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Note

Determination of aflatoxin M_1 in milk by reversed-phase high-performance liquid chromatography

NOBUYUKI TAKEDA
Public Health Institute of Hyogo Prefecture, Arata-Cho 2-1, Hyogo-Ku, Kobe 652 (Japan)
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Aflatoxin M_1, one of many animal biotransformation products of aflatoxin B_1[1], was first detected in the milk from cows[2]. M_1 has acute toxicity equivalent to that of B_1 and is a potent carcinogen to laboratory animals[3]; hence its presence in milk is a hazard to human health.

For the determination of M_1 in milk, thin-layer or high-performance liquid chromatography (HPLC) with fluorescence detection has been used for increased sensitivity and selectivity[4–6], but these methods are time-consuming, complex and expensive. Moreover, a large quantity of a toxic solvent such as chloroform is required to obtain a sufficiently clean extract for the sensitivity desired. Recently, Winterlin *et al.*[7] reported a simple on-column extraction method for M_1 in milk. However, no confirmatory test for M_1 was included in their method, resulting in the possibility of misleading background interferences at very low ppb levels.

The method described here involves a simple extraction and clean-up by SEP-PAK C_{18} cartridge prior to HPLC analysis coupled with a confirmatory test.

EXPERIMENTAL

Reagents and apparatus

A standard aflatoxin M_1 solution was prepared by dissolving 10 μg of commercially available M_1 (Makor Chemicals, Jerusalem, Israel) in 10 ml of chloroform–methanol (1:1) and the solution was diluted to 0.1 μg/ml. HPLC grade acetonitrile and methanol (Wako Pure Chemicals, Osaka, Japan) and glass-distilled water were used. A SEP-PAK C_{18} cartridge (Waters Assoc., Milford, MA, U.S.A.) was used for extracting M_1 from milk.

HPLC was performed using two Model 6000 pumps (Waters), a Model 660 solvent programmer (Waters) and a U6K injector (Waters). The HPLC column was a Radial PAK μBondapak C_{18} cartridge (10 μm, 8 mm × 10 cm) fitted in a Z-module radial compression separation system (Waters). A pre-filter (2 μm, Waters) was placed between the injector and the column. Detection was by a Model RF-530 fluorescence detector (Shimadzu Corporation, Kyoto, Japan), with excitation at 365 nm and emission at 425 nm, using the highest sensitivity of the instrument (range, × 1; sensitivity, high). The chromatograms were recorded by a Model VP-6521W 10-mV recorder (National Electrics, Osaka, Japan) at a chart speed of 0.5 cm/min.

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Operating conditions: temperature, ambient; flow-rate, 2.5 ml/min; mobile phase, 30% acetonitrile–methanol (1:1) in water; injection volume, $\leqslant 1500$ µl.

Extraction and clean-up

Ten millilitres of milk were diluted to 20 ml in water. The SEP-PAK C_{18} cartridge was prewashed with 10 ml of acetonitrile and then with 10 ml of water using a 20-ml glass syringe. The sample of milk was transferred to the syringe and injected into the cartridge at a flow-rate of *ca.* 10 ml/min. The sample-laden cartridge was washed with 10 ml of water, then with the same volume of basic 10% acetonitrile (ammonia–acetonitrile–water, 1:10:90), finally with 10 ml of acidic 10% acetonitrile (acetic acid–acetonitrile–water, 1:10:90). The washings were discarded. Five millilitres of acidic 30% acetonitrile (acetic acid–acetonitrile–water, 1:30:70) were then added to the cartridge. The eluate was collected into a 20-ml concentrating tube and evaporated to less than 1 ml under reduced pressure at 40°C. The solution was quantitatively transferred to a 10-ml graduated tube with 10% acetonitrile and brought to a final volume of 2 ml. An aliquot (1500 µl) of the solution was injected for HPLC. The M_1 fraction was collected manually into a 20-ml tube and evaporated to dryness.

Confirmation of M_1

Hexane (200 µl) and trifluoroacetic acid (TFA, 50 µl) were added to the residue, and the tube was capped tightly. The contents were mixed and allowed to react for 15 min at 40°C. The reaction mixture was evaporated to dryness, redissolved in 200 µl of 10% acetonitrile and then $\leqslant 100$ µl were injected for HPLC. The reaction product of M_1 is tentatively designated M_{2a}[8].

RESULTS AND DISCUSSION

Extraction and clean-up

In Winterlins' method[7], M_1 was not sufficiently separated from background interferences (Fig. 1). To remove them to permit detection of M_1 at lower levels, a clean-up on a SEP-PAK cartridge was attempted. The sample-laden cartridge was washed with 10 ml of basic then acidic 10% acetonitrile, and M_1 was eluted with 5 ml of acidic 30% acetonitrile.

The effective removal of fluorescent interferences by the present method is evidenced in the chromatogram shown in Fig. 1. This clean-up method requires significantly less time to carry out and provides a baseline separation between M_1 and the background.

Confirmation of M_1

Milk (10 ml) fortified with 1 ppb M_1 was extracted and separated by HPLC. The M_1 fraction was collected manually, divided into two equal portions and evaporated to dryness. One portion was redissolved in 1.5 ml of 10% acetonitrile and injected for HPLC. The other was treated with TFA and redissolved in 200 µl of 10% acetonitrile. Fifty microlitres of the solution were injected for HPLC. Fig. 2 shows the chromatograms of the non-treated (A) and TFA-treated M_1 fraction (B, designated M_{2a}). From the fact that the M_1 peak disappears, it can be concluded that the reaction with TFA is complete. The difference in retention time between M_1

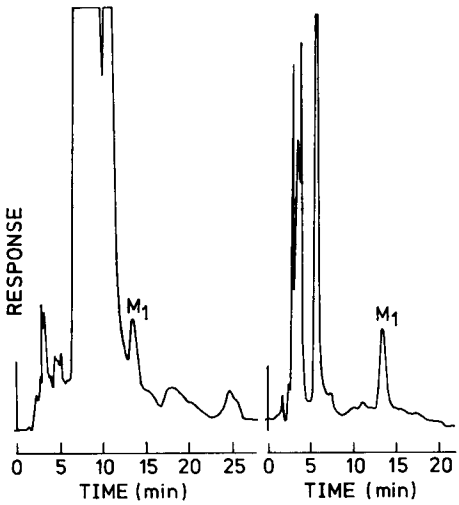

Fig. 1. Chromatograms of milk fortified with 0.5 ppb M_1: left, Winterlins' method; right, present method.

Fig. 2. Chromatograms of non-treated (A) and TFA-treated (B) M_1 fractions from HPLC.

(13.3 min) and M_{2a} (4.4 min) provides the confirmation of M_1. The conversion of M_1 into M_{2a} is quantitative (the calibration curve is linear for the concentration range 1–20 ng) and M_{2a} is approximately seven times more fluorescent than M_1 under the present conditions.

As the peak shift and the increase in peak height upon reaction with TFA are visible even if the M_1 fraction contains some background fluorescent materials, this method is highly selective and sensitive.

Re-use of SEP-PAK cartridge

In this experiment, four 10-ml portions of milk fortified with 0.5 ppb M_1 were extracted successively by the same cartridge. The cartridge was washed with 10 ml of water and acetonitrile, then activated with 10 ml of water prior to each extraction. The recoveries were 89.4, 88.5, 91.3 and 78.8%. These results demonstrate that a quantitative recovery is obtained with the same cartridge for each extraction and the operating cost is reduced by re-use of the cartridge.

Recovery of M_1

Table I shows the recoveries of 0.1–0.5 ppb M_1 added to 10 ml or 50 ml of milk. The present method gave an average recovery of 83.0% ranging from 71.9 to 95.5%. These values are of the same magnitude as obtained by other methods including the method of the Ass. Offic. Anal. Chem.[4].

Extraction capacity of SEP-PAK cartridge

Fifty millilitres of milk fortified with 0.1 ppb (5 ng) M_1 were extracted by two

TABLE I

RECOVERY (%) OF AFLATOXIN M_1 ADDED TO MILK

Volume of milk (ml)	*M_1 added*		*Recovery* (%)*	*No. of tests*
	ng	*ppb*		
10	5	0.5	84.1 (± 8.0)	7
	3	0.3	84.4	3
	2	0.2	87.5	3
	1	0.1	76.3	3
50	5	0.1	91.8	2

* Average value ± S.D.

cartridges connected with a small glass tube, overloading of organic materials having been presumed. When the entire sample had passed through the cartridges, a yellow pigment filled the upper cartridge and reached the lower one. The two cartridges were disconnected and washed separately. Fig. 3 illustrates the chromatograms of the eluate from each cartridge and shows that M_1 could be extracted by only the upper cartridge. The recovery was 91.8% (Table I). This result demonstrates that a large volume of milk (up to 50 ml) is quantitatively extracted by a single cartridge and the required sensitivity is obtained.

Sensitivity

The present method is capable of detecting $\leqslant 0.1$ ppb. Moreover, it should be noted that a large volume of sample (50 ml) coupled with the determination of M_{2a} enables a detection limit of less than 0.02 ppb.

Using the present method, thirteen samples of milk were analyzed and no M_1 was detected in any of the samples.

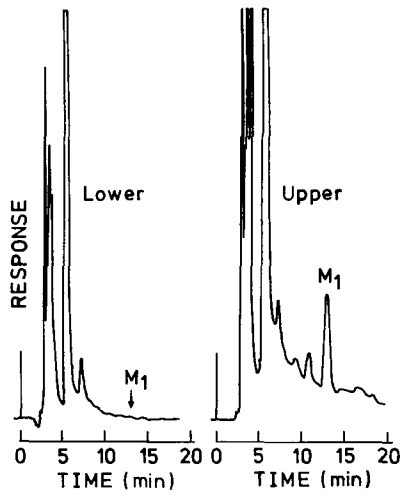

Fig. 3. Chromatograms of the eluates of milk (50 ml) fortified with 0.1 ppb M_1 from two SEP-PAK cartridges.

The proposed extraction and clean-up procedure coupled with a confirmatory test will be useful for analyzing numerous samples of milk with rapidity and accuracy, because it has the following advantages. A further clean-up prior to HPLC is not required. The time required for a complete analysis including confirmation is less than 1 h, in contrast to other procedures which require in excess of 3 h[4]. It is very selective, since only the collected M_1 fraction is converted into M_{2a}. Highly toxic or flammable solvents such as chloroform or diethyl ether are not used. It is economic, only small amounts of glassware and materials being needed. A single cartridge can be used for extraction of several samples. The use of a Radial-PAK (8 mm × 10 cm) column allows an increase in sample size for HPLC.

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* *Editor's note*: See also P. Chambon, S. D. Dano, R. Chambon and A. Grahchan, *J. Chromatogr.*, 259 (1983) 372, for a rapid HPLC method for aflatoxin M_1 in milk and dairy products using a zinc hydroxide precipitation to eliminate proteins, followed by a hexane clean-up.