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*Short Communication***Long-term Stability of Human Aflatoxin B<sub>1</sub> Albumin Adducts Assessed by Isotope Dilution Mass Spectrometry and High-Performance Liquid Chromatography–Fluorescence**

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**Abstract**

The measurement of the aflatoxin B<sub>1</sub>-lysine serum albumin adduct in human blood samples is the most facile biomarker for the assessment of chronic exposure to aflatoxin B<sub>1</sub>. Many technologies have been developed for the measurement of this protein adduct including immunoassays, high-performance liquid chromatography (HPLC) with fluorescence detection, and a newly developed isotope-dilution mass spectrometry method. Irrespective of the technology used to determine this adduct level, an important question remains about the long-term stability of this damage product in stored samples. To address this issue, 19 human serum samples that had been previously analyzed for the aflatoxin B<sub>1</sub>-lysine adduct by high-performance liquid chromatography–fluorescence in

1989 were re-analyzed by isotope dilution mass spectrometry after storage at  $-80^{\circ}\text{C}$ . The adduct concentrations measured by these two techniques were identical within 4% over the range 5 to 100 pg of aflatoxin B<sub>1</sub>-lysine/mg albumin. In addition, the specific chemical structure of the aflatoxin B<sub>1</sub>-lysine adduct in human samples was confirmed for the first time by collision-induced dissociation full scan mass spectrometry analysis of the protonated adduct molecular ion. These results illustrate that the aflatoxin B<sub>1</sub>-lysine serum albumin adduct can be stable in human serum stored at  $-80^{\circ}\text{C}$  since 1989, and this provides confidence for the measurement of this biomarker in repository samples from epidemiologic investigations. (Cancer Epidemiol Biomarkers Prev 2008;17(6):1436–9)

**Introduction**

Dietary exposure to the mycotoxin aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) is an important risk factor for the development of hepatocellular cancer in Asia and Africa (1). AFB<sub>1</sub> is oxidized by cytochrome P450s to two epoxides, one of which can react with double-stranded DNA to form mutagenic AFB<sub>1</sub>-N<sup>7</sup>-guanine adducts (2, 3). In addition, the hydrolysis products of these epoxides can react with the  $\epsilon$ -amino group of lysine in serum albumin (AFB<sub>1</sub>-lys; refs. 4, 5). Because the half-life of human serum albumin is  $\sim 20$  days, chronic AFB<sub>1</sub> exposure leads to the accumulation of albumin adducts up to 30-fold higher than that resulting from a single exposure (6–8).

Immunoassays, high-performance liquid chromatography (HPLC) with fluorescence detection, and isotope dilution mass spectrometry (IDMS) have been used to measure serum albumin adduct concentrations and assess aflatoxin exposure status in epidemiologic studies (9–12). Aflatoxin-albumin adduct concentrations mea-

sured by ELISA are well correlated with AFB<sub>1</sub>-lys concentrations measured by HPLC-fluorescence and IDMS, albeit the levels obtained in a recent study found that values using an ELISA were 2.6-fold higher than those measured in the same samples by IDMS (12, 13). The AFB<sub>1</sub>-lys adduct has been shown to be present in rat albumin by full scan and tandem mass spectrometry, such confirmatory spectra have not been reported for human albumin samples collected in exposed populations (8, 13). Thus, in the work reported in this study, we report the confirmation of this adduct by full scan collision-induced dissociation mass spectrometric analysis using a recently developed procedure (12, 14). Furthermore, we have explored the question of the long-term stability of this protein adduct in stored samples, in which the concentrations had been determined using HPLC with fluorescence detection (11). These results illustrate that the AFB<sub>1</sub>-lys serum albumin adduct can be stable in human serum stored at  $-80^{\circ}\text{C}$  since 1989, and provides confidence for the measurement of this biomarker in repository samples.

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**Materials and Methods**

**Chemicals.** AFB<sub>1</sub> and human serum albumin were purchased from Sigma-Aldrich. Pronase (120 kU/g) was purchased from Calbiochem-Novabiochem. Mixed mode

solid-phase extraction cartridges (Oasis MAX) were obtained from Waters, Corp.

**Synthesis of AFB<sub>1</sub>-lys and AFB<sub>1</sub>-D4-lys Standards.** AFB<sub>1</sub>-lys and the tetra-deuterated (D4) lysine internal standard AFB<sub>1</sub>-D4-lys were prepared for mass spectrometric assays and chromatographically purified as previously described (12, 13).

**Human Sample Collection and Preparation.** The remaining serum from 19 human samples from a previous study in the People's Republic of China were used in the current study (10). Dietary exposure to AFB<sub>1</sub> and the concentrations of albumin and AFB<sub>1</sub>-lys, determined by HPLC with fluorescence detection, were previously reported (11).

**Isotope Dilution Mass Spectrometric Determination of AFB<sub>1</sub>-lys.** Serum was analyzed using a minor variation of the method reported by McCoy and colleagues (15). Serum (100  $\mu$ L, ~4 mg albumin) was mixed with an internal standard (100  $\mu$ L  $\times$  2 ng AFB<sub>1</sub>-D4-lys/mL) and Pronase solution (250  $\mu$ L, 13 mg/mL PBS), and incubated for 4.5 h at 37°C. Solid-phase extraction-processed samples were analyzed by HPLC with mass spectrometric detection using a ThermoElectron TSQ Quantum Ultra operated in the positive electrospray ionization SRM mode. The internal standard parent molecular ion [(M + H)<sup>+</sup>, *m/z* 461.3] fragmented to yield an ion at *m/z* 398.2. The AFB<sub>1</sub>-lys molecular ion (*m/z* 457.2) fragmented to yield an ion at *m/z* 394.1. A 12-point isotopic dilution standard curve was generated by triplicate injection (100  $\mu$ L) of AFB<sub>1</sub>-D4-lys (200 pg) mixed with varying amounts of AFB<sub>1</sub>-lys (0-2.9 ng) prepared via 3-fold serial dilutions. The data was fitted using the method of least-squares with a 1/ $\times$  weighting factor. The isotope dilution standard curve was linear over the range of 0.5 pg to 2.9 ng AFB<sub>1</sub>-lys injected onto the column in 100  $\mu$ L ( $R^2 = 0.9995$ ). The coefficient of variation was 20%, when 0.5 pg of AFB<sub>1</sub>-lys was injected.

**Collision-Induced Dissociation Mass Spectra of Synthetic AFB<sub>1</sub>-lys and Authentic AFB<sub>1</sub>-lys Isolated from Archived Human Serum.** AFB<sub>1</sub>-lys was isolated from a Pronase digest of a pooled human serum sample (1 mL) using the same chromatographic conditions as those used to detect AFB<sub>1</sub>-lys in the IDMS assay. The pooled sample was prepared by combining aliquots of the 19 serum samples assayed for AFB<sub>1</sub>-lys. The average collision-induced dissociation profile spectrum of the parent molecular ion (*m/z* 457.2) was acquired over the range *m/z* 80 to 500 using a Thermo-Finnigan TSQ Quantum Ultra operated in the ESI-positive ionization mode.  $Q_1 = Q_3 = 0.7$  *m/z*; scan time, 0.33;  $Q_2$  gas (Ar) pressure, 1.5 mTorr;  $V_s = 4.2$  kV; collision energy, 33 eV; capillary temperature = 300°C; sheath pressure, 49; auxiliary pressure, 22; capillary offset, 35 V.

## Results

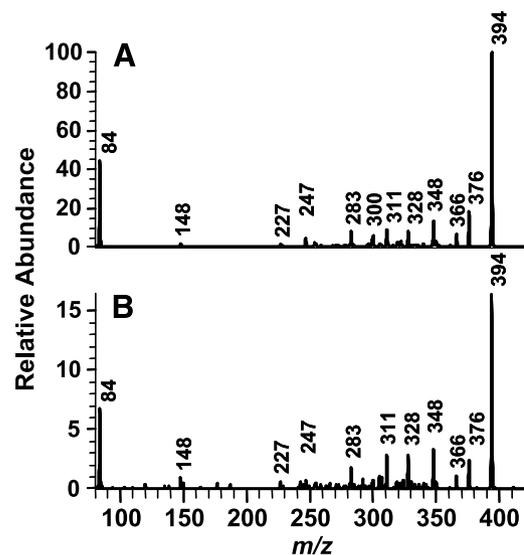
Collision-induced dissociation mass spectrometry was used to provide confirmation of the formation of the AFB<sub>1</sub>-lys in human samples obtained from a molecular epidemiologic investigation of a high-risk population for liver cancer. The collision-induced dissociation spectra of synthetic AFB<sub>1</sub>-lys and the authentic compound isolated

from pooled human sera are presented in Fig. 1. Fragmentation of the protonated molecular ion (M + H)<sup>+</sup> at *m/z* 457 from both the synthetic and *in vivo* samples produces (M + H-CO-H<sub>2</sub>O)<sup>+</sup> at *m/z* 411 and (M + H-CO-H<sub>2</sub>O-NH<sub>3</sub>)<sup>+</sup> at *m/z* 394. The fragment ion at *m/z* 328 is produced by cleavage of the C6-N $\epsilon$  bond and ion trap mass spectrometric studies previously revealed that it undergoes fragmentation with the loss of NH<sub>3</sub> to yield the ion at *m/z* 311 (13, 16). Fragmentation of the lysine moiety yields the immonium-NH<sub>3</sub> ion at *m/z* 84. The base peak (M + H-H<sub>2</sub>O-CO-NH<sub>3</sub>)<sup>+</sup> at *m/z* 394 was used to quantitatively analyze AFB<sub>1</sub>-lys in subsequent IDMS analyses.

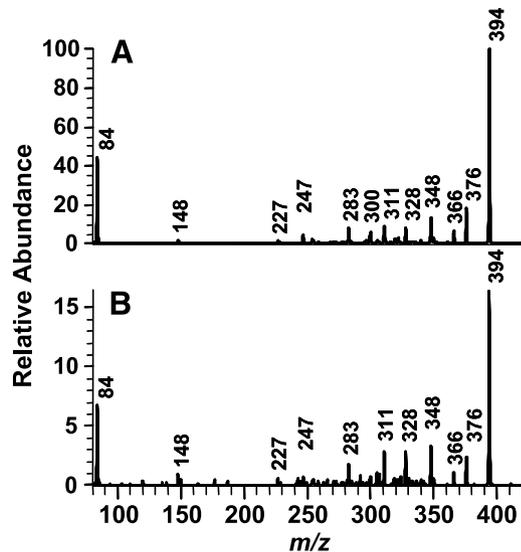
Using the mass spectrometry technique, the association of AFB<sub>1</sub>-lys concentrations measured by IDMS with those measured by HPLC with fluorescence detection was examined. Representative single-reaction monitoring chromatograms from IDMS analyses are presented in Fig. 2. The correlation of AFB<sub>1</sub>-lys concentrations previously measured by HPLC with fluorescence detection with those measured by IDMS is presented in Fig. 3 (11). A least-squares fit of the data shows that the IDMS method systematically detects 24% more AFB<sub>1</sub>-lys than reported in the original HPLC-fluorescence analysis. Nonetheless, the consistent association between these two analytic methods shows the stability of the aflatoxin adduct in serum albumin over a nearly 20-year period of storage and subsequent measurements.

## Discussion

The use of AFB<sub>1</sub> serum albumin adducts as exposure biomarkers in molecular epidemiologic studies of liver cancer has become a standard for these investigations over the past 20 years (17). The validation of this biomarker was first done in experimental models in rats (7, 8). Subsequently, mass spectrometric analysis of



**Figure 1.** Collision-induced dissociation mass spectra of the protonated molecular ion of AFB<sub>1</sub>-lys (*m/z* 457). **A**, synthetic samples; **B**, samples isolated from human serum.



**Figure 2.** Chromatographic separation and selected reaction monitoring chromatograms of AFB<sub>1</sub>-lys in an archival human serum sample. *Top*, archived human serum (139 pg AFB<sub>1</sub>-lys/mg albumin). **A1**, AFB<sub>1</sub>-lys selected reaction monitoring (*m/z* 457-394). **A2**, internal standard channel. AFB<sub>1</sub>-D4-lys selected reaction monitoring (*m/z* 461-398). Relative abundance (100%) =  $1 \times 10^5$ . *Bottom*, nondetection of AFB<sub>1</sub>-lys in archived human serum. **B1**, AFB<sub>1</sub>-lys-selected reaction monitoring (*m/z* 457-394). **B2**, AFB<sub>1</sub>-D4-lys selected reaction monitoring (*m/z* 461-398). Relative abundance (100%) =  $3 \times 10^4$ .

Pronase-digested albumin from rats treated with AFB<sub>1</sub> contributed to the identification of the adduct's chemical structure as AFB<sub>1</sub>-lys (4, 5, 8). These findings provided the rationale for the development of immunoassays, HPLC with fluorescence detection and mass spectrometry for this protein adduct detection and measurement. A very sensitive ELISA was developed for aflatoxin albumin adducts that has been validated and this method continues to have the highest throughput for large-scale studies (9, 12, 18, 19).

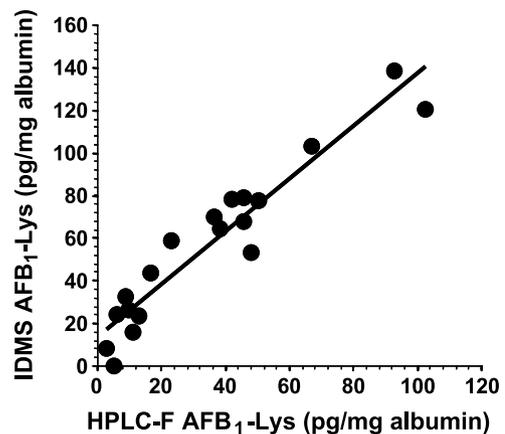
In the work reported here, we have taken advantage of human samples that had been analyzed by HPLC-fluorescence assay for IAFB<sub>1</sub>-lys in 1989 to provide a perspective for the long-term stability of these adducts. These serum samples were re-analyzed using IDMS after being stored at  $-80^\circ\text{C}$  for >15 years (10, 11). Intercomparison of concentrations measured using the two different methods provided a unique opportunity to evaluate AFB<sub>1</sub>-lys adduct stability and gain insight on the selection of analytic methods for use in aflatoxin exposure studies. The close tracking in the comparison of these two data sets provides confidence that the AFB<sub>1</sub>-lys adduct is stable in stored serum samples for long periods of time.

The least-squares fit of AFB<sub>1</sub>-lys concentrations measured by fluorescence and IDMS in this study provides descriptive information about the relative performance of these methods if the adduct did not significantly degrade during storage. The slope indicates that the IDMS method systematically detects, on average,

24% more adducts than the fluorescence method. The internal standard used in the IDMS assay provides adduct concentrations that are corrected for losses of AFB<sub>1</sub>-lys during sample handling and ion suppression effects that could otherwise affect its quantitative analyses. The HPLC-fluorescence method did not use an internal standard. Neither method is corrected for potential artifacts due to intersample differences in the efficiency of the Pronase digestion of albumin (15). Although the reported AFB<sub>1</sub>-lys concentrations measured by HPLC-fluorescence were not corrected for sample recovery, the method used in the original report was estimated to produce a recovery of  $\sim 80\%$  (11). After correction of the HPLC-fluorescence-measured adduct concentrations by an 80% recovery estimate, correlation of the IDMS data results in a slope of 0.99 without significantly changing the *y*-intercept, thus indicating that the same AFB<sub>1</sub>-lys concentrations were measured by both methods.

The measurement of equivalent AFB<sub>1</sub>-lys concentrations by IDMS and HPLC with fluorescence detection indicates that the AFB<sub>1</sub>-lys-albumin adduct is stable in human serum samples stored for 15 years at  $-80^\circ\text{C}$ . If more than  $\sim 6\%$  of the AFB<sub>1</sub>-lys degraded during storage in some or all of these samples, the systematic correspondence between concentrations measured using the two methods would not have been directly accounted for by the 80% recovery of the HPLC-fluorescence method. Degradation of AFB<sub>1</sub>-lys in these serum samples between the time of their initial collection in 1983 and their initial analysis by HPLC with fluorescence detection in 1989 cannot be ruled out. However, the correspondence between AFB<sub>1</sub>-lys concentrations measured after >15 years suggests that it has been stable for >24 years.

These samples also provided the opportunity to use a current method to determine the structure of the



**Figure 3.** Comparison of HPLC-fluorescence and IDMS measurement of AFB<sub>1</sub>-lys concentrations in samples stored for 19 years at  $-80^\circ\text{C}$ . The least-squares fit presented is described by the equation  $y = 1.24 + 13.74x$  ( $R^2 = 0.91$ ). Correction of the published AFB<sub>1</sub>-lys concentrations measured by HPLC-fluorescence for an 80% estimated method recovery yields a regression line described by the equation  $y = 0.99x + 13.74$  ( $R^2 = 0.91$ ).

aflatoxin protein adduct by mass spectrometry. The sensitivity of current mass spectrometry methods is at least 100-fold greater than the original studies reported for this adduct (8). The collision-induced dissociation spectrum of synthetic AFB<sub>1</sub>-lys in this report exhibits structurally salient fragment ions at *m/z* 84, 311, 328, 394, and 411. These ions were also detected during the structural characterization of AFB<sub>1</sub>-lys isolated from the serum of AFB<sub>1</sub>-treated rats (13). The virtually identical appearance of the collision-induced dissociation spectra of the coeluting material isolated *in vivo* supports the conclusion that AFB<sub>1</sub>-lys is present in human serum. Although aflatoxin albumin and AFB<sub>1</sub>-lys adducts have been studied for >20 years, this is the first report of the mass spectrum of AFB<sub>1</sub>-lys in human serum samples.

These data provide a basis for the selection of immunoassays, HPLC-fluorescence, and mass spectrometry methods for the measurement of albumin adducts and justification for the use of archived serum in aflatoxin exposure studies in people. Although the IDMS assay is more sensitive and more specific than the other assays, the high cost of mass spectrometric instrumentation makes it more expensive than the less sensitive HPLC-fluorescence method (9, 12, 20). Consequently, use of the IDMS method will result in higher study costs and therefore be less generally available to the research community. If the anticipated AFB<sub>1</sub>-lys serum albumin concentrations in a study population are sufficiently high (>5 pg AFB<sub>1</sub>-lys/mg albumin), the HPLC-fluorescence method may be a more cost-effective analytic tool. This study also shows that over the range 5 to 100 pg of AFB<sub>1</sub>-lys/mg albumin, these two techniques provide virtually identical results. When the requirement for specifically measuring AFB<sub>1</sub>-lys concentrations is not essential to the goals of a study, the sensitive, lower cost, and potentially higher throughput ELISA assay should be considered. Although the performance of the IDMS assay has been compared with both the ELISA and HPLC-fluorescence methods in separate reports, the intercomparison of all three assays using a single human serum sample set would be useful in guiding the selection of analytic methods for aflatoxin albumin adducts in future molecular epidemiology studies of liver cancer.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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