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Investigation of Aflatoxin M1 in Milk Powder Marketed in Karachi, Pakistan

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Abstract

Aflatoxins are strong toxic and cancer producing substance which can be excreted in lactating humans in the form of aflatoxin M1 (AFM1), exposed to food contaminated with mycotoxins. In the current study a total of 35 powdered milk samples (15 full cream instant milk powder and 20 infant milk powder) were analyzed for the detection of aflatoxin M1and results were compare with the standard upper allowable limits of AFM1. All samples of full cream powdered milk (branded and unbranded) and infant formula milk powder were found to be contaminated with AFM1 with an average level 104.3, 120.5, 125.0ng/kg respectively. The AFM1 contamination level were found in a range of 28–166ng/kg for branded full cream milk powder, 43–254ng/kg for unbranded full cream milk powder and 17-164ng/kg for infant formula milk powder, respectively. In total, three samples (60%) of full cream branded, 6 samples (60%) unbranded milk Powder and 15 samples (75%) of infant milk powder samples exceed European Union (EU) and the US regulations. Two of the samples (40%) full cream branded and five samples (50%) of unbranded milk Powder and seven samples (75%) of infant milk powder samples of were found exceeding the upper limits set for Pakistan by Pakistan Standards and Quality Control Authority *(PSQCA). Aflatoxin M1 is the most widely distributed mycotoxin in milk-based foodstuffs and has potential carcinogenic effects. Therefore, it is necessary to monitor milk and milk products for contamination levels of AFM1.*

Keywords: aflatoxin M1, HPLC, infant milk powder, milk powder

Introduction

Milk and dairy products provide important nutrients for humans; especially children are regularly included them in their diet (Başkaya *et al.*, 2006). However, along with healthful and immunologically advantageous components, some carcinogenic substances such as Aflatoxin M_1 (AFM_1) have also been observed in buffalo milk (Raza, 2006). AFB1 is converted into aflatoxin M1 (AFM1) during biotransformation process and excreted in milk of animals that have consumed feed contaminated with aflatoxin (Stubblefield *et al.*, 1983). Aflatoxins are typically found as secondary metabolites of *Aspergillus flavus* and *Aspergillus parasiticus* (Maragos, 2001). Aflatoxins frequently contaminate cereal crops, such as corn, beans, peanuts, and dried fruit under favourable conditions, and AFB1 has the highest toxicity (Hussein and Brasel, 2001). AFM1 is believed to be less toxic than AFB1 but it is a possible carcinogen to human according to IARC (Samet *et al.*, 2020). The epoxidation of AFM1 results in the formation of isomers accounting for nucleic acid and protein binding making it genotoxic and cytotoxic carcinogen (Neal, 1998). AFM1 is stable at 300^{o}C and is not affected by usual methods of pasteurization and sterilization; therefore, expected exposure to it through fresh, processed, powdered milk or infant milk formula can be harmful (Pohland and Trucksess, 2001). Maximum tolerable limits have been set for aflatoxin specific for various countries. The European

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Union EU has regulated the upper limit of aflatoxin as 50ng/L in fluid milk and 25ng/kg for infant milk products. In Austria and Switzerland, the maximum level is further reduced to 10ng/kg for infant food commodities In Pakistan, the maximum tolerable limit of AFM_1 is 10ppb in milk powder, while no particular legislation has been made for liquid milk (Byrne, 2004, Hussein and Brasel, 2001, Hussain *et al.*, 2010, Asi *et al.*, 2012).

Fresh and UHT treated fluid milk is mostly consumed in Pakistan, but the importance of powdered milk cannot be ignored as it is major raw material for chocolate and confectionery industries along-with the domestic users. In the Pakistan's National Nutrition Survey, which was conducted by Ministry of Health Pakistan, Aga Khan University and UNICEF, the proportion of women who were breastfeeding was estimated on the basis of feeding practices. It has been reported that 63.5% of Pakistani mothers have been practicing exclusive breastfeeding to their children up to 6 months of age and 43.7% of the children less than 5 years of age were found stunted. To reduce stunting the nursing mothers have been suggested in the survey to continue breastfeeding for 12-15 months (Afzal and Yusuf, 2013).

Keeping in view the availability of limited data on occurrence of AFM1 in powder milk and infant milk powder marketed in the city of Karachi, Pakistan, the current study was designed to determine the presence of aflatoxin M1 in both type of powdered milk in this megacity and to compare the results with the standard upper allowable limits of AFM1.

The study will be helpful in enforcing regulated standards for the safety of infant milk powder in Pakistan.

Materials and method

Chemicals and reagents

HPLC grade Methanol and acetonitrile (99.9%) were used for analysis. Standards of AflatoxinM_1 (analytical grade) were stored at 4^{o}C prior to use.

Sample collection

A total of 35 powdered milk samples (15 full cream instant milk powder and 20 infant milk powder) were purchased. 5 of the full cream milk powder were of branded and 10 samples were purchased from locally operated open markets located at various areas of Karachi.

Powder milk sample (20g) was dissolved in de-ionized water (200mL) at 50^{o}C with constant stirring on a magnetic stirrer for 30min, and then cooled to 20-25°C. The samples were then centrifuged with a speed 3000rpm for 15min at 5^{o}C. Before passage of milk sample; it was rinsed with 10mL acetonitrile and then 10mL water. Washing of cartridge was carried out with 10mL water then 10mL ammonia: acetonitrile: water (1:10:89, v/v/v) and 10mL acetic acid: acetonitrile: water (1:10:89, v/v/v).

HPLC analysis

Reverse-phase HPLC (model LC-10ADvp solvent delivery system; auto injection, Shimadzu, Japan) C_{18}Brownlee reverse phase column (220x4.6mm, particle size 5µm) with C_{18} guard column (Perkin Elmer) was used with fluorescence detection set at 440nm emission and 360nm excitation. The mobile phase was water: acetonitrile: methanol (66:17:17, v/v/v). The oven temperature

was maintained to 40°C with a flow rate of 1mL/min and injection volume for standard and sample extracts was kept 30μL. The calibration solution of AFM_1 ranging from 0.025-1ngmL^{-1}was prepared in 1mL 2:3vol/vol mixture of methanol and water and then it was filtered through PVDF membrane having pore size 0.45µm. Since aflatoxins are possible carcinogen, care has always been practiced to avoid exposure and 10% sodium hypochlorite was used for decontamination.

Statistical analysis

Standard deviation was estimated by using one-way analysis of variance ANOVA. Calibration curves and linear regression curve showed r^2values above 0.97 indicating good linearity.

Safety

Aflatoxin M1 is toxic and a liver carcinogen. To minimize exposure, it should be handled only in a fume hood while wearing protection (e.g., gloves, lab coat, and eye protection). The chemical itself, and all contacted materials, should be disposed of in a legal and environmentally safe manner.

Results and discussion

Validation of method and detection limit

HPLC method for the quantitative determination of aflatoxins M_1 has been validated as described earlier (Keskin *et al.*, 2009). The limit of detection (LOD) of Aflatoxins was estimated as three times signal-to-noise ratio. LOD of aflatoxin M_1 for the human breast milk samples was obtained as 20ng/kg. The calibration solutions of aflatoxins were prepared ranging from 0.025, 0.05, 0.1, 0.125 and 0.15ppb. Chromatogram of standard solution of aflatoxin M_1 is shown as Fig-1, and calibration curve constructed for aflatoxin M_1 is shown as Figure-2. A linear result was obtained for AFM_1 concentration and their corresponding peak heights with correlation coefficient 0.99.

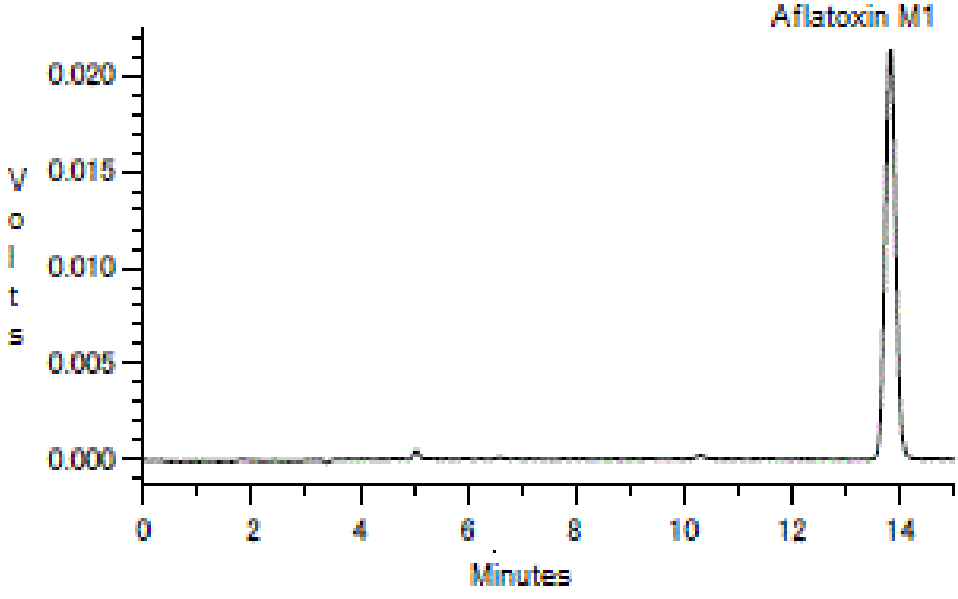

Figure 1: Chromatogram of Standard Solution of Aflatoxin M_1

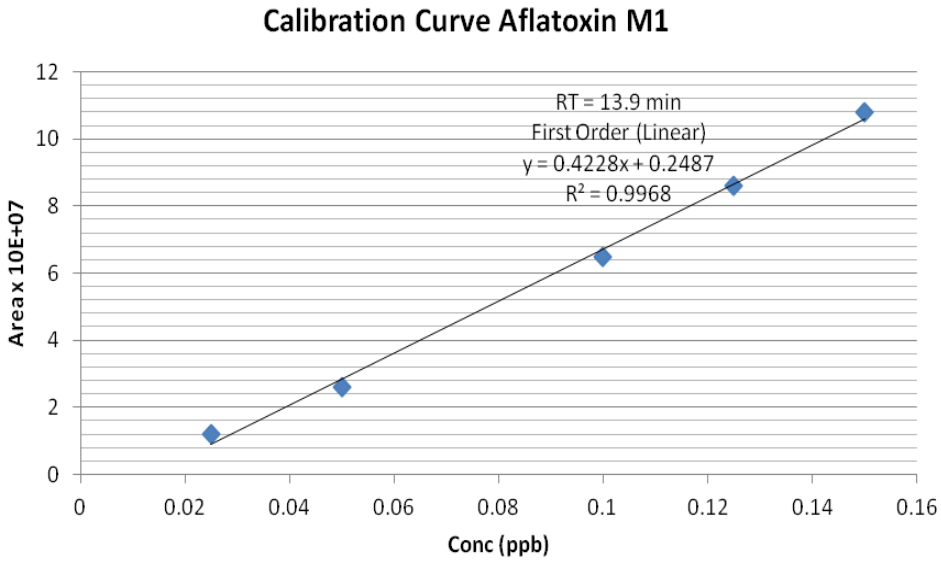

Figure 2: Calibration Curve Aflatoxin M_1

Table 1: Determination of AFM1 Contents in Powdered Milk Samples

Samples	No. of Samples	No. of Positive Samples (%)	AFM1contamination (ng/kg)	
			Range	**Mean ± SD**
Full Cream Milk Powder				
Branded	5	100	28 - 166	104.3 ± 35.1
Unbranded	10	100	43 - 254	120.5 ± 74.2
Infant Milk Powder	20	100	17 -164	125.0 ± 33.8

The analytical results of the occurrence of AFM_1 in full cream milk samples are summarized in Table-1. All samples of full cream powdered milk (branded and unbranded) and infant formula milk powder were found to be contaminated with AFM1 with an average level 104.3, 120.5, 125.0ng/kg respectively. The AFM1 contamination level were found in a range of 28–166ng/kg for branded full cream milk powder, 43–254ng/kg for unbranded full cream milk powder and 17-164ng/kg for infant formula milk powder, respectively. The contamination levels were found higher than the upper limits of AFM1 in milk 50ng/kg and 25ng/kg for infant milk products established by European Union and Codex Alimentarius. The average concentration of AFM1 was also found higher than the maximum tolerable limit of AFM_1 i.e. 10ppb in milk powder set by PSQCA (Byrne, 2004, Hussain *et al.*, 2010, Hussein and Brasel, 2001).

Table 2: Comparison of AFM1 Contents in Powdered Milk Samples with Standards

Samples	Positive Samples (%)	% Sample Exceeding Upper Limits		
		EU	US	PSQCA
Full Cream Milk Powder				
Branded	100	60	60	40
Unbranded	100	60	60	50

Infant Milk Powder	100	75	75	35

Comparison of AFM1 Contents in powdered milk samples with standards is described in Table-2. In total, three samples (60%) of full cream branded, 6 samples (60%) of unbranded milk Powder and 15 samples (75%) of infant milk powder samples exceed EU and the US regulation. Two of the samples (40%) full cream branded, five samples (50%) of unbranded milk Powder and seven samples (75%) of infant milk powder samples of were found exceeding the upper limits set for Pakistan by PSQCA.

To consider the hazard of AFM1 occurrence in infant foods, various studies have been carried out for the food hygiene in different countries. AFM1 was detected in 87 samples of infant food in India and 87.3% of them were positive for AFM1 residues (Rastogi *et al.*, 2004). AFM1 level was determined7.3ng/kg in 96.6% of infant formulas in Iran (Oveisi *et al.*, 2007). The AFM1 mean level was found 9.796 ± 1.036ng/L in 43.2% (125) of the positive infant formula milk powder in Egypt (Wael *et al.*, 2011). Aflatoxin M1 incidences of infant foods were higher in these studies.

Literature is also available about the occurrence of AFM1 in infant food samples, indicating lower levels of contamination. It was reported that the mean AFM1 level was 3.1ng/ kg in 37.7% of the 69 different infant formulas in Spain (Gomez-Arranz and Navarro-Blasco, 2010). In another study, AFM1 was found in 4 of 27 baby food samples between 0.017 and 0.041µg/kg in Portugal (Alvito *et al.*, 2010).

Conclusions

All samples of full cream powdered milk (branded and unbranded) and infant formula milk powder were found to be contaminated with AFM1 with an average level 104.3, 120.5, 125.0ng/kg respectively. The AFM1 contamination level were found in a range of 28–166ng/kg for branded full cream milk powder, 43–254ng/kg for unbranded full cream milk powder and 17-164ng/kg for infant formula milk powder, respectively. In total, three samples (60%) of full cream branded, 6 samples (60%) unbranded milk Powder and 15 samples (75%) of infant milk powder samples exceed EU and the US regulation. Two of the samples (40%) full cream branded and five samples (50%) of unbranded milk Powder and seven samples (75%) of infant milk powder samples of were found exceeding the upper limits set for Pakistan by PSQCA. In previous national and international studies, it was observed that variability existed in the AFM1 levels of infant foods. Therefore, many factors, including contamination of animal feedstuffs and seasonal differences, can affect the occurrence of AFM1. Aflatoxin M1 is the most widely distributed mycotoxin in milk-based foodstuffs and has potential carcinogenic effects. Therefore, it is necessary to monitor milk and milk products for contamination levels of AFM1. The analysis of AFM1 in risky foods is very important for human health. These results are valid for the obtained samples during our study and the data may change over time. For this reason, monitoring strategies should be considered for preventing contamination with AFM1 in food processing.

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