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Note

High-pressure liquid chromatography of oxidative aflatoxin metabolites

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The successful applications of high-pressure liquid chromatography (HPLC) in the analysis of aflatoxins (AF)[1–5] have shown great promise that this technique may become the basis for a method of choice for the routine monitoring of AF residues in food samples. This rapidly developing technique offers the advantages of speed, good resolution, and a high degree of precision and accuracy at a sensitivity comparable to the widely used thin-layer chromatographic (TLC) techniques[6–8].

In the present report we describe an application of HPLC to the analysis of some oxidative metabolites of AFB_1. The latter is known to be oxidized by animal tissues to form at least AFQ_1 (refs. 9–11), AFM_1 (ref. 12), AFB_{2a} (ref. 13) and aflatoxicol H_1 (AFH_1) (ref. 14). The use of HPLC analysis has considerably facilitated studies on the *in vitro* and *in vivo* metabolism of AFB_1.

EXPERIMENTAL

Aflatoxins

AFB_1 was purchased from Calbiochem, La Jolla, Calif., U.S.A. AFM_1 and AFQ_1 were prepared from monkey liver homogenates according to the method of Hsieh *et al.*[15] AFH_1 was prepared by the method of Salhab and Hsieh[14]. AFB_{2a} was chemically converted from AFB_1 with dilute acid[16]. The purity of all standards was checked by TLC–fluoridensitometric analysis before use.

Equipment

A Micromeritics Model 7000 high-pressure liquid chromatograph (Micromeritics, Norcross, Ga., U.S.A.) was used with a Schoeffel GM 770 monochromator and Schoeffel SF 770 spectroflow monitor (Schoeffel, Westwood, N.J., U.S.A.). An LDC Model 1309 fluorimonitor (Laboratory Data Control, Riviera Beach, Fla., U.S.A.) was connected in series proceeding the UV detector for most measurements. All separations were obtained on a Zorbax L (24 cm × 2.1 mm I.D.) column (DuPont, Wilmington, Del., U.S.A.).

TLC measurements were obtained on a Schoeffel Model SD 3000 recording spectrodensitometer operated in the transmission mode; excitation wavelength, 360 nm; emission wavelength, 410 nm; slit width, 1 × 10 mm; scanning speed, 4 in./min; attenuation, 0.4 absorbance units full scale (a.u.f.s.). Scans were made in the direction of development.

Mass spectral analysis was done on a Finnigan Model 3000 peak identifier (Finnigan, Sunnyvale, Calif., U.S.A.): beam current, 70 eV. The sample was introduced via a solid probe inlet system.

Thin-layer chromatography

Pre-coated (0.25 mm) 20 × 20 cm silica gel plates (E. Merck, Elmsford, N.Y., U.S.A.) were developed in unlined chambers containing chloroform–acetone–isopropanol (85:15:2.5).

High-pressure liquid chromatography

The mobile phase consisted of a solution of methylene chloride–chloroform (3:2), 50% saturated with water[5]. Methanol (0.9%) was used as a modifier unless otherwise noted. All solvents were nanograde quality. The saturation was accomplished by shaking the methylene chloride–chloroform mixture with water and then combining the organic layer with an equal volume of the dry solvent mixture. The chromatograph was operated in the constant-pressure mode (approx. 2360 p.s.i.) so as to maintain a flow-rate of approximately 0.7 ml/min.

Injections for standard curves were made on-column with the stop-flow technique. Solutions containing equal amounts of each compound (5 ng/μl) were injected at 50-, 100-, 150- and 200-ng levels. The lines were plotted using a least-squares fit for the data points and using (0,0) as an additional data point. Peak areas (mm^2) were calculated by multiplying the peak height by the width at half-height or the cut-and-weigh method.

RESULTS AND DISCUSSION

Isolation and detection

All four metabolites and AFB_1 were resolved by HPLC in 20 min as detected

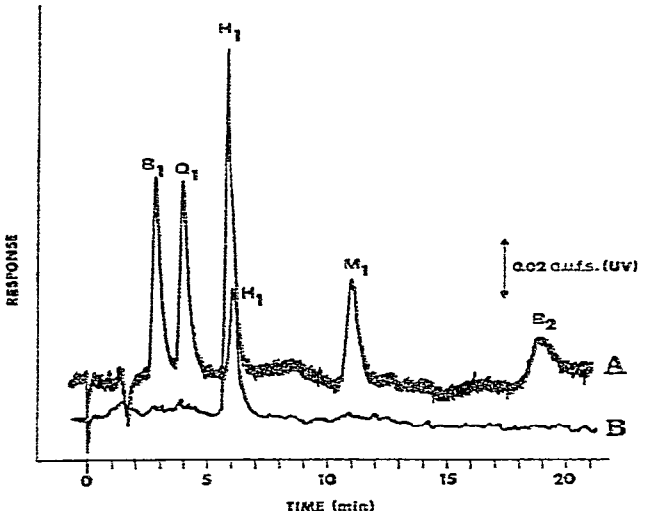

Fig. 1. High-pressure liquid chromatogram of aflatoxins B_1, Q_1, M_1, B_{2a}, and aflatoxicol H_1 (10 ng each) as detected by UV absorption at 345 nm (A) and by fluorescence (B).

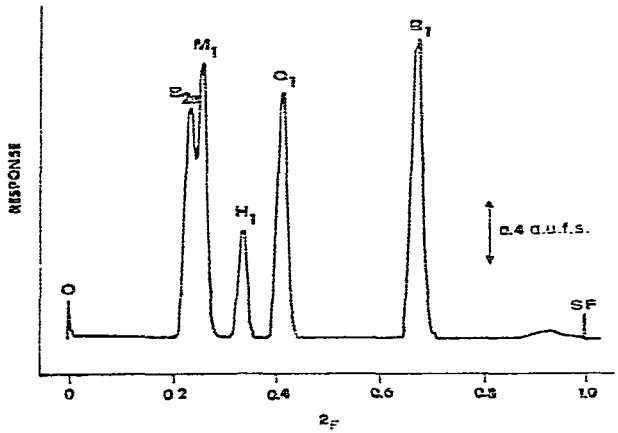

Fig. 2. Thin-layer chromatogram of aflatoxins B_1, Q_1, M_1, B_{2a}, and aflatoxicol H_1 (10 ng each) as detected by spectrofluoridensitometry.

by UV monitoring at 345 nm (Fig. 1). Compared to TLC (Fig. 2), on which AFM_1 and AFB_{2a} are only partially resolved, HPLC achieves a considerable improvement in the analysis of AFB_1 metabolites. All the compounds could be measured with good precision at the 5-ng level with HPLC although it was necessary to decrease the retention times for AFM_1 and AFB_{2a} for precision.

Since AFH_1 has its UV absorption maximum at 330 nm rather than 360 nm as for the other four compounds, AFH_1 was barely visible at the 5-ng level when detected by UV absorption at 360 nm (Fig. 3). At 345 nm, a compromise in detection was achieved in which all five compounds gave approximately the same response on an area basis with insignificant reduction in the overall sensitivity. Using a fixed-

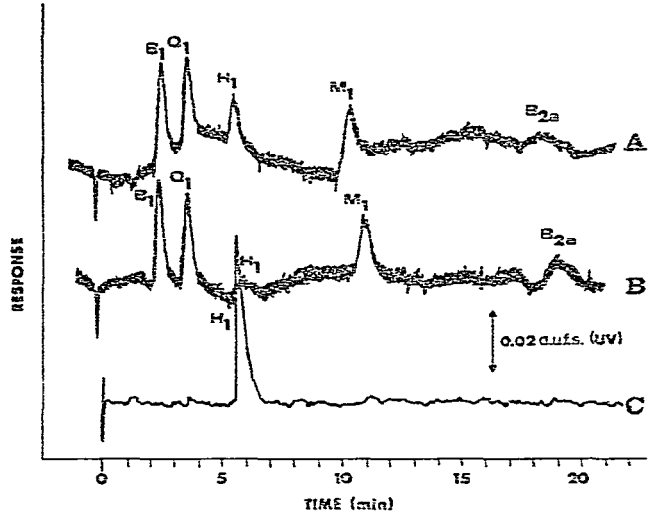

Fig. 3. Comparison of detection by absorption at 345 nm (A), 360 nm (B), or by fluorescence (C) of aflatoxin metabolites (at the 5-ng level) isolated by HPLC.

wavelength broad-band fluorescence detector, the response of AFH_1 was strongly enhanced, but the other compounds became barely detectable (Fig. 3). This response information is useful for confirming residues observed at a fixed wavelength. Using either 345 or 360 nm as the detection wavelength, all compounds gave linear responses in the range of 5–200 ng.

Reproducibility of AFB_1 injections was checked and found to be comparable to TLC–spectrodensitometric methods. Ten replicate injections were made and the mean value based on two different methods of integration (cut and weigh, and height times width at half height) had a 99% confidence limit of less than $\pm 6\%$. The mean of randomly chosen triplicate samples from the above ten injections was within 7% of the overall mean. The mean of triplicate injections of the metabolites with the exception of AFB_{2a} had a 95% confidence limit of $\pm 15\%$.

It should be noted that since the fluorescence detector was connected between the column and the UV detector, some tailing and peak broadening occurred in the UV signals which would not have happened with a single detector.

Application to metabolic studies

Using the parameters described above, HPLC was used to analyze chloroform extracts of the urine of rhesus monkeys previously dosed by oral administration with AFB_1. The chromatogram (Fig. 4) clearly indicates that AFM_1 is the predominate metabolite. Mass spectral analysis of the peak provided absolute confirmation. Identification of AFM_1 with TLC would have been unsatisfactory because of the poor resolution between AFM_1 and AFB_{2a}. HPLC was also used to analyze chloroform extracts of reaction mixtures containing AFB_1 and rhesus monkey liver homogenates. Results similar to those depicted in Figs. 1 and 3 were routinely obtained.

Once possible metabolites are identified, resolution and retention time can be optimized for a particular compound by adjusting the methanol content of the solvent. If there is a wide range of retention times for the peaks of interest, a preliminary run can be made to separate compounds by their relative retention times. Each fraction

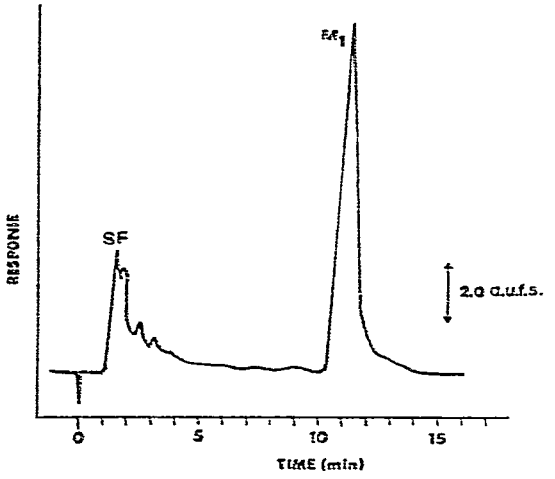

Fig. 4. High-pressure liquid chromatogram of aflatoxin metabolites in the chloroform extract of the urine excreted by rhesus monkeys orally given aflatoxin B_1.

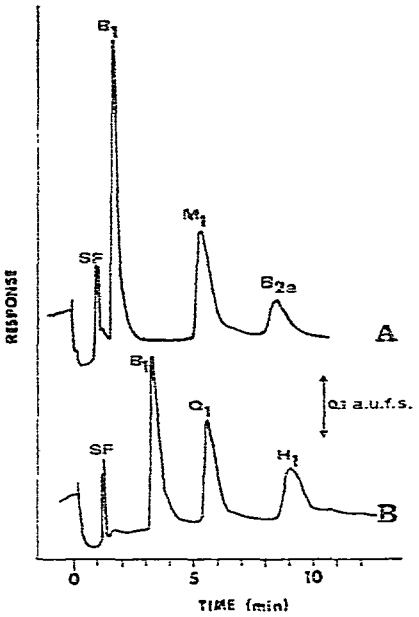

Fig. 5. Modification of retention times of various aflatoxin metabolites by adjusting the methanol content of the eluting solvent. (A) 1.3% methanol; (B) 0.5% methanol.

can then be re-injected under conditions suitable for optimal elution time and resolution (Fig. 5).

Comparison with TLC

HPLC offers many advantages over TLC for metabolic studies. It is, for example, especially valuable for preparative work since individual peaks can be collected without exposure to air or light during the chromatographic run. By using a preparative column, large quantities of extracts can be processed in a short time. In most cases HPLC also offers better resolution than TLC, and by slight modification of the solvent's polarity, the peaks of interest can be better resolved for purification purposes. HPLC also facilitates identification of AFM_1, AFB_{2a}, and AFH_1 as described previously.

HPLC on the other hand is not as efficient for analysis of a large number of samples. Multiple spottings on TLC only slightly increase the overall analysis time, whereas multiple injections on HPLC become time-consuming. Also there is the problem of peak broadening for the later eluting components on HPLC which makes it difficult to accurately measure peak areas. The delayed elution of very polar impurities can become overlapping contaminants in subsequently injected samples. Although this problem can be minimized by running a gradient elution to clean the column between runs, this expedient requires additional time to re-equilibrate the column to the original conditions.

The solvent system used in this study presented another minor problem in maintaining a constant amount of water on the HPLC column. After some time, excessive water may become adsorbed to the column packing resulting in deterio-

ration of resolution and peak shape. This problem may be easily corrected by running dry solvent through the system for a short period of time and then returning to the original solvent mixture having 50% water saturation.

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