The metabolites of cardiac glycosides in human urine

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A study of the urinary excretion of digitoxin in man was first attempted by Friedman and coworkers (1) by the use of the embryonic duck heart method of biological assay. These workers estimated that the amount of cardioactive substances (calculated as digitoxin) excreted in the urine of young adults over a period of 12 to 24 days after an oral dose was equivalent to approximately 40 per cent of the dose. However, no differentiation between digitoxin and possible cardioactive metabolites was attempted in this work. By using biosynthetically labeled C14-digitoxin, Okita et al. (2) found that 60 to 80 per cent of an intravenous dose was eliminated through the kidneys of humans over a long period, and that only 6 to 10 per cent of this appeared to be unchanged glycoside. The nature of the metabolites of the glycoside present in the urine was not investigated.

Some qualitative information has been obtained about the metabolic products of cardiac glycosides excreted in rat urine (3, 4). Digoxin and lanatoside C were both excreted in this animal, principally as the unchanged glycoside, but both were also converted to a metabolite product which has been designated Metabolite B. Investigation into the chemical nature of this substance (5) indicated that it was probably a conjugate of the aglycone digoxigenin. Digitoxin was excreted in only small amounts as the unchanged glycoside in this animal (4); the principal urinary product was a substance, Metabolite C, which was thought to be a conjugate of a derivative of digitoxigenin. With higher doses of digitoxin, other metabolites appeared in the urine, one of which was named Metabolite G.

We have now been able to make a qualitative study of the cardioactive substances present in human urine after oral administration of digoxin, digitoxin, and lanatoside C and have compared the metabolites present with those found in rat urine. A preliminary communication (6) outlined evidence which indicated that hydroxylation occurred at C-12 in the digitoxin molecule in humans and in rats, thereby producing a urinary metabolite which could not be separated from the glycoside digoxin. This evidence was based on experiments in which paper chromatography and C14-digitoxin were used, and the details of this work are also presented in this paper.
Methods

Extraction of Glycosides and Metabolites from Human Urine—Healthy adults were given oral doses of 1 mg. of each glycoside and the 12 hour urine collections were pooled. The pooled urine was treated with 40 per cent lead acetate solution until no further precipitation occurred. The precipitate was removed by centrifuging and the supernatant liquid was extracted by rolling approximately 200 ml. with three successive portions of 150 ml. of chloroform in 1 liter bottles for 2 hours. The chloroform extract was too heavily pigmented to allow direct application to paper chromatograms, and the following procedure was necessary to remove the bulk of the pigment. The chloroform extract was evaporated to small bulk under reduced pressure, absorbed on 250 mg. of washed Super-Cel, and dried. A partition column was prepared by adding 1 gm. of water to 1 gm. of Super-Cel and packed into a column 8 mm. in diameter, filled with water-saturated benzene, and allowed to equilibrate with this solvent. The urine extract was then packed on top of the column and eluted with 15 ml. fractions of each of the following water-saturated solvents, (a) benzene; (b) benzene 9, chloroform 1; (c) benzene 4, chloroform 1; (d) benzene 2, chloroform 1; (e) benzene 1, chloroform 2; (f) chloroform. The use of this partition column reduced pigment concentration and permitted the subsequent paper chromatography of the urinary extracts. Each fraction was evaporated to dryness, redissolved in chloroform-methanol, and chromatographed on paper strips 3 × 27 cm. Fractions obtained from solvents (a) to (d) were chromatographed by using System 3, and fractions (e) and (f) with System 1. The glycosides or metabolites were located by treating a narrow marginal strip cut from each chromatogram with alkaline m-dinitrobenzene (4), and these were used as markers for cutting away the metabolite or glycoside areas in the main strip. These areas were dried at 60° in a current of air and eluted with methanol for further tests or rechromatography.

Paper Chromatography—Systems 1, 2, and 3 were used to develop formamide-impregnated paper. Whatman No. 4 paper was dipped in a mixture of 25 per cent formamide in methanol or acetone and dried between sheets of absorbent paper. The chromatograms were run horizontally (3).

System 1—Benzene 78, chloroform 12, butanol 5, saturated with formamide (4).

System 2—Tetrahydrofuran 1, chloroform 1, saturated with formamide (7).

System 3—Methyl isobutyl ketone 4, isopropyl ether 1, saturated with formamide (8).

Systems 4 and 5 were reversed phase systems, according to the methods...
of Tschesche, Grimmer, and Seehofer (9), and were used for further identification.

System 4—Ethyl hexanol-amyl alcohol-water-formamide (6:2:8:2).
System 6—Chloroform, ethyl acetate, benzene, saturated with water (10).

Sulfuric Acid Absorption Spectra—Between 1 and 3 mg. of pure glycoside or aglycone were dissolved in 40 ml. of 98 per cent sulfuric acid and the ultraviolet absorption spectra recorded after 30 minutes and again about 16 hours later. The metabolite areas of the paper chromatograms being investigated were eluted with methanol and, after removal of the methanol, 2 ml. of 98 per cent sulfuric acid were added and the ultraviolet absorption spectra measured as for the glycosides or aglycones. The sulfuric acid spectra of a methanol extract of an equivalent area of blank paper were recorded for comparison.

C\textsuperscript{14}-Digitoxin—Two rats of 200 gm. were each injected with 46,000 d.p.m. of biosynthetically prepared C\textsuperscript{14}-digitoxin (11), and at the same time with 2 γ per gm. of non-radioactive digitoxin. The urine was collected for 12 hours, extracted as described previously (4), and counted on an internal gas flow Geiger counter. The extract was then chromatographed on formamide-impregnated strips 4 cm. wide with use of System 1. One-fifth of the strip was cut away and treated with alkaline m-dinitrobenzene. The areas on the untreated strip corresponding to the glycoside and metabolites were cut, dried, and eluted with methanol, and each solution was counted. To the eluate of Metabolite G (Fig. 3) 10 mg. of normal digoxin were added and the whole was recrystallized to radiochemical purity.

Results

Metabolites Excreted in Human Urine

Lanatoside C—A total of 12 mg. of lanatoside C was administered to twelve subjects and the pooled 12 hour urine collections were treated by the method described. Paper chromatography revealed the presence of two substances which produced a blue color with m-dinitrobenzene characteristic of the butenolide ring. The first of these metabolites was rechromatographed with digoxin on Systems 1 and 6 and did not separate. The second metabolite was rechromatographed with a sample of Metabolite B obtained from rat urine on System 1 and failed to separate.

Lanatoside C could not be detected in eluates by this method, owing to the retention of this glycoside on the water partition column. To overcome this difficulty, a portion of the chloroform extract of the urine was chroma-
toographed directly on paper, System 1 being used, and unchanged lanatoside C was detected free from urinary pigment.

**Digoxin**—A total of eleven persons received 11 mg. of digoxin. Paper chromatography revealed the presence of two substances, which were compared with authentic digoxin and with a sample of rat urine Metabolite B, respectively, on Systems 1 and 6. No separations were observed.

**Digitoxin**—A total of 7.5 mg. of this glycoside was administered to eight persons. Three substances could be detected on paper chromatograms. The first, which was present in only trace amounts, was found to be similar to unchanged digitoxin by paper chromatography comparison. The other two metabolites were compared with Metabolites C and G which are present in rat urine, and would not separate from them on Systems 1, 3, and 6 (Table I).

| Metabolites Present in Human Urine after Administration of Digitalis Glycosides |
|--------------------------------------|------------------------------------------|
| Glycoside               | Glycoside and metabolites detected in urine |
| Lanatoside C          | Lanatoside C, digoxin, Metabolite B     |
| Digoxin               | Digoxin, Metabolite B                   |
| Digitoxin             | Digitoxin, Metabolite C, digoxin, Metabolite G |

**Examination of Digitoxin Metabolite G**

**Paper Chromatography**—Although chromatography on formamide-impregnated paper does not in general give consistent $R_F$ values, it was noted that Metabolite G present in rat or human urine after digitoxin administration traveled very similarly to digoxin when chromatographed on System 1. This metabolite was eluted and chromatographed together with digoxin on Systems 1, 2, and 3 without any separation occurring. Comparisons were also made on the reverse phase Systems 4 and 5, and again no separation was obtained.

**Mild Acid Hydrolysis**—Metabolite G eluates were hydrolyzed by refluxing with 0.5 per cent HCl and 50 per cent ethanol. The chloroform extract was chromatographed on Systems 1 and 3 with an authentic sample of digoxigenin without any separation occurring.

**Ultraviolet Absorption Spectra in Sulfuric Acid**—The ultraviolet absorption spectra in sulfuric acid of digoxin, digoxigenin, Metabolite G, hydrolyzed Metabolite G, sarmentogenin, and episarmentogenin are shown in Figs. 1 and 2. Prominent maxima at 390 and 490 m$\mu$ are present for digoxin, digoxigenin, Metabolite G, and hydrolyzed Metabolite G. Sarmentogenin and episarmentogenin showed a characteristic maximum at 415 m$\mu$ which was absent in digoxin, digoxigenin, or Metabolite G.
Fig. 1. Ultraviolet absorption spectra in sulfuric acid. Curve 1, eluate of Metabolite G; Curve 2, eluate of hydrolyzed Metabolite G; Curve 3, eluate of filter paper blank; A, optical density (1 cm. cell).

Fig. 2. Ultraviolet absorption spectra in sulfuric acid. Curve 1, sarmentogenin; Curve 2, digoxigenin; Curve 3, digoxin; Curve 4, epissarmentogenin.
**C\textsuperscript{14}-Digitoxin** The radioactivity of the total chloroform extract of the bulked urine after injection of two rats with a total dose of 92,000 d.p.m. of C\textsuperscript{14}-digitoxin was 3110 d.p.m. This extract, when chromatographed on System 1, showed three areas of cardioactive material (Fig. 3). After drying and elution with methanol, the radioactive areas gave the following counts: (a) digitoxin area 240 d.p.m.; (b) Metabolite G area 408 d.p.m.; (c) Metabolite C area 980 d.p.m. To the Metabolite G area 10 mg. of digoxin were added, and the whole was recrystallized five times from chloroform-ether and gave the following specific activities (disintegrations per minute per mg.) after correction for self-absorption: first recrystallization (9 mg.), 22.6; second (7.0 mg.), 20.8; third (6.0 mg.), 22.0; fourth (5 mg.), 21.0; fifth (3.0 mg.), 20.4; m.p. of recovered digoxin, 255–260° (authentic sample m.p. = 255–257°).

**Comparison of Metabolite B (from Digoxin) and Metabolite C (from Digitoxin)**—Both metabolites showed a blue fluorescence in ultraviolet light after treatment with trichloroacetic acid and traveled similarly on paper chromatograms. When chromatographed together on System 1, no separation occurred. Both Metabolite B and Metabolite C after hydrolysis with dilute acid could not be separated from digoxigenin.

**DISCUSSION**

It is apparent that the metabolism of the cardiac glycosides digoxin, digitoxin, and lanatoside C is qualitatively similar in rats and in humans. We were unable to detect any metabolites in human urine which were not present in rat urine. Although quantitative estimation of the human urine metabolites was not attempted, the paper chromatograms indicated that, after digitoxin administration, the major cardioactive constituent of the urine was not unchanged glycoside, but was the substance which we have previously called Metabolite C in rat urine (5). Smaller amounts of the Metabolite G of rat urine and free digitoxin were detected.

The fact that Metabolite G could not be separated from the glycoside digoxin on five systems of paper chromatography indicates that it must be
very similar to, if not identical with, this substance. The apparent non-separation of digoxin from the C14-labeled Metabolite G excreted in rat urine after five recrystallizations is also strong evidence that these substances are the same compounds. Also, it appears from paper chromatographic evidence that Metabolite B found in human and rat urine after administration of digoxin and which has been shown to be a conjugate of digoxigenin (5) is identical with digitoxin Metabolite C.

The formation of digoxin from digitoxin involves hydroxylation of the steroid genin at C-12 in the β position. Although α hydroxylation at position 12 in the steroid nucleus occurs in the formation of bile acids and has been shown to take place in man after cholesterol administration (12), β hydroxylation in this position does not appear to have been previously recorded in animals. It must be pointed out, however, that the cardiac glycosides are unique among naturally occurring steroids in having rings C and D arranged cis to one another, and under these conditions the β position at C-12 is equatorial and hence the more favored configuration.

It is possible that hydroxylation could have occurred at position 11 in the digitoxin molecule and the resulting product be inseparable from digoxin by paper chromatography or recrystallization. A comparison of the ultraviolet absorption spectra of digoxigenin, digoxin, and Metabolite G in concentrated sulfuric acid with that of sarmentogenin (11-α-hydroxyl-digitoxigenin) with episarmentogenin (11-β-hydroxydigitoxigenin) indicates that Metabolite G is not an 11-hydroxyl compound.

The sulfuric acid absorption spectra of sarmentogenin and 11-episarmentogenin are identical. Also these two compounds, though separable, run very close to one another on paper chromatograms. This suggests that 12-α-hydroxydigitoxin and 12-β-hydroxydigitoxin (digoxin) would also be difficult to distinguish from each other. However, from the constant radioactivity upon recrystallization, and from a consideration of conformational factors, we prefer the identification of Metabolite G with digoxin rather than with 12-epidigoxin.

SUMMARY

1. The cardioactive metabolites excreted in human urine after the administration of lanatoside C, digoxin, and digitoxin are identical with those found in rat urine.
2. In the rat and in man the glycoside digitoxin undergoes hydroxylation at position 12 in the steroid aglycone, resulting in the formation of a metabolic product which cannot be distinguished from the glycoside digoxin.
3. Metabolite B, a conjugate of digoxigenin, found in human and rat urine after dosage with lanatoside C and digoxin, is identical with Metabolite C present in human and rat urine after digitoxin administration.
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