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Automated sample clean-up with solid-phase extraction for the determination of aflatoxins in urine by liquid chromatography

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ABSTRACT

An automated extraction and clean-up procedure was developed for the determination of aflatoxins in human urine at the 50 pg/ml level. Aflatoxins B1, B2, G1 and G2 are captured on C_2 extraction columns and simultaneously cleaned up with the aid of a robotic system. The processed samples are analysed by reversed-phase high-performance liquid chromatography. Fluorescence detection was enhanced for aflatoxins B1 and G1 using factorial design optimization of the post-column reactor. Silylation of the glass vials used in the robotic system was of the utmost importance. With non-silylated glass vials, up to 75% of the analytes were lost. Average aflatoxin recoveries were B1 95%, B2 90%, G1 93% and G2 89%.

INTRODUCTION

Aflatoxins are a group of secondary metabolites produced by the moulds *Aspergillus flavus* and *Aspergillus parasiticus*. Aflatoxins are highly toxic and carcinogenic compounds, and can be produced on a variety of plant products, ranging from peanuts, ground-nuts and almonds to cereals, such as maize, rice and wheat [1,2]. As a result of fungal attack, food products like milk, meat and eggs can also become indirectly contaminated with aflatoxins. In Sweden, aflatoxins have been found in acid-treated grain [3] and there is a risk of occupational exposure to aflatoxins during treatment of contaminated material.

A significantly elevated incidence of liver cancer has been observed among Swedish workers in grain mills, and it is postulated that aflatoxins may play a role [4]. A high incidence of liver cancer has also been noted among workers in live-stock-feed companies in Denmark [5] and among milkmaids in Finland [6]. Accordingly, a method for the analysis of aflatoxins in human urine is needed in order to monitor occupational exposure to aflatoxins.

The methods reported for the determination of aflatoxins, particularly in food and feeds, have been solid-phase extraction with high-performance liquid chromatography (HPLC) [7–9], or immunological methods, such as enzyme-linked immunosorbent assay (ELISA), immunological screening tests and the use of immunoaffinity columns [10,11]. Immunoaffinity columns are columns containing immobilized antibodies against aflatoxins that can be used for clean-up, followed by instrumental analysis, for example, by HPLC. Immunoaffinity columns were used by Sharman and Gilbert [12] for clean-up of aflatoxins in food

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and feeds and by Farjam *et al.* [13] for aflatoxin M1 in milk.

In the use of immunoaffinity columns for the determination of aflatoxins in urine, problems have been encountered on account of interaction of antigenic material in the urine with the antibodies in the column [14,15]. The various aflatoxins found in urine have been reviewed by Yourtee and Kirk-Yourtee [16]. The four mould products aflatoxins B1, B2, G1 and G2 (Fig. 1) and six other human metabolites have been found in urine. Bean *et al.* [17] reported levels of aflatoxins B1 and G1 in the range 70–120 pg/ml of urine.

The determination of aflatoxins can be carried out by HPLC with fluorescence detection. Post-column derivatization is used to enhance the fluorescence of aflatoxins B1 and G1 by addition of bromine [18] or iodine [19]. Aflatoxins B2 and G2 do not add halogens because of the absence of a double bond in the furanoid ring (see Fig. 1).

This paper describes a method for the analysis of aflatoxins B1, B2, G1 and G2 in human urine. A solid-phase extraction with Bond Elut C_2 columns was performed for sample clean-up, using a Waters robotic system. In order to enhance fluorescence, HPLC analysis was used with an optimized post-column system for derivatization by addition of bromine.

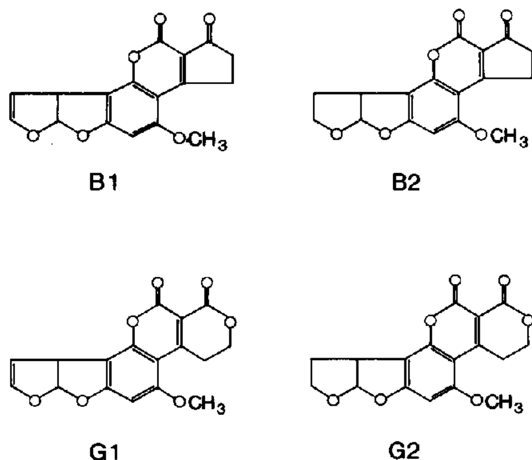

Fig. 1. Structures of aflatoxins B1, B2, G1 and G2.

EXPERIMENTAL

Chemicals and reagents

Aflatoxins B1, B2, G1 and G2 were purchased from Sigma (St. Louis, MO., USA) in crystalline form. The urine controls with specified content (batches 52201 and 58501) were of level 1 and were purchased from Bio-Rad (Anaheim, CA, USA). The chemicals used were methanol, tetrahydrofuran, acetone and dichloromethane (all p.a. from Merck, Darmstadt, Germany), HPLC-grade acetonitrile (Rathburn, Walkerburn, UK), analytical-grade toluene (May & Baker, Dagenham, UK), HPLC-grade hexane (Fisons, Loughborough, UK), chlorotrimethylsilane, hydrochloric acid and nitric acid from Merck and potassium bromide and sodium acetate from Riedel-de Haen (Hannover, Germany). For the solid-phase extraction, Bond Elut columns (lots 030345 and 030595) were used with 500 mg of C_2 bonded-phase material from Analytichem (Harbor City, CA, USA). The water used was purified with a Milli-Q system (Millipore, Milford, MA, USA). Nitrogen and helium plus grade were from AGA (Sundbyberg, Sweden).

Preparation of standards

All aflatoxin standards were prepared using a glove box. Each aflatoxin, 1 mg in a septum cap vial, was diluted in the vial with 2 ml of acetonitrile, using an injection syringe. Standards for method validation were prepared containing mixtures of aflatoxins B1, B2, G1 and G2 at three different concentrations. The concentrations were 4.90, 12.0 and 23.3 ng/ml, and 100 μl of each of the different solutions were used for spiking the samples for method validation. Solutions for the calibration curve were prepared in acetonitrile–water (1:1). The solutions were stored in refrigerator at 6°C and no significant sample loss was observed over two weeks.

Silanization

Test tubes with dimensions 100 × 16.0 mm were used for sample preparation. The tubes were washed by immersing in 2 *M* hydrochloric acid, followed by water and finally acetone. After

drying in air, the tubes were silanized by immersing in 2% chlorotrimethylsilane in toluene for 5 min. After silanization, the tubes were washed in methanol and dried at 70°C for 20 min. The tubes were stored for up to three weeks in a desiccator with blue gel prior to use.

To study loss of aflatoxin during evaporation, a mixture of 2.0 ng of each aflatoxin was added to 3.0 ml of dichloromethane in nine silanized test tubes and to nine tubes with no silanization. The dichloromethane was immediately evaporated from three tubes with and without silanization and the aflatoxins were reconstituted in 200 μl acetonitrile–water (1:1) for analysis by HPLC. The remaining tubes were stored at room temperature for 4 or 8 h until required for evaporation and analysis.

Sample clean-up

Sample preparation was performed with a Millilab 1A workstation (Waters, Milford, MA, USA) with adapters for the Bond Elut columns. The adapters were drilled with a conical drill, otherwise the probe tended to drop the Bond Elut column.

For sample preparation, the freeze-dried urine controls were reconstituted with 10 ml of water and diluted with 10 ml of sodium acetate buffer, pH 5. The samples were spiked with 100 μl from one of the three standards and then prepared by the Millilab workstation according to the following method:

(1) Condition the Bond-Elut column with 5 ml of acetonitrile followed by 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 10% acetonitrile in 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 10% tetrahydrofuran in hexane at 2 ml/min.

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

(7) Elute the aflatoxins with 3.0 ml of dichloromethane at 2 ml/min.

Each sample was prepared according to the scheme before the workstation started with the next sample. The eluate was collected in the silanized tubes and the solvent was manually evaporated under nitrogen at a flow-rate of 100 ml/min during 45 min at room temperature. The aflatoxins were then dissolved in 200 μl acetonitrile–water (1:1) using a vibrator (Wortex) for 15 s and then transferred to 200-μl plastic microvials (Waters) for HPLC analysis.

Chromatography

The liquid chromatograph consisted of a Waters Model 6000A pump and a Waters WISP 710A autoinjector. Detection was performed with an RF-530 fluorescence detector (Shimadzu, Kyoto, Japan) at an excitation wavelength of 365 nm and emmission wavelength of 440 nm. The post-column derivatization was performed with bromine at room temperature (21°C), using a KOBRA cell from Lamers & Pleuger (Den Bosch, Netherlands) at 20 μA [18]. The post-column reactor was knitted PTFE tubing, 500 × 0.55 mm I.D. (reaction time 6 s). The chromatogram was recorded with a Spectra Physics integrator (San Jose, CA, USA)

The chromatography was performed using a 250 × 4.6 mm I.D. Supelcosil LC-18 column (5 μm, Bellefonte, PA, USA), with a mobile phase consisting of acetonitrile–methanol–water (20:20:60). 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 20 μl. The mobile phase was continuously degassed by helium.

Derivatization

A fractional factorial design was used to study the post-column derivatization of aflatoxins B1 and G1. To study the influence of the derivatization of aflatoxins B2 and G2, a mixture of the four aflatoxins was used. Three factors were used: amount of potassium bromide in the mobile phase, length of the reaction coil and the current used for bromine generation. The potassium bromide level was 1 or 10 m*M*, the length of the reaction coil corresponded to a reaction time of 6

TABLE I

RANGE OF VARIATION OF EXPERIMENTAL FACTORS FOR POST-COLUMN DERIVATIZATION

Factor	Low level (−)	High level (+)
x_1: potassium bromide in mobile phase (mM)	1	10
x_2: reaction time (s)	6	71
x_3: current (μA)	20	200

TABLE II

EXPERIMENTAL DESIGN FOR OPTIMIZATION

y_1 and y_2 are the resulting responses as peak areas of aflatoxins B1 and G1, respectively.

Experiment	x_1	x_2	x_3	y_1 area B1	y_2 area G1
1	+	+	+	21191	17919
2	+	+	+	21014	17861
3	+	+	+	21069	17816
4	−	+	−	30646	27072
5	−	+	−	30132	27668
6	−	+	−	30588	27511
7	−	−	+	29532	26705
8	−	−	+	29725	27713
9	+	−	−	30902	26389
10	+	−	−	30410	25983
11	+	−	−	30572	26336

or 71 s and the current for bromine generation was 20 or 200 μA, as described in Table I. The aflatoxins in acetonitrile–water (1:1) were repeatedly injected into the chromatograph. The experimental design is shown in Table II.

RESULTS AND DISCUSSION

The final overall method was tested by spiking urine control samples containing aflatoxins B1, B2, G1 and G2 with known concentrations at three different levels. Recoveries are obtained by comparison with standards directly injected into the liquid chromatograph. The results of method validation are shown in Table III. When the urine sample of 10 ml was diluted and spiked with the standard mixture, average recoveries after extraction and clean-up were in the range 90–105% at

TABLE III

RECOVERIES OF AFLATOXINS B1, B2, G1 AND G2 FROM SPIKED HUMAN URINE AT THREE DIFFERENT LEVELS

Compound	Sample added (pg/ml)	Recovery (%)	R.S.D.[a] (%)	n	Average[b] recovery (%)
B1	49.0	99	7	6	95
	120	93	4	5	
	233	94	4	6	
B2	49.0	90	15	6	90
	120	87	7	5	
	233	93	2	6	
G1	49.0	95	7	6	93
	120	88	5	5	
	233	97	4	6	
G2	49.0	105	15	6	89
	120	76	5	5	
	233	87	8	6	

[a] Relative standard deviation.

[b] Mean recovery from the three different levels studied.

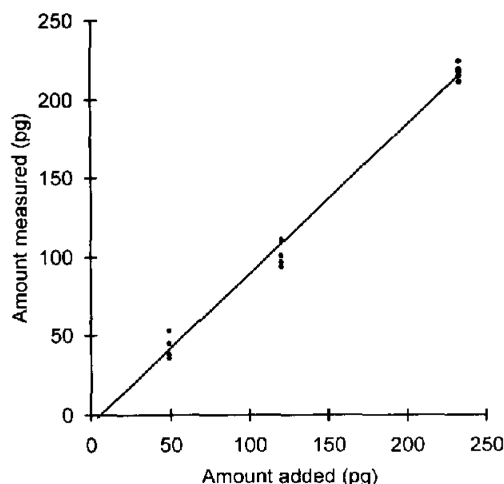

Fig. 2. Plot of recoveries of aflatoxin B2 in spiked human urine at three different levels.

49.0 pg/ml, 76–93% at 120 pg/ml and 87–94% at 233 pg/ml. The relative standard deviations, 2–15%, were all within accepted limits [20]. For example, in Fig. 2, which shows the results for aflatoxin B2, the amount of aflatoxin B2 measured is plotted against the amount added. Chromatograms of a spiked and unspiked urine control sample are shown in Fig. 3. The volume injected into the chromatograph corresponds to an amount of 1 ml of urine control sample. Despite a complex matrix, the automated procedure used gives an effective and reproducible clean-up of the urine sample. With the automated procedure up to twelve samples can be prepared and analysed in 24 h. The time for the preparation of each sample by the Millilab workstation was 63 min.

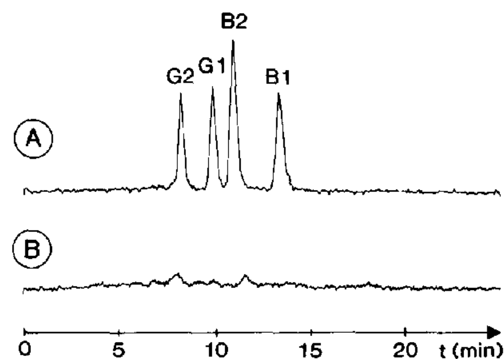

Fig. 3. Chromatogram of a urine sample extracted and cleaned up on Bond Elut C_2 columns. (A) Urine spiked to contain 120 pg/ml of each aflatoxin (B1, B2, G1 and G2). (B) Unspiked urine sample.

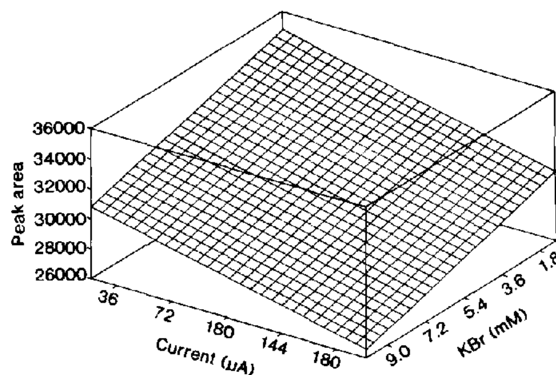

Fig. 4. Response surface of peak area of aflatoxin B1 against amount of potassium bromide in mobile phase against current for generation at a reaction time of 6 s.

The detection limits (signal-to-noise ratio of 3) per ml of urine were: B1 29 pg, B2 20 pg, G1 24 pg and G2 21 pg. Calibration curves for the different aflatoxins were linear with regression coefficients of 0.99 in the range 2.4–12 ng/ml with 20-μl injections ($n = 5$).

The recoveries were generally high, although it is known that automation of solid-phase extraction can result in low recoveries. Van Rhijn *et al.* [21] automated an extraction with two-column clean-up with intermediate solvent evaporation. The recoveries for the manual method gave recoveries of 80–90% for aflatoxin B1, compared with recoveries of 44% for the automated method.

Silanization of test tubes

Sources of recovery losses are adsorption to glass and losses during evaporation. The test tubes for the evaporation of dichloromethane were therefore silanized with trichlorosilane and the nitrogen flow was controlled. Table IV shows the recovery of the aflatoxins with and without silanized test tubes for the evaporation. When the test tubes were not silanized, a loss of aflatoxins of 15–75% was observed, the largest losses being noted for aflatoxins G1 and G2. No losses were seen when the tubes were silanized.

TABLE IV

EFFECTS OF SILYLATION OF TEST TUBES

A 2-ng aliquot of aflatoxin B1, B2, G1 or G2 in 3 ml of dichloromethane was stood in a test tube and evaporated after three different times.

Compound	Time (h)	Recovery (%)	
		Unsilanized tubes	Silanized tubes
B1	0	67	106
	4	77	100
	8	77	101
B2	0	75	101
	4	85	101
	8	84	103
G1	0	25	100
	4	44	99
	8	37	105
G2	0	33	103
	4	46	105
	8	45	101

Derivatization

The experimental design specified in Tables I and II was used to evaluate the influence of the variables on the derivatization of aflatoxins B1 and G1. In order to keep the experimental design as simple as possible, parameters such as temperature and mobile phase flow-rate were kept constant, and the experimental design for the variables studied was a 2^{3-1} fractional factorial design [22]. There were only four different settings in the design and the experiment could thus be performed within a short period of time. From the results in Table II, a linear response surface model that describes the variation in the peak area of aflatoxin B1 (y_1) as a function of the experimental settings (x_1–x_3) was computed by the method of least squares multiple linear regression. The model was: $y_1 = (27\,950 \pm 65) - (2091 \pm 65)x_1 - (2590 \pm 65)x_2 - (2177 \pm 65)x_3$.

The regression affords $r^2 = 0.998$. A normal probability plot of the residuals, $y_1^{Obs} - y_1^{Pred}$, shows a linear fit. Analysis of variance (ANOVA) of the regression did not indicate any lack of fit. This results showed that the reaction time should be short, that the current for generation should be low and that the concentration of potassium bromide should also be kept low. The settings of the variables chosen for observing the highest fluorescence were 6 s reaction time, a current of 20 μA and 1 m*M* potassium bromide in the mobile phase. Fig. 4 shows a three-dimensional projection of the response surface. The post-column derivatization did not affect aflatoxins B2 and G2 but gave 30-fold gain in sensitivity for B1 and G1.

CONCLUSIONS

A method for analysing aflatoxins B1, B2, G1 and G2 simultaneously in human urine has been described. Extraction and clean-up on Bond Elut C_2 columns was made automatic with a robotic system. This system enables a high sample throughput with average recoveries (>85%). The final analyses were performed by reversed-phase HPLC on C_{18} columns. Fluorescence detection (365/440 nm) was enhanced by optimizing the conditions for the post-column reactor by adding bromine to aflatoxins B1 and G1. It was observed that silylation of the glass vials used in the robotic system was of the utmost importance for preventing loss of analyte during the evaporation steps. Extraction and clean-up of aflatoxins is normally a very laborious task. Despite the chosen complex matrix, however, the automated procedure described here affords chromatograms with a minimum of impurities. The detection of small amounts (50 pg/ml) of aflatoxins B1, B2, G1 and G2 in human urine is thus made possible.

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