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Research Article



# STUDIES ON TRITIUM–LABELED DIGOXIN: TISSUE, BLOOD AND URINE DETERMINATIONS

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During the last 40 years extensive investigations have been reported on various aspects of the metabolism of cardiac glycosides. A detailed review describing the cellular basis of cardiac glycoside action has been reported by Hajdu and Leonard (1). One prime difficulty encountered in determining the metabolic fate of cardiac glycosides is in establishing a reliable and sensitive method for determining tissue, blood and urine levels of the glycosides as well as their derivatives in man and in experimental animals. The minute amounts present in blood, urine and tissues after oral or parenteral administration require extremely sensitive assay methods. Digitalis glycosides have been studied by utilizing bioassay (2–9), colorimetric (10–14), radioisotopic (15–17), and polarographic techniques (18, 19).

The behavior and fate of digitoxin in experimental animals and in man have been reviewed by Friedman, St. George and Bine (20). In Friedman's laboratory, bioassay techniques using embryonic duck hearts have detected 0.1 $\mu$g of digitoxin per ml of serum of humans who received 1.2 mg digitoxin intravenously (21). The method consists of the observation of cardiac irregularities in embryonic duck hearts immersed in test fluids (8, 22). This group has reported reproducible results in studies of experimental animals and humans (8, 20, 22–30).

Digitoxin labeled with carbon$^{14}$ has been shown to be an excellent tool for appraising the behavior and fate of the glycoside (15–17, 31–38). Studies utilizing carbon$^{14}$ digitoxin have included the fixation of cardioactive glycosides by isolated hearts (15); the tissue distribution and excretion in digitalis-sensitive and digitalis-resistant animals (16); the placental transfer of digitoxin in rats and guinea pigs (32); the renal excretion (33), blood levels (17), metabolic fate (34), and the placental transfer and fetal distribution (36) in humans.

The advantages of radioactive tracer methods for investigating the metabolic fate of digitoxin have been discussed by Sjoerdsma, Fischer and Johnson (15, 16), Okita and co-workers (17), and Geiling and Okita and co-workers (31–34, 36). The incorporation of a tracer substance with a higher maximal permissible radioactivity dose than that of carbon$^{14}$ would make it possible to study various aspects of the metabolic pathways of the parent compound in greater detail. Thus it would be feasible to perform long-term studies in humans utilizing the radioactive glycoside exclusively. Data obtained from acute experiments carried out in laboratory animals might be correlated with results in humans. Recently, tritium-labeled digoxin (digoxin-H$^3$)[1] became available for experimental use. Extensive data on the utilization of tritium-labeled compounds were presented at three annual symposia in New York City (39, 40). The physiology and toxicology of tritium in man were studied extensively by Pinson and Langham (41).

This report presents preliminary data from studies in dogs using digoxin-H$^3$ administered intravenously.

## EXPERIMENTAL METHOD

Unselected mongrel dogs weighing between 12 and 25 kg were anesthetized with Pentothal Sodium administered intravenously without prior medication. An endotracheal tube was inserted and respirations were assisted with a Phipps-Bird type respirator. Utilizing routine surgical techniques, polyethylene cannulas were inserted into a contralateral femoral artery and vein for the collection of blood samples. A superficial foreleg vein was cannulated for the administration of fluids and

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[1] Dr. Stanley T. Bloomfield, Burrough Wellcome, Ltd., kindly supplied the radioactive digoxin.

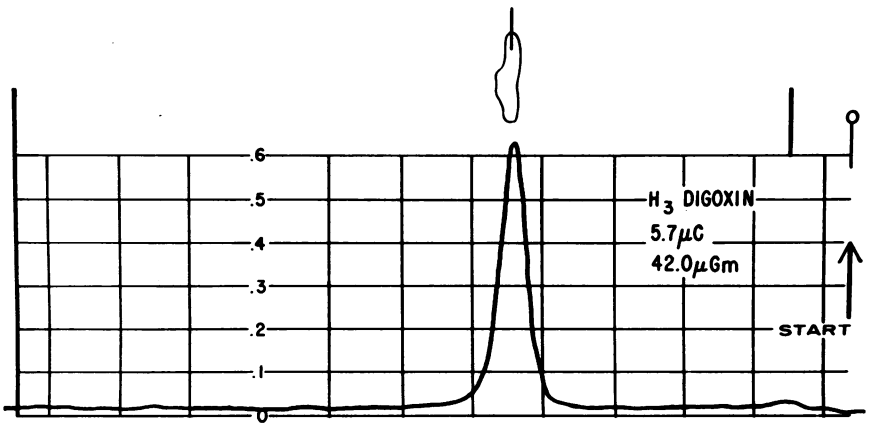


FIG. 1. COUNTING RECORD OF A CHROMATOGRAPHIC SPOT CONTAINING 42 μG OF DIGOXIN-$H^3$ (5.7 μC OF TRITIUM). Peak of radioactivity coincides with area of digoxin concentration.

drugs. The remaining superficial femoral artery was cannulated for recording intra-arterial blood pressures with a Statham pressure transducer. Needle electrodes were inserted and standard extremity electrocardiographic leads were recorded. Polyethylene catheters were introduced into the urinary bladder with 13 gage hypodermic needles or through a cystostomy wound. The right chest cavity was entered at the fourth intercostal space. The pericardium was incised and sutured to the chest wall. A purse string suture was placed on the right auricular appendage and a polyethylene catheter was guided into the coronary sinus. A transfixing suture was used to sustain the catheter in the coronary sinus with careful avoidance of blockage to the coronary sinus. The patency of the various catheters was maintained by a constant flow of heparinized saline. The arterial catheters were washed at repeated intervals with heparinized saline. Digoxin-$H^3$, and digoxin injection (Lanoxin) were mixed with 0.9 per cent saline in a syringe in the amounts specified in Table I. The tritium-labeled digoxin was made available in ampules containing 1 mg of digoxin labeled with 134 μc of tritium. The suspension was administered intravenously over one minute.

In order to make simultaneous collections, the serial collections from each blood vessel were made by three observers. Unless otherwise specified, samples were collected at 1, 3, 5, 10, 15, 30, 45 and 60 minutes after drug administration. Biopsy specimens were obtained from the right atrium and ventricle at predetermined intervals. Double mattress sutures were placed at the surgical site. At the termination of each experiment the animal was sacrificed. The liver, kidneys and heart were removed, weighed, and representative samples obtained. Total urine specimens were collected at various time intervals for measurement. The amount of fluid administered intravenously was determined for each study. Throughout the experiments, intra-arterial pressures and electrocardiograms were monitored and recorded.

*Preparation of samples for determination of radioactivity*

*1. Blood.* Ten ml of 95 per cent ethanol was added to 1 ml of heparinized blood in order to precipitate the protein. This was then centrifuged for 10 minutes at 2,500 rpm. Two ml of the clear supernate was removed and evaporated to dryness. The residue was dissolved in 0.4 ml of 95 per cent ethanol. Two-tenths ml was mixed with 15.0 ml of a solution made by mixing 0.01 g *bis*-phenyloxazolylbenzene (a wavelength shifter), 0.20 g 2,5 diphenyloxazole (a phosphor), and 100.0 ml toluene. The samples were transferred to special counting vials and cooled in a freezer at 0° C for at least 24 hours. All samples were counted in a Packard Tri-Carb liquid scintillation spectrometer with a two-channel pulse-height analyzer. This instrument was calibrated daily, utilizing a standard containing 134 mμc of tritium-labeled digoxin. All samples were counted for 1 hour. Unless otherwise specified, counts were expressed in millimicrocuries per gram of tissue. Recovery experiments were performed by adding digoxin-$H^3$ to blood and extracting as indicated. Approximately 95 to 98 per cent of the added radioactivity was recovered by the procedure used.

*2. Urine.* The urine samples were diluted with 95 per cent ethanol to a final dilution of 1:2. Two ml was evaporated to dryness and the residue treated as described above.

*3. Tissues.* The tissue samples were dried with filter paper to remove blood and extracellular fluid. They were weighed, and preserved in 5.0 ml of 95 per cent ethanol until homogenized. Two ml of the homogenate was removed, evaporated to dryness, and treated as described above.

*Chromatographic studies*

Chromatographic studies were performed, using multiple solvent systems (see below) to identify the glycoside or its metabolites in urine and blood. These studies were necessary, since the concentration of radioactive material in blood and urine does not necessarily indicate

the presence of unaltered digoxin $H^3$. Control chromatographic observations were performed using alcoholic solutions of radioactive and nonradioactive digoxin, and mixtures of these solutions with blood and urine in multiple proportions (42). Urine samples were precipitated with 40 per cent lead acetate and the supernate was extracted three times with chloroform (3 parts chloroform to 1 part urine). The chloroform extract was evaporated to dryness, and the residue dissolved in a 1:1 chloroform: methanol solution. This final suspension was evaporated to a small volume and examined with chromatographic techniques. Details of these techniques are given by Gisvold and Wright (43).

Chromatographic studies of digoxin solutions were carried out utilizing the following solvent systems: 1) chloroform-benzene-formamide (44); 2) chloroform-water-methanol (44); 3) benzene-chloroform-butanol (45); 4) benzene-ethyl acetate-chloroform (46); 5) methyl-isobutylketone-isopropyl ether-formamide (43). Optimal results were obtained with a slight modification of system 5. The proportions by volume were: methyl-isobutylketone, 8; isopropyl ether, 2; butanol, 1; and formamide, q.s. to saturate.

The chromatographic strips were allowed to develop in a closed jar saturated with the solvent system described. They were then passed quickly through a solution of 2.5 per cent meta-dinitrobenzene in chloroform and sprayed with a solution of 5 per cent KOH in methanol. The strips were examined under white and ultraviolet light. The Rf values were calculated for each chromatogram. The spots were sectioned off the paper, eluted with isopropanol, and processed for radioactivity counting as described previously.

## EXPERIMENTAL RESULTS

*Blood levels of digoxin-$H^3$.* Blood samples aspirated simultaneously from the femoral artery, femoral vein and coronary sinus showed a rapid decrease in radioactivity during the first 15 minutes (Table II).

*Urine levels of digoxin-$H^3$.* Significant levels of the radioactive isotope were measured in the urine samples collected within 30 minutes after the intravenous injection of tritium-labeled digoxin (Table III).

TABLE I

*Digoxin dose schedule*

| Dog | Weight | Digoxin-$H^3$ | Lanoxin | Vol. of injection |
|---|---|---|---|---|
| | *kg* | *mg* | *mg* | *ml* |
| 1 | 25.0 | 0.5 | 0.125 | 5.0 |
| 2 | 12.7 | 1.0 | 0.5 | 10.0 |
| 3 | 13.6 | 1.0 | 1.0 | 10.0 |
| 4 | 18.1 | 1.0 | 1.0 | 10.0 |
| 5 | 10.9 | 2.0 | 0.5 | 10.0 |

TABLE II

*Blood levels of digoxin-$H^3$*

| Dog | Time | Arterial | Venous | Coronary sinus |
|---|---|---|---|---|
| | *min* | | *mμc/ml blood* | |
| 1 | 1 | 184.14 | 49.84 | 117.56 |
| | 3 | 54.82 | 19.58 | 28.58 |
| | 5 | 34.90 | 13.34 | 21.40 |
| | 10 | 15.24 | 10.82 | 8.74 |
| | 23 | 8.88 | 7.34 | 4.60 |
| | 45 | 4.02 | 4.04 | 1.8 |
| 2 | 1 | 181.48 | 167.24 | 86.24 |
| | 3 | 81.56 | 75.38 | 96.94 |
| | 5 | 30.98 | 64.92 | 65.92 |
| | 10 | 26.92 | 41.64 | 43.06 |
| | 15 | 30.20 | 31.92 | 26.66 |
| | 30 | 15.44 | 14.32 | 14.46 |
| | 45 | 8.90 | 9.44 | 5.56 |
| | 60 | 10.60 | 7.80 | 10.26 |
| | 75 | 6.14 | 9.02 | 6.60 |
| | 90 | 4.46 | 4.64 | 4.20 |
| 3 | 1 | 238.30 | 153.40 | 158.20 |
| | 3 | 129.12 | 77.70 | 103.92 |
| | 5 | 112.02 | 60.16 | 79.94 |
| | 10 | 65.80 | 53.14 | 42.24 |
| | 15 | 26.80 | 34.26 | 38.50 |
| | 25 | 17.36 | 21.02 | 19.32 |
| | 35 | 12.32 | 17.14 | 13.26 |
| | 38 | 10.78 | 18.20 | 19.14 |
| 4 | 1 | 149.14 | 132.44 | 151.00 |
| | 3 | 69.26 | 80.16 | 105.46 |
| | 5 | 70.70 | 59.92 | 47.06 |
| | 10 | 47.58 | 47.38 | 36.86 |
| | 15 | 21.48 | 34.08 | 30.84 |
| | 30 | 26.82 | 27.72 | 15.04 |
| | 45 | 10.12 | 16.94 | 12.12 |
| | 60 | 12.44 | 12.48 | 12.70 |
| 5 | 1 | 324.00 | 119.58 | 199.82 |
| | 3 | 193.08 | 97.38 | 208.52 |
| | 5 | 90.24 | 214.32 | 142.18 |
| | 10 | 38.64 | 165.58 | 83.88 |
| | 15 | 20.38 | 66.78 | 29.90 |
| | 30 | 37.52 | 16.76 | 26.42 |
| | 45 | 8.70 | 19.98 | 32.58 |

*Tissue levels of digoxin-$H^3$.* Studies of the tissue levels of the isotope revealed significant amounts of radioactive digoxin in the liver, kidneys and heart. In tissue samples obtained at the end of the experiment, the highest concentration of radioactive digoxin per gram of tissue was found in the kidney. An average of 633.40 mμc was recovered per g of kidney. Studies of liver and heart revealed an average recovery of 128.48 and 72.35 mμc per g of tissue, respectively (Table IV). An average of 68.73 per cent of the total administered radioactive digoxin was recovered in the liver, heart and kidneys (Table V). These three organs accounted for an average of 4.1 per cent of the dog's body weight.

TABLE III

*Recovery of digoxin-H³ in urine*

| Dog | Time interval after i.v. injection of digoxin-H³ | Urine collected | Total recovered | Injected dose |
|---|---|---|---|---|
| | *min* | *ml* | *μc* | % |
| 1 | 0–22 | 13 | 2.926 | 8.06 |
| | 22–45 | 16 | 2.476 | |
| 2 | 0–60 | 38 | 2.528 | |
| | 60–75 | 44 | 2.020 | 6.28 |
| | 75–85 | 65 | 2.870 | |
| 4 | 0–10 | 42 | 0.125 | |
| | 10–30 | 200 | 3.374 | 6.45 |
| | 30–45 | 100 | 5.152 | |

*Chromatographic studies.* Controlled chromatographic studies of digoxin solutions showed that the Rf values of the glycoside were not altered by chloroform extraction. Chromatographic spots, satisfactory for identification studies, have been obtained using as little as 0.006 mg digoxin in 25 μl of 95 per cent ethanol.

The chromatographic strips obtained by using radioactive samples were cut lengthwise and studied, utilizing a Chromatogram scanner (Baird Atomic) with rectilinear galvanometric recorder (Texas Instrument, Inc.). A representative "curve" is depicted in Figure 1. The spots were later sectioned off the paper, eluted and processed for counting as described previously (42).

## DISCUSSION

A technique has been described which appears to be suitable for the appraisal of certain aspects of the metabolism of a cardiac glycoside in blood, urine and body tissues.

Forty minutes after the injection of tritium-labeled digoxin, approximately 68 per cent of the drug could be recovered from the heart, kidneys and liver. This percentage of recovery compares favorably with studies utilizing digitoxin-C¹⁴ (17, 33, 34). The relatively large amounts of the radioactive isotope recovered in the urine and kidneys corroborate data of other investigators who have shown that the major organ involved in the ultimate excretion of digitoxin is the kidney (34). Using the chromatographic techniques described, we have been able to identify the unaltered glycoside, digoxin, in dog urine in our acute, short-term experiments.

TABLE IV

*Recovery of digoxin-H³*

| Dog | Heart | Kidney | Liver |
|---|---|---|---|
| | | *mμc/g tissue* | |
| 1 | 47.00 | 147.48 | 14.26 |
| 2 | 80.30 | 899.32 | 48.40 |
| 3 | 113.62 | 525.70 | 124.54 |
| 4 | 64.30 | 388.34 | 82.80 |
| 5 | 156.51 | 1,206.18 | 372.44 |

Both digoxin and lanatoside C have been found to be excreted principally as the unchanged glysoside (38). A conjugate of digoxigenin has also been identified in rat urine after the administration of digoxin and lanatoside C (38, 47), and in rat bile after the administration of digoxin (48). Three separate bands have been obtained in chromatographic studies of rat urine after the administration of acetyldigitoxin (47). The band closest to the starting line could not be separated from metabolite B, a conjugate of digoxigenin.

TABLE V

*Recovery of digoxin-H³ from kidneys, liver and heart*

| Dog | Digoxin-H³ injected i.v. | Digoxin-H³ recovered | Digoxin-H³ recovered |
|---|---|---|---|
| | *μc* | *μc* | % |
| 1 | 67 | 40.886 | 61.02 |
| 2 | 134 | 97.628 | 72.10 |
| 3 | 134 | 98.680 | 73.64 |
| 4 | 134 | 78.572 | 58.63 |
| 5 | 268 | 209.753 | 78.26 |

After hydrolysis this product could not be separarated from digoxigenin. A second band which could not be separated from metabolite G, digoxin, was also hydrolyzed to digoxigenin. Further studies are being carried out in this laboratory to identify any metabolite that may be present in dog urine after the intravenous administration of radioactive digoxin. The nature of the radioactive material present in the kidney, heart and liver is being investigated in this laboratory.

From the data presented, it is evident that measurable levels of radioactive digoxin can be determined in blood, urine, heart, kidneys and liver of dogs receiving 3 to 7 μc of tritium per kg. According to published reports (49) "the maximal permissible amount of the radioisotope in the total body is $2 \times 10^3$ μc." Extrapolation of the data presented in this report suggests that satisfactory

levels of the isotope should be obtained in humans using digoxin-$H^3$ in dosages well within the permissible radioactivity. Preliminary observations regarded as satisfactory have been obtained in studies of urine and blood samples in one human (42). Apparently, studies of the metabolic pathways of digoxin in humans over long periods of time may be performed without dangerous exposure to radiation (50).

## SUMMARY

Blood, urine and body tissue levels of tritium-labeled digoxin were studied in five mongrel dogs. The isotopic concentrations were measured by the liquid scintillation technique. Utilizing chromatographic techniques, the radioactive material excreted in the urine after the intravenous administration of the drug could not be distinguished from unaltered digoxin.

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