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METABOLISM AND EXCRETION OF ^{3}H-DIGITOXIN IN THE RAT*

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Abstract—After oral administration of 25 μg/kg ^{3}H-labelled digitoxin (sp. act. 26·2 mCi/mg) to female rats, the total radioactivity in blood and in urine was eliminated with a half-life time of 2 and 1·7 days, respectively. The fecal elimination half-life time had a biphasic course. The chloroform-soluble and chloroform-insoluble metabolites excreted in urine and feces were determined in order to explain the much shorter half-life time of 0·4 days in feces during the early phase of elimination. In the feces, 45 per cent of the dose excreted within 5 days consisted of chloroform-soluble substances. In this fraction, the main excretion product was digoxigenin-bis-digitoxoside (20 per cent), whereas the percentages of the other glycosides, after the last collection period, amounted to significantly less: 9% digitoxin, 9% digoxin, 5% digitoxigenin-bis-digitoxoside, and 2% digitoxigenin-mono-digitoxoside. The chromatographic analysis of the chloroform-insoluble fraction, which accounted for 15 per cent of the dose, revealed a conjugation of glucuronic and sulfuric acid with digoxin, and digoxin, 5% digitoxigenin-bis-digitoxoside, and 2% digitoxigenin-mono-digitoxoside. The contrast, sulfuric acid alone was the main conjugation partner of 3-epi-digitoxigenin. In urine, 4·6 per cent of the administered radioactivity was represented by digoxin, 2 per cent by digitoxin, 1 per cent by digoxigenin-bis-digitoxoside, and 1·4 per cent by polar metabolites. Only traces of digitoxigenin-bis-digitoxoside and digoxigenin-mono-digitoxoside were detected. The much shorter half-life time of the eliminated radioactivity in feces seems to be due to the higher portion of poorly reabsorbed conjugation products and digoxigenin-bis-digitoxoside.

SEVERAL reports have appeared indicating the C_{12}-hydroxylation of digitoxin to digoxin and the formation of polar metabolites.[1–9] However, little work has been done to determine quantitatively the rate of excretion of digitoxin and its metabolites in urine and feces. For this reason the elimination of digitoxin and its chloroform-soluble and chloroform-insoluble metabolites were investigated at various time periods after oral administration of ^{3}H-digitoxin.

MATERIALS AND METHODS

Experiments were performed on female Sprague–Dawley rats (SPF rats from MUS-Rattus AG, Germany) weighing about 200 g. The animals were kept under normal laboratory conditions and fed on Altromin®. Food was withdrawn 12 hr before the animals received the glycoside, but water was available *ad lib*. The randomly-labelled tritiated digitoxin, obtained from New England Nuclear, with an initial specific activity of 26·2 mCi/mg, was diluted by the addition of 4·5 ml 50% ethanol in 0·9% NaCl to 0·5 ml of the labelled compound. From this solution each animal was intubated

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with 25 μg/kg in a volume of 2 ml/kg. At the appropriate time after administration of ^{3}H-digitoxin approximately 0·3–0·5 ml blood was withdrawn by cardiac puncture. Samples of urine and feces were collected every 3 hr during the first 12 hr, and once daily for the following 7 days.

Blood samples were weighed, centrifuged in plastic tubes, and the plasma layer was taken for counting. Aliquots of urine were directly transferred to a vial and counted for total radioactivity. Thereafter distilled water was added to urine specimens to a final volume of 2 ml. The mixture was extracted three times with chloroform. The chloroform layers were combined and evaporated to dryness at 37°. The radioactivity present in the water layers was measured. Feces were extracted three times with 20 ml acetone and the acetone layers were combined and counted for radioactivity. After evaporation to dryness the acetone residue was washed twice with petroleum ether and extracted three times with 10 ml chloroform. The aqueous phases were counted for radioactivity, the chloroform phases of urine and feces were redissolved in 50 μl chloroform–methanol (1 : 1) and applied to TLC-plates.

The aqueous phases of urine and feces (containing the chloroform-insoluble metabolites) were separately evaporated to dryness in three portions, then redissolved in 5 ml acetate buffer pH 5·2 containing 10 mg arylsulfatase from *Helix pomatia*, specific activity 5 U/mg or 10 mg β-glucuronidase from *Helix pomatia*, specific activity 3 U/mg (both from Boehringer Mannheim GmbH, with less than 2% contamination). Incubations were normally carried out for 24 hr at 37°. Samples without enzyme served as references. After the reactions had been stopped by adding 10 ml chloroform, the flasks were placed on a shaker for 15 min to facilitate extraction of the lipophilic splitting products. After repeating this process twice more with the same volume of chloroform, the organic phases were combined, evaporated to dryness, and analysed by thin layer chromatography. Aliquots of the remaining aqueous phase were counted for radioactivity.

Thin-layer plates of silica gel (Fa. Merck, Darmstadt) were routinely employed, 0·25 mm layer, flow distance 15 cm. Solvent system I: diisopropyl ether–methanol (1 : 9) five times development.[10] Solvent system II: chloroform–acetone (1 : 1) four times development. The glycoside bands were located with a radiochromatogram scanner (Thin-layer Scanner II, Berthold, Germany) and compared with the positions of the reference compounds* by spraying each plate with Kaiser's reagent[11] and subsequent examination under u.v.-light. Quantitative analysis was performed by automatic integration of the activity peaks (Integrator Berthold LB 2437, Germany).

Radioactivity of all samples was assayed in a Mark II liquid scintillation spectrometer. The counting efficiency was determined by the channel ratio method employing an external standard. To each liquid scintillation counting vial was added 10 ml of scintillation fluid (10% naphthalene, 0·98% diphenyl oxazole, 0·02% 2,2-*p*-phenylene-bis-(5-phenyloxazole) in dioxane).

RESULTS

Blood tritium levels and excretion rates. After oral administration of 25 μg/kg ^{3}H-digitoxin, tritium levels in the plasma reached their maximum at 2 hr and declined during and after the 8th hr with a half-life time of 2 days. While the excretion rate

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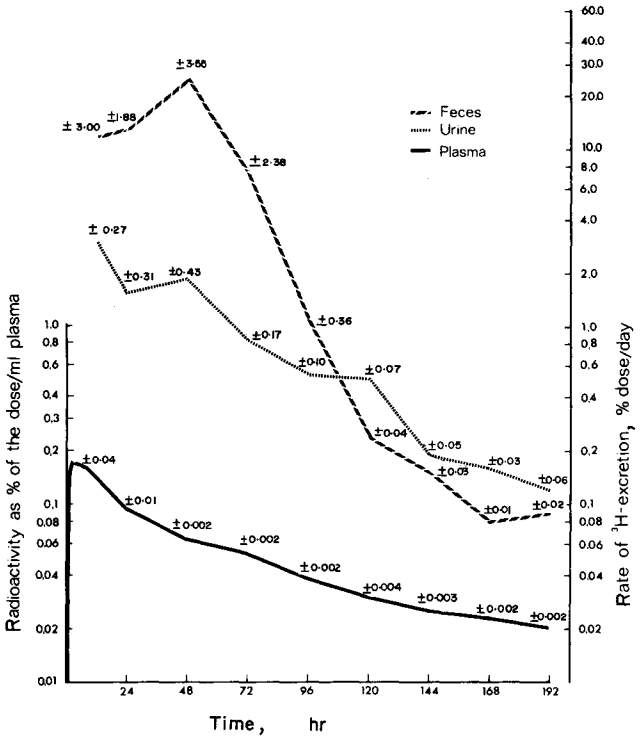

FIG. 1. Plasma concentration and rate of renal and fecal ^{3}H-excretion after p.o. administration of ^{3}H-digitoxin in rats. Mean ± S.E.M. (n = 7).

of radioactivity in urine had a nearly identical half-life time of 1·7 days, the half-life time of excreted radioactivity in feces was much shorter (0·4 days) during the period between 48 and 120 hr after administration of the glycoside. At later time periods, however, in feces a half-life time of 1·9 days was found, which is almost identical with that in blood and urine (Fig. 1). The accelerated excretion velocity of radioactivity in feces during the early phase of elimination may be due to metabolites which are

TABLE 1. CHLOROFORM-SOLUBLE AND -INSOLUBLE FRACTION IN % OF THE GIVEN DOSE DURING DIFFERENT COLLECTION PERIODS AFTER ORAL ADMINISTRATION OF 190 μCi/kg ^{3}H-DIGITOXIN IN RATS (n = 7).

	Time (hr) 0–24	24–48	48–72	72–96	96–120
			Urine		
$CHCl_3$-soluble	3·87 ± 0·25	1·42 ± 0·35	0·57 ± 0·13	0·37 ± 0·07	0·37 ± 0·06
$CHCl_3$-insoluble	0·63 ± 0·04	0·40 ± 0·08	0·26 ± 0·04	0·16 ± 0·03	0·13 ± 0·01
$CHCl_3/H_2O$	6·1	3·6	2·2	2·3	2·8
			Feces		
$CHCl_3$-soluble	16·1 + 2·03	19·3 ± 3·3	5·71 ± 2·2	0·71 ± 0·33	0·17 ± 0·03
$CHCl_3$-insoluble	8·23 ± 0·37	4·56 ± 0·31	1·52 ± 0·21	0·36 ± 0·03	0·07 ± 0·01
$CHCl_3/H_2O$	2·0	4·2	3·8	2·0	2·4

excreted more rapidly in feces than in urine. The metabolites of digitoxin, therefore, were separated and quantified.

Separation and characterization of chloroform-soluble and chloroform-insoluble fractions in urine and feces. The separation of the total radioactivity into a chloroform-soluble and a chloroform-insoluble fraction rendered an initial differentiation in the metabolism of digitoxin (Table 1). In the urine, the absolute amounts of both fractions decreased during the experimental period; 6·1 and 3·6 times more chloroform-soluble than chloroform-insoluble compounds were eliminated within the first and second 24 hr, respectively. This is compared with the ratio of 2·2–2·8 during the following collection periods from 48 to 120 hr. In the feces, the excretion of chloroform-soluble and -insoluble fractions was 5·4 and 10·5 times greater than in urine. In addition the accumulation of chloroform-insoluble polar compounds was distinctly increased during the 72 hr after oral administration of digitoxin; very little further accumulation occurred after 72 hr.

Figures 2a and 2b illustrate the thin-layer chromatographic separation of the chloroform-soluble fractions from urine and feces. Apart from smaller radioactive areas

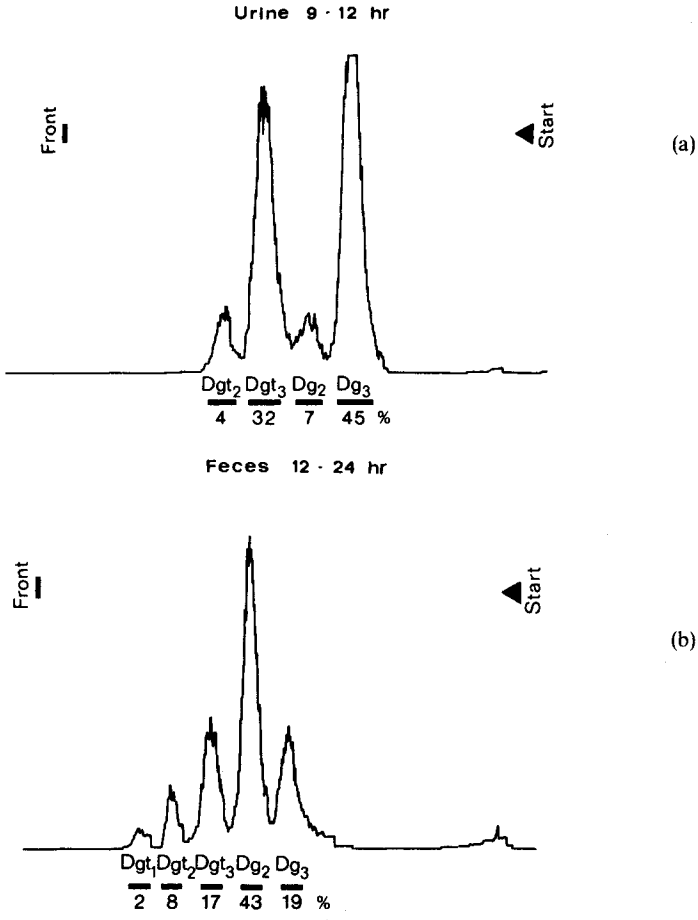

FIG. 2a and b. Thin-layer-chromatographic separation (system II) of $CHCl_3$-soluble fraction from urine (a) and feces (b) after p.o. administration of digitoxin.

which correspond to the *bis*-digitoxosides of digitoxigenin (Dgt_2) and digoxigenin(Dg_2), the main compounds in urine were digitoxin and digoxin (Fig. 2a). A different composition of metabolites was found in feces. The main component of the chloroform-soluble fraction was digoxigenin-bis-digitoxoside (Dg_2). Digoxin (Dg_3), digitoxin (Dgt_3) and the bis- and mono-digitoxosides of digitoxigenin were found in smaller amounts (Fig. 2b).

Incubation of the chloroform-insoluble fraction excreted in urine with β-glucuronidase and arylsulfatase revealed a splitting of 54 and 57 per cent, respectively. In feces, the same procedure yielded a cleavage for β-glucuronidase of 39 per cent and for aryl-sulfatase of 49 per cent. On the basis of the chromatographic behaviour, digoxin and digoxigenin-bis-digitoxoside seem to be the main conjugation partners of glucuronic acid, besides smaller amounts of some unidentified metabolites (Fig. 3a). No localized

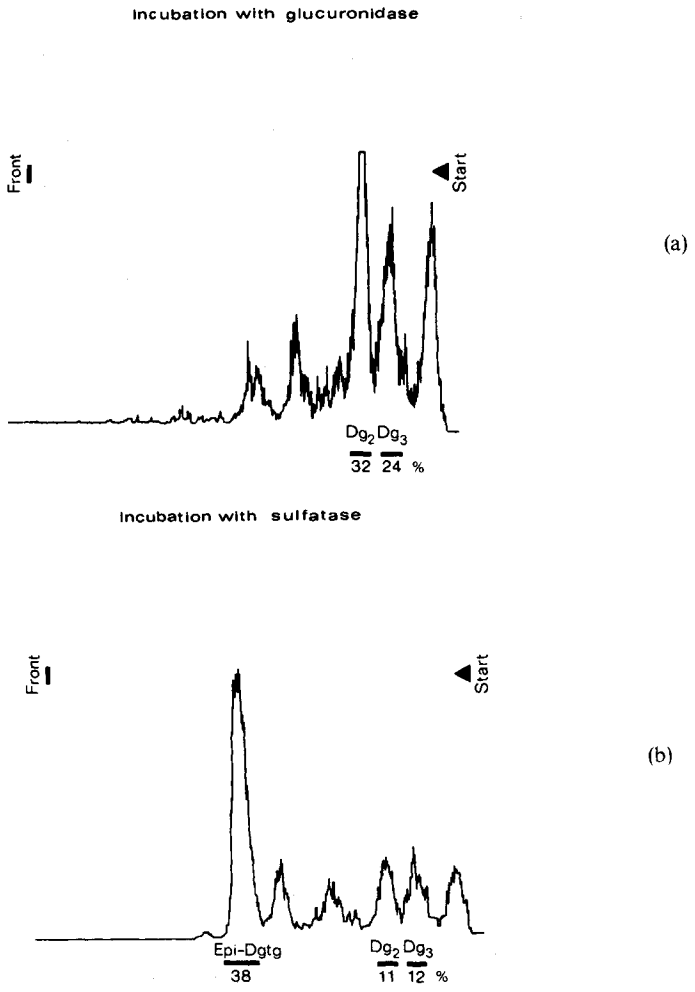

FIG. 3a and b. Thin-layer-chromatographic separation (system I) of $CHCl_3$-soluble splitting products. The $CHCl_3$-insoluble material excreted in feces after administration of digitoxin was incubated with β-glucuronidase (a) and aryl-sulfatase (b).

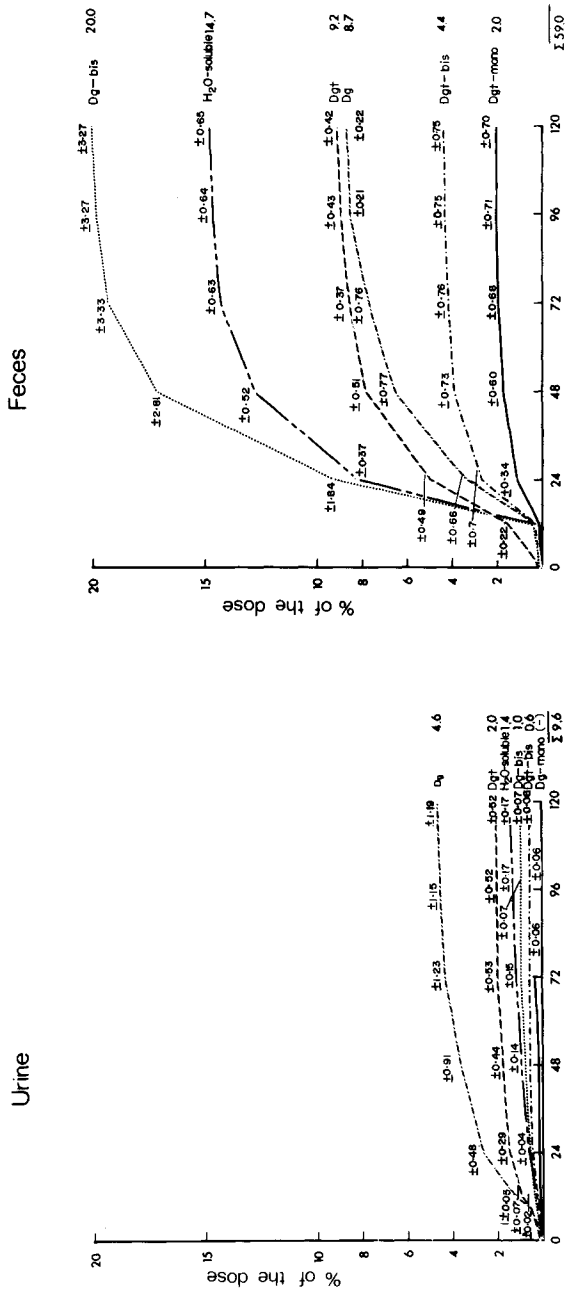

FIG. 4. Cumulative excretion of digitoxin and its metabolites in urine and feces within 120 hr after p.o. administration of digitoxin. Mean ± S.E.M. (n = 7).

radioactivity in regions of 3-epi-digitoxigenin or 3-epi-digoxigenin was observed in this experiment, indicating that compounds more polar, such as conjugation products of 3-epimers with glucuronic acid, were not formed. In contrast, after splitting with arylsulfatase 3-epi-digitoxigenin was found in overwhelming amounts, together with digoxin, digoxigenin-bis-digitoxoside and unidentified metabolites (Fig. 3b).

On the basis of quantitative evaluation (Fig. 4) of the chromatographic analysis of the chloroform-soluble fraction, 2 per cent of unchanged digitoxin was excreted in the urine. The major product was digoxin, which amounted to 4·6 per cent within 120 hr. Furthermore the metabolism of digitoxin resulted in the accumulation of smaller amounts of digoxigenin-bis-digitoxoside and digitoxigenin-bis-digitoxoside in urine. In the feces, collected during 120 hr, 59 per cent of administered radioactivity was recovered. Most of the radioactivity was associated with digoxigenin-bis-digitoxoside (20 per cent) and chloroform-insoluble compounds (14·7%) which may be poorly reabsorbed and excreted more readily in the feces. The remaining radioactivity included digitoxin (9·2%), digoxin (8·7%), digitoxigenin-bis digitoxoside (4·4%) and a smaller amount of digitoxigenin-mono-digitoxoside (2·0%).

DISCUSSION

The primary purpose of the present investigation was to examine the rate of excretion of digitoxin and its metabolites with the aim of gaining further insight into the metabolism of digitoxin. The metabolism of digitoxin is characterized first by an enzymatic cleavage of the sugar side chain[12] and by an epimerization of the genin moiety[13], secondly, by a reaction hydroxylating digitoxin to digoxin,[6,7,14,15] finally, in a turnover of chloroform-soluble compounds to chloroform-insoluble metabolites.[16] Although the steps in the metabolic degradation of digitoxin have been demonstrated in a qualitative sense, the information presented in the literature differs widely with regard to quantitative aspects.

Most of the orally administered radioactivity is apparently cleared very rapidly from the blood during the first 48 hr and thereafter more slowly. This is in accord with the findings of Spratt and Okita,[17] who showed that within 20 min after a single intravenous injection, digitoxin was almost completely eliminated from the blood of the rat. Additional experiments in our laboratory indicate that the rate of blood clearance in the early phase of elimination is conditioned by the ability of the rat liver to fix the glycoside. However, in contrast to digoxin, digitoxin persisted longer in the liver. This longer persistance in the liver could account for the fact that, in the case of digitoxin, an essentially greater degree of chemical change is observed.

The patterns of metabolic products in urine and feces differed greatly. In the urine it was evident that within 120 hr half of the eliminated radioactivity was due to digoxin, 10 per cent was digoxigenin-bis-digitoxoside, and there were traces of digoxigenin-mono-digitoxoside. These findings corroborate earlier observations of the efficiency of 12β-hydroxylase in rat liver.[7] Due to the nature of the experiment it cannot be decided, whether the removal of the digitoxose has a priority over the 12β-hydroxylation. In feces, one of the principal chloroform-soluble metabolites is digoxigenin-bis-digitoxoside which amounted to 34 per cent of the metabolic products excreted in a 120 hr period. This high percentage of digoxigenin-bis-digitoxoside can be interpreted as resulting from its intensive entero-hepatic circulation, as shown

in our laboratory.[18] On the one hand this metabolite appears to circulate between intestine, liver and intestine; on the other hand it comes to distal places of the intestine, which have a smaller capacity to reabsorb. This is analogous with findings of Greenberger *et al.*,[19] who demonstrated that significantly larger amounts of digoxin were absorbed from the proximal small intestine than from the distal. Other chloroform-soluble compounds include digitoxin, digoxin, digitoxigenin-bis-digitoxoside and digitoxigenin-mono-digitoxoside. The percentages of all these compounds for feces are consistently higher than for urine.

Furthermore, our results show that the chloroform-insoluble metabolites are also excreted in urine and feces. The predominant excretion was by the intestinal route, being 14·7% of the total administered radioactivity within 120 hr. Because of the extremely small amounts excreted in urine, it was not possible to obtain any further information about this fraction. From the splitting of the chloroform-insoluble fraction obtained from feces with β-glucuronidase and arylsulfatase, it is apparent that conjugation reactions with glucuronic acid and sulfuric acid take place in hydroxyls of digoxin and digoxigenin-bis-digitoxoside. The possibility of such direct conjugation of digoxin was first postulated by Kolenda *et al.*[20] When using isolated, perfused guinea pig liver, no free digitoxoses were detected during the metabolism of digoxin. According to Lauterbach and Repke[21] and Herrmann and Repke,[16] 3-epi-digitoxigenin should be rapidly conjugated with glucuronic and sulfuric acid. However, our results show that sulfuric acid alone is the conjugation partner of 3-epi-digitoxigenin. The failure to detect the glucuronide compound cannot be explained. Furthermore, the unidentified small peaks seen in the chromatograms of the chloroform-soluble splitting compounds favors the possibility that hydroxylation occurs at a site other than at carbon 12.

In conclusion, the data concerning the biotransformation of digitoxin can explain the pharmacokinetic behaviour of this glycoside. The shorter half-life time of radioactivity in feces may be due to the relatively higher percentage of chloroform-insoluble metabolites which were poorly reabsorbed in the gut.[18] In addition, higher amounts of digoxigenin-bis-digitoxoside apparently circulate in a short circuit between intestine, liver and intestine during the early phase of elimination. Therefore, it may be suggested that the gastrointestinal transit time is apparently the rate limiting step in the excretion of radioactivity in feces.

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