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Aflatoxins and ochratoxin A in baby foods and analysis of interactive cyto- and genotoxic effects in a human intestinal cell line

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Background

Aflatoxins
 B₁, B₂, G₁, G₂

Epoxide

Adduct

SSBs
 Bp substitution
 Frameshift mutations

Aspergillus flavus
Aspergillus parasiticus

Background

Aflatoxins
 M₁, M₂, B₁, B₂, G₁, G₂

Aspergillus flavus
Aspergillus parasiticus

Human carcinogen – group 1
 International Agency for Research on Cancer

Possible human carcinogen – group 2B
 World Health Organization

Aflatoxin M₁ (AFM₁)
 • Genotoxic
 • Hepatotoxic

Background

Ochratoxin A (OTA)

Aspergillus sp.
Penicillium sp.
Fusarium sp.

Evidences of genotoxic effects
in vivo and *in vitro*

Possible human carcinogen
 by IARC – group 2B
 International Agency for Research on Cancer
 World Health Organization

Background

EU maximum levels:
 0.10 µg/kg of AFB₁ for processed cereal-based foods and baby foods
 0.025 µg/kg of AFM₁ for infant formulae and follow-on formulae, including infant milk and follow-on milk
 0.5 µg/kg of OTA for cereal-based foods and baby foods

Restricted diet, rich in cereals
 More food per body weight
 Immature physiology

Recent evidences of baby food contamination with AFM₁ and OTA:

Baydar *et al.*, 2007
 Journal of Food and Drug Analysis, 19(1), 2007, Page 59-62
 Contamination of infant formulae marketed in Ankara with AFM₁, AFB₁ and OTA

Meucci *et al.*, 2010
 Food Additives and Contaminants, Vol. 27, No. 1, January 2010, 64-71
 Mycotoxin detection in infant formula milks in Italy
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 (Received 11 February 2009; final version received 17 July 2009)
 Presence of AFM₁ and OTA in infant formula powders marketed in Italy

Background

Gómez-Arranz and Navarro-Blasco, 2010
 Food Additives and Contaminants: Part B, Vol. 3, No. 3, September 2010, 193-199
 Aflatoxin M₁ in Spanish infant formulae: occurrence and dietary intake regarding type, protein-base and physical state
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 (Received 18 April 2010; final version received 20 June 2010)

Alvito *et al.*, 2010
 Food Anal. Methods 2010, 3, 225-230
 DOI: 10.1007/s12161-008-9964-x

Occurrence of Aflatoxins and Ochratoxin A in Baby Foods in Portugal
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AFM₁ was found in 26 infant formulae samples marketed in Spain

Occurrence AFM₁, AFB₁ and OTA in baby foods available in Portuguese market in 2007

Humans and animals might be exposed to mixtures rather than to individual compounds
 Interactions AFM₁ → OTA

Background

Models for interaction analysis:

Two conceptual models
Concentration Addition (CA): the joint action of a mixture is the summation of individual toxicities, assuming the same mode of action (MoA)
Independent Action (IA): the joint effects is estimated considering the probabilities of responses, assuming different MoA between chemicals

➔

- Synergism/antagonism
- Dose-ratio dependent
- Dose level dependent

Synergism or antagonism according to mixtures doses

Aims

1. a) Development and validation of an HPLC-FD method for simultaneous determination of various aflatoxins (AFM₁, AFB₁, AFB₂, AFG₁, AFG₂) and ochratoxin A in baby foods
 b) Application of the validated method to the detection of these mycotoxins in baby foods marketed in Lisbon, Portugal
2. Evaluation of cytotoxic and genotoxic effects of AFM₁ and OTA combinations, comparatively to their individual effects in a human-derived intestinal cell line

Methods Aflatoxins and ochratoxin A determination

Analytical method⁽¹⁾

Samples: 1. Chloroform extraction, 2. Liquid-liquid extraction, 3. Immunoaffinity cleanup, 4. HPLC-FD with post-column derivatisation

Validation parameters

1. Selectivity/Specificity
2. Linearity and working range
3. Analytical limits: Limit of detection (LOD) and Limit of Quantification (LOQ)
4. Precision: •Repeatability, •Intermediate precision
5. Recovery studies: using spiked samples and reference materials
6. Accuracy: • Reference materials, •Proficiency Tests

Internal quality control

Control charts

Samples

8 baby foods for infants and young children (>4 months age) purchased in big supermarkets, pharmacies and organic produce retail outlets of Lisbon, July-August 2010

[1] Alvim et al., (2010). Food Analytical Methods 3:22-30

Methods Cytotoxicity and genotoxicity studies

Cell line

Human colonic carcinoma Caco-2 cells

Viability assay: Neutral red assay⁽²⁾

48h exposure	Treatments for neutral red assays			
	Individual experiments		Mixture experiments	
	AFM ₁ (μM)	OTA (μM)	AFM ₁ (μM)	OTA (μM)
	0.5 - 10	0.0125 - 80	0.5 - 10	2.5 - 10

Genotoxicity assay: Comet assay^(3,4)

48h exposure	Treatments for comet assays			
	Individual experiments		Mixture experiments	
	AFM ₁ (μM)	OTA (μM)	AFM ₁ (μM)	OTA (μM)
	0.5 - 1	0.125 - 10	0.5 - 1	2.5 - 10

MIXTOX software

2) Repetto et al. (2008). Nature Protocols 3: 1125-1131
 3) Tice et al. (2000). Environmental and Molecular Mutagenesis 35: 206-221
 4) Sitch et al. (1998). Environmental Cell Research 175: 184-191

Results Aflatoxins and ochratoxin A determination

Validation

1. Selectivity/Specificity
 Good resolution of the method

Results Aflatoxins and ochratoxin A determination

Validation

1. Selectivity/Specificity
 Good resolution of the method
2. Linearity and working range

Results Aflatoxins and ochratoxin A determination

Validation

3. Analytical limits

All LOQ < lowest levels of the working range

4. Precision

- Repetibility: RSD from 5.3 to 9.6%
- Intermediate precision: RSD from 6.4 to 9.8%

RSD < 10%
Good precision of the method

Results Aflatoxins and ochratoxin A determination

Validation

5. Recovery studies

Mean recoveries for aflatoxins and ochratoxin A analysis ranged from 87% to 114%

Acceptable values according to Commission Regulation (EC) 401/2006 (recoveries of 70-110% for AFM₁ and 50-120% for aflatoxins B₁, B₂, G₁, G₂ and OTA)

6. Accuracy:

- Reference materials: quantified in FAPAS test materials were close to the assigned values and within the reported range
- Proficiency Tests: FAPAS proficiency trials gave z-scores between -1.1 and 1.4 for the different food matrix analyzed

Z-score between -2.0 and 2.0

Good accuracy of the method

Results Aflatoxins and ochratoxin A determination

Samples analysis

All the values detected < LOD value. No mycotoxins quantified in all the analyzed samples

Results Cytotoxicity studies

Individual experiments

Viability reduction in Caco-2 cells

Dose-dependent decrease in cell viability after exposure to AFM₁ and OTA

Results Cytotoxicity studies

Cytotoxicity of mixtures similar to cytotoxicity of OTA

Results Genotoxicity studies

Exposure to OTA or AFM₁ did not induce significant DNA damage ($p > 0.05$)

Results Genotoxicity studies

Mixtures experiments

Exposure to mixtures of OTA and AFM₁ did not induce significant DNA damage ($p > 0.05$)

Results Cytotoxicity and genotoxicity studies

MIXTOX analysis – mixtures modelling

According to Jonker *et al.*, 2005 criteria:

- Antagonism was observed after the CA model fit ($a > 0$)
- Dose level deviation was observed after IA modeling, where antagonism was observed at low dose levels and synergism at higher dose levels ($a > 0$).

MIXTOX not applicable to genotoxicity data of mixtures experiments

Conclusions

- ❖ Development and validation of an HPLC-FD method for simultaneous detection of aflatoxins M₁, B₁, B₂, G₁, G₂ e ochratoxin A in baby foods
- ❖ No mycotoxins were detected in the analysed samples
- ❖ AFM₁ and OTA impaired Caco-2 cell viability in a dose-response relationship
- ❖ Mixtures of AFM₁ and OTA showed a general antagonistic effect on Caco-2 cells viability, especially at low dose levels. However, a synergistic effect might be observed at higher doses
- ❖ AFM₁ and OTA did not cause significant genotoxic effects in Caco-2 cells DNA, individually or in mixtures

Evaluation of interactive effects of both toxins when present in the same baby food and health effects in children's future life

Analysis of more baby food samples from the portuguese market

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Thank you for your attention!

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