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Occurrence of aflatoxin M_1 in milk determined by HPLC with derivatization method in Korea (1999-2000)

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Abstract : In this study, the levels of aflatoxin M_1 (AFM_1) in milk were determined by HPLC with derivatization method. Milk samples were purified using C_{18} disposable cartridge followed by derivatization with trifluoroacetic acid and analysed using HPLC with fluorescence detection. The recoveries of AFM_1 from milk samples added AFM_1 at a level of 0.025~0.1 ng/ml were 94.7~98.0% with detection limit of 0.009 ng/ml. The amounts of AFM_1 were determined below 0.05 ng/ml for all tested samples of commercial milk collected in 1999 and 2000.

Key words : Aflatoxin M_1, HPLC, milk

Introduction

Aflatoxin M_1 (AFM_1) is a hydroxylated derivative of aflatoxin B_1 (AFB_1) metabolized in lactating animal by liver cytochrome P450, so it can be found in milk of diary cattle fed with feed contaminated with AFB_1 [5, 10, 12]. The exposure of AFM_1 to infants has been most particular concern because it is hepatotoxic and carcinogenic compound in a number of species. Infants and children who, are uniquely vulnerable and more sensitive than adults, are major consumers of milk and milk products [2]. Many researchers have carried out the inspection on AFM_1 contamination in food and many countries established the levels of regulation for AFB_1 in food or feed and AFM_1 in milk by considering each country's condition [1, 4, 6, 8].

The level of AFM_1 in milk should not be exceed 0.5 ng/ml according to the United State Food and Drug Administration guideline. Most European countries apply the limit of 0.05 ng/ml for AFM_1 to milk. The level of international regulation in milk was determined as 0.5 ng/ml by CODEX in 2001. The maximum residue level for AFM_1 milk was determined as 0.5 ng/ml in harmonization with CODEX regulation in 2003.

The aim of this study was to determine the amounts of AFM_1 by HPLC-FLD (High performance liquid chromatography-Fluorescence detector) with derivatization method and a solid phase extraction method in commercial milk and diary raw milk in Korea between 1999 and 2000.

Materials and Methods

Materials

Commercial milk samples were purchased from local markets near the Anyang city in 1999 and 2000. Diary raw milk samples were kindly provided by local milk companies collecting from individual diary farms.

Reagents

Aflatoxin M_1 and trifluoroacetic acid (TFA) were obtained from the Sigma (USA). Organic solvents such as acetonitrile, benzene, and hexane were used with HPLC grade. Cartridges (Sep-Pak plus C_{18}) were obtained from Waters Inc. (USA) and preconditioned with 5 ml of acetonitrile and 5 ml of distilled water (D.W), sequentially, just before use.

Condition of Analysis

A chromatographic equipment consisted of a IsoChrom LC pump, a FL 3000 fluorescent detector (Spectra-physics, USA) set at the wavelengths of 360 nm for excitation and 420 nm for emission and a Supelcosil LC-18 liquid chromatographic column (250 ×

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4.6 mm; 5 μm; Supelco, USA). The mobile phase consisted of water-acetonitrile-isopropyl alcohol (80 + 8 + 12, v/v/v) and a flow rate was 1.0 ml/min.

Preparation and derivatization of standard solution

Standard solution were prepared according to Lee *et al.* [7] and derivatized according to the Stubblefield *et al.* [9]. Standard solution (50 μl) was dried with a nitrogen gas at 50°C and then added each of 200 μm hexane and TFA. Then the test tubes were capped, vortex and reacted for 10 min at 40°C. The derivatized solution were dried under nitrogen gas at 50°C, reconstituted with 0.5 ml of water-acetonitrile (75 + 25, v/v) solution and filtered through 0.45 μm. The filtered solution were injected 50 μl to the HPLC.

Extraction and clean up

The method of extraction of AFM_1 from milk was modified from that of Lee *et al.* [7]. Raw milk was centrifuged at 2,500 × g for 20 min at 4°C to remove cream layer. The 20 ml of aqueous layer at the lower part was applied to preconditioned Sep-Pak C_{18} cartridge with a flow rate of 4-6 ml/min and then washed with each of 5 ml D.W and 20 ml of 10% acetonitrile solution, followed by elution of AFM_1 with 2 ml acetonitrile. The eluant including AFM_1 was dried, derivatized with TFA, evaporated again and reconstituted according to the above preparation and dervatization of standard solution. AFM_1 amounts in sample were calculated by regression curve obtained from AFM_1 spiked into milk samples at the concentration of 0.025, 0.05 and 0.1 ng/m. Blank milk was prepared by passing commercial milk through activated C_{18} cartridge.

Table 1. Recoveries of AFM_1 from fortified milk samples

Fortified amount (ng/ml)	Recoveries (%, mean ± SD, n = 3)	Coefficient variation
0.025	97.79 ± 11.86	12.12
0.05	98.03 ± 5.89	6.00
0.1	94.70 ± 9.27	9.78

R for regression curve of aflatoxin M_1 derivatives in spiked milk is 0.999.

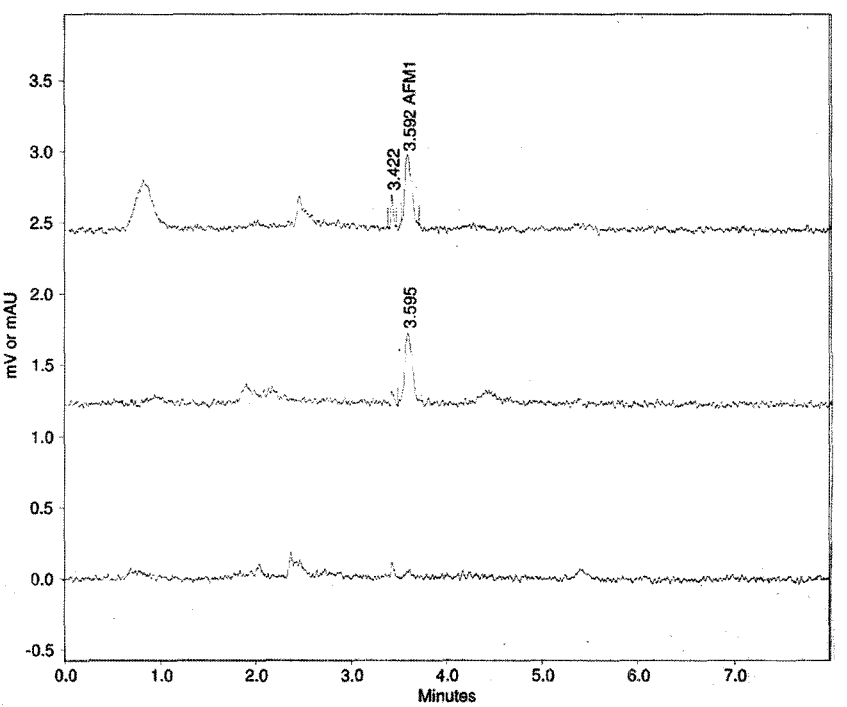

Fig. 1. Chromatogram of AFM_1 derivative in milk.
Bottom chromatogram is blank milk, middle is standard 0.025 ng/ml and upper is milk fortified with AFM_1 at 0.025 g/ml. Chromatography condition as described in materials and method.

Table 2. Occurrence levels and ranges of AFM_1 in commercial and dairy fresh milk in 1999-2000

Sample	Year[1)]	No. of sample	Ranges (ng/ml, (%))				Mean (ng/ml)
			< 0.01	0.01-0.05	0.05-0.5	> 0.5	
Dairy raw milk	'00	65	6 (9.2)	48 (73.8)	11 (16.9)	0	0.0311
	'99	48	5 (10.5)	42 (87.5)	1 (2.1)	0	0.0242
Commercial milk	'00	13	0	13 (100)	0	0	0.0376
	'99	10	0	10 (100)	0	0	0.0186
Total	'00	78	6 (7.7)	61 (78.2)	11 (14.1)	0	0.0320
	'99	58	5 (8.6)	52 (89.7)	1 (1.7)	0	0.0242

[1)]Commercial milk samples were purchased near Anyang city, dairy raw milk samples were collected only at Gyeonggi province in spring 1999 and Gyeonggi, Gangwon, Chungcheong, Gyeungsang, Jeolla province in fall 2000.

Results

Recoveries of Aflatoxin M_1

Recoveries for AFM_1 were 97.8%, 98.0% and 94.7% when AFM_1 standard solution were spikied in triplicate in the blank milk at the concentrations of 0.025, 0.05, and 0.1 ng/ml (Table 1) and a linear regression equation was obtained for fortified AFM_1 concentrations and their corresponding peak area in blank milk ($R = 0.999$). There was also no interference peak in spiked sample when treated with above procedure (Fig. 1) and detection limit was 0.009 ng/ml in milk.

Occurence of AFM_1 in milk

The levels and ranges of AFM_1 in commercial milk and dairy raw milk are represented in Table 2. The content of AFM_1 in commercial milk were between 0.01 and 0.05 ng/ml in 1999 and 2000. Dairy raw milk samples as collected in Gyeonggi province at dairy farms in spring 1999 and the amounts of AFM_1 were mostly below 0.05 ng/ml (47 cases) and one was more than 0.05 ng/ml. In the year 2000, dairy raw milk samples were collected in Gyeonggi, Gangwon, Chungcheong, Gyeongsang, Jeolla province in the fall and 54 cases (83%) were below 0.05 ng/ml and 11 cases (17%) were more than 0.05 ng/ml in the AFM_1 amount.

Discussion

AFM_1 is a hydroxylated metabolite of potent carcinogen AFB_1, classified as a class 2B human carcinogen in the IARC (International agency for research on cancer), and it is secreted in milk and urine of animals fed with AFB_1 contaminated feed. The excreted amount of AFM_1 ranges from 0~4% with an average ratio of below 1% as a percentage of fed AFB_1. AFM_1 is disappeared within 2-4 days after withdrawal from the contaminated feed. Maximum residue limit in milk was determined by considering regulation level of AFB_1 in feed, feed consumption amount per day, milk production amount per day and metabolic rate (1~2%) [10, 11].

Detection limit and repeatability are the most important factors in the development of analysis method because the regulation level in milk is very low. In our study, fluorescence intensity is increased 5 times compared to parent compound by derivatization of AFM_1 with TFA to TFA-AFM_1 (AFM2a) according to the Dominguez *et al.* [3]. Using derivatization method with TFA and solid phase extraction with C_{18} cartridge, detection limit for AFM_1 in milk is enhanced to 0.009 ng/ml (3 times of standard deviation). This detection limit is similar to other's report [6, 7] and this method can be sufficiently applied for the detection of EU (European union) residue limit. Average recoveries of 94.70~98.03% and CVs of 9.78~12.12% for AFM_1 added to 20 ml of blank milk were obtained when samples were extracted and purified with solid phase extraction and derivatezed with TFA according to the above procedure. These results are satisfied with CODEX recommendation guideline, 50~120% for recovery and 35% for CV. So, this used analysis method is sufficient to determine EU regulation level of AFM_1 in milk (0.05 ng/ml).

Most European countries support that limit of 0.05 ng/ml for aflatoxin AFM_1 should be of application for liquid milk and this limit be also applicable for milk products, taking into account the concentration caused by the drying process. as maximum residue limit in

milk. They concluded that lowest level should apply to limit for AFM_1 to protect the infant or young children because it is very genotoxic compound (1 ng/kg body weight), milk and milk products are primary food for them and they are more sensitive than adults [13].

On the other hand, other countries like US, Brazil suggest that limit of 0.5 ng/ml for aflatoxin AFM_1 is applicable for liquid milk because it is very difficult to detect 0.05 ng/ml with reported analysis method, carcinogenicity of AFM_1 is lower 10 times than that of AFB_1 and 0.05 ng/ml result in severe disruption to international feed trade. Limit of 0.05 ng/ml in liquid milk was proposed to CODEX by European countries, it was returned to step 6 after 32nd session of the codex committee on food additives and contaminants in 2000 [13]. After concentrated discussion at 33rd session of the codex committee on food additives and contaminants in 2001, committee concluded that there are no difference in carcniogenicity between 0.05 ng/ml and 0.5 ng/ml according to the 56th report of Joint FAO/WHO expert committee on food additives in 2001. The limit of 0.5 ng/ml in liquid milk was endorsed as maximum residue for the aflatoxin M_1 at CODEX alimentarius commission in 2001. The amounts of AFM_1 in commercial milk in Korea were below 0.05 ng/ml and average concentration were 0.0128 and 0.0310 ng/ml in 1999 and 2000, respectively. The result in 2000 is similar to Kim's report in Korea and is lower than Turkey 0.043 ng/ml [1] and Iran 0.105~0.525 ng/ml [2].

In case of individual dairy farm milk, the incidence above 0.05 ng/ml is increased from 2.1% to 16.9% in 2000. It is inferred that seasonal variation showed in survey results, because samples were collected only in Gyeonggi province in March 1999 and mostly in Jeolla province in September 2000. Exposure to AFM_1 by milk is below regulation of EU. It is inferred that commercial milk in Korea is safe for infant and young children. Only AFB_1 limit in feed is regulated by government, most feed are dependent on imported grains in Korea, and most important impact on the amount AFB_1 occurrence level in feed was temperature and moisture because *Aspergillus flavus* can easily grow in feed having moisture 13% and environmental moisture 50%. Furthermore, it can produce aflatoxin under condition of 25°C and 80% of relative humidity [11]. Much interest should be concentrated on the AFB_1 contamination of importing countries of grain and storage of feed in rainy summer season.

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