



Determination of Aflatoxin M1 in whey powder by HPLC and ELISA methods

Mahboubeh Shaneshin^{a,b}, Hoda Noursalehi^a, Zahra Mousavi^c, Zeinab Pourjabbar^a, Mannan Hajimahmoodi^{a,b*}

^a Drug and Food Control Department, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

^b Food and Drug Administration, Tehran University of Medical Sciences, Tehran, Iran

^c Pharmacology & Toxicology Department, Faculty of Pharmacy & Pharmaceutical sciences, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran

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ABSTRACT

Whey powder is one of the dairy products that is used widely in the food industry. Therefore, it is necessary to make sure that it is healthy and is not contaminated with various toxins such as Aflatoxin M1, in order to protect the health of the consumers. Producing Whey powder from contaminated milk (with Aflatoxin M1) results in the products containing Aflatoxin M1. This study was conducted to determine the contamination of whey powder to Aflatoxin M1 in two domestic factories. Whey powder samples were collected during 8 months from December 2014 to July 2015. Regarding the Aflatoxin M1, the samples were evaluated by ELISA. Additionally, 26 samples were also analyzed by HPLC-FLD method. According to ELISA method, the contamination level of 44 samples (52.4%) was less than the permitted value (1000 ppt) and 40 samples (47.6%) were higher than the maximum tolerance limit recommended by Iranian national standard (Number: 5925). The average concentration of toxin in the studied samples was 1100.2 ± 734.8 (ppt). The range of changes in concentration was between 200 and 7000 ppt. In the current study, seasonal impact on the level of Aflatoxin M1 was not significant among the samples. The results of HPLC and ELISA methods did not show significant differences in the detection of Aflatoxin M1 contamination. Seasonal impact on the level of Aflatoxin M1 was not significant among the samples. Contamination of whey powder with Aflatoxin M1 is harmful to human health.

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1. Introduction

Aflatoxins are an important group of fungal toxins, produced by some species of *Aspergillus*, (especially *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus niger*) in agricultural products, in the presence of moisture and heat and the lack of suitable storage conditions. They can be found in most plant products including peanut, soy, rice and wheat (1,2).

Eighteen different types of Aflatoxins are known in nature, among them, aflatoxins G2, G1, B2, B1 are important, and their metabolites are called M1 and M2 (1, 3).

Aflatoxin B1 (AFB1) is more common and toxic

than Aflatoxin M1 (AFM1) (4). AFM1 is a metabolite of AFB1 found in milk when dairy cattle are fed with contaminated foodstuff. It is relatively stable and the pasteurization process cannot degrade it. Aflatoxins are shown to be immunosuppressive, mutagen, teratogen and carcinogen in both animals and human (5,6).

AFB1 and M1 have been classified as primary and secondary groups of carcinogenic compounds by International Agency for Research on Cancer (IARC) and the World Health Organization (WHO) respectively (7).

Whey powder is one of the milk products which is widely used in the food industry. The presence of AFM1 in whey powder is due to the use of AFM1 infected milk in its production (8).

* Corresponding author. Tel/Fax: +98 2164121219
E-mail address: hajimah@sina.tums.ac.ir

Moreover, heat treatments, such as sterilization and pasteurization, are not effective on destroying the poison (9). According to the WHO and other organizations the maximum allowed level of AFM1 in milk and dairy products is 50-500 ng/kg (7,10). Whey powder is widely used in food industry in Iran. The maximum acceptable amount was determined 1000 ng/Kg (11). In the present study, the AFM1 was measured by ELISA method in whey powder for the first time in Iran. ELISA is a biochemical diagnostic method. This method does not need much sample preparation. Additionally, it is easy to conduct and is not expensive (12).

In the current study, the ELISA test was used to measure the AFM1 in whey powder, then 26 samples were randomly analyzed by HPLC method.

2. Materials and methods

2.1. Samples

The 84 whey powder samples were purchased from two Iranian factories and were analyzed with ELISA technique (December 2014 to July 2015). Twenty six of the samples were also analyzed by HPLC method.

2.2. Chemicals and reagents

The quantitative analysis of AFM1 in the whey powder samples was performed by competitive enzyme immunoassay using Ridascreen AFM1 (Company R-Bio pharm, Germany, Art. No.: R1121) test kit. For HPLC analysis, acetonitrile (HPLC grade, purity $\geq 99.9\%$) was purchased from Merck (Darmstadt, Germany) and de-ionized water was prepared through the Thermo Scientific Branstead Easypure II system. AFM1 standard was purchased from Sigma-Aldrich (34031 Company, USA). The stock solution of AFM1 was prepared by acetonitrile at a concentration of 10 $\mu\text{g}/\text{kg}$ and was stored at -10°C . Working solution was prepared by dilution of acetonitrile and water.

2.3. ELISA Procedure

In the present study, ELISA was used for screening contaminated samples as one of the most sensitive, low cost and simple immunoassays available.

According to the kit manufacturer company, 10 g of each sample was weighed and dissolved by adding 100 ml water. Then, a homogenous suspension was prepared by shaking samples on Rotator for 15 min. The suspension was heated in 50°C water bath for 30 min. when the samples became cool, they were

centrifuged at 10°C , 3500 g for 10 min and the upper layer oil was removed totally. The lower skimmed milk was used for ELISA test. AFM1 kit was prepared and the test was done according to the manufacturer's protocol. One-hundred micro-liters of the standard solutions or the defatted samples were added to the cells to occupy the binding sites, and then conjugated enzyme was added and incubated. It led to convert the chromogen to a blue product. Then, the stop solution was added to the wells which caused the color to become yellow. Lately, the absorbance was measured at 450 nm by photometer (Hyperion ELISA reader, Biotek Instruments, USA) against blank within 15 min. Concentration of AFM1 was calculated by calibration curve which was obtained using 6 standards for ELISA with the following concentrations: 0, 5, 10, 20, 40, 80 ppt and the results were compared with the Iranian national standards (11).

2.4. HPLC Procedure

2.4.1. Preparation of samples

Whey powder (5 g) was dissolved by adding 50 ml water. The suspension was shaken to make the samples homogenous. Homogenous suspension was placed in an ultrasonic bath for 2 min. The suspension (40 ml) was centrifuged at 4°C , 8000 rpm for 15 min. The upper oil layer was removed completely and the liquid was collected for immunoaffinity column (IAC). After preparation of the solid phase of IAC, 20 ml liquid content was passed to IAC for purification and AFM1 separation. The column was washed with 20 ml water. Acetonitrile (500 μl) at the speed of 2-3 ml/min was passed through the column and the solution was collected in a vial. The vial ingredients were put on the heater at 50°C until the solution was dried and concentrated. After evaporation of acetonitrile, 1 ml of acetonitrile and distilled water solution used as the mobile phase, were added to the sediments in the glass tube and mixed for 1 min by shaker until all residues well dissolved and clear solution was obtained. This solution was then injected into the HPLC. Concentration of AFM1 was calculated by calibration curve which was obtained by 5 standards for HPLC with the following concentrations: 50, 100, 500, 1000, 2000 ppt and the results were compared with the Iranian national standards (11).

2.4.2. Apparatus

The HPLC determination of AFM1 was conducted using an Agilent 1200 series liquid chromatograph

equipped with a gradient pump capable of mixing four solvents, 20 μ L loop injector, vacuum membrane degasser and an RF detector. Analysis was performed with a ZORBAX Eclipse-XDB, C18 column (150mm \times 4.6mm, 5 μ m). The mobile phase was composed of acetonitrile and water (75:25). The flow rate 0.8 ml per min and the injection volume was 20 μ L. The excitation wavelength was 360 nm and emission wavelength was 440 nm.

2.5. Method validation

The method was validated according to the ICH guidelines. Linearity was constructed with five different concentrations of AFM1 and the correlation coefficient (R^2) was assessed (0.999). The detection limit (LOD) and quantification limit (LOQ) may be expressed as: $DL = 3.3 \sigma/S$ and $QL = 10 \sigma/S$, where, σ is the standard deviation of the response and S is the slope of the calibration curve of the analyte. The estimate of σ may be carried out by using standard deviation of blank. In order to verify the feasibility of

the method, recovery was carried out by analyzing samples before and after the addition of known quantities of aflatoxin standard solution to samples (13).

2.6. Statistical analysis

Data were statistically analyzed using t-test and ANOVA to determine significant differences among the groups. Statistical tests were conducted using SPSS 16. The P values less than 0.05 were considered significant. The values were expressed as means \pm SD.

3. Results

The relation between the absorption percentage and concentration of AFM1 (in the range of 0-80 ppm) was evaluated and the results are shown in the curve below (Figure 1).

The calibration curve of AFM1 (within the concentration range of 50-2000 ppm) is shown in the Figure 2.

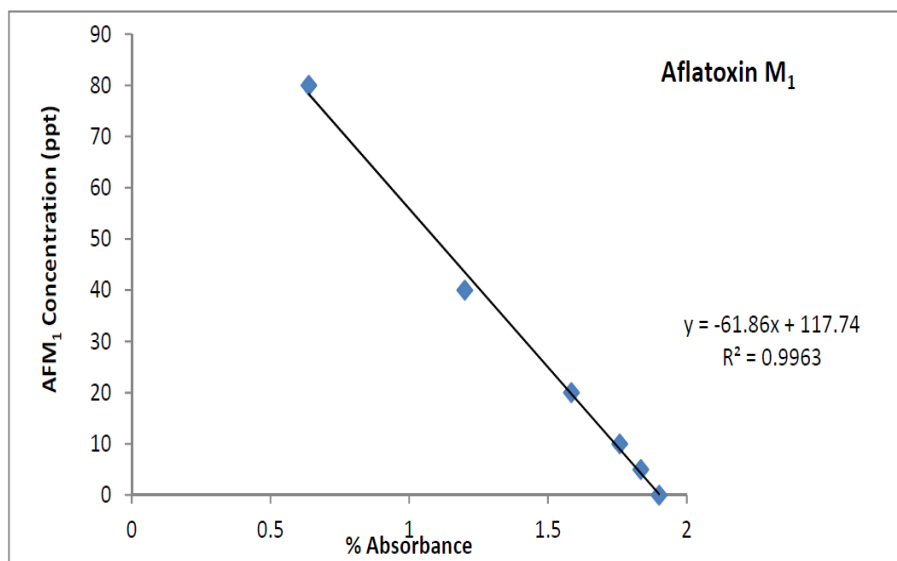


Figure 1. The linear fitted curve of AFM1 standards by ELISA

The results of the calibration data, LOD, LOQ and recovery of AFM1 by HPLC are shown in Table 1.

The contamination levels of samples are shown in Table 2. The results of the contamination of whey protein powder samples by ELISA and HPLC methods are demonstrated in Table 3. The contamination of samples of two factories producing whey powder in first and second half of the year is shown in Table 4.

4. Discussion

AFM1 is a dangerous metabolite of AFB1. Although AFM1 has 10 times less mutagenic and carcinogenic effects than AFB1, the IARC (International Agency for Research on Cancer) categorized AFM1 in group 1 of carcinogenic agents (7).

This toxin compound can be found in the milk of the cows that are fed with aflatoxin contaminated feeds.

In addition, it can also be found in cheese, milk, yogurt, butter, etc (3).

The quality of milk is the most important factor which may influence the level of contamination of dairy products by AFM1. Thus, manufacturers should avoid using contaminated milk in order to improve the

quality of their products. The contamination of products of various factories differs according to the level of contamination in different animal husbandries milk.

Therefore, in order to maintain the community health, it is necessary to experiment the milks and its

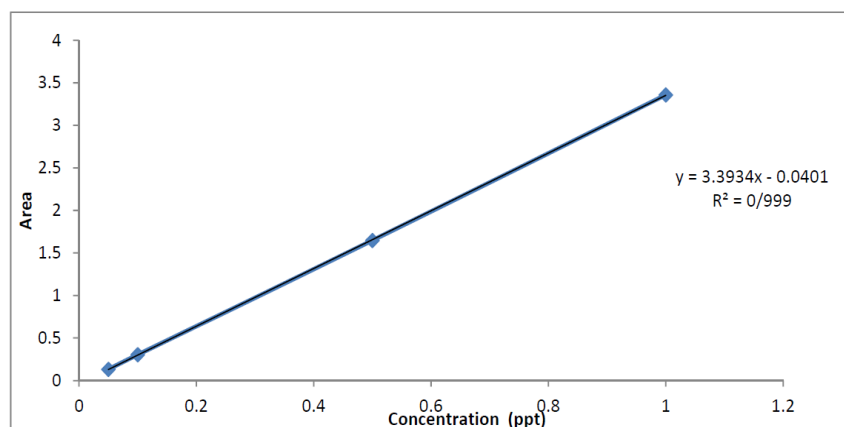


Figure 2. The linear fitted curve of AFM1 standards by HPLC

Table 1. Analytical characteristics of the method

method	calibration equation	R ²	linear range (ppt)	LOD (ppt)	LOQ (ppt)	Recovery (%)			
						ELISA		HPLC	
						Toxin added (ppt)	Recovery (%)	Toxin added (ppt)	Recovery (%)
HPLC	Y=3.393x-0.04	0.9999	50-2000	5.9	18.01	100	111	50	71.4
ELISA	y = -61.86x + 117.74	0.9963	0-80	-	-	200	117	100	69.5
						400	121	200	77.02

Table 2. The AFM1 contamination level of protein powder samples at different levels

Affluence	Aflatoxin M1 (ppt)			
	1000<		<1000	
	percentage	Number	percentage	Number
	47.6	40	52.4	44

Table 3. The contamination of whey protein powder samples by two methods (ELISA and HPLC)

Method	Total sample	The number of cases in accordance with the limit	The number of cases of non-compliance with the limit	Mean± Standard deviation (ng/kg)	Non-compliance Range (ng/kg)	p-value
ELISA	84	44	40	1100.0±734.0	1000-7000	p>0.05
HPLC	26	20	6	1006.1±315.5	1078-6609.2	p>0.05

Table 4. The contamination of samples of the two factories producing whey powder in first and second half of the year

	Category	Sample Frequency	Relative frequency%	Mean± S.D (ppt)	Range of concentration (ppt)
Factory	Brand A	48	57.1	1059.6±925.0	200-7000
	Brand B	36	42.9	1153.9±355.1	330-1660
Season	First half year	36	42.8	1100.3±374.8	200-1640
	Second half year	48	57.2	1099.8±921.35	330-7000

products so that make sure they are not contaminated by aflatoxin (14).

Galvano et al. (1996) carried out a research on effect of climatic condition on contamination of milk and dairy products. It was revealed that the amounts of AFM1 in milk and dairy products vary in different regions and seasons (14). In contrast, our results indicated no significant difference on the level of AFM1 due to seasonal impact. Kamkar A. (2005) has conducted an experiment on detection of AFM1 in UHT milk samples by ELISA. The seasonal effects on the contamination level were investigated and it was revealed that the variance among the contamination level of the samples was not significant (15).

Rahimi et al. (2012) carried out an investigation on measuring the AFM1 in pasteurized milk and white cheese. The results did not show significant differences among pasteurized milk samples (16).

Kim EK et al. (2009) measured the incidence of aflatoxin in 180 samples (pasteurized milk, infant formula, powdered milk and yoghurt) from Seoul, Korea by HPLC and ELISA. The results gain by the two methods were closely similar (17).

Rodriguez Velasco ML et al. (2003) measured AFM1 in cow's milk by ELISA and HPLC. According to the results, the concentration of AFM1 in all samples was within the acceptable limits of European Union (18). Pournourmohammadi et al. (2015) determined the level of AFM1 in pasteurized milks of Kerman. The measured concentration of AFM1 in all samples was lower than the maximum acceptable limit by FDA (19).

Behfar A et al. (2012) measured AFM1 in consumed pasteurized milks of Ahwaz by HPLC. The concentration range of AFM1 in all 100 samples was within 0.45 to 9.760 ng/l (20).

Some studies measured the level of AFM1 in milk in winter compared to summer (21-23). Differences between AFM1 content of milk in various seasons can be caused by dissimilar animal nutrition in hot and cold seasons. During the summer, the animals are fed by green grass and weeds. In the winter, animals are fed by corn, wheat and cotton seed due to lack of or unavailability of fresh green grass (24).

In the current study, the measurement of AFM1 in Whey powder was performed by using the HPLC and ELISA. The maximum acceptable level of AFM1 in milk and dairy products varies in different countries based on their internal rules. In the present study, the Iranian National Standard for pollution index was used (11).

According to the results obtained by ELISA, all 84 samples were contaminated with AFM1. The contamination levels of 44 samples (52.4%) were less

than the permitted value (1000 ppt) and 40 samples (47.6%) were higher than the maximum tolerance limit recommended by Iranian National Standard (11).

According to statistical analysis, it was observed that the contamination level was not significantly different according to the season. Additionally, no significant differences were observed in the average of AFM1 contamination of the two dairy producer factories ($p>0.05$).

In recent years, the use of ELISA screening test is more common because of the easy method and low cost. Other advantages of this method are the analysis of large number of samples in short time and simple extraction methods (25,26).

In a study performed on pasteurized milks in Athena, the presence of AFM1 was determined by ELISA and HPLC. According to the results, the samples which showed contamination level upper than the acceptable limit by ELISA were detected not to be contaminated by HPLC method (25).

In order to analyze by HPLC, 26 samples were selected randomly. The concentration of AFM1 in 20 samples (76.9%) was less than the maximum limit and 6 samples were above the determined limit by Iranian Standard.

HPLC results did not show significant difference in the detection of AFM1 contamination ($p>0.05$). The results of this study showed the presence of AFM1 contamination in produced whey powder which the amount in half of them was over the standard limits. Further investigation is recommended to improve the supervision.

5. Conclusion

Storage conditions, animal feeding, the country's geographical situation and environmental conditions may influence the growth of fungi which produce AFB1. According to the results of the present study, there is no significant difference in AFM1 levels due to the season. The findings of this study showed the occurrence of AFM1 in produced whey powder samples which was considered to be possible hazard for public health. Since pasteurization and sterilization have negligible impact on reducing the toxicity of AFM1, it would be a wise suggestion to use the high quality milk in production of whey powder.

Conflict of interest

The authors have no conflict of interest.

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