ANALYSIS OF AFLATOXIN B_1-LYSINE ADDUCT IN INFANT DRIED BLOOD SPOT SAMPLES USING HPLC-FLUORESCENCE METHOD

by

WENJIE CAI

(Under the Direction of Jia-Sheng Wang)

ABSTRACT

Aflatoxins (AF) are group I human carcinogen and potent mycotoxins that commonly contaminate peanuts, corns, and their products during growth, harvest and storage. Exposure to AF is linked to growth stunting in children, which has been extensively studied in recent years. With increasing involvement of young children as study subjects, dried blood spot (DBS) rose in popularity as an important alternative blood collection strategy. In this study, an HPLC-fluorescence method measuring exposure biomarker of AFB_1, AFB_1-lysine adduct, was developed and validated. For initial method development and validation, DBS and serum samples were obtained from AFB_1-treated F344 rats, as well as selected samples from human field study, which were analyzed and compared, and has found good correlation between serum and DBS samples (0.997 for the single-dose exposure and 0.996 for repeated dose exposure). DBS samples (n=393) from Nepal were analyzed and compared with the results from matching serum samples. The result suggested that the HPLC-florescence method is effective in detecting AFB_1-lysine adducts and can potentially be applied in epidemiological studies.

INDEX WORDS: Aflatoxin B_1; dried blood spots; HPLC-fluorescence

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DEDICATION

This work is dedicated to my parents, who have helped me become who I am today and always be there to support me. I would also like to dedicate this to family and friends who showed endless care and patience during every step of the way.

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TABLE OF CONTENTS

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Aflatoxins (AFs) are the toxic secondary metabolites of common fungus *Aspergillus flavus* and the closely related species *A. parasiticus.* A "turkey X" disease spread in England in the late 1950s, killing thousands of young turkeys, chicks, and ducklings in farms. The reason was unknown at first, but soon studies linked the disease to the mold-contaminated meal given to the poultry before the outbreak. Researchers found *Aspergillus flavus* in the peanuts and further extracted the toxic agent from the cultures of this mold, which was then named Aflatoxin (A. flavus toxin) (Kensler et al., 2011). The major hosts of *Aspergillus flavus* among food are maize, groundnuts and cottonseed, while lesser amounts of aflatoxins can be existed in a wider range of food commodities. Humid and warm ambient favor the growth of *Aspergillus flavus* and encourages the production of aflatoxins (IARC, 2002). Most areas and countries with environment favorable for *Aspergillus flavus* in the world have established standards to regulate the concentration of aflatoxins in crops, but some developing countries failed to limit the contamination under the harmful level due to lack of money, technology, supervision etc. (Egmond et al., 2007). Aflatoxin B_1 gives a blue color under the ultraviolet light while aflatoxin G_1 gives a green color under the same situation, thus, they are named by the colors observed (Bennett & Klich, 2003). Among the different families, aflatoxin B_1, B_2, G_1, G_2 and M_1 are the most commonly studies species. This study would mainly focus on the aflatoxin B_1. Aflatoxin B_1 has been classified as group I carcinogen

by IARC, can cause both acute and chronic toxic effects in both human and animal models (IARC, 1993). Acute exposure to high dose aflatoxin B_1 can cause immediate death while chronic exposure can lead to hepatocellular carcinoma (Hussein & Brasel, 2001).

This thesis focuses on the validation of a high-performance liquid chromatography (HPLC)-fluorescence method used in detecting AFB_1-lysine adducts in the DBS samples and the application of this method into the infant DBS samples. The literature review (Chapter 2) provides a detailed review about AFs, including their chemistry and their impact on crop, animal, and human health and how it specifically affects the growth and development of children. This chapter will also provide background information on dried blood spot sampling method. Chapter 3 and chapter 4 describe studies that developed and validated the HPLC-fluorescence method used for analyzing DBS samples. The study described in chapter 3 focuses on the animal studies that validated the HPLC-fluorescence method while the study described in chapter 4 applies the method developed and validated in chapter 3 into field-collected infant DBS samples from Nepal.

CHAPTER 2

LITERATURE REVIEW

Introduction

Mycotoxins are toxic secondary metabolites produced by filamentous fungi, and can be toxic to vertebrates and other animal groups in very low concentration (Bennett & Klich, 2003). Aflatoxins (AFs) were discovered in 1962 after the outbreak of mysterious turkey X disease in England. At first more than 100,000 young turkeys died from the unknown cause, then similar incidents occurred in ducklings and chickens (Asplin & Carnaghan, 1961), as well as in swine (Harding et al., 1964; Loosmore & Harding, 1961), and calves(Loosmore & Markson, 1961). All the episodes were eventually linked together by feeding with the same shipment of peanut meal imported from Brazil. Active component was extracted from the meal (Sargent et al., 1961), which then was then proven to be aflatoxins. Mainly produced by *Aspergillus flavus*, AFs cause toxic effect to susceptible species(Wogan, 1966). Peanut has been suggested to be the most frequently contaminated among all the crops (Hiscocks, 1965). Contamination can happen in the field or during the harvest, storage and transportation of the crop commodities, thus approximately 100 countries in the world have established detailed regulations for mycotoxins in food and feed (Egmond et al., 2007). For detection of AFs, the chromatographic methods are considered the golden standard, such as thin-layer

chromatography (TLC) and high-performance liquid chromatography (HPLC), though methods like ELISA, mass spectroscopy and electrochemical immunosensors are being developed and applied in the field as well (Wacoo et al., 2014).

Chemistry and Biology of Aflatoxins

Aflatoxins are strongly fluorescent under the ultraviolet light, which greatly facilitated the isolation of aflatoxins from the contaminated peanut meals (Wogan, 1966). This property also provides an effective way to monitor the isolation and purification processes. Aflatoxins are extractable using methanol (Loosmore & Harding, 1961; Sargent et al., 1961), aqueous methanol, aqueous acetone (Goldblatt & Pons, 1965), and a hexane-acetone-water azeotrope (Goldblatt, 1965). The aflatoxins produced in cultures on liquid media can be removed by partitioning into chloroform (Adye & Mateles, 1964); while extraction of aflatoxins from cultures on solid substrate involves chloroform, followed by subsequent precipitation in petroleum ether to concentrate the extracts (Asao et al., 1963, 1965). Extracts produced by these procedures are separable by chromatographic techniques into their individual components. The conditions most widely used involve separation on silica gel developed with 3 to 5% methanol in chloroform (Asao et al., 1965). Further improvement in isolation of aflatoxins using kieselguhr plates developed with formamide-benzene water has been reported (Mateles & Adye, 1964).

When chromatograms of aflatoxins are viewed under the ultraviolet light, a complex array of fluorescent compounds is clearly present (Wogan, 1966). Of the four most well-known aflatoxins, two emit blue visible light, thus named aflatoxins B_1 and B_2;

while the other two show yellow-green color, and are named aflatoxins G_1 and G_2 (Goldblatt, 1970). The relative migrate rates (RF) of aflatoxins B_1, B_2, G_1, G_2 on silica gel plates developed in chloroform-methanol (97:3) are 0.56, 0.53, 0.48, and 0.46, respectively (Asao et al., 1965). Spectral data and some physical properties of the compounds are summarized in Table 2.1 (Marin et al., 2013).Molecular data of the four aflatoxins are shown in Table 2.2. The Büchi group has determined the five-ring structures of aflatoxins B_1, B_2, G_1 and G_2 in 1963, which are shown in Figure 2.1 (Asao et al., 1963, 1965; Chang et al., 1963; van Dorp et al., 2010). Structurally, aflatoxins consist of five rings, including a furfuran moiety, an aromatic six-membered ring, a six-membered lactone ring, and either a five-membered pentanone or a six-membered lactone ring (Brase et al., 2013).

Aflatoxins are acutely toxic compounds. The liver is the main target organ, followed by the kidneys (Bennett & Klich, 2003; Marin et al., 2013), but the pancreas, gall bladder, lung, and gut may also be affected (Reiss, 1982). Aside from their acute toxicity, aflatoxins are also highly carcinogenic. In fact, aflatoxin B_1 is the most potent known liver carcinogen for mammals (Brase et al., 2013). When taken orally over a long period, the aflatoxins are absorbed from the gut and are transported to liver where they are metabolized, inducing tumors and metastases (Lilly, 1965). Due to the high toxicity and carcinogenicity, it is impractical to used aflatoxins as therapeutic agents, although they did show ability in inhibiting sporulation by reducing the activity of crucial enzymes (Reiss, 1971).

Contamination of Food Commodities with Aflatoxins

The *Aspergillus fungi* are ubiquitous contaminants in the food crops, particularly maize, groundnuts, oilseeds and tree nuts (IARC, 1993). As the secondary metabolites of the fungi *Aspergillus flavus*, *Aspergillus parasiticus*, and occasionally other *Aspergillus species*, aflatoxins are also largely produced in these agriculture commodities, especially in tropical and subtropical regions worldwide (Khlangwiset et al., 2011). These fungi can also produce aflatoxins during the postharvest conditions such as food storage, transportation, and food processing. Worldwide aflatoxins contamination levels in maize and maize products are shown in Table 2.3 (IARC, 1993).Among all these fungi, the *Aspergillus flavus* group of species is a normal constituent of the microflora in air and soil, and is found on or in living or dead plants and animals throughout the world (Raper & Fennell, 1965). It has been found to be associated with peanut soils and peanuts wherever they are grown (Diener, 1973). *Aspergillus flavus* is also an important storage fungus associated with the deterioration of wheat, maize, rice, barley, bran, flour, soybeans, and other seeds (Christensen, 1957). Naturally contaminated lots of maize associated with losses of swine in Alabama in 1965 contained only aflatoxins B_1 and B_2, while other naturally contaminated lots of peanuts, soybeans, maize, and cottonseed contain aflatoxins B_1, B_2, G_1 and G_2 (Diener & Davis, 1977).

Approximately 90 countries have regulations that establish maximum aflatoxin limits in food and feed products due to the high exposure of aflatoxins in food and agriculture commodities (Adams & Whitaker, 2004).Table 2.4 shows the number of isolates of *Aspergillus flavus* that contaminated groundnuts in eight U.S. states in a range

of 1 to 17,000 ppb aflatoxin B_1(Diener & Davis, 1977). The Canadian and European Union limit is 15 ng/g as compared to the United States Food and Drug Administration limit of 20 ng/g (Adams & Whitaker, 2004). Table 2.5 shows the aflatoxins level in imported groundnuts into US (IARC, 1993). Due to the heterogeneity of aflatoxin contamination and the lability presented in the aflatoxins analysis procedures, agriculture commodities can be classified as beyond the limitation by mistake and then rejected by the importer (Whitaker, 1993).

The most crucial factor in *Aspergillus flavus* growth and aflatoxin production is the moisture of a natural substrate such as peanut (Austwick & Ayerst, 1963). Few fungi grow at substrate moistures equals to 70 percent relative humidity (Diener & Davis, 1977). In pure laboratory culture conditions, the *Aspergillus flavus* growth is limited by the 80 percent relative humidity (Austwick & Ayerst, 1963). Interruption and retardation of the field drying cycle by rain, overcast humid weather, or a regain of moisture after picking and storage usually result in the development of *Aspergillus flavus* with subsequent toxin formation (Austwick & Ayerst, 1963; Bampton, 1963). When placed in tropical conditions, toxin can be detected in 48 hours from peanuts that were free of toxin at digging (Bampton, 1963). In 1963 and 1964, peanut crops in northern Nigeria harvested at or earlier than normal were free of aflatoxins, while late harvesting peanut resulted in some level of toxin (McDonald & Harkenss, 1976). Aside from the moisture, temperature, time, and drought stress are also closely related to the aflatoxin occurrence in peanut before digging (Bampton, 1963; Diener & Davis, 1966; McDonald, 1969; Petrtit et al., 1971; Porter & Garren, 1968). During commercial storage and transportation, a potential used of 20 to 60 percent CO2 in controlling fungi growth has

been indicated by the researches (Lenders et al., 1967). Prevention of aflatoxin contamination is considered the best way to mitigate the aflatoxin problem (Dickens, 1975). Harvesting with minimal damage to pods, rapid drying and storage with aeration in low moisture environment can further reduce the production of aflatoxins in food commodities (Diener & Davis, 1977).

Effects of Aflatoxins in Animals and Human Population

Effects of Animal Exposure to Aflatoxins

Toxicity effects of aflatoxins on domestic animals were first recognized in England in 1960 and 1961 (Wogan, 1966). Several outbreaks were reported in young turkeys, ducklings and chickens, as well as in swine and calves (Loosmore & Harding, 1961). All these incidents used the same peanut imported from Brazil as animal rations. Subsequently, peanut samples were tested and found that samples from over 13 producing countries were contaminated by aflatoxins (Allcroft & Carnaghan, 1963). The aflatoxins are largely recognized as acutely toxic in most animal species. Table 2.6 summarized its lethality to various experimental animals. The LD50 presented were calculated from mortality over 7-day period (Wogan, 1966). The adverse effects of aflatoxins exposure on various growth indicators have long been demonstrated in multiple animal species (Khlangwiset et al., 2011). Reduced feed intake and subsequent weight loss have been reported in ducklings (Cheng et al., 2001). Principle histological changes in rat liver have also been reported by Butler, with compromised development of a perioral zone of necrosis marked biliary proliferation (Butler, 1964). Feeding a high level of an aflatoxin mixture to poultry could reduce their body weight and increase their

liver and kidney weight (Smith et al., 1992). When aflatoxin B_1 was added to the culture (0.1-10,000 ng/ml of medium), the cellular immunosuppression and the inhibition of DNA synthesis in porcine lymphocytes could be observed (Pang & Pan, 1994). Horses are jaundiced and anorexic before death after consuming aflatoxin B_1-contaminated feed (58. 4 μg / kg) (Asquith, 1991). Post-mortem examination of horses reveled severe hepatic lesions (Vesonder et al., 1991). Mice are generally resistant to the hepatocarcinogenic effect compared to rats, which can be further explained by the high level of glutathione-S-transferase activity in mice challenged with aflatoxin B_1 (Quinn et al., 1990). Ruminants have generally been more tolerance to the adverse effects of aflatoxins because their rumen microbiota is capable of degrading aflatoxins (Hussein & Brasel, 2001).

Effects of Aflatoxins on Human Population

Aflatoxin B_1 is the most toxic specie of the aflatoxins, classified as Group 1 carcinogens by IRAC (IARC, 1987). Occurring ubiquitously in nature, exposure to aflatoxins occur mostly by ingestion, but also through some less common routes such as dermal and inhalation routes (Peraica & Pavlovic, 1999). The mainly targeted organ for toxicity and carcinogenicity is the liver, followed by the kidney (Bennett & Klich, 2003; Marin et al., 2013). Aflatoxin consumption increases the risk of hepatocellular carcinoma synergistically in people who are chronically infected with hepatitis B virus (Groopman & Kensler, 2005). Table 2.7 shows several aflatoxicosis outbreaks in tropical countries. Clinical evaluations of these cases indicated acute liver injury (Tandon, 1977). In tropical countries, aflatoxins have been detected in the blood of pregnant women, in neonatal

umbilical cord blood, and in breast milk (human 3-30, 31). Significant geographic and seasonal variations are frequently observed in their field researches (Hendrickse, 1991; Maxwell, 1989).

Furthermore, studies have demonstrated that exposure to aflatoxin can cause various adverse health effects, such as growth retardation in children. Abdulrazzaq et al. collected 166 pairs of cord/maternal blood from two hospitals in Al Ain, United Arab Emirates, and found that aflatoxin M_1 can be detected from all 43 samples from low-birthweight infants (< 2500g). Also, strong negative correlation was suggested between aflatoxin levels and birthweight ($r = - 0.565$, $p < 0.001$) and between maternal serum aflatoxin M_1 concentration and birthweight ($r = - 0.654$, $p = 0.0001$) (Abdulrazzaq et al., 2004). Turner et al. conducted a cross-sectional study in Gambian infants, measuring height and weight for 138 infants monthly for 12 months consecutively (Turner et al., 2007). Aflatoxin-albumin adduct levels were measured in maternal blood, in cord blood and in infants at 16 weeks. They found that AF-albumin adduct level in maternal blood was a strong predictor of weight ($p = 0.012$) and height ($p = 0.044$). A reduction of maternal aflatoxin-albumin from 1100 pg. /mg to 10 pg. /mg would lead to a 0.8 kg increase in weight and 2 cm increase in height during the first year of growth. Another cross-sectional study carried out in Kisumu District, Kenya (Okoth & Ohingo, 2004) examined 242 children from the household. Weight, height and aflatoxin level of the flour were measured. Only 28% of non-wasted children had flour with detectable aflatoxin level while about 54% of the wasted children were from household with aflatoxin contaminated flour, suggesting a significant association of aflatoxin exposure and child wasting ($p = 0.002$).

History of Dried Blood Spot Sampling Method

First described by Ivar Bang, a new blood sampling method based on the use of dry matrix intrigues the continuously interest in exploring the dried blood spot technology (Lehmann et al., 2013). This alternative sample collecting method is called "dried blood spot" (DBS). For the longitudinal studies, repeated samplings are usually conducted, meaning a single participant may need to provide about 20ml to 30ml blood. Although using serum or plasma is the golden standard for currently biomarker measurement, the costs, participant burden and storage and transportation of the venipuncture blood samples pose great barriers to the field study, especially in the remote field settings where access to experimental instrument are limited (McDade, 2014). Another concern about the venipuncture method is that since it needs 5-10ml whole blood per time from per subject, the relatively large amount of blood required makes the serial sampling difficult (Sharma et al., 2014).

In 1963, Guthrie et al. applied the DBS in the analysis of phenylketonuria in neonates (Guthrie & Susi, 1963), and since then the DBS method gained popularity in bio-analysis (Sharma et al., 2014). Filter paper has been used to collect blood samples from public health purpose for almost 40 years now. In 2011 more than 35,000 DBS samples has been collected for research purpose in the US, including application in large surveys like the National Longitudinal Study of Adolescent Health and the Health and Retirement Study (Mei et al., 2001). Worthmann et al. described direct immunofluorometric assays for luteinizing hormone and follicle-stimulating hormone in finger-stick blood spots dried on filter paper, based on modified commercially available

kits (Worthman & Stallings, 1994). They found that values in dried blood spots are highly correlated with plasma results, therefore results from DBS could be directly converted to plasma equivalents. Furthermore, they measured hormones from DBS samples, and good sample stability for a range of steroids and proteins were documented. They also found a good convertibility from DBS results to plasma/serum equivalent concentrations (Worthman & Stallings, 1997). Hejiden et al. successfully performed the therapeutic drug monitoring of renal transplant patients using DBS. They used HPLC-electrospray-tandem mass spectrometry method to analyze samples and found that the DBS sampling of everolimus gave higher results than venous sampling, though the differences were not significant (Heijden et al., 2009). A group from UK developed a quantification method for dexamethasone in DBS samples using high-performance liquid chromatography (HPLC-MS). The overall recovery of dexamethasone from DBS samples was 99.3% (94.3%-105.7%). The accuracy (related error) and precision (coefficient of variation) values were within the pre-defined limits of ≤ 15% at all concentration. Variations in the volume of blood spotted were found not to affect the performance of the measurement. In addition, the detected concentrations were successfully evaluated using a simple 1-compartment pharmacokinetic model (Patel et al., 2010). Riley's group also reported a good correlation between the results of DBS samples and urinary samples in sphingoid base 1-phosphates level, a putative biomarker for fumonisin B_1 inhibition of ceramide synthesis in humans, in animal model (Riley et al., 2015). In 2015, Cramer et al. measured mycotoxin ochratoxin A (OTA) and its thermal degradation product 2'R-ochratoxin A for the first time in biological samples from coffee consumers. Only in the blood of coffee drinkers was 2'R-ochratoxin detected, with mean concentration of 0.11

μl/L and maximum concentration of 0.414 (Cramer et al., 2015). The same group then established and validated a multi-mycotoxin approach for the detection of 27 mycotoxins and metabolites in both dried blood spots and dried serum spots based on a fast sample preparation followed by sensitive HPLC-MS/MS analysis (Osteresch et al., 2017).

Studies have demonstrated that exposure to aflatoxin can cause various adverse health effects, such as growth retardation in children. Abdulrazzaq et al. collected 166 pairs of cord/maternal blood from two hospitals in Al Ain, United Arab Emirates, and found that aflatoxin M_1 can be detected from all 43 samples from low-birthweight infants (< 2500g). Also, strong negative correlation was suggested between aflatoxin levels and birthweight ($r = - 0.565$, $p < 0.001$) and between maternal serum aflatoxin M1 concentration and birthweight ($r = - 0.654$, $p = 0.0001$) (Abdulrazzaq et al., 2004). Turner et al. conducted a cross-sectional study in Gambian infants, measuring height and weight for 138 infants monthly for 12 months consecutively (Turner et al., 2007). Aflatoxin-albumin adduct levels were measured in maternal blood, in cord blood and in infants at 16 weeks. They found that AF-albumin adduct level in maternal blood was a strong predictor of weight ($p = 0.012$) and height ($p = 0.044$). A reduction of maternal aflatoxin-albumin from 1100 pg. /mg to 10 pg. /mg would lead to a 0.8 kg increase in weight and 2 cm increase in height during the first year of growth. Another cross-sectional study carried out in Kisumu District, Kenya (Okoth & Ohingo, 2004), examined 242 children from the household. Weight, height and aflatoxin level of the flour were measured. Only 28% of non-wasted children had flour with detectable aflatoxin level while about 54% of the wasted children were from household with aflatoxin contaminated flour, suggesting a significant association of aflatoxin exposure

and child wasting ($p = 0.002$). In a more recent cross-sectional study conducted in Kumasi, Ghana (Shuaib et al., 2010), the adverse health effect of aflatoxin exposure to children had been further proved. 785 women were included in this study and their blood aflatoxin B_1-albumin levels ranging from 0.44 to 268.73 pg/mg. Participants were divided into 4 quartiles base on the blood concentrations of aflatoxin B_1-lysine adduct and all the data were adjusted by sociodemographic variables. Shuaib et al. found that mothers in the quantile with highest aflatoxin B_1-lysine level had a significantly higher risk of having low-weight babies (< 2.5 kg, odds ratio = 2.09). Although the association was not significant, mothers in the highest quantile also suggested an increased risk of having preterm deliveries and stillbirths. In a successional study conducted in West Africa, Gong et al. measured 480 children from 4 geographic areas and found that negative correlations between aflatoxin-albumin level and all the three growth parameters were significant ($p=0.001$ for height for age, $p=0.005$ for weight for age, and $p=0.047$ for weight for height) (Gong et al., 2003). A subsequent longitudinal study (Gong et al., 2004) measured 200 children aged 16-37 months from 4 different villages in Benin. After adjusting for age, sex, height at recruitment, socioeconomic status, village, and weaning status, the data showed that there is a strong association between aflatoxin-albumin level and the height increase ($p<0.0001$) over the 8-month follow-up. Children with the lowest aflatoxin exposure were 1.7cm higher on average than children with the highest exposure. Due to the special characteristics of children, DBS sampling could potentially be applied in these studies.

DBS sample collection method only needs 5 to 6drops of capillary blood from each participant (finger or heel prick, about 50ul per blood) to be placed on the blotting

paper. Aside from the small volume, DBS method also offers many other advantages. Firstly, blood collection process is easy to perform and relatively non-invasive(Sharma et al., 2014). DBS cards can even be made by the patients/participants at home without the presence of medical specialists. Secondly, the sampling procedure takes far less blood volume with minimized pain, making the repeat blood sampling possible, especially from elderly or infants. After drying for hours in the room temperature, the DBS cards then can be packed with desiccant and transported to destination by regular mail with a reduced risk of contamination. Also, the thin cards are easy to store at the laboratories or biobanks. Concerning sample stability, many studies have shown that most components from whole blood are stable at room temperature for at least 7 days. In some cases, such as opiates, stability can even increase during storage (Lehmann et al., 2013).

Method of Determination of Aflatoxins

Detection of aflatoxins in various substrates is greatly facilitated.by their fluorescence under the ultraviolet light (Wogan, 1966). Further investigations show that aflatoxins are extractable with methanol (Braithwaite et al., 1985). A variety of extraction procedures have since been developed for the determination of aflatoxins (Wogan, 1966). Rapid, on-site tests can only determine the possible presence of aflatoxin providing no quantitative or definitive results (Hurbraugh, 2005). However, toxins are mostly produced within the kernels for food crops like corn, therefore their presence must be determined by analytical tests (Hurbraugh, 2005).

On-site test for aflatoxins

The ultraviolet light test is a visual inspection for the presence of a greenish-yellow fluorescence under light at wave length of 365 nanometers. A color standard is required in this method. More than four glowing particles per 5-pound sample indicate a likelihood of a 20-ppb level of aflatoxin. Laboratory analysis is required to verify the accuracy since this test is only an initial screening. In previous aflatoxins outbreaks, very few samples with over 20ppb of aflatoxin were non-detectable (Wacoo et al., 2014).

Commercial test kits with immunoassay (or ELISA) techniques are available for on-site tests for aflatoxin. Through identifying specific proteins found in aflatoxin with antibodies, immunoassay analysis can quantify the presence of aflatoxin. Entire grains should be ground before testing, though a benefit of the test kit is that it would require a very small amount of ground grain.

Laboratory analysis of aflatoxins

Chromatographic techniques are based on the physical interaction between a mobile phase and a stationary phase. The components to be separated are distributed into the two phases (Braithwaite et al., 1985). The various constituents in the analytes travel at different speeds thus resulting in different distribution between the mobile and the stationary phases. Thin-layer chromatography was first used by Iongh et al. and has been recognized by the Association of Official Analytical Chemistry (AOAC) as the method of choice in 1990 (Iongh et al., 1964). The stationary phase of a thin-layer chromatography is usually made of silica or alumina; while the mobile phase is consist of methanol,

acetonitrile and water mixture (Betina, 1985). Aflatoxins as low as 1-20 ppb have been reported of detectable (Trucksess et al., 1984). High-performance liquid chromatography is one of the most popular techniques for aflatoxin separation and determination (Li et al., 2011). It makes use of the stationary phase confined to either a glass or a plastic tube, and a mobile phase comprising aqueous/organic solvents, which flow through the solid absorbent. The analytes are attributed differently within the stationary phase (Malviya et al., 2010) through chemical as well as physical interactions with the stationary and mobile phases (Rahmani et al., 2009). The sensitivity of high-performance liquid chromatograph can be as low as 0.1 ng/kg (Herzallah, 2009).

Immunochemical techniques are also popular in aflatoxin detection. These methods rely on the specificity binding between antibodies and antigens. Radioimmunoassay, enzyme-linked immunosorbent assay, immune-affinity column assay, and immunosensors are the frequently used immuno-chemical methods in aflatoxin analysis (Wacoo et al., 2014). Enzyme-linked immunosorbent assay is a safer alternative of radioimmunoassay. Avrameas achieved the separation of enzyme-antigen conjugates and enzyme-antibody conjugates bin 1969 (Avrameas, 1969), which underlined the development of enzyme-linked immunosorbent assay. This technique is largely used in the detection of aflatoxins in agriculture products nowadays (Anjaiah et al., 1989; Kawamura et al., 1988).

Tables

Table 2.1 Spectral data summary for aflatoxins.

Aflatoxin	**Ultraviolent absorption** *265nm* *363nm*	**Fluorescence emission (nm)**
B_1	13,400 21,800	425
B_2	9,200 14,700	425
G_1	10,000 16,100	450
G_2	11,200 19,300	450

Table 2.2 Molecular data summary for aflatoxins.

Aflatoxin	**Molecular formula**	**Molecular weight**	**Melting point (°C)**
B_1	$C_{17}H1_2O_6$	312	268-269
B_2	$C_{17}H_{14}O_6$	314	286-289
G_1	$C_{17}H_{12}O_7$	328	244-246
G_2	$C_{17}H_{14}O7$	330	237-240

Table 2.3 FAO/WHO/UNEP Monitoring Program: aflatoxins in maize and maize products.

Country	Year	No. of samples	Median (µg/kg)	90th percentage (µg/kg)
Maize				
Brazil	1981	228	< 8.0	< 8.0
Canada	1976	25	< 4.0	< 4.0
Guatemala	1976-79	231	< 4.0	4.0-360.0
Kenya	1978-79	78	< 0.1-70	30-1920
Mexico	1979-80	96	< 2.5-10.0	< 2.5-30.0
United Kingdom	1978	29	5.0	8.0
USA	1978-83	2633	< 1.0-80.0	10.0-700.0
USSR	1981-82	219	< 1.0	< 1.0-662.0
Maize products				
Brazil	1983	20	4.0	6.0
Canada	1977-79	22	< 4.0	10.0-20.0
Guatemala	1978-79	283	< 10.0	< 10.0
Kenya	1978	217	< 2.0	Not reported
Mexico	1978	40	< 0.5	2.0
United Kingdom	1978	13	< 0.1	< 0.1
USA	1978-83	174	< 1.0	< 1.0-56.0
USSR	1976	87	< 1.0	5.0

Table 2.4 Number of *Aspergillus flavus* isolates that contaminated peanuts in eight different U.S. states.

State or Country	No. of Isolates	Aflatoxin B_1 in peanuts (ppb)
Alabama	7	2,870-17,000
Florida	1	6,900
Georgia	2	70-2,450
New Mexico	1	0
North Carolina	1	2,760
Texas	12	280-610
Uganda	1	2,400
Virginia	11	180-9,200

Table 2.5 Aflatoxins in raw, shelled groundnuts imported into the USA, 1981.

Origin	No. of lots	Lots (%)	
		Determinable	**> 20 µg/kg**
China	2585	15	2.5
India	1453	92	58.0
Sudan	932	94	78.0
Argentina	446	40	4.5
South Africa	112	41	21.0
Malawi	80	60	10.0
Australia	52	10	4.0
Brazil	44	100	95.0
Egypt	41	14	2.0
Taiwan	37	27	0.0
USA	172669	20	3.0

Table 2.6 Comparison of lethality of single doses of aflatoxin B_1.

Animal	**Age (or weight)**	**Sex**	**Rout**	**LD_{50}** mg/kg
Dcukling	1 day	M	PO	0.37
	1day	M	PO	0.56
	1 day	M-F	PO	1
Rat	21 days	M	PO	5.5
	21 days	F	PO	7.4
	100g	M	PO	7.2
	100g	M	ip	6.0
	150g	F	PO	17.9
Hamster	30 days	M	PO	10.2
Guinea pig	Adult	M	ip	ca. 1
Rabbit	Weanling	M-F	ip	ca. 0.5
Dog	Adult	M-F	ip	ca. 1
	Adult	M-F	PO	ca. 0.5
Trout	100 g	M-F	PO	ca. 0.5

Table 2.7 Outbreak of aflatoxicosis.

	No. of Subjects	Symptoms and signs	Exposure			Material analyzed	Liver histopathology
			Source	Duration	Toxin		
Uganda	1;**1**[a]	Abdominal pain, edema of legs, palpable liver, on ECG prolongation of P-R interval, right bundle branch block	Cassava	5-30 days	Aflatoxin 1.7 ppm	_	Centrilobular necrosis, polymorphonuclear infiltration and fibrin in sinusoids, fatty changes in midzonal region
India	397; **106**	Brief febrile episode, vomiting, anorexia, jaundice, ascites, edema of legs, massive gastrointestinal bleeding	Maize	Several weeks	Aflatoxin B_1 6.25-15.6 ppm	Serum Urine	Bile duct proliferation with periductal fibrosis, multinucleated giant cells, foamy cytoplasm, bile stasis in bile ducts, dilated biliary canaliculi
Kenya	21; **12**	Brief febrile episode, vomiting, anorexia, jaundice, ascites, vomiting, abdominal discomfort, anorexia, jaundice, edema of legs, ascites, tachycardia, tenderness of liver, melaena, gastrointestinal bleeding	Maize	Several weeks	Aflatoxin B_1 1.6-2.7ppm	Liver (autopsy)	Marked centrilobular necrosis, slight fatty infiltration, and no proliferation of bile ducts
USA	1; **0**	Non-pruritic macular rash, nausea, headache	Purified aflatoxin B_1	2 days	Aflatoxin B_1 5.5 mg	_	normal

Figures

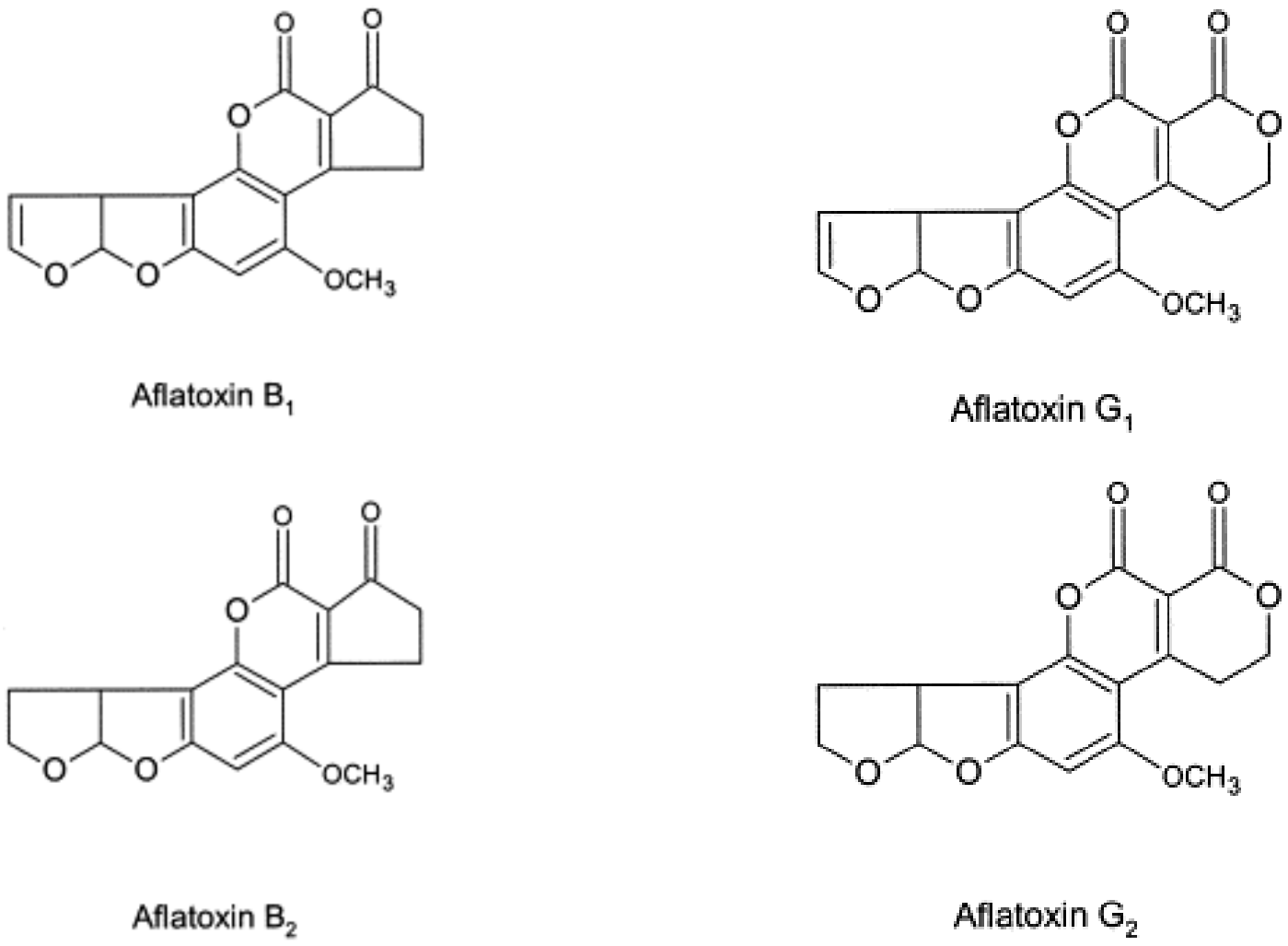

Figure 2.1 Molecular structures of aflatoxins B_1, B_2, G_1, and G_2.

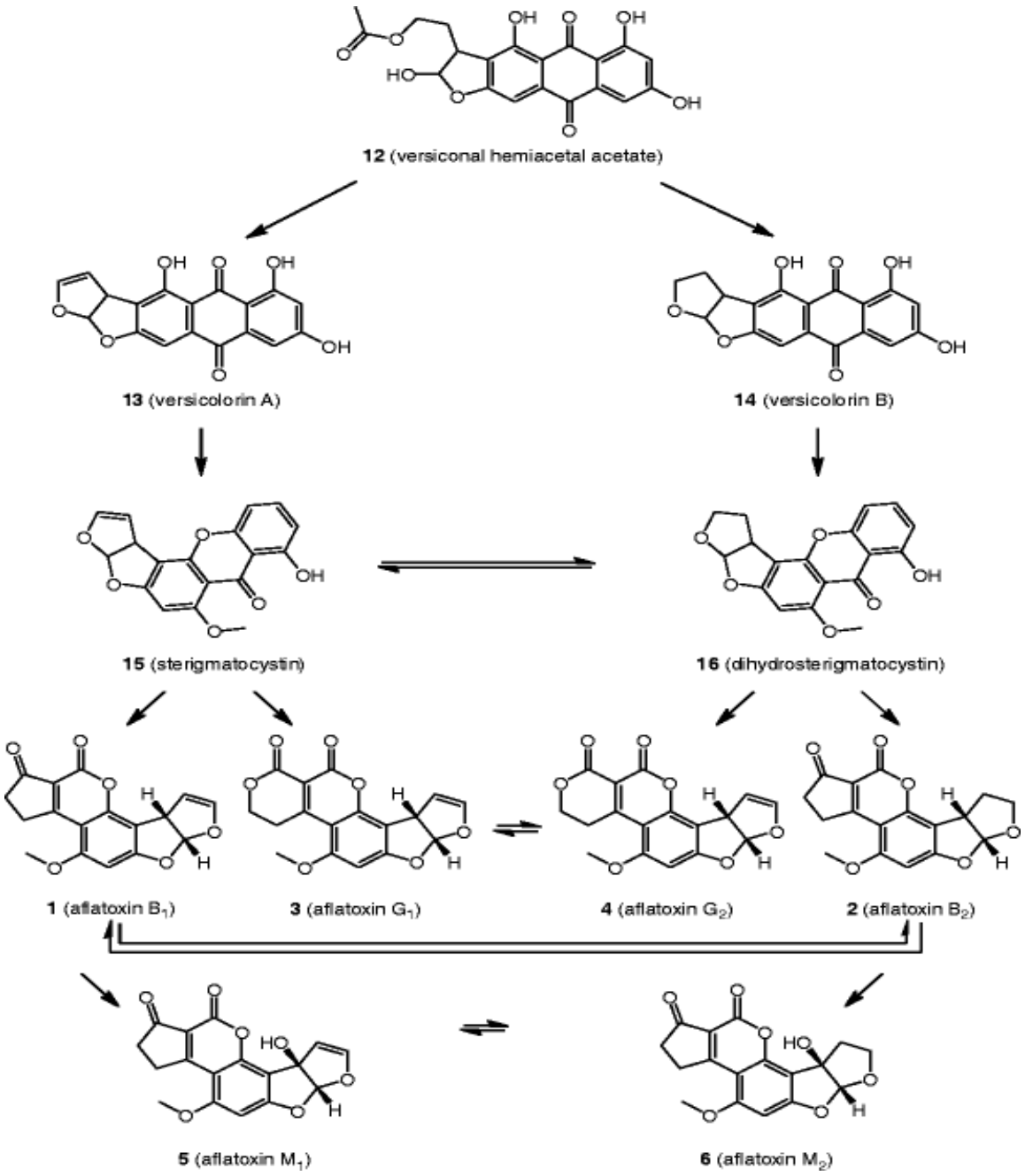

Figure 2.2 Biosynthesis of aflatoxins B_1, B_2, G_1, G_2, M_1 and M_2.

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CHAPTER 3

ANALYSIS OF AFLATOXIN B_1-LYSINE ADDUCT LEVELS IN DRIED BLOOD SPOT SAMPLES OF ANIMALS AND HUMANS

Introduction

Since their discovery in the 1960s, aflatoxins have been studied, monitored, and regulated in the United States and worldwide. *Aspergillus flavus* and *A. parasiticus* are the two main strains of fungi that produce aflatoxins (Bennett & Klich, 2003). Contamination may occur during growing, harvesting, storage or even processing. Aflatoxins are very heat stable and are not destroyed in many cooking and processing methods. Food contaminated with aflatoxins may cause harm to humans and animals, especially if consumed regularly. Exposure to aflatoxins has been shown to be carcinogenic in studies of different animal species and can lead to human hepatocellular carcinoma (HCC) (Jackson & Groopman, 1999). Aflatoxins also have mutagenic, toxic, and immunosuppressive effects and potentiate other diseases (Brase et al., 2013).

First described by Ivar Bang, a new blood sampling method based on the use of dry matrix intrigues the continuously interest in exploring the dried blood spot technology (Lehmann et al., 2013). This alternative sample collecting method is called "dried blood spot" (DBS). Compared to the traditional vein puncture sampling method, DBS sampling is less invasive to the participants and breaks the barriers to filed studies at remote areas (McDade, 2014). After drying three hours at room temperature, filter papers

that contain the whole blood can be easily stored and transported to the laboratories. Due to these unique benefits, the DBS method gained popularity in bio-analysis(Sharma et al., 2014). In 2011 more than 35,000 DBS samples has been collected for research purpose in the US, including application in large surveys like the National Longitudinal Study of Adolescent Health and the Health and Retirement Study (Mei et al., 2001).

However, to our knowledge, there are currently few studies on assessing aflatoxin exposure using DBS samples in young populations. The aims for the current study is thus to validate a previously developed method for quantification of aflatoxins B_1-lysine levels in DBS samples, using both samples from animals treated with single- and repeated-doses of aflatoxin B_1, as well as human DBS samples of known aflatoxin B_1 exposure

Materials and methods

Reagents and chemicals

Albumin standard was purchased from Point Scientific (Canton, MI). Albumin DMA reagent was purchased from Thermo Fisher Scientific (Waltham, MA). The total protein standard was purchased from Sigma-Aldrich (St. Louis, MO). Dye reagent concentrate coomassie brillian blue was obtained from Bio –RAD (Hercules, CA). Ammonium phosphate monobasic powder purchased from Honeywell Fluka, Fisher Scientific (Waltham, MA). Sodium azide and normal human serum were obtained from Sigma-Aldrich (St. Louis, MA). Pronase protein was purchased from Calbiochem (San Diego, CA). The Dulbecco's phosphate buffer saline was purchased from American Type Culture Collection (Manassas, VA). Ammonium hydroxide, formic acid and phosphoric

acid from Fisher Scientific (Hampton, NH). HPLC grade water and 2-propanol were purchased from J.T. Baker Chemical Company. Methanol and acetonitrile were purchased from Honeywell (Morris Plains, NJ). Dimethyl sulfoxide (DMSO); Unlabeled aflatoxin B_1 standard (>98% purity); aflatoxin B_1-lysine was synthesized and purified separately in laboratory. ELISA kit for human albumin analysis (ab108788) was obtained from Abcam (Cambridge, MA). Normal human whole blood was purchased from Biological Specialty Corporation (Colmar, PA).

Sample preparation and extraction

Seven commercial DBS cards, Ahlstrom 226, Munktell TFN, GE Whatman DMPK, C-31 ETF base paper, GE What man 903 protein saver, GE Whatman DMPK A, and GE Whatman DMPK B card, were purchased separately from different commercial sources. After testing the capacity of holding whole blood and serum, GE Whatman 903 protein saver was selected in this study.

50µl whole blood was added onto the filter paper to saturate the circular spaces for a typical blood sample, while for serum and other less viscous samples 20µl was added to prepare the dried serum spot. Store the DBS cards in air-tight Ziploc bags in 4 °C until use after drying at room temperature for 4 hours. All the DBS cards were purchased from GE Healthcare Bio-Science (Pittsburgh, PA).

The extraction and analysis method used are shown in Figure 3.1. Firstly, the spot discs were cut and dispensed into the cell culture cluster, two discs per well. Then 500µl PBS was added into each well and the plate was shaken with plate shaker at speed level 6 for 30 minutes. The eluents were then extracted and stored in Eppendorf tubes. PBS

elution was then repeated once and the eluents from the two washes were combined. To optimize the washing strategy, 50 ml of whole blood, as well as 20, 40, and 60 ml normal human serum were spotted onto filter paper. The spots were cut out and washed with 1 × PBS using plate shaker for three times, and individual washes were collected separately, and analyzed for albumin and total protein based on method described previously(Qian et al., 2013).

Sample extraction was performed using a method previously reported by our lab(Qian et al., 2013). First, 500µl of the eluent were aliquoted and then digested in water bath at 37 degrees Celsius for three hours with 70 ml 10mg/ml pronase. The contents were then purified by solid phase extraction, using Oasis MAX 1cc extraction cartridge purchased from Waters Corporation. Final eluent in 1ml 2% formic acid in methanol was vacuum-dried with a Labconco Centrivap concentrator (Kansas City, MO). The contents were then reconstituted with 150µl 25% methanol in water and transferred to sample vials for HPLC injection.

HPLC conditions for aflatoxin B_1-lysine analysis

The Agilent Technology 1100 Series HPLC value system was used for separation and quantification of target analytes. Detection was completed using the fluorescence detector. The HPLC and detector conditions are shown in Table 3.1. Excitation and emission wavelengths for fluorescence detection were 470 nm and 405 nm, respectively. Chromatographic separations were achieved using Zorbax Eclipse XDB-C18 reverse phase column (5 mm, 4.6 × 250 mm), with a gradient of Buffer A and B (20 mM $NH_4H_2PO_4$, pH 7.2, and 100% methanol, respectively), to achieve separation within 25

min at flow rate of 1.0 mL/min. Injection volume for all samples were 100 ml. Retention time for aflatoxin B_1-lysine adduct peak was 13.1 min. Quality assurance and quality control procedures were taken during analyses, which included simultaneous analysis of one authentic standard and a quality control sample per sequence. Final results were manually integrated and calculated using a standard curve and adjusted by albumin content of corresponding samples. Adducts in DBS was further confirmed and validated using LC/MS/MS technique.

Method Validation

The accepted criteria for biological method validation were followed in validating the method in this study. Seven key parameters were evaluated, including selectivity, recovery, matrix, linearity, precision, accuracy, and limits of detection and quantification. Aflatoxin B_1-lysine working solutions was diluted from purified aflatoxin B_1-lysine, ranging from 10 to 5000 pg/ml. Separate calibration curves were generated for DBS and serum on different days. Linearity follow the extraction was tested using these calibration curves. Results were analyzed using weighted least-squares regression analysis. A series of diluted standards were injected into the PHLC, to determine the limit of detection (LOD) and the lowest limit of quantification (LLOQ). The LOD was defined as the lowest aflatoxin B_1-lysine standard concentration with signal-to-noise (S/N) ratio of 3:1 in 3 consecutive injections. The LLOQ was defined as the lowest aflatoxin B_1-lysine standard concentration with signal-to-noise (S/N) ratio of 10:1. Five replicates each for three different concentrations of standards were added to blank samples, extracted as described above, and analyzed to determine intra-assay precision and accuracy. The inter-

assay precision and accuracy were determined for three independent experiments of the replicates. Inter-run precision was expressed as the relative standard deviation (RSD) of concentrations calculated for quality control samples. Inter-run accuracy was expressed as the relative error of the calculated concentrations. Recovery rate was determined by analyzing spiked DBS, prepared via adding known amounts of aflatoxin B_1-lysine adduct (at concentration of 50, 200 and 1000 pg/mL) to whole blood before creating the DBSs. The peak area of standard without processing was represented as 100 percent. The recovery rate was calculated as the ratio between the mean peak areas of three processed samples and that of unprocessed standards. Stability of aflatoxin B_1-lysine in dried blood spots was determined using batches of DBS aliquots obtained from aflatoxin B_1-treated rats. Room temperatures (18-23 °C) stability for up to 24 h was assessed by analyzing samples left at room temperature for 12 and 24 h, while the long-term stability storage at 4 °C refrigerator was assessed via analyzing samples stored for 1, 6 and 12, months. The stability was expressed as percentages of the initial concentration of the analytes spiked, quantified just after preparation.

Validation study designs of animal and human samples

Male F344 rats (120-130 g) purchased from Harlan Laboratories Incorporation (Indianapolis, IN) were used in this study. Animals were acclimated one week prior to treatment and maintained under controlled conditions. All animal use and care and experimental protocol were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Georgia and in compliance with NIH regulations. The detailed experimental design for animal validation studies were outlined

in Fig. 3.2. 50µl blood was applied to the DBS card and dried at room temperature for 4 hours, and the rest of the blood was centrifuged to prepare the serum. Results from DBS samples were compared to those of corresponding serum adjusted to equivalent volumes. DBS cards and serum samples for human validation study were collected from a previously conducted cohort study focusing on the linkage between aflatoxins exposure with various birth outcomes in Kenya. Participant samples were grouped into 3 categories: low, medium and high aflatoxin B_1 exposure in DBS samples and serum samples, respectively. Statistical analysis was achieved by using SAS 9.4 (SAS Institute, Cary, NC, USA).

Results

Whatman 903 protein saver card was used for the highest holding capacity and lowest interference among all the tested commercial DBS cards. The total protein recoveries for normal human spots were 112.3% after first wash, 92.6% after second wash, and 84.4% after third wash; 74.3%, 95.2%, and 103.3% for albumin recoveries, respectively. In all the cases, the first and second eluents constituted up to 100% of the analytes, with content in the third wash close to 0%. Results in whole blood spots were the same. Consequently, only two washes were applied in the subsequent extractions.

Within the range of 10 - 5000 ng/mL, the quantitation of this method is subjected to linear regression. The correlation coefficient for the standard curve is 0.9997. The LOD was determined to be 0.2 pg. /mg albumin and the LLOQ was 0.4 pg/mg albumin. Inter- and intra-assay data for accuracy and precision in DBS were shown in Table 3.2. The recovery rates were 68.01%, 76.00%, and 71.67% for three concentrations 0.05, 0.2

and 1.0 ng/ml, respectively. The stability of AFB_1-lysine was tested in DBS samples collected over 12 months and data shown in Fig. 3.3.

F344 rats were single- or repeated dosed with aflatoxin B_1; and DBS and serum samples were collected from the rats. Aflatoxins B_1-lysine levels of both samples types were measured and compared, as shown in Table 3.3 and Fig. 3.4. When treated with single- or repeated dose, the correlation between aflatoxin B_1 doses and aflatoxins B_1-lysine levels in serum are positive, and there is dose-response in aflatoxin B_1-lysine levels to aflatoxins B_1 doses, too. According to the linear regression model, at 2 hours post-treatment every 1.0 mg/kg increase in aflatoxin B_1 concentration was associated with an increase of 24.34% (CI: 21.97-26.71%) in aflatoxin B_1-lysine level.

The agreement analysis of aflatoxin B_1-lysine adduct levels for DBS samples of single- and repeated-dose studies were further conducted, and outcomes were shown in Fig. 3.5 A and B. Predicted regression lines for individual time points all have positive slopes, showing good dose-response in the AFB_1-lysine levels obtained via DBS samples for both single- and repeated-dose studies. The regression lines for different time points for both single- and repeated-dose studies are approximately parallel, with different intercepts predicted by the expected patterns of dose designs. The pattern observed for DBS samples were similar to that of serum samples (Fig. 3.4). The Pearson coefficient of correlation is 0.997 for single-dose and 0.996 for repeated-dose ($p < 0.0001$ for both; Fig. 3.5 A and B). Bland-Altman plot analysis of the log difference showed that for both dosing regiments, the distribution of the log differences between DBS and serum aflatoxin B_1-lysine levels is within 95% confidence interval, thereby showing good agreement between the two sample types.

Discussion

There is a trend in directly assessing aflatoxin B_1 exposure to infant and young children instead of quantifying the maternal levels of serum aflatoxin B_1-lysine or milk aflatoxin M_1. Sampling from the infant or young children requires drawing blood directly from the young participants. This DBS procedure would be naturally non-invasive and easy to practice. Blood sampling with DBS therefore could be performed even in absence of the specialists. Another benefit would be sample storage and transportation as most of the population studies are conducted in poor economic regions and have limited resources. Lastly, studies like longitudinal design, which require repeated sampling at different time points during initial stages could also benefit from the dried blood sampling method. Therefore, DBS-based measurement is highly valued and largely recommended.

Study presented here focused on the development and validation of a novel HPLC-fluorescence method to accurately detect the aflatoxin B_1-lysine adduct level in DBS samples. Overall the DBS results are highly correlated with the matched serum samples results, which is the most common matrix for aflatoxin B_1-lysine measurement. Washing procedure also showed a good recovery of albumin and total proteins contained in the DBS samples, further demonstrated the holding capacity of the DBS cards. For the animal validation study, aflatoxin B_1-lysine adduct level from DBS showed a good dose- and time- response for both sing- and repeated-dose treatments, which is matched with the previously reported kinetics studies (Qian et al., 2013). For the human validation studies, aflatoxin B_1-lysine adducts could be detected in majority of the samples and showed a good correlation with the results of the corresponding serum samples.

Two concerns about the method would be the interference of the hemoglobin and the relatively small volume of blood (Li & Tse, 2010; McDade et al., 2007; O'Mara et al., 2011). For the first concern, the final aflatoxin B_1-lysine adducts level in samples were normalized by albumin level as this adduct is found predominantly on serum albumin. As for the small sample volume of DBS, it would be vital to recover the maximum of protein from the blood spot card. A recovery rate over 100% has been achieved through a second wash of the DBS card. Due the complex analytes in whole blood, there could be matrix effect that interfering the measurement of aflatoxin B_1-lysine adducts and the albumin. Thus, further confirmation of the results might be required, especially in the low adduct concentration group.

Studies have demonstrated that exposure to aflatoxin can cause various adverse health effects, such as growth retardant in children. Turner et al. conducted a cross-sectional study in Gambian infants, measuring height and weight for 138 infants monthly for 12 months consecutively (Turner et al., 2007). Aflatoxin-albumin adduct levels were measured in maternal blood, in cord blood and in infants at 16 weeks. They found that AF-albumin adduct level in maternal blood was a strong predictor of weight ($p = 0.012$) and height ($p = 0.044$). A reduction of maternal aflatoxin-albumin from 1100 pg. /mg to 10 pg. /mg would lead to a 0.8 kg increase in weight and 2 cm increase in height during the first year of growth. In a successional study conducted in West Africa, Gong et al. measured 480 children from 4 geographic areas and found that negative correlations between aflatoxin-albumin level and all the three growth parameters were significant ($p=0.001$ for height for age, $p=0.005$ for weight for age, and $p=0.047$ for weight for height) (Gong et al., 2003). A subsequent longitudinal study (Gong et al., 2004) measured

200 children aged 16-37 months from 4 different villages in Benin. After adjusting for age, sex, height at recruitment, socioeconomic status, village, and weaning status, the data showed that there is a strong association between aflatoxin-albumin level and the height increase ($p<0.0001$) over the 8-month follow-up. Children with the lowest aflatoxin exposure were 1.7cm higher on average than children with the highest exposure. The results from these reported adverse effects of exposing aflatoxin B_1 to children. Due to the special characteristics of children, DBS sampling has been largely applied in the recent studies.

Conclusion

Recovery of protein in the DBS cards is largely improved with the novel modification in sample washing out. Aflatoxin B_1-lysine adduct levels showed a good dose- and time-response to the treatment of aflatoxin B_1 in animal studies. Adducts level in human DBS samples also had a good correlation with the level in corresponding serum samples. Recent studies have suggested the potential adverse effects of aflatoxin B_1 exposure in early childhood, causing stunting and growth retarded. Considering the less invasive nature of DBS sampling method, especially when the repeated sampling is required, DBS sampling is ready to be applied into the measurement of mycotoxin exposure in large filed studies, especially suitable with infant and young children. Overall, this study was successful in developing and validating the HPLC-fluorescence method for aflatoxin B_1-lysine adducts measurement in both animal and human DBS samples.

Tables

Table 3.1 HPLC conditions, gradient program, and column information.

Column	Zorbax Eclipse® XDB-C18 5 μm, 4.6×250mm Agilent, Santa Clara, CA		
Mobile phase A	20mM $HN_4H_2PO_4$		
Mobile phase B	100% Methanol		
Flow Rate	1.0 mL/min		
Column Temperature	25 °C		
Injection Volume	100 μl		
Gradient	Time (min)	A%	B%
	0.00	30	70
	5.00	30	70
	15.00	65	35
	18.00	65	35
	20.00	30	70
	25.00	30	70

Table 3.2 The intra- and inter-assay precision and accuracy for analysis of aflatoxin B_1-lysine adduct in DBS samples (n=5).

AFB_1-lysine (ng/ml)	Precision		Accuracy (%)
	Mean ± SD	RSD (%)	
Intra-assay			
0.05	0.05 ± 0.01	4.10	101.2
0.2	0.21 ± 0.01	6.83	103.4
1	0.99 ±0.04	3.97	99.8
Inter-assay			
0.05	0.05 ±0.00	1.91	98.0
0.2	0.20 ± 0.01	1.59	105.0
1	1.01 ± 0.05	5.00	106.0

Table 3.3 Association between aflatoxin B_1 exposure and aflatoxin B_1-lysine levels in DBS samples based on linear regression model.

Single-dose treatment			Single-dose treatment		
Time	β^a (95% CI)	*p*	Time	β^a (95% CI)	*p*
2 h	24.34 (21.97-26.71)	<0.001	1 week	94.71 (89.24-100.17)	<0.001
24 h	19.8 (18.29-21.3)	<0.001	2 weeks	123.09 (114.65-131.54)	<0.001
3d	19.82 (18.33-21.32)	<0.001	3 weeks	117.18 (109.66-124.70)	<0.001
5 d	23.11 (20.35-25.88)	<0.001	4 weeks	106.47 (104.17-108.77)	<0.001
7 d	7.40 (6.22-8.57)	<0.001	5 weeks	93.67 (88.76-98.59)	<0.001

Figures

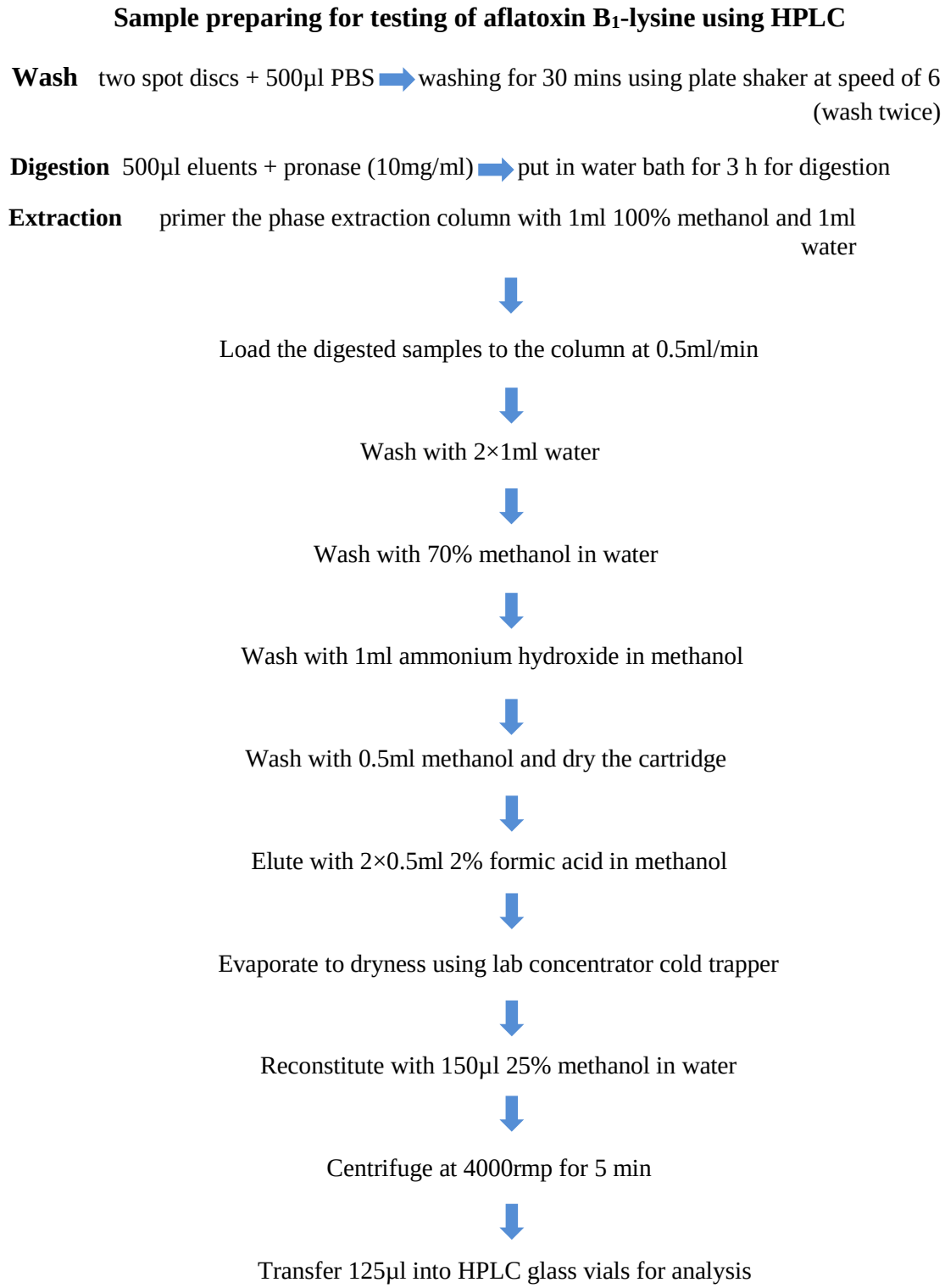

Figure 3.1 Flowchart of basic procedure for sample preparation and HPLC method.

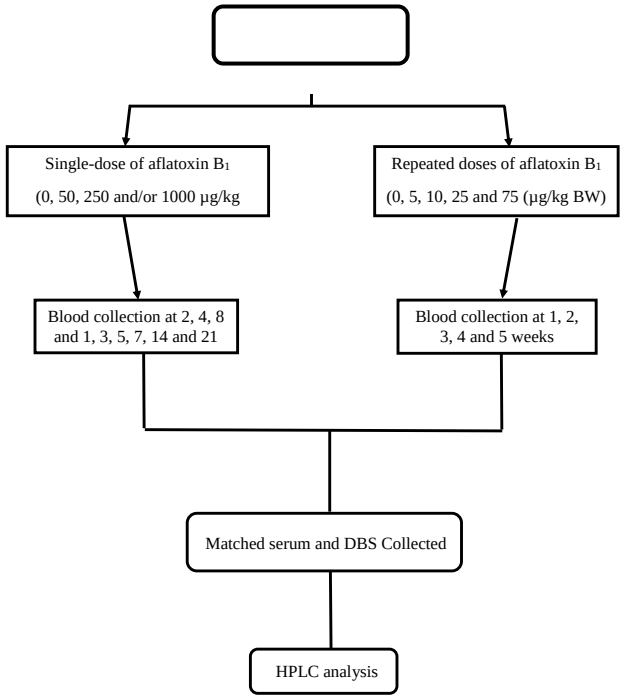

Figure 3.2 Experimental design of F344 rat animal model treated with aflatoxin B_1 for DBS method validation.

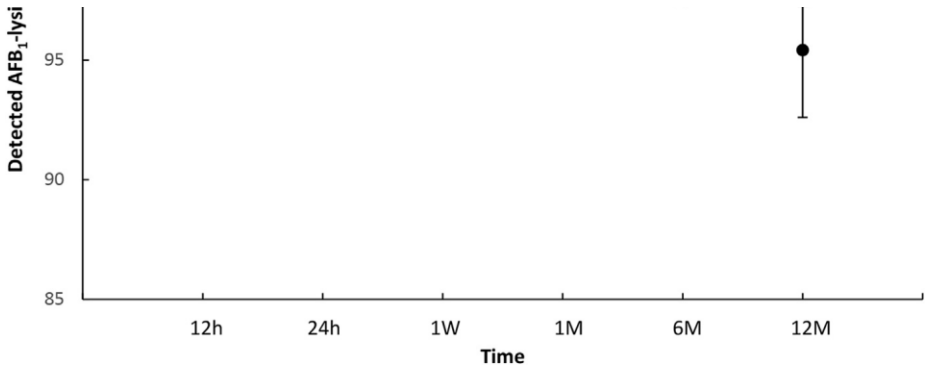

Figure 3.3 Stability test of aflatoxin B_1-lysine in DBS samples over 12-month period. Data expressed as percentage of detected AFB_1-lysine as compared to amount of standard spiked into samples.

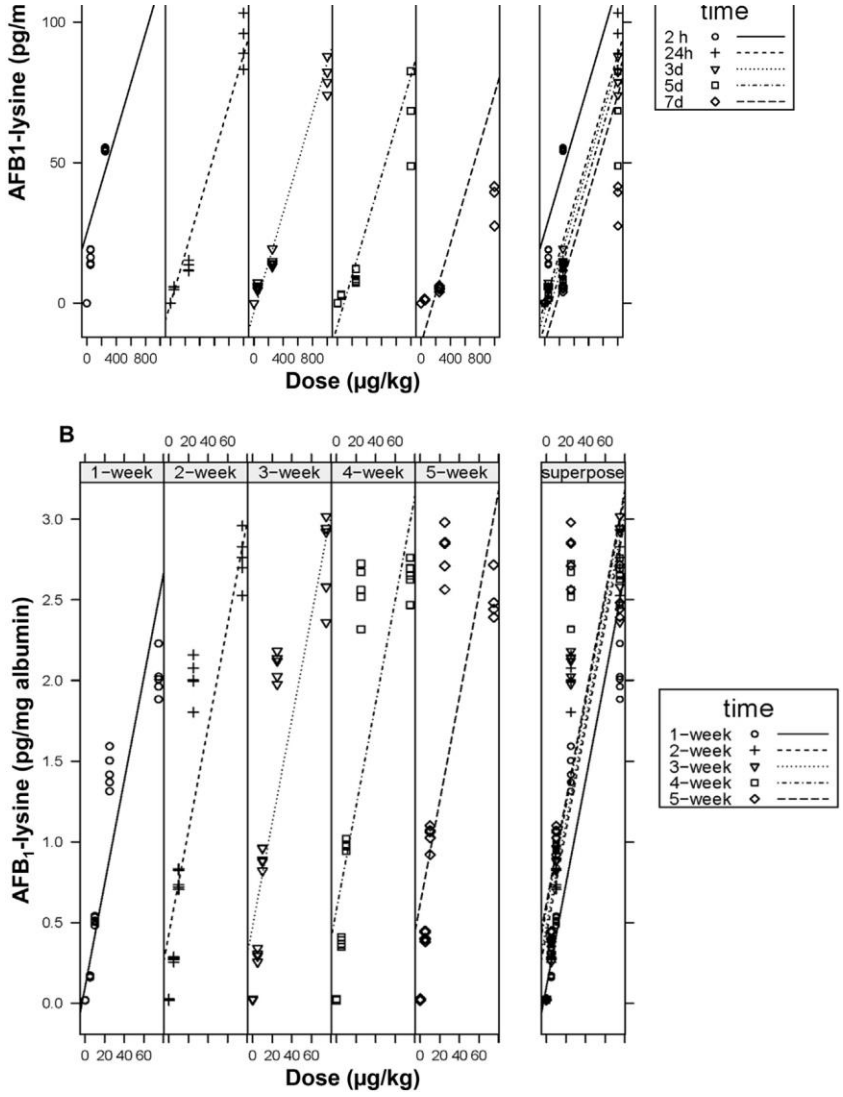

Figure 3.4 Aflatoxin B_1-lysine levels (pg/mg albumin) in DBS samples from F344 rats treated with different doses of aflatoxin B_1. A: Single-dose at 2, 24, 72, 120, and 168 h after treatment; B: Repeated-dose at 1, 2, 3, 4, and 5 weeks after initial treatment.

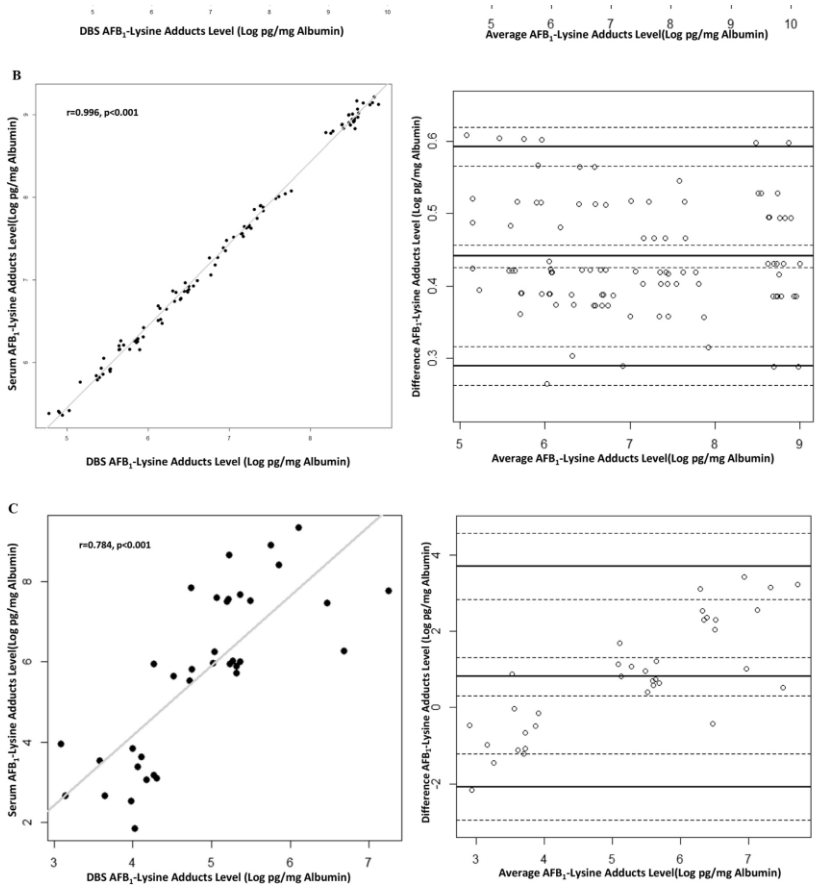

Figure 3.5 Scatter plot and Bland-Altman plot analyses of aflatoxin B_1-lysine levels in DBS samples as compared to corresponding serum samples. A: Animal single-dose; B: Animal repeated dose; C: Human samples. In Bland-Altman plots, center solid lines indicate mean difference between two samples, while upper and lower lines indicate 95% confidence interval.

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CHAPTER 4

ASSESSMENT OF AFLATOXIN EXPOSURE IN DRIED BLOOD SPOT SAMPLES OF INFANTS SUING HPLC-FLUORESCENCE METHOD

Introduction

Aflatoxins are carcinogenic agents that are commonly found in crops during growth, harvest and storage. Exposure to aflatoxins is suggested to cause growth retardation in children, which has been examined in recent years (Khlangwiset et al., 2011). Venipuncture is the most commonly used sample collection technique; however, it causes pains and anxiety in the study subjects. With increasing involvement of young children, dried blood spot sampling gained popularity for sample collection. Compared to the traditional venipuncture method, the DBS sampling is a less invasive way to collect the field samples, especially from children.

Nepal is a landlocked country in South Asia located in the Himalaya with a warm and humid climate. Nepal is also a country with limited resources, so it relies largely on importing food commodities to fees the needs (Koirala et al., 2005). Peanuts, raw materials of vegetable oil (palm oil) and peanut butter are some of the main imports. A study was conducted in 16 districts of the eastern region of Nepal collected food samples from 1995 to 2003, which found the highest contamination of aflatoxins in peanut butter and vegetable oil (42.5%). The concentrations also exceeded the recommended level (20 μg/kg) in Nepal (Gautam et al., 2008). Time to time there has been outbreaks of aflatoxin

toxicity caused by the consumption of moldy grain. Presences of aflatoxin in human blood have also been reported in Nepal. In a study conducted in Bhaktapur, Nepal, a chronic exposure to aflatoxins among children was reported with a geometric mean of 3.62 pg aflatoxin B_1-lysine/mg albumin (Mitchell et al., 2017). Another study conducted in rural South Asia also found exposure to aflatoxin, with 94% detectable samples ranging from 0.45 to 2939.30 pg aflatoxin B_1-lysine/mg albumin in pregnant women (Groopman et al., 2014). Since exposure to aflatoxin B_1 can pose adverse effects in children such as stunting and growth retardation, conducting aflatoxins research in Nepal is of great importance.

Previously our lab has developed a HPLC based method to assess the aflatoxin B_1-lysine adducts from DBS samples. In this study, DBS samples from Nepal were used to validate the detection method. DBS discs were cut and washed with phosphate buffer saline, the eluents were purified and concentrated subsequently, and then the HP LC-fluorescence method was used to detect the concentration of the aflatoxin B_1-lysine adducts in the eluents. The mobile phases were the same as that used to measure the serum samples. The higher the aflatoxin B_1-lysine adducts concentrations in the DBS samples, the better data correlation was observed. Future works should be focused on improving the method's sensitivity at the low dose level.

Materials and methods

Study design

The overall purpose of this study was to further validate the detection method of aflatoxin B_1-lysine adducts, as well as to measure the aflatoxin B_1–lysine adduct level in

dried blood samples and the corresponding serum samples. A larger population was involved in this study. Both dried blood spot samples and serum samples were collected from over 800 participants in Nepal. Aflatoxin B_1-lysine adducts levels in dried blood spot samples from infants were determined and then compared with the results from their matched serum sample. After analysis of all samples, results were collected and compared. The robustness of the method was also improved.

Chemicals and Reagents

Albumin standard was purchased from Point Scientific (Canton, MI). Albumin DMA reagent was purchased from Thermo Fisher Scientific (Waltham, MA). The total protein standard was purchased from Sigma-Aldrich (St. Louis, MO). Dye reagent concentrate coomassie brillian blue was obtained from Bio –RAD (Hercules, CA). Ammonium phosphate monobasic powder purchased from Honeywell Fluka, Fisher Scientific (Waltham, MA). Sodium azide and normal human serum were obtained from Sigma-Aldrich (St. Louis, MA). Pronase was purchased from Calbiochem (San Diego, CA). The Dulbecco's phosphate buffer saline was purchased from American Type Culture Collection (Manassas, VA). Ammonium hydroxide, formic acid and phosphoric acid from Fisher Scientific (Hampton, NH). HPLC grade water and 2-propanol were purchased from J.T. Baker Chemical Company. Methanol and acetonitrile were purchased from Honeywell (Morris Plains, NJ). Aflatoxin B_1-lysine was synthesized and purified separately in laboratory.

Supplies

Whatman 903 drying rack with Velcro was purchased from Sigma-Aldrich (St. Louis, MO). Cell culture clusters (24-well) were purchased from Corning Inc. (Corning, NY). Oasis MAX 1cc extraction cartridge was purchased from Waters Corporation (Milford, MA).Targeted DP vials were purchased from National Scientific Company (Rockwood, TN).Crimp caps for micro vial, sliver crimp cap and DP blue cap with T/RR Septa were purchased from Agilent Technologies (Santa Clara, CA). 12 × 75mm glass tubes were purchased from Fisher Scientific (Waltham, MA). Zorbax Eclipse XDB-C18 reverse phase column was purchased from Agilent Technologies (Santa Clara, CA).

Sample collection and processing

Samples were collected and transported from Nepal. All the dried blood spot samples and serum samples were stored at -80 degrees Celsius upon arrival. There are four shipments of dried blood spot samples in total, with the corresponding serum sample came separately. Sample extraction and elution were completed within hours of HPLC analysis in order to preserve the aflatoxin B_1-lysine structure and concentration in each extraction that was injected into HPLC.

HPLC conditions

The Agilent Technology 1100 Series HPLC Value system was used for separation and quantification of target analytes. Detection was completed using the fluorescence detector. The HPLC and detector conditions are shown in Table 3.1. Excitation and emission wavelengths for fluorescence detection were 470 nm and 405 nm, respectively.

Chromatographic separations were achieved using Zorbax Eclipse XDB-C18 reverse phase column (5 mm, 4.6 × 250 mm), with a gradient of Buffer A and B (20 mM NH4H2PO4, pH 7.2, and 100% methanol, respectively), to achieve separation within 25 min at flow rate of 1.0 mL/min. Injection volume for all samples were 100 ml. Retention time for aflatoxin B_1-lysine adduct peak was 13.1 min.

Sample extraction and analysis using HPLC-fluorescence method

The extraction and analysis method used are shown in Figure 4.1. For dried blood spot samples, firstly blood spot discs were cut into the cell culture cluster, two discs per well. Then 500µl PBS was added into each well and plate was shake with plate shaker at speed level 6 for 30 minutes. The eluents were collected and stored in Eppendorf tubes. The PBS elution was repeated once, and the eluents were combined. For measuring albumin, 5µl eluent was transferred into a separate glass tube, and then added with 1ml DMA reagents and vortexed. The mixed solution was then transferred into plastic cuvette and measured at the spectrophotometer under UV wavelength of 630 nm. For total protein measurement, 5µl diluted eluent was transferred into another glass tube and 200µl coomassie brilliant blue reagent was added into the tube. Total protein was measured via spectrophotometer under the UV wavelength of 595 nm. Both albumin and total protein concentration were calculated according to corresponding standard curves. For HPLC analysis of DBS samples, 500µl of the eluents was digested in water bath at 37 degrees Celsius for three hours with 70 ml 10mg/ml pronase. The contents were then purified by solid phase extraction, using Oasis MAX 1cc extraction cartridge purchased from Waters Corporation. Final eluent in 1ml 2% formic acid in methanol was vacuum-dried with a

Labconco Centrivap concentrator (Kansas City, MO), and then reconstituted with 150µl 25% methanol in water. The content was vortexed, centrifuged at 4000rmp for 5 minutes, and the supernatant was transferred to sample vials for HPLC injection. For serum samples, the thawed samples were first deactivated via incubating for 30 minutes at 56 degrees Celsius. The deactivated samples were then aliquoted for albumin and total protein measurements (Qian et al., 2013). An aliquot of 150 µl was then digested, purified and analyzed according to protocol used in previous studies (Qian et al., 2013).

Statistical Analysis

STATA 12, SAS 9.4 and Microsoft Excel were used to determine geometric mean and standard deviation of the levels of albumin, total protein, and aflatoxin B_1-lysine adduct in the dried blood spot samples and corresponding serum samples. Summary statistical were performed for each sample type as well.

Results

Two waves of DBS samples, 393 samples in total, were received and analyzed between June 2016 and August 2017. The results of DBS samples were shown in Tables 4.2 and 4.3. For the first batch of 171 DBS samples, the detection rate was 99%. The highest aflatoxin B_1-lysine adduct level in this batch was 16.03 pg/ml albumin, while the lowest level was 0.4 pg/ml albumin. The median concentration of the first batch was 4.95 pg/ml albumin, with a standard deviation of 3.16. The geometric mean of this batch was 4.24 pg/ml albumin, with the 95% confidential interval from 3.85 to 4.68 pg/ml albumin. For the second batch of 222 DBS samples, the detection rate was 100%. The highest

aflatoxin B_1-lysine adducts level in this batch was 95.58 pg/ml albumin, while the lowest level was 0.71 pg/ml albumin. The median concentration of the second batch was 9.25 pg/ml albumin, with a standard deviation of 14.09. The geometric mean of this batch was 10.73 pg/ml albumin, with the 95% confidential interval from 8.88 to 12.58 pg/ml albumin.

The same batch of samples also contains 2220 serums samples, which were measured between March 2016 and October 2017. These samples consist of 810 samples for the first batch and 1410 for the second batch. The results of the serum samples were shown in Tables 4.2 and 4.3. The detection rates were 95% and 100% for first and second, respectively. The highest concentration detected in the first batch of serum samples was 94.49 pg/ml albumin, while the lowest concentration was 0.41 pg/ml albumin. The geometric mean of aflatoxin B_1-lysine adduct of the first batch was 2.20 pg/ml albumin, with a 95% confidential interval from 1.89 to 2.56 pg/ml albumin. For the second batch of 1410 serum samples, the highest aflatoxin B_1-lysine concentration was 41.60 pg/ml albumin, while the lowest concentration was 0.40 pg/ml albumin. The mean level of this batch was 1.43 pg/ml albumin, with the standard deviation of 2.05. The geometric mean was 1.05 pg/ml albumin, with a 95% confidential interval from 0.60 to 1.54 pg/ml albumin.

For evaluating the correlation between the two types, results were divided into 5 groups based on aflatoxin B_1-lysine adduct levels. Pearson's correlation was analyzed comparing the results from DBS samples and the matched serum samples in each group (Tables 4.4 and 4.5). For the first batch, there were a total of 165 pairs of matched DBS and serum samples. The Pearson's correlation was negative in the group with the lowest

serum aflatoxin B_1-lysine adduct levels, while for the other groups, from the lowest to highest quartiles, the Pearson's correlation were 0.3479 ($p \leq 0.005$), 0.6871, 0.1666 and 0.9903, respectively. For the second batch, there was 193 pair of matched DBS and serum samples. For samples with an aflatoxin B_1-lysine adduct level less than 4.90 pg/ml albumin, the Pearson's correlation was 0.82, with a p value less than 0.005. For the sample group with an aflatoxin B_1-lysine level larger than 5.00 pg/ml albumin, the Pearson's correlation was unable to be calculated, since the sample size within this area was small. The overall Pearson's correlation was 0.66.

Discussion

Aflatoxins exposure among children in Asian and African population has typically been associated with the weaning status, coinciding with increased consumption of maize- and peanut-based weaning foods (Khlangwiset et al., 2011). Typical weaning foods in Nepal included rice prepared and dipped in sauces that differed in composition, depending on ingredient availability. This introduction of solid foods, which would be the primary sources of aflatoxin exposure, has largely influenced the results. However, the weaning time and food amount give to the children can vary (Khlangwiset et al., 2011). Exposure could be higher with earlier introduction and greater intake of the solid food after weaning. A study that included 15-month old children found that there was no association between aflatoxin exposure and weaning status, age, or consumption of grain-based food, although this early introduction of solid food should be a primary source of aflatoxin exposure (Mitchell et al., 2017). Due to the heterogeneity within Nepal country, the study location would be a key factor. In this Nepal study, the exact location was not

indicated in the original materials. Considering the special characteristics of young children, DBS sampling method exceeded the traditional venipuncture blood sampling in many ways. The less invasive nature of DBS sampling method facilitates the field study with large population size, as well as allows the researches to be conducted in the remote areas.

For longitudinal studies, repeated samplings are usually conducted, meaning a single participant may need to provide about 20ml to 30ml blood. Although using serum or plasma is the golden standard for currently measurement of aflatoxin B_1 exposure biomarker, the costs, participant burden and storage and transportation of the venipuncture blood samples pose great barriers to the field study, especially in the remote field settings where access to experimental instrument are limited. Another concern about the venipuncture method is that since it needs 5-10ml whole blood per time from per subject, the relatively large amount of blood required makes the serial sampling difficult. Unlike the downsides of using vein blood sampling, all the above-mentioned researches used small volume of whole blood because DBS sample collection method only needs 5 to 6drops of capillary blood from each participant (finger or heel prick, about 50ul per blood) to be placed on the blotting paper. Blood sampling through DBS can be called non-invasive. However, the use of DBS samples to evaluate exposure is relatively new, and more intensive researches and method validations are needed.

In this study, the aflatoxin B_1-lysine adducts levels in DBS samples were comparable to the levels in the corresponding serum samples. Method developed in our laboratory is proved to be efficient in measuring the aflatoxin B_1-lysine adduct levels in DBS samples. Better correlations are observed in samples with higher aflatoxin B_1-lysine

levels in the exposed population. Potential factors affecting the correlations in different exposure groups in our population include the decreased sample volume, the interference of hematocrit effect and matrix effect of extra components in DBS samples. Final aflatoxin B_1-lysine adducts levels are normalized by the matched albumin concentrations as majority of the aflatoxin B_1-lysine adduct is contained in serum albumin. The hematocrit effect would be minimized by normalization. The complicated components in the DBS samples would be responsible for the decreased detection rate in the low exposure group. Matrix effects can affect the quantification of the aflatoxin B_1-lysine adduct and the albumin. Due to the method sensitivity, LC/MS techniques are considered in verifying adduct and albumin results.

More than 800 infant DBS samples and 4000 serum samples were shipped from Nepal in 4 shipments. Aflatoxin B_1-lysine adducts was measured in 393 DBS samples and 2220 serum samples using the HPLC-fluorescence method. The overall detection rate for DBS samples was over 98% and nearly all detectable in serum samples, indicating aflatoxin B_1 was extensively exposed in infants. As mentioned above, aflatoxin B_1 exposure is highly associated with the weaning time and the food after weaning. Due to the limited resources, major food commodities such as maize and peanut are mainly imported in Nepal. Long transportation time, improper storage, and the humid climate favor mycotoxin growth together resulted in the contamination of the crop commodities. This method to measure the aflatoxin B_1-lysine adduct in DBS sample could be a good alternative to quantifying aflatoxin B_1 exposure in infant and young children.

Conclusion

In conclusion, this study updated the chronic exposure data in certain parts of Nepal via an updated HPLC-fluorescence method. The novel modification of this method is successful in improving the accuracy of measuring aflatoxin B_1-lysine adduct levels in DBS samples. Correlations between the DBS samples and the matched serum samples are good. However, since the correlation improves with higher the aflatoxin B_1-lysine adducts concentrations in the DBS samples, the method accuracy in measuring samples with low aflatoxin B_1-lysine adducts needs to be improved. The results from this study also proved the feasibility of using the dried blood spot sampling method to collect the blood samples in filed studies with large population size.

Tables

Table 4.1 HPLC conditions, gradient program, and column information.

Column	Zorbax Eclipse® XDB-C18 5 μm, 4.6×250mm Agilent, Santa Clara, CA		
Mobile phase A	20mM $HN_4H_2PO_4$		
Mobile phase B	100% Methanol		
Flow Rate	1.0 mL/min		
Column Temperature	25 °C		
Injection Volume	100 μl		
Gradient	Time (min)	A%	B%
	0.00	30	70
	5.00	30	70
	15.00	65	35
	18.00	65	35
	20.00	30	70
	25.00	30	70

Table 4.2 Summary of data from determination of aflatoxin B_1-lysine levels in DBS and serum samples in shipment 1.

Parameters	DBS	Serum
Number	171	165
Detection rate (%)	99%	95%
Median	4.95	1.60
Mean	5.33	5.65
Std dev.	3.16	12.97
Minimal	0.31	0.41
Maximal	16.03	96.49
Geomean	4.24	2.20
95% CL	3.85, 4.68	1.89, 2.56

Table 4.3 Pearson's correlation between DBS and serum samples in different aflatoxin B_1-lysine adduct level groups in shipment 1.

AFB_1-Lysine level (pg/mg albumin)	Pearson's correlation	t	p
0.29-4.90	-0.1216	-1.53	/
5.00-9.90	0.3479	4.62	8.02E-06
10.00-19.9	0.6871	11.77	2.94E-23
20.00-49.90	0.1666	2.10	3.70E-02
50.00+	0.9903	88.82	7.75E-135

Table 4.4 Summary of data from determination of aflatoxin B_1-lysine levels in DBS and serum samples in shipment 2.

Parameters	DBS	Serum
Number	222	193
Detection rate (%)	100%	97%
Median	9.24	0.89
Mean	14.57	1.60
Std dev.	14.09	3.32
Minimal	0.71	0.42
Maximal	95.58	41.60
Geomean	10.73	1.07
95% CL	8.88, 12.58	0.29, 1.85

Table 4.5 Pearson's correlation between DBS and serum samples in different aflatoxin B_1-lysine adduct level groups in shipment 2.

AFB_1-Lysine level (pg/mg albumin)	Pearson's correlation	t	p
0.40-0.49	0.8228	20.01	9E-49
5.0-9.9	1.0000	/	/
10.00-19.9	1.0000	/	/
20.0-49.9	/	/	/
50+	/	/	/

Table 4.6 Summary of data from determination of aflatoxin B_1-lysine levels in DBS and serum samples in two shipments.

Parameters	DBS	Serum
Number	393	358
Detection rate (%)	100%	96%
Median	7.38	1.22
Mean	10.89	3.44
Std dev.	12.30	9.29
Minimal	0.31	0.41
Maximal	95.58	96.49
Geomean	7.23	1.48
95% CL	5.94, 8.52	0.51, 2.45

Table 4.7 Pearson's correlation between DBS and serum samples in different aflatoxin B_1-lysine adduct level groups in two shipments.

AFB_1-Lysine level (pg/mg albumin)	Pearson's Correlation	t	p
0.29-4.9	0.45627	7.09	2.58224E-11
5.0-9.9	0.01512	0.21	0.834733626
10.00-19.9	0.50004	7.98	1.32252E-13
20.0-49.9	0.41492	6.30	1.97939E-09
50+	0.99032	98.59	9.3742E-166

Figures

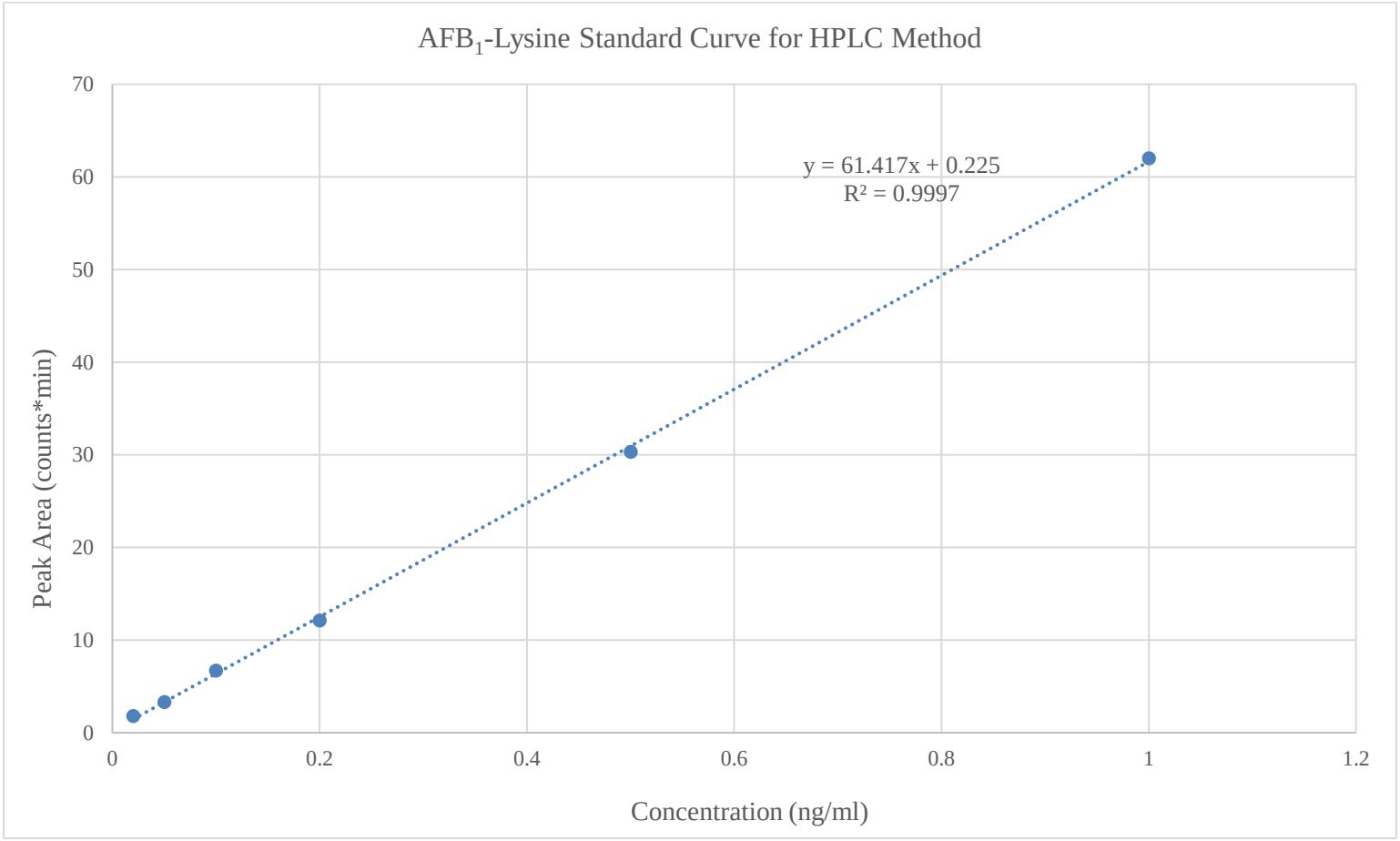

Figure 4.1 Standard curve for aflatoxin B_1-lysine for HPLC using conditions shown in Table 4.1.

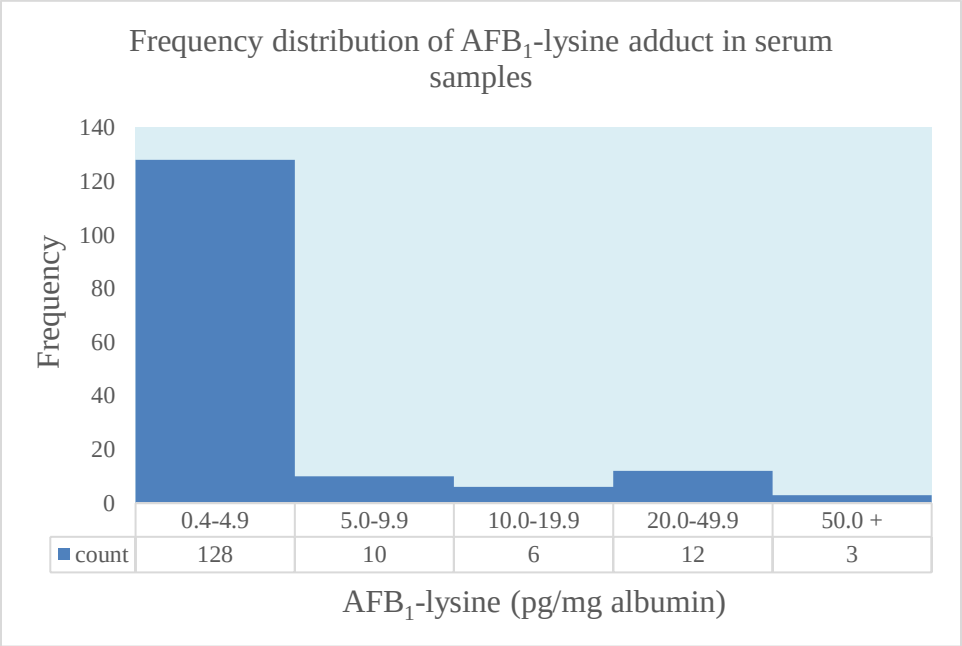

Figure 4.2 Frequency distribution of aflatoxin B_1-lysine adduct in serum samples in shipment 1.

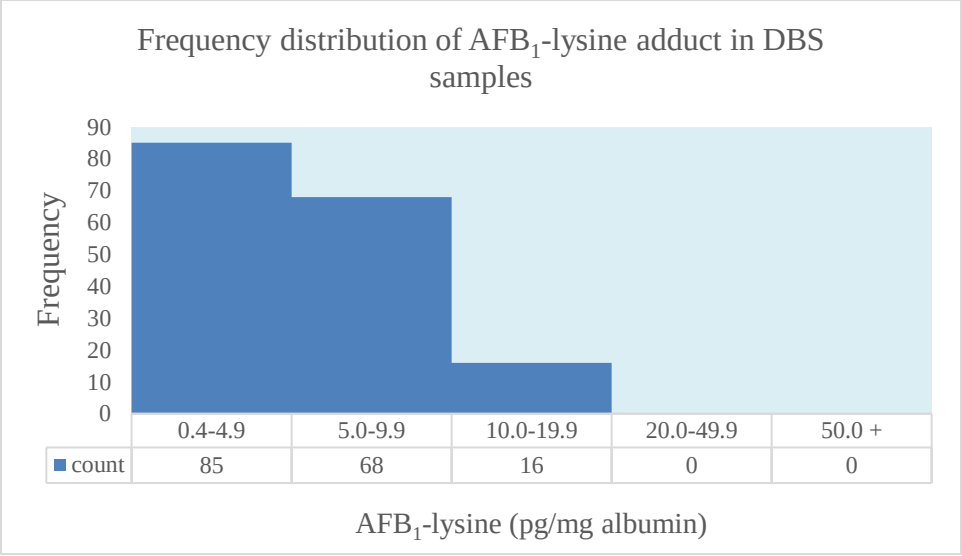

Figure 4.3 Frequency distribution of aflatoxin B_1-lysine adduct in DBS samples in shipment 1.

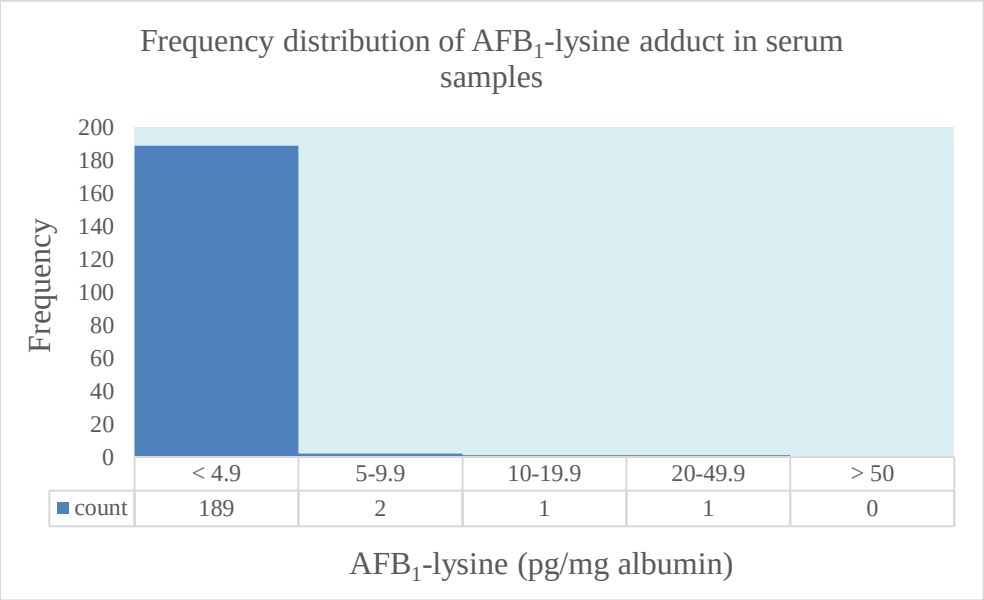

Figure 4.4 Frequency distribution of aflatoxin B_1-lysine adduct in serum samples in shipment 2.

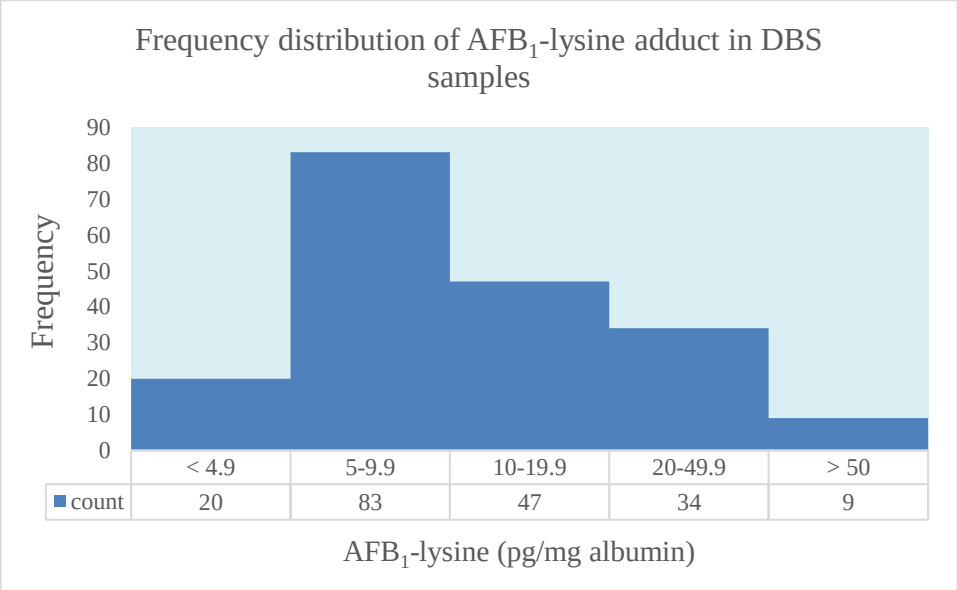

Figure 4.5 Frequency distribution of aflatoxin B_1-lysine adduct in DBS samples in shipment 2.

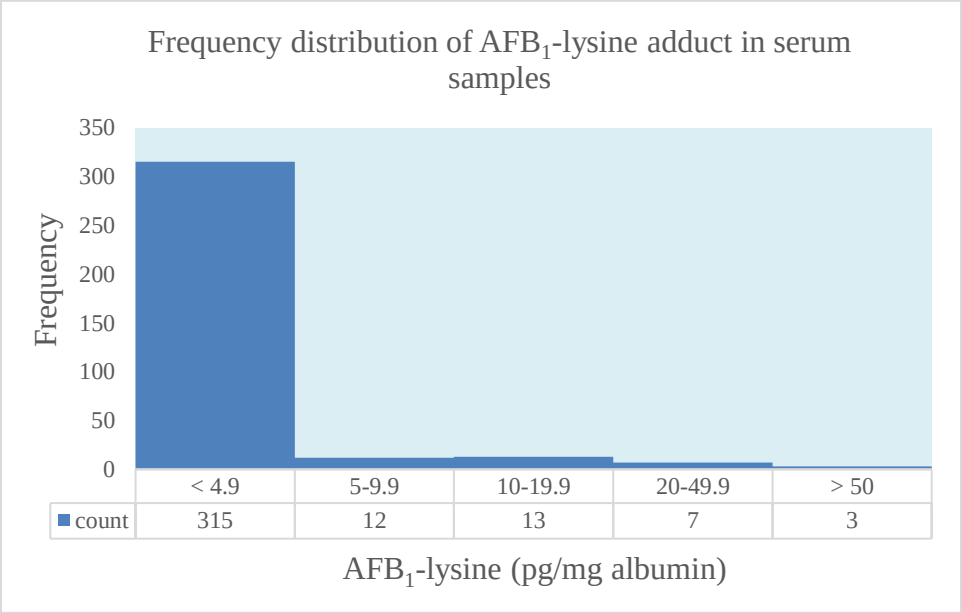

Figure 4.6 Frequency distribution of aflatoxin B_1-lysine adduct in serum samples in two shipments.

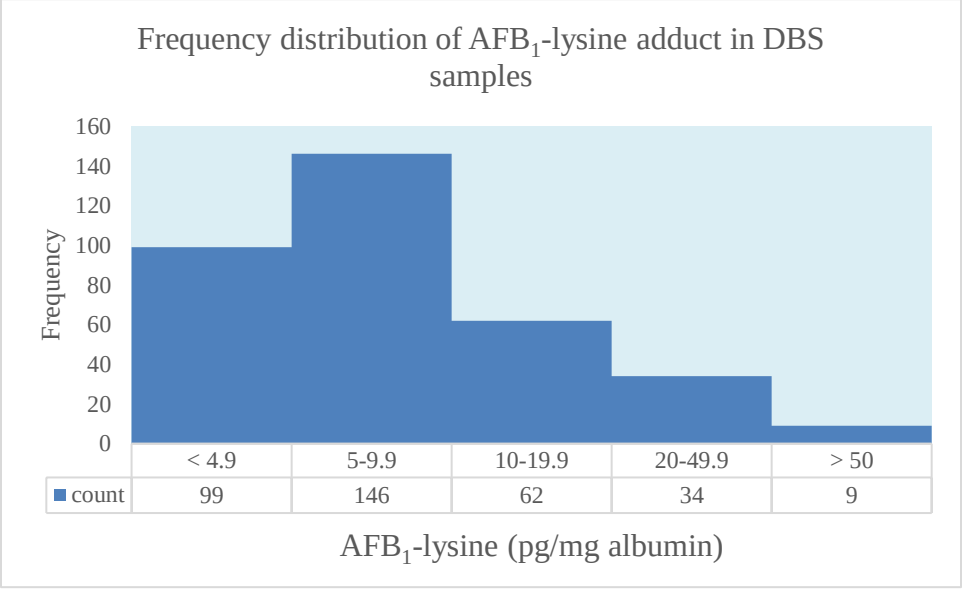

Figure 4.7 Frequency distribution of aflatoxin B_1-lysine adduct in DBS samples in two shipments.

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CHAPTER 5

SUMMARY AND FUTURE DIRECTION

An HPLC-fluorescence method to measure aflatoxin B_1-lysine adducts in DBS samples was developed and validated in this research. Animal study and human design were used to validate this method. Good dose- and time-responses were observed in both single- and repeat-dosed rats. Both validation studies indicated that this HPLC-fluorescence method was accurate and reliable in quantifying the exposure biomarkers of aflatoxin B_1. Over 400 infant DBS samples from Nepal were measured using the HPLC-fluorescence method. The corresponding serum samples collected from the same participants were also quantified with a HPLC method. Pearson's correlations between the results of DBS samples and the results of matched serum samples were calculated. Good correlation in the high exposure group was observed while no significant correlation was achieved in the low exposure group.

All results were normalized by albumin concentration, and the overall correlation between DBS sample results and serum sample result was good. Aside from the normalization, there were other concerns about the method. First is the small amount of blood contained in the DBS, which was resolved by modifying the washing process. Another concern would be the matrix effect from the whole blood. Matrix effect could influence the measurement of aflatoxin B_1-lysine adducts and the albumin. Thus, further confirmation of the results might be required, especially in the low adduct concentration

group. Consider the current sensitivity of technique, LC/MS techniques would be required to further validate the results.