Human Aflatoxin Albumin Adducts Quantitatively Compared by ELISA, HPLC with Fluorescence Detection, and HPLC with Isotope Dilution Mass Spectrometry

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Abstract

Essential to the conduct of epidemiologic studies examining aflatoxin exposure and the risk of heptocellular carcinoma, impaired growth, and acute toxicity has been the development of quantitative biomarkers of exposure to aflatoxins, particularly aflatoxin B_1. In this study, identical serum sample sets were analyzed for aflatoxin-albumin adducts by ELISA, high-performance liquid chromatography (HPLC) with fluorescence detection (HPLC-f), and HPLC with isotope dilution mass spectrometry (IDMS). The human samples analyzed were from an acute aflatoxicosis outbreak in Kenya in 2004 (n = 102) and the measured values ranged from 0.018 to 67.0, nondetectable to 13.6, and 0.002 to 17.7 ng/mg albumin for the respective methods. The Deming regression slopes for the HPLC-f and ELISA concentrations as a function of the IDMS concentrations were 0.71 (r^2 = 0.95) and 3.3 (r^2 = 0.96), respectively. When the samples were classified as cases or controls, based on clinical diagnosis, all methods were predictive of outcome (P < 0.01). Further, to evaluate assay precision, duplicate samples were prepared at three levels by dilution of an exposed human sample and were analyzed on three separate days. Excluding one assay value by ELISA and one assay by HPLC-f, the overall relative SD were 8.7%, 10.5%, and 9.4% for IDMS, HPLC-f, and ELISA, respectively. IDMS was the most sensitive technique and HPLC-f was the least sensitive method. Overall, this study shows an excellent correlation between three independent methodologies conducted in different laboratories and supports the validation of these technologies for assessment of human exposure to this environmental toxin and carcinogen. (Cancer Epidemiol Biomarkers Prev 2008;17(7):1653–7)

Introduction

Aflatoxins have been shown to be potent toxic, mutagenic, and carcinogenic compounds in humans and animals (1). Epidemiologic studies have clearly linked dietary exposure with increased risk of hepatocellular carcinoma (1). These investigations have been markedly improved by mechanism-based biomarkers that provide individual exposure assessment (2, 3). Among these biomarkers, aflatoxin serum albumin adducts have been shown to be highly correlated with dietary aflatoxin intake and resultant cancer risk (3, 4).

Over the past 20 years, several different validated analytical strategies have been devised to quantify aflatoxin albumin adducts, including ELISA, high-performance liquid chromatography (HPLC) with fluorescence detection (HPLC-f), and isotope dilution mass spectrometry (IDMS; refs. 5-9). Each of these methods has inherent strengths and weaknesses that affect sensitivity and sample throughput. In this report, we have undertaken a careful evaluation of these methods using identical sample sets from individuals from Kenya involved in an aflatoxicosis outbreak. The study permits evaluation of the relative analytical strengths of each of these methods and provides a foundation for quantitative comparisons of biomarker concentrations employing different analytical techniques.

Materials and Methods

Chemicals and Standards. Pronase (nuclease-free) was obtained from Bio-Rad. Aflatoxin B_1 (AFB_1), bromocresol purple, L-lysine, and human serum albumin were purchased from Sigma-Aldrich. Human sera were from either Sigma-Aldrich or Tennessee Blood Services. The Coomassie protein assay was purchased from Bio-Rad. Preparation of the aflatoxin immunoaffinity resins has been described (10, 11). Methanol and acetonitrile were HPLC grade; all other reagents were reagent grade quality or better.

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Table 1. Comparison of blind quality-control results for the analysis of aflatoxin albumin adducts from a diluted Kenya patient sample

Method		Level*			Overall mean
		1	2	3	
IDMS					
	Mean †	13.0	31.3	105	
	SD	0.87	2.52	11.8	
	CV (%)	6.7	8.1	11.3	8.7
HPLC-f					
	Mean †	8.1 ‡	23.4	66.5	
	SD	1.4	2.6	2.4	
	CV (%)	17.1	10.9	3.6	10.5
ELISA					
	Mean§	61.8	149	571 ‡	
	SD	7.5	8.5	59.7	
	CV (%)	12.1	5.7	10.5	9.4
Ratios					
	HPLC-f/IDMS	0.62	0.75	0.64	0.67
	ELISA/HPLC-f	7.63	6.36	8.60	7.53
	ELISA/IDMS	4.76	4.76	5.46	4.99

NOTE: AFB_1-lysine adducts (or equivalents) per milligram of albumin were measured by three different laboratories using different types of analysis.
* Level 2 is 0.294 of level 3, and level 1 is 0.119 of level 3.
† pg/mg albumin, $n = 6$ per level. Samples were analyzed in duplicate on 3 d.
‡ One assay value was not included in the calculations.
§ pg/mg albumin equivalents, $n = 6$ per level. Samples were analyzed in duplicate on 3 d.

The aflatoxin-lysine adduct (AF-lys) and the internal standard prepared with Ld4-(4,4,5,5)-lysine hydrochloride (Cambridge Isotope Laboratories) were synthesized and purified as described (9, 12).

Sample Preparation. Of the 113 samples from Kenya analyzed previously by IDMS (13), 102 were divided into aliquots and submitted for analysis by HPLC-f and ELISA. The 11 samples not available for HPLC-f and ELISA analyses were deleted from the IDMS data set such that each method contained an identical sample set. The Kenyan participants were divided into cases and controls and selection criteria are presented elsewhere (13). Two controls were randomly selected from each case patient's village because they shared similar soil, microclimate, and farming practices.

Samples to be used for the determination of assay precision were prepared by dilution of a human sample obtained from the Kenya aflatoxicosis outbreak (13) with normal serum from the Tennessee Blood Services Bank. The sample was diluted to three concentrations and duplicate aliquots at each concentration were analyzed on 3 days ($n = 18$). Duplicate samples of the diluent serum were also analyzed on each day. All analysts were masked to the identity of these samples.

Quality-control samples containing two levels of aflatoxin-albumin adducts were prepared by dilution of rat plasma obtained from AFB_1-dosed rats (9) with human serum. These samples were analyzed on each of three different days ($n = 12$).

Additional serum samples ($n = 28$) were obtained from Tennessee Blood Services, and aliquots were analyzed by each of the laboratories. The U.S. Centers for Disease Control and Prevention also provided to each laboratory a stock solution of AF-lys reference material to determine if there was agreement among laboratories as to its concentration.

Methods of Analysis and Statistics. All of the serum samples were analyzed by ELISA, HPLC-f, and IDMS by methods as described previously (5, 8, 9). Deming and linear regressions of the AF-lys concentrations were done using Analyse-It for Microsoft Excel. Clinical data from the Kenya investigation were analyzed using SAS version 8.02 (SAS Institute). Conditional logistic regression models (adjusted for age, sex, and district) were used to explore the relationship between case status and AF-lys serum concentrations. One value by HPLC-f was less than the limit of quantitation and was assigned a value of 0.0.

Results

Because AF-lys is not commercially available, each of the laboratories was provided a stock solution to be measured as a calibration reference. Identical concentrations were determined at the U.S. Centers for Disease Control and Prevention and the University of Leeds, with the Johns Hopkins University laboratory measurement ~4% lower. In the data presented here, no correction for these differences has been made.

A human specimen from the Kenya outbreak was diluted with human serum to three levels and analyzed in duplicate on three separate days to determine assay precision. A summary of the results is presented in Table 1. In both the HPLC-f and ELISA assays, one sample was excluded from statistical calculations because it was more than four SD from the mean. After excluding these two results, the between-day imprecision for all of the assays at all adduct levels was <18%. The diluent serum obtained from a U.S. blood blank was assayed at less than quantifiable levels, except for one sample assayed as 10.9 pg/mg albumin as measured by HPLC-f (data not shown). The ratios of concentrations determined by each method are also presented in Table 1.

Quality-control samples prepared from diluted dosed rat plasma were also analyzed in duplicate on 3 days by each laboratory. The higher level gave mean [coefficient of variation (CV)] concentrations of 25.3 (5.2), 19.8 (16.1), and 13.9 (15.8) pg/mg albumin for the IDMS, HPLC-f, and ELISA methods, respectively. The corresponding mean concentrations for the lower level were 4.4 (9.8) pg/mg albumin, not detected, and 2.7 (24.1) pg/mg albumin. For the lower quality-control sample, the IDMS between-day CV of 9.8% showed that the assay was quantitative at 4.4 pg/mg albumin. The ELISA method CV for the same sample was 24.1%, indicating that the concentration was near the limit of quantitation. The HPLC-f method did not detect AF-lys at this level. Therefore, the relative sensitivities of the methods as indicated by this study were evaluated as IDMS > ELISA > HPLC-f. This is consistent with the limits of detection reported by IDMS of 0.25 pg/mg (9), by ELISA of 3 pg/mg albumin (14, 15), and by HPLC-f of ~9 pg/mg (6).

The Kenya aflatoxicosis outbreak resulted in 317 acute hepatic failures of which 125 persons eventually died (13). Serum samples collected from that study were first analyzed for AF-lys by IDMS. With permission from the Kenya government, residual sample aliquots from 102 subjects (19 case subjects, 61 control subjects, and

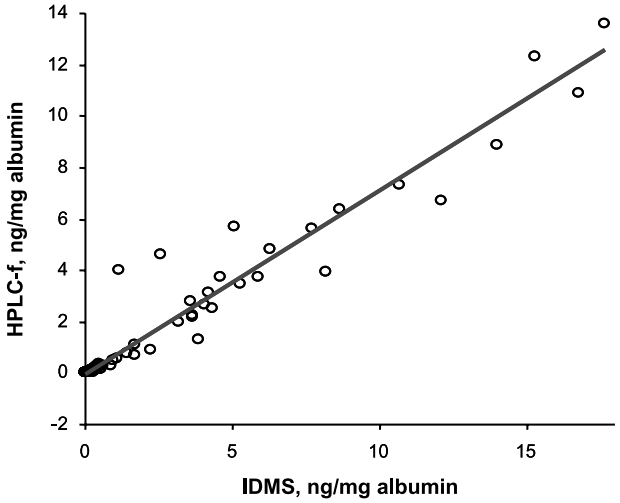

Figure 1. Deming regression of the 2004 Kenya patient sample assay results obtained by HPLC-f and IDMS.

22 subjects without case status) were further analyzed by ELISA and HPLC-f. A comparison of the IDMS and HPLC-f results from these human samples is shown using a Deming regression in Fig. 1. The concentration ranges obtained by the IDMS assay were from 0.002 to 17.7 ng AF-lys/mg albumin, and the ranges for the HPLC-f assay were from nondetectable to 13.6 ng/mg. The slope of the regression line was 0.71 with a 95% confidence interval (95% CI) from 0.68 to 0.75. The regression intercept was -0.004 ng/mg albumin with a 95% CI of -0.18 to 0.10. When analyzed by linear regression, the slope was 0.70 with a correlation coefficient of 0.95 and an intercept of -0.014 ng/mg albumin. The Deming regression of the samples as analyzed by IDMS and ELISA analytical methods is presented in Fig. 2. The concentrations determined by the ELISA method ranged from 0.018 to 67.0 ng/mg albumin. The slope of the regression line was 3.3 (95% CI, 3.18-3.46) with an intercept of -0.316 ng/mg albumin (95% CI, -0.89 to 0.26). The corresponding linear regression had a slope of 3.2 with an intercept of 0.076 ng/mg albumin and a correlation coefficient of 0.96. Figure 3 presents the Deming regression for the ELISA and HPLC-f assay results. The slope was 4.76 with a 95% CI from 4.44 to 5.08, and an intercept of -0.264 ng/mg albumin (95% CI, -1.21 to 0.68). The linear regression slope was 4.31 with an intercept of 0.341 ng/mg albumin and a correlation coefficient of 0.90. Thus, all three methods were highly correlated across these different human samples.

In addition to the samples collected from the aflatoxicosis outbreak area, 28 serum samples obtained from U.S. blood donors were analyzed. The ELISA reported two positive results (confirmed by repeat analysis) as 34.3 and 10.1 pg/mg and an additional four subjects had levels at or near the limit of detection. IDMS analysis and HPLC-f analysis did not detect quantifiable levels in these samples.

As a final assessment of the three methods, a repeat of the Kenyan aflatoxicosis case-control study was done (13). In the previous work by IDMS, those subjects having concentrations at or above the median serum AF-lys were shown to be at a significantly higher risk for developing aflatoxicosis (adjusted odds ratio, 14.8; 95% CI, 3.0-72.2). In the present study with IDMS using 19 cases and 61 controls, the adjusted odds ratio was 31.7 with a 95% CI of 2.9 to 350.9. Using the HPLC-f technique, the adjusted odds ratio was 13.8 with a 95% CI of 2.0 to 93.4. The ELISA method yielded an adjusted odds ratio of 142.6 with a 95% CI of 3.3 to 6357. Based on the geometric means, the case levels were 6.4 to 6.5 times higher than controls when analyzed by IDMS or HPLC-f and 11.6 times higher if analyzed by ELISA.

Discussion

This study provides valuable information on the comparability of three alternative assays for a key exposure biomarker, the aflatoxin-albumin adduct, in the context of a study of aflatoxicosis in Kenya.

The aflatoxin-albumin adduct Deming regression data were significantly skewed; therefore, the levels were initially log transformed. However, Deming equations based on log-transformed data were not more predictive than ones based on nontransformed data, and using the slopes of Deming equations based on nontransformed data provided quantitative estimates. Intercepts were insignificant and could be ignored.

Because the HPLC-f and IDMS analyses are expected to quantitate only AF-lys, the Deming regression slope of 0.71 from the Kenya samples and the overall ratio of 0.67 (HPLC-f/IDMS) from the diluted Kenya patient sample likely reflect the differences in analytical recovery between the methods. The IDMS method uses a stable-isotope internal standard to normalize for analyte loss, whereas no correction is made for the HPLC-f assay. The uncorrected recovery for the IDMS extraction procedure was reported as 78 $\pm$ 6.4% (9). The uncorrected Deming slope is calculated as 0.78, which is close to the Deming regression of 0.71, suggesting that recovery may account for most, if not all, of the differences in the two methods.

In a previous study of IDMS and ELISA analyses (16), the reported regression slope was 2.6 compared with

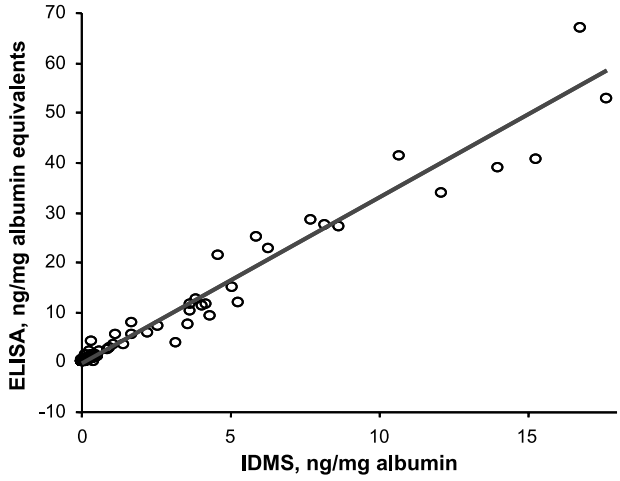

Figure 2. Deming regression of the 2004 Kenya patient sample assay results obtained by ELISA and IDMS.

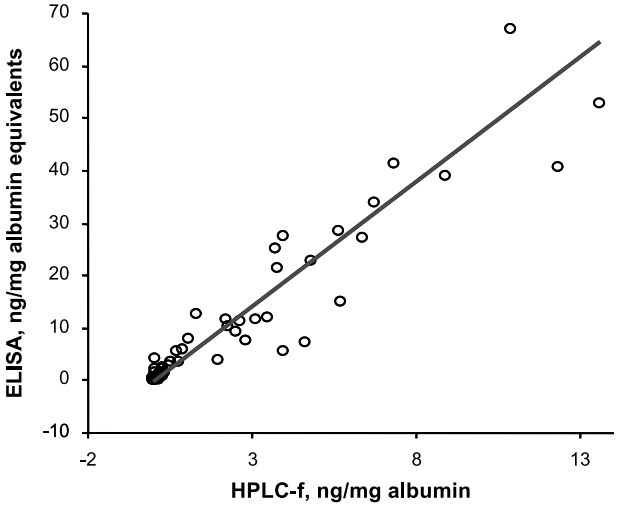

Figure 3. Deming regression of the 2004 Kenya patient sample assay results obtained by HPLC-f and ELISA.

the present value of 3.2 estimated by linear regression. However, in the latter study, the highest adduct concentration as measured by IDMS was ~25 pg/mg albumin, whereas in the present investigation the highest concentration was 3 orders of magnitude higher (17.7 ng/mg). Because the present study represents an extreme range of adduct levels, the curve was truncated to ELISA concentrations of 500 pg/mg albumin for the purpose of comparison and the regression analysis was repeated (n = 39). The slope of this line was 2.53 with a correlation coefficient of 0.86 and an intercept of 0.014 ng/mg albumin, within experimental error of the slope obtained previously (16).

A comparison of the HPLC-f and ELISA methods has been made previously by Wild et al. (17), and the regression slope was of the order of 10. This is somewhat higher than in the current study, although there are methodologic differences between the two studies. In addition, the AF-lys levels were again far lower in the earlier study (17). The higher response of the ELISA method relative to chromatographic methods has been reported previously (16-18). The chromatographic methods are expected to measure only the concentration of AF-lys. Other adducts resulting from incomplete proteolysis digestion, formation of adducts with different amino acids, and ingestion of structurally related aflatoxins, including aflatoxin G_1, could all potentially contribute to the higher ELISA measurements. Multiple chromatographic peaks related to AFB_1 adducts, of which AF-lys is a comparatively small percentage of the total products, have been detected when radiolabeled AFB_1 was administered to rats (6).

The 2004 Kenya study represents an acute aflatoxicosis outbreak and the adduct levels reflect a degree of exposure not encountered previously in epidemiologic studies. The highest previous reported level in a human sample (a child from Benin) was 1.06 ng/mg albumin by ELISA (19), whereas in the present study levels 65 times (67 ng/mg) greater were observed. Despite difference in the quantitative results, all of the methods are able to distinguish between cases and control subjects at $P < 0.01$; subjects with AF-lys levels above the median value for each method were between 13.8 and 143 times more likely to develop aflatoxicosis than were subjects with values below the median.

Each of these methods offers merit depending on the requirements of the study. ELISA offers a high degree of sensitivity and relative ease of analysis. It is likely the least expensive of the methods. It will also tend to have the least specificity for AF-lys. HPLC-f will offer specificity for AF-lys at a relatively low cost, but the sample preparation is extensive and time-consuming. In addition, for many epidemiologic studies, it may not be sufficiently sensitive. IDMS has the greatest sensitivity and specificity of the methods and will tend to be more precise, but it requires expensive instrumentation and technically advanced personnel. Depending on the number of samples to be analyzed, costs may be partly offset by increased analytical speed with 96-well plate technology.

Overall, this study shows excellent correlation between three independent methodologies conducted in different laboratories; the data indicate that each method is valid for assessment of human exposure to AFB_1. Nevertheless, the methods have slightly different characteristics, and these need to be considered in relation to the particular study being conducted.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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