Monitoring of Aflatoxin M₁ in Market Raw Milk in Lahore City, Pakistan

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Abstract.- Aflatoxin M₁ (AFM₁) was determined in market raw milk samples collected from four towns of city Lahore, Pakistan. Total 84 samples were collected during a period of 4 months (April through July 2007) and were processed for purification of AFM₁ through an immunoaffinity column. Each sample was analyzed for AFM₁ using fluorescent detector of high performance liquid chromatography (HPLC). Eighty one percent milk samples contained AFM₁ levels exceeding the American and European tolerance limits. The mean value of AFM₁ was 17.38µg/L ranged from 0.69 to 100.04 µg/L. High levels of AFM₁ in the raw milk samples is an enormous health risk factor for end consumers. There is need to improve storage conditions of feed ingredients that will mitigate the AFB₁ production in the feed/ration and ultimately decrease the AFM₁ levels in the animal milk.

Key Words: Aflatoxin M₁, HPLC, fluorescent detector, market raw milk samples.

INTRODUCTION

Milk and its products are fundamental components of human diet. Milk is mainly contaminated with aflatoxin M₁ (AFM₁). Consumption of such milk may be the principle way for entrance of AFM₁ into the human body (Galvano et al., 2001). AFM₁ is found in milk of the animals that are fed on aflatoxin contaminated feed (Van Egmond, 1989). Aflatoxin consists of B₁, B₂, G₁ and G₂ components and is produced as a result of growth of Aspergillus flavus and Aspergillus parasiticus species on feed ingredients during storage and/or transportation (Ali et al., 2005; Rizzi et al., 2003). Animals fed on aflatoxin contaminated feed metabolize in their liver AFB₁ into its hydroxylated product such as AFM₁ that is excreted in milk, feces and urine (Guerre et al., 2000). About 1-3% ingested AFB₁ is converted into AFM₁ (Ali et al., 1999; Barbieri et al., 1994).

The AFB₁ or AFM₁ toxins enter into food chain and accumulate in the adipose tissues of the end consumers. These toxins are considered the most serious threat to animal/human health due to their potential of carcinogenic, hepatotoxic, teratogenic and mutagenic (Maqbool et al., 2004; Wang et al., 1996; Wild and Turner, 2002).

The study was designed to monitor the AFM₁ in fresh raw milk retailed in Lahore city, Pakistan in terms of its compliance with international aflatoxin limits using an optimized HPLC method.

MATERIALS AND METHODS

Source of milk samples

Middle men collect fresh milk from dairy farmers from suburban and rural areas in morning and evening times and supply to retail milk shops of city Lahore, Pakistan. Eighty four samples of raw milk (250 ml each) were collected randomly from the markets of Gulberg Town, Ravi Town, Gujranwala Town and Shalimar Town, during April 2007 to July, 2007. The samples (15 samples from each town) were collected once a year. The milk samples in sterilized polythene bags were transported in ice-packed cooler to the laboratory where were stored at -20°C until analyzed for AFM₁.

Chemicals and reagents

Acetonitrile (HPLC grade) of Sigma-Aldrich (Steinheim, Germany) was used for AFM₁ analysis. The immunoaffinity columns AflaM₁ TM HPLC were obtained from VICAM (Watertown, MA, USA). The water used for analysis was double distilled with Millipore water purification system.
Standard of AFM1 (10 µg/mL in acetonitrile) was purchased from Supelco (Bellifonte, PA, USA). All the other chemicals used were of analytical grade.

**Extraction procedure**

The AFM1 in milk was extracted using the method as described by Dragacci et al. (2001). Each of the stored milk samples was warmed in water bath at 37°C and centrifuged at 600x g for 10 minutes. The top layer with fat contents was removed and the milk was passed through filter paper (Whatmann No. 4). Fifty mL of the milk was loaded in a syringe barrel which was attached with immunoaffinity columns (IAC). The test portion was passed at the flow rate of 2-3 mL/min. The column was washed with 20 mL water and then air dried. Acetonitrile (4 mL) was passed through the column for at least 60 seconds and aflatoxin M1 was eluted. The eluate was dried using water bath in fume hood. It was diluted with the mobile phase at the time of its determination through liquid chromatography (LC).

**Determination of AFM1 with fluorescence detection**

Each sample was processed for determination of AFM1 through HPLC system of Agilent 1100 series (Agilent, USA), using AOAC Official Method, 2000.08 (Dragacci et al., 2001). The HPLC system was equipped with an auto sampler LAS G1313A, and a fluorescence detector FLD G1321A with excitation and emission wavelength of 365 nm and 435 nm, respectively. The ZORBAX Eclipse XDB-C18 (Octadecyl silane chemically bonded to porous silica) column (Agilent, USA), 4.6 ×150 mm with particle size 5 µm in diameter and acetonitrile in ratio of 25% to 75% of water was used as mobile phase. The flow rate was set to be 0.8 mL/min. Standard solutions AFM1 with concentrations of 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 µg/L in acetonitrile were used to obtain the calibration curve. The retention time for AFM1 was 6.1 min.

**Statistical analysis**

The results regarding AFM1 levels in milk samples were statistically analyzed by applying one way analysis of variance (ANOVA) (Steel and Torrie, 1997).

**RESULTS AND DISCUSSION**

Aflatoxin M1 (AFM1) was detected in raw market milk supplied to Lahore city, Pakistan. Presence of AFM1 in milk and milk products is a worldwide concern since these products are main source for introducing aflatoxins in the human diet and are largely consumed by children including infants who are highly susceptible to adverse effects of the toxins (Rastogi et al., 2004).

Amount of AFM1 contamination in raw milk samples was more than 50 ng/kg (Table I). The levels ranged from 0.69 to 100 µg/L in analyzed samples. Hussain and Anwar (2008) analysed milk samples from dairy animals at Punjab level and found 96.4 % samples having toxin level below the US tolerance limit. Only 3% samples were having the levels higher than the tolerance limits. Due to serious health concerns, many countries have set maximum permissible standards for levels of AFM1 in milk and dairy products and AFB1 in animal feeds. However, regulatory limits of permissible standards are influenced by economic considerations world wide (Stoloff et al., 1991; Van Egmond, 1989). The US regulation has prescribed a limit of 500 ng/kg of AFM1 in milk and milk products (Codex Alimentarius Commission, 2001). However, European Communities has fixed the limit to a maximum of 50 ng/kg of AFM1 (Commission Regulation EC No.466/2001). Similar high levels of the HPLC based AFM1 levels were detected in Indonesia, Philippines and Thailand (Henry et al., 2001). In Iran, 98 samples were positive for AFM1 with an overall mean level of 0.053 µg/L. Levels of the toxin were also higher in winter and spring than in summer and autumn (Tajkarimi et al., 2007) while in Sarab City of Iran, 77% (total 111 raw milk samples) were contaminated with AFM1 levels (range between 0.015 and 0.280 µg/L) and 40% of the positive samples exceeded the tolerance limit of 0.050 µg/L (Kamkar, 2005). In Turkey, 47% of the 129 analyzed samples contained AFM1 levels exceeding the EU accepted limit (Unusan, 2006). In North African countries, randomly selected samples of raw cow milk were contaminated with AFM1 (range between 30 and 3130 ng/L) (Elgerbi et al., 2004). In India, the incidence of contamination of AFM1 in...
Table I.- Incidence of Aflatoxin (M1) contaminated milk samples in different towns* of Lahore city, Pakistan.

<table>
<thead>
<tr>
<th>Area (n=21)</th>
<th>Contaminated samples (%)</th>
<th>Minimum (µg/L)</th>
<th>Maximum (µg/L)</th>
<th>Mean (µg/L) ± SD</th>
<th>± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gulberg Town</td>
<td>15 (71.4)</td>
<td>1.47</td>
<td>56.02</td>
<td>19.75 ± 18.29</td>
<td>4.89</td>
</tr>
<tr>
<td>Ravi Town</td>
<td>18 (85.7)</td>
<td>1.30</td>
<td>73.58</td>
<td>15.43 ± 16.78</td>
<td>4.07</td>
</tr>
<tr>
<td>Gungbuksh Town</td>
<td>17 (80.9)</td>
<td>0.98</td>
<td>40.46</td>
<td>12.13 ± 11.24</td>
<td>2.81</td>
</tr>
<tr>
<td>Shalimar Town</td>
<td>18 (85.7)</td>
<td>0.69</td>
<td>100.04</td>
<td>22.31 ± 26.18</td>
<td>6.35</td>
</tr>
</tbody>
</table>

* There are nine towns of Lahore city, Pakistan but milk samples were randomly collected from four towns of the city. Figures having similar superscript are not significantly different (P>0.05).

infant milk, milk based cereal weaning food and liquid milk samples was almost in the magnitude of 87% (Rastogi et al., 2004), with 99% of contaminated samples exceeding the EU/Codex recommended limits.

The incidence of AFM₁ contamination in the milk samples collected from Gulberg Town, Ravi Town, Gunjbuksh Town and Shalimar Town was 71, 86, 81 and 86 percent, respectively. Milk samples from either of the town contained AFM₁ levels exceeding the permissible limits (Fig.1). Hussain et al. (2008) reported 42.5% and 52.5% milk samples were found contaminated from buffalo and cow, respectively, with the toxin. AFM₁ levels in almost all the contaminated milk samples exceeded 50 ng/kg. These results are in accordance with El-Sayed et al. (2000), Salem (2002) and Elgerbi et al. (2004) who reported high levels of AFM₁ in bovine raw milk. Moreover, there is a strong correlation between AFM₁ level in milk and AFB₁ content in the animal’s ration (Van Egmond, 1989). The most important factors affecting the amount of AFB₁ occurrence in feed was undoubtedly temperature and moisture. The toxin producing fungi such as Aspergillus flavus and A. parasiticus species show enormous growth in feeds having water contents between 13% and 18% and environmental moisture between 50% and 60%. Furthermore, these moulds can produce the toxin under conditions of 25°C and 85–90% relative humidity (Bakirci, 2001). The results indicate that feeds/ration for the dairy animals might be heavily contaminated with AFB₁.

Keeping in view high levels of AFM₁, there is dire need to improve storage conditions of animal ration/feed that will mitigate the AFB₁ levels in feed/feed ingredients and ultimately decrease the toxin in animal milk.

It is concluded that levels of AFM₁ in 81% raw milk samples collected from Lahore market exceeds the permissible limits. In Pakistan, more than 90 percent milk is consumed as raw so could be main source of the toxin for end users.

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Evaluation and application of a simple and rapid method for the analysis of aflatoxins in commercial


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