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6β-HYDROXY-3-EPIDIGITOXIGENIN—THE MAJOR METABOLITE OF DIGITOXIGENIN BY RABBIT LIVER HOMOGENATES

WILLIAM H. BULGER, SIDNEY J. STOHS and DESMOND M. S. WHEELER

Department of Medicinal Chemistry and Pharmacognosy, and the Department of Chemistry, University of Nebraska, Lincoln, Neb. 68508, U.S.A.

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Abstract— ^{3}H-digitoxigenin was metabolized *in vitro* by rabbit liver homogenates. The major metabolite resulting from this biotransformation was isolated and identified as 6β-hydroxy-3-epidigitoxigenin. Approximately 50 per cent of the substrate was converted to this metabolite after 1 hr of incubation. The previously identified 5β-hydroxydigitoxigenin, 3-dehydrodigitoxigenin and 3-epidigitoxigenin were also present as metabolites. This is the first confirmation of the hydroxylation of digitoxigenin at C_6.

THE PRESENT literature pertaining to the biotransformation of cardiac glycosides has been reviewed by Repke,[1,2] Okita[3] and Doherty.[4] Digitoxin is biotransformed by way of a C_{12}-hydroxylation to digoxin.[5,6] The three digitoxose moieties comprizing the sugar side chains of digitoxin and digoxin are removed in a stepwise manner ultimately yielding the aglycones, digitoxigenin and digoxigenin.[7–10] In addition, polar metabolites of digitoxin other than C_{12}-hydroxylation products have been recovered from the urine of rabbit[11] and man,[12,13] as well as the perfusate of guinea pig liver.[14] These metabolites have not been identified, and the rabbit products were found to be more toxic than digitoxin.[11]

Employing liver slices from 10 species (including rabbit, rat and man), the major pathway for the biotransformation of digitoxigenin was established by Herrmann and Repke[15] to be an epimerization at the C_3-hydroxyl group from the β to the α orientation by way of a 3-keto intermediate. Inversion at the C_3-position has been demonstrated to decrease the biological activity of digitoxigenin.[16] Herrmann and Repke[15] also found that small amounts of several more polar derivatives were formed and that only the rat hydroxylated digitoxigenin at the 12β-position. These authors also observed that liver slices from seven species (including rabbit and man) hydroxylated digitoxigenin at a position thought to be the C_6.

In this laboratory, a positive identification of 5β-hydroxydigitoxigenin as one of the polar metabolites resulting from the biotransformation of digitoxigenin by rabbit liver homogenate was accomplished.[17] A tentative identification was also effected of 5β-hydroxydigitoxigenin and 16β-hydroxydigitoxigenin as trace, more polar metabolites resulting from the incubation of digitoxigenin with rat adrenal homogenates.[18] Additional studies indicate that rat liver homogenates can hydroxylate digitoxigenin at the 5β-, 16β- and 1β-positions as well as the previously reported 12β-positions.[19]

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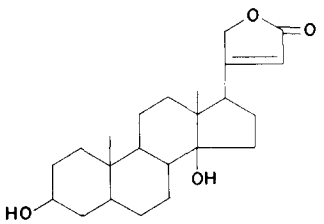
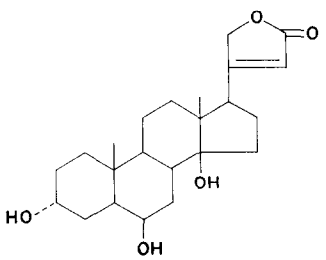


(I) Digitoxigenin

FIG. 1. Structure of digitoxigenin (I).

We here wish to report that the major metabolite of digitoxigenin (I) (Fig. 1) after incubations with rabbit liver homogenates has been demonstrated to be the 6β-hydroxy-3-epi derivative (II) (Fig. 2).



(II) 6β-Hydroxy-3-epidigitoxigenin

FIG. 2. Structure of 6β-hydroxy-3-epidigitoxigenin (II).

EXPERIMENTAL

^{3}H-digitoxigenin. Randomly labeled ^{3}H-digitoxigenin (I) was generously provided by the Gesellschaft Fur Kernferschung M.B.H. Karlsruhe, Germany. The genin was purified by preparative thin-layer chromatography (TLC) with subsequent removal from the Silica gel by Soxhlet extraction according to a previously described method,[20] and was found to have a radiochemical purity of 98 per cent when chromatographed in the two TLC systems described below.

5β-OH-digitoxigenin. Reference amounts of 5β-OH-digitoxigenin (periplogenin) were generously provided by Dr. Yoshio Nozaki, Shionogi Research Laboratories, Shionogi & Co., Fukushima-ku, Osaka, Japan.

3-Dehydrodigitoxigenin and 3-epidigitoxigenin. Reference amounts of 3-dehydrodigitoxigenin (3-ketodigitoxigenin) and 3-epidigitoxigenin were prepared by the method of Tamm and Gubler[21] as modified by Stohs *et al.*[22] By this method, digitoxigenin was oxidized with Jones reagent to 3-dehydrodigitoxigenin. Subsequent reduction with $NaBH_4$ yielded the 3-epi derivative.

Reagents. The NAD^+, $NADP^+$, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide, bovine serum albumin (BSA), Tris buffer and digitoxigenin were from the Sigma Chemical Co. The 3β-hydroxysteroid oxidoreductase was purchased from the Worthington Biochemical Corp., and the acetone-d_6 (100 per cent) was obtained from Diaprep Inc. All other chemicals were Fisher reagent grade.

Animals. Male rabbits (2·2 kg, 10 weeks of age) derived from the California strain were employed. The animals were maintained in our animal colony on a diet of Purina Laboratory Chow and tap H_2O *ad lib.* for 1 week prior to experimentation and were fasted overnight before use.

Liver homogenates. The rabbits were stunned by a blow on the back of the neck, exsanguinated, and the livers excised. The desired amount of tissue was placed in a 30° solution of 0·25 M sucrose plus 0·05 M Tris chloride, pH 7·4, 0·005 M $MgCl_2$, and 0·01 M NaCl (TMN). A Potter–Elvehjem homogenizer was employed to prepare 10 per cent liver homogenates.

Incubation. Each incubation flask contained a total volume of 10 ml. The medium consisted of rabbit liver homogenate equivalent to 0·75 g of tissue, $1·7 \times 10^{-2}$ M glucose-6-phosphate, $4·0 \times 10^{-4}$ M $NADP^+$, 5 units glucose-6-phosphate dehydrogenase, $1·5 \times 10^{-2}$ M nicotinamide, 0·50% BSA and 0·50 μCi of ^{3}H-digitoxigenin ($2·7 \times 10^{-4}$ M). The ^{3}H-digitoxigenin was added to each flask in 0·20 ml of 70% ethanol. All other reagents were dissolved in 0·25 M sucrose plus TMN. Two types of controls were employed: controls without tissue which contained an equivalent volume of 0·25 M sucrose plus TMN in place of the liver homogenate, and boiled tissue controls in which the liver homogenate was heated for 10 min in boiling water bath and brought to 37° before being added to the incubation medium. The incubation mixtures were maintained on ice prior to incubation. Incubation was effected at 37° under O_2–CO_2 (95:5) with constant shaking in a H_2O bath shaker (Eberbach) for 60 min.

Extraction. A modification of a previously described extraction procedure was employed.[17] At the end of the incubation period, the contents of each flask were transferred to separate 250-ml glass-stoppered flasks containing 40 ml of methylene dichloride. The extraction mixtures were agitated for 24 hr. The organic phase of each mixture was removed, dehydrated over anhydrous $NaSO_4$, evaporated to dryness *in vacuo*, taken up in 3 ml of methylene dichloride–methanol (3:2) and transferred to separate 10-ml vials. From 92 to 95 per cent of the initial radioactivity was routinely recovered by this procedure. Exhaustive extraction of initially unrecovered radioactivity revealed, by TLC analysis and liquid scintillation counting, that the distribution of metabolites (within experimental error) was identical to that obtained by the routine extraction procedure described above.

Chromatography. Two Silica gel (0·05–0·2 mm, EM reagents) columns, 41 × 1·5 cm, were prepared by adding a slurry of 35 g Silica gel in methylene dichloride to a Pyrex glass column and allowing the Silica gel to equilibrate. The columns were flushed with approximately 200 ml of methylene dichloride (100 ml/hr). Approximately 150 extracts of individual incubation mixtures were pooled, divided into two portions, concentrated in methylene dichloride to approximately 3 ml, loaded on the columns, and the columns eluted with 200 ml each of the following methylene dichloride–methanol mixtures: 100:0, 95:5 and 92:8. A total of 64 10-ml fractions were collected on an automated fraction collector (ISCO 272) for each column. Approximately 75 mg of digitoxigenin and its metabolites was chromatographed on each column. Radioactive bands were localized by counting 100-μl aliquots of alternate fractions. Appropriate fractions were subsequently subjected to TLC analysis and pooled according to metabolite content. The metabolites thus obtained were subjected to additional purification by preparative TLC.

Thin-layer plates were prepared by casting a 0·375 mm thick (0·5 mm for preparative plates) layer of Silica gel H (EM reagents) on 20 × 20 cm glass plates. Two solvent systems were employed: solvent system I consisted of chloroform–isopropanol (9:1) with two developments: solvent system II utilized cyclohexane–acetone–acetic acid (65:33:2) with four developments. In all cases, the solvent front was allowed to reach the top of the plate during each development. Anisaldehyde reagent, 20% H_2SO_4 and Kedde reagent were used as chromogenic agents.[17,23]

Aliquots of each of nine individual incubation mixture extracts and control extracts (no tissue and boiled controls) equivalent to 7500 dis/min were co-chromatogramed with the reference standards, digitoxigenin, 3-dehydrodigitoxigenin, 3-epidigitoxigenin and 5β-hydroxydigitoxigenin in TLC system II. Liquid scintillation counting was employed according to the method of Talcott *et al.*[20] to determine the localization of radioactivity on the resulting chromatograms. The results are expressed in Table 1 as the percentage of total radioactivity ± the standard error of the mean. The extracts were subjected to additional TLC analysis in TLC systems I and II. The R_f (relative to digitoxigenin) values and the reaction of the metabolites with the chromogenic agents are also reported in Table 1 and represent the mean values for the nine extracts.

Metabolite identification. The methods of Brown and Wright[24] and Herrmann and Repke[15] were employed to obtain the anhydrous H_2SO_4 dehydration absorption spectrum of the rabbit-generated major metabolite from digitoxigenin. Absorption spectra were also obtained for digitoxigenin, 12β-hydroxydigitoxigenin (digoxigenin), 16β-hydroxydigitoxigenin (gitoxigenin), 5β-hydroxydigitoxigenin (periplogenin), and Δ^5-digitoxigenin (xysmalogenin). Each sample was prepared by adding 3 ml of concentrated H_2SO_4 to approximately 100 μg of cardenolide and developing for 30 min. A double beam recording spectrophotometer (Beckman DB) was employed to record each spectrum between 220 and 500 nm.

Mass spectra of the major metabolite and digitoxigenin were determined on a Hitachi RMU 6D mass spectrometer.

Evidence for the configurations of the hydroxy group at the C-3 of the major metabolite was obtained by incubating the rabbit metabolite, 3-epidigitoxigenin, and a digitoxigenin control with 3β-hydroxysteroid oxidoreductase (from *Pseudomonas testosteroni*). Each incubation flask contained a total volume of 5 ml of medium which consisted of 1 unit of enzyme, 1 mg substrate (metabolite, 3-epidigitoxigenin or digitoxigenin), and $9{\cdot}0 \times 10^{-4}$ M NAD^+ in 0·25 M sucrose plus TMN buffer. The incubations were conducted at 37° with constant shaking for 2 hr. The flasks were left open to the atmosphere. The reactions were terminated with 20 ml methylene dichloride, extracted for 2 hr on a shaker, and the organic phases removed and evaporated to dryness *in vacuo*. The resulting extracts were taken up in 2 ml methylene dichloride–methanol (3:2) and transferred to a 10-ml vial. Twenty microliters of each extract were chromatogramed with appropriate standards in the aforementioned TLC systems. A two-dimension chromatogram was obtained by developing a TLC plate four times in TLC system I with four subsequent developments in TLC system II in the second direction. The chromogenic agent was 20% H_2SO_4.

Nuclear magnetic resonance (NMR) spectra for 1-mg amounts of the metabolite, digitoxigenin, and 3-epidigitoxigenin were determined in acetone-d_6 on a Varian XL-100 spectrometer in the Fourier–Transform mode. The positions of the peaks were

TABLE 1. METABOLITES RESULTING FROM THE BIOTRANSFORMATION OF DIGITOXIGENIN BY RABBIT LIVER HOMOGENATES*

Cardenolide	% Total extractable radioactivity	R_f (relative to digitoxigenin)		Color reaction	
		TLC system I†	TLC system II‡	20% H_2SO_4	Anisaldehyde
3-Dehydrodigitoxigenin	1·70 ± 0·21	1·22	1·08	Tan	Red
Digitoxigenin	4·11 ± 1·73	1·00	1·00	Blue–green	Blue–green
3-Epidigitoxigenin	21·14 ± 3·56	0·86	0·90	Blue–gray	Blue
5β-OH-digitoxigenin	4·95 ± 0·40	0·38	0·62	Lt. green	Dk. green
3-Epi-6β-OH-digitoxigenin	50·68 ± 5·58	0·08	0·20	Dk. blue	Dk. violet

* Incubation extracts were co-chromatographed with reference standards on Silica gel H plates. The plates were developed in TLC system II, the cardenolide bands were visualized, and the radioactivity associated with each band was assayed by liquid scintillation counting.[20] The per cent total cpm values presented are the mean of nine individual incubation extracts with the standard error of the mean. No metabolism was observed in the case of the controls (no tissue and boiled). The metabolites were co-chromatographed with reference standards in TLC systems I and II. The R_f (relative to digitoxigenin) values for each metabolite and corresponding standard were identical and are given. No reference standard for the major metabolite, 3-epi-6β-hydroxydigitoxigenin, was available. All standards and metabolites were positive to Kedde reagent.

† TLC system I: chloroform 2-propanol (9:1).

‡ TLC system II: cyclohexane–acetone acetic acid (65:33:2).

Table 2. Portions of major peaks in NMR spectra of digitoxigenin and derivatives*

Compound	Chemical shifts of protons on selected carbon atoms					
	3-H	6-H	18-H	19-H	21-H†	22-H‡
Digitoxigenin	4·02 (8)§		0·916	0·956	4·91	5·83
3-Epidigitoxigenin	3·58 (20)¶		0·913	0·928	4·92	5·86
Major metabolite	3·46 (24)¶	3·74 (8)§	0·941	1·106	4·92	5·84

* The chemical shifts are expressed in terms of δ units relative to tetramethylsilane (TMS). The numbers in parentheses for the protons on C_3 and C_6 are the peak widths at one-half peak height ($W_{\frac{1}{2}}$) and are given in Hz. The NMR spectra were determined in acetone-d_6 on a Varian XL-100 spectrometer in the Fourier Transform mode.

† Quartet.

‡ Triplet.

§ Broad singlet.

¶ Multiplet.

measured relative to tetramethylsilane (TMS) and are recorded in Table 2. We thank Dr. D. Thoennes for these measurements.

RESULTS AND DISCUSSION

Three radioactive bands were recovered from the Silica gel column. Band I (fractions 21–23) yielded three cardenolides when subjected to additional purification by preparative TLC in solvent system I (three passes). Co-chromatography with appropriate reference standards demonstrated that the three cardenolides were 3-dehydrodigitoxigenin, digitoxigenin and 3-epidigitoxigenin. Band II (fractions 26–29) contained the previously identified 5β-hydroxydigitoxigenin. The major metabolite of digitoxigenin was recovered from Band III (fractions 37–53). The R_f (relative to digitoxigenin) values in both TLC systems for the cardenolides recovered from the column are reported in Table 1.

All the above-mentioned metabolites were positive to Kedde reagent (pink color) indicating the presence of an unsaturated lactone ring which is characteristic of cardenolides.[25,26]

The amount of radioactivity associated with each metabolite after a 1-hr incubation is given in Table 1. Each value represents the mean of nine individual incubations with the S. E. M. No metabolism was observed in any of the control flasks without tissue or the heated tissue control flasks. Approximately 50 per cent of the recovered radioactivity was localized in a single metabolite subsequently identified as 3-epi-6β-hydroxydigitoxigenin.

The absorption spectra of the major metabolite, 5β-hydroxydigitoxigenin, and Δ^5-digitoxigenin in concentrated sulfuric acid were identical (absorption maxima 236 and 411 nm, absorption minima 274 nm). However, they were different from the spectra of digitoxigenin and its 12 and 16β-hydroxy derivatives which were in agreement with previously reported spectra for these latter three compounds.[15,24] Digitoxigenin derivatives bearing a hydroxyl group at the C_5- or C_6-position are believed to be dehydrated in H_2SO_4 to Δ^5-digitoxigenin, and the resulting spectrum is identical to that for authentic Δ^5-digitoxigenin.[15] Employing the above principle, Herrmann and Repke[15] reasoned that the cardenolide they designated metabolite III was a C-6 hydroxyl derivative of digitoxigenin. Metabolite III had an absorption spectrum in

H_2SO_4 that was superimposable upon the spectrum of Δ^5-digitoxigenin and was chromatographically distinct from 5β-hydroxydigitoxigenin.[15]

The mass ion values for digitoxigenin and the rabbit metabolite were 374 and 390, respectively, suggesting that the metabolite was a mono hydroxyl derivative of digitoxigenin. In addition, the fragmentation patterns of both digitoxigenin and the major metabolite exhibited ion peaks of 203, 231 and 246 which have been described by Fayez and Negm[27] as peculiar to cardenolides having a C_{14}-hydroxyl group on the steroid nucleus. Both cardenolides displayed an ion peak of 111, which can be attributed to the lack of a C_{16}-hydroxyl group on the cardenolide nucleus. An ion peak of 127 (not present in the major metabolite) would indicate the presence of a C_{16}-hydroxyl group.

The incubation of digitoxigenin with 3β-hydroxysteroid oxidoreductase yielded a single metabolite that was indistinguishable from reference 3-epidigitoxigenin when chromatogramed in TLC systems I and II. When 3-epidigitoxigenin was used as the substrate, no metabolites were recovered. The rabbit metabolite incubated with the oxidoreductase was similarly unaltered as evidenced by chromatography in both TLC systems.

The two-dimensional chromatogram failed to separate untreated rabbit metabolite from metabolite incubated with the 3β-hydroxysteroid oxidoreductase. However, this system can resolve digitoxigenin and 3-epidigitoxigenin. This evidence coupled with the observation that 3-epidigitoxigenin is generated by rabbit liver homogenate (Table 1), and the fact that TLC systems I[17] and II[17,23] have previously separated the 3-dehydro and 3-epi derivatives of digitoxigenin, 12β-hydroxydigitoxigenin and 5β-hydroxydigitoxigenin from their parent cardenolides indicates that the hydroxyl group at the C_3 is in the α (epi) configuration in the major metabolite of digitoxigenin.

The above evidence suggests that the major metabolite is the 3α-hydroxy epimer of digitoxigenin with an additional hydroxyl group in ring A or B. A comparison of the NMR spectrum of the metabolite with those of digitoxigenin (I) and its three epimer leads to the unambiguous conclusion that the major metabolite is 3α,6β,14β-trihydroxy-5β-card-20(22)-enolide (II). Since the metabolite is poorly soluble in chloroform, the spectra were run in deuteroacetone. The major peaks in the spectra and their assignments are shown in Table 2.

The appearance and positions of the C_{21}-H and C_{22}-H signals in the spectra of digitoxigenin and 3-epidigitoxigenin are similar to those described previously for spectra run in CCl_3.[28] The narrow singlet at 3·74 in the major metabolite does not correspond to the C_3-H signal at 4·02 in digitoxigenin: apart from the difference in chemical shifts, the two peaks (in spite of their similar half widths) differ in appearance with that in digitoxigenin being broader at its base. The peak at 3·46δ in the metabolite corresponds both in position and shape with the C_3-H in 3-epidigitoxigenin. This result confirms the enzymatic evidence that the transformation of digitoxigenin into the major metabolite has involved epimerization at C_3. The signal at 3·74δ in the metabolite is due to the hydrogen attached to the carbon bearing the newly introduced hydroxyl group, and from the width of the 3·74 peak this hydroxyl group is axially oriented. Comparison of the spectra of 3-epidigitoxigenin and the metabolite shows that the new hydroxyl has shifted the C_{18}-H and C_{19}-H signals downfield by 0·028 and 0·178 ppm respectively. Although it is dangerous to directly

use values of shifts measured in chloroform[28,29] to evaluate shifts measured in deuteroacetone, we can say that the magnitude of the shift in the C_{19}-H signal is consistent only with the hydroxyl being at the 1β-, 6β- or 11β-positions. The 11β-position can be eliminated as the shift of C_{18}-H signal is so much less than that of C_{19}-H. Placing the hydroxy at 1β is also inconsistent with the evidence: a hydroxyl at that position would only affect the C_{18}-H slightly ($\pm$0·01 ppm), and would cause a downfield shift of the 3-hydrogen. In addition, authentic 1β-hydroxydigitoxigenin failed to chromatograph relative to the major metabolite in TLC system II in the position expected if the hydroxyl group of the major metabolite was 1β. We, therefore, conclude the new hydroxyl in the major metabolite is in the 6β-position.

The digitoxigenin derivative produced by homogenized rabbit liver is 3-epi-6β-hydroxydigitoxigenin (3α,6β,14β-trihydroxy-5β-card-20(22)-enolide) (II). This is the first positive confirmation of the hydroxylation of digitoxigenin at C_6. In addition to the report by Hermann and Repke,[15] Titus *et al.*[30] tentatively identified 6β-hydroxydigitoxigenin as a biotransformation product of digitoxigenin by *Tricothecium roseum*. However, this metabolite proved to be identical to the 7β-hydroxydigitoxigenin reported by Ishii *et al.*,[31] and Scherrer-Gervai *et al.*[32] as a metabolic product of several micro-organisms.* Samples of 7β-hydroxydigitoxigenin obtained from both Titus' and Ishii's groups proved to be chromatographically identical, and both were chromatographically distinct from our rabbit-derived 3-epi-6β-hydroxy derivative of digitoxigenin. The significance of the C_6-hydroxylation of digitoxigenin is yet to be determined. Whether it constitutes one of the as yet unidentified metabolites of digitoxin in the urine of man and several animals is not known. The physiological activity of this metabolite must also be investigated.

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