Excretion of an Aflatoxin-Guanine Adduct in the Urine of Aflatoxin B<sub>1</sub>-treated Rats

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ABSTRACT

Administration of aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) to rats resulted in the urinary excretion of 2,3-dihydro-2-(N'-guanyl)-3-hydroxyaflatoxin B<sub>1</sub>. This is the major product formed by the interaction in vivo of AFB<sub>1</sub> with rat liver nucleic acids. The adduct was isolated from urine by the combined use of preparative and analytical high-pressure liquid chromatography and was quantitated by measurement of absorbance at 365 nm. The method allowed reproducible quantitation of adduct in urine samples from rats treated with AFB<sub>1</sub> by i.p. injection at levels as low as 0.125 mg/kg.

Application of the method to urine samples from rats given injections of AFB<sub>1</sub> (1 mg/kg) revealed the presence of a compound chromatographically identical to authentic 2,3-dihydro-2-(N'-guanyl)-3-hydroxyaflatoxin B<sub>1</sub>. Spectral and chemical analysis of mg quantities of this compound provided strong evidence that this compound is identical to authentic adduct.

Measurement of this adduct in the urine of rats given injections of different doses of AFB<sub>1</sub>, showed that excretion occurs in a dose-dependent manner. Comparison of the dose-response curve for adduct excretion with that previously observed for adduct formation in rat liver DNA in vivo revealed a high degree of qualitative similarity, with the levels of adduct excreted in urine representing 30 to 40% of the levels seen initially in liver DNA.

INTRODUCTION

Recent investigations of the molecular aspects of aflatoxin carcinogenesis have revealed the existence of a relationship between the carcinogenic potency of AFB<sub>1</sub> and the extent of its covalent binding to hepatic nucleic acids in various animal models (4, 9, 11). It has also been demonstrated that the covalent binding of aflatoxin to rat liver DNA occurs in a dose-dependent manner (2, 9). These findings suggest the possibility that measurement of the levels of covalent binding of aflatoxin to rat liver nucleic acids in vivo may allow an indirect estimation of the extent of exposure of these animals to AFB<sub>1</sub>.

Currently, the only means of quantitating the extent of binding of aflatoxin to liver nucleic acids in vivo involves removal of the liver, isolation and purification of the nucleic acids, and measurement of the amount of bound carcinogen. This procedure does not allow measurement of binding over the prolonged periods of carcinogen dosing usually needed for tumor development. A noninvasive means of estimating nucleic acid binding by and, consequently, extent of exposure to AFB<sub>1</sub> would circumvent this difficulty.

The major product formed by the interaction of AFB<sub>1</sub> with rat hepatic nucleic acids in vivo is AFB<sub>1</sub>-N<sup>7</sup>-Gua (2, 7). This compound or a derivative thereof is lost rapidly from nucleic acids in vivo (9, 11) and is presumably not reutilized in nucleic acid synthesis. The possibility therefore exists that this adduct is excreted in the urine of AFB<sub>1</sub>-treated rats and that measurement of its excretion could indirectly provide an estimate of the extent of exposure of these animals to AFB<sub>1</sub>.

The research described herein indicates that an aflatoxin-guanine adduct indistinguishable from authentic AFB<sub>1</sub>-N<sup>7</sup>-Gua is excreted in the urine of AFB<sub>1</sub>-treated rats. The excretion of this compound occurs in a dose-dependent manner, allowing an estimate of the body burden of AFB<sub>1</sub>, in these animals. Furthermore, the quantity of adduct excreted in the urine during a 48-hr period following AFB<sub>1</sub> administration represents a relatively fixed proportion of the total amount of AFB<sub>1</sub>-N<sup>7</sup>-Gua initially formed in the hepatic DNA.

MATERIALS AND METHODS

Chemicals. Unlabeled AFB<sub>1</sub> was purchased from Makor, Inc. (Jerusalem, Israel) and used without further purification. DNA (type I) was obtained from Sigma Chemical Co. (St. Louis, Mo.) and nuclease P<sub>1</sub> was purchased from the Yamasa Shoyu Co., Ltd. (Choshi, Japan). Solvents for chromatography were all HPLC grade and were obtained from either Burdick & Jackson Laboratories, Inc. (Muskegon, Mich.) or Fisher Scientific Co. (St. Louis, Mo.). Water was purified through a Milli-Q reagent-grade water system (Millipore Corp., Bedford, Mass.). All other chemicals were of reagent quality.

Production of Authentic AFB<sub>1</sub>-N<sup>7</sup>-Gua. Authentic AFB<sub>1</sub>-N<sup>7</sup>-Gua was synthesized essentially by the method of Martin and Garner (8). AFB<sub>1</sub> was dissolved in dichloromethane to give a solution of 100 µg AFB<sub>1</sub> per ml. An equal volume of a solution of DNA (1.5 mg DNA per ml in 20 mM potassium phosphate, pH 7.4) was added to the vial, and the mixture was rapidly stirred to form an emulsion. m-Chloroperoxybenzoic acid was added to the emulsion to give a final concentration of 230 µg/ml, and the mixture was allowed to react for 24 hr. After this time, the reaction was stopped by centrifugation of the reaction mixture at 320 × g for 15 min to separate the phases.

DNA was precipitated from the aqueous phase by the addition of 4 M NaCl to obtain a solution 1 M in NaCl, with the subsequent addition of 3 volumes of cold (−20°) ethanol. The precipitated DNA was wound out on glass rods and dried in a vacuum at ambient temperature. The DNA was redissolved in 0.05 M potassium acetate, pH 5.25, and heat denatured at 100° for 10 min. After cooling on ice, the DNA was hydrolyzed.
by the addition of 20 μg nuclease P₁ per mg DNA, followed by incubation at ambient temperature overnight.

Following hydrolysis, methanol was added to the sample to obtain a final concentration of 5%. The adduct produced in this procedure was isolated and purified by the chromatographic methods described below.

Animals. Male Fischer rats were obtained from the Charles River Breeding Laboratories (North Wilmington, Mass.). Animals were obtained at 4 weeks of age and housed individually in suspended wire-bottomed cages in a controlled environment until use. Animals were fed a nutritionally complete semisynthetic agar gel diet (12) and given drinking water ad libitum. Rats were used at 7 weeks of age; their weights at this time were 140 to 170 g.

Instrumentation. Ion-exchange HPLC was performed as described previously (3). All other HPLC was performed on a Model 204 liquid chromatograph (Waters Associates, Inc., Milford, Mass.), equipped with a Model U6K injector and 2 Model 6000A pumps controlled by a Model 660 solvent programmer to allow gradient elution. Detection was accomplished by means of a Model 440 dual-channel absorbance monitor (Water Associates) at 254 and 365 nm. The columns used on this instrument were a C₃₅-Bondapak analytical reversed-phase column and a µPorasil analytical silica column (both products of Waters Associates). Quantitation of the adduct was accomplished by measuring the 365-nm peak height with an electronic integrator (Columbia Scientific Industries Corp., Austin, Texas) which was calibrated with (14C)AFB₁-N⁷-Gua. UV absorption spectra of the adduct were recorded on a Model 557 UV-visible spectrophotometer (Hitachi, Ltd., Tokyo, Japan).

Collection of Urine Samples. Urine samples were collected by housing animals individually in glass metabolic cages, allowing separate collection of urine and feces. Urine was collected frozen in a vessel immersed in dry ice-isopropyl alcohol. Quantitative collection of urine samples was accomplished by washing down the cage surfaces with 50 ml of water. The average sample contained 50 ml water and approximately 10 ml urine.

Determination of Adduct in Urine Samples. Samples were adjusted to pH 5 by the addition of 1 N HCl and then deproteinized by the addition of 2.3 volumes of isopropyl alcohol and storage at 4° overnight. Precipitated material was removed by centrifugation of the samples at 800 × g for 10 min at 4°. The supernatants were then rotary evaporated at 50° to a volume of approximately 5 ml. Samples were then adjusted to be approximately 7% in methanol by the addition of 10 ml of 10% methanol to each.

Samples were next submitted to an initial cleanup step by preparative chromatography on C₁₈-Sep-Pak cartridges. Cartridges were attached to a 3-way valve at the outlet of a 20-ml glass syringe. The syringe acted as the sample reservoir during the chromatography, and the 3-way valve facilitated the loading of liquid onto the Sep-Pak without the introduction of air. Samples and 3 consecutive 5-ml 10% methanol rinses of the urine collection vessel were then sequentially passed through the cartridge at a flow rate of approximately 2 ml/min. Weakly retained sample components were eluted from the Sep-Pak by passing 5 ml 10% acetonitrile through the cartridge; these components and those not retained in the loading steps were discarded.

The retained components, which included AFB₁-N⁷-Gua, were eluted from the cartridge with 12 ml 80% methanol; the eluted material was collected. This fraction of each sample was concentrated by rotary evaporation to 1 to 2 ml and subjected to further cleanup on a C₁₈-Bondapak column. The sample and a vessel rinse of 1 ml 0.1 N HCl (brought to a pH of 5 with 0.1 N NaOH) were loaded onto the column at 0.5 ml/min and 50° in a mobile phase of 10% methanol. Elution of AFB₁-N⁷-Gua was accomplished by increasing the flow rate to 1.0 ml/min and running a gradient from 10 to 80% methanol in a period of 1 hr. A fraction was collected which eluted 3 min on either side of the retention time of authentic adduct (28 min). This fraction was concentrated by rotary evaporation to 0.1 to 0.2 ml and analyzed on a µPorasil column.

The sample and a vessel rinse of 0.15 ml 0.1 N HCl (brought to pH 5 with 0.1 N NaOH) were co-injected onto the µPorasil column and eluted isocratically with a mobile phase of 4.5% acetonitrile in water at 1.0 ml/min and 20°. This normal-phase chromatographic system was chosen for use after a systematic investigation of a variety of reversed-phase systems failed to establish conditions which resulted in a satisfactory separation of adduct from 365 nm-absorbing urinary compounds. Preliminary results obtained on a silica-based cation exchange column indicated that such a system could effect the desired separation but that the separation was due mostly to differential adsorption of adduct and urinary compounds and not to ion exchange. This observation was confirmed on µPorasil and led directly to development of the admittedly unorthodox method reported herein. The adduct eluted at a retention time of 11 to 12 min and was quantitated by measuring the 365 nm peak height and referring this value to the calibration curve for the digital integrator. A representative chromatogram is shown in Chart 1. The performance of the method was evaluated by measuring the adduct content of spiked samples that consisted of 5 ml control urine and 25 ml water to which known amounts of adduct had been added.

Characterization and Measurement of the Excretion of Aflatoxin Adducts

Chart 1. Chromatogram showing the final purification of AFB₁-N⁷-Gua from the urine of a rat given an injection of AFB₁. The sample was previously subjected to sequential cleanup through a C₁₈-Sep-Pak reversed-phase cartridge and a C₁₈-Bondapak analytical reversed-phase column. Cleanup through the Sep-Pak was accomplished by loading the sample onto the cartridge, followed by the removal of nonretained and weakly retained components by sequential elution with 10% methanol and 10% CH₃CN. The remaining components were eluted with 80% methanol and submitted to more thorough cleanup by elution through the analytical column by means of a 10 to 80% methanol-H₂O gradient. Details are given in the text.
Urinary Adduct. A total of 15 rats were given i.p. injections of AFB\textsubscript{1} (1.0 mg/kg) in 50 \( \mu \)l dimethyl sulfoxide and housed in metabolic cages for urine collection. Urine was collected for 48 hr after injection; these samples were then pooled and processed in portions through the procedure described above. The compound eluting from the \( \mu \)Porasil column at the retention time of authentic adduct was collected and rechromatographed on the analytical reversed-phase system described above. The purified compound was first subjected to analysis by UV absorption spectroscopy in both acid and base. Following spectroscopic analysis, the compound was reisolated chromatographically and subjected to methylation by dimethyl sulfate. This methylation was performed under conditions which have been shown to result in the selective methylation of the imidazole ring nitrogen atoms of guanine. Following methylation, the urinary compound was subjected to perchloric acid hydrolysis under conditions that cleave bonds connecting aflatoxin moieties to the guanine base but leave methyl groups intact. The hydrolysate was submitted to chromatographic analysis on a system which affords complete resolution of all of the methylated guanines which may have been formed in the methylation step. This chemical derivatization procedure indicates whether or not the compound in question contains guanine and, if it does, whether or not either of the imidazole nitrogen atoms was blocked by covalent attachment to the aflatoxin moiety at the time of methylation. Thus, if the urinary compound was in fact AFB\textsubscript{1}-N\textsuperscript{7}-Gua, the only major product formed in the methylation-hydrolysis procedure would be expected to be 9-methylguanine. A similar amount of authentic AFB\textsubscript{1}-N\textsuperscript{7}-Gua was subjected to spectroscopic analysis and chemical derivatization under identical conditions.

Adduct excretion as a function of both time and dose was determined by injecting groups of rats i.p. with 0.125, 0.25, 0.5, or 1.0 mg AFB\textsubscript{1} per kg in 50 \( \mu \)l dimethyl sulfoxide. Urine samples were collected for various times following injection, and their adduct contents were determined by the method described above.

RESULTS

Performance of the Method. Results of the evaluation of the method for isolation of AFB\textsubscript{1}-N\textsuperscript{7}-Gua from urine are shown in Chart 2. Recoveries range from a low of 69% at 25 ng adduct per sample to essentially quantitative at 200 ng adduct per sample. Analysis of variance of the data presented in Chart 2 indicates that the recovery of adduct from these samples is linear for all but the smallest amount of adduct added (25 ng), where recovery deviates significantly from linearity \((p < 0.001)\). As a result of this characteristic of the method, the regression line shown is applicable for accurately quantitating adduct in samples containing 50 ng adduct or more. Reported adduct levels which are less than this are regarded as semi-quantitative estimates due to a lack of information regarding the shape of the curve at these concentrations of adduct.

Characterization of the Urinary Adduct. Preliminary investigation of the possible urinary excretion of adduct by rats given injections of [\textsuperscript{14}C]AFB\textsubscript{1} indicated that a labeled compound chromatographically identical to authentic AFB\textsubscript{1}-N\textsuperscript{7}-Gua was excreted by these animals. This compound was subsequently isolated from the urine of 15 rats given i.p. injections of AFB\textsubscript{1} (1 mg/kg) to give an estimated total of 3.6 \( \mu \)g of adduct. This compound was then subjected to UV spectroscopic analysis; the results of this analysis are shown in Chart 3.

The spectra of the urinary compound in both acid and base are similar to those of the authentic compound for wavelengths greater than 300 nm, suggesting that the urinary compound contains aflatoxin. This observation is in accordance with the appearance of radiolabel in this compound following the injection of labeled AFB\textsubscript{1}. The lack of a bathochromic shift in base indicates that the putative urinary adduct is not a 2-hydroxy-aflatoxin derivative. Reacidification of both samples resulted in the regeneration of the spectra observed in acid.

The presence of guanine in the putative urinary adduct was demonstrated by methylation of the adduct and subsequent perchloric acid hydrolysis of the methylated product. The methylation was performed under conditions which afford selective methylation of the imidazole nitrogen atoms of guanine and therefore would have resulted in the formation of 9-methylguanine if the urinary compound was identical to the authentic adduct as has been demonstrated previously. The only detectable methylated guanine derivative formed from either authentic or putative urinary adduct was 9-methylguanine. This result is consistent with the assumption that the urinary compound contained guanine and that the guanine was substituted by the aflatoxin moiety at position 7 at the time of methylation.

Taken together, the results of the methylation and the spectral analyses argue strongly that the urinary compound contains an AFB\textsubscript{1} derivative covalently bound to the N\textsuperscript{7} atom of guanine. Thus, the urinary compound is identical to authentic AFB\textsubscript{1}-N\textsuperscript{7}-Gua by these criteria.

Excretion of Adduct by AFB\textsubscript{1}-Injected Rats. Determination of the amounts of adduct excreted by rats at various times following i.p. AFB\textsubscript{1} administration indicated that, at the 1-mg/kg dose, 80% of the total excretion of adduct occurred during a 48-hr period after dosing. The balance of adduct excretion...
Chart 3. Spectra of authentic AFB\textsubscript{1}-N\textsuperscript{7}-Gua and putative urinary adduct in acid and base. The urinary compound was isolated from the 48-hr urines of 15 rats given injections of AFB\textsubscript{1} (1 mg/kg). The 2 compounds were present in similar quantities. A, spectra of authentic adduct and putative urinary adduct at pH 1; B, the same compounds at pH 13.

occurred between 48 and 144 hr. In the case of the 0.25-mg/kg dose, no detectable levels (i.e., less than 5 ng) of adduct were observed at times later than 48 hr. Investigation of adduct excretion at shorter times indicated that the excretion of this compound occurs to an equal extent in the first and second days following AFB\textsubscript{1} administration.

Because the excretion of adduct was nearly complete in 48 hr, the amounts of adduct excreted by this time at various dose levels were used to generate the dose-response curve shown in Chart 4. The data presented in Chart 4 show that the excretion of adduct occurred in a dose-dependent manner. It is also apparent that the animal-to-animal variation in the amount of adduct excreted at a given dose of AFB\textsubscript{1} is small. It is therefore possible, under the conditions used here, to estimate the total body burden of AFB\textsubscript{1} by measuring the adduct levels in the urine of exposed animals.

DISCUSSION

The major product formed by the interaction of AFB\textsubscript{1} with the hepatic nucleic acids of rats is AFB\textsubscript{1}-N\textsuperscript{7}-Gua (2, 7). Although it is not yet known what role the formation of this product plays in aflatoxin carcinogenesis, the extent to which it is formed appears to correlate well with the sensitivity of AFB\textsubscript{1}-treated animals to the toxic and carcinogenic effects of aflatoxin (4, 9, 11). Measurement of the extent of formation of this adduct in liver nucleic acids of AFB\textsubscript{1}-treated animals would therefore be expected to allow estimation of the risk presented to these animals as a result of their exposure.

The method presented herein was developed for quantitating AFB\textsubscript{1}-N\textsuperscript{7}-Gua in the urine of AFB\textsubscript{1}-treated rats. This method was shown to be both sensitive and reproducible, allowing quantitation of urinary adduct from rats given injections of as little as 0.125 mg AFB\textsubscript{1} per kg. Application of the method to the urine of animals dosed with different amounts of AFB\textsubscript{1} showed that the excretion of AFB\textsubscript{1}-N\textsuperscript{7}-Gua is dependent on the administered dose of AFB\textsubscript{1}. This dose dependence of adduct excretion under the experimental conditions used here allows an estimate of the administered AFB\textsubscript{1} dose to be made on the basis of the amount of adduct excreted in urine.

The observation that AFB\textsubscript{1}-N\textsuperscript{7}-Gua is excreted in a dose-dependent manner is consistent with earlier observations that the covalent binding of AFB\textsubscript{1} to liver nucleic acids in the rat exhibits a dose response (2, 9). As was noted above, the risk to animals (or to organs within a given animal) from exposure to AFB\textsubscript{1} correlates with the degree of nucleic acid binding of this compound. It was therefore of interest to determine whether or not a relationship existed between the amount of AFB\textsubscript{1}-N\textsuperscript{7}-Gua formed initially in rat liver DNA and the amount of this adduct subsequently appearing in urine. The results of this comparison are shown in Chart 5.

The dose response curves for the formation and the excretion of adduct are similar in shape; it can be seen that the amount of adduct excreted in 48 hr represents 30 to 40% of that initially present in hepatic DNA. Since the removal of aflatoxin from DNA is essentially complete by 24 hr after injection, the remainder of the bound material is either excreted in bile or it is removed and excreted as another product.

The similarity in the shapes of the 2 curves, taken together with evidence showing that the administration of AFB\textsubscript{1} causes
the formation of far greater amounts of adduct in the liver than in other organs of the rat, suggests that a precursor-product relationship may exist between adduct formed in hepatic DNA and the urinary adduct. However, it has been demonstrated previously that \( \text{AFB}_1 \) binds to rat liver RNA and DNA to a nearly equal extent on a per ng basis following i.p. injection of \( \text{AFB}_1 \) (9). Also, the qualitative distribution of adducts in RNA has been shown to be similar to that in DNA (7). RNA is present in rat liver in a quantity approximately 5 times greater than DNA (5); therefore, rat liver RNA is expected to contain approximately 5 times as much \( \text{AFB}_1 \)-N\(^7\)-Gua as DNA. Aflatoxin bound to RNA is rapidly removed from this macromolecule (9), although the nature of the removed material is not known. It is therefore not presently possible to determine the extent to which RNA contributes to the total amount of adduct excreted in the urine of \( \text{AFB}_1 \)-treated animals.

It is nonetheless apparent that measurement of the excretion of adduct in urine can provide a means to estimate the extent of binding of \( \text{AFB}_1 \) to liver DNA \textit{in vivo}. While the dose-response characteristics are not known for present for RNA binding as they are for DNA, it may also be possible to estimate RNA binding, and therefore total nucleic acid binding, by measuring urinary \( \text{AFB}_1 \)-N\(^7\)-Gua levels.

It has been previously demonstrated that the administration of 40 i.p. doses of \( \text{AFB}_1 \), each 25 \( \mu \)g, over an 8-week period causes a 100\% incidence of hepatocellular carcinoma in male Fischer rats (10). If the rats used are of 150-g initial weights, these doses are equivalent to doses of 0.167 mg/kg. Since the excretion of adduct is easily determined after doses of 0.125 mg/kg, it should be possible to monitor adduct excretion by rats maintained on this carcinogenic regimen.

The results presented above were obtained under a set of specific and well-defined conditions. It is not presently known how generally applicable this approach to risk assessment will be, either with \( \text{AFB}_1 \) or with other carcinogens. It will be necessary to show that the relationship demonstrated above between the formation and excretion of adduct holds under different conditions of dose route and dose protocol (i.e., acute or chronic dosing) and for different species. If the measurement of urinary adduct levels allows an estimate to be made of the degree of \( \text{AFB}_1 \) binding to liver nucleic acids under all circumstances, then the procedure described above may prove to be applicable in the general sense as a tool for noninvasive assessment of the risk presented to an animal or a human by aflatoxin exposure.

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REFERENCES

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