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# Immunoaffinity column clean-up for the high-performance liquid chromatographic determination of aflatoxins $B_1$, $B_2$, $G_1$, $G_2$, $M_1$ and $Q_1$ in urine

Anders Kussak, Barbro Andersson*, Kurt Andersson

*National Institute of Occupational Health, Analytical Chemistry Division, P.O. Box 7654, S-907 13 Umeå, Sweden*

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**Abstract**

A method for the determination of aflatoxins $B_1$, $B_2$, $G_1$, $G_2$, $M_1$ and $Q_1$ in human urine has been developed. The 10-ml urine samples were automatically cleaned up on immunoaffinity columns and analysed by high-performance liquid chromatography (HPLC), including post-column derivatization with bromine and fluorescence detection. Average aflatoxin recoveries were: $B_1$ 103%, $B_2$ 106%, $G_1$ 98% and $G_2$ 96% in the range 6.8–73 pg/ml of urine and $M_1$ 103% and $Q_1$ 100% in the range 18–97 pg/ml of urine. The relative standard deviations were all between 1% and 21%. The determination limits of aflatoxins in urine were 6.8 pg/ml for $B_1$, $B_2$, $G_1$ and $G_2$ and 18 pg/ml for $M_1$ and $Q_1$.

## 1. Introduction

Research carried out after disease outbreaks among turkeys, ducklings, pigs and cattle resulted in the discovery of aflatoxins in the early 1960s. Aflatoxins were found to be produced by the food-borne moulds *Aspergillus flavus* and *A. parasiticus* [1]. They were named aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ (Fig. 1). Of these, aflatoxin $B_1$ is the best studied, and it is the most toxic, mutagenic and carcinogenic [1]. Aflatoxins are further converted to different metabolites with cytochrome P-450 enzymes. Aflatoxin $B_1$ is converted to metabolites like aflatoxin $B_1$-epoxide and the hydroxylated aflatoxins $M_1$, $P_1$ and $Q_1$ (Fig. 2) [2]. The hydroxylated metabolites can

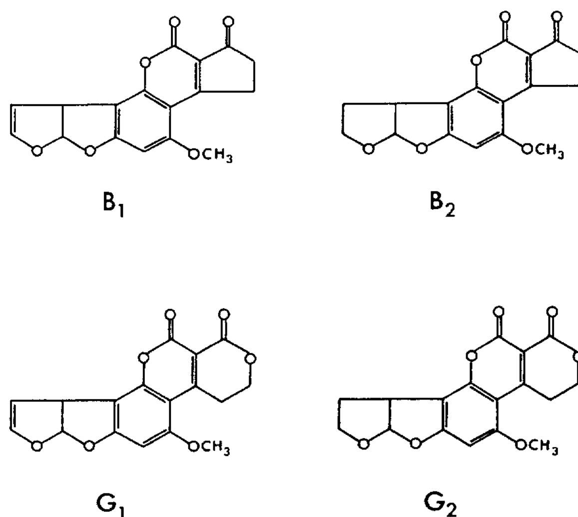


Fig. 1. Structures of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$.

* Corresponding author.

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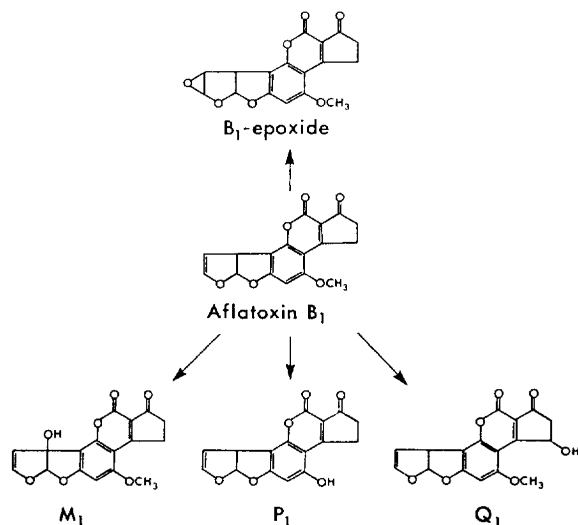


Fig. 2. Metabolic pathways of aflatoxin $B_1$ by cytochrome P-450 enzymes.

form glucuronide and sulphate conjugates, and the conjugates can be enzymatically hydrolysed at analysis by β-glucuronidase and sulphatase [3].

The various aflatoxins and their levels in human urine have been reviewed [4,5]. The aflatoxins found in human urine consisted of aflatoxins $B_1$, $B_2$, $G_1$, $G_2$, $M_1$, $Q_1$, $P_1$ and $B_{2a}$, aflatoxin-$N^7$-guanine and aflatoxicol. In China, corn meal and peanut oil in particular were found to be contaminated with aflatoxin $B_1$, with levels in the range 23–500 μg/kg. This gave an estimated level of dietary intake of 58 μg $B_1$ per day. The urine samples were found to contain aflatoxin $M_1$ in the range 0.03–0.3 ng/ml as analysed by enzyme-linked immunosorbent assay (ELISA) with a previous solid-phase extraction clean-up [6]. In urine samples from Zimbabwe the average level of aflatoxin $M_1$ reported was 4 ng/ml, based on thin-layer chromatography [7]. In Gambia the average dietary intake of the total amount of aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ during one week was estimated to be 12 μg. The exposure was positively correlated with a urinary excretion of 48–7100 ng aflatoxin-$N^7$-guanine per day [8].

With such methods as ELISA or radioimmunoassay (RIA), the aflatoxins are determined as a total amount and expressed as aflatoxin $B_1$ equivalents, if the antibodies used recognize many different aflatoxins [4]. From Denmark, aflatoxin levels of 0–3.0 ng/ml aflatoxin $B_1$ equivalents were reported [9]. Collected human urine samples have been loaded onto $C_{18}$ Sep-Pak cartridges and then transported for further analysis, using immunoaffinity column clean-up followed by chromatographic separation and UV detection [8]. The latter was sufficiently sensitive for the detection of the levels of aflatoxins excreted, but different derivatization reactions for the fluorescence detection of the four aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ in combination with HPLC have recently been reviewed [10].

In addition to dietary intake, exposure to aflatoxins carried by airborne dust in the working environment is possible [4]. In Denmark serum-bound aflatoxins have been found in samples from feed factory workers [11]. We therefore developed a method for determining aflatoxins in airborne dust from feed factories [12]. This paper describes a method for the simultaneous determination of aflatoxins $B_1$, $B_2$, $G_1$, $G_2$ and the hydroxylated aflatoxins $M_1$ and $Q_1$ in human urine, using automated immunoaffinity column clean-up and liquid chromatography with post-column derivatization. The aim was the detection of ppt levels of aflatoxins in urine from feed factory workers exposed to dust.

## 2. Experimental

### 2.1. Chemicals and reagents

Aflatoxins $B_1$, $B_2$, $G_1$, $G_2$, $M_1$ and $Q_1$ as well as β-glucuronidase with glucuronidase and sulphatase activity were purchased from Sigma (St. Louis, MO, USA). The chemicals used were HPLC-grade acetonitrile (Rathburn, Walkerburn, UK), potassium bromide and sodium acetate, both p.a. from Riedel–de Haen (Hanover, Germany), methanol, acetone, chlorotrimethylsilane, hydrochloric acid, acetic acid and nitric acid, all p.a. from Merck (Darmstadt, Germany), analytical-grade toluene from May and Baker (Dagenham, UK), potassium hydrox-

ide from Eka Nobel (Gothenburg, Sweden) and 95% ethanol from Kemetyl (Stockholm, Sweden). The water used was purified with a Milli-Q system (Millipore, MA, USA). Nitrogen and helium plus grade were obtained from AGA (Sundbyberg, Sweden). For sample clean-up, Aflaprep immunoaffinity columns (batch EF 192/2) were purchased from Rhône-Poulenc (Glasgow, UK).

Urine controls (batches 63601 and 62011) were purchased from Bio-Rad (Anaheim, CA, USA).

### 2.2. Safety precautions

Aflatoxins are toxic, mutagenic and carcinogenic compounds. As a safety precaution, all neat aflatoxin reagents were handled in a glovebox. Destruction of aflatoxin solutions was performed by reaction in 10% potassium hydroxide in ethanol and subsequent dilution. Contaminated glassware, vials, tubes, etc. were sealed in high-security disposals and thereafter incinerated.

### 2.3. Preparation of standards

Individual aflatoxins, in crystalline form, were diluted to solutions containing 10 ng/$\mu$l of each aflatoxin in acetonitrile and stored in a refrigerator at 8°C for up to two months. These solutions were diluted to a standard containing 0.15 ng/$\mu$l aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ and 0.20 or 0.40 ng/$\mu$l aflatoxins $M_1$ and $Q_1$, stored for up to one week at 8°C. This standard solution was then simultaneously diluted to obtain spiking solutions and solutions for the calibration curve by means of an injection syringe into 4-ml septum-capped HPLC vials. Spiking solutions were diluted in acetonitrile and 99 $\mu$l (calibration of syringe) were used to spike the different urine samples. The solutions for spiking urine control samples had levels of 1.8, 4.9 and 9.9 pg/$\mu$l for aflatoxins $M_1$ and $Q_1$ and 0.69, 3.6 and 7.4 pg/$\mu$l for aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$. Solution for spiking authentic urine samples, collected at feed factories, had levels of 1.8, 2.8 and 3.8 pg/$\mu$l for aflatoxins $M_1$ and $Q_1$ and 1.4, 2.1 and 2.9 pg/$\mu$l for aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$. Calibration curves were in the range 0.42–11.4 pg/$\mu$l for aflatoxins $M_1$ and $Q_1$ and 0.32–4.26 pg/$\mu$l for aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$, and diluted in acetonitrile–water (30:70, v/v). Solutions for the calibration curve and spiking were stored in a refrigerator at 8°C for no longer than 24 h.

### 2.4. Sample clean-up

The lyophilised urine control samples were dissolved in 10 ml of water and then diluted with 10 ml of 0.1 *M* sodium acetate buffer, adjusted to pH 5.0 by the addition of 0.1 *M* acetic acid. The 20-ml samples were cleaned up on immunoaffinity columns with the use of a Millilab work station (Waters, Milford, MA, USA). The immunoaffinity column was conditioned with 10 ml of water before the 20-ml sample was loaded on the column. After the column had been washed with 10 ml of water, it was dried with nitrogen at a flow-rate of about 1.2 l/min for 1 min. The aflatoxins were then eluted with 2 ml of acetonitrile, with a pause of 30 s after 1 ml and 1.5 ml, into a silanized test-tube. The test-tubes of soda-lime glass (No. 109.986-14; Kebo Lab., Stockholm, Sweden) had previously been washed in 2 *M* hydrochloric acid, followed by water and acetone, air-dried and silanized with 2% chlorotrimethylsilane in toluene for 5 min and washed in methanol and dried at 70°C for 20 min [13]. All flow-rates used were 2 ml/min. The eluate was evaporated to dryness under a flow of nitrogen at 120 ml/min and then redissolved in 200 $\mu$l of acetonitrile–water (30:70, v/v) with the use of a vortex mixer (Scientific Industries, Bohemia, NY, USA) and then transferred to 200-$\mu$l vial inserts (Waters) for HPLC analysis.

### 2.5. Chromatography

Standards and cleaned up urine samples were analysed on a HPLC system containing a Waters Model 6000A pump, a Waters WISP 712 injector, a Spectra Physics integrator (San Jose, CA, USA) and an RF-530 fluorescence detector (Shimadzu, Kyoto, Japan) with an excitation wavelength set at 365 nm and emission wavelength at 440 nm. The column was a 4-$\mu$m,

100 × 8 mm I.D. Nova-Pak Phenyl column (Waters) with a mobile phase of acetonitrile–water (30:70, v/v). The water contained 1 m*M* potassium bromide and 1 m*M* nitric acid. The flow-rate was 1.5 ml/min, and the injection volume 150 $\mu$l. 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 20 $\mu$A. The post-column reactor consisted of PTFE tubing, 500 × 0.55 mm I.D. The length of the reaction tube, the current at the KOBRA cell and the amount of potassium bromide in the mobile phase were at the same settings for the derivatization that we previously optimized in a factorial design [13].

### 2.6. Quantitation

Quantitation of aflatoxins $B_1$, $B_2$, $G_1$, $G_2$, $M_1$ and $Q_1$ from spiked urine samples was performed by comparison with standard solutions containing the mixture of aflatoxins by HPLC. Recovery studies were performed by spiking 99 $\mu$l of the different standards described above to (a) urine control samples at three levels and (b) three different authentic urine samples from workers at feed factories, at three levels.

### 2.7. Authentic urine samples

Urine samples were analysed from sixteen workers in feed factories, exposed to dust suspected to be contaminated with aflatoxins. The workers had been working for two days before a morning urine sample was obtained.

The urine samples were frozen in 12-ml batches, stored at −20°C until analysis or analysed directly. If the thawed urine contained precipitates, it was inverted three times, and 10 ml were then immediately transferred for analysis. The 10-ml urine samples were diluted with 10 ml of pH 5.0, 0.1 *M* sodium acetate buffer and analysed (unspiked and spiked with aflatoxins) as above. Nine of the samples were also analysed after enzymatic hydrolysis. The urine samples were hydrolysed with 500 U/ml $\beta$-glucuronidase and 24 U/ml sulphatase in sodium acetate buffer for about 17 h in a water-bath at 37°C. After hydrolysis, the 20-ml samples were cleaned up on the immunoaffinity columns.

## 3. Results and discussion

The current method is a further development of our previous technique for analysing aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ in urine [13]. The selectivity of the method has been enhanced by using an immunoaffinity column instead of solid-phase extraction. The present method is used for the simultaneous determination of aflatoxins $B_1$, $B_2$, $G_1$, $G_2$, $M_1$ and $Q_1$. Aflatoxin $P_1$ cannot be analysed by this method since it loses its fluorescence on post-column derivatization with bromine [14]. The method for the immunoaffinity column clean-up was automated by a Millilab work station, and the time for clean-up of each sample was 60 min. After the clean-up was completed, the solvent containing the cleaned up extract was analysed for aflatoxins using HPLC, and all six metabolites were separated within 15 min.

The method was used with authentic urine samples collected at those feed factories where airborne dust samples had been collected on a previous occasion. The dust samples contained aflatoxin $B_1$ in levels up to 53 $\mu$g/kg [12].

### 3.1. Quantitation

The method was validated by spiking urine control samples with standards containing a mixture of aflatoxins at different levels. The results are presented in Table 1. The recoveries of the different aflatoxins were all high and ranged on average from 96% to 106% with relative standard deviations of 1% to 21%. It has been shown that authentic urine may contain interfering compounds that interact with the antibodies [9,15]. We therefore repeated the recovery studies with three authentic urine samples (Table 2). These contained no aflatoxins, as determined in a previous analysis. The aflatoxin-free urine from each individual was separated into three samples, each spiked at a different

Table 1
Recovery of six different aflatoxins from spiked urine control samples

| Compound | Added (pg/ml urine) | Recovery (%) | R.S.D. (%) | $n$ | Average recovery[a] (%) |
|---|---|---|---|---|---|
| $B_1$ | 6.8 | 102 | 15 | 5 | |
| | 36 | 106 | 2 | 6 | 103 (±4) |
| | 73 | 101 | 3 | 6 | |
| $B_2$ | 6.8 | 108 | 4 | 5 | |
| | 36 | 108 | 2 | 6 | 106 (±2) |
| | 73 | 103 | 1 | 6 | |
| $G_1$ | 6.8 | 99 | 21 | 5 | |
| | 36 | 98 | 6 | 6 | 98 (±6) |
| | 73 | 98 | 2 | 6 | |
| $G_2$ | 6.8 | 103 | 3 | 5 | |
| | 36 | 94 | 7 | 6 | 96 (±3) |
| | 73 | 93 | 3 | 6 | |
| $M_1$ | 18 | 100 | 16 | 5 | |
| | 48 | 107 | 2 | 6 | 103 (±5) |
| | 97 | 101 | 2 | 6 | |
| $Q_1$ | 18 | 100 | 12 | 5 | |
| | 48 | 103 | 3 | 6 | 100 (±4) |
| | 97 | 98 | 2 | 6 | |

[a] The 95% confidence limits in brackets, $n = 17$.

Table 2
Recovery of six different aflatoxins from three different spiked urine samples, $n = 3$

| Compound | Added (pg/ml urine) | Recovery (%) | R.S.D. (%) | Average recovery[a] (%) |
|---|---|---|---|---|
| $B_1$ | 14 | 97 | 5 | |
| | 21 | 102 | 4 | 100 (±3) |
| | 28 | 102 | 1 | |
| $B_2$ | 14 | 100 | 5 | |
| | 21 | 98 | 6 | 99 (±3) |
| | 28 | 101 | 2 | |
| $G_1$ | 14 | 102 | 5 | |
| | 21 | 93 | 2 | 98 (±4) |
| | 28 | 100 | 2 | |
| $G_2$ | 14 | 86 | 6 | |
| | 21 | 82 | 7 | 84 (±4) |
| | 28 | 84 | 8 | |
| $M_1$ | 18 | 97 | 8 | |
| | 28 | 95 | 4 | 96 (±4) |
| | 38 | 96 | 7 | |
| $Q_1$ | 18 | 105 | 1 | |
| | 28 | 93 | 6 | 98 (±5) |
| | 38 | 96 | 5 | |

[a] The 95% confidence limits in brackets, $n = 9$.

level. When the average recoveries were compared between authentic urine and urine controls, a slight difference was observed. The largest difference in average recovery was 12% and was observed for aflatoxin $G_2$, followed by aflatoxins $B_2$ and $M_1$ with 7%. Despite the differences, all individual recoveries were over 82%.

The equations for the calibration curves of the different aflatoxins in seventeen spiked urine controls and nine authentic urine samples are presented in Table 3. The calibration curves were linear, despite the lower reproducibility for aflatoxins $B_2$, $G_2$ and $M_1$.

The determination limits of aflatoxins in urine are 6.8 pg/ml for aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$, and 18 pg/ml for aflatoxins $M_1$ and $Q_1$. Compared with our previous method, the current method gives a six-fold increase in sensitivity for aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ [13]. The detection limits (signal-to-noise ratio of 3) were as follows: for aflatoxins $B_1$, $G_1$ and $G_2$ 0.13 pg/$\mu$l, for aflatoxin $B_2$ 0.07 pg/$\mu$l and for aflatoxins $M_1$ and $Q_1$ 0.34 pg/$\mu$l, with 150-$\mu$l injections of the standard aqueous samples analysed by HPLC.

Aflatoxins that are excreted in the urine can be in either free form or bound as glucuronide or sulphate. Unfortunately, there are no commercially available aflatoxins in this conjugated form, and the aflatoxins are therefore usually analysed in the free form. Conjugated aflatoxins can be transformed into free form using enzymatic hydrolysis with $\beta$-glucuronidase and sulphatase [3]. In Fig. 3 chromatograms are presented from the same urine sample, hydrolysed and non-hydrolysed, respectively. In addition, a non-hydrolysed urine sample spiked with 21 pg/ml aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ and 28 pg/ml aflatoxins $M_1$ and $Q_1$ is shown.

No aflatoxins could be found in the sixteen urine samples collected at feed factories, i.e. there must be less than 6.8 pg/ml aflatoxins $B_1$, $B_2$, $G_1$, or $G_2$ and less than 18 pg/ml aflatoxins $M_1$ or $Q_1$. By comparison with injected standards, all aflatoxin peaks were well separated

Table 3
Calibration curves and correlation coefficients of aflatoxins in control and authentic urine

| Compound | Concentration (pg/ml) | $a$ [a] | $b$ [b] | $r$ |
|---|---|---|---|---|
| $B_1$ | 6.8–73 | $1.01 \pm 0.03$ | $0.37 \pm 1.10$ | 0.998 |
| $B_2$ | 6.8–73 | $1.03 \pm 0.02$ | $0.07 \pm 0.81$ | 0.999 |
| $G_1$ | 6.8–73 | $0.97 \pm 0.03$ | $0.14 \pm 0.94$ | 0.998 |
| $G_2$ | 6.8–73 | $0.93 \pm 0.03$ | $-0.64 \pm 1.42$ | 0.996 |
| $M_1$ | 18–97 | $1.02 \pm 0.03$ | $-0.27 \pm 1.92$ | 0.997 |
| $Q_1$ | 18–97 | $0.97 \pm 0.03$ | $0.80 \pm 1.55$ | 0.998 |

[a] Slope of the calibration curve $y = ax + b$, with 95% confidence limits.
[b] Intercept of the calibration curve with 95% confidence limits.

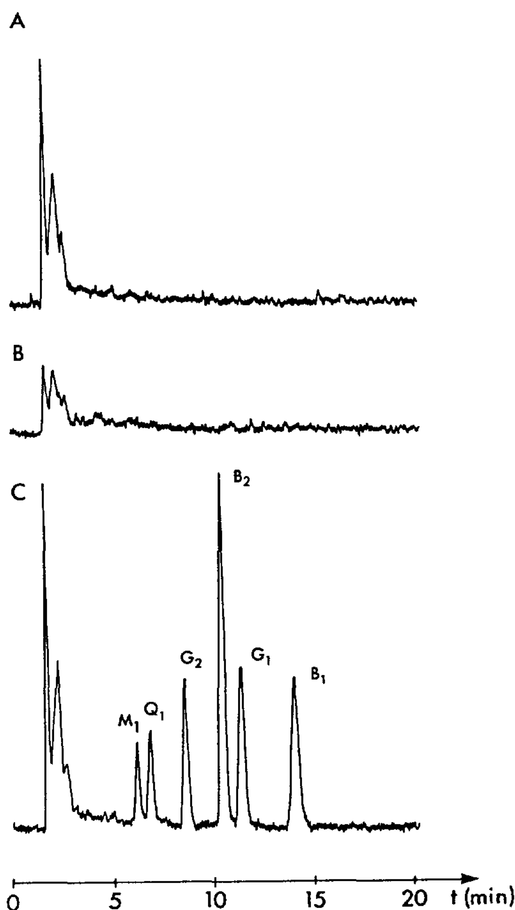


Fig. 3. Chromatograms (fluorescence detection) of the same urine sample cleaned up on immunoaffinity columns. (A) Non-hydrolysed. (B) Hydrolysed. (C) Spiked with 21 pg/ml aflatoxins $B_1$, $B_2$, $G_1$ and $G_2$ and 28 pg/ml aflatoxins $M_1$ and $Q_1$.

from interference in all urine samples. Aflatoxins could not be detected in either hydrolysed or non-hydrolysed urine.

## 4. Conclusions

A method for the automated immunoaffinity column clean-up of aflatoxins in urine and determination by HPLC is described. This method enables the simultaneous determination of aflatoxins $B_1$, $B_2$, $G_1$, $G_2$, $M_1$ and $Q_1$ in 10-ml urine samples at low ppt levels in a simple manner. If the urine samples were hydrolysed before the clean-up, no extra interference took place. The method was used with urine from feed factory workers in a place where aflatoxins were found in airborne dust on a previous occasion.

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