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Physiologically Based Toxicokinetics of Serum Aflatoxin B₁-Lysine Adduct in F344 Rats

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Abstract

Aflatoxin B₁-lysine adduct (AFB-Lys) is a reliable biomarker for aflatoxin exposure; however, a systematic toxicokinetic evaluation has not been reported. In this study, male F344 rats were orally exposed to single, or repeated, doses of AFB₁ and the toxicokinetics of serum AFB-Lys that followed treatments were investigated. A single-dose of AFB₁ increased serum AFB-Lys levels rapidly peaking at 4 h, followed by first-order elimination, through which the half-life was estimated to be 2.31 days. A physiologically based pharmacokinetic model showed that approximately 3.00-3.90% and 1.12-1.98% of the administered AFB₁ doses were converted to serum AFB-Lys adducts at 2 h and 24 h post treatment, respectively. Repeated AFB₁ exposure at 5-25 µg/kg body weight linearly increased serum AFB-Lys levels for 5 weeks in animals, resulting in a 1-1.5 times higher AFB-Lys level overall. This indicates the potential of this adduct as a reliable biomarker for repeated low dose exposure. Higher dose exposure at 75 µg/kg increased the level of AFB-Lys to a maximum at 2 weeks, followed by a gradual decrease to near plateau level up to 5 weeks. In conclusion, this study systematically evaluated the toxicokinetics of serum AFB-Lys adduct in F344 rats using a physiologically based pharmacokinetic model and robust statistical modeling analysis and provided a firm and clear understanding of the toxicokinetics of this biomarker.

Keywords

aflatoxin B₁; aflatoxin B₁-lysine adduct; biomarker; toxicokinetics

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Conflict of Interest

The authors declare no conflict of interest.

1. Introduction

Aflatoxins (AF) are ubiquitous food contaminants produced mainly by *Aspergillus flavus* and *A. parasiticus*. Humans exposed to AF contaminated food are subject to a wide range of health effects, including acute poisoning (aflatoxicosis), hepatocellular carcinoma (HCC) and stunting in children (IARC, 1993; Liu and Wu, 2010; Smith et al., 2012; Williams et al., 2004). The uncovering of the etiological role of AF exposure is closely associated with the development and application of molecular markers relevant to AF exposure or risk (Kensler et al., 2011; Qian et al., 1994).

The toxicology of aflatoxin B₁ (AFB₁) has been detailed elsewhere (Eaton and Groopman, 1994). AFB₁ is metabolically activated by liver cytochrome P450 (CYP450) 1A2 and 3A4 to AFB₁-8,9-epoxide which can readily bind DNA to form AFB₁-N⁷-guanine adduct (Essigmann et al., 1977) or react with albumin to produce AFB₁-albumin adduct if not conjugated with glutathione (Sabbioni et al., 1987). It has been shown that albumin is the only protein in serum that binds AFB₁ to a significant level (Skipper et al., 1985). Lysine is the primary amino acid group for the covalent binding of AFB₁ to albumin. Under enzyme digestion, AFB₁-lysine (AFB-Lys) is released from the adducted albumin (Sabbioni et al., 1987). This biomarker has been widely used to indicate human exposure status (Azziz-Baumgartner et al., 2005; Wang et al., 1996) and to assess human chemoprevention strategies (Tang et al., 2008; Wang et al., 2008). Several previous studies have reported the partial toxicokinetics of the AFB-Lys adduct in animal models (Sabbioni et al., 1987; Scholl et al., 2006; Wild et al., 1986; Wild et al., 1996). However, a complete study design relevant to human exposure (route, dose and duration) is lacking, and further studies are warranted to demonstrate the physiologically based toxicokinetics of the serum AFB-Lys biomarker.

2. Materials and methods

2.1. Chemicals and materials

Unlabelled aflatoxin B₁ (> 98% purity), dimethyl sulfoxide (DMSO), albumin determination reagent (bromocresol purple), and normal rat serum were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). Pronase (25 kU, Nuclease-free) was purchased from Calbiochem (La Jolla, CA). Protein assay dye reagent concentrate and protein standards were purchased from Bio-Rad Laboratories Inc. (Hercules, CA). Mixed mode solid phase extraction (SPE) cartridges were purchased from the Waters Corp. (Milford, MA). Authentic AFB-Lys was synthesized as previously described (Sabbioni et al., 1987). All other chemicals and solvents were of highest grade and purity available.

2.2. Animals

Male F344 rats (120-130 g) were purchased from Harlan Laboratories Inc. (Indianapolis, IN) and maintained under controlled conditions of temperature (22-25°C), humidity (50-70%), and light/dark cycles (12 h/12 h). Animals were fed with AIN-76A diet (Teklad, Madison, WI) and acclimated for one week before treatment. Feed and water were provided *ad libitum*. Animal use and care and the experimental protocol were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia.

2.3. Experimental design

For the single-dose study, rats (5 per group) were gavaged with 0, 50, 250, or 1,000 µg AFB₁/kg body weight (BW). AFB₁ was dissolved in DMSO with a gavage volume of 50 µL/100g BW. Animals were anesthetized and blood was collected by cardiac puncture at 2, 4, 8 h, and 1, 3, 5, 7, 14, and 21 days after treatment. For the repeated-dose study, rats (25 per group) were gavaged daily with 0, 5, 10, 25, or 75 µg/kg BW for up to 5 weeks (5 days

per week). Animals (5 per group) were anesthetized and blood samples were collected at weekly intervals and 24 h after the last dose. Serum samples were prepared and stored at -20°C until analysis.

2.4. Serum sample processing and quantification of AFB-Lys adducts

The method for serum processing and quantification of AFB-Lys adducts has been reported previously (Qian et al., 2010). Albumin and total protein levels in serum were determined as previously described (Wang et al., 1996). Aliquots of each serum sample (150 μ L) were digested by pronase (pronase:total protein, 1:4, w:w) at 37°C for 3 h to release AFB-Lys adducts. The digests were purified using Waters MAX SPE cartridges. After priming with methanol and equilibration with water, the loaded cartridge was sequentially washed by water, 70% methanol, and 1% ammonium hydroxide in methanol at a flow rate of 1 mL/min. The eluate in 2% formic acid in methanol was vacuum-dried with a Labconco Centrivap concentrator (Kansas City, MO) and reconstituted for HPLC-fluorescence detection. The average recovery rate was 90%.

The Agilent 1200 HPLC-fluorescence system (Santa Clara, CA) was used for quantification of the AFB-Lys adducts. The mobile phases consisted of buffer A (20 mM $\text{NH}_4\text{H}_2\text{PO}_4$, pH 7.2) and buffer B (100% Methanol). The Zorbax Eclipse XDB-C18 reverse phase column (5 micron, 4.6 $\times$ 250 mm) was used and 100 μ L was injected at a flow rate of 1 mL/min. A gradient was generated to separate the AFB-Lys adduct within 25 min and the retention time of AFB-Lys was equal to approximately 12.3 min. AFB-Lys adduct was detected by fluorescence at the excitation and emission wavelengths of 405 nm and 470 nm, respectively. Quality assurance and quality control procedures were taken during analyses, which included simultaneous analysis of one authentic standard and a quality control sample daily. The limit of detection was 0.4 pg/mg albumin. The serum AFB-Lys level of each sample was adjusted by its albumin content accordingly.

2.5. Statistical analysis

The curves of serum AFB-Lys formation and elimination after a single dose were fitted with zero-, first-, and second-order kinetic functions, which were used to determine the appropriate model for AFB-Lys kinetics. The lowest-order model with acceptable residuals ($r^2 > 0.90$) was considered to optimally describe the elimination curve. All modeling and residual determinations were performed with Sigma Plot 10.0 software (San Jose, CA) using the least sum of squares method. The half-life was computed from the fitting of the model selected.

3. Results

3.1. Single dose study

Following a single-dose of AFB_1 , the levels of AFB-Lys in serum rapidly increased at 2 h and reached a peak at 4 h, followed by a non-linear decrease (Fig. 1). Linear dose-response relationships were found between AFB_1 doses and serum AFB-Lys levels at both 2 and 24 h post treatment (See supplementary Figs. 1A and B, $r^2 = 0.98$ and 0.99 , respectively). In addition, the elimination of serum AFB-Lys followed first-order kinetics for both 50 ($r^2 = 0.9829$, $p < 0.001$) and 250 $\mu\text{g/kg}$ groups ($r^2 = 0.9745$, $p < 0.0001$) (See supplementary Table 1). Non-linear decreases of serum AFB-Lys after 4 h were demonstrated based on model analysis for the 50 and 250 $\mu\text{g/kg}$ groups (Fig. 2A and B). Significant linear decreases were also observed with the log (AFB-Lys) over time for the 50 and 250 $\mu\text{g/kg}$ groups. Half-lives of AFB-Lys were estimated to be 2.35 and 2.27 days from the curve fittings of the data from the 50 and 250 $\mu\text{g/kg}$ groups, respectively. The half-life was estimated based on the integrated slope (λ , as the regression coefficient of the fitted model) of the log (AFB-Lys)

over time curves, i.e., $t_{1/2} = 0.693/\lambda$ (Figs. 2C and D). The conversion ratios of AFB₁ to AFB-Lys following a single dose treatment were determined to be 3.00-3.90% at 2 h or 1.12-1.98% at 24 h post treatment (See supplementary Tables 2 and 3), as estimated by a physiologically based pharmacokinetic model.

3.2. Repeated dose study

Repeated dosing with AFB₁ increased serum AFB-Lys for up to 5 weeks at all doses (Fig. 3). A dose-dependent temporal increase in the AFB-Lys level was found in animals that received 5-25 $\mu\text{g/kg}$ AFB₁ over a 5-week study period. The level of AFB-Lys reached a maximum after 2 weeks, followed by a gradual decrease over time in the 75 $\mu\text{g/kg}$ group. To further examine the kinetics of the serum AFB-Lys after the repeated-dose treatment, we conducted different model analyses and found that the linear regression model best fit the data for the 5 ($r^2 = 0.96$, $p < 0.01$, Fig. 4A), 10 ($r^2 = 0.97$, $p < 0.01$, Fig. 4B) and 25 $\mu\text{g/kg}$ groups ($r^2 = 0.98$, $p < 0.01$, Fig. 4C), respectively. A Gaussian curve was the best fit for the 75 $\mu\text{g/kg}$ group ($r^2 = 0.86$, $p = 0.1634$, Fig. 4D). Linear dose-response relationships were also found between cumulative doses and serum AFB-Lys levels (See supplementary Fig. 2A, $r^2 = 0.99$, $p < 0.01$), as well as between the cumulative doses and the conversion ratio (AFB-Lys formation)/(AFB₁ dosed) after a 1-week treatment (See supplementary Fig. 2B, $r^2 = 0.99$, $p < 0.01$).

4. Discussion

In this study we evaluated the toxicokinetics of serum AFB-Lys adduct in F344 rats with special focus on doses relevant to acute (single) or chronic (repeated) human exposure. The peak concentration of serum AFB-Lys was found at 4 h in the current study. The mean AFB-Lys concentration of 2.56 $\pm$ 0.38 ng/mg albumin at 24 h after a single dose of 250 $\mu\text{g/kg}$ was comparable to previously reported values in animals (Vinitketkumnuen et al., 1999; Wild et al., 1990). The highest dose of 1000 $\mu\text{g/kg}$ was selected to simulate the extreme condition in which human acute aflatoxicosis occurred, as found in 2004-2005 outbreaks in Kenya, where a mean serum level of 3.2 ng/mg albumin for this biomarker has been reported in cases of aflatoxin poisoning before death (Azziz-Baumgartner et al., 2005).

Our physiological model based estimation of the conversion ratio of AFB₁ to AFB-Lys at 24 h after a single dose (1.12-1.98%) was similar to previously reported values of 1-3% (Sabbioni et al., 1987; Wild et al., 1986). The model generated half-life of serum AFB-Lys in this study (2.31 days, mean value of 2.35 and 2.27) was close to the previously reported values of 2.5 days (Sabbioni et al., 1987), 55 h (Wild et al., 1986), and 2.6 days (Scholl et al., 2006) in rats. The elimination kinetics of serum AFB-Lys followed a first-order kinetic decay model, as determined by comparisons of different model fittings.

It is worth noting that the dose range selected in the repeated experiment (5-75 $\mu\text{g/kg}$ BW) was relevant to the exposure levels in high-risk human populations, such as Kenya, Ghana, and Guangxi, China, where the mean AFB₁ levels were found to be 100-1000 $\mu\text{g/kg}$ in corn (Azziz-Baumgartner et al., 2005; Groopman et al., 1992; Tang et al., 2009). We observed a linear increase of serum AFB-Lys for animals that received 5-25 $\mu\text{g/kg}$ AFB₁ daily, leading to a 1.0-1.5 times increase after 5 weeks compared to that after 1 week, which supports its potential use as a long-term biomarker. In addition, we observed that serum AFB-Lys reached a maximum after 2 weeks for animals that received a high daily dose of AFB₁ (75 $\mu\text{g/kg}$ BW). The pattern observed for the 75 $\mu\text{g/kg}$ group (Gaussian curve) may reflect a variation in the metabolic balance between AFB-epoxide formation and detoxification (Knight et al., 1999) or enzymatic induction of glutathione S transferase (GST) (Strelac et al., 2003), and this coincided with the alternative signs of toxicity found at this dose.

It has to be pointed out that data for toxicokinetics presented in this study are different from previous studies in the following ways: (1) the current study employed a human relevant route of exposure (orally) rather than the i.p. injection adopted by Scholl et al (Scholl et al., 2006), which could better mimic the toxicokinetics of the biomarker *in vivo*; (2) we exposed animals with unlabelled AFB₁ rather than ³H- (Wild et al., 1986) or ¹⁴C- labeled AFB₁ (Sabbioni et al., 1987); (3) we used a physiologically based model to estimate the conversion of AFB₁ doses to serum AFB-Lys adducts rather than expression of the conversion ratio as AFB-Lys level (ng/mg albumin)/AFB₁ dose (μg/kg body weight). This conversion ratio as previously reported by Scholl et al. (2006) is not straightforward (Scholl et al., 2006) given the different units involved; (4) the current study used both single and repeated treatment procedures with relatively longer duration as compared to previous repeated treatment protocols of 14 days (Wild et al., 1996) or 9 days (Scholl et al., 2006). One more highlight of this study is the use of robust statistical modeling analysis that greatly facilitates the understanding of the toxicokinetics of the AFB-Lys. This work also represents the first systematic evaluation of this biomarker using a complete design that incorporates relevant physiological basis into the toxicokinetic estimation. Data from this study provides a firm and clear understanding of the toxicokinetics of serum AFB-Lys adduct and will benefit future studies using this biomarker for linking to human health effects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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List of abbreviations

AF	aflatoxins
AFB₁	aflatoxin B ₁
AFB-Lys	aflatoxin B ₁ -lysine
CYP450	cytochrome P-450
DMSO	dimethyl sulfoxide
GST	glutathione S transferase
HCC	hepatocellular carcinoma
HPLC	high performance liquid chromatography
SPE	solid phase extraction

References

- Azziz-Baumgartner E, Lindblade K, Giesecker K, Rogers HS, Kieszak S, Njapau H, Schleicher R, McCoy LF, Misore A, DeCock K, Rubin C, Slutsker L. Case-control study of an acute aflatoxicosis outbreak, Kenya, 2004. *Environ. Health Perspect.* 2005; 113:1779–1783. [PubMed: 16330363]
- Eaton, DL.; Groopman, JD. *The Toxicology of aflatoxins: Human health, veterinary, and agricultural significance.* Academic Press. New York, NY; San Diego: 1994.

- Essigmann JM, Croy RG, Nadzan AM, Busby WF Jr, Reinhold VN, Buchi G, Wogan GN. Structural identification of the major DNA adduct formed by aflatoxin B1 in vitro. *Proc. Natl. Acad. Sci. U. S. A.* 1977; 74:1870–1874. [PubMed: 266709]
- Groopman JD, Zhu JQ, Donahue PR, Pikul A, Zhang LS, Chen JS, Wogan GN. Molecular dosimetry of urinary aflatoxin-DNA adducts in people living in Guangxi Autonomous Region, People's Republic of China. *Cancer Res.* 1992; 52:45–52. [PubMed: 1727385]
- IARC. Aflatoxins. IARC Monogr. Eval. Carcinog. Risks Hum. 1993; 56:245–395. [PubMed: 8411624]
- Kensler TW, Roebuck BD, Wogan GN, Groopman JD. Aflatoxin: a 50-year odyssey of mechanistic and translational toxicology. *Toxicol. Sci.* 2011; 120(Suppl 1):S28–48. [PubMed: 20881231]
- Knight LP, Primiano T, Groopman JD, Kensler TW, Sutter TR. cDNA cloning, expression and activity of a second human aflatoxin B1-metabolizing member of the aldo-keto reductase superfamily, AKR7A3. *Carcinogenesis.* 1999; 20:1215–1223. [PubMed: 10383892]
- Liu Y, Wu F. Global burden of aflatoxin-induced hepatocellular carcinoma: a risk assessment. *Environ. Health Perspect.* 2010; 118:818–824. [PubMed: 20172840]
- Qian G, Tang L, Liu W, Wang JS. Development of a non-antibody method for rapid detection of serum aflatoxin B1-lysine adduct. *Toxicologist.* 2010; 114:248.
- Qian GS, Ross RK, Yu MC, Yuan JM, Gao YT, Henderson BE, Wogan GN, Groopman JD. A follow-up study of urinary markers of aflatoxin exposure and liver cancer risk in Shanghai, People's Republic of China. *Cancer Epidemiol. Biomarkers Prev.* 1994; 3:3–10. [PubMed: 8118382]
- Sabbioni G, Skipper PL, Buchi G, Tannenbaum SR. Isolation and characterization of the major serum albumin adduct formed by aflatoxin B1 in vivo in rats. *Carcinogenesis.* 1987; 8:819–824. [PubMed: 3111739]
- Scholl PF, McCoy L, Kensler TW, Groopman JD. Quantitative analysis and chronic dosimetry of the aflatoxin B1 plasma albumin adduct Lys-AFB1 in rats by isotope dilution mass spectrometry. *Chem. Res. Toxicol.* 2006; 19:44–49. [PubMed: 16411655]
- Skipper PL, Hutchins DH, Turesky RJ, Sabbioni G, Tannenbaum SR. Carcinogen Binding to Serum-Albumin. *P. Am. Assoc. Canc. Res.* 1985; 26:90–90.
- Smith LE, Stoltzfus RJ, Prendergast A. Food chain mycotoxin exposure, gut health, and impaired growth: a conceptual framework. *Adv. Nutr.* 2012; 3:526–531. [PubMed: 22797988]
- Strelac NJ, Saicic ZS, Magic ZM, Spasic MB, Trutic NV, Krtolica KV. Aflatoxin B1-induced changes of glutathione-S-transferase activity in the plasma and liver of the rat. *Vojnosanit Pregl.* 2003; 60:415–420. [PubMed: 12958799]
- Tang L, Tang M, Xu L, Luo H, Huang T, Yu J, Zhang L, Gao W, Cox SB, Wang JS. Modulation of aflatoxin biomarkers in human blood and urine by green tea polyphenols intervention. *Carcinogenesis.* 2008; 29:411–417. [PubMed: 18192689]
- Tang L, Xu L, Afriyie-Gyawu E, Liu W, Wang P, Tang Y, Wang Z, Huebner HJ, Ankrah NA, Ofori-Adjei D, Williams JH, Wang JS, Phillips TD. Aflatoxin-albumin adducts and correlation with decreased serum levels of vitamins A and E in an adult Ghanaian population. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 2009; 26:108–118. [PubMed: 19680878]
- Vinitketkumnuen U, Chewonarin T, Dhumtanom P, Lertprasertsuk N, Wild CP. Aflatoxin-albumin adduct formation after single and multiple doses of aflatoxin B(1) in rats treated with Thai medicinal plants. *Mutat. Res.* 1999; 428:345–351. [PubMed: 10518006]
- Wang JS, Qian GS, Zarba A, He X, Zhu YR, Zhang BC, Jacobson L, Gange SJ, Munoz A, Kensler TW, et al. Temporal patterns of aflatoxin-albumin adducts in hepatitis B surface antigen-positive and antigen-negative residents of Daxin, Qidong County, People's Republic of China. *Cancer Epidemiol. Biomarkers Prev.* 1996; 5:253–261. [PubMed: 8722216]
- Wang P, Afriyie-Gyawu E, Tang Y, Johnson NM, Xu L, Tang L, Huebner HJ, Ankrah NA, Ofori-Adjei D, Ellis W, Jolly PE, Williams JH, Wang JS, Phillips TD. NovaSil clay intervention in Ghanaians at high risk for aflatoxicosis: II. Reduction in biomarkers of aflatoxin exposure in blood and urine. *Food Addit. Contam. Part A Chem. Anal. Control Expo. Risk Assess.* 2008; 25:622–634. [PubMed: 18478481]
- Wild CP, Garner RC, Montesano R, Tursi F. Aflatoxin B1 binding to plasma albumin and liver DNA upon chronic administration to rats. *Carcinogenesis.* 1986; 7:853–858. [PubMed: 3085966]

- Wild CP, Hasegawa R, Barraud L, Chutimataewin S, Chapot B, Ito N, Montesano R. Aflatoxin-albumin adducts: a basis for comparative carcinogenesis between animals and humans. *Cancer Epidemiol. Biomarkers Prev.* 1996; 5:179–189. [PubMed: 8833618]
- Wild CP, Jiang YZ, Sabbioni G, Chapot B, Montesano R. Evaluation of methods for quantitation of aflatoxin-albumin adducts and their application to human exposure assessment. *Cancer Res.* 1990; 50:245–251. [PubMed: 2104776]
- Williams JH, Phillips TD, Jolly PE, Stiles JK, Jolly CM, Aggarwal D. Human aflatoxicosis in developing countries: a review of toxicology, exposure, potential health consequences, and interventions. *Am. J. Clin. Nutr.* 2004; 80:1106–1122. [PubMed: 15531656]

Fig. 1. Serum concentrations of AFB-Lys adduct after a single-dose of AFB₁ exposure. The inset is an enlarged view of 0 to 24 h. Values shown are mean ± SD (n = 5).

Fig. 2. Regression analysis of the elimination kinetics of serum AFB-Lys adduct after a single-dose of AFB₁ exposure. Nonlinear curve fitting of AFB-Lys over time was tested for the 50 (A) and 250 µg/kg (B) groups. Linear curve fitting of the log (AFB-Lys) over time was tested for 50 (C) and 250 µg/kg (D) groups. The slopes generated from Figs. C and D were used for calculating the half-life for serum AFB-Lys. Dashed lines indicate 95% CI.

Fig. 3.

Serum concentrations of AFB-Lys adduct after repeated AFB₁ exposure. Values shown are mean ± SD (n = 5).

Fig. 4.
 Regression analysis of serum concentrations of AFB-Lys adduct over repeated exposure.
 (A), 5 $\mu\text{g/kg}$ group; (B), 10 $\mu\text{g/kg}$ group; (C), 25 $\mu\text{g/kg}$ group and (D), 75 $\mu\text{g/kg}$ group.
 Dashed lines indicate 95% CI.