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HPLC–MS/MS method for the simultaneous determination of aflatoxins in blood: toxicokinetics of aflatoxin B_1 and aflatoxin M_1 in rats

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Abstract

Mycotoxins are highly toxic fungal metabolites that can pose health threats to humans and animals. Aflatoxins are a type of mycotoxin produced mainly by *Aspergillus flavus* and *A. parasiticus*. A sensitive high performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) method with multiple reaction monitoring (MRM) modes was developed for the determination of aflatoxins in blood after acetonitrile precipitation extraction. The limits of quantification of aflatoxins ranged from 0.05 to 0.2 ng/mL. Intra-day accuracy ranged from 92 to 111.0%, and intra-day precision ($n=6$) ranged from 1 to 8%. Inter-day accuracy and precision were 94.0–102.0% and 2.0–8.0%, respectively. The toxicokinetics of AFB1 and its metabolite AFM1 after a single oral administration (AFB1 1 mg/kg body weight) were studied in male Sprague–Dawley rats. The blood AFB1 and AFM1 profiles could be adequately described by a noncompartmental model. The highest concentration of AFB1 (C_{max} 93.42 ± 23.01 ng/mL) was observed with T_{max} at 0.15 ± 0.034 h. AFB1 was rapidly metabolized to AFM1 which reached its peak blood concentration (C_{max} 53.86 ± 12.12 ng/mL) at 0.33 ± 0.11 h. The HPLC–MS/MS method was simple and sensitive, appropriate for studying the in vivo toxicokinetics of aflatoxins.

Keywords: Aflatoxins, AFB1, AFM1, HPLC–MS/MS, Toxicokinetic

Introduction

Mycotoxins are toxic secondary metabolites produced mainly by *Aspergillus, Fusarium, Penicillium, Alternaria,* and *Claviceps* genera (Arroyo-Manzanares et al. 2021). More than 450 mycotoxins and their metabolites are known, which can lead to toxicological effects of varying severity, from mild gastroenteritis to fatal cancer (Benkerroum 2020). Humans and animals are mainly exposed to mycotoxins through their diets (Slobodchikova and Vuckovic 2018). Mycotoxins generally have appreciable thermal stability and can be present at all levels of the food chain, from untreated seeds to processed foods and feed (Santis et al. 2017; Andrade et al. 2013). Current estimates indicate that 25% of the world's food supply is contaminated by mycotoxins (Slobodchikova and Vuckovic 2018).

One class of mycotoxins of concern in humans and animal diets are the aflatoxins, which are the most toxic mycotoxin (Benkerroum 2020). Aflatoxins are produced primarily by strains of the fungi *Aspergillus flavus* and *A. parasiticus* (Benkerroum 2020; Ardic et al. 2008). Aflatoxin poisoning can be acute, subacute, or chronic, depending on the amount of toxin consumed, exposure time, and species specificity and sensitivity (Bastianello et al. 1987). Chronic aflatoxicosis is predominantly perceived as a promoter of liver cancer and compromised

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immunity (Bruchim et al. 2012). The most common clinical signs of acute aflatoxicosis include icterus, hematemesis, hematochezia, diffuse hemorrhage and ascites, nausea, abdominal swelling, constipation alternating with diarrhea and abdominal pain, as well as signs associated with disseminated intravascular coagulation (DIC) (Bruchim et al. 2012; Benkerroum 2020; Martínez-Martínez et al. 2021). In 1991, the consumption of a traditional Chinese dish contaminated with both boric acid and aflatoxins resulted in 17 severe poisoning cases and 13 deaths. Autopsies detected abnormally high levels of aflatoxin B1(AFB1), aflatoxin B2(AFB2), aflatoxin G1(AFG1), aflatoxin M1(AFM1), and aflatoxin M2(AFM2), as well as aflatoxicol, in various organs, including the liver, kidney, heart, spleen, lung, and brain (Chao et al. 1991).

AFB1 is the most commonly reported aflatoxin and is also the most carcinogenic due to its genotoxic and teratogenic properties and its ability to damage the liver, kidneys, and spleen (Huang et al. 2021; Klvana and Bren 2019; Mupunga et al. 2016). The World Health Organization (WHO), based on records of worldwide aflatoxicosis outbreaks and in vitro tests, considers that regular consumption of food contaminated with AFB1 at levels of 1 mg/kg or higher for a short period can cause acute intoxication in humans, while daily consumption of AFB1-contaminated food at a dose of 0.02–0.12 mg/kg body weight (BW) over 1 to 3 weeks can cause life-threatening aflatoxicosis (Benkerroum 2020). AFB1 has also been associated with growth impairment in children and suppression of immune function (Xia et al. 2020). AFB1 is metabolized to AFM1 via hydroxylation, and AFM1 has been proposed as a biomarker for acute aflatoxin exposure, which is excreted in the feces, urine, and milk of lactating mammals, including humans (Mupunga et al. 2016; Schrenk et al. 2020). AFM1 is primarily considered a detoxification product of AFB1 metabolism, showing only 10% of mutagenicity compared to AFB1 (Wogan and Paglialunga 1974). However, AFM1 are carcinogenic when delivered orally via the diet or by gavage (Schrenk et al. 2020). Several studies reported immunosuppressive effects of AFM1 was similar for that of AFB1, on both humans and other animals (Marchese et al. 2018). It has been reported that Cytochromes P450 activation is not required for AFM1 to exert cytotoxic effects (Marchese et al. 2018; Neal et al. 1998). AFM2 is similarly produced by the hydroxylation of AFB2 (Bianco et al. 2012).

The methods for the detection of aflatoxins in biological sample have included enzyme-linked immunosorbent assays (ELISAs) (Seetha et al. 2018), high-performance liquid chromatography–fluorescence detection (HPLC-FD) (Andrade et al. 2013), high-performance liquid chromatography–isotope dilution mass spectrometry (HPLC-IDMS) (McMillan et al. 2018; Han et al. 2012), high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) (Cao et al. 2018; Ediage et al. 2012), and high-performance liquid chromatography–high resolution mass spectrometry (HPLC-HMRS) (Slobodchikova and Vuckovic 2018; Rubert et al. 2014). HPLC–MS/MS is commonly used for determining aflatoxins because of its high selectivity and sensitivity (Arroyo-Manzanares et al. 2021; Tkaczyk and Jedziniak 2021); Osteresch et al. 2017) proposed an HPLC–MS/MS method for the determination of aflatoxins in dried blood spots or dried serum spots following extraction with water/acetone/acetonitrile (30:35:35, v/v/v) as the extraction solvent. This method is sensitive, with a limit of quantification (LOQ) for aflatoxins ranging from 0.05 to 0.1 ng/mL. De Santis et al. (2017) developed an HPLC–MS/MS method using pronase treatment in combination with acidified ethyl acetate liquid–liquid extraction (LLE) and QuEChERS for the analysis of aflatoxin in serum. The concentrations for AFM1, ranged from 0 to 1.91 ng/mL, and AFB1 ranged from 0 to 0.73 ng/mL in serum from children with autism. However for toxicological analysis, the sample pretreatment was time-consuming. Devreese et al. (2012) developed an HPLC–MS/MS method that included protein denaturation with acetonitrile for simultaneous determination of 13 mycotoxins, including AFB1, in pig plasma. Following administration of an intragastric bolus of AFB1, deoxynivalenol (DON), T-2 toxin (T-2), zearalenone (ZON), and ochratoxin A (OTA) (all 0.05 mg/kg BW) to six piglets, AFB1 was detected at levels ranging from 0.5 to 2.2 ng/mL in pig plasma. Moreover, the widespread existence and toxicity of AFB2, AFG1, AFG2 and AFM1 make it very important to develop analytical methods that can be used for the toxicokinetic study of the aflatoxins besides AFB1.

The aim of the present study was to establish a sensitive a HPLC–MS/MS method to determine the concentrations of common aflatoxins (AFB1, AFB2, AFG1, AFG2, AFM1, and AFM2) in blood and study the toxicokinetics of AFB1 and AFM1 in rats. The chemical structures of common aflatoxins are shown in Fig. 1.

Methods

Chemicals and reagents

Aflatoxin B1 (AFB1, 3 mg/L in acetonitrile), AFB1(5 mg, purity > 99%), Aflatoxin B2 (AFB2, 3 mg/L in acetonitrile), Aflatoxin G1 (AFG1, 3 mg/L in acetonitrile), Aflatoxin G2 (AFG2, 3 mg/L in acetonitrile), Aflatoxin M1 (AFM1, 10 μg/mL in acetonitrile), Aflatoxin M2 (AFM2, 0.50 μg/mL in methanol), aflatoxin B1-$^{13}C_{17}$ (AFB1-$^{13}C_{17}$, 0.50 μg/mL in acetonitrile), and aflatoxin M1-$^{13}C_{17}$ (AFM1-$^{13}C_{17}$, 0.50 μg/mL in acetonitrile) were purchased from ANPEL (Shanghai, China). Methanol

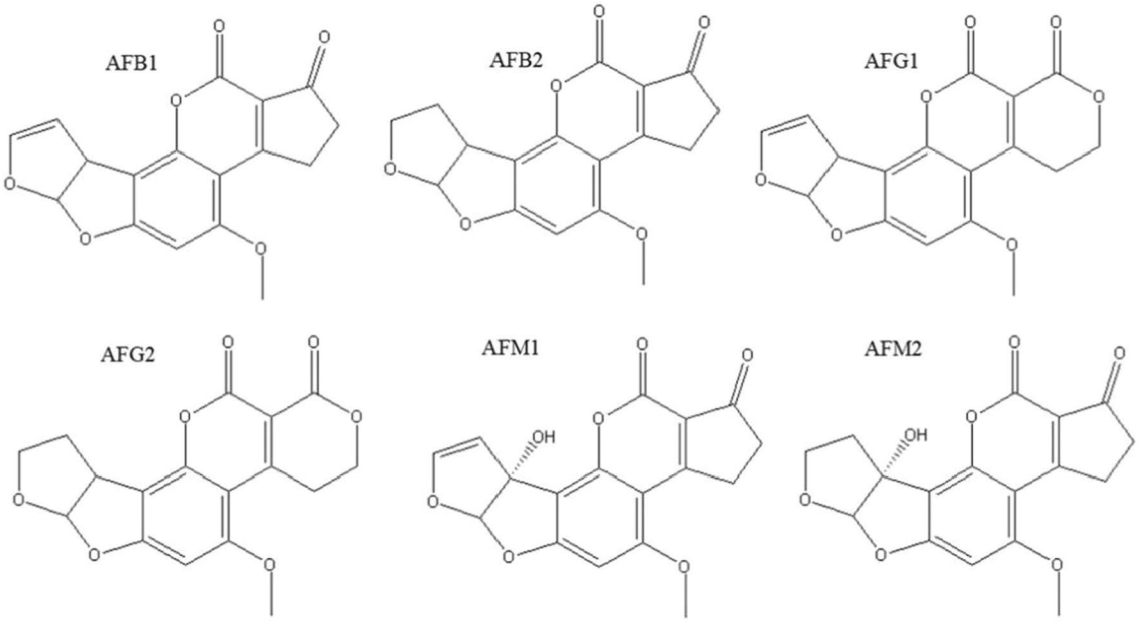

Fig. 1 Chemical structure of aflatoxins

and acetonitrile (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (98%) and ammonium acetate (98%) were obtained from Fluka (Buchs, Switzerland). Ultrapurified water was produced with a Milli-Qadvantage A10 system (Merck KGaA, Darmstadt, Germany). Filter membranes (0.22 μm pore size) were purchased from Sinopharm Chemical Reagent (Co., Ltd., China). Sunflower oil was purchased from Mighty (Standard Food, China).

Preparation of working solutions

The individual stock solutions were used to prepare a standard mixture in methanol with concentrations of 200 ng/mL (AFB1, AFB2, AFG1, AFG2, AFM1, AFM2). Working solutions with concentrations of 1, 10, and 100 ng/mL were prepared by dilution in methanol. The individual stock solutions were used to prepare an internal standard mixture in methanol at the following concentrations: AFB1-$^{13}C_{17}$ (30 ng/mL) and AFM1-$^{13}C_{17}$ (90 ng/mL). Internal standard (IS) working solutions with concentrations of 6 ng/mL (AFB1-$^{13}C_{17}$) and 18 ng/mL (AFM1-$^{13}C_{17}$) were prepared by dilution in methanol. The standard mixture and internal standard mixture were stored at − 20 °C and renewed every 2 weeks. Calibration curves and quality control samples were prepared from the working solutions.

Animals

Male Sprague–Dawley (SD) rats ($n = 6$, 230 ± 10 g) were purchased from Shanghai Jihui Experimental Animals Breeding Co., Ltd. Before the experiment, the animals were randomly distributed to cages for one week to allow acclimatization to the following environmental conditions: 12 h day/night cycle, temperature 25 °C, standard diet and water. The study was approved by the Animal Ethics Committee of the Academy of Forensic Science of China (2021-SJYLL-126). For the kinetics studies of aflatoxins in blood, six rats were administered a single dose at 1 mg/kg BW. AFB_1 was gavaged in about 1 mL of sunflower oil (0.2 mg/mL), depending on the weight of each rat. Blood samples (200 μL) were collected through the fundus vein prior to dosage (0 min) and again at 5, 10, 15, 20, 30, and 45 min, and 1, 4, 8, 12, and 24 h. The blood samples were immediately transferred to anticoagulant tubes and stored at − 20 °C until analysis.

Sample preparation

A 5 μL volume of IS working solution (6 ng/mL AFB1-$^{13}C_{17}$ and 18 ng/mL AFM1-$^{13}C_{17}$) and 900 μL acetonitrile were added to 100 μL whole blood, followed by vortex mixing (1 min) and centrifuging (1249 × g, 5 min). The supernatant was transferred to another tube and evaporated using a gentle nitrogen (N_2) stream (35 °C). The dry residue was reconstituted in 100 μL water/acetonitrile (80:20, v/v). After vortex mixing for 30 s, the sample was passed through a 0.22 μm filter membrane. The filtrate

was transferred to an autosampler vial, and 10 µL was injected into the LC–MS/MS system.

Samples with concentrations that exceeded the linear range were appropriately diluted with blank blood and then treated as described above.

HPLC–MS/MS

The HPLC–MS/MS system consisted of an Acquity™ Ultra Performance LC (Waters Corporation, USA) equipped with an AB Sciex 5500 Triple quadrupole ™ quadrupole mass spectrometer (AB Sciex, Foster City, USA). Data acquisition and quantification were performed using Analyst 1.7.1 and SCIEX OS 1.4.0 software.

Chromatographic separation was achieved on an Allure PFPP column (100 × 2.1 mm, 5 µm i.d., Restek, USA). Samples were eluted with a gradient of 20 mmol/L ammonium acetate, 5% acetonitrile, and 0.1% formic acid in water (phase A) and methanol (phase B) at a flow rate of 0.3 mL/min. The following step gradient was used: 5% B for the first 1.0 min, increased to 50% B from 1.0 to 3.0 min, increased to 60% B from 3.0 to 3.5 min, isocratic at 60% B from 3.5 to 4 min, increased to 80% from 4 to 8 min, decreased to 50% B from 8 to 11 min, decreased to 5% B from 11 to 13 min, and a final column equilibration at 5% B for 1 min. The autosampler temperature was 4 °C and the autosampler needle was thoroughly washed twice between injections.

The mass spectrometer system was operated using electrospray ionization in positive ionization mode with multiple reaction monitoring (MRM). The optimum mass spectra were obtained under the following conditions: curtain gas (CUR), 20 psi (nitrogen); ion spray voltage (IS), 4500 psi; collision cell exit potential (CXP), 14 V; entrance potential (EP), 10 V; ion source gas 1 (GS1), 35psi; ion source gas 2 (GS2), 35 psi; and source temperature (TEM), 500 °C. A summary of the MRM parameters and retention times is shown in Table 1, and product ion mass spectra from protonated molecules of aflatoxins are shown in Fig. 2.

Table 1 MS/MS conditions for the detection of target analytes by the MRM method

Analyte	Precursor ion *(m/z)*	Product ions *(m/z)*	RT (min)	CE (eV)	DP(V)
AFB1	313.1	241.1* 214.1	6.8	52 48	41
AFB2	315.1	259.3* 286.9	6.6	45 39	34
AFG1	329.1	243.0* 200.1	6.2	39 55	18
AFG2	331.0	189.0* 245.1	5.9	54 36	51
AFM1	328.9	273.1* 228.9	5.4	38 58	17
AFM2	330.9	272.9* 229.1	5.1	39 59	29
AFB1-$^{13}C_{17}$	330.0	255.1* 301.1	6.8	54 43	48
AFM1-$^{13}C_{17}$	346.2	288.2* 242.0	5.4	38 59	55

*The quantifier ions

RT retention time, *CE* collision energy, *DP* declustering potential

Method validation

The analytical method was validated according to international guidelines (Peters et al. 2007; Matuszewski et al. 2003). The following parameters were examined: selectivity, limits of detection (LODs), LOQs, calibration curves, precision, accuracy, matrix effects, extraction efficiency, freeze–thaw stability, and dilution integrity.

Selectivity

Selectivity was evaluated using six different blank blood samples to assess the interference of the matrix components with the analytes and the internal standards at the corresponding retention times.

LOD, LOQ, and linearity

Triplicate samples with concentrations of 0.01, 0.02, 0.03, 0.05, 0.08, 0.1, and 0.2 ng/mL were prepared for detection in blank samples. The LOD was determined as the lowest concentration of mycotoxin that could be detected with a minimum signal-to-noise ratio of 3. The LOQ defined as the lowest concentration that meets minimum signal-to-noise ratio of 10 and the requirements for accuracy in the range of 80–120% and precision of ≤ 20% relative standard deviation (RSD) based on intra-day and inter-day experiments.

Linearity was evaluated by preparing matrix-matched calibration curves in rat blood with concentrations of 0.05, 0.5, 2, 10, 16, and 20 ng/mL for AFB1, AFB2, AFG1, and AFM1. Matrix-calibration standards in rat blood with concentrations of 0.2, 0.5, 2.0, 5.0, 10.0, 16.0, and 20.0 ng/mL were prepared for AFG2 and AFM2. Weighted linear regression ($1/x^2$) was used for all aflatoxins to construct calibration curves.

AFB1-$^{13}C_{17}$ was used as the internal standard for AFB1, AFB2, AFG1, AFG2. AFM1-$^{13}C_{17}$ was used as the internal standard for AFM1 and AFM2.

Inter-day and intra-day precision and accuracy were determined by analyzing quality control (QC) samples at four concentration levels (0.05, 0.5, 2, and 16 ng/mL) for AFB1, AFB2, AFG1, and AFM1, at four concentration levels (0.2, 0.5, 2, and 16 ng/mL) for AFG2 and AFM2. Each concentration was prepared in six replicates, and the experiments were repeated over four consecutive days. Intra-day accuracy and inter-day accuracy were

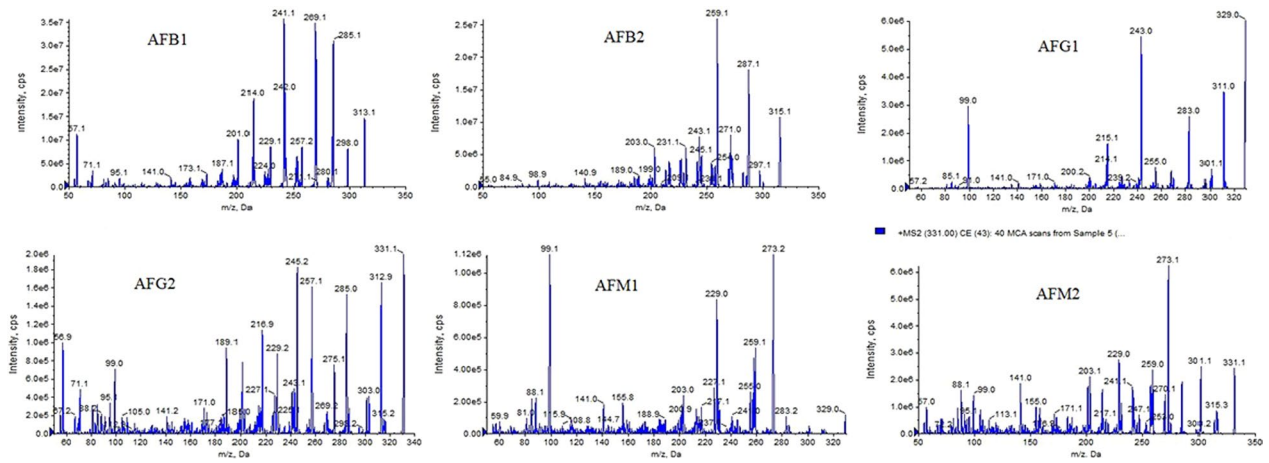

Fig. 2 Product ion mass spectra from protonated molecules of aflatoxins

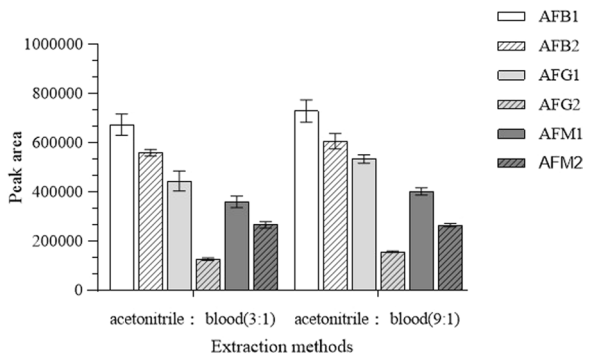

Fig. 3 Comparison of the peak areas of aflatoxins (4 ng/mL) in spiked blood sample using acetonitrile extraction at different volumes ($n=3$)

evaluated by comparing the calculated concentrations of the QC samples with the theoretical concentrations. Values from 85 to 115% were accepted. Precision was evaluated by RSD%, with a value less than 15% considered acceptable.

Matrix effect and extraction recovery

The matrix effect was determined according to formula 1:

$$\text{matrix effect} = \left(A_{\text{post-extracted}}/A_{\text{std.}}\right) * 100\% \quad (1)$$

where $A_{\text{post-extracted}}$ is the peak area of aflatoxins in post-extracted spiked blood, and A std. is the peak area of a standard solution with the same concentration.

Extraction recovery was determined according to the following formula:

Table 2 Calibration, LOD, and LOQ of aflatoxin in blood

Analyte	Curve equation	*r*	LOD (ng/ml)	LOQ (ng/ml)
AFB1	Y = 3.37X + 0.036	0.9992	0.02	0.05
AFB2	Y = 2.53X + 0.010	0.9993	0.02	0.05
AFG1	Y = 2.62X − 0.0036	0.9991	0.01	0.05
AFG2	Y = 0.78X-0.016	0.9991	0.03	0.2
AFM1	Y = 1.14X + 0.0079	0.9995	0.02	0.05
AFM2	Y = 0.75X − 0.015	0.9992	0.03	0.2

$$\text{extraction recovery} = \left(A_{\text{pre-extraction}}/A_{\text{post-extracted}}\right) * 100\% \quad (2)$$

where $A_{\text{pre-extraction}}$ is the peak area of the spiked sample pre-extraction.

Freeze–thaw stability

The freeze–thaw stability was investigated with blood samples spiked at 0.05, 0.5, 2, and 16 ng/mL for AFB1, AFB2, AFG1, and AFM1 and at 0.2, 0.5, 2, and 16 ng/mL for AFM2 and AFG2. Three freeze/thaw cycles ($n=3$ replicates per condition) were used, with each cycle consisting of freezing at − 20 °C for 21 h, thawing, and leaving at room temperature for 3 h. The freeze-thawed samples were processed and analyzed at the same time as the standard curve samples, and the concentrations of aflatoxins in the freeze-thawed samples were calculated from the standard curves. The stability acceptable accuracy was 85–115% and acceptable RSD was set at ± 15%.

Dilution integrity

The dilution QC (100 ng/mL) was diluted 250-fold using blank matrix in six replicates for blood sample

Table 3 Precision, accuracy, recovery, and matrix effects of aflatoxins in blood

Analyte	Spiked concentration (ng/mL)	Intraday($n=6$)		Inter-day (6×4 days)		Recovery (mean ± SD, %)	Matrix effects (mean ± SD, %)
		accuracy (%)	Precision (%)	accuracy (%)	Precision (%)		
AFB1	0.05	95	4	99	3	78 ± 3	67 ± 1
	0.5	102	1	100	5	85 ± 4	65 ± 2
	2	100	2	99	5	79 ± 6	66 ± 1
	16	97	1	99	3	70 ± 1	63 ± 1
AFB2	0.05	104	4	100	5	80 ± 1	82 ± 1
	0.5	106	4	99	6	86 ± 3	68 ± 2
	2	93	4	98	7	80 ± 5	67 ± 1
	16	106	2	99	4	72 ± 2	63 ± 1
AFG1	0.05	105	1	100	4	75 ± 1	80 ± 1
	0.5	105	4	99	4	93 ± 4	76 ± 3
	2	95	4	99	6	82 ± 5	79 ± 0.4
	16	106	3	100	4	70 ± 2	74 ± 1
AFM1	0.05	104	5	102	4	75 ± 3	107 ± 2
	0.5	98	2	98	5	88 ± 2	99 ± 1
	2	92	1	94	5	89 ± 6	103 ± 2
	16	98	1	98	2	73 ± 3	96 ± 2
AFG2	0.2	105	5	100	4	75 ± 2	72 ± 2
	0.5	97	6	98	5	95 ± 2	70 ± 2
	2	111	3	99	8	81 ± 2	74 ± 1
	16	90	4	98	6	68 ± 3	67 ± 1
AFM2	0.2	111	6	101	4	73 ± 1	100 ± 2
	0.5	96	8	101	4	96 ± 5	105 ± 2
	2	103	5	95	5	89 ± 1	100 ± 0.5
	16	96	2	99	3	73 ± 3	102 ± 3

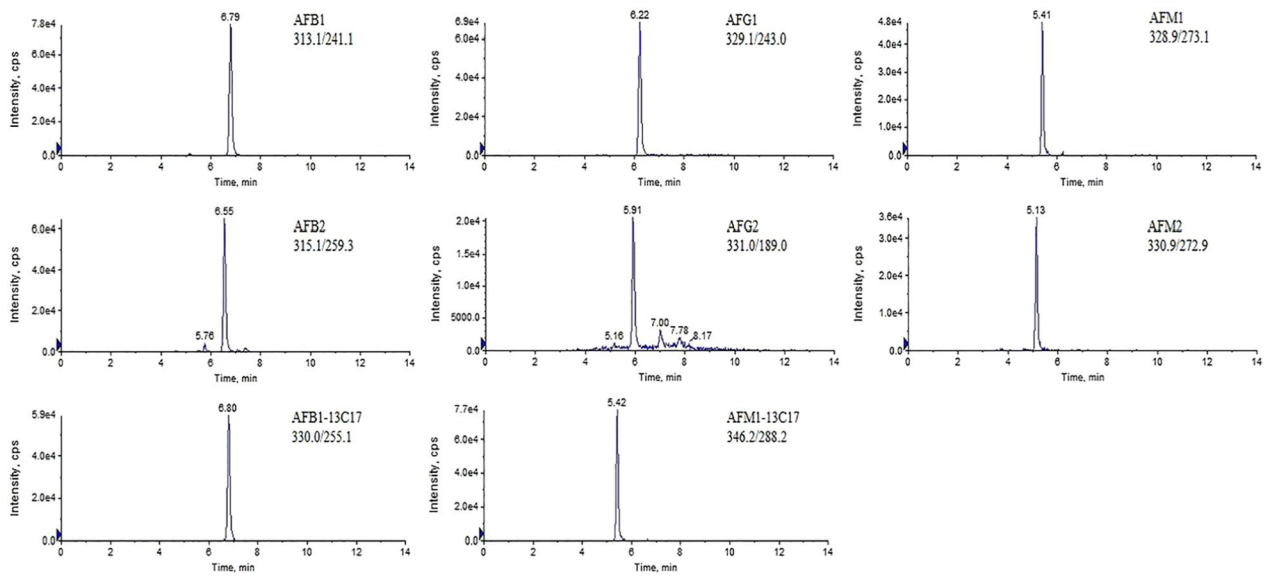

Fig. 4 MRM chromatograms of quantitative ions for aflatoxins (spiked at 0.5 ng/mL) and IS (AFB1-$^{13}C_{17}$ spiked at 0.3 ng/mL and AFM1-$^{13}C_{17}$ spiked at 0.9 ng/mL)

Table 4 Freeze–thaw stability

Analyte	Spiked concentration(ng/mL)	Accuracy (%)	Precision (%)
AFB1	0.05	97	5
	0.5	102	1
	2	118	3
	16	111	3
AFB2	0.05	100	7
	0.5	98	1
	2	114	1
	16	106	0.3
AFG1	0.05	107	3
	0.5	92	1
	2	114	1
	16	105	2
AFG2	0.2	98	5
	0.5	97	3
	2	109	5
	16	105	3
AFM1	0.05	108	4
	0.5	99	0.4
	2	119	2
	16	115	1
AFM2	0.2	103	1
	0.5	100	1
	2	117	1
	16	113	4

analyses. The diluted samples (0.4 ng/mL) and calibration curve were processed and analyzed on the same day. The dilution integrity acceptable accuracy was 85–115% and acceptable RSD was set at ± 15%.

Data analysis

The toxicokinetics parameters were determined by noncompartmental analysis using DAS 3.2.8 (BioGuider Co., Shanghai, China). The following parameters were calculated: peak concentration (C_{max}), time of maximum blood concentration (T_{max}), area under the curve (AUC_{0-t}), area under the curve from zero to infinity ($AUC_{0-\infty}$), half-life time ($t_{1/2}$), apparent total clearance (CLz/F), and apparent distribution volume (Vz/F). Data for all response variables were reported as mean ± SD. The significance level (a) was set at 0.05.

Results and discussion

Sample preparation

The acetonitrile protein precipitation method was applied to develop a sensitive HPLC–MS/MS method for screening and quantifying aflatoxins, since it is a simple, rapid, inexpensive, and suitable method for screening mycotoxins in large numbers of samples (Devreese et al. 2012). The efficiency of extraction of blood with different volumes of acetonitrile was compared. Extraction was more effective with a 9:1 (v/v) ratio of acetonitrile: blood than with a 3:1 (v/v) ratio, as shown in Fig. 3.

Method validation

No significant peaks or responses were detected in the blank blood samples at the retention times of the target compounds or the IS. The chromatograms of samples spiked at 0.5 ng/mL and IS (AFB1-$^{13}C_{17}$ spiked at 0.3 ng/mL and AFM1-$^{13}C_{17}$ spiked at 0.9 ng/mL) are shown in Fig. 4. Calibration curves were constructed for each compound and showed good linearity ($r > 0.999$). The LODs ranged from 0.01 to 0.03 ng/mL. The LOQs for aflatoxins were 0.05 ng/mL for AFB1, AFB2, AFG1, and AFM1 and 0.2 ng/mL for AFM2 and AFG2. The calibration curves, LODs, and LOQs are shown in Table 2. The LOQ was lower than that previously reported for the protein precipitation method for aflatoxin determinations in plasma or serum. Fan et al. (2019) reported a HPLC–MS/MS method based on plasma aflatoxin extraction in acetonitrile containing 1% formic acid with LOQs between 0.1 and 0.2 ng/mL. Cao et al. (2018) developed a method using enzymatic hydrolysis and protein precipitation. The LOQs for aflatoxin ranging from 0.21 to 0.43 ng/mL, the detectable concentrations of AFB_1 and AFB_2 in hepatocellular carcinoma (HCC) patients were reported as 1.23–4.56 ng/mL and 1.16–3.75 ng/mL, respectively. Because our method is sensitive enough, it is expected to be applied to aflatoxins exposure assessment in the future.

The accuracy and precision are shown in Table 3. The results of intra-day and inter-day precision were within 20%, and the accuracies were 92–111% and 94–102%, respectively. The recoveries and matrix effects are listed in Table 3. The recoveries of QC samples ranged from 68.0 to 96% for all analytes. Matrix effects varied between 63 and 107% for all compounds. The blood matrix had no significant ion suppression or ion enhancement on AFM1 or AFM2 but it had a significant ion suppression effect on AFB1, AFB2, AFG1, and AFG2. Nevertheless, the choice of AFB1-$^{13}C_{17}$ as an IS offset this interference with acceptable accuracy and precision. The accuracy range of stability was between 92 and 119%, and the RSD results were within 7%. The investigation of stability during the 3 freeze/thaw cycles showed that all analytes were stable under these conditions, as shown in Table 4. The dilution integrity results for aflatoxins are shown in Table 5. The accuracies of

Table 5 Concentration, precision, and accuracy of diluted samples

Analyte	Target concentration(ng/mL)	Calculated concentration(ng/mL)	Accuracy (%)	Precision (%)
AFB1	0.4	0.39	100	3
		0.41		
		0.39		
		0.38		
		0.39		
		0.40		
AFB2	0.4	0.39	99	2
		0.41		
		0.39		
		0.38		
		0.39		
		0.40		
AFG1	0.4	0.40	101	1
		0.41		
		0.40		
		0.40		
		0.39		
		0.40		
AFG2	0.4	0.36	98	6
		0.41		
		0.42		
		0.39		
		0.35		
		0.36		
AFM1	0.4	0.40	100	2
		0.41		
		0.39		
		0.40		
		0.39		
		0.41		
AFM2	0.4	0.35	93	5
		0.40		
		0.36		
		0.37		
		0.34		
		0.35		

the 250-fold diluted QC samples ranged from 93 to 101% and the precision (RSD) was < 6%. This confirmed that samples with concentrations higher than the maximum concentration in calibration curve could still be analyzed after appropriate dilution with blank matrix.

Toxicokinetic study

The calculated kinetic parameters, expressed as mean ± SD, are shown in Table 6. The concentration–time profiles are presented in Fig. 5.

After oral administration of 1 mg/kg BW of AFB1, both AFB1 and AFM1 were found in the blood at 5 min. The blood levels of AFB1 reached a maximum concentration (93.42 ± 23.01 ng/mL) of approximately at 0.15 h and AFM1 reached a maximum concentration (53.86 ± 12.12 ng/mL) at 0.33 h. The concentrations of AFB1 and AFM1 in blood peaked quickly and then declined. However, the concentration of AFB1 fluctuated at 8 h. AFB1 and AFM1 were rapidly eliminated from the blood, with $T_{1/2}$ of 7.62 h and 4.73 h, respectively. Due to its lipophilicity and low molecular weight, most of the ingested AFB1 was readily absorbed in the gastrointestinal tract (Bastaki et al. 2010). A previous study on intestinal absorption kinetics showed that the

Table 6 Pharmacokinetic parameters using noncompartmental analysis of AFB1 and AFM1 in rat blood after a single oral dose of AFB1

Pharmacokinetic parameters	Unit	AFB1	AFM1
Tmax	h	0.15 ± 0.034	0.33 ± 0.10
Cmax	ng/ml	93.42 ± 23.01	53.86 ± 12.12
$T_{1/2}$	h	7.62 ± 3.61	4.74 ± 2.58
$AUC_{(0\text{-}24\ h)}$	h*ng/ml	49.59 ± 17.19	110.26 ± 36.28
$AUC_{(0\text{-}\infty)}$	h*ng/ml	51.26 ± 18.43	112.48 ± 36.19
CLz/F	l/h/kg	22.33 ± 9.79	9.98 ± 4.21
Vz/F	l/kg	226.46 ± 100.76	65.96 ± 38.47

Data are presented as mean ± SD

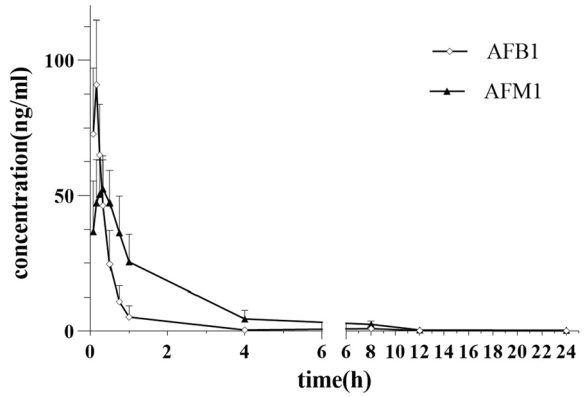

Fig. 5 Mean blood concentration–time curves for AFB1 and AFM1 following oral administration (1 mg/kg BW) in rats ($n = 6$)

absorption of AFB1 in the rat small intestine is a very rapid process that follows first-order kinetics, with an absorption rate constant (ka) of 5.84 + 0.05 (Ramos and Hernández 1996). Ingestion of AFB1 administered at high doses could cause acute toxic symptoms immediately (McKean et al. 2006). Similar to our results, the oral administration of AFB1 (0.5 mg/kg BW) to rats resulted in the highest concentration of AFB1 at 0.17 h in a previous study (Han et al. 2012). However, the target analytes may have been confused with the metabolites in previous studies, as the obtained time-to-peak for AFB1 ranged from 2–3 h (Han et al. 2012). Jubert et al. (2009) used the two-compartment model to simulate the toxicokinetic parameters of AFB1 in plasma and obtained values of $T_{1/2\alpha} = 2.86$ h and $T_{1/2\beta} = 64.4$ h. $T_{1/2\alpha}$ was speculated to represent the half-life of free aflatoxin. AFB_1 and its metabolites or conjugates could not be distinguished by the protocol used in Jubert's study. Our study shows that the half-life of free aflatoxin in blood is longer than the time speculated by Jubert.

AFM1 appeared rapidly in the blood after oral ingestion, and the peak concentration was rapidly reached, showing that the metabolism of AFB1 occurs immediately. Therefore, rapid metabolism may be one of the reasons for the rapid elimination of AFB1 from the blood. Lactating Holstein dairy cows (Gallo et al. 2008) and pregnant mice (Bastaki et al. 2010) given a single oral dose of AFB1 showed detectible plasma AFM1 at 5 min and peak values at 25 min, in agreement with the present findings. The half-life of AFM1 is 4.73 h, indicating that AFM1 was quickly removed from the blood. The AFM1 amounts reflect acute aflatoxin exposure in the past 2–3 days and excretion mainly in urine and milk (Mupunga et al. 2016; Schrenk et al. 2020). The Vz/F is wider for AFB1 than for AFM1, and the high volume of distribution inferred a relatively high tissue concentration of AFB1 (Wong and Hsieh 1980). Blood concentrations of AFB1 fell quickly after 0.15 h, except for a fluctuation at 8 h, when the blood concentration of AFB1 (0.99 ± 0.52 ng/mL) was higher than at 4 h (0.42 ± 0.14 ng/mL). This may indicate a redistribution of AFB1 in the peripheral blood, as reported previously by Wong et al. (1980). The redistribution of AFB1 in rats may reflect that the rate of AFB1 entering the tissue is greater than the rate of metabolism or elimination from the tissue (Wong and Hsieh 1980). This redistribution was not observed in monkeys through active metabolism in their tissues. The effective uptake and metabolism of AFB1 may explain the greater sensitivity to acute toxicity in monkeys than in rats (Wong and Hsieh 1980).

Conclusion

An HPLC–MS/MS method was specifically developed for simultaneous determination of aflatoxins (AFB1, AFB2, AFG1, AFG2, AFM1 and AFM2) in blood. AFB1 and AFM1 were found in all rat blood samples between 5 min and 24 h after a single administration of AFB1. AFB1 was rapidly absorbed after intragastric administration and showed a peak at 0.15 h. AFM1 reached a peak concentration at 0.33 h, indicating that AFB1 was rapidly metabolized. AFB1 and AFM1 were rapidly eliminated with half-life times ($t_{1/2}$) in blood of 7.62 h and 4.73 h, respectively. The kinetic parameter values presented here might be helpful in predicting the toxicokinetics and toxicity of AFB1 and AFM1 in animals and humans.

Abbreviations

HPLC: High-performance liquid chromatography; MS: Mass spectrometry; AFB1: Aflatoxin B1; AFB2: Aflatoxin B2; AFG1: Aflatoxin G1; AFG2: Aflatoxin G2; AFM1: Aflatoxin M1; AFM2: Aflatoxin M2; QC: Quality control; MRM: Multiple reaction monitoring; DIC: Disseminated intravascular coagulation; WHO: World Health Organization; BW: Body weight; ELISAs: Enzyme-linked immunosorbent assays; HPLC-HMRS: High-performance liquid chromatography–high resolution mass spectrometry; HPLC-FD: High-performance liquid

chromatography–fluorescence detection; HPLC-IDMS: High-performance liquid chromatography–isotope dilution mass spectrometry; LLE: Liquid–liquid extraction; DON: Deoxynivalenol; T-2: T-2 toxin; ZON: Zearalenone; OTA: Ochratoxin A; HCC: Hepatocellular carcinoma; SD: Sprague–Dawley; RT: Retention time; CE: Collision energy; DP: Declustering potential; LOD: Limit of detection; C_{max}: Peak concentration; T_{max}: Time of maximum blood concentration; AUC_{0-t}: Area under the curve,; $AUC_{0-\infty}$: Area under the curve from zero to infinity; $t_{1/2}$: Half-life time; CLz/F: Apparent total clearance; Vz/F: Apparent distribution volume.

Author contributions

MBC and HY contributed to the design of the study. MBC performed the experiments and analyzed the data. SY, ZNC, XZL, BD and WL gave their valuable suggestions during the course of validation. MBC, HY wrote the manuscript. HY and WL involved in writing–review and editing and supervision. All authors read and approved the final manuscript.

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Availability of data and materials

Almost all details of experimental data are presented in the article.

Declarations

Ethics approval and consent to participate

This study was approved by the institute ethics committee.

Consent for publication

The authors have no objection regarding publication of the article.

Competing interests

The authors declare that they have no competing interests.

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