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Determination of aflatoxin Q_1 in urine by automated immunoaffinity column clean-up and liquid chromatography

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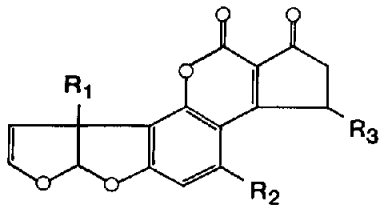
Abstract

A liquid chromatographic system with an automated clean-up procedure for aflatoxin Q_1 in human urine is described. The samples were cleaned up by using immunoaffinity columns originally designed for aflatoxin M_1. The chromatographic system was a C_{18} column with an acidic mobile phase of acetonitrile–water containing potassium bromide. Fluorescence detection (365/440 nm) of aflatoxin Q_1 was enhanced by addition of bromine, using post-column derivatization, which was studied by factorial designs. Average recovery of aflatoxin Q_1 in spiked 10-ml urine samples was 88% (R.S.D. = 6.4%) at a level of 50 pg/ml. The determination limit was 49.5 pg/ml urine.

1. Introduction

Aflatoxins are a group of carcinogenic mycotoxins produced by the moulds *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins can occur as toxic contaminants of food and feeds [1]. The naturally occurring aflatoxins are aflatoxin B_1, B_2, G_1 and G_2. Aflatoxin B_1 is known to be hydroxylated by human liver enzymes to aflatoxin M_1, P_1 and Q_1 [2] (Fig. 1).

When fungal-infected material is handled, aflatoxins can constitute an occupational hazard. In Denmark, aflatoxin B_1 has been found in dust at a livestock-feed company, and the workers at this place showed serum-bound aflatoxin B_1 [3]. We recently described a method employing solid-phase extraction and high-performance liquid



Aflatoxin	R_1	R_2	R_3
B_1	-H	$-OCH_3$	-H
M_1	-OH	$-OCH_3$	-H
P_1	-H	-OH	-H
Q_1	-H	$-OCH_3$	-OH

Fig. 1. Chemical structures of aflatoxins B_1, M_1, P_1 and Q_1.

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chromatography (HPLC) for determination of aflatoxin B_1, B_2, G_1 and G_2 in urine [4].

For food and feed analysis of aflatoxin, antibodies are being used to an increasing extent for clean-up or detection and are now commercially available from several manufacturers [5]. Immunochemical methods for analysis have been described and reviewed [5–7]. Aflatoxins are a group of mycotoxins with similar structures and cross-reactions with the antibodies may occur. The antibodies are bound to a support and packed in a column that is used for clean-up and the various aflatoxins are separated by HPLC. Immunoaffinity columns in combination with HPLC have been used for the determination of aflatoxin B_1, B_2, G_1 and G_2 in foods and feeds [8], in peanut butter [9], in corn, peanuts and peanut butter [10] and for the determination of aflatoxin M_1 in milk [11].

Using immunoaffinity column clean-up, Groopman *et al.* [12] found aflatoxin M_1, P_1 and Q_1 in urine from people living in Gambia, but levels of these hydroxylated metabolites were not reported. In China, chloroform-extracted human urine samples have been found to contain levels of 0.01–2.09 ng/ml of aflatoxin M_1 [13]. Despite the fact that aflatoxin M_1 is the hydroxylated metabolite most frequently observed in humans [2], *in vitro* studies of human liver samples have shown that aflatoxin B_1 is metabolised to aflatoxin Q_1 to a greater extent than to aflatoxin M_1 [14].

Aflatoxin M_1 has a higher native fluorescence than aflatoxin Q_1 [15]. To increase the fluorescence of aflatoxin Q_1, the metabolite can be converted to hemiacetals by acid-catalysed addition of water, employing the method previously reported for aflatoxin B_1 and G_1 [15]. Another method to enhance the fluorescence of aflatoxins B_1 and G_1 is to add bromine in post-column derivatization reaction [4,16].

This work describes the determination of aflatoxin Q_1 in urine by means of an immunoaffinity column packed with antibodies for aflatoxin M_1, taking advantage of the cross-reactivity with aflatoxin Q_1. Aflatoxin Q_1 was derivatized by addition of bromine in an on-line post-column derivatization reaction.

2. Experimental

2.1. Chemicals and reagents

Aflatoxins M_1, Q_1 and P_1 were purchased from Sigma (St. Louis, MO, USA) in crystalline form. For sample clean-up, Aflaprep M columns (KIT bx: AFM 23) were used from Rhône-Poulenc (Glasgow, Scotland, UK). Aflatoxin-free urine controls (batch 58501) were of level 1 and were purchased from Bio-Rad (Anaheim, CA, USA). The chemicals used were tetrahydrofuran (p.a. from Merck, Darmstadt, Germany), HPLC-grade acetonitrile (Rathburn, Walkerburn, UK), HPLC-grade hexane (Fisons, Loughborough, UK), p.a. nitric acid from Merck and potassium bromide and sodium acetate, both p.a. from Riedel-de Haen (Hannover, Germany). The water used was purified with a Milli-Q system (Millipore, Milford, MA, USA). Nitrogen and helium plus-grade were obtained from AGA (Sundbyberg, Sweden). For silanization, the chemicals used were methanol, acetone, chlorotrimethylsilane and hydrochloric acid (p.a., Merck) and analytical-grade toluene (May and Baker, Dagenham, UK).

2.2. Preparation of standards

All aflatoxin standards were prepared in a glove box. Aflatoxin Q_1 (0.1 mg) was dissolved by means of an injection syringe in 1 ml of acetonitrile in the septum capped vial in which it was delivered. Standards used to spike urine samples were prepared in acetonitrile at concentrations of 4.95, 14.6 and 30.5 ng/ml. Solutions used for the calibration curve were prepared in acetonitrile–water (1:1, v/v) in the range 1.25–16.9 ng/ml of aflatoxin Q_1. The calibration curve and the standards for spiking urine samples were prepared on the same day and stored in a refrigerator at 6°C up to two weeks. The original aflatoxin solution was stored in a freezer at −18°C for a period up to six months. The standard solution used for the derivatization study of aflatoxin Q_1 was prepared in acetonitrile–water (1:1, v/v) at a concentration of 12.2 ng/ml. Aflatoxin P_1 (5 μg) was

dissolved in 0.5 ml acetonitrile and aflatoxin M_1 (10 μg) in 1 ml acetonitrile in the same way as aflatoxin Q_1. The solutions were further diluted in acetonitrile–water (1:1, v/v) to concentrations of 0.05 and 0.005 ng/μl, respectively.

2.3. Sample clean-up

Sample preparation was performed with a Millilab 1A workstation (Waters, Milford, MA, USA). One-millilitre syringe barrel inserts (part number WAT064698, Waters) were used to connect the Aflaprep M columns with the probe moving the clean-up columns. The test-tubes used for the sample clean-up were silanized by the following method [4]: washed in 2 *M* hydrochloric acid, followed by water and acetone, air-dried, silanized with 2% chlorotrimethylsilane in toluene for 5 min, and finally washed in methanol and dried at 70°C for 20 min.

For sample preparation, the freeze-dried urine controls were reconstituted with 10.0 ml of water and diluted with 10.0 ml of sodium acetate buffer, pH 5.0. The samples were spiked with 100 μl from one of the three spiking standards and then processed by the Millilab workstation according to the following method:

(1) Condition the Aflaprep M column with 10 ml of water at 20 ml/min.

(2) Load the 20.0-ml sample (urine + buffer) onto the column at 2 ml/min.

(3) Wash the column with 10.0 ml of water at 2 ml/min.

(4) Dry the column with nitrogen for 5 min at 100 kPa.

(5) Wash the column with 3.0 ml of 25% tetrahydrofuran in hexane at 2 ml/min.

(6) Dry the column for 60 s with nitrogen at 100 kPa.

(7) Elute the aflatoxin with 1.5 ml of acetonitrile at 2 ml/min, with a hold of 30 s after 0.5 ml.

Each sample was completely processed before the workstation started with the next sample. The eluate was collected in the silanized tubes and the solvent was manually evaporated under nitrogen with a flow of 80 ml/min during two hours at room temperature. The aflatoxin was then redissolved in 200 μl acetonitrile–water (1:1, v/v) using a vibrator (Vortex, Bohemia, NY, USA) for 15 s and then transferred to 200-μl plastic microvials (Waters) for HPLC analysis.

2.4. HPLC analysis

The liquid chromatograph consisted of a Waters Model 6000A pump and a Waters WISP 712 autoinjector. Detection was performed with an RF-530 fluorescence detector (Schimadzu, Kyoto, Japan) at an excitation wavelength of 365 nm and emission wavelength of 440 nm. The post-column derivatization was performed with bromine at room temperature (21°C), using a Kobra cell from Lamers and Pleuger (Den Bosch, Netherlands) at 100 μA. The post-column reactor consisted of PTFE tubing, 500 × 0.55 mm I.D. (reaction time 6 s). Chromatograms were recorded with a Spectra Physics integrator (San Jose, CA, USA).

Chromatography was performed with a 4-μm, 100 × 5 mm I.D. Nova-pak C_{18} column (Waters) with a mobile phase of acetonitrile–water (25:75, v/v). The water contained 1 m*M* potassium bromide and 1 m*M* nitric acid. The flow-rate was 1 ml/min, and the injection volume 30 μl. The mobile phase was continuously degassed with helium.

2.5. Derivatization of aflatoxin Q_1

Factorial designs [17] were used to study the post-column derivatization of aflatoxin Q_1. The experiment was used to study the effects of the most important variables on the derivatization: the amount of potassium bromide in the mobile phase, the current used and the reaction time. A first design was a 2^3 factorial design that was performed with a potassium bromide concentration of 0.01 or 5 m*M*, a current of 10 or 200 μA, and 3 or 9 s reaction time. The design was augmented with centre-point experiments in which the levels were set at 2.5 m*M*, 200 μA and 6 s respectively. The second design was a 2^2 factorial design with a potassium bromide concentration of 0.6 or 1.4 m*M* and a current of 50

Table 1
Experimental design and peak areas of aflatoxin Q_1 in the factorial design

Experiment	Variables[a]		Area Q_1
	x_1	x_2	y_1
1	1	1	4914
2	1	1	5020
3	1	1	5114
4	1	−1	5761
5	1	−1	5552
6	1	−1	5837
7	0	0	5599
8	0	0	5380
9	0	0	5562
10	−1	1	5347
11	−1	1	5394
12	−1	1	5526
13	−1	−1	5801
14	−1	−1	6143
15	−1	−1	5888

[a]Coding of variables: x_i, definition, [(−1)-level, (0)-level, (+1)-level]: x_1, potassium bromide (mM), [0.6, 1.0, 1.4]; x_2, current (μA), [50, 100, 200].

or 200 μA, augmented with centre-point experiments with 1 mM and 100 μA, as described in Table 1. The reaction time was set at 6 s and the injection volume was 20 μl of the aflatoxin Q_1 standard solution of 12.2 ng/ml.

3. Results and discussion

It has been found that, to achieve high recoveries with immunoaffinity columns, it is necessary to use a slow, steady flow for sample loading and elution. This can be very tedious and time consuming when the columns are run manually [8,9]. The method used for sample clean-up was therefore automated, using the Millilab workstation. The time needed for each sample preparation was one hour. To obtain the total analysis time, the times needed for evaporation and HPLC analysis are added, resulting in a total time of 24 h for analysis of *ca.* 10 samples. Prior to HPLC analysis, the acetonitrile eluate was evaporated in order to reduce the volume of the sample. For rapid evaporation, without heating in order to prevent loss of the aflatoxins, there should be as little water in the eluate as possible. To remove water from the column, a flow of nitrogen was used, followed by a wash with 25% tetrahydrofuran in hexane. Using these drying steps, no loss of aflatoxin Q_1 was observed. The test-tubes used for evaporation were silanized, which has been shown to be most important for preventing loss of aflatoxins [4].

Chromatograms of a spiked and an unspiked urine control sample are shown in Fig. 2. The clean-up procedure gives chromatograms free from interferences.

3.1. Post-column derivatization

The first design indicated a lack of fit after fitting to a second-order interaction model. Hence data are not presented. However it was noted that, with all settings at medium values (2.5 mM, 100 μA and 6 s), the highest fluorescence was achieved and that with lower or higher settings (0.01 mM, 10 μA and 3 s or 5 mM, 200 μA and 9 s) there was less fluorescence. It was therefore concluded that, with all settings at a low level, not enough bromine was available to react with the toxin, and with all settings at a high level, an excess of bromine was present and a second reaction could follow the first, reducing

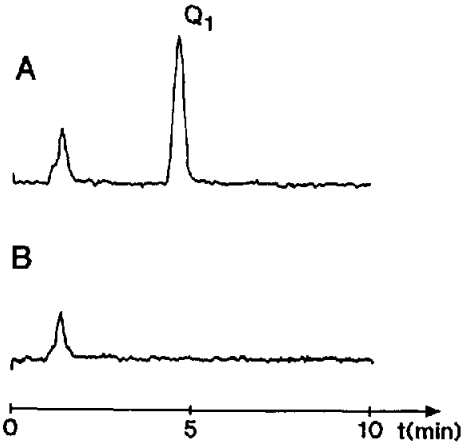

Fig. 2. Chromatogram of a urine sample extracted and cleaned up on Aflaprep M columns. (A) Urine spiked to contain 146 pg/ml of aflatoxin Q_1. (B) Unspiked urine sample.

the fluorescence [16]. For optimum fluorescence of aflatoxin Q_1 the selected values should therefore not be extreme but settings close to the average should be used: 2.5 m*M* potassium bromide in the mobile phase, 100 μA for bromine generation, and a reaction time of 6 s.

A factorial design was then performed with a centre point near the average settings of the initial experiment. In the experiment the potassium bromide content in the mobile phase and the current were altered, as these variables are both expected to influence the amount of bromine generated. The reaction time was kept constant at 6 s to limit the number of variables. The values used in the experiment were 1 m*M* potassium bromide in the mobile phase and 100 μA for bromine generation. The design was performed with small changes around these settings in order to ascertain the stability of the derivatization around these values. From the results in Table 1, a second-order interaction model was computed that described the variation in the peak area of aflatoxin Q_1 (y_1) as a function of the experimental settings ($x_1 - x_2$). The model was: $y_1 = (5503 \pm 77) - (158 \pm 86)x_1 - (298 \pm 86)x_2 - (45 \pm 86)x_1x_2$. Analysis of variance (ANOVA) of the regression did not indicate any lack of fit. From the model it was concluded that when the concentration of potassium bromide or the current for generation of bromine was changed in either direction, the peak area changed at most 10% from the results obtained using the centre point parameters. Maximal deviation was observed when both the potassium bromide concentration and the current were set at their high or low levels at the same time. Using 1 m*M* potassium bromide in the mobile phase, 100 μA for bromine generation and a reaction time of 6 s, a 20-fold increase in fluorescence was achieved compared with the underivatized aflatoxin Q_1.

Attempts were also made to derivatize aflatoxin P_1 using post-column derivatization with bromine, but aflatoxin P_1 lost its fluorescence completely, probably because of bromination of the aromate at the phenol site (see Fig. 1). No increase in fluorescence was observed for aflatoxin M_1 with post-column derivatization. Of the underivatized aflatoxins, aflatoxin M_1 has the highest fluorescence, followed by aflatoxins P_1 and Q_1 [15].

The method described was developed for aflatoxin Q_1 in spiked urine samples, so the chromatographic method used was selected although it did not separate the different hydroxylated metabolites. If there is a need to separate all three metabolites, another chromatograhic method can be used [18].

3.2. Quantitation

The method employing automated immunoaffinity clean-up and post-column derivatization was validated with spiked urine samples. The values used for the post-column derivatization were those obtained from the factorial design. Recoveries are computed by comparison with standards injected directly onto the HPLC system. The average recoveries of replicated analyses ($n = 5$) were 88% at 49.5 pg/ml, 90% at 146 pg/ml, and 86% at 305 pg/ml, with relative standard deviations of 6.4, 3.6 and 4.8% respectively.

Calibration curves for aflatoxin standards were linear, with regression coefficients of 0.9992 over the range 1.25–16.9 ng/ml with 30-μl injections ($n = 6$). The detection limit (signal-to-noise ratio of 3) was 1.25 ng/ml with 30-μl injections of the standard aqueous samples analyzed by HPLC. The equation for the standard curve of the spiked urine samples was $y = 0.85x + 3.6$ with a 95% confidence limit of 0.04 for the slope and 8.4 for the intercept. The determination limit was 49.5 pg/ml of urine.

4. Conclusions

A method for the analysis of aflatoxin Q_1 in human urine has been described using immunoaffinity columns for clean-up, determination by HPLC, post-column derivatization and fluorescence detection. We showed here that aflatoxin Q_1 in urine can be extracted on Aflaprep M columns originally designed for the analysis of aflatoxin M_1 in milk. Reproducible clean-up

results were obtained by using the Millilab workstation. Post-column derivatization with the addition of bromine enhanced the fluorescence detection (365/440 nm) of aflatoxin Q_1. This was not possible for the other hydroxylated metabolites (aflatoxin M_1 and P_1). Employing the described automated immunoaffinity column clean-up and post-column derivatization, determination of aflatoxin Q_1 in a 10-ml urine sample can be performed at a level of 50 pg/ml.

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6. References

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