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Quantitative determination of aflatoxin B1-oxime by column liquid chromatography with ultraviolet detection

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Abstract

Liquid chromatography was used for the quantification of aflatoxin B1-oxime (AFB1-oxime). The yield of AFB1-oxime in the reaction mixture was 89%, while after purification on silica gel it was 72%. LC analysis of the reaction mixture after silica gel fractionation revealed a retention time of 0.84 min for AFB1-oxime, 8.42 min for AFB1, 1.21 min for unknown 1 and 1.61 min for unknown 2. UV–visible analysis of the reaction mixture after silica gel fractionation showed a λ_{max} of 269 and 361 nm for AFB1-oxime, 263 and 360 nm for AFB1, 273 nm for unknown 1 and 275 nm for unknown 2. Excitation and emission wavelengths were found to be 269 and 368/438 nm for AFB1-oxime, 359/424 nm for AFB1, 270 and 367/450 nm for unknown 1 and 273 and 416/447 nm for unknown 2. The method may find versatile application in monitoring reactions for the preparation of oximes of various analytes for the synthesis of their immunogens.

Keywords: Aflatoxins; Aflatoxin B1-oxime

1. Introduction

Aflatoxins are secondary metabolites of *Aspergillus flavus* and *A. parasiticus* consisting of a dihydrodifurano or tetrahydrodifurano moiety fused to a substituted coumarin. Among the various aflatoxins, AFB1 is the most potent teratogen, mutagen and hepatocarcinogen [1]. The International Agency for Research in Cancer (IARC) has classified it as a Group 1 carcinogen [1,2]. The analytical techniques used to monitor AFB1 in edible products are column chromatography [3,4], thin layer chromatography [5], high-performance liquid chromatography [6,7], high-performance thin layer chromatography [8], ELISA [9,10] and immunoaffinity cleanup methods coupled to liquid chromatography [11]. The simplicity, sensitivity and rapid detection of AFB1 by ELISA has made possible the monitoring of several samples simultaneously [12]. ELISA and other immunochemical methods require highly specific polyclonal or monoclonal sera for specific and sensitive detection of antigens [13].

AFB1, being a small molecule of low molecular mass, is unable to elicit an immune response when injected into animals. To raise polyclonal antisera against AFB1, binding to a high molecular mass protein such as bovine serum albumin (BSA) is a prerequisite [14]. This step requires activation of the AFB1 molecule by introduction of a reactive carboxyl group from carboxymethoxyl amine, leading to the formation of aflatoxin B 1-1-(*o*-carboxymethyl)

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oxime (AFB1-oxime) [15]. The addition of a free carboxyl group changes the polarity of the AFB1 molecule, which facilitates the separation and qualitative detection of AFB1 and AFB1-oxime by silica gel column chromatography and TLC, respectively [15]. Until now, quantitative analysis of the product(s) of this reaction has not been attempted and hence the products of the reaction as well as the quantity of AFB1-oxime to be used for preparation of the immunogen have not been ascertained.

In the present study, attempts were made to quantify the conversion of AFB1 into AFB1-oxime by reversed-phase liquid chromatography together with a study of the UV–visible and fluorometric characteristics of the reaction mixture containing unreacted AFB1, AFB1-oxime and unknown by-products, as well as the individual characteristics of these components after purification over a silica gel column. The method may find versatile application in the monitoring of oximes of various analytes for the synthesis of their immunogens.

2. Experimental

2.1. Chemicals

Standard AFB1 was procured from Sigma (St. Louis, MO, USA). Carboxymethoxyl amine hemihydrochloride was obtained from Aldrich (Milwaukee, WI, USA). Solvents were of the highest purity available commercially. Precoated silica gel plates, methanol and acetonitrile (Omnisolv), liquid chromatography grade, were the products of E. Merck (Dermstadt, Germany).

2.2. Reaction of AFB1 with carboxymethoxyl amine

Aflatoxin B1 was converted into AFB1-oxime essentially by the method of Chu et al. [15]. Four milligrams of AFB1 was refluxed for 2.5 h at 110°C with 6.36 mg carboxymethoxyl amine dissolved in 3.2 ml of a mixture of pyridine–water–methanol (1:1:4, v/v) and left at room temperature overnight in the dark. The reaction mixture was dried on a rotary evaporator and redissolved in chloroform–methanol (9:1, v/v).

2.3. TLC of the reaction mixture

TLC was performed on precoated silica gel G glass plates to confirm the conversion of AFB1 into AFB1-oxime in either of two solvent systems: (I) chloroform–acetone (9:1, v/v), (II) benzene–acetic acid (9:1, v/v).

2.4. Purification of AFB1-oxime

The reaction mixture dissolved in chloroform–methanol (9:1, v/v) was loaded onto a silica gel (mesh size 60–120) column (20 cm×1 cm) pre-equilibrated with chloroform. The column was initially eluted with chloroform followed by a mixture of chloroform–methanol (9:1, v/v). Fractions (2 ml) were collected on a LKB 2070 UltroRac II Fraction Collector (Bromma, Sweden) and the absorbance of each fraction was read at 365 nm in a double-beam spectrophotometer (Perkin-Elmer Lambda Bio 20). The column was finally washed with methanol. The fractions were pooled according to the absorbance peaks and dried on a rotary evaporator for UV–visible, fluorescence and liquid chromatographic analysis.

2.5. UV–visible analysis

Spectra of the reaction mixture, pooled fractions following silica gel chromatography and that of standard AFB1 were recorded from 220 to 500 nm on a double-beam spectrophotometer.

2.6. Fluorescence analysis

Excitation and emission spectra of the reaction mixture, pooled fractions following silica gel chromatography and that of standard AFB1 were recorded at a bandwidth of 5 and 10 nm for excitation and emission, respectively, on a Perkin-Elmer LS-50B Luminescence Spectrometer.

2.7. Liquid chromatographic analysis

A liquid chromatography (LC) instrument (Waters) equipped with a Waters dual pump Model 510, an Octadecyl C_{18} guard column of pellicular media (Whatman, Clifton, NJ, USA) and a reverse-phase

column (Lichrospher R100 RP-18, 5 μm, E. Merck) was used for analysis. LC analysis of the pooled fractions was performed at ambient temperature under isocratic conditions with a mobile phase containing water–methanol–acetonitrile (60:20:20, v/v) at a flow-rate of 1 ml/mm. The eluate was monitored on a Waters UV–Vis absorbance detector (Model 486) at 365 nm and chromatograms were recorded on a Waters Integrator (Model 746).

3. Results and discussion

TLC of the reaction mixture (containing AFB1, carboxymethoxyl amine and the products, including AFB1-oxime) developed in solvent system I and viewed under 365 nm in a UV dark room chamber revealed three fluorescent spots, one at the site of application representing AFB1-oxime, the second at R_F 0.53, which was comparable to standard AFB1, and, third, an unknown spot at R_F 0.73. In solvent system II the spots representing AFB1-oxime and AFB1 showed respective R_F values of 0.13 and 0.21, while the value for the unknown was 0.57 (data not shown).

The LC resolution of the reaction mixture at 365 nm is depicted in Fig. 1. The standard AFB1 showed a single peak at RT 8.36 min which matched the unreacted AFB1 peak of the reaction mixture. The chromatogram of the reaction mixture showed three distinct peaks: (1) AFB1-oxime at RT 0.88 min, (2) an unknown at RT 1.55 min, and (3) unreacted AFB1 at RT 8.33 min (Fig. 1). The three peaks in the chromatogram of the reaction mixture displayed the following yields: peak (1) 3.56 mg (89%), peak (2) 0.26 mg (6.5%), and peak (3) 0.15 mg (3.75%) (Table 1).

Fig. 2 shows the UV–visible spectral characteristics of the reaction mixture and standard AFB1. The spectra of the reaction mixture and standard AFB1 show a λ_{max} at 360 nm, while an additional peak at 251.8 nm was observed for the reaction mixture, but not in the AFB1 spectra (Fig. 2). Calculation according to absorbance at 360 nm showed a yield of 3.86 mg of the fluorescent material including AFB1 and AFB1-oxime (Table 1).

The fluorescence properties of the reaction mixture and standard AFB1 are depicted in Table 2. The

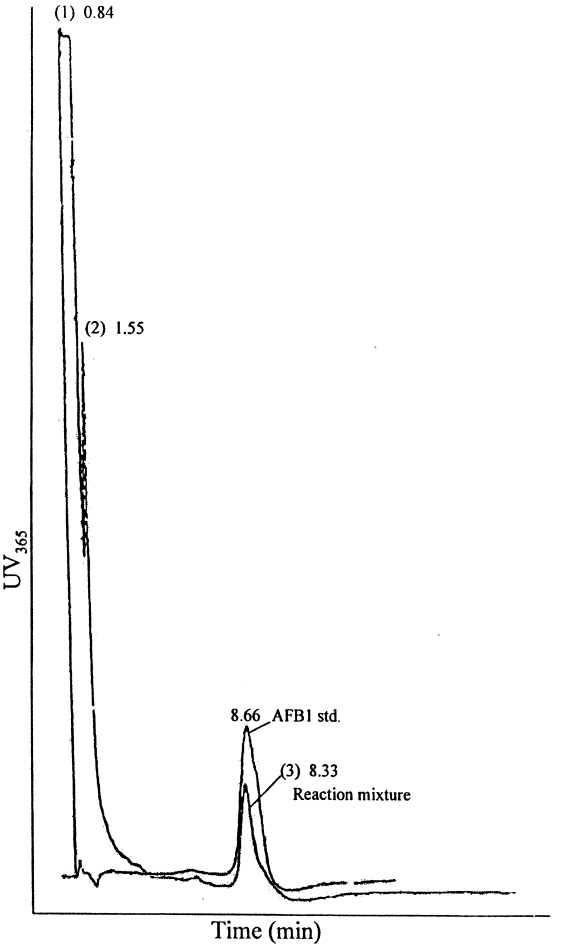

Fig. 1. LC–$UV_{365\ nm}$ trace of a reaction mixture containing AFB1 and carboxymethoxyl amine and a comparison with standard AFB1.

reaction mixture showed excitation maxima at 269 and 366 nm with emission at 431 nm, while AFB1 showed excitation and emission maxima at 359 and 425 nm, respectively. Quantitation of the products could not be performed due to lack of reference standards.

Purification of AFB1-oxime from the reaction mixture by silica gel column fractionation is shown in Fig. 3. The eluted fractions when analysed at 365 nm showed four distinct absorbance peaks. TLC of different fractions in a solvent system containing chloroform–acetone (9:1, v/v) was carried out to identify the fractions containing AFB1-oxime. Four peaks were eluted from the column: peak 1, fractions

Table 1
Comparative yield of AFB1-oxime detected by liquid chromatography and UV–visible analysis

	Liquid chromatography coupled with UV detection[a] (mg)		UV–visible analysis (mg)	
	Reaction mixture	Purified fractions	Reaction mixture	Purified fractions
AFB1	0.15	0.097	3.86	0.087
AFB1-oxime	3.56	2.88		2.32
Unknown 1	0.26	0.171		NC[c]
Unknown 2	ND[b]	0.089		NC[c]

[a] Yields calculated according to percent peak area in the chromatogram.
[b] ND, not detected.
[c] NC, not calculated.

1 to 28, representing AFB1; peak 2, fractions 46 to 100, representing AFB1-oxime; peak 3, fractions 109–118 of unknown 1; and peak 4, fractions 119–127 of unknown 2. Peaks 1, 2 and 3 were eluted by chloroform–methanol (9:1, v/v) while peak 4 was eluted by 100% methanol.

The LC–$UV_{365\ nm}$ trace of the four individually pooled peak fractions eluted from the silica gel column are shown in the inset of Fig. 4. Eluted peaks 1 and 2 show a RT of 8.42 and 0.84 min, corresponding to AFB1 and AFB1-oxime, respectively. Peaks 3 and 4 eluted from the silica gel column show RT of 1.21 and 1.61 min, respectively, representing the two unknown compounds (inset of Fig. 4). The yields of AFB1-oxime, unreacted AFB1 and the two unknown peaks were 2.88 mg (72%), 0.097 mg (2.4%), 0.171 mg (4.3%) and 0.089 mg (2.2%), respectively (Table 1).

UV–visible analysis of the four peaks eluted from the silica gel column is shown in Fig. 4. Peak 1 revealed a typical AFB1 spectrum with λ_{max} at 360

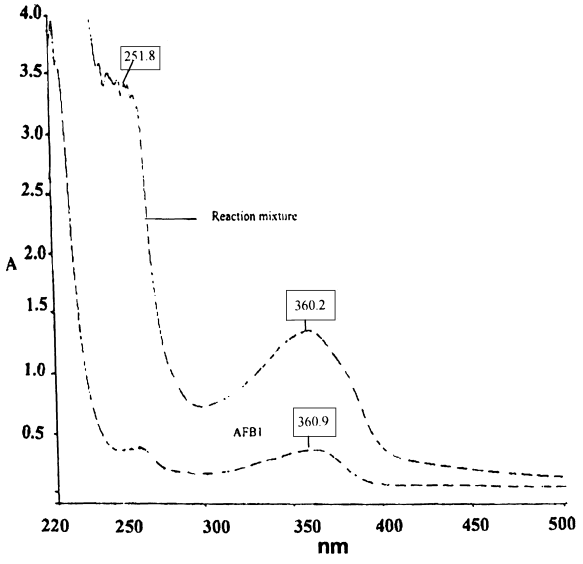

Fig. 2. UV–visible spectra of a reaction mixture containing AFB1 and carboxymethoxyl amine and a comparison with standard AFB1.

Table 2
Excitation and emission wavelengths of standard AFB1, the reaction mixture and purified fractions collected following silica gel column chromatography

Sample	Excitation λ_{max} (nm)	Emission λ_{max} (nm)
Standard AFB1	359.1	432.1
Reaction mixture	269.1	431.1
	365.8	430.9
Eluted silica fractions		
Peak 1, unreacted AFB1	359.1	424.6
Peak 2, AFB1-oxime	268.9	433.6
	367.9	430.9
Peak 3, unknown 1	270.2	450.3
	366.9	450.3
Peak 4, unknown 2	272.8	446.9
	416.3	446.9

The wavelengths were recorded from the spectra using an excitation slit of 5 nm and an emission slit of 10 nm.

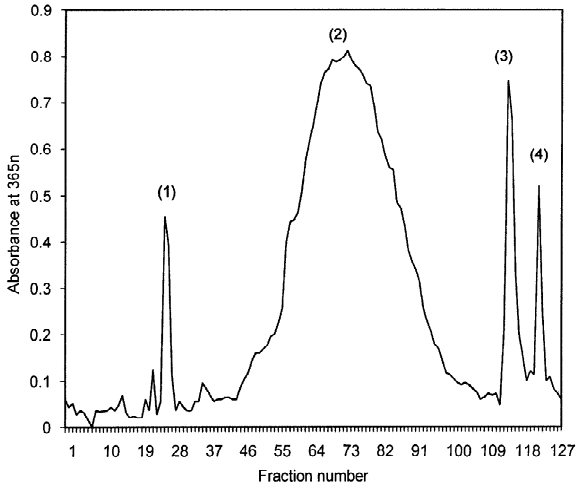

Fig. 3. Absorbance (A_{365}) of fractions collected following silica gel column chromatography of a reaction mixture containing AFB1 and carboxymethoxyl amine.

nm (Fig. 4a). Peak 2, identified as AFB1-oxime, showed a spectrum having absorbance maxima at 361 and 269 nm (Fig. 4b). Peaks 3 and 4 eluted from the column showed λ_{max} at 273.6 and 275.7 nm, respectively, corresponding to unknowns 1 and 2 (Fig. 4c and d). The yield of purified AFB1-oxime and the quantity of unreacted AFB1 estimated by absorbance were found to be 2.32 mg (58%) and 0.087 mg (2.2%), respectively (Table 1).

The fluorescence characteristics of the four peaks eluted from the silica gel column are shown in Table 2. Peak 1, representing AFB1, exhibited excitation and emission maxima at 359 and 425 nm, respectively. Peak 2, identified as AFB1-oxime, showed excitation maxima at 269 and 368 nm with an emission maximum at 432 nm for both excitations. Peaks 3 and 4 eluted from the column showed excitation λ_{max} at 270 and 272 nm, respectively, with an emission maximum at 450 nm for both fractions, corresponding to unknowns 1 and 2. Quantitation of the products could not be performed due to lack of reference standards.

Several methods have been described for the quantitative estimation of AFB1 [16]. However, analysis of AFB1-oxime is performed either by TLC or by comparing the absorbance at 362 nm with the molar absorptivity of 20 950 [14]. The results presented in the present communication show that quantification of AFB1-oxime in the reaction mixture itself could be performed by LC, which is not possible using existing analytical methods.

UV–visible analysis of the reaction mixture suggests that unreacted AFB1 and AFB1-oxime have a common λ_{max}. Hence, the absorbance observed at 360 nm is the combined result of both the reactant and the product. However, LC–$UV_{365\ nm}$ gives a clear resolution in the form of different peaks for unused reactant, AFB1, and products, including AFB1-oxime. The individual products were quantified on the basis of peak area, which is not possible by UV–visible measurements.

LC–$UV_{365\ nm}$ analysis of the fractions collected after column chromatography helped in estimating the final recovery of AFB1-oxime and other unknown products. The results of UV–visible analysis of purified AFB1-oxime are quite similar to that for standard AFB1, suggesting that the heterocyclic chromophore of AFB1 may not be altered during the reaction [15].

It is interesting to note that unknown peak 4 eluted from the silica gel column by methanol was not observed on TLC, indicating it to be of non-fluorescent nature. LC of peak 4 showed a RT of 1.61 min. Unknown peaks 3 and 4 with a λ_{max} at 270 nm indicate similar structures. However, the differences in the UV–visible characteristics of the unknown from that of AFB1 suggest that the structure of the unknown may be different from AFB1 or AFB1 derivatives. Attempts are being made to characterise the structures of these unknown peaks eluted from the silica gel column in order to understand the reaction mechanism of AFB1-oxime formation.

Overall, the results suggest that the method may find versatile application in the monitoring of the oximes of various analytes for the synthesis of their immunogens.

4. Nomenclature

AFB1	aflatoxin B1
AFB1-oxime	aflatoxin B 1-1-(*o*-carboxymethyl) oxime
TLC	thin layer chromatography

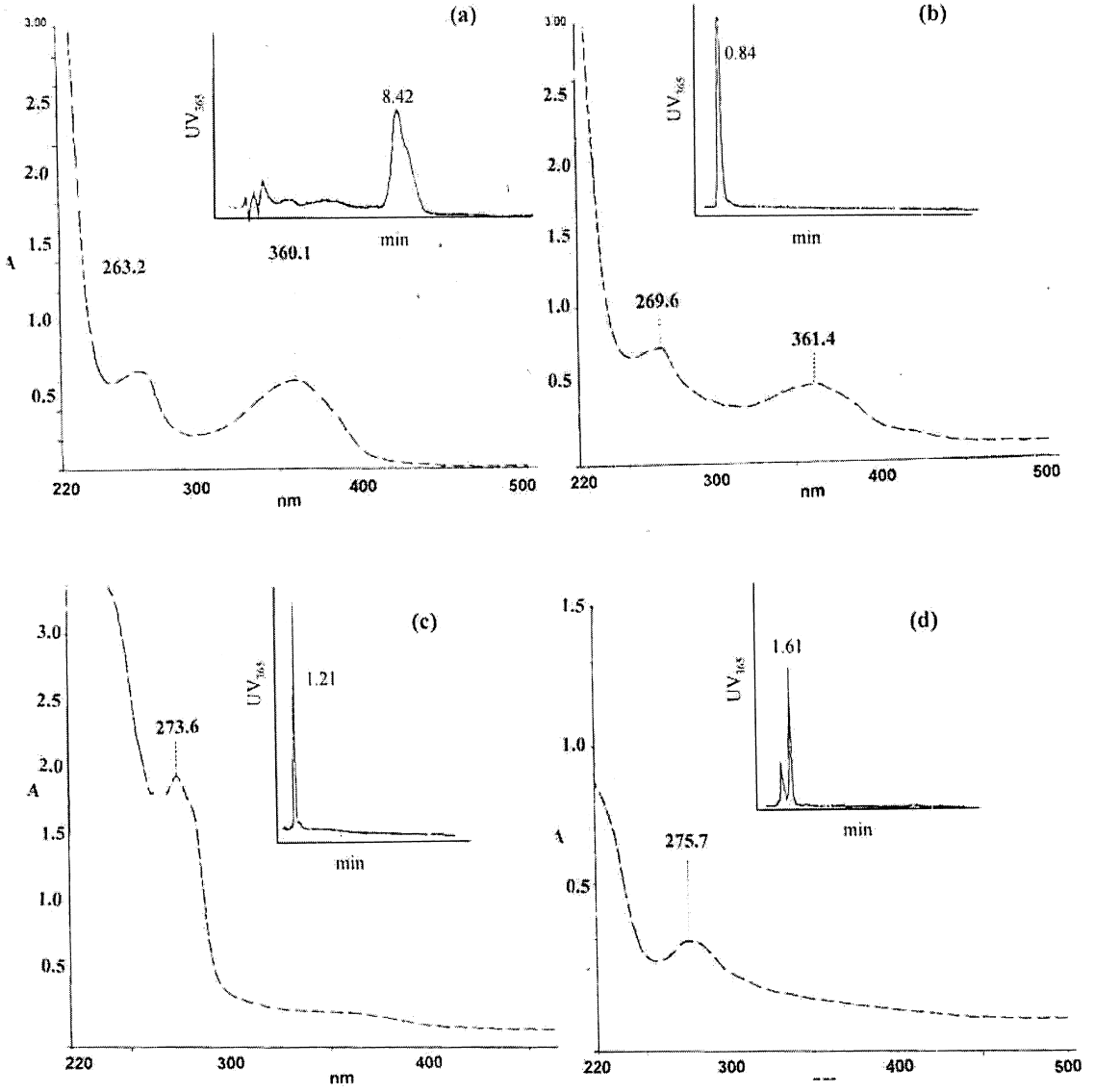

Fig. 4. UV–visible spectra and LC–UV_{365} nm trace (the inset shows the LC–UV trace) of purified fractions collected following silica gel column chromatography of a reaction mixture containing AFB1 and carboxymethoxyl amine: (a) peak 1 of Fig. 3, unreacted AFB1; (b) peak 2 of Fig. 3, AFB1-oxime; (c) peak 3 of Fig. 3, unknown 1; (d) peak 4 of Fig. 3, unknown 2.

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