Bovine Hepatic Metabolism of Aflatoxin B_1

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Differences in the expression and catalytic activity of hepatic biotransformation enzymes account for species differences in xenobiotic metabolism, including that of aflatoxin B_1 (AFB_1). The main objectives of this study were (1) to define the procedure for isolation and culture of bovine hepatocytes, (2) to characterize the biotransformation capacity of bovine hepatocytes for AFB_1, and (3) to develop an HPLC method for the simultaneous analysis of AFB_1 and its metabolites. Bovine hepatocytes were isolated and cultured in monolayers. Metabolic function of these hepatocytes was assessed by measuring total cytochrome P450 (CYP450) content, glutathione *S*-transferase (GST) activity, ethoxyresorufin O-deethylation (EROD), testosterone hydroxylation, and α-naphthol glucuronidation. When using these cultures to study the biotransformation of AFB_1, the principal metabolites of AFB_1 were AFM_1 and AFB_1-dihydrodiol (AFB_1-dhd). Minor amounts of AFB_1−glutathione conjugate (AFB_1−GSH) and a polar metabolite were also detected. The polar metabolite was not specifically identified as a glucuronidation product of AFB_1. No AFP_1, AFQ_1, AFB_{2a}, or aflatoxicol (AFL) was detected. The HPLC method developed provided the simultaneous detection of AFB_1 and the metabolites AFB_1-dhd, AFM_1, AFP_1, AFQ_1, AFL, and AFB_1−GSH as well as AFB_{2a}.

Keywords: *Bovine; hepatocytes; biotransformation; AFB_1*

INTRODUCTION

Hepatic enzymes, referred to as drug metabolizing enzymes (DMEs), play an essential role in the biotransformation, sequestration, and elimination of xenobiotics including food components and contaminants, medicinal products, and environmental pollutants. The term DME refers in particular to the cytochrome P450 (CYP450) enzyme family as well as to the major conjugating enzymes such as the glutathione *S*-transferase (GST) family and the UDP-glucuronosyl transferases (UDPGT) (Gonzalez and Nebert, 1990; Nebert, 1994). Originating from closely related genes, CYP450 enzymes show an extensive variability in catalytic activity and substrate specificity, which can be attributed to minor changes in amino acid sequence between species or even individuals (Smith et al., 1991; Belpair and Bogaert, 1996; Nelson et al., 1996).

Whereas DMEs have been intensively studied in laboratory animal species and human organ specimens, information on the specific expression and substrate specificity in bovine species is limited [for a review, see Fink-Gremmels and Miert (1996)]. Species differences in hepatic drug metabolism may not only account for differences in drug disposition and drug interactions of veterinary medicinal products but may also result in different metabolite patterns in edible tissues, milk, and eggs of farm animals intended for human consumption.

Aflatoxin B_1 (AFB_1) is a mycotoxin produced by toxicogenic strains of *Aspergillus flavus* and *Aspergillus parasiticus* and is found as a contaminant in food and feed commodities in several regions of the world. AFB_1 has been shown to be a potent liver carcinogen in experimental animals and is classified as a Group I carcinogen in humans (IARC, 1987). Cows fed AFB_1-contaminated feed develop general health problems, including immunosuppression and reproductive dysfunction. Acute effects include reduced feed consumption and associated weight loss, reduction in milk production (Robens and Richard, 1992), and sudden death (Cockroft, 1995). Although these health problems are of economic importance in animal husbandry (Shane, 1994), the main concern about AFB_1 in animal feeds is the occurrence of AFB_1 and associated metabolites in milk and meat products (Van Egmond, 1994).

Feeding experiments with contaminated feed commodities or directly administered aflatoxin indicated that besides AFB_1, AFM_1 and aflatoxicol (AFL) could be measured in liver, kidney, and muscle tissue (Stubblefield et al., 1983; Trucksess et al., 1983). In addition, AFM_1 could be detected in cow's milk (Munksgaard et al., 1987). However, other metabolites of AFB_1, including AFP_1, AFQ_1, and AFB_1-dihydrodiol (AFB_1-dhd), and many conjugated products have been identified by in vitro and in vivo experiments in other species including mice, rats, and humans [for a review, see Gorelick (1990)] but not in bovine species.

Here we describe the techniques for isolation and the culture conditions for bovine hepatocytes allowing investigations on species-specific biotransformation processes. The series of experiments indicates the overall activity of CYP450, GST, and UDPGT and, by application of established model substrates, the catalytic functionality and conjugating activity of the isolated hepatocytes. Cell cultures were exposed to AFB_1 to establish the bovine pattern of hepatic aflatoxin me-

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tabolites. An HPLC method is introduced, allowing the simultaneous analysis of AFB_1 and its metabolites AFM_1, AFP_1, AFQ_1, AFB_1-dhd, AFB_1−GSH, and AFL as well as AFB_{2a}. The method developed avoids extensive sample preparation, which might result in quantitative losses of certain metabolites.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin (BSA; fraction V), glutamine, hydrocortisone-21-hemisuccinate sodium, bovine pancreas insulin, gentamicin sulfate, collagenase (type IV), powdered Williams' E medium containing phenol red (10.7 mg/L), HEPES, EGTA, testosterone, 11β- and 16α-testosterone, androstenedione, AFB_1, AFQ_1, AFP_1, AFB_{2a}, and AFL were obtained from Sigma (St. Louis, MO). Fetal bovine serum was provided by Gibco BRL Life technologies (Eggenstein, Germany). 2α-, 6α-, and 15α-testosterone were gifts from Prof. D. N. Kirk (Steroid Reference Collection, London, U.K.). 12β- and 15β-testosterone were gifts from G. D. Searle & Co. (Skokie, IL). 2β-, 6β- 7α-, 11α-, 16β- and 19-hydroxytestosterone were purchased from Steraloids (Wilton, NH). Resorufin was obtained from Eastman Kodak (Rochester, NY). β-Glucuronidase was purchased from Boerhinger Mannheim GmbH, Germany. Ring-labeled [^{14}C]AFB_1 with a specific activity of 179.5 mCi mmol^{-1} (6641.5 Bq nmol^{-1}) was obtained from Movarek Biochemicals (Brea, CA). Dr. H. P. van Egmond (National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands) kindly provided AFM_1. All other chemicals were of HPLC or analytical grade.

AFQ_1 was prepared from AFB_1 using liver cells of marmoset monkeys, kindly provided by Dr. A. S. A. M. van der Burght (Ritox, Utrecht, The Netherlands). AFQ_1 obtained from commercial sources contained two epimers resolved as separate HPLC peaks. The second of these two peaks cochromatographed with AFQ_1 generated by marmoset monkey cells, whereas the first was not formed in this in vitro system. AFB_1−GSH conjugate was synthesized according to the method of Raney et al. (1992). AFB_1-dhd was prepared using avian microsomes (Neal et al., 1986).

Isolation of Hepatocytes. The caudate liver lobe of dairy cows (n = 3, 3−5 years old, 400−500 kg) was obtained from the slaughterhouse within 10 min of exsanguination. After removal, the lobe (≈100 g of tissue) was rinsed with ice-cold Eurocollins buffer (15 mM KH_2PO_4, 42 mM K_2HPO_4, 15 mM KCl, 1 mM $NaHCO_3$, 0.2 M glucose, pH 7.4) supplemented with 1 mM EGTA to remove all of the blood and to deplete calcium, by inserting a cannula, connected to a peristaltic pump, into the veins. After transport of the lobe in fresh buffer on ice to the laboratory, hepatocytes were isolated according to the method of Van't Klooster et al. (1992), based on the method of Seglen (1976) with some modifications. Briefly, the lobe was placed on a perfusion funnel and a retrograde three-step perfusion was performed via four cannulas. The buffers used were sterile, adjusted to pH 7.4 and saturated with O_2/CO_2 (95:5) (v/v) at a temperature of 37 °C. The liver lobe was first perfused with 1 L of buffer I containing 142 mM NaCl, 0.5 mM KCl, 9.2 mM HEPES, and 0.5 mM EGTA at a rate of 40 mL/min. Buffer II (1 L) had the same composition but lacked the EGTA. Finally, the lobe was perfused by recycling buffer III containing 9.2 mM HEPES, 66.7 mM NaCl, 6.7 mM KCl, 4.74 mM $CaCl_2$, and 0.05% collagenase for 15−20 min. After the collagenase digestion, the lobe was transferred to a laminar flow cabinet, where the liver capsule was carefully removed as well as the nonperfused tissue. Subsequently, cells were released by gently agitating the lobe in a buffer containing 9.3 mM HEPES, 9.91 g/L modified Hanks' balanced salts solution (HBSS), and 3% bovine serum albumin (pH 7.65). The cell suspension was sieved through a nylon mesh (125 μm) and centrifuged (80g, 5 min). The cell pellet was washed three times with Williams' E medium. The final pellet was weighed to estimate the number of cells isolated (1 g contained ≈1.2 × 10^8 cells), and the viability of the cells was assessed by trypan blue exclusion. Lactic dehydrogenase was determined according to the method of Bergmeyer et al. (1965).

Cell Culture. Cells were cultured at a density of 4 × 10^6 cells/60 mm culture dish (Greiner, Alphen a/d Rijn, The Netherlands) in 4 mL of Williams' E containing 4% new-born calf serum, glutamine (1.67 mM), gentamicin sulfate (50 μg/mL), hydrocortisone (1 μM), insulin (1 μM), $CaCl_2$ (0.5 mM), and $MgCl_2$ (0.5 mM). Cells were incubated in a humidified atmosphere of air (95%) and CO_2 (5%) at 37 °C and left to attach for 4 h. The medium was then replaced by medium without serum, $CaCl_2$, and $MgCl_2$.

Enzyme Assays. Ethoxyresorufin O-deethylation (EROD) was assayed essentially as described by Wortelboer et al. (1990). Briefly, cells were incubated with 5 μM ethoxyresorufin in HBSS for 20 min. One hundred microliters of incubation medium was diluted 1:1 with 0.01 M NaOH in a 96 well plate and measured with a Cytofluor 2300 (Millipore Corp., Bedford, MA) at λ_{ex} = 530 nm and λ_{em} = 590 nm. Testosterone hydroxylation was determined by incubating hepatocytes with 250 mM testosterone in HBSS according to the method of Funae and Imoaka (1987). After 20 min, 2.5 mL of medium was stored at −20 °C until use or extracted immediately with 6 mL of dichloromethane after 11β-hydroxytestosterone was added as internal standard. After extraction, the aqueous layer was removed and the dichloromethane fraction was dried under nitrogen. The residue was reconstituted in 130 μL of 50% aqueous MeOH and analyzed by HPLC. Testosterone metabolites were separated using a 200 × 3 mm (5 μm) Chromsep C_{18} column (Chrompack, Middelburg, The Netherlands) at 60 °C. Elution was performed using (A) water/methanol 75:25 (v/v) and (B) water/methanol/acetonitrile 30:64:6 at a flow rate of 0.8 mL/min. The gradient started with 100% A decreasing linearly to 42% in 45 min and a further decrease of 1% in 5 min. Finally, the gradient was restored to 100% A in 3 min. UV detection was performed at 254 nm. Metabolites were quantified by comparing peak areas to those of authentic standards. CYP450 determination was conducted according to the method of Rutten et al. (1987) using the dithionite difference spectrum method. Samples were stored at −70 °C until analysis.

UDPGT-dependent enzyme activities were determined by incubating hepatocyte cultures with 250 μM α-naphthol for 30 min. Medium samples were analyzed for α-naphthol glucuronide by means of HPLC, essentially as described by Redegeld et al. (1988).

Total GST activity was assayed according to the method of Habig et al. (1974). Briefly, cells were scraped off the culture dishes in 1 mL of Cord buffer (2 mM $NaH_2PO_4 \cdot 2H_2O$, 1 mM $Na_2HPO_4 \cdot H_2O$, 0.14 M NaCl, 8.5 mM $MgCl_2$, and 11 mM glucose, pH 6.3) and stored at −20 °C until analysis. The cells were thawed, sonicated for 5 min, and centrifuged. Ten microliters of supernatant was added to 990 μL of a 0.2 M potassium phosphate buffer (pH 6.5) containing 2 mM EDTA, 1 mM GSH, and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) as substrate. The reaction was followed at 340 nm for at least 3 min using a spectrophotometer (Shimadzu UV-2101 PC). The activity was calculated by comparing the change in absorption of a sample with that of a reference from which the supernatant was omitted. Protein in cells and supernatant was assayed according to the method of Lowry et al. (1951) using BSA as a standard. All incubations were performed in triplicate.

Incubation with AFB_1. Twenty hours after isolation, incubations with AFB_1 commenced after the cell culture medium was replaced with Williams' medium E containing AFB_1 in DMSO (maximum concentration of DMSO = 0.1%). After incubation, 1 mL samples of medium were stored at −20 °C until analysis by HPLC. To study the sequestration of AFB_1 in cells and medium, parallel incubations were conducted with [^{14}C]AFB_1.

HPLC Analysis of Aflatoxin Metabolites. Medium samples were thawed, centrifuged (10000g), and analyzed by reversed-phase HPLC using an ODS-2 Spherisorb C_{18} (250 × 4.6 mm, 5 μm) column (Chrompack) fitted with a C_{18} guard column. Elution consisted of a gradient of (A) 5 mM aqueous sodium acetate (pH 3.5) and (B) acetonitrile/5 mM aqueous sodium acetate (pH 3.5) (70:30) (v/v) with a flow rate of 1.5

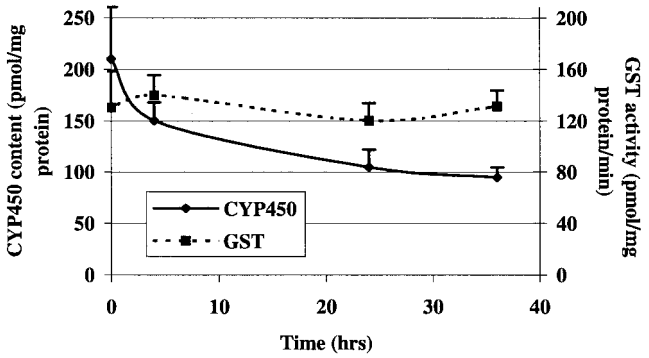

Figure 1. Time course of total CYP450 content and GST activity in cultured bovine hepatocytes. Data represent mean ± SD of cultures in triplicate obtained from three cows.

mL/min at ambient temperature. The multistep gradient started at 30% B for 5 min and increased linearly to 45% B in 35 min, followed by a further linear increase to 49% B in 3 min. After 10 min, the percentage of B was further increased to 100% in 2 min and held at this level for 11 min to elute AFB_1 and AFL. The initial conditions were re-established over 2 min and, after another 7 min, the next run was started. Metabolites were detected using fluorescence at $\lambda_{ex} = 365$ nm and $\lambda_{em} = 420$ nm (FP-920 Jasco, Japan) and UV absorption at $\lambda = 365$ nm (LKB VWM 2141, Pharmacia, Sweden). Typically, 25−50 μL samples were analyzed. Peak areas were determined using Gynkosoft 5.5 data analysis package (Gynkotek, Germany) and a calibration curve of AFB_1. Sample injections were performed using a cooled autosampler (Promis II, Spark Holland, The Netherlands).

Determination of concentration and purity of aflatoxin standards was performed according to IUPAC/AOAC methods (Scott, 1990). The limit of detection (LOD) for AFB_1 for fluorescence and UV detection was calculated according to the method of Taylor (1987).

Metabolites in medium following incubation of cells with AFB_1 were subjected to β-glucuronidase treatment. Samples were assayed using the method described above.

The distribution of AFB_1 over cells and medium was assayed by incubating hepatocyte cultures with [^{14}C]AFB_1. Fifty microliter samples of medium and cells scraped in PBS were counted separately (β-counter, Minaxi, Tri-Carb 4000 series, United Technologies Packard). Samples of medium were analyzed using HPLC directly followed by scintillation counting (β-counting, LSC Berthold LB 506 C, MaxiFluor, Backer) as detector.

RESULTS

Isolation of bovine hepatocytes performed as described above yielded 5.3−10.4 g of cells per liver lobe with a viability of 73−82%, as assessed by trypan blue exclusion. Average plating efficiency was 82−87% 3−4 h after plating. Lactic dehydrogenase leakage was 30% just after the isolation and remained at this level for 24 h. After incubation of the cells in serum-free medium overnight, hepatocytes formed a monolayer with a flattened appearance.

The metabolic functionality of the isolated hepatocytes was assayed by measuring total CYP450 content, testosterone hydroxylation, EROD, α-naphthol glucuronidation, and GST activity. The initial level of CYP450 was 210.4 ± 50 pmol/mg of protein immediately after isolation, decreasing to 94.6 ± 10.1 pmol/mg of protein within 36 h after isolation (Figure 1). The initial total GST activity amounted to 130.5 ± 28.3 pmol/mg of protein/min and remained stable for at least 36 h. The total CYP450 content and phase I and II enzyme activities assayed 24 h after isolation are shown in Table 1.

Table 1. Total CYP450 Content and DME Activities in Cultured Bovine Hepatocytes Measured 24 h after Isolation[a]

enzyme assay	activity (pmol/mg of protein/min)
CYP450[b]	104.7 ± 17.3
EROD	11.9 ± 4.0
testosterone hydroxylations[c]	
2β	15.7 ± 3.1
6β	32.8 ± 10.0
11α	2.2 ± 0.8
12b	16.3 ± 4.1
15β	4.3 ± 1.1
androstenedione	162.4 ± 36.2
GST[d]	120.3 ± 13.6
α-naphthol glucuronidation	275.0 ± 90.0

[a] Data are expressed as means ± SD obtained from triplicates from three different cows. [b] Cytochrome P450 (CYP450) content in picomoles per milligram of protein. [c] No other hydroxytestosterone metabolites were found. [d] GST, total activity toward CDNB as substrate.

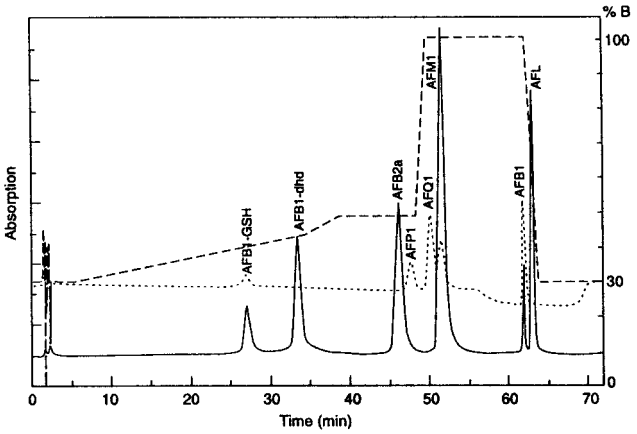

Figure 2. HPLC chromatogram showing the separation of AFB_1 and its metabolites: (—) fluorescence signal; (• • •) UV signal; (- - -) gradient eluents B. For details on HPLC method, see Materials and Methods.

EROD activity was 11.9 ± 4.0 pmol/mg of protein/min. Metabolites of testosterone formed by bovine hepatocytes were 6β-, 12β-, and 2β-hydroxytestosterone and androstenedione, the latter not being a CYP450 metabolite. α-Naphthol glucuronidation amounted to 275 pmol/mg of protein/min.

HPLC Analysis of Aflatoxin Metabolites. Figure 2 shows a typical HPLC separation of AFB_1, AFM_1, AFQ_1, AFP_1, AFB_1-dhd, AFL, AFB_{2a}, and AFB_1−GSH using both fluorescence and UV detection. Separation was achieved by using a gradient of aqueous sodium acetate (pH 3.5) and 35% acetonitrile in the same buffer. Several solvent compositions and gradients had been examined. A methanol−water gradient did not provide a separation of the AFB_1 hydroxy metabolites AFM_1 and AFQ_1. Although a gradient of acetonitrile and water was able to separate those, separation and peak shape of the more polar metabolite, AFB_1−GSH, was not satisfactory. Acidification of the eluent improved peak shapes and was employed in the separation described. Ultrafiltration during sample preparation was apparently responsible for losses of ~80%. However, centrifugation resulted in high yields. It was evident that AFB_1 and its metabolites are unstable in the presence of UV light, resulting in inaccurate measurements. Therefore, the use of amber vials in the autosampler is essential. However, those precautions still resulted in losses of AFM_1 (30%), AFB_1-dhd (65%), and AFB_1 (40%) if the

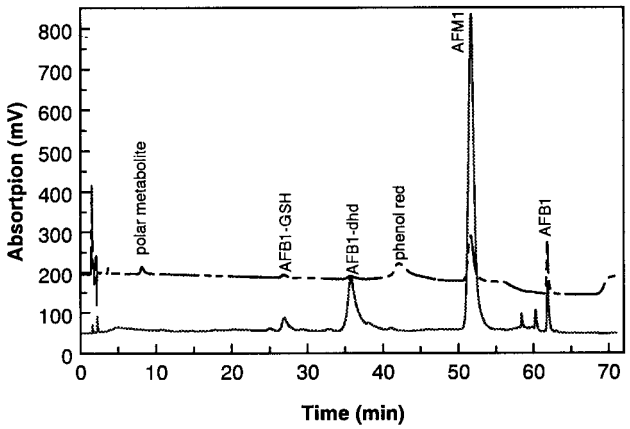

Figure 3. Typical HPLC chromatogram of cell culture medium obtained from incubations of bovine hepatocytes with AFB_1: (—) fluorescence signal; (- - -) UV signal. Incubation with 8 μM AFB_1 for 24 h started 20 h after isolation of the hepatocytes.

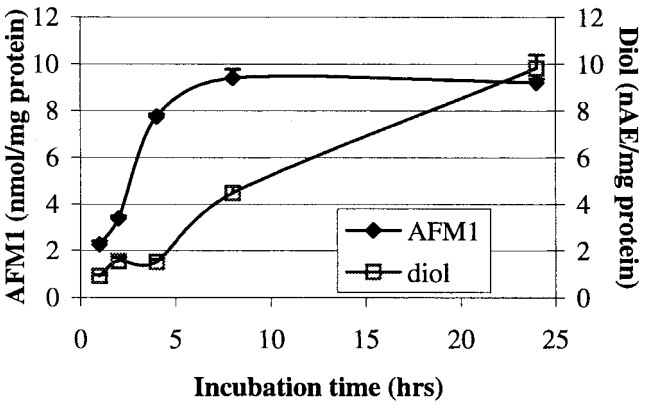

Figure 4. Time course of the metabolism of AFB_1 into AFM_1 and AFB_1-dhd by cultured bovine heptaocytes. AFB_1-dhd is expressed in 10^{-9} aflatoxin B_1 equivalents.

samples were kept at room temperature overnight in a standard autosampler. Cooling the samples on the autosampler to 4−8 °C reduced overnight losses of AFB_1 and AFB_1-dhd to 20 and 8%, respectively, and preserved AFM_1 levels totally. Cooling also improved the peak shape of AFM_1. AFB_1−GSH appeared to be stable at room temperature or 4 °C.

The LOD of AFB_1 using fluorescence detection was 0.8 pmol, whereas UV detection was less sensitive (LOD = 5.5 pmol).

Figure 3 shows a typical HPLC chromatogram of medium following the incubation of bovine hepatocytes with 8 μM AFB_1 for 24 h. AFB_1 was metabolized predominantly to AFM_1 with a smaller amount of AFB_1-dhd; some AFB_1−GSH was formed as well as an unknown metabolite at t_R = 7.3 min. Attempts to deglucuronidate this compound were not successful. The small peak between 40 and 45 min in the UV chromatogram is caused by phenol red, the pH indicator of the cell culture medium. Parallel incubations with [^{14}C]-AFB_1 revealed no other metabolites in the medium and >80% of the radioactivity added was present in the cell culture medium after the incubations (data not shown). Figure 4 shows the time-dependent formation of AFM_1 and AFB_1-dhd by bovine hepatocytes incubated with 8 μM AFB_1 over 24 h.

DISCUSSION

The first aim of the present study was to establish the procedure for isolation and culture of bovine hepatocytes in confluent monolayers. The method described yielded cells with good biotransformation capacity. Eurocollins buffer supplemented with EGTA was used for rinsing and transport of the liver lobe, with the aim to prevent acidosis and Kupffer cell activation (Sumimoto et al., 1991; Ar'Rajab et al., 1994). Preliminary experiments in which the medium was supplemented with pentoxifylline to prevent Kupffer cell activation activity (Currin et al., 1993) did not result in an increase in viability and CYP450 content.

To characterize the biotransformation capacity of the bovine hepatocyte cultures, basic enzyme activities, including typical CYP450-dependent reactions such as testosterone hydroxylation and EROD, were measured, as well as total CYP450 content, GST activity, and α-naphthol glucuronidation. The total CYP450 content declined by 50% within 24 h, compared with ≈80% decrease in CYP450 content in rats (Paine, 1990). In contrast, the GST activity was found to be constant for 36 h after isolation. Shull et al. (1986) found a loss of activity of other phase II enzymes, for example, sulfatase and glucuronidase, in cultured bovine hepatocytes. It was suggested that this decline occurred due to detachment of cells. The GST activity expressed in bovine hepatocytes is much lower than that of rat hepatocytes (Smith et al., 1984; Woutersen-Van Nijnanten et al., 1997). The major CYP450 metabolites of testosterone were 6β-, 12β-, and 2β-hydroxytestosterone. In contrast, 2α-, 6β-, and 16α-hydroxytestosterone are the main metabolites produced by rat hepatocytes. The bovine-specific metabolism of testosterone is in agreement with results presented by Van't Klooster et al. (1993) and Longo et al. (1991), the latter using bovine microsomes instead of hepatocytes. EROD and glucuronidation activities were found to be comparable with results in rat (Wortelboer et al., 1990) and bovine hepatocytes (Van't Klooster et al., 1994).

Bovine hepatocyte cultures were established to study the species-specific metabolism of AFB_1, and an HPLC method was developed allowing the simultaneous analysis of the most prominent AFB_1 metabolites. Few studies have dealt with metabolism of AFB_1 in hepatocytes, and even fewer combined controlled cell cultures with HPLC analyses. In earlier studies, hepatocyte cultures or suspensions were extracted with chloroform followed by TLC determinations (Loury et al., 1984; Wei et al., 1985). Conjugates were assessed by incubating the aqueous phase with glucuronidase and sulfatase followed by extraction (Ch'ih et al., 1989). Subsequently, extracts of medium following incubation of rat hepatocytes with AFB_1 were analyzed by HPLC (Lotlikar et al., 1989; Jennings et al., 1994). Langouët et al. (1995) described the analysis of medium of human hepatocytes exposed to AFB_1. Using fluorescence detection they identified *exo*- and *endo*-AFB_1−GSH, AFB_1, and AFM_1. AFP_1 and AFQ_1 were not detectable in medium from human hepatocytes incubated with AFB_1 due to their poor fluorescence.

The method described here has the advantage of allowing the analysis of medium of hepatocytes incubated with AFB_1 in the presence of UV-absorbing constituents in the cell culture medium, by using both UV and fluorescence detection. AFB_1 itself as well as AFM_1, AFP_1, AFQ_1, AFB_1-dhd, AFB_1−GSH, AFL, and AFB_{2a} could be separated and analyzed simultaneously

without an extensive cleanup bearing the risk of quantitative losses of certain metabolites.

Incubation of bovine hepatocytes with AFB_1 resulted in the formation of AFM_1, AFB_1-dhd, AFB_1−GSH, and an unknown polar metabolite, which is not amenable to β-glucuronidase treatment. Parallel incubations with [^{14}C]AFB_1 confirmed that no other metabolites were formed. Moreover, it was justified to analyze only the incubation medium as most radioactivity was found to be extracellular after short- and long-term incubations. Time course studies showed that AFM_1 was predominantly formed within the first 8 h of incubation, whereas the diol appeared in the medium only after prolonged incubation times (Figure 4).

Larsson et al. (1989) showed that crude bovine liver tissue homogenate converts AFB_1 into AFM_1, AFP_1, and traces of AFQ_1. In addition, their experiments with [^{3}H]-AFB_1 indicated the reduction of AFB_1 to AFL. The difference in biotransformation between liver tissue homogenate and controlled hepatocyte cultures might indicate the contribution of nonparenchymal liver cells to AFB_1 metabolism (Schlemper et al., 1991; Liu and Massey, 1992; Jennings et al., 1994; Roy and Kulkarni, 1997). Furthermore, these differences might reflect the loss of CYP450 activity under cell culture conditions. AFB_{2a} formation, as described by Larsson et al. (1989), was probably due to nonenzymatic degradation known to occur under the influence of UV light or at acidic conditions.

In vivo experiments demonstrated that AFM_1, which is excreted into milk, is a major metabolite in bovines (Frobish et al., 1986; Munksgaard et al., 1987; Van Egmond et al., 1994; Sabino et al., 1995). In addition, AFL could be detected in plasma, erythrocytes, and milk of lactating cows (Trucksess et al., 1983). As no AFL could be detected in bovine hepatocyte cultures, the reduction of AFB_1 to AFL might be attributed to the microbial activity of the rumen or gut flora. Experiments in this laboratory have shown a conversion rate of ≈1% for the ruminal flora (Auerbach et al., 1998). However, additional formation of AFL by cytosolic reductases, as observed in avian species, cannot be excluded completely, as minor amounts of AFL might be retained in the cells (Chen et al., 1981).

Compared to results obtained with rat hepatocytes, bovine hepatocytes seem to have a reduced capacity to conjugate AFB_1 phase I metabolites. In incubations with rat hepatocytes not only were AFM_1, AFP_1, and AFQ_1 detected but also their conjugated forms (Wei et al., 1985; Ch'ih et al., 1991). In addition, AFB_1−GSH conjugate and AFB_1-mercapturate were formed. However, no AFB_1-dhd was found using rat hepatocytes.

AFB_1-dhd is a major metabolite of AFB_1 in avian species (Neal et al., 1981) and is described here for the first time as a metabolite of bovine AFB_1 metabolism. The quantification of AFB_1-dhd is hindered by its ability to bind to intracellular proteins (Schiff base reaction). Thus, it can be suggested that in bovines AFB_1-dhd is a major metabolite arising from the hydrolysis of the AFB_1-epoxide.

In summary, our results with defined cultures of bovine hepatocytes indicate that these metabolize AFB_1 into AFM_1, the AFB_1-epoxide, measurable in its hydrolyzed form as AFB_1-dhd, and the AFB_1−GSH conjugate as well as an unknown polar metabolite. No other metabolites were formed as confirmed in incubations with ^{14}C-labeled AFB_1. In consideration of the typical species-dependent expression of CYP450 as well as GST activity, further studies will be directed toward the contribution of individual bovine CYP450 isoenzymes in AFB_1 metabolism.

ABBREVIATIONS USED

AF, aflatoxin; AFB_1-dhd, aflatoxin B_1 dihydrodiol (8,9-dihydroxy-8,9-dihydroaflatoxin B_1); AFB_1−GSH, AFB_1−glutathione conjugate [8,9-dihydro-8(*S*-glutathionyl)-9-hydroxyaflatoxin B_1]; AFL, aflatoxicol; DME, drug metabolizing enzyme; EROD, ethoxyresorufin O-deethylation; GST, glutathione *S*-transferase; HBSS, Hanks' buffered salts solution; LOD, limit of detection; UDPGT, UDP glucuronosyl transferase.

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