Journal of Chromatography, 227 (1982) 256—261
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 1052

Note

Screening method for the detection of aflatoxin and metabolites in human urine: aflatoxins B_1, G_1, M_1, B_2a, G_2a, aflatoxicols I and II

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(First received March 23rd, 1981; revised manuscript received August 3rd, 1981)

Ingestion of aflatoxins has been associated with the occurrence of hepatocellular carcinoma (HCC) [1], and the identity of the eventual carcinogenic agent has been closely linked to the way in which aflatoxin is metabolised in the liver [2]. It has been shown that the sensitivity of animal species to the carcinogenic effects of aflatoxin B_1 can be directly correlated with hepatic activity and its transformation into aflatoxicol [3]. In man, hepatic metabolism and detoxification of aflatoxins may be related to a number of environmental influences [1]. Among these, the presence of acute or chronic liver pathology (cirrhosis, HCC, hepatitis) might influence the metabolism of aflatoxin in the liver, and this effect may be manifested in the type and concentration of metabolites excreted. Identification and quantification of aflatoxin metabolites may yield information concerning the mechanisms of aflatoxin metabolism in man.

This paper describes the successful extension of a simple technique for the extraction and quantification of aflatoxin M_1 from human urine [4] to aflatoxin B_1 and the metabolites aflatoxicol I and II, aflatoxin B_2a, G_2a and the derivative tetrahydrodeoxyaflatoxin B_1.

EXPERIMENTAL

The method used for aflatoxin extraction was based on the technique described by Messripour and Nesheim [4]. Aflatoxin M_1, aflatoxicols I and II, aflatoxins B_2a, G_2a and tetrahydrodeoxyaflatoxin B_1 were supplied by Makor Chemicals (Jerusalem, Israel). Aflatoxins B_1 and G_1 were supplied by Aldrich (Milwaukee, WI, U.S.A.). The recovery experiments used human urine spiked with a known amount of standard aflatoxin in the range 0.2—1.0 ng/ml for aflatoxins B_1, G_1, and 2.0—4.0 ng/ml for the metabolites. Celite 545 filter

0378-4347/82/0000—0000/$02.75

aid (10 g) (Johns Manville, Denver, CO, U.S.A.) was added to 100 ml urine. The mixture was stirred continuously for 10 min with a magnetic stirrer whilst 200 ml of distilled acetone were added slowly, then the mixture was filtered into a 600-ml beaker.

The filtrate was stirred and 100 ml of distilled water added, followed by 10 ml of a 20% lead acetate solution. The mixture was allowed to stand for 5—10 min, until the precipitate coagulated, and 5 ml of saturated sodium chloride slowly added with stirring, followed by 5 g of Celite. Stirring was continued for a further 1—2 min before the mixture was filtered into a 600-ml beaker. The filtrate was stirred and a freshly prepared ferric hydroxide slurry (30 ml 6.7% $FeCl_3$ and 170 ml 0.2 *M* sodium hydroxide) added, followed by 5 g Celite 545. The mixture was transferred into two 500-ml separating funnels and 50 ml of 0.1% sulphuric acid added to each. The filtrate in each flask was extracted with two portions of distilled chloroform (15 ml and 10 ml) and the two extracts combined and washed by shaking with 100 ml of 5% sodium chloride solution. The washed extract was evaporated to dryness over a water bath under a gentle stream of nitrogen. It was then quantitatively transferred to a small sample bottle and again evaporated to dryness. The final mixture was redissolved in 0.6 ml AR chloroform (Merck, Darmstadt, G.F.R.).

The extract was analysed by thin-layer chromatography (TLC). The 0.5 mm plates 20 cm × 20 cm, were prepared from silica gel G-HR (Macherey, Nagel and Co., Düren, G.F.R.), activated for 1 h at 105°C and stored in a desiccator. The chromatography tank was equilibrated overnight with acetone (distilled)—chloroform (distilled)—propan-2-ol (AR) (10:85:5). The chloroform extract (30 µl) was spotted in parallel with the standard aflatoxin metabolite at different concentrations. The original concentration of aflatoxin in the urine which would be expected from 30 µl of a 0.6-ml extract was calculated and different percentages of this used. To allow for the background fluorescence of other urine constituents, an aflatoxin-free urine extract was prepared as a control, and this was spotted on the same spot as the standards, so that they ran together. All plates were developed up to 15 cm from the bottom edge. The temperature was in the range 23—29°C, and the relative humidity in the range 40—80%. The concentration of aflatoxin metabolite was estimated by visual comparison of the fluorescence with the intensity of the standard spots plus control urine under 365 nm UV light (Hanovia Fluorescence 16).

The Messripour and Nesheim [4] method includes a detection column, however our experience showed that some interfering fluorescent substances were contained in the charcoal and acid alumina. These could be removed by washing with chloroform—methanol (97:3, v/v), however after this treatment some of the aflatoxin M_1 was retained by the first part of the column, which consisted of a mixture of acid-treated charcoal, magnesia, Celite 545 and glasswool in the ratio 2:4:8:1. In view of this and considering the large volume of extract required (0.5 ml out of a total of 0.6-ml sample) the column detection technique was not used in these studies.

TABLE I

RECOVERY OF AFLATOXIN METABOLITES EXTRACTED FROM HUMAN URINE

Metabolite	Limit of detection on TLC plate (ng)*	Recovery (%)			Mean value ± S.D. (%)	R_F**	Lowest detectable concentration in urine*** (ng per 100 ml)
		Run 1	Run 2	Run 3			
Aflatoxin B_1	0.3	60	75	75	70 ± 9	0.73 ± 0.08	9
Aflatoxin G_1	0.5	75	78	75	76 ± 2	0.59 ± 0.06	13
Aflatoxin M_1	1.0	80	85	60	75 ± 15	0.44 ± 0.08	27
Aflatoxin B_2a	1.0	25	10	12	16 ± 6	0.39 ± 0.08	125
Aflatoxin G_2a	2.0	60	55	50	55 ± 5	0.29 ± 0.07	73
Aflatoxicol I (natural isomer)	1.0	25	30	50	35 ± 13	0.69 ± 0.07	57
Aflatoxicol II (unnatural isomer)	1.0	45	48	50	48 ± 3	0.62 ± 0.06	42
Tetrahydrodeoxyaflatoxin B_1	1.0	60	60	60	60 ± 0	0.79 ± 0.06	33

*Estimated in presence of urine with a high fluorescent background.
**The R_F values are quoted to 1 S.D. of all estimations carried out in this study using standard metabolites.
***For a 30-µl application from a 0.6-ml concentrate per 100 ml urine.

RESULTS

The percentage yield obtained of each aflatoxin metabolite is presented in Table I. A representative thin-layer chromatogram is shown in Fig. 1, demonstrating the clear separation of metabolites. The limit of visible detection of aflatoxin on the TLC plate was measured in the presence of urine extract. These data, together with the average recovery obtained for each metabolite were used to calculate the lowest aflatoxin concentration detectable by this method. If the sample is concentrated to 0.2 ml rather than 0.6 ml sensitivity is increased, but this increases the amount of background fluorescence from the urine and reduces the volume available for confirmatory tests.

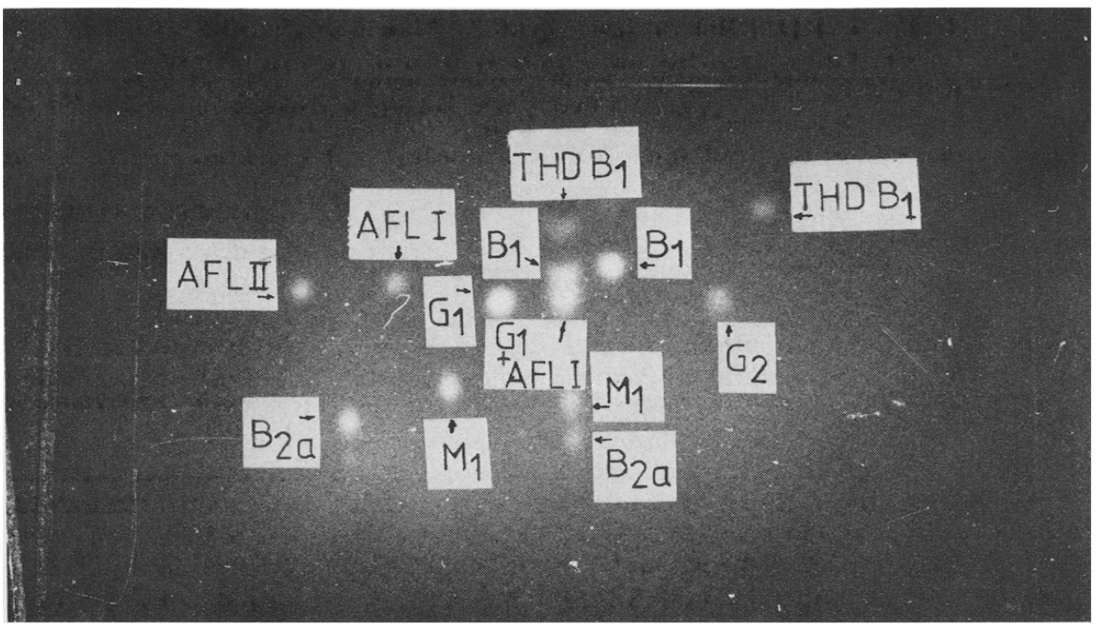

Fig. 1. A representative thin-layer chromatogram showing separation of aflatoxins B_1, G_1, M_1, B_2a, G_2a, aflatoxicols I (AFL I) and II (AFL II) and tetrahydrodeoxyaflatoxin B_1 (THD B_1); 0.5-mm silica gel G-HR plate equilibrated overnight, and run in acetone—chloroform—propan-2-ol (10:85:5).

Derivatisation with trifluoroacetic acid on the TLC plates was attempted for all the compounds in Table I [5]. Apart from aflatoxins B_1, G_1 and M_1 only the tetrahydrodeoxyaflatoxin B_1 responded to a slight degree.

This method has been used in this laboratory to analyse 200 samples of human urine in duplicate of which six have been shown to contain aflatoxin M_1 at greater than 30 ng per 100 ml [6]. This was confirmed by formation of the derivative with trifluoroacetic acid, seen on two-dimensional chromatography. No other suspected metabolite was observed. The urine samples were collected from hospital patients just after admission, in Lusaka.

DISCUSSION

A number of extraction and detection methods has been used to examine foodstuffs, animal tissue, human and animal milk, excreta and urines. In many reports, total recovery rates for the whole extraction and detection procedures are not stated, however the following values have been noted: 79—84% [7], 90—100% [8], 85% [9] and 80—83% [10] for aflatoxin B_1; 90—100% [8] and 100% [10] for aflatoxins G_1, G_2 and 87—88% [11], 92% [12] and 65% [9] for aflatoxin M_1. These compare reasonably with the data presented in this paper. This method uses simple instrumentation and a technique which is suitable for countries where aflatoxin is a problem and screening would be advocated.

However, it is necessary to assess the suitability of this method in relation to the local aflatoxin levels suspected. A previous study of plate-food samples collected in the dry season in the Eastern Province of Zambia gave the mean level of contamination of positive samples equal to 10 μg aflatoxin B per kg food [13]. Assuming an intake of 1 kg of food daily and a urine output of 1 l daily, and using the data available that 3.8% of aflatoxin is detectable as metabolites in vitro [3], the expected concentration of aflatoxin metabolites will be 38 ng per 100-ml urine sample. If 75% recovery is assumed approximately 1.5 ng would be present in the urine extract on the TLC plate for 30 μl of an 0.6-ml concentrate. Table 1 demonstrates that this metabolite concentration is at the lower levels of detection of this technique.

Using this method, urine samples obtained both from patients newly admitted to hospital with acute or chronic liver disease and from control patients without evidence of liver pathology, are being examined for aflatoxin metabolites. This is to assess the frequency of exposure to aflatoxins and the possible influence of liver disease on metabolite excretion patterns.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the advice and assistance of Dr. A.D. Campbell, Food and Drug Administration, Washington DC, U.S.A., and wish to thank the University of Zambia for a Research Grant.

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