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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF AFLATOXINS IN HUMAN URINE

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SUMMARY

A method was developed for the extraction and determination of unconjugated aflatoxins in human urine by high-performance liquid chromatography. The analysis is based on the elimination of lipid-soluble constituents other than unconjugated aflatoxins in urine by light petroleum extraction. The unconjugated aflatoxins were subsequently extracted from the aqueous phase with chloroform—acetone. Chromatography was performed isocratically with a silica column at 40°C. The resolved aflatoxins were detected and identified by ultraviolet and fluorometric detectors. The recoveries of aflatoxins B_1 and G_1 added prior to the extraction were 72% and 83%, respectively. This procedure is simple, sensitive and practically useful for epidemiological survey of unconjugated aflatoxins in human urine from areas with a high risk of aflatoxin consumption.

INTRODUCTION

Aflatoxins are major metabolites produced by some strains of *Aspergillus flavus*. Their role as a potent hepatotoxic agent as well as a hepatocarcinogen is evident in a number of animal species [1, 2]. The contamination of human food by these mycotoxins and the detection of aflatoxins in autopsy specimens as reported elsewhere have suggested a role for aflatoxins in human diseases, in particular, acute encephalopathy with fatty degeneration of viscera and hepato-

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cellular carcinoma [2–7]. Nevertheless, none of the reports have provided direct evidence for the existence of aflatoxins in living human tissues or biological fluids.

Data from experimental animals show that the major portion of aflatoxin B_1 consumed is metabolized in the liver. Both native aflatoxins and their metabolic products are excreted mainly in urine and bile [1, 8]. Approximately 0.2% of the oral dose of aflatoxin B_1 was excreted unchanged in the urine of primates within 24 h [9]. The detection of aflatoxins and their metabolic products in human urine will serve as unquestionable proof of the presence of aflatoxin in humans. In addition, the identification of aflatoxin derivatives in urine may help in the understanding of aflatoxin metabolism in man.

In order to detect the presumably small amount of native aflatoxins in human urine, extremely sensitive methods for extraction and detection are required. Gregory and Manley [10] described a procedure for the high-performance liquid chromatographic (HPLC) determination of aflatoxins in animal tissues and products. The recoveries of aflatoxins B_1 and G_1 blended with whole milk before the extraction were 72 and 75% respectively, with a detection limit of 0.05 ± 0.1 ng/g for each aflatoxin. Reversed-phase as well as normal-phase HPLC was used for the analysis of aflatoxins, with the minimum detectable amount of each aflatoxin in the range 1–2 ng using an ultraviolet (UV) detector and 0.3 ng with a fluorescence detector [10–14].

In this paper, we describe a simple method for the extraction and sensitive detection of unconjugated aflatoxins in human urine, based on normal-phase HPLC equipped with both UV and fluorometric detectors.

EXPERIMENTAL

Chemicals

Aflatoxins B_1 and G_1 were purchased from Makor Chemical (Jerusalem, Israel). They were dissolved in an appropriate volume of absolute methanol. Standard solutions containing 0.06 and 0.02 μg of aflatoxins B_1 and G_1, respectively, and 3.3 μg each of aflatoxins B_1 and G_1 per ml of elution solvent were subsequently prepared and used for HPLC analysis.

Extraction of aflatoxins from urine

Five millilitres of human urine were used for each extraction. The urine sample was extracted twice, each time with 50 ml of light petroleum (b.p. 40–60°C), to eliminate lipid- and fat-soluble constituents. Aflatoxins in the aqueous portion were extracted with 50 ml of chloroform. After a second extraction with 50 ml of chloroform–acetone (1:1, v/v), the organic phases were combined, shaken with 10 g of anhydrous sodium sulfate, and filtered through Whatman No. 40 paper under vacuum. The aliquot was evaporated to dryness at approximately 50°C in a rotary evaporator and stored at –20°C in nitrogen atmosphere and protected from exposure to light. Each extracted sample was dissolved in 2 ml of elution solvent and filtered through a Millipore filter (pore size 0.45 μm) before analysis.

Equipment

Normal-phase chromatography was performed with a DuPont Model 870 HPLC system, equipped with a universal septumless injector, temperature-controllable column compartment and a variable-wavelength spectrophotometer. The fluorescence of resolved samples was determined with a connected Jasco Model FP 550 spectrofluorometer. Separations were achieved with a Zorbax Sil column (25 cm × 4.6 mm I.D., particle size 6 μm, DuPont Co., Wilmington, DE, U.S.A.) at an elution rate of 1 ml/min and a nominal pressure of 10.4 MPa. The temperature in the column compartment was maintained at 40°C. Detector wavelength was 362 nm for spectrophotometric absorption. With fluorescence detection, the samples were excited at 365 nm and the fluorescence was recorded at 430 nm. The amount of aflatoxins in the extract was calculated from the known concentration of standard aflatoxins and the area under the UV-absorbing peak measured by a Hruden planimeter (the use of peak height analysis yielded similar results).

Elution solvent system

The elution system consisted of analytical grade toluene—ethyl acetate—absolute methanol—90% formic acid (89:7:2:2, v/v) [13]. Ethyl acetate, absolute methanol and 90% formic acid were mixed prior to the addition of toluene. The solvent was filtered through a Millipore filter (pore size 0.45 μm) and degassed by stirring under vacuum.

RESULTS AND DISCUSSION

A chromatogram of standard aflatoxins B_1 and G_1 is shown in Fig. 1. Aflatoxins B_1 and G_1 were eluted with retention times of 9.5 and 16.5 min, respectively. The extremely high fluorescence of aflatoxins B_1 and G_1 was also demonstrated in the same chromatogram and was used for the identification of aflatoxins. Under the conditions employed in this study, the minimum detectable amounts of aflatoxins B_1 and G_1 were 3 and 1 ng per 50 μl of injected amount, respectively (Fig. 2).

Analysis of aflatoxins in urine

A chromatogram of an extract from normal urine is shown in Fig. 3. Seta et al. [15] observed more than 100 UV-absorbing constituents in human urine by anion-exchange HPLC. Among these, 33 components were identified. In the present report, several lipid- and fat-soluble constituents in urine were removed by extraction with light petroleum and only a few UV-absorbing constituents were left and resolved by normal-phase HPLC; none of them exhibited fluorescence.

Aflatoxins that were added to urine prior to extraction remained in the aqueous phase after light petroleum extraction and were subsequently recovered in the chloroform extract. The chromatogram of an extract from human urine with added aflatoxins (Fig. 4) explicitly shows the UV-absorbing and concomitant fluorescent peaks of aflatoxins B_1 and G_1. The recoveries were 72% and 83% of the added amounts for aflatoxins B_1 and G_1, respectively.

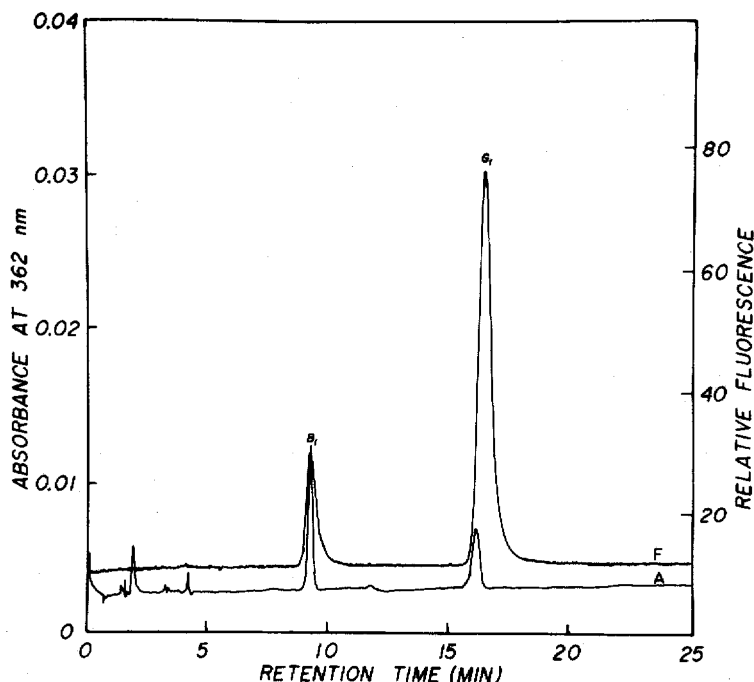

Fig. 1. HPLC resolution of aflatoxins B_1 and G_1 (0.16 μg of each) on Zorbax Sil column. Mobile phase toluene—ethyl acetate—methanol—90% formic acid (89:7:2:2), flow-rate 1.0 ml/min, column temperature 40°C, UV detection at 362 nm (A) at 0.04 a.u.f.s., fluorescence detection (F) as described in the text. The injected amount was 50 μl.

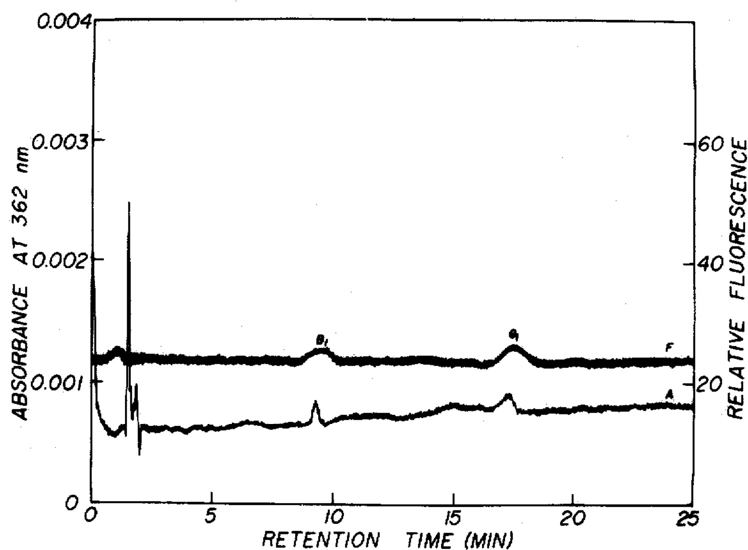

Fig. 2. HPLC resolution of aflatoxins B_1 (3 ng) and G_1 (1 ng). The operating conditions for HPLC were similar to those described in the legend to Fig. 1. The UV chromatogram was recorded at 0.005 a.u.f.s.

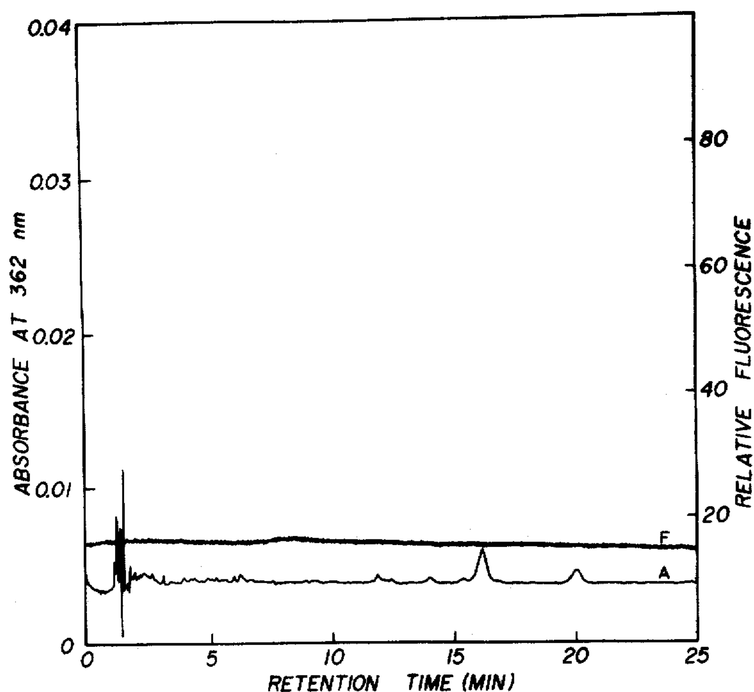

Fig. 3. HPLC separation of an extract from normal human urine. The injected amount was 100 μl, which was equivalent to 250 μl of unextracted urine. The operating conditions for HPLC were similar to those described in the legend to Fig. 1.

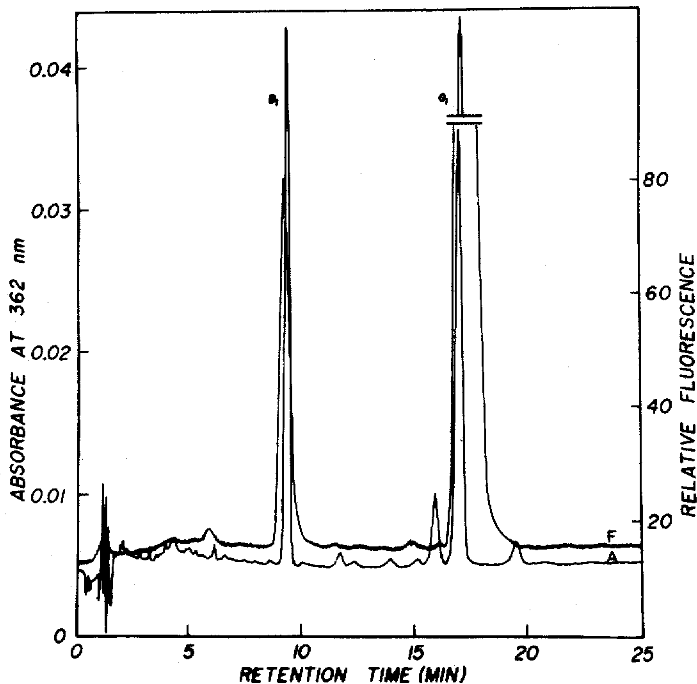

Fig. 4. HPLC separation of extract from human urine with added aflatoxins. Amounts of 6 and 10 μg of aflatoxins B_1 and G_1, respectively, were added to each ml of urine before extraction. The subsequent operating conditions were similar to those in Fig. 3.

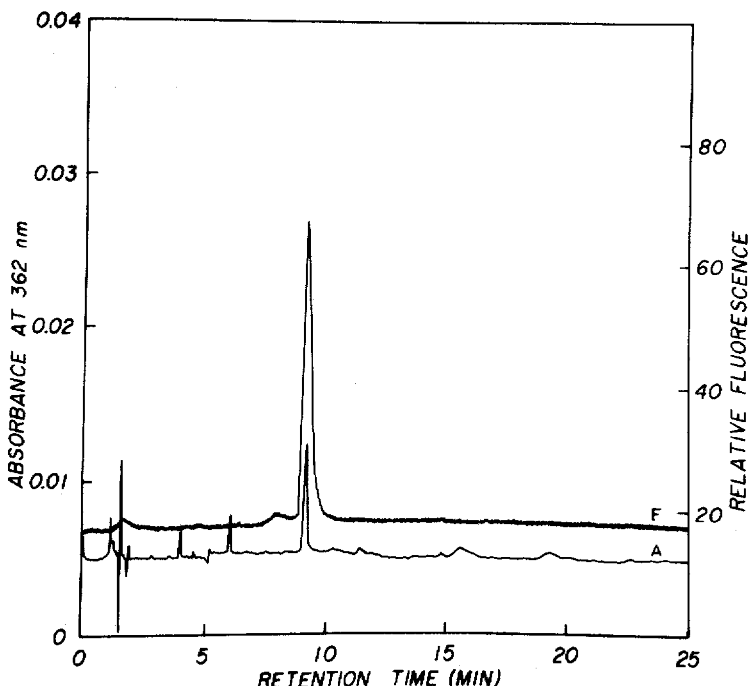

Fig. 5. HPLC separation of an extract from urine of a child in Songkhla province, using the same extraction procedure and HPLC operating conditions as for Fig. 3.

The observation of a UV-absorbing peak in chromatograms of urine extracts from children in Songkhla Province with a retention time in the vicinity of aflatoxin B_1, suggested the presence of aflatoxin B_1 in the urine (Fig. 5). The extremely high fluorescence of this compound served as a convincing proof for aflatoxin B_1. Similar chromatograms were observed in 30 of 106 urine samples from the same area. The finding is consistent with the epidemiological study on aflatoxin contamination of prepared food in the Songkhla area [16].

Since human urine contains several UV-absorbing constituents, the removal of these constituents by light petroleum without disturbing aflatoxins is simple and effective [17]. As a result, interference in aflatoxin separation from constituents with similar retention times during HPLC analysis was clearly reduced. With the presumably small amount of native aflatoxin B_1 excreted in human urine, an extremely sensitive detection device is essential. HPLC equipped with UV and fluorescence detectors offers a system to fit the requirement. The fluorescence of the resolved sample also served as a confirmation for aflatoxins.

Based on data obtained from the study in primates, the major portion of aflatoxins excreted in human urine should be conjugated derivatives rather than native aflatoxins and unconjugated metabolites. The conjugated derivatives are water-soluble compounds which can be converted back to the unconjugated aflatoxins by enzyme digestion or mild hydrolysis [18]. With the presumed large quantity of conjugated aflatoxins in human urine, the subsequent detection and identification can be achieved by conventional thin-layer chromatography. In comparison, the method described in this paper provides a quick, sensitive and simple procedure for the extraction and identification of uncon-

jugated aflatoxins in urine. This procedure can be employed in the study of in vivo metabolism of aflatoxins in humans as well as in the epidemiological study of aflatoxins in human biological fluids.

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