乳中のアフラトキシンM1測定法

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Determination of aflatoxin M_1 in powdered formula: an inter-laboratory study and the surveillance in Japan

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Abstract

A method to determine aflatoxin M_1 (AFM_1) levels by using an immunoaffinity column-based clean-up procedure and HPLC with fluorescence detection was validated by an inter-laboratory study among ten laboratories in Japan. Using the validated method, we surveyed AFM_1 contamination in powdered formula. Samples for validation included a blank, three levels (blind pairs) of AFM_1 spiked into liquid milk, naturally contaminated liquid milk, and naturally contaminated powdered formula. All samples were frozen and sent to the ten participating laboratories. For the liquid milk spiked at 1.0, 0.5, and 0.05 μg/kg levels, recoveries were 89.9, 91.6, and 88.2%, respectively. The repeatability relative standard deviation (RSD_r) and reproducibility relative standard deviation (RSD_R) were less than 7.4 and 8.1%, respectively. The recovery, RSD_r, and RSD_R of the powdered formula were 94.5, 8.9, and 11.9%, respectively. The RSD_r and RSD_R of the naturally contaminated milk were 13.3 and 20.9%, respectively. The Horwitz ratio (HorRat) values of all six samples were less than 1.0. For surveillance, 108 commercial powdered formulae were obtained in Japan. The average value of AFM_1 in the powdered formulae was 0.002 μg/L, as ready-for-infant liquid milk (14 g powdered formula in 100 mL water). The highest contamination was 0.025 μg/L.

Introduction

Aflatoxins, a group of potent genotoxic carcinogenic compounds, are secondary metabolic products of *Aspergillus flavus*, *A. parasiticus*, and *A. nomius* that may contaminate various agricultural commodities[1)]. Aflatoxin M_1 (AFM_1) is an aflatoxin B_1 (AFB_1) metabolite that is readily transferred to mammalian milk[2)].

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Aflatoxins containing AFM_1 are classified as possibly carcinogenic to humans (Group 1) by the International Agency for Research on Cancer[3]. In 2001 , the Codex Alimentarius Commission established a maximum residue level (MRL) of 0.5 μg/kg for AFM_1 in milk[1]. Many countries have established regulations for AFM_1 levels in bovine milk. The MRLs for bovine milk are 0.5 and 0.05 μg/kg in the United States[4] and the European Union[5], respectively. For infants, the MRL for bovine milk is 0.025 μg/kg in the European Union[5].

In Japan, the surveillance of AFM_1 have been carried out in raw bulk milk in 2004[6] and in commercial liquid milk in 2001 and 2002[7]. The average level of AFM_1 in raw bulk milk was less than 0.011 μg/kg and that in commercial liquid milk was 0.009 μg/kg. These levels were below those deemed permissible by the Codex Alimentarius Commission. However, the surveillance of powdered formula has not yet been undertaken in Japan. Before such monitoring takes place, the analytical methods for powdered formula and liquid milk must be validated with an inter-laboratory study. Several validated analytical methods that include TLC[8], HPLC[9,10], and lateral flow assay[11] have been reported for the determination of AFM_1. Immunoaffinity columns (IACs)[9] are the most popular method for the clean-up of AFM_1 samples from milk. Regulations regarding AFM_1 have not existed in Japan yet. In this study, we conducted an inter-laboratory study for the validation of AFM_1 in powdered formula and liquid milk. Using this method, the surveillance of 108 powdered formulae was conducted for the first time in Japan.

Materials and Methods

Standard and reagents An AFM_1 (Wako Pure Chemicals, Osaka, Japan) standard stock solution (1.0 μg/mL) was in a sealed amber glass bottle. The AFM_1 concentration was determined according to the molar absorptivity of AFM_1 in acetonitrile (19,000) at the maximum adsorption near 350 nm[12]. AFM_1 standard stock solutions were stored at −20°C until use. HPLC grade acetonitrile and water were used. The IACs (Horiba, Kyoto, Japan) were stored at 4°C.

Fortification procedure and samples for inter-laboratory study To evaluate recovery, AFM_1 solutions at three different concentrations were added to liquid milk (20.0 g, blank), which was purchased from a supermarket located in Tokyo, and stirred gently. The final concentrations of AFM_1 in the liquid milk were 1.0 μg/kg (A), 0.5 μg/kg (B), 0.05 μg/kg (C), and blank (D). Naturally contaminated raw milk was prepared by feeding cows with AFB_1 contaminated feed. Naturally contaminated powdered formula (0.473 μg/kg), which was a surplus sample of the food analysis performance assessment scheme (FAPAS), was purchased from GSI Creos Corporation (Tokyo, Japan).

Pretreatment The artificially and naturally contaminated liquid milk samples and the blank were warmed to 37 °C, stirred gently to mix using a glass rod or magnetic stirrer, and sonicated for 5 min. At least 40 mL of milk was transferred to a 50 mL plastic centrifuge tube. After 5 min centrifugation at 3000 rpm at 25 °C or room temperature, an upper layer of fat was removed. The milk was filtered through a glass fiber filter in a glass funnel and transferred to an Erlenmeyer flask or beaker. Exactly 20.0 g of filtrate was weighed for purification by IAC. To spike, AFM_1 solutions (20 μL) were added and gently stirred and sonicated in 5 minutes and loaded onto an IAC.

Powdered formula (5.0 g) was weighed, mixed with water (30 mL, 50°C), and sonicated for 5 min to

obtain a homogeneous mixture. This was allowed to cool to room temperature (~ 25°C). The sample was diluted to 50 mL with water. The solution was filtered through a glass fiber filter. If necessary, the milk was centrifuged for 5 min at 3000 rpm at 25°C or room temperature. The filtrate sample (20mL) was loaded on an IAC immediately after filtration.

Purification by IAC The loaded samples of pretreated liquid milk and powdered formula solution were dropped at a flow rate of 1-2 drops·s^{-1}. The IAC was then washed with water (15 mL). AFM_1 was eluted with acetonitrile (3 mL) and the eluate was collected in a silanized amber screw top vial. After solvent evaporation under nitrogen gas, HPLC injection solution (1 mL, acetonitrile:water (2 : 8 , v/v)) was added and agitated using a mixer. The solution was transferred to a silanized amber vial for HPLC injection. The silanized amber screw top and HPLC vials were washed with 20-30% acetonitrile solution before use.

HPLC conditions The HPLC column was octadecyl silylied gel (3-5 μm particle size; diameter: 3-4.6 mm; length: 150-250 mm) maintained at 40 °C in a column oven. The mobile phase was acetonitrile:water (25:75, v/v), used at a flow rate of 0.6-1.0 mL/min. The injection volume was 20-100 μL, and detection was with a fluorometric detector by an excitation wavelength of 365 nm and emission wavelength of 435 nm.

AFM_1 standard solution for HPLC The AFM_1 standard solution (1.0 μg/mL) was diluted in acetonitrile, and dried with a nitrogen gas stream or an evaporator. One mL of acetonitrile: water (2:8, v/v) was added to the residue and mixed well for AFM_1 calibration standard solutions. A seven point calibration curve covering the range of interest for the test sample (0.1-20.0 ng/mL) was established. The calibration curve was to be linear.

Table 1. Inter-laboratory study results of aflatoxin M_1 in milk with limits of detection (LOD) and limits of quantification (LOQ)

laboratory	Sample												LOD (μg/kg) luquid milk	LOQ (μg/kg) luquid milk
	A : 1.0 μg/kg		B : 0.5 μg/kg		C : 0.05 μg/kg		D : blank		Powdered formula (0.473 μg/kg)		Naturally contaminated milk			
1	0.767	0.868	0.426	0.448	0.039	0.042	0.010	0.010	0.497	0.425	0.564	0.491	0.003	0.011
2	0.932	0.918	0.507	0.483	0.049	0.042	0.006(t)	0.017(t)	0.381	0.487	0.550	0.586	0.002	0.008
3	0.976	1.014	0.500	0.489	0.054	0.044	0.010(t)	0.010(t)	0.449	2.582	—[a]	0.558	0.025	0.083
4	0.903	0.946	0.473	0.473	0.046	0.045	0.009	0.009	0.479	0.423	0.542	0.555	0.004	0.013
5	0.854	0.855	0.427	0.440	0.042	0.044	0.010(t)	0.010	0.446	0.419	0.474	0.473	0.012	0.040
6	0.868	0.913	0.450	0.450	0.044	0.046	0.010	0.011	0.489	0.475	0.327	0.567	0.003	0.008
7	0.859	0.916	0.455	0.468	0.041	0.042	0.009(t)	0.009(t)	0.458	0.452	0.484	0.479	0.010	0.033
8[c]	0.941	0.882	0.417	0.321	0.020	0.278	—[b]	0.000	0.226	0.121	0.261	0.364	0.010	0.033
9	0.887	0.510	0.342	0.404	0.039(t)	0.153	0.000	0.032(t)	0.387	0.320	0.269	0.293	0.050	0.167
10	0.861	0.929	0.424	0.417	0.045	0.042	0.011	0.011	0.503	0.511	0.484	0.489	0.002	0.007

(t) : trace

a) IAC chocked

b) sample vial breakage

c) all data eliminated for statistical anaylsis

d) data of 1.0, 0.5, 0.05 μg/kg were raw data minus 0.010

Calculation The AFM_1 mass concentration of the test sample was calculated using the following equation for liquid milk:

$Wm = Wa/Ws$

Where, Wm is the numerical value of the AFM_1 in the test sample (μg/kg), Wa is the numerical value of the AFM_1 in the HPLC injection test sample (ng/mL), and Ws is the weight of the test sample.

For the powdered milk sample, the AFM_1 mass concentration of the test sample was calculated using the following equation.

$Wm = Wa/ (Ws/2.5)$

The AFM_1 mass concentrations in the surveillance samples as liquid milk (powdered formula/water = 14 g/100 mL by the conventional manufacturer's manual) were calculated using the following equation.

$Wl = Wa/ (Ws/2.5) \times 14/100.$

The limit of detection (LOD) was calculated with a signal/noise (S/N) ratio of 3 : 1 , and the limit of quantification (LOQ) was calculated with an S/N ratio of 10:1.

Inter-laboratory study To validate the method, an inter-laboratory study was carried out using six samples (a blank, three spiked liquid milks, one naturally contaminated liquid milk, and one naturally contaminated powdered formula) with duplicate blind samples, according to the protocols of the Association of Official Analytical Chemists (AOAC)[13]. Ten laboratories participated in the inter-laboratory study: the Kawasaki City Institute for Public Health, Kewpie Corporation, Food Analysis Technology Center SUNATEC, Japan Ecotech Co., Ltd., Japan Food Research Laboratories, Hamamatsu City Health and Environment Research Institute, Mie Prefecture Health and Environment Research Institute, Meiji Dairies Corporation, Morinaga Corporation, and Snow Brand Milk Products Co., Ltd.

Surveillance of powdered formula The 108 samples for the surveillance comprised 24 brands with different product lot numbers that were purchased or obtained from six manufacturers of infant powdered formulae in 2010 in Japan. The samples were dissolved in hot water in the same manner as in the validated inter-laboratory method.

Statistical analysis The precision parameters; the inter-laboratory relative standard deviations for repeatability (RSD_r) and for reproducibility (RSD_R) were deduced as recommended by the AOAC[13].

Results and Discussion

Validation of method for detection of AFM_1 In Table 1, the results of the inter-laboratory study are shown. Ten laboratories returned results, but because of the incomplete results from laboratory 8, its data were omitted from the statistical analysis. Outliers were determined by the Cochran and the Grubbs tests[13]. For samples A, B, C (spiked AFM_1 in liquid milk), and D (blank), laboratory 9 was an outlier by the Cochran test. For the powdered formula, laboratory 3 was an outlier by the Cochran test. For the naturally contaminated milk, the results from laboratory 3 were omitted because only one result for duplicate samples was reported.

The data for the blank sample (D) showed that the AFM_1 concentration of commercial liquid milk in Japan was 0.010 μg/kg (average). This value was nearly equal to the average for Japanese commercial liquid milk reported by Nakajima *et al.* (0.009 μg/kg)[7]. All spiking data (A: 1.0 ; B: 0.5 ; C: 0.05 μg/kg) were subtracted from the blank (0.010 μg/kg). The LODs for the majority of the participants were under 0.010 μg/kg or equal to, except for laboratory 3, 5 and 9.

Table 2 shows the average levels, precision parameters, and Horwitz ratio (HorRat) values for this method. The recoveries of the spiked samples (A, B, and C) were acceptable, in the range 88.2-91.6%. The recovery for the powdered formula (94.5%) was also acceptable. The RSD_rs of samples A, B, and C were very low, <7.4%. The RSD_rs of the naturally contaminated powdered formula and liquid milk were 8.9% and 13.3%, respectively. The RSD_Rs of the spiked samples (A, B, and C) were less than 8.1%, whereas those for the naturally contaminated powdered formula and milk were 11.9% and 20.9%, respectively. The HorRat values were calculated using $RSD_R/22$[14]. HorRat values for the spiked samples (A, B, and C) were less than 0.37, whereas those of the naturally contaminated powdered formula and liquid milk were 0.54 and 0.95, respectively. According to the criteria of the EU[15], the precision parameters and HorRat values in this method are adopted as the method for the surveillance of powdered formula as well as liquid milk.

In another inter-laboratory study of an analytical method for the determination of AFM_1 using an immunoaffinity column as a clean-up procedure and HPLC with fluorescence detection, the recovery of a 0.05 μg/kg spiked sample of liquid milk[9] was 74%. The RSD_rs and RSD_Rs of spiked and naturally contaminated samples were in the ranges 8-18% and 21-31%, respectively. In the cases of low and high fat powdered formulae[10], the RSD_Rs for the AFM_1 concentration range of 0.08-0.6 μg/kg were 11-23%. Compared to the other two inter-laboratory studies, this method is superior in terms of the analysis for both liquid milk and powdered formula as a validated method.

Surveillance Using the method validated in this study, a survey of AFM_1 levels in powdered formulas was performed. The LOD was 0.003 μg/L (as ready-for-infant liquid milk; 14 g powdered formula in 100 mL water). The average value was 0.002 μg/L. The distribution of AFM_1 contamination showed that 72 samples were lower than the LOD; 16 samples were at the LOD ~0.05 μg/L; 12 samples were 0.005-0.010 μg/L; 6

Table 2. Summary of statistical of inter-laboratory study results for aflatoxin M1 in milk

Sample	A: 1.0 μg/kg	B: 0.5 μg/kg	C: 0.05 μg/kg	D: Blank	Powdered formula (0.473 μg/kg)	Naturally contaminated milk
Participants	10	10	10	10	10	10
Removed Labs.	2	2	2	2	2	2
Retained Labs.	8	8	8	8	8	8
Average (μg/kg)	0.899	0.458	0.044	0.010	0.447	0.477
True value, %	89.9	91.6	88.2		94.5	
Repeatability SD [S_r]	0.038	0.010	0.003		0.040	0.064
Repeatability relative SD [RSD_r, %]	4.3	2.1	7.4		8.9	13.3
Repeatability value [r ($2.8S_r$)]	0.107	0.028	0.009		0.111	0.178
Reproducibility SD [S_R]	0.059	0.029	0.004		0.053	0.100
Reproducibility relative SD [RSD_R, %]	6.6	6.3	8.1		11.9	20.9
Reproducibility value [R ($2.8S_R$)]	0.166	0.081	0.010		0.149	0.279
HorRat [$RSD_R/22$]	0.30	0.29	0.37		0.54	0.95

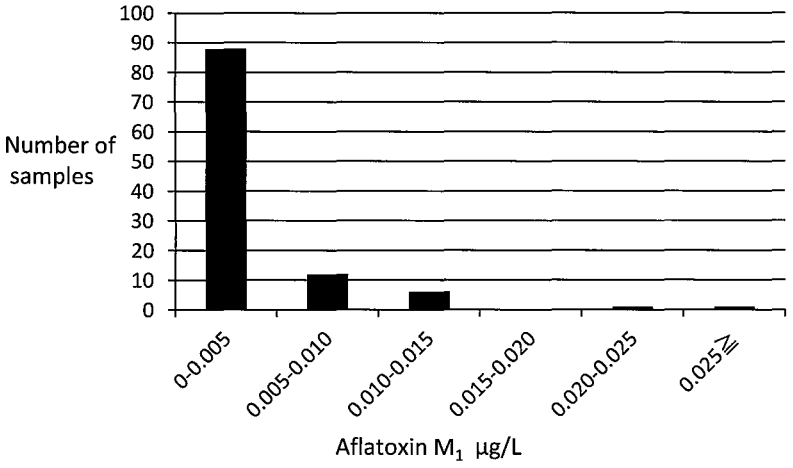

Fig. 1. Surveillance of aflatoxin M_1 in powdered formula μg/L as ready-for-infant liquid milk (14 g of powdered formula in 100 mL of water)

samples were 0.010-0.015 μg/L; one sample was 0.020-0.025 μg/L; and one sample was 0.025 μg/L. The strictest regulatory limit is 0.025 μg/kg of the EU for infant milk[5]. This study demonstrates that the powdered formula supplied in Japan contains extremely low levels of AFM_1, as determined using the analytical method validated by inter-laboratory study.

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乳中のアフラトキシン M_1 測定法：日本における室間実験と乳児用粉乳の実態調査

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日本の 10 機関による，乳中アフラトキシン M1（AFM_1）測定の室間実験を行った．イムノアフィ

ニティカラムにより AFM_1 を精製し，高速液体クロマトグラフィー- 蛍光検出で測定した．各 1.0, 0.5, 0.05 μg/kg の AFM_1 を添加した乳，ブランク乳，汚染粉乳，自然汚染乳の 6 種類の牛乳材料を用いた．3 種類の AFM_1 添加した乳の添加回収率は 88.2–91.6%，汚染粉乳の回収率は 94.5%であった．ブランク乳を除く 5 種類の牛乳材料の室内再現相対標準偏差は 13.3%以下，室間再現相対標準偏差は 20.9%以下，修正 HorRat 値は 1 以下であった．この方法を用いて，日本国内で入手した 108 の乳児用粉乳の試料を測定した．平均値は 0.002 μg/L（乳幼児に与える液体換算：14 g 粉乳 /100 mL 水）であり，最高値は 0.025 μg/L だった．

キーワード：aflatoxin M_1; inter-laboratory study; liquid milk; powdered formula; surveillance