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Health risk assessment and determination of bisphenol A and aflatoxin M1 in infant formula

Ghazal Mirzaei¹, Najmeh Yazdanfar², Nabi Shariatifar¹, Ebrahim Molaei -Aghaei¹ and Parisa Sadighara^{1*}

Abstract

Background Bisphenol A (BPA) is one of the chemical compounds used in food packaging, so it can migrate from the packaging into food. Also, environmental pollution of this compound is high due to its high use. Therefore, it may enter food chains through the environment. Aflatoxin M1 (AFM1) is one of the common mycotoxins in milk. Its presence has been reported worldwide. Infant formula is an alternative to human milk. The main ingredient of this product is cow's milk.

Aims This study aimed to investigate the levels and risk assessment of BPA and aflatoxin M1 in infant formula.

Methods Samples were purchased from 7 leading brands of infant formula in pharmacies. The samples were extracted according to common protocols and then injected into HPLC and analyzed with a fluorescence detector for both contaminants.

Results BPA wasn't detected in infant formula samples, but the presence of AFM1 was confirmed in 11% of the samples. Of course, there is no risk in this regard with the risk assessment.

Conclusion Infant formula samples are not of concern for infants in terms of BPA and aflatoxin M1. However, continuous monitoring is recommended for this product.

Keywords Bisphenol A, Aflatoxin M1, Infant formula, HPLC, Food packaging, Environmental pollution, Mycotoxins

Introduction

In children, disruption in endocrine glands and metabolism causes disturbances in the growth of the children and their mental development and sexual organs. Children's detoxification systems are less efficient. Children's blood-brain barriers are also immature, making them

more susceptible to nerve damage [1]. Their endocrine, reproductive, and renal systems are also immature [2]. One of the endocrine disruptors is bisphenol A (BPA). BPA is an estrogenic environmental pollutant that binds to the estrogen receptor even in very low doses. In children, BPA causes a decrease in IQ, interference with the thyroid and gonads, interference with brain development, and behavioral changes [3]. The tolerance daily intake (TDI) in the European Food Safety Organization was estimated at 50 micrograms per kilogram of body weight per day, which was reduced to 2 µg/kg body weight (bw) per day [4].

Milk and dairy products are major components of the diet of infants and young children, which may be

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contaminated with BPA. It remains in sediments and soil and enters the animal feed and can enter milk and dairy products [5]. Furthermore, studies have shown that BPA is likely to increase during milk processing and reach the highest levels in the final product [6, 7]. Packaging plays an important role in food preservation. It maintains both the quality and safety of food [8]. BPA can enter food through migration from food packaging such as polycarbonate bottles and cans with epoxy [9, 10].

Milk and dairy products in the diet of infants and young children may be a route for mycotoxins to enter their bodies [11]. Mycotoxins are produced by molds, and various types of mycotoxins can contaminate food. One of the most common mycotoxins is aflatoxins. Aflatoxins are produced by various *Aspergillus* species, particularly *Aspergillus flavus* and *Aspergillus parasiticus* [12]. Aflatoxins are divided into four types: B1, B2, G1, and G2. Aflatoxins type B1 are highly toxic and carcinogenic and are identified in a variety of plant food products [12]. AFM1 has been repeatedly detected in milk and its products. This mycotoxin is a hydroxylated metabolite of aflatoxin B1, which enters the animal body through fodder and feed. These two mycotoxins belong to group 1 of the IARC (International Agency for Research on Cancer) and are carcinogenic to humans [13, 14]. Every year, a significant number of liver cancers are reported due to exposure to aflatoxins [15]. Therefore, the presence of AFM1 in milk cannot be ignored, and this product should be regularly evaluated in this regards. This mycotoxin is resistant to heat and is not destroyed by sterilization and pasteurization processes in milk [16].

Infant formula replaces human milk in cases where the mother cannot or does not want to breastfeed her child [17]. Infant formula is available on the market in two forms: powdered infant formula and liquid infant formula. This product undergoes pasteurization, homogenization, and drying processes and is generally enriched with minerals such as iron [18].

Considering the toxic effects of BPA and AFM 1, the sensitivity of infant formula consumers, and the possibility of contamination of these types of products with these two contaminants, this study examined samples of prominent infant formula brands sold in Tehran.

Method and materials

Materials

Standards of Bisphenol A (purity > 99.0%) was obtained from Sigma- Aldrich and Immunoaffinity columns for AFM1 was purchased from aoki immunoclean (Berlin, Germany). The HPLC grade hexane, methanol, and acetonitrile were purchased from Samchun (Seoul, Republic of Korea). Forty-two samples of 7 brands of infant formula were purchased from pharmacies in Tehran.

The extraction of BPA from infant formula samples

Initially, a 10 ppm stock solution for BPA was prepared. 0.01 g of BPA was weighted and made up to volume 10 with methanol. The stock solution of 10ppm can be stored at a refrigerator temperature of 4 °C for 1 month. To draw the calibration curve, we prepared different concentrations of 10 to 50 µg/L from this stock solution. The standard solutions were stored at 0–4°C.

To extract bisphenol A from the samples, the method of Santonicola et al. was used with some modification [19]. First, 12.5 g of infant formula powder was weighed and mixed with boiled water to a volume of 100 mL. We poured about 20 mL of milk solution into an Erlenmeyer flask. Then 15 mL of acetonitrile and 15 mL of hexane were added. The mixture of sample and solvents was completely mixed with a magnetic shaker for 10 min. The samples were transferred to a 15 mL Falcon and centrifuged at 15 °C for 10 min at 6500 rpm. The solution was removed from the centrifuge in two phases, and the upper layer was a gel containing hexane and fat. The acetonitrile layer was introduced into the Erlenmeyer flask and, for further extraction, the layer on the ashless paper was washed with 5 mL of acetonitrile. The samples were filtered with a syringe filter and the sample was transferred to a vacuum oven at a temperature of 40 °C. Then we dissolved the dried sample with 0.5 mL of the mobile phase (methanol /water/ 70:30 v/v).

The extraction of AFM1 from infant formula samples

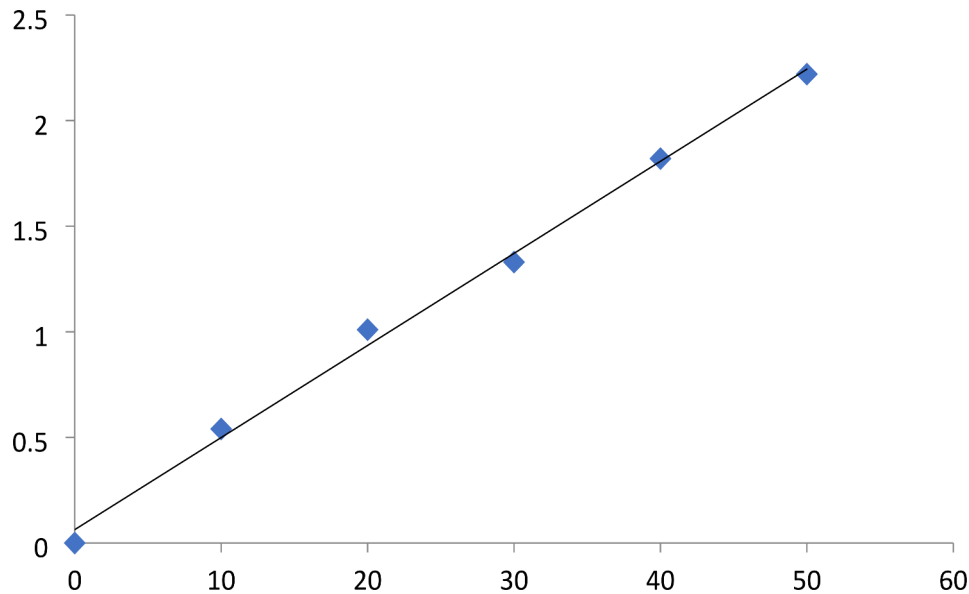
Previously published methods were used for extraction [20]. The procedure began with the combination of 20 g of milk sample with 80 ml of HPLC grade water, and was adjusted to a final volume of 100 ml. A portion of this solution, amounting to 60 ml, was then subjected to centrifugation. Subsequently, immunoaffinity columns were utilized for the extraction and separation process. Initially, the column was primed with an acetate buffer solution, followed by water. Next, 20 ml of the defatted sample was introduced into the column, followed by another rinse with HPLC grade water. In the subsequent step, 2.5 ml of acetonitrile solvent was passed through the column. Following this, the sample underwent drying. Once dried, the sample was dissolved in 1.0 ml of mobile phase, consisting of water, acetonitrile, and methanol at a ratio of 60:20:20 v/v/v, before being introduced into the HPLC for analysis.

High performance liquid chromatography system

Chromatographic analysis was carried out according previous studies [21, 22] using an Agilent HPLC-FLD system (Wilmington, USA) equipped with Agilent G1321B fluorescence detector (FLD) with a 1200 series quaternary pump, an Eclipse-XDB-C18 analytical column (25 cm-4.6 mm, 5 µm) and auto sampler was used. The excitation

Table 1 Validation parameters

Parameters	Analytical feature (BPA)	Analytical feature (AFM1)
Limit of detection ($\mu\text{g}/\text{kg}$)	0.3	0.05
Limit of quantification ($\mu\text{g}/\text{kg}$)	1	0.125
Recovery percent	101.85	89.12%
Linear dynamic range ($\mu\text{g}/\text{kg}$)	2.0–50.0	-
Regression equation	$y = 0.0372x - 0.0144$	$y = 3828x - 36.89$
r^2	0.9968	0.9994

**Fig. 1** Bisphenol A standard curve

wavelength was 275 nm and the emission wavelength was 313 nm for BPA, and the excitation wavelength 360 nm and the emission wavelength were 440 nm for AFM1. The mobile phase was maintained at a flow rate of 1 mL/min., and the volume of the injection was 20 μL .

Statistical analysis

The mean and standard deviation were calculated using SPSS software (version 20). Two-way analysis of the variance test was used for comparison between groups. *P*-value of less than 0.05 was considered a significant difference.

Results

Analytical performance of method

Analytical method performance including limit of detections and quantifications (LODs and LOQs, respectively), linear dynamic ranges (LDRs), extraction relative recoveries and intra and inter-day precision (RSDs) were calculated by two spiking levels of BPA (Table 1). Linearity dynamic ranges of 10.0–50.0 $\mu\text{g}/\text{kg}$ ($R^2 = 0.99$) were obtained. The LOD and LOQ for BPA were obtained at 0.3 and 1 $\mu\text{g}/\text{kg}$ practically based on signal-to-noise ratios of 3 and 10. RSDs for intra and inter day precision were

Table 2 The amount and Health risk assessment for BPA and AFM1

(Total)	BPA	AFM1
Mean \pm SD	ND	0.002 ± 0.008 $\mu\text{g}/\text{kg}$
Positive percentage	0	11%
HQ (0–6 months)	-	0.2
HQ (6–12 months)	-	0.17
HQ (1–2 years)	-	0.16

obtained in the range of 2.92–3.11%, respectively. The obtained relative recoveries of BPA at a spiking level of 20 ($\mu\text{g}/\text{kg}$) were from 96.4 to 103.9%. To draw a standard curve and linear equation, concentrations of 0, 10, 20, 30, 40, and 50 ppb bisphenol A were injected into the device (Fig. 1).

Determination of BPA and AFM1 in infant formula samples

In our study, it was found that in the evaluated infant formula samples, the measurable amounts of BPA were lower than the detection limit of the HPLC device, and BPA was not detected in any of the samples. However, aflatoxin M1 was detected in 11% of the samples, and its average level was determined to be 0.002 $\mu\text{g}/\text{kg}$ (Table 2).

A significant difference was also observed between brands in the amount of aflatoxin M1. The risk assessment for aflatoxin M1 was calculated using the determined average.

The risk assessment for AFM1 in infant formula

Milk consumption for infants is calculated as follows [23]. Infants (0–6 months): 120 gr with an average weight of 5.9 kg, Children (6–12 months): 160 g with an average body weight of 9.3 kg, and Children (1–2 years): 200 g with an average weight of 12.2 kg.

The estimation of daily intake (EDI) for AFM1 was determined using the following formula: $EDI = C_i \times C_c / BW$ or body weight. C_i represents the mean concentration of AFM1, C_c is the ingestion rate of milk, and BW is the average body weight of children.

TDI (tolerable daily intake) is considered 0.2 ng/kg/day [24]. Hazard quotient (HQ) is calculated as EDI/TDI. The results of risk based on three age groups are summarized in Table 2. It was calculated as less than 1 in all age groups (Table 2).

Discussion

In this study, BPA was not detected in any of the infant formula samples. In the past, an epoxy resin layer was used in infant formula cans [25]. But, due to the toxicity of BPA and the harmful effects on the health of babies, in recent years, most brands of infant formulas removed the epoxy resin layer in the cans. Therefore, the amount of BPA in infant formula has decreased significantly.

The results of this study were compared with the results of other similar studies. In one study conducted in China in 2017 on 76 milk powder samples across China, BPA was not detected in any of the samples, which is very similar to our study [26], in which BPA was not found in any of the samples in this study. Similar results were also observed in another study conducted in Spain in 2012 on infant formula samples. In this study, the amount of BPA was lower than the permissible limit [27]. The permissible limit is set at 0.6 mg/kg according to European Union regulations [27].

In 2010, Ackerman et al. conducted a study in the United States of America to investigate the amount of BPA in infant formula cans by LC-MS/MS. Based on the results obtained on 36 different samples of liquid and powdered infant formula, only one sample showed the amount of BPA above the permissible limit. The amount of BPA detected ranged from 0.48 to 11 ng/g [28]. Furthermore, in 2004, a study was conducted in Spain to determine the amount of bisphenol in infant food. None of the samples exceeded the permissible limit. The amount of bisphenol in the samples was reported to be $0.28 \pm 0.02 \mu\text{g/kg}$ [29]. In that year, the ban on the use of BPA in infant food packaging had not yet been

implemented. In 2021, Karsauliya et al., conducted a study on the measurement of bisphenols in powdered infant formulas available in Indian markets. The presence of seven bisphenols (A, AF, E, ZC, FL, S, Z) in infant formula was evaluated and liquid chromatography and tandem mass spectrometry (UPLC-MS/MS) were used. The highest concentration was for BPA with an average of 5.46 ng/g, and then the highest value was estimated for bisphenol Z and S. Other bisphenols were not detected in any of the samples. In this study, the estimated daily intake (EDI) was also determined, and its value was calculated to be less than the TDI [30]. A study was also conducted to evaluate the presence of BPA in infant formula samples in Brazil. In this study, the measured concentrations were lower than the migration limit set by the European Union and Brazil (600 $\mu\text{g/kg}$) and the concentration range was 0.2 to 10.2 $\mu\text{g/kg}$ [31].

In 2015, Cirillo et al. investigated the contamination of powdered infant formula and liquid infant formula with phthalates and bisphenol in the United States. The samples included 28 infant formula powders, and the method of performing was gas chromatography with a flame ionization detector and high performance liquid chromatography with the fluorometric detector. Contrary to the results of the current study, BPA was detected in milk samples in this study. The concentration of BPA was 0.003 up to 0.375 $\mu\text{g/g}$ with an average of 0.015 $\mu\text{g/g}$ reported for the samples [32].

Another contaminant measured in infant formula samples in this study was AFM1. The results of the levels of this mycotoxin were also compared with other studies. Sartori et al., 2023 in Brazil carried out research on AFM1 in 123 Infant Formulas by ultra-high performance liquid chromatography-tandem mass spectrometry (UHPLC-MS/MS). AFM1 was detected in 18 (14.6%). The concentration levels ranged from 0.016 to 0.057 $\mu\text{g/kg}$. Three of the examined samples (0.040, 0.044, and 0.057 $\mu\text{g/kg}$) had AFM1 values higher than the threshold set by EU limits (25 ng/kg) [33]. The detected ranges of AFM1 in this study were higher than in the current study (Table 2).

In 2023, Demir and Agaoglu determined the level of AFM1 in powdered infant formula (72 samples for premature, hypoallergenic, 0–6, 6–9, 9–12 and 12–36 months) by Enzyme Linked Immunosorbent Assay (ELISA) in Turkey. Results showed that AFM1 was quantified in 49% of the analyzed samples and the group of infant formulae aged 12–36 (8 samples) months had the highest level of AFM1 contamination. Five samples of baby formula exhibited AFM1 levels exceeding the local maximum permissible concentration (Türkiye Food Codex Regulation standards: 0.025 $\mu\text{g/kg}$). The percentage of contamination of infant food was higher than in the current study [34].

Hooshfar et al., 2020 in Iran measured the amount of AFM1 in 29 infant formula samples using HPLC-FLD and also performed a risk assessment with the resulting amount. AFM1 was determined in 3.4% of evaluated samples. The calculated HQ values were less than one, similar to the results of the current study. These findings suggest that there is no significant health concern related to this level of exposure [35].

Quevedo-Garza et al., 2020 studied the AFM1 contamination in infant formula ($n = 55$) using HPLC with fluorimetric detection in Mexico. AFM1 was detected in eleven of the analyzed samples (20%), which was higher than the allowable limit set by the European Union, ranging from 40 to 450 ng/L. The determined value is higher than the current study. The carcinogenic risk value revealed a high risk for the all groups of age (ranges of 0–6 and 6–12 months, and 1–2 years) [36].

Conclusion and future research

In this study, BPA and AFM1 in powdered milk available in pharmacies were quantified by HPLC containing a fluorescence detector after extraction with appropriate solvents. The amount of bisphenol A was not detected in all samples and the amount of aflatoxin M1 was lower than the permissible level. The results obtained are due to the absence of BPA in the packaging of infant formula and also the safety of raw milk used for infant formula. Overall, with the risk assessment conducted for AFM1, the most prominent and widely consumed brands of infant formula in pharmacies are safe in terms of the presence of BPA and AFM1.

Abbreviations

BPA	Bisphenol A
AFM1	Aflatoxin M1
TDI	Tolerance daily intake
ELISA	Enzyme linked immunosorbent assay
UHPLC-MS/MS	Ultra-high performance liquid chromatography-tandem mass spectrometry
EDI	Estimated daily intake
HQ	Hazard quotient
LOD	Limit of detections
LOQ	Limit of quantifications
LDRs	Linear dynamic ranges
FLD	Fluorescence detector
BW	Body weight
IARC	International agency for research on cancer

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Author contributions

The samples was extracted by two authors G.M and E.M.A. The analysis were performed by N.Y. Writing and editing was done by P.S, G.M, and N.S.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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