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Reduction of Digoxin to 20*R*-Dihydrodigoxin by Cultures of *Eubacterium lentum*

LARRY W. ROBERTSON,* APPAVU CHANDRASEKARAN, RICHARD H. REUNING, JAMES HUI, AND BHUPAT D. RAWAL†

College of Pharmacy, The Ohio State University, Columbus, Ohio 43210

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The anaerobic bacterium *Eubacterium lentum*, a common constituent of the intestinal microflora, inactivates digoxin by reducing the unsaturated lactone ring. Reduction of the cardiac glycoside by growing cultures of *E. lentum* ATCC 25559 proceeded in a stereospecific manner, with the 20*R*-dihydrodigoxin constituting more than 99% of the product formed. This is in contrast to the 3:1 ratio of 20*R* and 20*S* epimers formed in the chemical catalytic hydrogenation. Formation of the reduced glycosides proceeded quantitatively when an overall concentration of 10 μg/ml was added to the cultures. *E. lentum* did not hydrolyze the digitoxose sugars from C-3 of the parent glycoside. However, the synthetically prepared sugar-hydrolyzed metabolites (digoxigenin, digoxigenin monodigitoxoside, and digoxigenin bisdigitoxoside) were reduced to the corresponding dihydro metabolites. Repetition of the experiments with a feces sample from a volunteer who was known to be a converter of digoxin to dihydrodigoxin gave results identical to those obtained with pure *E. lentum* cultures.

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The cardiac glycoside digoxin (DG3) (Fig. 1) is the most widely used drug for the treatment of congestive heart failure and cardiac arrhythmias. It is known that an important route of metabolism of DG3 is through the formation of the bisdigitoxoside (DG2), monodigitoxoside (DG1), and aglycone (digoxigenin) (DG0), by the cleavage of the sugar moieties attached at the C-3 position of the steroid nucleus (Fig. 1) (8, 9, 12). Although there is evidence for additional metabolism of the aglycone, the metabolites are incompletely characterized (12). Even though the formation of large amounts of the reduced metabolites of DG3 (for example, dihydrodigoxin [DHDG3]) (Fig. 1) in a significant minority of patients was known for some time (1, 2, 4, 18, 21–23), it was only recently discovered that this conversion of DG3 to DHDG3 occurs within the gastrointestinal tract (17). Further, the organism *Eubacterium lentum*, a gram-positive, obligate anaerobe and normal member of the intestinal flora, was identified as a bacterial species that can perform the reduction (5, 6). *E. lentum* and other *Eubacterium* species are capable of performing a variety of transformations on xenobiotic and native molecules, including dehydroxylation of corticosteroids (3), biotransformations of linoleic acid and bile acids (7, 15), and reduction of cholesterol (19). The intensified study of *E. lentum* and other intestinal bacteria is part of an increasing awareness of the important role of these procaryotic residents in drug metabolism (13).

Reduction of the unsaturated ring of DG3 leads to the introduction of a new chiral center at C-20 (1, 2). Standard chemical catalytic hydrogenation results in the formation of two DHDG3 epimers: 20*R* and 20*S*. These epimers are formed in a ratio of about 3:1. Recent investigations indicate that the DHDG3 epimer found in human urine is 20*R* (20). Since the stereochemistry of the reduced metabolites formed by incubating *E. lentum* with DG3 or other cardiac glycosides has not been studied before, this investigation was designed in part to determine the stereochemistry of the product(s).

Although it has been shown that *E. lentum* can convert DG3 to one or more reduction products, the products have only been detected and quantitated by a radioimmunoassay (5, 6), wich gives no information as to whether the digitoxose sugars at C-3 are present or have been hydrolyzed. Likewise, it was not known whether the bacterium could convert the sugar-hydrolyzed metabolites to their respective dihydro products. Gastric juice of patients who have an acidic pH can hydrolyze the sugar side chain from DG3 in a reaction that leads to the formation of DG2, DG1, and DG0 (9–11). Such hydrolysis products would be available to the intestinal bacteria to perform further conversion (reduction) reactions. To investigate the latter possibility, the sugar-hydrolyzed metabolites were incubated with *E. lentum* cultures and with fecal samples obtained from a volunteer who was known to be a DHDG3 excretor.

MATERIALS AND METHODS

Chemicals and media. Reference DG3 and its synthetically prepared sugar-hydrolyzed metabolites and dihydro derivatives were obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and Burroughs Wellcome Co. (Research Triangle Park, N.C.). The derivatizing reagent (1-naphthoyl chloride) was purchased from Fluka (Hauppauge, N.Y.). The media for growing the bacterial and fecal samples were thioglycolate medium without dextrose or indicator (TG) (Difco Laboratories [Detroit, Mich.] or BBL Microbiology Systems [Cockeysville, Md.]) and brain heart infusion broth (Difco or BBL) with added cysteine (BHIC) (3).

The *E. lentum* strain was obtained from the American Type Culture Collection (ATCC 25559). The cultures were maintained by serial transfer in Lee tubes (16) filled with TG medium with 1.5% agar added.

Bacterial transformation. The steroids were dissolved in dimethyl formamide (DMF) and added to tubes containing 15 ml of freshly autoclaved medium (TG or BHIC) to give an overall concentration of 10 μg of DG3 per ml. Equimolar concentrations of other compounds were used. The volume

* Corresponding author.
† Present address: Pharmacy Department, University of Queensland, St. Lucia, Queensland, Australia 4067.

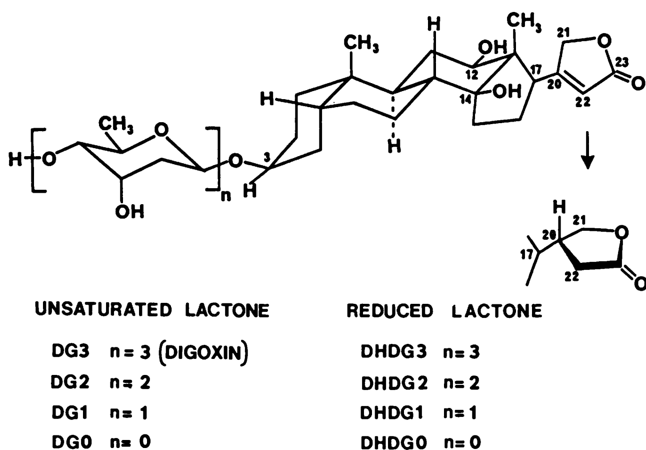

FIG. 1. Structures of DG3 and its sugar-hydrolyzed and reduced metabolites.

of DMF added was 0.16 ml or less per tube. The tubes were then inoculated with 1 ml of a 2-day-old *E. lentum* culture. After mixing, the tubes were capped tightly and incubated at 37°C. After 7 days of incubation the tubes were centrifuged, and a 1-ml sample of the supernatant was removed from each tube and extracted with methylene chloride. After derivatization the extract was analyzed for the parent cardiac glycoside and the corresponding dihydro compounds (both epimers) by the chromatographic method described below. (T. A. Shepard, J. Hui, A. Chandrasekan, R. A. Sams, R. H. Reuning, L. W. Robertson, J. H. Caldwell, and R. L. Donnerberg, J. Chromatogr., in press). To determine the time course of bacterial reduction of DG3, cultures prepared and inoculated as described above were sampled at 1-h intervals for a period of 30 h.

Fecal samples. Fecal specimens were obtained from a normal volunteer, who was known to be a DHDG3 excretor, but who had not taken DG3. A sterile spatula was used to add 3.0 g of fecal sample to a tube containing 15 ml of sterile distilled water. After mixing for 15 s on a Vortex mixer, 1-ml samples were transferred to tubes holding 15 ml of the culture medium (TG or BHIC) containing 10 μg of the compound of interest per ml. The tubes were incubated for 7 days at 37°C. After incubation the contents of the tubes were mixed, the tubes were centrifuged, and a 1-ml sample of the supernatant from each tube was analyzed for parent and dihydro compounds.

Controls. Apart from the above bacterial and fecal incubations the following control experiments were performed. First, *E. lentum* was inoculated into each of the media used in the above experiments and incubated for 2 days. The cultures were then autoclaved, DG3 (10 μg/ml) was added, and the tubes were incubated for 7 days. After incubation the tubes were analyzed for conversion of DG3 to DHDG3. Second, DG3 (10 μg/ml in DMF) was added to a freshly inoculated *E. lentum* culture, extracted immediately (0 h), and analyzed. Finally, uninoculated sterile media and blank *E. lentum* cultures with DMF (0.16 ml) alone were incubated at 37°C for 7 days. The cultures were then extracted and analyzed for any possible peaks at the retention times of the dihydro metabolites (either epimer). The same types of controls, including autoclaving the feces-containing media, adding digoxin, and analyzing for conversion to DHDG3, were performed with fecal incubations.

Extraction procedures. To each 1-ml sample of supernatant was added 1 ml of methylene chloride containing digitoxigenin (DT0) (100 ng) as an internal standard and 1 ml of methylene chloride. The tubes were hand-shaken and centrifuged, and the aqueous layer was removed by aspiration. A 2-ml volume of an aqueous 5% sodium bicarbonate solution was added to the methylene chloride extract. The tube was then shaken and centrifuged, and the aqueous layer was again aspirated. The organic phase was transferred to a clean test tube and evaporated to dryness under nitrogen before derivatization.

Derivatization. To the evaporated sample was added 25 mg of 4-dimethyl aminopyridine, 10 μl of 1-naphthoyl chloride, and 100 μl of acetonitrile. The sample was mixed well with a Vortex mixer and was allowed to react for 1 h at 50°C.

Preparation for chromatography. The derivatized samples were evaporated to dryness under nitrogen and the excess derivatizing agent was hydrolyzed with 2 ml of an aqueous 5% sodium bicarbonate solution. After the tubes were shaken for 5 min, 2 ml of chloroform was added to solubilize the derivatives, and the tubes were rocked for 1 min on a mechanical mixer. The aqueous layer was aspirated, and the organic phase was mixed with 2 ml of an aqueous 5% sodium bicarbonate solution. The aqueous layer was aspirated again, and the organic phase was mixed with 3 ml of a 0.05 N hydrochloric acid solution containing 5% sodium chloride for 1 min to remove any remaining 4-dimethyl aminopyridine. The aqueous layer was aspirated, and the acid wash was repeated three times. The chloroform layer was evaporated to dryness under nitrogen.

Chromatography. The high-pressure liquid chromatograph was equipped with a model 110A pump (Beckman Instruments, Inc., Fullerton, Calif.), a model FS970 fluorometer (Kratos), a 200-μl injection loop, a dual-channel recorder, and a normal-phase column (particle size, 5 μm; length, 25 cm; internal diameter, 4.6 mm) (Lichrosorb Si60; Jones Chromatography). The mobile phase consisted of hexane-methylene chloride-acetonitrile (5:1:1). The samples were reconstituted with a small volume of mobile phase, and a sample of this mixed medium was injected onto the column and chromatographed at a solvent flow rate of 2.2 ml/min. The separation of internal standard (DT0), DG3, and reference metabolites is shown in Fig. 2.

Standard curves. Samples containing 25 to 200 ng of DG3, DG2, DG1, DG0, and DHDG3 in 2-propanol were pipetted into test tubes (15 ml), and the solvent was evaporated under nitrogen. To each of the samples was added 1 ml of blank broth or broth containing feces. Methylene chloride (1 ml containing 100 ng of internal standard [DT0] and 1 ml of plain solvent) was added to each tube, and the compounds were extracted, derivatized with 10 μl of 1-naphthoyl chloride, and chromatographed as described above. Standard curves were prepared by plotting the peak height ratios against the amount of glycoside analyzed. The correlation coefficients (r) obtained for each compound were: DG0, 0.989; DG1, 0.970; DG2, 0.985; DG3, 0.998; 20*S*-DHDG3, 0.996; and 20*R*-DHDG3, 0.997.

RESULTS AND DISCUSSION

Incubation of DG3 (10 μg/ml) with growing cultures of *E. lentum* ATCC 25559 for 7 days at 37°C resulted in a nearly quantitative reduction of the cardiac glycoside to its dihydro metabolite, 20*R*-DHDG3 (Fig. 2). Examination of chromatogram II in Fig. 2 shows clearly that most of the starting material was converted to a single product. Very small amounts of the starting material remained in the extract, and

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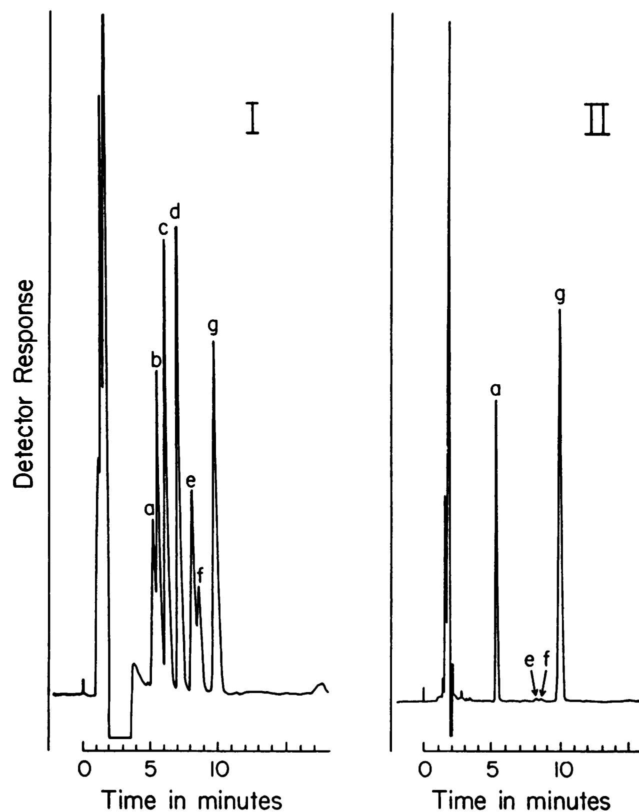

FIG. 2. High-pressure liquid chromatograms of derivatized reference standards, 100 ng each (I), and an extracted and derivatized sample of *E. lentum* culture incubated for 24 h with DG3 (II). (a) DT0 (internal standard); (b) DG0, (c) DG1; (d) DG2; (e) DG3; (f) 20*S*-DHDG3; and (g) 20*R*-DHDG3.

one additional product was also present. Comparison with chromatogram I and the high-pressure liquid chromatography retention times shown in Table 1 indicate that the major product was 20*R*-DHDG3. The 20*R* epimer is identical to the major product found in the urine of those persons who are capable of converting DG3 to its dihydro metabolite (2, 20). More recently, scale-up and isolation of the major conversion product from cultures of *E. lentum* followed by mass spectral analysis has confirmed that the product is indeed DHDG3 (R. E. Shomo II, A. Chandrasekaran, A. G. Marshall, R. H. Reuning, L. W. Robertson, and C. R. Weisenberger, submitted for publication). The dihydro derivative shows a peak in the mass spectrum corresponding to an ion with a molecular weight of 782, which represents an addition of two mass units (reduction of the C-20 = C-22 double bond) (Fig. 1) to DG3 (molecular weight, 780).

That the reduction proceeds with high stereospecificity in *E. lentum* cultures may be seen by comparing chromatograms I and II in Fig. 2. Reference commercial DHDG3 (peaks f and g in chromatogram I), when derivatized and chromatographed according to the procedure described, showed two separate peaks, one with a retention time of 10.2 min (major, 20*R*-DHDG3) and one with a retention time of 8.6 min (minor, 20*S*-DHDG3). The commercial DHDG3 is prepared by chemical catalytic hydrogenation, and the 20*R* and 20*S* epimers are formed in a ratio of 3:1 (2). The stereospecificity was found to be the same when samples from an incubation of DG3 with feces from a volunteer, who was known to excrete the reduction product in urine, were

TABLE 1. High-pressure liquid chromatography detection of reduced products from DG3 and sugar-hydrolyzed metabolites[a]

Compound derivatized	Retention time (min)
DTO	5.2
DGO	5.6
20*S*-DHDGO	6.1
20*R*-DHDGO	6.5
DG1	6.3
20*S*-DHDG1	6.5
20*R*-DHDG1	7.2
DG2	7.4
20*S*-DHDG2	7.6
20*R*-DHDG2	7.9
DG3	8.2
20*S*-DHDG3	8.6
20*R*-DHDG3	10.2

[a] Normal-phase column was Lichrosorb Si60. Mobile phase consisted of hexane-methylene chloride-acetonitrile (5:1:1).

analyzed. The 20*R*-DHDG3 accounted for greater than 99% of the reduction products formed.

The controls did not show any conversion, and there were no interfering peaks from the microorganism or culture media which had retention times near either of the DHDG3 epimers. A time course study in which samples were analyzed every hour for 30 h from the time of inoculation and addition of the DG3 indicated that the accumulation of DHDG3 began after approximately 16 h of incubation and growth and that conversion was essentially complete in 24 h. Therefore, although our original incubation studies were carried out for 7 days, in the manner of previous protocols (5, 6), it was possible to obtain almost quantitative conversion in 24 to 36 h.

The high-pressure liquid chromatography method used in this study is also capable of detecting and quantitating the sugar-hydrolyzed metabolites of DG3 and their reduction products, which show characteristic retention times of their naphthoyl derivatives (Table 1). Examination of chromatogram II in Fig. 2, as well as other incubations of DG3 with *E. lentum*, shows no evidence of the hydrolysis of the deoxy sugars from the C-3 position of the steroidal nucleus.

E. lentum not only converts DG3 to DHDG3, but also converts the sugar-hydrolyzed metabolites of DG3 (DG2, DG1, and DG0) to their respective dihydro metabolites. Analysis of samples from incubation of *E. lentum*, or of feces of a producer of the reduction compound, with DG2, DG1, or DG0 resulted in formation of pairs of reduction products which were identified by cochromatography with reference compounds. The 20*R* epimer constituted greater than 99% of the products formed in each case. Repesentative retention times are shown in Table 1.

Thus, the sugar-hydrolyzed DG3 metabolites are good substrates for reduction by *E. lentum*. Preliminary studies also indicate that the bacterium is also capable of converting digitoxin (a cardiac glycoside that differs from DG3 by lacking a hydroxy group at C-12) to dihydrodigitoxin. These studies indicate that the enzyme that catalyzes the reduction of the unsaturated glycosides to their saturated derivatives has specificity primarily for the unsaturated lactone ring and perhaps for the steroid nucleus, but not for the specific hydroxylation pattern or for the sugar side chain. This represents an important addition to the contribution of Dobkin et al. (5, 6), who detected the formation of DG3 reduction products with a radioimmunoassay, which could distinguish between the unsaturated and reduced lactone

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ring, but told nothing about the status of the remainder of the cardiac glycoside molecule.

In several DG3 metabolism studies in humans (9–12), the total recovery of unchanged drug and metabolites has been considerably less than 100%. It is possible that the reduced sugar-hydrolyzed metabolites may account for some of the missing material in some patients. In addition, DHDG3 is known to show considerably less cardiac activity than DG3 (14), and in some patients up to 52% of the total glycosides in the urine are in the form of DHDG3 (2). Therefore, in certain patients formation of dihydro metabolites represents a major route of drug inactivation. A better understanding of the reduction of DG3 and related cardiac glycosides by intestinal bacteria may contribute to improved therapy of cardiac patients.

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