nuclear magnetic resonance and infrared absorbance were used to confirm the hemiacetal structure for aflatoxin B_1 and sterigmatocystin. The ultraviolet absorbance, fluorescence, and chromatographic properties of the metabolites were investigated. Using these data, we optimized the detection and solvent conditions for high-performance liquid chromatography. We determined that, of the conditions studied, maximum sensitivity and resolution for the native aflatoxins were achieved with a mobile phase of methanol, tetrahydrofuran, and water, a C_8 column in series with a C_{18} column, and fluorescence detection with 365 nm excitation and 430 nm emission wavelengths for aflatoxins B_1 and M_1 and with 500 nm emission wavelength for aflatoxins P_1 and Q_1. For the analysis of the hemiacetals, a mobile phase of methanol, acetonitrile, and water provided better chromatography and fluorescence detection. Sterigmatocystin and O-methylsterigmatocystin were readily converted to the hemiacetal forms, which, like the aflatoxins, were more polar and, therefore, earlier eluting by reversed-phase HPLC (methanol, acetonitrile, and water, 236 nm absorbance). These data are important to maximize the sensitivity and confidence for detecting the mycotoxin metabolites in biological specimens.

INTRODUCTION

In 1966, the hemiacetals of aflaloxins B_1 and G_1 were first reported[1]. These hemiacetals could be synthesized by the acid-catalyzed addition of water across the vinyl ether (C2,3) double bond (Fig. 1)[2] and were found to be more intensely fluorescent than the corresponding naturally occurring mycotoxins[3]. The ease of produc-

tion and the enhanced fluorescence of the hemiacetals have encouraged investigations of the hydrated form rather than the native compound by high-performance liquid chromatography (HPLC)[4–7]. When aflatoxin B_1 is acted on by the liver enzymes (rat, mouse, monkey, and human), it is hydroxylated to the metabolites aflatoxins M_1, P_1 and Q_1 (refs. [8–12]) (Fig. 1), which are conjugated with glucuronic acid when excreted in the urine[9].

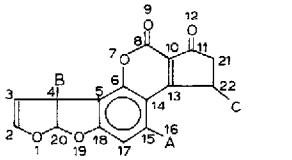
Sterigmatocystin (STR) is similar to aflatoxin: it is produced by species of *Aspergillus* that contaminate stored nuts and grain, it has the 7,8-dihydrofurano-[2,3-*b*]furan moiety where a vinyl ether double bond is located, and it is conjugated with glucuronic acid when excreted in the urine[13]. Unlike aflatoxin, however, unless it is dissolved in a strong acid such as sulfuric acid, STR in not intensely fluorescent[14]. Typically, HPLC of STR uses ultraviolet (UV) absorbance for detection[15–17].

We have been interested in analyzing for the mycotoxins in human specimens[18,19]. We present chromatographic and spectroscopic properties of the hemiacetals of aflatoxin B_1 (AFB), aflatoxin M_1 (AFM), aflatoxin P_1 (AFP), aflatoxin Q_1 (AFQ), sterigmatocystin (STR), and O-methylsterigmatocystin (OMS). The results of this study were used to enhance sensitivity and confidence in the HPLC determination for these mycotoxin metabolites in human urine[19].

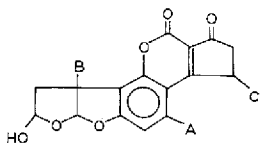
EXPERIMENTAL

Equipment and reagents

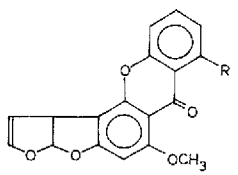
Standards of AFB, AFM, AFP, AFQ, STR and OMS (Sigma, St. Louis, MO U.S.A.), were prepared in acetonitrile. Because these mycotoxins are highly toxic and carcinogenic, standards were prepared in a glove box. Trifluoroacetic acid (TFA) (Aldrich, Milwaukee, WI, U.S.A.) and orthophosphoric acid (Fisher Scientific, Fair

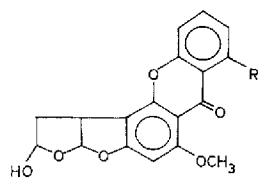


aflatoxin	A	B	C
B1	-OMe	-H	-H
M1	-OMe	-OH	-H
P1	-OH	-H	-H
Q1	-OMe	-H	-OH



aflatoxin	A	B	C
B2a	-OMe	-H	-H
M2a	-OMe	-OH	-H
P2a	-OH	-H	-H
Q2a	-OMe	-H	-OH





	R
sterigmatocystin	-OH
O-methylsterigmatocystin	-OMe

sterigmatocystin-hemiacetal

O-methylsterigmatocystin-hemiacetal

Fig. 1. Chemical structures of the native and hemiacetal forms of the mycotoxins studied. Me = Methyl.

Lawn, NJ, U.S.A.) were reagent grade. All solvents were HPLC-grade (Burdick & Jackson, Muskegon, MI, U.S.A.).

HPLC was performed with a Waters system, incorporating an M6000A pump, an M721 data module, an M720 system controller, and an M710B WISP injector (Millipore, Milford, MA, U.S.A.). Detectors were a Perkin-Elmer LS-4 and a Perkin-Elmer 650-10S fluorescence spectrophotometers (Norwalk, CT, U.S.A.) and a Waters 480 spectrophotometer. Either a Sepralyte C_8 (25 cm × 2.1 mm I.D., 10-μm) column (Analytichem, Harbor City, CA, U.S.A.) or a Spherisorb C_8 (15 cm × 4.0 mm I.D., 5 μm) (Chromanetics, Sci-Con, Winter Park, FL, U.S.A.) in series with a Spherisorb C_{18} (15 cm × 4.0 mm I.D., 5 μm) column were used as the stationary phases. A Waters 990 photodiode array detector was used for determining the UV absorbance spectra of the mycotoxins. A Nicolet 170SX Fourier transform infrared (FT-IR) spectrometer (Nicolet, Madison, WI, U.S.A.) was used for the IR analyses. Samples were analyzed as KBr pellets. The instrumentation and conditions for the nuclear magnetic resonance (NMR) analyses are described elsewhere[20].

Preparation of the mycotoxin hemiacetals

Hexane (200 μl) and TFA (50 μl) were added to 2 μg of the mycotoxin. After 15 min at 60°C, 950 μl of water–acetonitrile (9:1) was added, the solution was vortexed, and the lower aqueous layer was transferred to a vial for HPLC analysis[18,21]. By convention, the hemiacetal of aflatoxin B_1 is called aflatoxin B_{2a} (AFB_{2a})[1]; following this convention the hemiacetals of AFM, AFP, and AFQ have been designated AFM_{2a}, AFP_{2a} and AFQ_{2a}.

RESULTS AND DISCUSSION

The purity of the standards was evaluated by HPLC. When only one fluorescent (365 nm excitation, 430 nm emission) peak was present in the HPLC chromatogram, the standard was regarded as pure. We evaluated the standards in this fashion because very small amounts of aflatoxins P_1 and Q_1 were available. When the standards were dissolved in methanol, they deteriorated in a few weeks, this has also been observed by other investigators[22]. Aflatoxin Q_1 deteriorated most quickly and began to show multiple HPLC peaks after two weeks. Greater stability was obtained when the aflatoxins were dissolved in acetonitrile. Aflatoxin Q_1 produced one HPLC peak for more than a month.

Hemiacetal synthesis

The conversion of the native mycotoxin to the hemiacetal was evaluated by the disappearance of the native peak and the appearance of a fluorescent HPLC peak for the hydrated structure. As shown in Table I, using reversed-phase isocratic HPLC, the hemiacetals eluted faster than their corresponding native mycotoxins. Table II shows minimum detectable amounts of each aflatoxin and its hemiacetal under various conditions. The aflatoxins were generally more fluorescent in the hemiacetal form. The reduction in the retention time and the increased fluorescence of the hemiacetals serve to confirm the parent mycotoxin. Three variations of the TFA-catalyzed addition of water to aflatoxin were evaluated: in method 1[21], 200 μl of dry hexane and 50 μl of TFA were mixed with the mycotoxin. The reaction proceeded for 15 min at

TABLE I

RELATIVE RETENTION TIMES OF THE HEMIACETAL STRUCTURES OF AFLATOXIN AND STERIGMATOCYSTIN METABOLITES

Mobile phase: acetonitrile–methanol–water (3:2:5), 0.3 ml/min. Column: Sepralyte C_8, 250 mm × 2 mm I.D., 10 μm. Relative retention times calculated as retention time (hemiacetal)/retention time (native mycotoxin).

	Relative retention time
Aflatoxins	
B	0.56
M	0.65
P	0.63
Q	0.84
Sterigmatocystins	
STR	0.43
OMS	0.48

room temperature, and then 950 μl of water–acetonitrile (9:1) was added and the solution vortexed. The lower, aqueous layer was transferred to another vial for analysis. In method 2[23], hexane and TFA were added as in method 1, but the solution was incubated at 40°C for 15 min. After incubation, the reaction mixture was evaporated to dryness with a gentle stream of nitrogen and 40°C heat, then dissolved in 1 ml of

TABLE II

INFLUENCE OF MOBILE PHASE AND EMISSION WAVELENGTH ON THE RELATIVE FLUORESCENCE OF AFLATOXINS*

(a) Methanol–THF–0.3% H_3PO_4 (35:5:60), 0.5 ml/min. (b) Methanol–THF–water (35:5:60), 0.5 ml/min. (c) Methanol–acetonitrile–water (35:5:60), 0.5 ml/min.

Aflatoxin	*Mobile phase*	*Emission wavelength (nm)***			
		430		*500*	
		Native (ng)	*Hemiacetal (ng)*	*Native (ng)*	*Hemiacetal (ng)*
B	a	0.43	0.03	2.8	0.07
	b	0.34	0.05	2.0	0.11
	c	1.7	0.03	5.6	0.06
M	a	0.15	0.07	0.27	0.19
	b	0.15	0.08	0.24	0.19
	c	0.08	0.06	0.27	0.19
P	a	16	12	1.4	1.7
	b	8.0	3.0	0.59	0.77
	c	25	2.8	1.0	1.1
Q	a	17	0.16	7.5	0.05
	b	12	0.17	3.0	0.06
	c	12	0.05	5.6	0.02

* Shown as the minimum detectable amount in ng, signal-to-noise ratio is 3:1.
** 365 nm was used as the excitation wavelength.

acetonitrile. In method 3[7], 1 ml of TFA–water (9:1) was added to the dried standard material and the mixture reacted for 15 min at room temperature. Each sample was then analyzed by HPLC. HPLC determined that AFB was easily and completely converted to its hemiacetal by all three methods, but the other metabolites (AFM, AFP, AFQ, STR and OMS) were not as reactive and, therefore, not completely hydrated. Other investigators have noted the incomplete reaction by AFM with TFA[24]. Similarly, substituting 50% aqueous trichloroacetic acid for TFA readily converted AFB but not the other metabolites. Moderate heat (60°C) was necessary to ensure complete reaction of all of the metabolites. The method described in *Preparation of the mycotoxin hemiacetals* completely converted each of the six mycotoxins to their hemiacetal structure.

The hemiacetal of sterigmatocystin

IR and NMR analyses confirmed the hemiacetal structure of sterigmatocystin (Fig. 1). After determining the IR and NMR spectra for aflatoxin B_{2a}, we compared the spectra for sterigmatocystin. The IR spectrum of AFB_{2a} demonstrated the addition of water to the C2,3 double bond by the appearance of a broad O–H stretching band at 2458 cm^{-1} and by the disappearance of the double bond 1620 cm^{-1} band. Additionally, a new band at 1086 cm^{-1} was observed for the hemiacetal C–O stretching, while the band from 1199 cm^{-1} vinyl ether C–O–C stretching in five membered ring disappeared. This spectrum closely agreed with published results[3,25]. In the IR spectra for STR hemiacetal, the presence of 3220 cm^{-1} band (aliphatic O–H stretch) and the 1442 cm^{-1} and 1420 cm^{-1} bands (O–H bend), and the absence of the 1620 cm^{-1} band, supported addition of water across the vinyl ether double bond. The NMR spectra of AFB, STR and their hemiacetals, and selective homodecoupling experiments used to make assignments, are described elsewhere[20]. These spectra verify that (a) water was added across the double bond of the vinyl ether, (b) the hydroxyl group was attached to the alpha carbon, and (c) two hemiacetal configurations were produced by the hydroxyl group occurring in an up (beta structure) or a down (alpha structure) orientation. These data confirm that the hemiacetal of sterigmatocystin can be easily synthesized.

The UV, fluorescent, and chromatographic properties of the hemiacetals of sterigmatocystin and O-methylsterigmatocystin were investigated. Fig. 2 shows that the UV spectra of STR and OMS hemiacetals were not different from the spectra for the native mycotoxins. STR is reported to be fluorescent under strong acidic conditions[13]; however, because strong acids cannot be used as a mobile phase for HPLC, UV absorbance is used as the method of detection. Converting the parent structure of STR or OMS to the hemiacetal, likewise, did not produce a structure that was suitably fluorescent when using HPLC. Table I gives the relative retention times of hemiacetals of STR and OMS. The hydrated structures clearly are more polar than the native forms and, therefore, earlier eluting by reversed-phase liquid chromatography (RPLC). These results parallel the observed chromatography of the aflatoxin hemiacetals.

The hemiacetal of aflatoxin B_1 (AFB_{2a})

The UV spectrum of aflatoxin B_1 was not significantly affected by the conversion to the hemiacetal (Fig. 2), although, the fluorescence intensity was. As shown

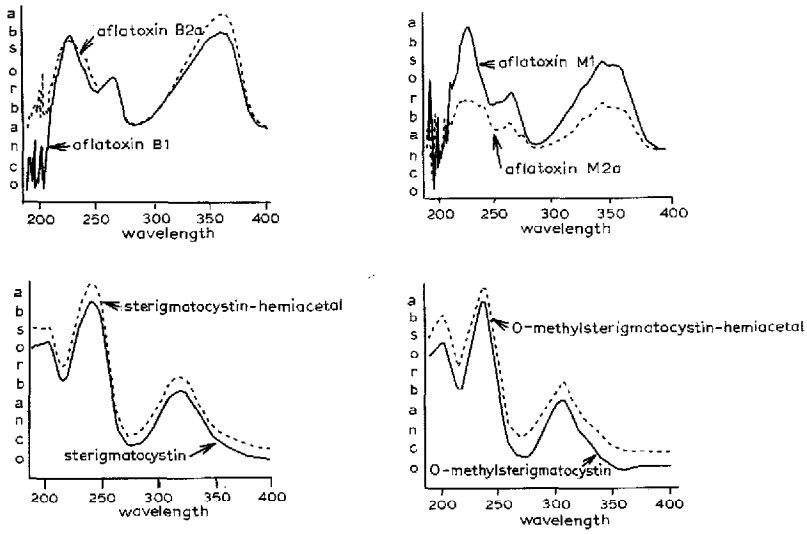


Fig. 2. UV spectra of native and hemiacetal structures of aflatoxin B_1, aflatoxin M_1, sterigmatocystin, and O-methylsterigmatocystin. See text for details. Wavelength in nm.

in Table II, depending on the mobile phase and the emission wavelength used, AFB_{2a} was nearly seven times more fluorescent than AFB. Maggon and Gopal[26] reported that the excitation maximum for AFB_{2a} dissolved in methanol shifted to 405 nm. When we tried to use 405 nm as the excitation wavelength with 430 nm as the emission wavelength, however, the signal-to-noise ratio increased significantly, reducing the overall detection limit. Because of the better signal-to-noise ratio with 365 nm excitation wavelength, this setting was used. By using the excitation wavelength of 365 nm and emission wavelength of 430 nm, 340 pg of AFB could be detected in 35% methanol mobile phase modified with THF; its hemiacetal could be detected at 50 pg in acetonitrile-modified mobile phase. The synthesis of the hemiacetal by the method we used was confirmed by NMR and IR. The hemiacetal was more polar and, therefore, earlier eluting by RPLC. In fact, relative to the parent molecule, AFB_{2a} was the most polar of the aflatoxin hemiacetals. The combination of increased fluorescence and reduced retention time can be used to confirm the presence of the native mycotoxins in a sample.

The hemiacetal of aflatoxin M_1 (AFM_{2a})

As with AFB, the shape of the UV spectra curves of AFM and its hemiacetal, as shown in Fig. 2, was not significantly different. Unlike AFB, the fluorescence of AFM was less affected by the mobile phase or the conversion to the hemiacetal. The greatest sensitivity for AFM (see Table II) was in an acetonitrile-modified methanol mobile phase where 80 pg could be detected when an emission wavelength of 430 nm was used. Under these same conditions, the greatest sensitivity for the hemiacetal could be obtained (60 pg). Fig. 3 shows the detector response to 10 ng of AFM and AFM_{2a} under these conditions. The conversion to the hemiacetal did not improve the detection limit for this mycotoxin by more than two times under any of the conditions tested. The native toxin, however, is detectable at lower levels (80 pg) than any of the

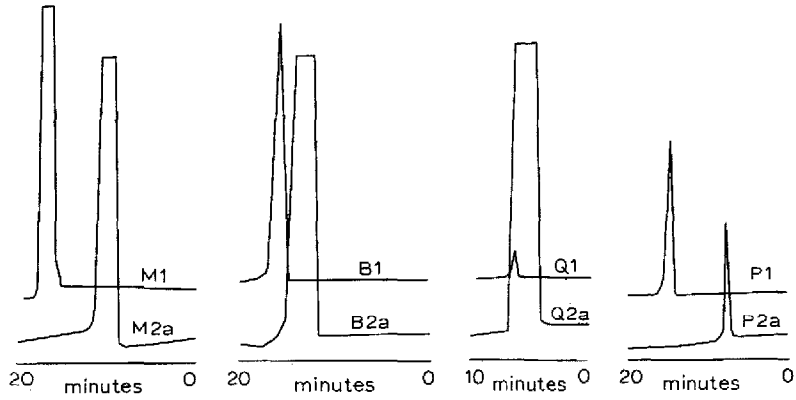


Fig. 3. Detector responses to 10 ng of analyte. With the fluorescence wavelength set at 365 nm, the conditions for optimal detection of aflatoxin metabolites are shown. See text for details.

other aflatoxin metabolites. The lower detection limit for the hemiacetal could be simply due to the fact that it is more polar and, therefore, earlier eluting than the native structure. Table III shows that of the aflatoxin metabolites, the conversion to the hemiacetal had the least effect on the retention time for AFM (relative retention times of the hemiacetal to the native compound ranged from 0.58 to 0.63). Investigators using RPLC for AFM analysis have been converting the native toxin to the hemiacetal to enhance fluorescence[23,24,27–29]. Using an excitation wavelength of 365 nm and emission wavelengths greater than 400 nm with mobile phases of methanol and/or acetonitrile, researchers were able to achieve between a two-fold and six-fold increase in fluorescence of AFM_{2a} over AFM[27–29]. Although we did not see such a marked improvement in fluorescence of the hemiacetal, it is not clear if this was because of improved detection for AFM by us or diminished detection for AFM_{2a}. Differences in detectors, filters, diffraction gradings, and chemical interaction greatly influence the fluorescence production and detection.

TABLE III

EFFECT OF THE MOBILE PHASE MODIFIER ON RETENTION TIMES

Column: Spherisorb C_8, 150 mm × 4 mm I.D. plus Spherisorb C_{18}, 150 mm × 4 mm I.D. Mobile phase: water–methanol–modifier (60:35:5). Modifiers: (a) THF + 0.3% H_3PO_4; (b) THF; (c) acetonitrile. Flow-rate: 0.5 ml/min.

Alfatoxin	*Mobile phase*					
	a		*b*		*c*	
	Native (min)	*Hemiacetal (min)*	*Native (min)*	*Hemiacetal (min)*	*Native (min)*	*Hemiacetal (min)*
B	23.0	10.0	16.4	8.2	38.0	13.6
M	12.0	7.6	9.8	6.2	16.8	9.8
P	17.4	8.4	13.4	6.8	19.0	9.0
Q	10.2	5.8	8.4	5.2	13.6	6.2

The hemiacetal of aflatoxin Q_1 (AFP_{2a})

As the native structure, the detection limit of aflatoxin Q_1 was nearly 100 times less sensitive than that of aflatoxin M_1. When 430 nm excitation was used, the detection limit was 12 ng. An emission wavelength of 500 nm, however, gave more sensitivity for this analyte with THF-modified methanol mobile phase providing the best sensitivity (detection limit 3 ng). As shown in Table II, synthesis of the hemiacetal improved the sensitivity 150 times (detection limit 20 pg; acetonitrile modifier, 500 nm wavelength). Of the four aflatoxins, AFQ demonstrated the greatest enhancement in fluorescence by the hydration reaction and was changed from one of the least fluorescent of the native toxins to the most fluorescent of the hemiacetals (see Fig. 3). Thus, we believe that the key to detecting AFQ by HPLC is to analyze for the hemiacetal.

The hemiacetal of aflatoxin P_1 (AFP_{2a})

Under similar conditions, the hemiacetals of AFB, AFM, and AFQ were always more fluorescent than the native toxin; however, this was not true for AFP. Table II shows that while AFP_{2a} was more fluorescent than the native toxin at the 430 nm emission wavelength, at the 500 nm wavelength the fluorescent signal for the native toxin was stronger. Because the fluorescence signal at 500 nm emission (0.6–1.4 ng) was more than ten times more intense than at 430 nm (8–25 ng), the longer wavelength should be used to analyze for the native toxin. By using 500 nm as the emission wavelength, however, conversion to the hemiacetal would not improve the detection limit for AFP (0.8–1.7 ng), but the shift in retention time would confirm its presence. Of the four aflatoxin metabolites, we found the hemiacetal of AFP to be the least fluorescent under the conditions tested (see Fig. 3).

Many HPLC methods use gradient elution to resolve the very similar structures of these mycotoxins. We were able to improve peak resolution by using two types of reversed-phase columns in series; when a C_{18} was coupled with a C_8 column, all of the peaks (four native aflatoxins and four hemiacetals) were resolved (Fig. 4 and 5). To optimize resolution, we evalutated three ternary mobile phases. Methanol modified with acetonitrile has been used by several investigators for RPLC[4–6], and others have added acid to the mobile phase[7]. THF was investigated as a modifier because it is less polar than either methanol or acetonitrile. Although the hemiacetals for AFB, AFM, and AFQ were more intensely fluorescent in the mobile phase modified with acetonitrile, the native toxins for AFB, AFP, and AFQ were more fluorescent when THF was used as the modifier. Acidification of the mobile phase with orthophosphoric acid modified with THF did not significantly enhance the fluorescence. More intense fluoresence signals were obtained for AFB, AFM, and their hemiacetals (AFB_{2a} and AFM_{2a}) by using 430 nm as the emission wavelength, whereas an emission wavelength of 500 nm allowed more intense signals for AFQ, AFP, and their hemiacetals (AFQ_{2a} and AFP_{2a}) (Fig. 4 and 5). Of those tested, a mobile phase of methanol–THF–water (35:5:60) provided the best resolution of the mycotoxins on the double-column system (Table III). In routine laboratory analysis for aflatoxins, the native structures are analyzed separately from the hemiacetals. We found that the conditions to best analyze for the native aflatoxins are a mobile phase of methanol, THF, and water, a C_{18} plus a C_8 column, an excitation wavelength of 365 nm, and an emission wavelength of 430 nm for detection of AFB and AFM and an emission

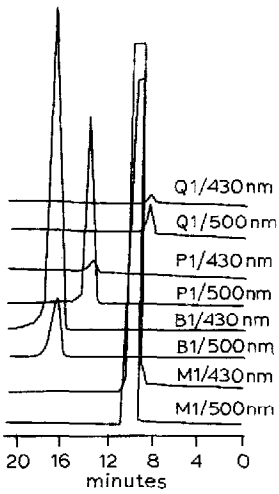


Fig. 4. Composite of chromatograms of 10 ng samples of native aflatoxins. A Spherisorb C_8 column was used in series with a Spherisorb C_{18} column. The mobile phase was a 35% aqueous methanol solution modified with 5% THF. The flow-rate was 0.5 ml/min. Fluorescence detection used 365 nm as the excitation wavelength; the emission wavelength was as indicated.

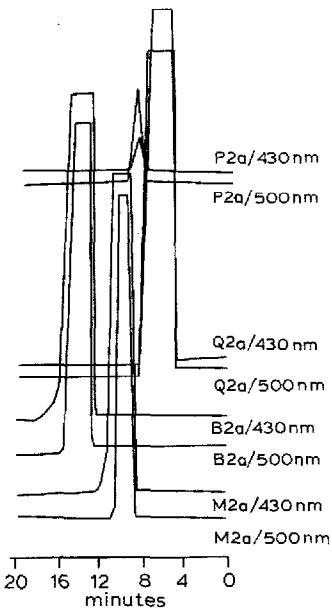


Fig. 5. Composite of chromatograms of 10-ng samples of the hemiacetal forms of aflatoxins. Column, flow-rate and detector conditions are as described in Fig. 4. The mobile phase was 35% aqueous methanol solution modified with 5% acetonitrile.

wavelength of 500 nm for detection of AFP and AFQ (Fig. 4). For detection of the hemiacetals, a mobile phase of methanol, acetonitrile, and water with the same detector settings provided maximum sensitivity, resolution, and confirmation (Fig. 5). Although these conditions may not be optimal for each of the mycotoxins, they are the good compromise of those tested.

CONCLUSION

In this study we showed that the hemiacetals for the metabolites of aflatoxin and sterigmatocystin can be easily prepared, and we confirmed this with NMR and IR for AFB and STR. The hemiacetals for AFP, AFQ and OMS have not been reported before. These hemiacetals have distinctive chromatographic and fluorescence properties (in the case of the aflatoxins) that can be used to confirm the identification and improve the detection of the mycotoxins. The native mycotoxins and the more polar hemiacetals can be resolved by HPLC. Many investigators use emission wavelengths of 435 nm, but for the fluorescence of AFP and AFQ, a wavelength of 500 nm results in greater sensitivity. The fluorescence is also influenced by the composition of the mobile phase. Maximum sensitivity for detection of these mycotoxins is obtained by optimizing the emission wavelength and the mobile phase composition. The ease in preparing the hemiacetal of these mycotoxins, the improved sensitivity for three aflatoxin metabolites, and the reduced retention time make this a good confirmatory test for the HPLC analysis.

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