An approach to the toxicity and toxicokinetics of aflatoxin B1 and ochratoxin A after simultaneous oral administration to fasted F344 rats

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Abstract

Humans are exposed to the hepatotoxic aflatoxin B1 (AFB1) and nephrotoxic ochratoxin A (OTA) through diet. However, kinetic and toxicological data after their co-administration are scarce.

In this study, a single oral dose of AFB1 (0.25 mg/kg bw)+OTA (0.5 mg/kg bw) was administered to fasted F344 rats. Blood, liver and kidney were harvested at different timepoints for mycotoxins quantification, relative weight calculation, clinical biochemistry and histopathology analysis.

Toxicity parameters pointed to acute toxicity in liver due to AFB1. No remarkable toxicity was observed in kidneys or immunological organs. Maximum observed concentrations in plasma (Cmax) were at 10 min and 2 h for AFB1 and OTA, respectively. AFB1 plasma concentration could indicate a rapid absorption/ metabolism of the mycotoxin; and AFB1 liver and kidney concentrations were lower than LOQ and LOD, respectively. For OTA, Cmax was 4326.2 μg/L in plasma. In kidney and liver Cmax was reached at 8 h and concentrations were very similar between both organs at all timepoints.

Due to the low levels of AFB1, the effect of OTA on AFB1 kinetics could not be assessed. However, AFB1 seems not to affect OTA kinetics, as its profile seems very similar to kinetic studies performed only with OTA in similar conditions.

1. Introduction

Mycotoxins are secondary metabolites produced by different fungal species that can be found in many agricultural commodities and processed food (Bennett and Klich, 2003). Aflatoxin B1 (AFB1) and ochratoxin A (OTA) are some of the most relevant mycotoxins due to their toxic effects and demonstrated human exposure (EFSA, 2006; EFSA, 2007).

AFB1 causes acute hepatotoxicity in humans and animals, and in severe intoxications may cause death. The International Agency for Research on Cancer (IARC) concluded that there was sufficient evidence of carcinogenicity of naturally occurring AFB1 in humans (IARC, 1993, 2002); therefore, the authorities cannot estimate a safe intake following the ALARA principle (as low as reasonably achievable) (EC, 2002). It has been described that, in humans, orally administered AFB1 follows a two-compartment model of absorption and elimination, with a rapid distribution phase followed by a slower elimination phase (EFSA, 2007). In rats, intestinal absorption of AFB1 is very fast and follows first-order kinetics (Ramos and Hernandez, 1996). Absorbed AFB1 reaches the liver through the portal system and is bioactivated by P450 cytochromes. The resulting epoxide attacks DNA forming an adduct (McLean and Dutton, 1995). AFB1 metabolism is well known (McLean and Dutton, 1995; El-Khatib *et al.*, 1998; Smela *et al.*, 2002; Bedard and Massey, 2006); on the contrary, data regarding kinetics of AFB1 in humans and in laboratory animals are sometimes contradictory or incomplete (IARC, 2012). In the majority of the studies carried out, the authors used methods that could not detect the AFB1 molecule or differentiate it from the resulting metabolites (Wong and Hsieh, 1980; Coulombe Jr and Sharma, 1985; Cupid *et al.*, 2004; Jubert *et al.*, 2009; Firmin *et al.*, 2010).

With regard to OTA, this mycotoxin has been related with the spontaneous avian and porcine nephropathy, and with the Balkan endemic nephropathy in humans. It is a potent nephrocarcinogenic compound in rodents. However, its mechanism of genotoxicity remains controversial despite the increasing number of studies devoted to this issue. Some studies have reported formation of DNA adducts (Pfohl-Leskowicz and Castegnaro, 2005; Pfohl-Leskowicz and Manderville, 2007; Mantle *et al.*, 2010) while others have proposed indirect genotoxic or epigenetic mechanisms (Arbillaga *et al.*, 2004; 2007; Mally *et al.*, 2004; 2005; Mally and Dekant, 2005; Turesky, 2005; Marin-

Kuan *et al.*, 2008). Some of the hypothesis regarding the mechanism of action of OTA would completely account for tumor formation, whereas others have been considered as possible contributors to it (WHO, 2008). For these reasons, the IARC classified OTA as a possible carcinogenic compound (IARC, 1993). In most animal species, the kinetic behavior of OTA has been described as a two compartment open model, although recent data regarding the accumulation in kidneys suggests that these models are too simple and should be re-analyzed using multi-compartment models (EFSA, 2006). Upon absorption from the gastrointestinal tract, OTA binds to serum proteins (approximately 99%). This binding mostly determines its half-life in the body and considerable variations across species have been reported due to different affinity and degrees of protein binding (O'Brien and Dietrich, 2005). Reabsorption of OTA from the intestine back to the circulation can take place as a consequence of biliary recycling and may also explain some of the inter-species differences observed. Once in circulation, OTA mainly accumulates in kidney, liver and muscle (Ringot et al., 2006). In addition, reabsorption of OTA can occur in the kidney proximal and distal tubules (Ringot et al., 2006) which may also contribute to the long half-life of OTA and increased nephrotoxicity. Regarding its elimination, there are several publications supporting a different contribution of the urinary, biliar or faecal excretion routes depending on several factors such as route of administration, dose and inter-species differences in degrees of entero-hepatic circulation (Dietrich *et al.*, 2005; Kuiper-Goodman and Scott, 1989). Vettorazzi *et al.* (2009, 2010, 2011) have performed a series of kinetic studies and reported that sex, age and fasting conditions may have an impact on the kinetic profile of OTA.

Human and animal populations are exposed to multiple mycotoxins because the same food might be contaminated by more than one mycotoxin, and mycotoxins might reach humans from different sources. Co-exposure to different mycotoxins, could originate synergic or additive toxic effects on human or animal health; however, knowledge regarding this aspect or regarding the influence of co-occurrence on toxicokinetic or toxicological characteristics of the mycotoxins is still limited. There are few *in vivo* toxicology studies with AFB1 and OTA mixtures which gave contradictory results with respect to the endpoint studied: mortality, histopathology findings, blood parameters… In most of them the doses administered are some orders of magnitude higher than those potentially in nature and that increases the difficulty in making proper comparisons. In poultry, high doses of these mycotoxins increased mortality in a synergetic way but on

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the contrary, OTA inhibited lipid accumulation normally induced by AFB1 (Huff and Doerr, 1981; Huff et al., 1983, 1988, 1992). Other authors could not find any interaction between these mycotoxins with regard to mortality (Micco et al., 1988), relative weight of most organs, blood parameters or immunological status (Ringot et al., 2006). In swine, AFB1 and OTA had additive interactions according to liver weight and blood chemistry but they were antagonists with regard to the degree of renal cortical interstitial fibrosis and relative kidney weight (Harvey et al., 1989). On the contrary, Tapia and Seawright (1985) reported in pigs no interactions in liver and kidney toxicity at low doses, similar to real exposure. In rats, AFB1 and OTA showed no interaction regarding the measurement of mortality, weight gain, or most serum biological parameters but the anaplastic and hyperchromatic nuclei, necrosis and bile duct proliferation observed were more pronounced in the combined toxin group after 4 months (Rati et al., 1981). In rats and rabbits, the combination resulted less teratogenic than OTA alone, although some new manifestations appeared (Wangikar et al., 2004; 2005).

The main objective of this work was to study the kinetic behavior of AFB1 and OTA after a single oral dose of both mycotoxins in rats, in order to study possible changes in their pharmacokinetic profiles in relation to those reported in previous studies, due to the presence of both mycotoxins; another objective was to determine whether or not this kinetic behavior could have a role in their interaction. Moreover, toxicity parameters like clinical signs, clinical biochemistry parameters or histopathology were studied so as to obtain a more ample approach to the mixture interaction (ICH, 1994).

2. Material and methods

2.1. Safety precautions

Aflatoxin B1 and ochratoxin A are toxic substances. They were always manipulated in solution, avoiding the formation of dust and aerosols. Nitrile gloves were used for all the procedures that were carried out. During the manipulation of treated animals or contaminated samples, FPP3 masks were used.

2.2. Chemicals and reagents

For administration to the animals, AFB1 and OTA were purchased in powder form Sigma (Steinheim, Germany). AFB1 was dissolved in dimethyl sulfoxide (DMSO) to an initial concentration of 4.73 g/L. OTA was dissolved in 0.10 M $NaHCO_3$ (pH 7.4) (Riedel-deHaën, Seelze, Germany) to an initial concentration of 1.00 g/L. All the solutions were maintained at -20ºC until use. Aflatoxin B1 was kept in the dark to avoid degradation. The mixture of AFB1 and OTA that was administered (0.025 g/L and 0.050 g/L respectively) was prepared from the initially concentrated forms.

For analytical quantification of the mycotoxins, AFB1 was purchased as a solution of 2 mg/L in acetonitrile (ACN) and OTA was purchased as a solution of 10 mg/L in ACN, both from OEKANAL® Fluka (Schnelldorf, Germany) as certified reference materials. Different reference solutions were prepared mixing AFB1 and OTA in a mixture of acetonitrile: methanol 50:50 v/v. For the tissue homogenates, $NaH_2PO_4.H_2O$ (Merck, Darmstadt, Germany) at 0.05 M, pH 6.50, was used. All the reagents used for the HPLC analysis were of analytical grade. ACN and methanol (MeOH) HPLC grade and formic acid (HCOOH) were obtained from Sigma Aldrich (St. Quentin Fallavier, France).

2.3. Animals

All the animals used, ten-week-old male Fisher 344 (F344) rats, were purchased from Harlan (Horst, The Netherlands). On the day of arrival, the animals were weighed (weight variation did not exceed ± 20% (OECD, 1984; OECD, 2009) and then distributed into polycarbonate cages with stainless steel covers for one week in order to allow acclimatization to the environmental conditions: 12 h day/night cycle, temperature 22 ± 2ºC, relative humidity 55 ± 10%, standard diet (Harlan Iberica, Spain) and water *ad libitum*. The experiments were performed under fasting conditions, so the food was removed 12 h before administration. On the day of the administration, the mean weight of the animals was 187.2 ± 5 g. The *in vivo* experiments were approved by the Ethics Committee on Animal Experimentation of the University of Navarra.

2.4. Study design and sample collection

The animals were randomly distributed into 5 groups of 3 animals per group. After at least five days of acclimatization, the animals received oral administration of a single dose of a mixture of 0.25 mg/kg bw of AFB1 and 0.5 mg/kg bw of OTA in $NaHCO_3·H_2O$ (0.1M pH 7.4) (0.5% DMSO). The volumes of administration were 10

mL/kg bw; therefore, the volume and dose administered were adjusted to the animal weight. A control group with two rats was added in order to obtain control samples and assure that no cross-contaminations occurred during the study. They received oral administration of a mixture of $NaHCO_3.H_2O$ (0.1 M pH 7.4) with 0.5% DMSO.

In order to determine the AFB1 and OTA concentrations in plasma at 10 min, 30 min, 2 h and 4 h, approximately 2 mL of blood from 3 animals per time point were collected from the retro-orbital sinus under isoflurane anesthesia. At 8 h, 24 h, 48 h, 72 h and 96 h, plasma was obtained by decapitation (n=3 per endpoint). Blood was extracted from each animal only once before sacrificing; extraction time points for each rat were chosen taking into account the time for volemia recovery (Diehl *et al.*, 2001). After retro-orbital extraction or decapitation, blood was collected into heparinized tubes (BD Vacutainer system) for clinical biochemistry analysis and AFB1 and OTA determination. Blood samples were centrifuged (1085 ×g for 15 min at 4°C) in order to obtain plasma, which was stored at −80°C.

The livers and kidneys were extracted from the animals, washed with water until the external blood was removed, blotted on filter paper, and finally weighed. Kidneys were sliced longitudinally into two halves (in order to have a representative sample of all kidney parts) and the liver was cut into five pieces. One half of each kidney and a piece of the biggest lobe of each liver were fixed in 4% formaldehyde solution, dehydrated and embedded in paraffin for histopathological analysis. The other three halves of kidney and the rest of the liver pieces were packed individually (each piece), flash-frozen in liquid N_2 and stored at -80°C for mycotoxin determination. In order to prevent cross contamination between samples, all the dissection material was cleaned with water and rinsed with ethanol after each animal necropsy.

2.5. *Clinical biochemistry and histopathology*

In order to analyze possible weight changes in the target organs due to administration of the mycotoxins, the relative weights (RW) of liver and kidneys were calculated dividing the weight of each organ by the total weight of the animal.

Biochemical analyses of plasma samples were performed with a Hitachi 911™ (Roche Diagnostics) analyzer using the protocols for determining standard patterns in plasma obtained from Roche: total protein (g/dL), albumin (g/dL), glucose (mg/dL), aspartate

transaminase (AST) (U/L), alanine transaminase (ALT) (U/L), alkaline phosphatase (U/L) and urea (mg/dL).

For the histopathological examination, paraffin sections (3 μm) were cut, mounted onto glass slides, and dewaxed and stained with hematoxylin and eosin (H&E). In the observation and evaluation of each sample, the systemic anatomopathological protocol was applied, with special attention to:

- Normalcy or alteration of the architecture and proportions of the cutaneous structures.

- Presence of circulatory phenomena.

- Evaluation and quantification of degenerative or necrotic phenomena.

- Existence or absence of inflammatory phenomena, types and intensity.

- Abnormal growths: atrophy, hyperplasia, hypertrophy, neoplasia.

- Particular or special findings.

The evaluation of some of these alterations (circulatory, degeneration and/or necrosis, inflammation and growth abnormalities) was carried out by calculating the different "fields" with the adequate increases for their correct observation. Whenever necessary, measurements were taken by means of the calibrated digital system.

2.6. *Determination of mycotoxins in plasma, liver and kidney*

The concentration of mycotoxins in plasma and tissues was determined by UHPLC with fluorescence detection. The extraction procedure and the UHPLC-FLD quantification method were previously set up and validated for these biological samples (Corcuera *et al.*, 2011). One piece of kidney or liver was homogenized in a round-bottom plastic tube with 4μL of cold sodium phosphate buffer (0.05 M, pH 6.50) per mg of tissue. The tissue homogenates were aliquoted and stored for at least one day at -80ºC until mycotoxin extraction was carried out. The plasma samples or the tissue homogenates were kept at room temperature for 30 min before the extraction step. Next, 100 μL of plasma or tissue homogenate were treated with 300 μL of the extractive solution (ACN acidified with formic acid (HCOOH) 1%) which precipitated the proteins and released the mycotoxins. After mixing this in a vortex during 2 min approximately, it was

centrifuged at 6200 x g for 15 min at 4ºC in order to separate the protein fraction from the supernatant that contained the mycotoxins. The supernatant (200 µL) was evaporated under vacuum and the solid residue was resuspended in 200 µL of: H_2O (1% HCOOH): MeOH:ACN 50:50 (0,1%HCOOH), 60:40.

Analyses were performed on a total of 63 samples (29 plasma, 17 liver and 17 kidney) in an Agilent Technologies 1200 liquid chromatographic system equipped with a fluorescence detector (G1321A model) controlled by ChemStation B.03.02 software (Hewlett-Packard). Mycotoxins were separated on an Ascentis® Express C18 column (150 mm x 2.1 mm; 2.7 µm) from Supelco (PA, USA). The injection volume was 40 µL and the flow rate was 0.9 mL/min. Chromatography was performed at 60ºC. The mobile phase was a mixture of an organic phase (A) (MeOH-ACN, 50:50, v/v) and water (B), both acidified with 0.5 % of formic acid. Proportions of both organic and aqueous phases were switched between isocratic and gradient profiles during the entire analysis procedure. The elution program starts with the isocratic profile until minute 2.4 with 30% of A, then from minute 2.4 to 2.5 min the organic phase increases up to 43%, from minute 2.5 to 8.3 min another isocratic profile at 43% of A, from minute 8.3 to 10.0 there is a last increase up to 65% of A and finally, from minute 10.0 the system returns to 30% of A to restore the starting conditions during 5 minutes. The retention times under these conditions were 2.5 minutes for AFB1 and 8.4 minutes for OTA. Before the sample entered the fluorescence detection cell, and in order to increase sensitivity for AFB1, a photoderivatization device (AURA Industries, NY, USA) with a mercury lamp (λ = 254 nm) and a knitted reactor coil of 0.25 mL (5 m x 0.25 mm) was included. During the first 4 minutes of analysis, fluorescence conditions were optimized for AFB1 (excitation 366 nm and emission 433 nm wavelengths), and after that, for OTA (excitation 225 nm and emission 461 nm wavelengths).

The LOQ were 2 µg/L in plasma and 8 µg/kg in liver and kidney for both mycotoxins. The LODs for AFB1 were as follows: 0.1 µg/L in plasma and 0.01 µg/kg in kidney and liver; the LODs for OTA were: 0.3 µg/L in plasma and 0.01 µg/kg in kidney and liver. Recovery was very efficient for both mycotoxins in plasma and tissues (between 93% and 96% for AFB1 and between 94% and 96% for OTA), and the relative standard deviation (RSD) obtained within and between day experiments was below 10% in all the matrices studied. All the mycotoxin levels obtained have been corrected by the recovery value for each matrix.

2.7. Statistical analysis

Data are presented by descriptive analysis as mean ± standard deviation (SD) of three animals. The distribution of the data was checked for normality using the Shapiro-Wilks test. The homogeneity of the variance was verified by the Levene test. The comparisons were performed using the Kruskal-Wallis test followed by the DMS test. P-values equal to or below 0.05 were accepted as the level of significance.

3. Results

3.1. Clinical biochemistry and histopathology

The treated animals did not show any clinical signs of toxicity such as weakness, anorexia or abdominal distension during the experiment. However, during the necropsies, after 48 h, 72 h and 96 h, the livers of the treated animals were light red with a visible loss of color in comparison to the control animals. Moreover, in treated animals, the relative weight of the livers after 48 h, 72 h and 96 h was significantly higher than in the control animals (table 1).

The biochemical parameters of the treated animals were comparable to the control values with the exception of the transaminases: ALT and AST (table1). The increases in ALT and AST are signs of hepatocyte death due to hypoxia, fatty change or necrosis (Smith *et al.*, 2002). Their values increased after administration of the mycotoxins, and AST reached a maximum after 48 h, while ALT reached the maximum after 72 h. Afterwards, the levels returned to control levels, showing a recovery of the liver after acute damage.

The most evident alterations were detected in liver while the renal alterations are the least significant. The lymphoid organs represented by the spleen, thymus and Peyer's patches showed no differences between the control and treated groups at the different observation times.

In the liver, a progressive lesional state was observed. It is characterized as follows: at 8 h diffuse hepatocyte necrosis, accompanied by focal hepatitis, stands out. At 24 h hepatocyte necrosis continues to be observed in porta spaces and parenchyma (figure. 1 B). At the same time, discreet degeneration begins to be observed in the cytoplasm of the hepatocytes and a few of these appear to be binucleated or with large nuclei. At 48 h

a large number of hepatocytes present intense tumefaction and degeneration in the cytoplasms (figure. 1 C), while others show necrosis surrounded by inflammatory infiltrates. In the porta spaces, they proliferate with bile canaliculi and a process of fibrosis begins (figure. 1 C). At 72 h, diffuse hepatocyte necrosis and the inflammatory response in the parenchyma are maintained. Degeneration of the hepatocytes in the cytoplasm is scarcely evident. Fibrosis and proliferation of bile canaliculi in the porta spaces as well as focal fibrosis of the parenchyma are notable (figure. 1 D). This fibrosis is interpreted as small areas of cicatrization due to the loss of hepatocytes. Together, these findings can be defined as the very initial stages of cirrhosis. At 96 h hepatocyte necrosis is no longer observed and the inflammatory response is either stabilized or has decreased. The cytoplasm of the hepatocytes presents tumefaction and moderate degeneration. Proliferation of bile canaliculi and portal fibrosis are largely present (figure. 1 E) as well as regenerative phenomena in the hepatocytes with numerous binucleated cells (figure. 1 F).

In the kidney, the proximal convoluted tubules progressively lost the renal glucogen. At the same time, in the glomerules, hypercellularity was observed due to the infiltration of inflammatory cells, resulting in glomerulonephritis. Over time, sclerosis and moderate glomerular atrophy were observed. The alterations in the renal tubules were not evident and even though there was an increase of interstitial nephritis and interstitial fibrosis observed as of 48 h, no previous serious tubular lesion was found.

3.2. *Plasma and tissue mycotoxin concentrations*

After administration of mycotoxins, OTA was found in plasma and tissues at all the timepoints, and very low levels of OTA were detected in control samples (<LOQ), presumably due to OTA contamination of standard animal diet (Vettorazzi *et al.*, 2009; Zepnik *et al.*, 2003; Mantle, 2008). The amount in plasma increased as of 10 minutes and reached a maxima at 2 h (C_{max} obs = 4326.6 µg/L) (figure 2A). In tissues, the OTA concentration was similar in liver and kidney, reaching maximum levels at 8 h (figure 2B).

AFB1 could only be quantified in plasma at 10 and 30 minutes (24.8 and 9.5 µg/L, respectively). In liver, levels below the LOQ were observed 8 h after administration, and no AFB1 was detected in kidney. In plasma, chromatograms showed a wide front peak, very different from the control or AFB1-spiked plasma samples. This indicated

the presence of new compounds with more hydrophilic properties than AFB1 or OTA. In order to enlarge the front peak, chromatographic conditions were modulated and at least 8 different peaks appeared (figure 3). They had maxima at 10 min and then decreased in a time-dependent manner until 24 h. After 48 h, no peaks appeared in the front.

4. Discussion

The ICH guideline S3A highlights the need to integrate pharmacokinetics into toxicity testing, which should aid in the interpretation of the toxicology findings (ICH, 1994). With this aim, our study attempted to learn more about the toxic and kinetic behavior of mycotoxins AFB1 and OTA when they are administered together. Wong and Hsieh, (1980) described that rats are one of the most sensitive species to AFB1 acute toxicity and carcinogenic effects. OTA has been described as a potent nephrocarcinogen in male rodents and its kinetic profile could be influenced by sex, age and fasting conditions (Vettorazzi *et al.*, 2010; 2011). Due to the aforementioned, young male rats were selected for this experiment. To avoid the interaction of food, the mycotoxins were administered in fasting conditions. In rats, doses from 0.2 to 12.5 mg/kg bw were used in single or repeated oral dose toxicity studies of AFB1 toxicity (Wong and Hsieh, 1980; Coulombe Jr and Sharma, 1985; Rati *et al.*, 1981; Bannasch *et al.*, 1985; Raj *et al.*, 1998; Ellinger-Ziegelbauer *et al.*, 2006; Theumer *et al.*, 2010). A low dose of AFB1 (0.25 mg/kg bw) which corresponds to approximately 3% of LD_{50} (Wong and Hsieh, 1980) (EFSA, 2007) was administered in order to avoid strong acute toxicity of AFB1. The single OTA dose selected (0.5 mg/kg bw) was slightly higher than carcinogenic doses (NTP, 1989; Castegnaro et al, 1998; Mantle et al 2005). It corresponds to 2.5% of the LD_{50} (NTP, 1989), has been used in recent studies (Zepnik *et al.*, 2003), and was the same as that used by Vettorazzi *et al.* 2009; therefore, some comparisons could be made.

Although there were no signs of general toxicity during the experiment, the biochemical and histopathological results pointed to acute toxicity in liver and no remarkable toxicity in kidney or other organs. The signs of hepatotoxicity appeared 48 h after administration, with paleness of livers, increases in transaminases, cell necrosis and inflammatory infiltration. In concordance, the relative liver weight showed significant increases due to inflammation processes. However, the liver started a repairing process

after 72 h and its effects were evident after 96 h because AST and ALT almost returned to normal values. At that time, the regenerative phenomena were evident by the numerous binucleated cells present. These findings coincide with the observations made by Rati *et al.*1981 regarding acute toxicity of AFB1, and it appears that the AFB1 and/or its toxic metabolites are eliminated (metabolized and excreted) in a range of 48 h-96 h. No remarkable toxicity was observed in kidneys or immunological organs (spleen, thymus and Peyer's patches) so OTA did not cause acute toxicity at this dose,. Therefore, we can assume that the acute toxic effects observed after the combined treatment of AFB1 and OTA were mostly due to AFB1 effect.

Very low levels of AFB1 were found in plasma after 10 or 30 min (1% and 0.4% of the administered dose, respectively). The liver AFB1 levels were lower than the LOQ (8 µg/kg) at all time points and AFB1 was undetectable in kidney. For this reason, it was not possible to calculate any kinetic parameter. These findings suggest that the absorption of AFB1 was very fast, and that the molecule was rapidly metabolized in the liver. Moreover, almost none of the unmetabolized AFB1 was distributed outside the liver, into the plasma and onto the kidneys. The compounds detected in the front peak are assumed to be AFB1 metabolites because they have not been detected in the spiked plasma sample with AFB1 and OTA nor in recent OTA kinetic studies (Vettorazzi *et al.*, 2009; 2010; 2011). These compounds had a maxima at 10 min, decreased in a time-dependent manner until 24 h, after which they were undetectable. Coulombe and Sharma (1985) described a two-compartmental model for AFB1 kinetics in Sprague-Dawley rats, reaching the maximum concentration after 3 h and with the plasma half-life being 91.8 h. A more recent study found the maximum concentration 4 h after administration, with plasma half-life of 53 h (Firmin *et al.*, 2010). These data describe long plasma half-lives, with long elimination phases after quantifying only AFB1 or mixed with its metabolites. These observations do not coincide with our findings in which AFB1 disappeared from plasma in 30 minutes, and its metabolites in 24h, and this suggested an extremely fast uptake and metabolism of AFB1. Jubert *et al.* 2009, observed a rapid uptake (T max = 1 h) in humans, with urinary elimination of 95% of the dose in 24 h, which is closer to our findings; in their work, they could not differentiate among AFB1 and its metabolites. It might be possible that OTA modifies/accelerates AFB1 metabolism in liver into very hydrophilic compounds that are rapidly excreted (24 h) through the kidney, but there are no studies available about

AFB1 molecule kinetics without taking into account its metabolites, so it was hard to make proper comparisons.

With regard to OTA, the concentration profiles in plasma, liver and kidney and some of the kinetic parameters are comparable to the ones that Vettorazzi *et al.* (2010, 2011) obtained under similar conditions (figure 2A) but with OTA alone. The maximum concentration in plasma was obtained after 2 h and corresponded to 87% of the initial dose, which was similar to the 83% that Vettorazzi *et al.*2010 had reported. Moreover, as in Vettorazzi *et al.* (2011), the same parallelism between plasma and tissue concentrations was observed. No significant differences were observed in the OTA tissue concentrationss after 24 h between previous kinetic studies performed with OTA alone and the current results of OTA and AFB1 co-administration. Comparing the obtained OTA profile with other experiments and models of OTA kinetics, the differences seem to be more closely related to the age of the rats than to the presence of AFB1 in the system (Vettorazzi *et al.*, 2009; 2010; 2011).

5. Conclusions

In conclusion, more specific information has been obtained regarding the behavior of the mycotoxins AFB1 and OTA in F344 rats after one oral administration. Toxicity has been observed in liver, while no remarkable toxic effects were observed in kidney. The acute toxic effects observed in liver, which were attributed to AFB1, together with the fact that AFB1 and its metabolites disappeared from plasma in 24 h, suggest a rapid absorption and metabolization of the mycotoxin. The low AFB1 plasma levels prevented to have kinetic data and therefore to assess the influence of OTA on AFB1 kinetics.

With regard to OTA, it appeared that its plasma and tissues levels were not affected by the presence of AFB1 as the kinetic profile seemed very similar to studies performed only with OTA in similar conditions. So further investigations to confirm this hypothesis would be of great interest.

Acknowledgments

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Legend to figures:

Figure 1: Microscopic images of liver samples. A progressive lesional state can be observed. A) Normal liver of a control sample. B) Focal cell necrosis and discreet degeneration of cytoplasm after 24 h. C) Intense tumefaction of hepatocytes, cell proliferation and initial fibrosis in porta spaces after 48 h. D) Necrosis and inflammatory response maintained fibrosis around bile canaliculi in porta spaces after 72 h. E) Moderate tumefaction of cytoplasms, proliferation of bile canaliculi and portal fibrosis after 96 h, and also F) binucleated hepatocytes in regenerative phenomena. Magnification of E x100. Magnification of A, C and D x200. Magnification of B and F x400.

Figure 2: Plasmatic (A) or tissue (B) concentrations of OTA overtime after a single oral administration of AFB1+OTA (0.25 mg/kg bw of AFB1 and 0.5 mg/kg of OTA in NaHCO$_3$·H$_2$O (0.1 M pH 7.4)). The plasma Cmax and Tmax and tissue values obtained at 24h in this study and in Vettorazzi *et al*. (2010, 2011) under similar experimental conditions have been included.

Figure 3: Superimposed chromatograms of plasma samples collected 10 and 30 minutes, 4, 8 and 24 h after administration. Chromatographic conditions were modulated in order to show the AFB1 metabolites that appeared in plasma samples. Chromatograms of a control plasma sample and a spiked plasma sample with AFB1 and OTA have been superimposed in the plot.