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Analysis of Norwegian milk and infant formulas for ochratoxin A.

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Abstract

Samples of organic cow's milk, conventional cow's milk, and cow's milk-based infant formulas were analysed for the occurrence of ochratoxin A by means of a HPLC method. The detection limit was 10 ng/l. Ochratoxin A was detected in 6 out of 40 conventional cow's milk samples (range 11 - 58 ng/l), and in 5 out of 47 organic milk samples (range 15 - 28 ng/l). No ochratoxin A was detected in any of the 20 infant formula samples. The ochratoxin A levels in cow's milk found in this investigation are sufficient to cause a higher intake of ochratoxin A than the suggested TDI of 5 ng/kg bw/day, e.g. in small children who consume large quantities of milk.

Keywords: cow's milk, infant formula, Norway, ochratoxin A, organic milk

Introduction

The detection of a high incidence of ochratoxin A (OA) contamination of human blood in Europe and Canada indicates that there is a continuous and widespread human exposure to this mycotoxin (HALD 1991). Humans are exposed to OA by consumption of food which has been directly contaminated through growth of fungi, by consumption of food-products derived from exposed animals, and probably also by inhalation of contaminated dust (HENDRY and COLE 1993, DI PAOLO et al. 1993). OA has been detected in various human food commodities, such as cereals, coffee, and pork and poultry meat (SPEIJERS and Van EGMOND 1993).

Consumption of cow's milk has not previously been regarded as a possible source of OA intake, because feeding experiments with OA contaminated feed to milking cows have shown low biotransfer of OA into the milk (HULT et al.1976; RIBELIN et al. 1978). This is because the microflora in the rumen of the cow degrades OA to the less toxic metabolite ochratoxin α. However, Breitholtz-Emanuelsson et al. (1993) reported OA in 5 of 36 cow's milk samples from Sweden, in the range from 10 to 40 ng/l.

Even low concentrations of OA in cow's milk can be of importance for consumers of large quantities such as children, and contribute to a significant portion of the total dietary intake. As OA is classified by The International Agency for Research on Cancer (IARC 1993) as a possible human carcinogen (category 2B), there is reason for concern about the potential long term effects from continuous exposure to this toxin. In addition, OA has been shown to have nephrotoxic, immunotoxic, genotoxic and teratogenic properties (KUIPER-GOODMAN and SCOTT 1989, KUIPER-GOODMAN 1996).

The level of OA contamination of foodstuffs and feed varies significantly between countries and with season (SPEIJERS and Van EGMOND 1993). Analyses of human milk samples from Sweden (BREITHOLTZ-EMANUELSSON et al. 1993) and Norway (SKAUG et al. in press) indicate that the level of OA contamination could be higher in Scandinavia than in some other European countries. The aim of the present study was to examine the occurrence of OA contamination of cow's milk samples from Norway.

Experimental

Milk samples

Forty conventionally produced cow's milk samples, 47 cow's milk samples produced by organic farms, and 20 cow's milk-based infant formula samples were analysed for OA. The conventional milk samples were obtained from groceries located in different counties in Norway during January - July, 12 samples in 1995 and 28 samples in 1996. The organic milk samples were kindly provided by the regional dairy in Skarnes, Hedmark county, from June 1997 to February 1998. The infant formulas were produced by two different manufacturers. All milk samples were stored at - 20 °C.

Reagents

OA was purchased from Sigma (St Louis, MO, USA). A solution of OA (10 μg /ml in methanol) was calibrated spectrophotometrically at 333 nm using the value of 6640 for the extinction coefficient (BAUER and GAREIS 1987). The OA solution was diluted to

1 x 10^{-8} M in methanol, and stored at -20 °C. Silica gel cartridge, 500 mg Bond Elut LRC, was obtained from Varian. Nylon membrane filter (Gelman Cameo 3N 0.45 μm Syringe Filter) was obtained from Micron Separations Inc.

Extraction and clean-up

The method described by Breitholtz-Emanuelsson et al. (1993) was used with slight modifications of the purification and HPLC conditions, and with another confirmation method for OA. A volume of 5 ml methanol, 0.5 ml 1 M HCl, and 5 ml chloroform were added to a 2 ml portion of liquid milk. Instead of a self-packed silica gel column in a Pasteur pipette, a commercially available silica gel cartridge with 500 mg silica was employed for clean up of the chloroform extract. The silica gel was washed with 5 ml chloroform before the chloroform phase from the extraction was added. After evaporating the purified extract to dryness at 40-45 °C under a stream of N_2 gas, the residue was immediately dissolved in 300 μl of mobile phase by ultrasonication for 15 minutes. All sample extracts were filtered through a 0.45 μm membrane filter prior to injection onto the HPLC column. OA was extracted from the liquid infant formula prepared according to its directions for use.

HPLC analysis

The chromatographic system consisted of a Merck Hitachi L-6200 A Intelligent Pump, fluorescence detector (Merck Hitachi F-1080), interface (Merck Hitachi D-6000), a Rheodyne manual injector, and chromatography software (model Hitachi D-6000 HPLC

Manager). Separations were carried out on a Spherisorb S3ODS2 (C-18) column, 4.6 x 150 mm, with

3 μm particles. A 50 μl aliquot of the sample extract was injected onto the column. The mobile phase consisted of 10 mM tetrabutyl ammonium bromide in a methanol-potassium phosphate buffer (pH 7.5) mixture. The ratio of methanol to potassium phosphate buffer was 51:49. Flow rate was 0.8 ml/min. The determinations were performed at 380 nm (excitation wavelength) and 450 nm (emission wavelength).

Confirmation

The confirmation of positive samples were performed in two ways:1) As a routine all sample extracts shown to contain OA during initial HPLC analysis were analysed in duplicate, with a direct spiking of the second aliquot. This was performed by adding an amount of a stock solution of OA to the aliquot. The chromatograms of the unspiked and the spiked sample extract were then compared. 2) Derivatization of OA through methylation of the extracts with subsequent HPLC analysis was also used for qualitative confirmation of positive samples (ZIMMERLI and DICK 1995): A 200 μl aliquot of the purified sample extract was evaporated to dryness, and the residue was dissolved in 2.5 ml methanol and 0.1 ml conc. HCl. The mixture was kept overnight at room temperature. After evaporating the mixture to dryness, the residue was dissolved in 200 μl mobile phase. 50 μl of it was injected onto the HPLC column and analysed (disappearance of the OA peak, and, in samples containing more than 40 ng/l OA, appearance of a new peak with the same retention time as that of the metyl ester).

Analytical quality control

OA standard solutions (70 -700 ng/l) for calibration were prepared every day by dilution of the 10^{-8}M OA stock solution with HPLC mobile phase. For quantitation, peak heights were measured by a Merck Hitachi integrator. The 10^{-8}M OA stock solution was added to milk samples for recovery experiments and for calculation of the detection limit. The mean recovery of OA for the whole procedure, calculated from spiked milk samples (100 - 700 ng/l), was 70 %. The detection limit (signal-to-noise ratio of 3) was 1.5 pg OA. The quantitation limit for spiked milk samples was 10 ng/l OA. The standard curves used for quantitation were calculated by the least-squares method. The coefficient of variation of the peak height of OA from spiked milk samples was 13.5% (between days).

Results and discussion.

OA was detected in 6 of the 40 conventional cow's milk samples (range 11 - 58 ng/l OA), and in 5 of the 47 organic cow's milk samples (range 15 - 28 ng/l OA). This is approximately the same contamination incidence as previously found in cow's milk from Sweden (BREITHOLTZ-EMANUELSSON et al.1993). In the Swedish investigation, 14 % (5 out of 36) of the cow's milk samples were found to contain OA (range 10 - 40 ng/l), compared to a total of 13 % (11 out of 87) in this study (range 11 - 58 ng/l). However, no OA was detected in 121 cow's milk samples from northern Germany (VALENTA and GOLL 1996). The detection limit was the same in all three studies. The weather conditions during growth, harvest and storage has great influence on OA levels in the crop (JØRGENSEN et al. 1996), which is probably also reflected in OA levels in feedstuff. Climatic differences, as well as differences in farming procedures may explain the variation in OA contamination of the milk between countries.

No difference in OA contamination between milk from organic farms and milk from conventional farms was found in the present study. The number of organic farms in Norway is increasing, and organic milk has been available on the market since 1995. Organic dairy farming practices differ from those of conventional farming in several ways that may affect the risk of mycotoxin exposure of the milking cows. For example, cows are kept outdoors for a longer period throughout the year as compared to conventional farming. This reduces the inhalation exposure to dust and fungal spores. Also, the feed used on organic farms is mostly produced locally, without use of artificial fertilizer or agricultural chemicals such as fungicides. The restricted use of fungicides may cause an increased risk of fungal infection, and thus, higher levels of mycotoxin contamination of the feed. Although the OA levels found in organic milk in the present study do not indicate a higher risk of contamination, more data are needed.

If cow's milk used for the production of infant formulas is subject to OA contamination, the same is to be expected for the infant formula. OA was not detected in any of the 20 infant formula samples tested. However, the number of infant formula samples analysed was less than the number of cow's milk samples, and the result is therefore subjected to larger uncertainty.

Direct contamination of the milk with OA can occur by growth of toxigenic *Aspergillus* and *Penicillium* species in the raw milk, if the hygienic quality of milk handling is poor (ŠKRINJAR et al. 1995). However, as the hygienic quality in Norwegian dairy farming is high, direct growth of fungi is not likely to be a source for OA contamination.

In view of the low biotransfer of OA into milk seen in feeding experiments with cows, other routes than peroral exposure of the cow should be considered. The spores of many species of toxigenic fungi have been shown to contain mixtures of mycotoxins (LACEY et al.1994). Exposure to mycotoxins may therefore occur through inhalation of airborne particles containing mycotoxins, such as dust and fungal spores (HENDRY and COLE 1993). In recent years, growing attention has been given to occupational inhalation exposure to mycotoxins in agriculture. Dairy farmers have a high occupational exposure to airborne fungi, especially when tending cows (EDUARD 1997), and the same exposure is to be expected for the cows in the cowshed. *Penicillium* and other toxigenic fungi have been found in airborne dust samples in the agricultural environment (LAPPALAINEN et al. 1996), and inhalation exposure of farmers, and thus of milking cows, is possible.

The results of this study, and the previous study in Sweden show that cow's milk should be considered as a possible source of OA in the human diet. The presence of contaminants in milk is likely to have greater implications for infants and young children than for adults having a more varied diet. Different risk assessments of the intake of OA have been performed, and different tolerable daily intakes (TDIs) have been suggested (WHO 1991, KUIPER-GOODMAN and SCOTT 1989, KUIPER-GOODMAN 1996). However, risk assessments that have been carried out do not differentiate between risk to children and to adults. Children represent a particularly sensitive population group for which a specific TDI should be evaluated, especially in consideration of the unfavourable dose/body-weight ratio. The Nordic Working Group on Food Toxicology and Risk Evaluation (1991) has suggested a maximum TDI of OA in humans of 5 ng per kg body weight, based on carcinogenicity studies with OA in adult rats. The OA levels in cow's milk found in this investigation are

sufficient to cause a higher intake of OA than the suggested TDI of 5 ng/kg bw/day in small children who consume large quantities of milk.

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