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Determination of Aflatoxin M_1 in Powdered Milk Consumed by Infants in Jeddah, Saudi Arabia Using Three Methods

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Abstract. Thirty-one samples of infant powdered milk formula marketed in Jeddah, Saudi Arabia were collected from different pharmacies and supermarkets then screened for aflatoxin M_1 using three different techniques including thin layer chromatography, high performance liquid chromatography, and radioimmunoassay. These methods showed various results. Aflatoxin M_1 was not detected in the samples by radioimmunoassay procedure while both thin layer chromatography and high performance liquid chromatography showed positive results. Aflatoxin M_1 was detected in nine samples. Twenty-two per cent of the positive samples recorded high levels of aflatoxin M_1. The levels detected by the high performance liquid chromatography ranged from 67.7 to 173.0 ng/kg.

Introduction

Aflatoxin M_1 is a hydroxylated metabolite of aflatoxin B_1, found in milk of animals that consume feed contaminated with aflatoxin B_1 [1-4]. On the other hand, aflatoxin B_1 (AFB_1) and aflatoxin M_1 (AFM_1) are known as hepatoxins and hepatocarcinogens. Their harmful effects in human, especially infants, are vital [5, 6]. The fact that milk, being one of the most important and valuable foods, might be contaminated with aflatoxins, has initiated a boom of ongoing scientific research. Yet, numerous reports available all over the world indicate presence of AFM_1 in infants powdered pasteurized dry milk as well as dairy products [1, 7-11].

In Saudi Arabia, as it is the case in many developing countries, financial reasons have forced women to be outside the house for work. Consequently, there has been heavy reliance on infant powdered milk for formula mostly imported from abroad. In 1999, Saudi daily newspapers have warned of the presence of very toxic compounds in

baby milk locally sold regardless of the absence of scientific evidence. Department of Health at Ministry of Commerce has accordingly withdrawn this type of milk from pharmacies and markets.

International researchers have developed several techniques and procedures for aflatoxin M_1 detection from infant formula of powdered milk, which vary in their sensitivity and requirements. The objective of this work was to prove the presence of AFM_1 in infant powdered milk and to find out the most simple, economical and reliable method for the analysis of baby milk for aflatoxin M_1 content using thin layer chromatography, high performance liquid chromatography and radioimmunoassay.

Materials and Methods

Collection and preparation of powdered milk samples for aflatoxin M_1 analysis

A total of thirty-one samples of commercial pasteurized powder formula baby milk for newborn to three-year old babies, were obtained from pharmacies and supermarkets in Jeddah, Saudi Arabia, between 1999-2000. They were stored at room temperature until being analyzed for aflatoxin M_1. Before taking sub-samples for extraction, the samples were thoroughly mixed. The methods used for analysis were thin-layer chromatography (TLC) plates, high performance liquid chromatography (HPLC), and radioimmunoassay (RIA).

Extraction and clean up procedures for TLC and HPLC

Powdered milks were reconstituted as 10% (wt/vol) solutions by stirring with distilled warm water. The solution was filtered through fluted Whatman filter papers (No. 2 cat. No. 1002 150). Then, C_{18} Sep-Pak cartridge of water (Water Assoc., Milford, MA, USA) or C_{18} of Whatman cartridge (Whatman solid phase-extraction device ODS-5 octadyl; 18% E) was pre-washed twice with 10 ml of methanol, followed by 10 ml of water. Fifty ml of milk solution was loaded on the column. After loading for around 15 min, the column was washed with an additional 10 ml of hexane. Then the column was left to dry. The milk extract was eluted with 4 ml of dichloromethane-acetone (80:20, vol/vol) [12] and the elute was divided into equal portions in two different vials then evaporated to dryness under a gentle stream of nitrogen. The content of the first vial was re-dissolved with 0.5 ml of water-acetonitrile (75:25, vol/vol) for AFM_1 detection and determination by using TLC method. The content of the second vial was used for determination of AFM_1 by HPLC according to the method described by Stubblefield [13].

Analysis by thin layer chromatography (TLC)

TLC equipment

a) Aflatoxin M_1 standard: 10 mg AFM_1, purchased from Sigma Chemical Co., St. Louis, Mo, USA were dissolved in benzene-acetonitrile (98:2, vol/vol) to give concentrations of 1 µg/ml, 2 µg/ml, and 3 µg/ml. Working solutions were prepared in two types: in water-acetonitrile (9:1, vol/vol) and AFM_{2a} (derivatives AFM_1) in water-

acetonitrile (75:25, vol/vol).

Derivatization of AFM_1 was carried out as described by Stubblefield [13] by adding 200 µl of hexane and 200 µl of trifluoroacetic acid (TFA) to the evaporated solution of AFM_1 sample extract in silylated vials, heating at 40ºC in a water bath for 10 min, evaporating to dryness under nitrogen, re-dissolving with 40-100 µl of water-acetonitrile (75:25, vol/vol), and analyzing by HPLC or TLC.

b) Thin layer chromatographic plates: For detection of aflatoxin M_1 in the samples applying thin-layer chromatography methods, aluminum-backed (kieselgel 60) plates (Cat. No. 5553/7) was used.

c) Developing solvents: The solvent systems used for developing on one dimension TLC were as follows:

1) Chloroform-acetone-isopropanol (87:10:3, vol/vol/vol). The R_f value of AFM_1 was 0.17.

2) Isopropyl alcohol-acetone-chloroform (5:10:85, vol/vol/vol) as recommended by Stubbleffield *et al.* [14] as the best system. The R_f value of AFM_1 was 0.39.

3) The solvent system used for two-dimensional TLC was ethyl ether-methanol-water (91:4.5:1.5, vol/vol/vol) for first direction. After drying for 15 min in the dark, chloroform–acetone-methanol (90:10:2, vol/vol/vol) was used for the second direction.

The quantity sample extract and standard spotted on the TLC plates used No. 2.

TLC conditions: The plates were removed and air-dried in fume cabinet, then examined in a chromato-vue cabinet (Model Mfd. Inc., San Gabriel, California, USA) under visible light or long wavelength (363 nm) UV light. Aflatoxin M_1 was determined by comparing the chromatograms of sample aliquots with the standard [14].

Analysis of aflatoxin M_1 by HPLC

HPLC equipment

Immunoaffinity columns (Afla-M_1) containing monoclonal antibodies against aflatoxin M_1 (No. G 1007) were purchased from VICAM Science Technology (313 Pleasant St. Watertown, MA 024712, USA). For the analysis, modular VIAL used was Amber, WO/Cap CS-200 1.5 ml - MFG. No. 224810; a Beckman solvent delivery module Pumps 110B was used; the flow rate 0.5 ml/ min equipped with C_{18} Column Ra 5µ x 25 cm. A Beckman 421 controller; linked to a Varian 4270 Chromato-integrator and a pen recorder, Attenuation: 4/8; sensitivity 100 Varian Fluorescence detector, the flow rate of the solvent was 0.5 ml/min^{-1} [15]. The sample (20 µl) was injected into the instrument using a 25 µl glass syringe (Hamilton, Reno, Nevada, USA).

HPLC conditions

Mobile phase: The mobile phases for HPLC used were as follows:

1) Water-methyl alcohol-acetonitrile (5: 4: 1, vol/vol/vol) (3.2-3.5),

2) Water- isopropyl alcohol-acetonitrile (80:12:8, vol/vol/vol) (4.02-4.04), and
3) 30% acetonitrile-methanol (1:1, vol/vol) (2.6-3.5).

A peak indicated the presence of aflatoxin M_1 with the same retention time (4.02±0.02 min) as the standard. The mean peak area was determined from on the printout for 3-5 measurements.

Analysis of aflatoxin M_1 by radioimmunoassay

RIA equipment and conditions

This method was essential according to that came in operation manual with eleven steps [15]**.** The time for screening radioimmunoassay for detection and identification of M_1 is rapid, and also very sensitive because it could measure between 0.05 and 05 ppb.

The analysis used for the detection of aflatoxin M_1 in milk was Charm II test using table reagents [15]. By using the charm radioimmunoassay, aflatoxins could be detected through their active functional groups. In the Charm II test for aflatoxin in milk, a microorganism containing antibody binding sites for aflatoxin was added (in the form of a binder tablet) to the sample. Then a labeled tracer aflatoxin (^{3}H) aflatoxin M_1 was added to compete for the binding sites with any contaminating aflatoxins already in the sample. The amount of labeled tracer bound to the sites was measured and the result was compared to the control point. The more labeled tracer bound to the sites, the higher the result and the lower of aflatoxin concentration in the sample [15].

Results and Discussion

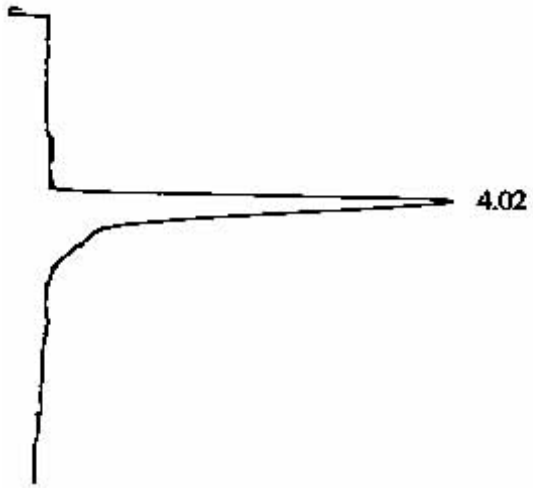
Using thin-layer chromatography (TLC) method, the results in Table 1 showed that aflatoxin M_1 was not detected on the majority of the tested powdered infant samples. However, AFM_1 was detected on nine samples with differences among the concentration of samples as expressed in terms of brightness of fluorescence. Two of these samples (1 and 6) were highly concentrated with aflatoxin M_1 (84.6 and 173.0 ng/kg), two samples (26 and 28) were of moderate concentration (32.37 and 46.13) and five samples (4, 9, 11, 24, and 25) were of low concentration (4.12, 2.37, 4.68, 4.50 and 10.87 respectively). This method is always considered a quick and sensitive means for quantitative determination of aflatoxins. In accordance with these results, Pestka *et al.* [12] compared TLC, HPLC and RIA and found that TLC method exhibited high sensitivity, but required extensive extraction and cleanup procedures. They also mentioned that most analytical methods for determining aflatoxins used thin-layer chromatography either for visual or densitometric measurement of their fluorescent zones. Yet, Stubblefield and Shotwell [17] found that a comparison of corn extract assays (6 – 98 ppb B_1) with TLC and HPLC revealed that HPLC values averaged 25% less than TLC values. This difference was insignificant.

With respect to high performance liquid chromatography method, the data presented in Table 1 showed that aflatoxin M_1 quantitative estimation by this method (Fig. 1) was detected in nine of the powdered milk samples. There was almost a clear direct

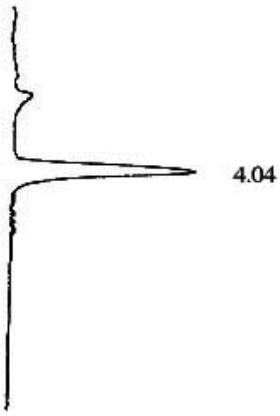
relationship between the concentration of aflatoxin M_1 as indicated by TLC and its quantity as it was estimated by HPLC (Table 1). Two samples (1 and 6) were with high concentrations of aflatoxin M_1 (173.0 and 67.7 ng/kg, respectively). In accordance with these results, Van Egmond [18] indicated that the tolerance level for AFM_1 in milk varied among countries, from 0.05 ppb in most European countries to 0.5 ppb in the United States. Contrary to these results, Jalon *et al.* [19] surveyed raw and heated milk in Spain and found that 80% of raw milk samples and 85% of heated milk samples contained AFM_1 less than 0.01 ppb and no samples contained more than 0.04 ppb. Moreover, Markaki and Melissari [20] analyzed 81 samples of commercial pasteurized milk from Athens markets for the presence of aflatoxin M_1 using HPLC procedure. Thirty-two of these samples contained aflatoxin M_1 at levels of 2.5–5 ng/l while 31 contained only traces of aflatoxin (0.5–1 ng/l). In nine samples no AFM_1 was detected [19].

Table 1. Occurrence of aflatoxin M_1 detected on powdered infant milk using three different methods: Thin-layer chromatography (TLC), high performance liquid chromatography (HPLC) and radioimmunoassay (RIA)

No	Type of infant powdered milk sample	AF M_1 Content in ng/kg		
		TLC	HPLC	RIA
1	Aptamil 2	84.62	67.7	0.0
2	Aptamil+ F	0.0	0.0	0.0
3	Babina L. GF	0.0	0.0	0.0
4	Babina plus	4.12	3.3	0.0
5	Babina progress	0.0	0.0	0.0
6	Bebelac	216.25	173.0	0.0
7	Bebelac 1	0.0	0.0	0.0
8	Bebelac 2+ F	0.0	0.0	0.0
9	Bebelac FL	2.37	1.9	0.0
10	Biomil 1	0.0	0.0	0.0
11	Biomil 2	4.68	3.75	0.0
12	Guigo 21	0.0	0.0	0.0
13	Guigo 21F	0.0	0.0	0.0
14	Guigoz 2+ F	0.0	0.0	0.0
15	Isomil	0.0	0.0	0.0
16	Maeil	0.0	0.0	0.0
17	Maeil Mam'mA	0.0	0.0	0.0
18	Milupa HN25	0.0	0.0	0.0
19	Nektarmil+ Hony	0.0	0.0	0.0
20	Nido Nestle	0.0	0.0	0.0
21	Nursie	0.0	0.0	0.0
22	Nursie 2 (DANONE)	0.0	0.0	0.0
23	Nutrilon fllow-on	0.0	0.0	0.0
24	Nutrilon low lactose	4,5	3.6	0.0
25	Nutrilon premium	10.87	8.7	0.0
26	Promil	32.37	25.9	0.0
27	S-26	0.0	0.0	0.0
28	S-26 Gold	46.13	36.9	0.0
29	Similac	0.0	0.0	0.0
30	Similac advance+ F	0.0	0.0	0.0
31	Similac LF	0.0	0.0	0.0



Standard of aflatoxin M_1 (R. T. 4.02)



Aflatoxin M_1 separation from milk sample (R.T. 4.04)

Fig. 1. High performance liquid chromatographic separation of aflatoxin M_1.

All the tested powdered infant milk samples were uncontaminated with aflatoxin M_1 when using radioimmunoassay (RIA) method as indicated in Table (1). This result was in disagreement with that obtained by Saitanu [11] who found aflatoxin M_1 in most of the raw milk samples and off-the-shelf milk products using radioimmunoassay. On the other hand, he found that all powdered milk samples were negative for aflatoxin M_1

except two samples with concentration of less than 0.1 ppb. When Galvano *et al.* [21] checked 97 samples of dry milk for infant formula from four Italian cities for aflatoxin M_1 (AFM_1) by immunoaffinity column extraction, they found AFM_1 in 81 (84%) of the dry milk samples in amounts ranging from < 1 ng/L to 496.5 ng/L at mean level: 18.08 ng/L). They also indicated that only one sample of the dry milk had levels of AFM_1 exceeding the Swiss legal limits. Pestka *et al.* [12] suggested that aflatoxin M_1 might be best quantified by RIA because most analytical methods for aflatoxin M_1 used thin layer chromatography or TLC. These techniques exhibited high sensitivity, but required extensive extraction and cleanup procedures. They also found RIA was sensitive in the range of 5 – 50 ng per assay but was subjected to interference by whole milk.

In conclusion, these results indicate that the efficient control of aflatoxin M_1 in dairy products requires precise and easily performed analytical methods. It is necessary for full care of reliable detecting of aflatoxin M_1 presence in powdered infant milk to use different methods. Because the data indicated that there was a variation between the results of each method, it was better to evaluate aflatoxin M_1 in powdered milk samples by TLC and HPLC methods than by RIA, which gave negative results for all samples.

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تقدير أفلاتوكسن م١ في حليب البودرة المستهلكة من الأطفال الرضع في المملكة العربية السعودية باستخدام طرق كشف مختلفة

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ص.ب ١٢١٦١ ، جدة ٢١٤٧٣

(قدم للنشر في ١٤٢٤/٢/١٠هـ ؛ قبل للنشر في ١٤٢٤/٨/٨هـ)

ملخص البحث : في هذا البحث تم دراسة حوالي ٣١ عينة حليب بودرة أطفال تسوق في المملكة العربية السعودية، جمعت من مختلف الصيدليات والسوبر ماركت في الفترة ما بين ١٩٩٩م–٢٠٠٠م، حيث فحصت لتقدير محتواها من سم الأفلاتوكسن وذلك باستخدام ثلاثة طرق مختلفة شملت هذه الطرق طريقة الألواح الكروماتوجرافية (السيلكا جل) (Thin layer chromatography (TLC، طريقة الإشعاع المناعي (Radioimmunoassay (RIA ، وطريقة التحليل الكيميائي الكروماتوجرافي عال الأداء (performance liquid chromatography (HPLC . عند فحص العينات السابقة بواسطة طريقة (RIA) لم يتم تعيين أي كمية من الأفلاتوكسن م١ في العينات حيث كانت جميع النتائج سالبة. بينما باستخدام الطريقتين الأخريتين (TLC) و (HPLC) كانت النتائج إيجابية حيث لوحظ تلوث ٩ عينات بالأفلاتوكسن م١، وهذا يعني أن ٢٢% من العينات كانت ملوثة بالأفلاتوكسن م١. وحددت طريقة (HPLC) المستخدمة كمية التلوث بهذا السم وحيث كانت عينتين منهم تمثل نسبة عالية حيث قدرت الكيمة ١٧٣ ميكروجرام/كجم والأخرى ٦٧,٧ ميكروجرام/كجم.