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Determination of aflatoxins in medicinal herbs and plant extracts

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Abstract

The occurrence of the aflatoxins B_1, B_2, G_1 and G_2 in common medicinal herbs and plant extracts was examined. The aflatoxins are toxic metabolites of the fungal strains *Aspergillus flavus* and *Aspergillus parasiticus*. The method involves the implementation of high-performance liquid chromatography (HPLC) together with fluorescence detection. Aflatoxins B_1 and G_1 are determined as their bromine derivatives, produced in an on-line, postcolumn electrochemical cell (KOBRA cell). The aflatoxins are extracted using a mixture of methanol and water and isolated and concentrated by means of immunoaffinity column chromatography. The aflatoxins are separated and detected by HPLC using an RP-18 column and a scanning fluorescence detector. The method permits the detection of aflatoxins with a detection limit of 0.05 ppb and recoveries in a range 70–100% for a variety of medicinal herbs. The method was tested on capsicum, potatoes, stinging nettles, birch leaves, senna leaves, etc.

1. Introduction

Aflatoxins are toxic metabolites of fungal origin (*Aspergillus flavus* and *Aspergillus parasiticus*). These mycotoxins are known to be highly toxic and carcinogenic. Therefore, the contamination of foods and animal feed with these mycotoxins is controlled by legal limits worldwide. In Germany these limits are 4 ppb (w/w) for the sum of aflatoxins B_1, B_2, G_1 and G_2 and 2 ppb for aflatoxin B_1. For human dietary products, such as infant nutriment, there are stronger legal limits: 0.05 ppb for aflatoxin B_1 and the sum of all aflatoxins.

Much has been published about the occurrence of aflatoxins in nuts (peanuts), nut-like products, corn and other natural products [1–3]. Only a few papers have described the contamination of spice (e.g., capsicum) [4] and dried fruit [5]. In this study, we examined the occurrence of aflatoxins in medicinal herbs, plant extracts and herbal remedies using HPLC with fluorescence detection after electrochemical derivatization in a KOBRA cell [6]. Especially birch leaves, senna fruits, *Rauwolfia serpentina*, etc., could be naturally contaminated. Previously, most determinations of aflatoxins have involved two dimensional high-performance thin-layer chromatography (HPTLC) with fluorescence detection by means of a TLC scanner. Enzyme-linked immunosorbent assay (ELSIA) tests and most often HPLC methods have been described.

Fluorescence detection with iodine postcolumn derivatization has two distinct disadvantages:

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first it requires the use of two HPLC pumps and second there is the problem of the instability of the iodine solution. The method using trifluoroacetic acid (TFA) as derivatizing agent has poor reproducibility and is difficult to automate.

The official methods in Germany [9] using two-dimensional TLC is time consuming and cannot be used for every matrix. For example, the medicinal herb *Rauwolfia serpentina* is known to give wrong results using TFA derivatization or TLC techniques because of its content of fluorescent alkaloids such as reserpine and rescinnamine.

2. Experimental

This method was employed for tests on a variety of medicinal herbs and their extracts: capsicum, stinging nettles, ginger, cardamom, hawthorn (*Crataegi folium cum flore*), mistletoe (*Visci herba*), cone flower (*Echinaceae angustifoliae radix*), deadly nightshade (*Belladonnae folium*), birch leaves, senna leaves, potatoes, beans, ivy leaves (*Hedera folium*), horse chestnut (*Hippocastani semen*), fennel seek (*Foeniculi fructus*), valerian (*Valerianae radix*), gourd seed (*Cucurbitae semen*), melissa (*Melissae folium*), cress (*Nasturtii herba*), passion flowers, etc.

2.1. Principle

The aflatoxins are extracted using a mixture of methanol and water by sonification and isolated and concentrated by the means of immunoaffinity column chromatography. The aflatoxins are separated and detected by HPLC using an RP-18 column and a scanning fluorescence detector.

2.2. Reagents

All reagents used were of analytical-reagent grade. No interference with the aflatoxins was observed. The extraction solvent was a mixture of methanol (LC grade; Baker, Gross-Gerau, Germany) with water (70:30, v/v). The mobile phase was water–methanol (58:42, v/v) with addition of 119 mg/l of potassium bromide (Merck, Darmstadt, Germany) and nitric acid (65%, 100 μl/l) (Merck).

The aflatoxin working standard solutions were prepared by dilution of a stock standard solution (Mix Kit-M; Supelco, Bellefonte, PA, USA) with methanol (1:100, v/v). The resulting absolute concentration was determined by comparison of the HPLC signals with standard solutions of single aflatoxins. For this purpose pure aflatoxins were dissolved in methanol and determined by means of UV spectrophotometry, the UV absorption of the standard solutions being measured at 362 nm against pure methanol. The aflatoxin concentration was calculated using the following equation.

$$c = A \cdot 1000 \cdot F \quad (1)$$

where c = concentration (ng/ml), A = absorption and $F = 14.31$ for aflatoxin B_1, 13.08 for aflatoxin B_2, 15.53 for aflatoxin G_1 and 15.53 for aflatoxin G_2.

This solution was further diluted with water (1:20, v/v). The resulting concentrations were 0.15 ng/ml for aflatoxin B_2 and G_2 and 0.50 ng/ml for aflatoxin B_1 and G_1. The solutions can be stored at −18°C in the dark.

All work was carried out in the absence of daylight. All glassware was cleaned with dilute hydrochloric acid and water. Used glassware was decontaminated using sodium hypochlorite solution (4% active chlorine; Rühl-Chemie, Germany).

2.3. Apparatus

A sonification bath was used for the extraction of the aflatoxins. As a clean-up step immunoaffinity column chromatography (Aflaprep columns; Coring System, Darmstadt, Germany) was applied. The HPLC system was purchased from Waters (Eschborn, Germany). The HPLC equipment consists of a system interface module (SIM box, Waters), an HPLC pump (W510; Waters), an injection valve (Rheodyne Model 7125 or W717 autosampler; Waters, a precolumn (Nova-Pak C_{18}; Waters), the analytical column (Radial-Pak cartridge packed with RP-18 Type 8

NVC, 4 μm, 100 × 8 mm I.D.; Waters), the KOBRA cell and a scanning fluorescence detector (W470; Waters). The KOBRA cell was purchased from Coring System.

2.4. Extraction

A 5-g amount of a homogenous sample, which was collected by official methods [7], was mixed and homogenized in a mill (<0.5 mm, Retschmill). To this sample 20 ml methanol–water (70:30, v/v) was added and extracted by sonification for 30 min. After centrifugation an aliquot of the supernatant (5 ml) was diluted with water to 25 ml and thoroughly mixed.

2.5. Immunoaffinity column chromatography

The solution obtained was passed through the Aflaprep immunoaffinity column, which was conditioned with 10 ml of water. After washing with 20 ml of water, the aflatoxins were eluted in six successive steps with 0.5 ml of methanol, with a stop of 30 s after each step, under reduced pressure using a Vac-Elut system. The solution obtained was diluted with water to 10 ml and was used directly for injection after membrane filtration.

2.6. HPLC conditions

The HPLC analysis was carried out as follows. The isocratic HPLC system consisted of a pump (W510; Waters), an injection valve (Rheodyne Model 7125) with a 200-μl sample loop or an autosampler (W717; Waters). Separation was carried out on a Radial-Pak cartridge, Type 8 NVC C-18 (4 μm, 100 × 8 mm I.D. (Waters) with a precolumn (Nova-Pak C_{18}; Waters). The eluent was water–methanol (58:42, v/v) with the addition of 119 of mg potassium bromide and 100 μl of nitric acid (65%) per litre at a flow-rate of 0.8 ml/min (isocratic). The aflatoxins were detected using a scanning fluorescence detector ($\lambda_{ex} = 360$ nm, $\lambda_{em} = 440$ nm; filter, 1.5 s; attenuation, 8; gain, ×100; emission band width, 30 nm). Aflatoxins B_1 and G_1 were derivatized using a KOBRA cell with a current of 100 μA and a length of the reaction coil of 28.2 cm. The chromatograms were evaluated using a chromatography workstation (Waters) with Maxima or Millennium software. A system interface module (SIM box) served to control the pump and to digitize the signals of the fluorescence detector.

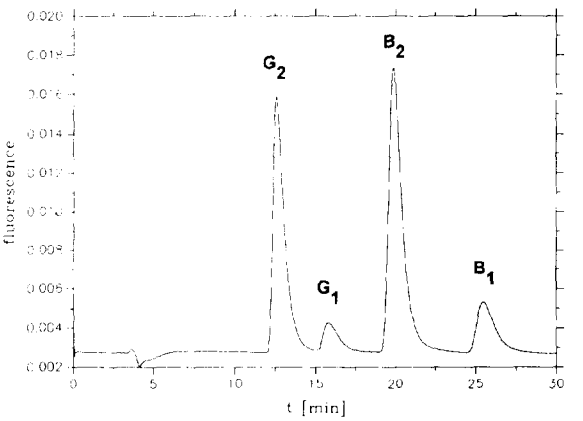

Fig. 1. Chromatogram of a standard pool without KOBRA cell.

3. Results and discussion

Fig. 1 shows a chromatogram of a standard solution of the four aflatoxins without the KOBRA cell (current 0 μA) and Fig. 2 one with the KOBRA cell (current 100 μA).

The signals represent an amount of 35–57 pg

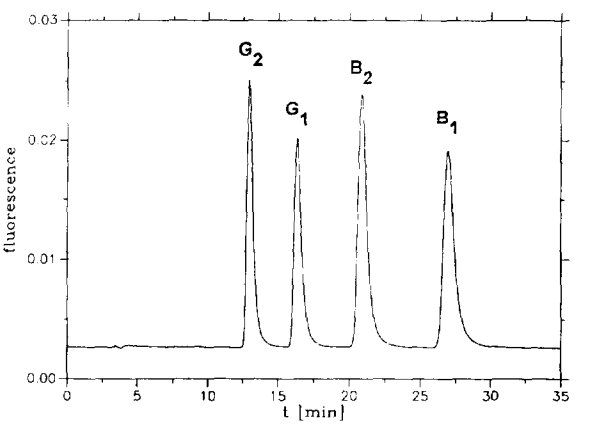

Fig. 2. Chromatogram of a standard pool with KOBRA cell.

of each aflatoxin. Using the KOBRA cell, the sensitivity for the detection of aflatoxin B_1 and G_1 is drastically increased. The developed method can be used for determination of aflatoxins in complex plant materials (medicinal herbs, plant extracts) and in herbal remedies, such as tinctures and coated and uncoated tablets. Also herbs containing fluorescent components, such as citrus pulps, *Rauwolfia serpentina* and licorice (*Liquiritiae radix*) could be successfully examined.

The method was validated corresponding to the EC guideline "Analytical Validation" and the Tripartite ICH Text on Validation of Analytical Procedures by the CPMP working party on Quality of Medicinal Products [8]. This document is concerned with the discussion of the characteristics that must be considered during the validation of analytical procedures included as part of registration applications submitted within the EC, Japan and USA. Typical validation characteristics for impurity tests are specificity, precision (repeatability), accuracy, linearity and detection limit of the method.

3.1. Specificity

The specificity of the analysis is given, on the one hand, by the comparison of the retention times of the signals in the sample and the standard pool, and on the other, by the excitation and emission spectra of the signals. Fig. 3 shows as an example the excitation spectrum of aflatoxin B_1 and Fig. 4 the corresponding emission spectrum.

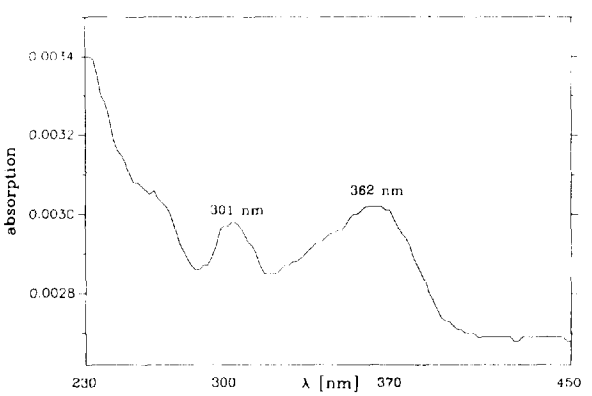

Fig. 3. Excitation spectrum of aflatoxin B_1.

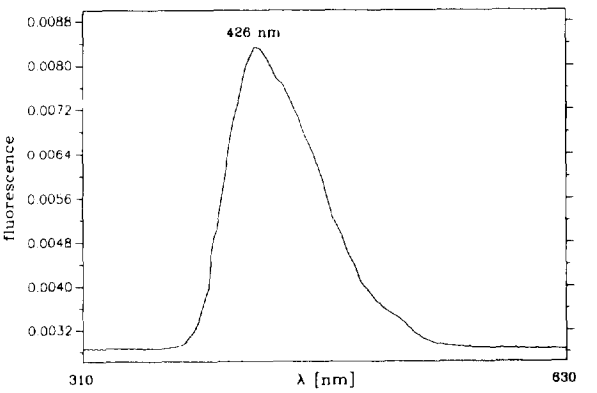

Fig. 4. Emission spectrum of aflatoxin B_1.

In addition, samples that were not contaminated by aflatoxins were measured without the

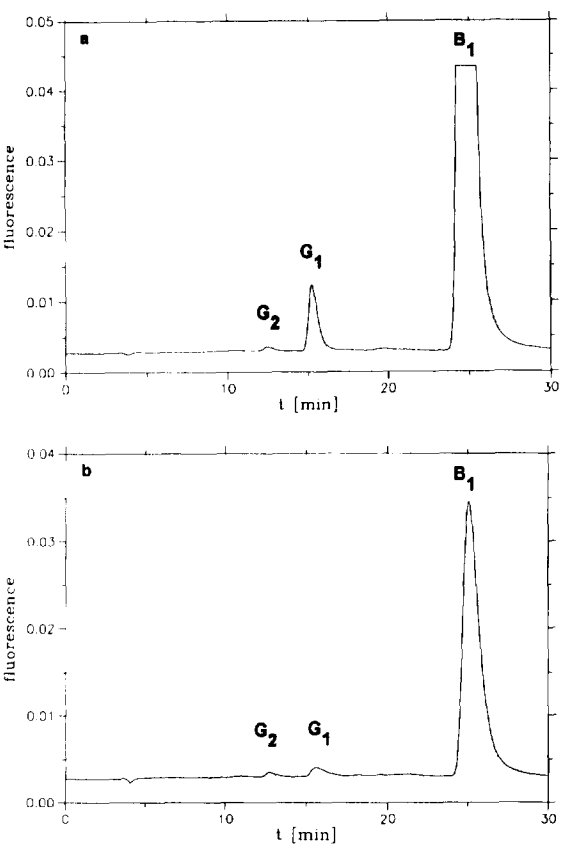

Fig. 5. Chromatograms of a natural contaminated extract of *Rauwolfia serpentina* (a) with and (b) without KOBRA cell.

Table 1
Reproducibility of the analytical procedure ($n = 6$; $P = 95\%$)

Aflatoxin	R.S.D. (%)	C.I. (ppb)
B_1	5.15	0.0962
B_2	2.43	0.0340
G_1	2.43	0.0368
G_2	3.20	0.0221

KOBRA cell. In the case of natural contamination the signals for aflatoxin B_1 and G_1 will be decreased.

Fig. 5a and b show chromatograms of a natural contaminated extract of *Rauwolfia serpentina* with and without the KOBRA cell. The signals of the aflatoxin B_1 and G_1 are decreased without the KOBRA cell. This is indirect proof of the specificity of the method.

3.2. Precision

The precision was determined by a multiple analysis of a spiked sample (capsicum). The precision is expressed by the relative standard deviation (R.S.D.) and the confidence interval of the mean value (C.I.; $n = 6$; $P = 95\%$; Table 1).The level of contamination was 1.780 ppb for aflatoxin B_1, 1.333 ppb for aflatoxin B_2, 1.444 ppb for aflatoxin G_1 and 0.659 ppb for aflatoxin G_2.

3.3. Accuracy

The accuracy of the method was examined by the determination of the recoveries of the aflatoxins (Table 2). The recoveries of different matrices were determined by the addition of an aflatoxin pool to the sample (0.24 ng/ml aflatoxin G_2, 0.29 ng/ml aflatoxin G_1, 0.18 ng/ml aflatoxin B_2, 0.26 ng/ml aflatoxin B_1).

3.4. Linearity

The linearity of the measurements was checked for a standard solution containing aflatoxins in a range from the limit of detection up to a concentration of 10 ng/ml. The calibration graphs can be described by the following equations:

aflatoxin B_1: $y = 0.843x$ ($R^2 = 0.999$)

aflatoxin B_2: $y = 0.897x$ ($R^2 = 1.000$)

aflatoxin G_1: $y = 0.843x$ ($R^2 = 1.000$)

aflatoxin G_2: $y = 0.843x$ ($R^2 = 1.000$)

where y = fluorescence intensity and x = concentration (ng/ml).

4. Conclusions

A postcolumn procedure using a KOBRA cell to generate bromine electrochemically has been described. The sensitivity of the method is substantially increased with the use of the cell. The method can be applied to various plant materials and herbal remedies and with high selectivity for all aflatoxins; no matrix effects are observed.

Table 2
Recoveries of aflatoxins in different medicinal herbs

Sample	Recovery (%)			
	Aflatoxin B_1	Aflatoxin B_2	Aflatoxin G_1	Aflatoxin G_2
Potatoes	96.1	92.6	97.1	88.7
Valerian	78.6	84.7	82.0	57.8
Fennel seed	78.6	79.6	87.8	68.5
Coriander	66.4	70.9	78.2	59.1
Gourd seed	99.4	93.5	97.1	78.7

The procedure offers several advantages over other methods: high reproducibility, good recoveries for a variety of plant materials, easy automation, no need to use aggressive or unstable reagents, such as TFA or iodine, and inexpensive equipment (only one HPLC pump is used).

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