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Note

Comparison of three methods for the extraction of aflatoxins from human serum in combination with a high-performance liquid chromatographic assay

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Four major aflatoxins B_1 (AFB_1), B_2, G_1 and G_2, produced by certain strains of *Aspergillus flavus* and *Aspergillus parasiticus*, occur as frequent contaminants of many food commodities such as peanuts, corn and rice with AFB_1 usually found in the greatest concentration [1]. The AFB_1 metabolites aflatoxins M_1, M_2 and aflatoxicol have also been identified, aflatoxins M_1 and M_2 are excreted in the milk of animals fed rations containing aflatoxins B_1 and B_2 [1].

The ability of aflatoxin B_1 and its related compounds to act as potent carcinogens in many species has been well documented [2, 3] and there is considerable evidence that these compounds may be primary causes of human liver cancer in certain areas [4—8]. AFB_1 has also been cited in the aetiology of Reye—Johnson syndrome [9—11].

In order to facilitate investigation of the role of ingested aflatoxin in human disease a sensitive method for the detection of aflatoxin in small volumes of human sera is required. Radioimmunoassay [12] and ELISA techniques [13, 14] fulfill the requirements but both methods involve the use of specific antibodies. These antibodies are not commercially available and their production requires special resources including facilities for animal work, which are not available in many laboratories, especially in countries where aflatoxin contamination of food is most prevalent.

In order to identify the most consistently reliable method for the detection of AFB_1 in small serum samples comparisons were made between the methods of Nelson et al. [11], Romer [15], and Van Egmond et al. [16] for extracting AFB_1 from 1 ml or less of human sera spiked with known amounts of AFB_1 using high-performance liquid chromatography (HPLC) as the detection

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system. This paper reports the results of these comparisons and the applicability of the chosen method for the extraction of other aflatoxins B_2, G_1, G_2, M_1, M_2 and aflatoxicol from serum.

MATERIALS AND METHODS

Aflatoxin B_1 (20 μg/ml in chloroform) was obtained from Dr. Van Egmond, Rijksinstituut voor de Volksgezondheid (Bilthoven, The Netherlands). Aflatoxicol (0.1 mg/ml) in methanol and aflatoxins B_2, G_1, G_2, M_1 and M_2 in solid form were obtained from Sigma (Poole, Great Britain). Standard stock solutions were prepared by dissolving solid standards in a mixture of acetonitrile—benzene (1:9) to give concentrations of 0.1 μg/ml or by diluting the aflatoxin B_1 and aflatoxicol solutions to give concentrations of 0.1 μg/ml. All other chemicals were obtained from BDH (Poole, Great Britain).

Blood specimens were obtained, by antecubital venipuncture, from normal healthy adult volunteers. After allowing the blood to clot at room temperature for 2 h serum was separated by centrifugation. Spiked sera samples were prepared by transferring aliquots of the aflatoxin standard solutions containing known amounts of aflatoxin to tubes and evaporating to dryness under a stream of nitrogen. Serum (10 ml) was added to the tubes and incubated at 30°C for 1 h with gentle swirling. The resultant serum containing known amounts of aflatoxin standard was either used immediately or stored at 4°C until use.

HPLC

The sample components were separated on an ODS 5-μm column, 25 cm × 5 mm (HPLC Technology, Macclesfield, Great Britain) and detected by fluorescence detector (Kratos, Schoeffel Instruments) fitted with a 365-nm excitation filter and a 418-nm emission filter. The mobile phase consisted of water—methanol (50:50) at a flow-rate of 2 ml/min and pressure of 241 bar.

Extraction techniques

(1) Hexane—chloroform extraction. This is the method according to Nelson et al. [11]. Initially, 1 ml hexane was added to a 1-ml aliquot of aflatoxin spiked serum and gently mixed for 2 min, the mixture was centrifuged for 5 min at 2000 *g* and the upper hexane layer containing the serum lipids, removed. This procedure was repeated twice with further 1-ml aliquots of hexane, and the hexane layer removed after each centrifugation. The serum was then extracted four times with 1-ml aliquots of chloroform, by the addition of 1 ml of chloroform to the serum, vigorous shaking for 4 min, centrifugation for 10 min at 2000 *g* and removal of the lower chloroform layer. The chloroform extracts were pooled, evaporated to dryness under a stream of nitrogen, redissolved in 50 μl methanol—water—acetonitrile (25:25:50) and 20 μl of the resultant solution analysed by HPLC.

(2) Chloroform extraction with pentane clean-up. This method is that used by Van Egmond et al. [16] for determination of aflatoxins in liver samples.

Serum (1 ml) was mixed on a wrist-action shaker with 20 ml chloroform for 30 min. Phosphoric acid (4 mol/l), 0.4 ml, was added and the mixture shaken

for a further 1 min, then filtered and the filtrate blown to dryness. The extract was cleaned by sequential washings with (a) 7 ml *n*-pentane, 2.5 ml methanol and 0.25 ml 1.2 *M* sodium chloride; (b) 3.5 ml 1.2 *M* sodium chloride and 2.5 ml pentane; (c) 1 ml pentane, 1.5 ml diethyl ether, and 0.05 ml 4 *M* hydrochloric acid. After each addition the extract was shaken for 1 min and the pentane layer discarded. Finally 0.45 ml hydrochloric acid was added and the aflatoxin extracted with 1-ml aliquots of chloroform four times. The chloroform extracts were pooled, and then analysed by HPLC as described in (1) above.

(3) Acetone—ferric gel—chloroform extraction. This method is based on the Romer method [15] as used by the Tropical Products Institute (London, Great Britain).

Serum (1 ml) was added to 5 ml acetone and shaken on a wrist-action shaker for 30 min. The mixture was filtered and 0.3 g basic cupric carbonate added, mixed with a glass rod and then allowed to stand for 2.5 min. Ferric chloride (0.41 *M*, 3 ml) was added to 17 ml 0.2 *M* sodium hydroxide to form a gel which was immediately added to the sample, mixed well and allowed to stand for 2 min with occasional swirling. The mixture was filtered. The filtrate was placed in a separating funnel with 22 ml 0.0054 *M* sulphuric acid and 1 ml chloroform and shaken vigorously for 1 min. The layers were allowed to separate and the chloroform layer run off through a filter funnel containing anhydrous sodium sulphate. The upper aqueous layer was further extracted with three 1-ml chloroform aliquots. The extracts were pooled, blown to dryness and then analysed by HPLC as described above.

Procedure

Unspiked serum samples were extracted by all three methods and analysed by HPLC to detect fluorescing compounds which might interfere with aflatoxin B_1 analysis. None were detected, as shown in Fig. 1.

The lower limits of detection were measured by the lowest concentration at which the aflatoxin would produce a peak 2% of full scale deflection on the recorder. For each extraction method the lower limit of detection of AFB_1 was checked by extracting and analysing serum samples spiked with AFB_1 in concentrations ranging from 200 to 800 pg/ml. The lower limits of detection for methods (1), (2) and (3) were 250, 300 and 400 pg/ml, respectively.

Following the above procedures six serum samples spiked with AFB_1 in concentrations ranging from 571 to 8000 pg/ml were analysed by the three methods. One additional serum sample containing AFB_1, 296 pg/ml was analysed by method (1) only. The reproducibility of each method was checked by replicate analysis at each concentration. The standard deviation associated with the mean value for six estimations was expressed as the coefficient of variation.

RESULTS

Results are presented in Tables I, II and III. Recovery of AFB_1 by method (1) ranged from 80—95%, by method (2) from 59—82% and by method (3) from 33—67%. The mean coefficient of variation was similar for the three

TABLE I

REPRODUCIBILITY OF AFLATOXIN MEASUREMENTS USING EXTRACTION METHOD (1)

In all cases $n = 6$.

Aflatoxin concentration (ng/ml)		Recovery (%)	Coefficient of variation (%)
Actual	Recovered (mean ± S.D.)		
8.0	6.43 ± 0.21	80.4	3.3
8.0	7.61 ± 0.302	95.1	4.0
4.0	3.51 ± 0.07	87.8	2.0
4.44	4.09 ± 0.27	92.1	6.6
2.96	2.65 ± 0.31	89.7	11.6
0.571	0.471 ± 0.054	82.5	11.5
0.296	0.28 ± 0.022	94.8	7.8
	Mean	88.9	Mean 6.7

TABLE II

REPRODUCIBILITY OF AFLATOXIN MEASUREMENTS USING EXTRACTION METHOD (2)

In all cases $n = 6$.

Aflatoxin concentration (ng/ml)		Recovery (%)	Coefficient of variation (%)
Actual	Recovered (mean ± S.D.)		
8.0	4.77 ± 0.25	59.6	5.2
8.0	4.86 ± 0.31	60.7	6.3
4.0	2.57 ± 0.08	64.2	3.1
4.44	3.46 ± 0.15	77.9	4.3
1.0	0.87 ± 0.12	87.0	13.8
0.571	0.47 ± 0.04	81.8	8.3
	Mean	71.9	Mean 6.8

methods. The coefficient of variation was slightly greater for the lowest concentrations of AFB_1, by all methods. Percentage recovery of AFB_1 by method (2) seemed to be inversely proportional to the serum concentration of AFB_1 rising from ca. 60% in the highest concentration to ca. 80% in the lowest concentrations.

HPLC analysis of the hexane discarded in method (1) and *n*-pentane discarded in method (2) showed no AFB_1.

Storage of the spiked sera and the extracts at 4° C for periods up to one week did not affect the recovery or measurement of AFB_1.

TABLE III

REPRODUCIBILITY OF AFLATOXIN MEASUREMENTS USING EXTRACTION METHOD (3)

In all cases $n = 6$.

Aflatoxin concentration (ng/ml)		Recovery (%)	Coefficient of variation (%)
Actual	Recovered (mean ± S.D.)		
8.0	3.78 ± 0.21	47.3	5.6
8.0	4.76 ± 0.41	59.5	8.6
4.0	2.67 ± 0.19	66.8	7.1
4.44	1.46 ± 0.15	32.8	10.3
2.96	1.33 ± 0.07	44.9	5.3
0.571	0.36 ± 0.04	62.7	11.1
	Mean	52.3	Mean 8.0

TABLE IV

REPRODUCIBILITY, SENSITIVITY AND RECOVERY OF AFLATOXINS FROM SPIKED SERA SAMPLES USING EXTRACTION METHOD (1)

Aflatoxin	Mean recovery (%)	Limit of detection (pg/ml)	Reproducibility mean variation (%)
B_1	88.9	250	6.7
B_2	79.1	25	5.7
G_1	67.3	500	10.2
G_2	77.0	25	6.3
M_1	76.4	100	8.2
M_2	69.3	25	11.1
Aflatoxicol	81.2	100	9.8

Method (1) was also investigated for the extraction of aflatoxins B_2, G_1, G_2, M_1, M_2, and aflatoxicol from human sera samples spiked with these other aflatoxins. Using replicate analysis of six sera specimens the recoveries, reproducibilities and limits of detection are shown in Table IV. A chromatogram of a serum sample spiked with a mixture of aflatoxins B_1, B_2, G_1, G_2, M_1, M_2 and aflatoxicol is shown in Fig. 1.

DISCUSSION

In the appraisal of the suitability of laboratory techniques for use in clinical practice or in human research, sensitivity and reproducibility of the method and its applicability to small samples of body fluids or tissue are some of the most important considerations.

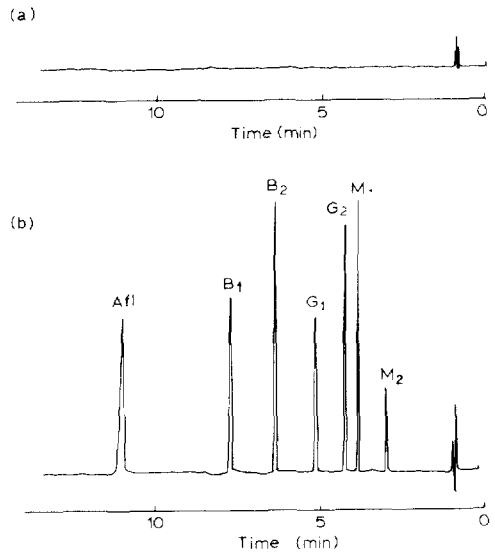

Fig. 1. HPLC chromatograms of human sera extracts. (a) Extract from unspiked serum, (b) extract from 1 ml serum spiked with aflatoxin M_2, 0.3 ng; M_1, 4 ng; G_2, 1.0 ng; G_1, 25 ng; B_2, 1.0 ng; B_1, 13 ng; and aflatoxicol, 2.5 ng. Column: Spherisorb 5 μm ODS; mobile phase: methanol—water (50:50); flow-rate: 2 ml/min; fluorescence detector, excitation 365 nm, emission 418 nm.

Method (1) [11] was found to be more sensitive than the other methods and also gave better recovery of AFB_1 from sera. The mean recovery of AFB_1 with method (1) was 88.9% compared to 71.9% and 52.3% with methods 2 and 3, respectively. It has been demonstrated that aflatoxin B_1 can adhere to the walls of glass vessels [17] and the variation in the recoveries observed could be a reflection of the number of vessels used in processing the sample in each method.

For ease of handling, 1 ml of serum was the preferred volume but all three extraction methods could be performed using as little as 0.1 ml serum.

There is a possibility that some drugs may interfere with this technique and this is at present under investigation.

As a result of these studies method (1) [11] has been adopted as the method of choice in our laboratory for aflatoxin detection in human fluids and tissues and has proved satisfactory also for the detection of aflatoxin B_2, G_1, G_2, M_1, M_2 and aflatoxicol.

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