# Demonstation of Enhanced Lethality of Drugs in Hypoexcretory Animals

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**Hypoexcretory states were produced in mice: anuria by penile ligation, extrahepatic cholestasis by bile duct ligation, intrahepatic cholestasis by phenylisothiocyanate treatment, and reduced bile flow by α-naphthylisothiocyanate treatment. Intraperitoneal administration of ouabain to mice with no or diminished bile flow resulted in enhanced mortality rates. Digoxin and digitoxin effected enhanced lethality in anuric mice. Lanatoside-C was not more toxic to hypoexcretory mice. The use of hypoexcretory mice in toxicologic evaluations of pharmacologic agents is suggested.**

THE PURPOSE of this paper is to compare the acute lethalities of certain drugs in hypoexcretory animals with acute lethalities obtained in normal animals. While drugs may be excreted by several routes, the major routes of excretion are the urinary and biliary routes. Urinary excretion can be prevented in male mice by the production of anuria (1). Biliary excretion may be totally blocked by ligation of the common bile duct which produces extrahepatic cholestasis. A condition resembling intrahepatic cholestasis can be induced by treatment with aryl isothiocyanates in mice (2) or rats (3). Only two aryl isothiocyanates, phenylisothiocyanate (PIT) and α-naphthylisothiocyanate (ANIT), possess cholestatic activity (4); both ANIT and PIT have been used in these investigations. The drugs selected as test agents in these studies are four cardiac glycosides: ouabain, lanatoside-C, digoxin, and digitoxin. The excretion of these agents has been studied by Cox and Wright (5), who show that ouabain is excreted primarily *via* bile, lanatoside-C mainly *via* bile with a small degree of urinary excretion, digoxin excreted approximately equally in the bile and urine, and digitoxin excreted primarily in the urine.

## METHODS

Adult, male, Swiss-Webster-type mice weighing 25–40 Gm., were housed in groups of 10 in stainless steel "shoebox" cages and allowed food and water *ad libitum* before and after treatment. Groups of 20 mice were anesthetized with ether and rendered anuric by the penile ligation (PL) method of Becker and Gibson (1). Other groups of 20 mice, anesthetized and sham-ligated (PL-sham), served as controls. Other mice were anesthetized with ether and the common bile duct isolated. The common bile duct was ligated near its junction with the duodenum and the incision closed with wound clips. Cholestasis or reduced bile flow was also induced by treating groups of 20 mice with 85 mg./Kg. of PIT[1] or ANIT.[1]

Total cholestasis was demonstrated 24 hr. after treatment by the method of Plaa and Becker (6) in which treated mice are injected intravenously with a saturated solution of fluorescein in isotonic sodium bicarbonate. Fifteen minutes after fluorescein was injected, the bile ducts were exposed and examined under ultraviolet light. A cholestatic animal showed no fluorescence in the bile duct. Actual flow of bile in ANIT treated mice was determined by cannulation of the common bile duct according to the method of Plaa and Becker (6) 24 hr. after oral administration of ANIT.

Groups of PL, PL-sham, and normal animals were injected intraperitoneally 2 hr. after operation with varying concentrations of the selected glycoside. Twelve hours after glycoside treatment, the number dead in each group was counted. Ouabain was dissolved in water, the other glycosides were dissolved in ethanol, and the concentration adjusted so that the desired drug dosage was administered intraperitoneally in 0.1 ml. of 47.5% ethanol and the final average dosage (mg./Kg.) was calculated from the average body weight per group. Preliminary tests showed that 0.1 ml. of 47.5% ethanol was not lethal to mice. In other experiments, groups of 20 BDL and 20 BDL-sham mice were treated 24 hr. after surgery, along with groups of 20 PIT, ANIT, corn oil vehicle treated, and normal mice, by intraperitoneal injection of varying dosages of the selected glycosides. The number dead in each group 24 hr. after glycoside treatment was recorded.

For both the anuria and cholestasis studies, the number dead in each group was expressed as percentage of the number treated and the standard error calculated by the binomial expansion method. Statistical significance ($P < 0.05$) between test groups and sham-operated and normal groups was also found by the binomial expansion method (7). Statistical analysis of bile flow data was made by Student's $t$ test with the level of significance chosen as $P < 0.05$.

## RESULTS

**Cholestasis and Impaired Bile Flow**—Twenty four hours after PIT, 85 mg./Kg., ten treated mice were tested for bile flow by the fluorescein method; nine (90%) showed no fluorescein, indicating total cholestasis. On the other hand, ANIT, 85 mg./Kg., effected total cholestasis in only 12 of 33 animals (35%). However, actual bile flow as measured in 4 mice 24 hr. after ANIT treatment was found to be definitely reduced in all but one animal (Table I). The percentage of animals shown in Table I which produced no bile agrees well with the 35% value obtained by the indirect (fluorescein) method.

**Lethality in Anuric Mice**—Digitoxin in three dosages, 2.5, 3.5, and 6.0 mg./Kg., effected significantly higher mortality rates in anuric mice than in sham-operated or normal mice. Similarly, digoxin in two dosages, 5.0 and 9.2 mg./Kg., also

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[1] K&K Laboratories, Inc., Jamaica, N. Y.

TABLE I—EFFECT OF ANIT, 85 mg./Kg. ORALLY, ON BILE FLOW IN THE MOUSE 24 hr. AFTER ADMINISTRATION

| Mouse No. | Bile Flow, μl./hr. hr. After Bile Duct Cannulation 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| | | Control[a] | | |
| 1 | 115 | 105 | 85 | 90 |
| 2 | 115 | 110 | 100 | 95 |
| 3 | 60 | 90 | 115 | 85 |
| 4 | 65 | 80 | 130 | 55 |
| $\bar{X}$ ± S.E. | 89 ± 15 | 96 ± 7 | 108 ± 10 | 81 ± 9 |
| | | Anit | | |
| 1 | 75 | 60 | 30 | 55 |
| 2 | 75 | 0 | 0 | 0 |
| 3 | 150 | 90 | 85 | 70 |
| 4 | 0 | 0 | 0 | 0 |
| $\bar{X}$ ± S.E. | 75 ± 31 | 38 ± 22[b] | 29 ± 20[b] | 31 ± 18[b] |

[a] Control animals received corn oil p.o., 0.01 ml./Gm. body weight, 24 hr. before bile duct cannulation. [b] Significantly less than controls at $P < 0.05$.

TABLE II—TWELVE-HOUR ACUTE LETHALITY OF OUABAIN, LANATOSIDE-C, DIGOXIN, AND DIGITOXIN IN ANURIC AND NORMAL MALE MICE

| Dosage,[a] mg./Kg. | Lethality[b] Anuric | Normal[c] | Significance (P) |
|---|---|---|---|
| | Ouabain | | |
| 2.2 | 15.0 ± 8.0 | 5.0 ± 4.8 | N.S. |
| 4.2 | 25.0 ± 9.9 | 25.0 ± 9.9 | N.S. |
| 8.5 | 45.0 ± 11.3 | 50.0 ± 11.2 | N.S. |
| | Lanatoside-C | | |
| 3.4 | 33.3 ± 15.7 | 8.4 ± 8.0 | N.S. |
| 6.8 | 70.0 ± 14.5 | 70.0 ± 14.5 | N.S. |
| 10.2 | 78.0 ± 14.0 | 90.0 ± 9.5 | N.S. |
| | Digoxin | | |
| 4.0 | 35.0 ± 10.6 | 10.0 ± 6.7 | N.S. |
| 5.0 | 50.0 ± 11.2 | 10.0 ± 6.7 | <0.01 |
| 9.2 | 75.0 ± 6.9 | 37.5 ± 7.7 | <0.001 |
| | Digitoxin | | |
| 2.5 | 70.0 ± 14.5 | 0.0 | <0.001 |
| 3.5 | 80.0 ± 12.7 | 10.0 ± 9.5 | <0.002 |
| 6.0 | 86.0 ± 6.6 | 43.0 ± 9.0 | <0.001 |

[a] Intraperitoneal administration. Minimum group size: 20 mice. [b] Per cent dead ± standard error. [c] Sham operated groups were not significantly different from the normal groups. [d] Not significantly different ($P > 0.05$).

ouabain or lanatoside-C (Table II). Lethality in sham-operated animals did not differ significantly from that in normal mice.

**Lethality in Cholestatic or Impaired Bile Flow Mice**—Ouabain was the only cardiac glycoside tested which effected increased mortality in mice with experimentally induced extrahepatic (BDL), intrahepatic cholestasis (PIT), or reduced bile flow (ANIT). Results are given in Table III.

## DISCUSSION

The enhanced lethality of ouabain in animals with poor or no bile flow is a reasonable result as ouabain is believed to be excreted only in the bile. Similarly, the enhanced lethality of digitoxin in anuric animals is reasonable since digitoxin is excreted primarily *via* the urinary route. The increase in mortality rates seen in anuric animals treated with digoxin suggests that the urinary route of excretion is more important in mice than the biliary route, although the drug is believed to be excreted by both routes in the normal animal. Lanatoside-C, which according to Cox and Wright (5) is about 70% excreted in the bile and to a small degree in the urine, failed to effect enhanced lethality in either biliary or urinary hypoexcretory mice. This unexpected and challenging finding indicates that lanatoside-C can be eliminated, apparently, by either route of excretion with equal facility. The mechanism of the change in excretory routes remains to be investigated. The polarity of lanatoside-C is approximately that of ouabain as both compounds have five free hydroxyl groups. Another question which arises, then, is why the biliary hypoexcretory mouse cannot shift the elimination of ouabain to the urinary route.

Enhancement of toxicity of drugs in hypoexcretory animals other than the mouse has also been demonstrated. Enhanced lethality of ouabain in bile duct ligated cats has been reported by Kusakari and Uchida (8). Marcus *et al.* (9) studied the distribution of digitoxin in anuric dogs and humans in

TABLE III—TWENTY-FOUR HOUR ACUTE LETHALITY OF OUABAIN, LANATOSIDE-C, DIGOXIN, AND DIGITOXIN IN CHOLESTATIC AND NORMAL MALE MICE

| Drug | Dosage,[a] mg./Kg. | Lethality[b] ANIT[c] | PIT[c] | BDL[d] | Normal[e] | Significance[f] (P) |
|---|---|---|---|---|---|---|
| Ouabain | 2.2 | 33.3 ± 12.2 | 26.6 ± 4.4 | 26.6 ± 11.4 | 0.0 | <0.05 |
| | 4.2 | 47.0 ± 12.1 | 60.0 ± 12.9 | 67.0 ± 10.3 | 16.0 ± 4.6 | <0.05 |
| | 8.5 | 96.0 ± 4.2 | 92.0 ± 5.4 | 74.0 ± 9.4 | 52.0 ± 10.0 | <0.05 |
| Lanatoside-C | 3.4 | 15.0 ± 8.0 | 5.0 ± 4.8 | 20.0 ± 9.0 | 10.5 ± 7.0 | N.S.[g] |
| | 6.8 | 52.6 ± 12.0 | 45.0 ± 11.0 | 52.6 ± 12.0 | 55.5 ± 12.0 | N.S. |
| | 10.2 | 85.0 ± 8.0 | 75.0 ± 10.0 | 85.0 ± 8.0 | 85.0 ± 8.0 | N.S. |
| Digoxin | 4.0 | 26.3 ± 10.1 | 0.0 | 29.4 ± 11.1 | 5.6 ± 5.4 | N.S. |
| | 5.0 | 42.0 ± 11.3 | 45.0 ± 11.1 | 26.4 ± 10.1 | 35.0 ± 10.6 | N.S. |
| | 9.2 | 63.2 ± 11.1 | 65.0 ± 10.6 | 63.2 ± 11.1 | 35.0 ± 10.6 | N.S. |
| Digitoxin | 2.5 | 53.0 ± 12.1 | 16.7 ± 8.8 | 25.0 ± 9.7 | 22.2 ± 10.4 | N.S. |
| | 3.5 | 67.0 ± 9.6 | 60.0 ± 12.7 | 58.0 ± 11.3 | 50.0 ± 7.1 | N.S. |
| | 6.0 | 87.0 ± 5.5 | 85.0 ± 8.0 | 85.0 ± 8.0 | 80.0 ± 9.0 | N.S. |

[a] Intraperitoneal administration. Minimum group size: 20 mice. [b] Per cent dead ± standard error. [c] Eighty-five mg./Kg. in corn oil by mouth, 24 hr. before test drug administration. [d] Twenty-four hours before test drug administration. [e] Sham-treated animals of the various cholestatic groups were not significantly different from normals. [f] Probability of no difference between the smallest lethality in any cholestatic group and the normal group. [g] N.S., no significant differences between the various groups ($P > 0.05$).

effected enhanced lethality in anuric mice. The lowest digoxin dosage, 4.0 mg./Kg., effected higher but nonsignificant increases in lethality. No significant changes in mortality rates were effected by renal failure and found increased storage of digitoxin in these species when urinary flow was diminished.

The possibility of enhanced lethality of therapeutic agents in hypoexcretory humans, therefore,

exists. Physicians have intuitively recognized the need to reduce dosages of some agents, notably certain antibiotics, in patients with renal insufficiency. Toxicologists might properly concern themselves with the need to evaluate the toxicity of new pharmacologic agents in hypoexcretory animals as the mouse preparations described here. Such evaluation might be made as a part of drug safety evaluation during the preclinical testing period.

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# Determination of Benzoyl Peroxide Stability in Pharmaceuticals

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**Spectrophotometric, titrimetric, and polarographic techniques were compared for stability testing of commercially available benzoyl peroxide pharmaceuticals. Initial testing revealed comparable results but significant discrepancies were observed on prolonged testing. The titrimetric procedure employed showed little degradation of the benzoyl peroxide at elevated temperatures, while both the spectrophotometric and polarographic methods demonstrated radical potency losses under the conditions of testing. Agreement was excellent between the polarographic and spectrophotometric procedures above 70 per cent of the original assay with a significant divergence being noted only below this level. A rapid, reproducible procedure of sufficient accuracy for pharmaceutical product stability and control purposes is presented.**

WITH THE INTRODUCTION of benzoyl peroxide as a topical medicinal, this laboratory began an investigation into the published methods of analysis with a view to pharmaceutical stability evaluation. Previous methods have been employed to establish the absence of residual peroxides and associated by-products in the manufacture of plastics, polymers, fats, oils, breads, and flours. Since the primary purpose of any pharmaceutical test is specificity, it was believed that a more intensive investigation of the available methods was necessary prior to the adoption of any particular technique.

Titrimetric, spectrophotometric, and polarographic methods were compared for the determination of benzoyl peroxide stability in the dry form and in commercially available pharmaceutical preparations. The titrimetric method employed in this comparison was a modification of Wheeler's (1) procedure, a variation of the technique originally proposed by Lea (2).

Many spectrophotometric procedures have been described for the determination of organic peroxides, and the relative merits and disadvantages of these techniques have been thoroughly evaluated by Kolthoff and Medalia (3) and others (4, 5). The spectrophotometric procedure employed in this comparison was a modified form of the method of Banerjee and Budke (6). They adapted the iodimetric procedure of Heaton and Uri (7), using a 2 to 1 mixture of acetic acid and chloroform, instead of the proportions suggested by Lea (2). This technique proved to be the most promising due to the reduction of any tendency toward continued autooxidation, catalyzed oxidation, and/or induced oxidation. These properties were associated with the higher polarity of the solvent system. This procedure was designed for trace quantities of peroxide, but it was found applicable, by judicious choice of sample size and dilutions, to pharmaceutical preparations containing up to 10% benzoyl peroxide, with excellent accuracy and reproducibility.

A polarographic method was utilized for comparison as a result of the work of Ricciuti, Coleman, and Willets (8), who performed a statistical evaluation of a modified Wheeler procedure compared to a polarographic method for the analysis of tetralin hydro-peroxide and found the polarographic technique to be more reliable where impure products were encountered, due to its greater specificity. The various methods for polarographic measurement of organic peroxides are closely related in procedure and similar in results; therefore, a modification of the procedure of Bezuglyi and Dmitrieva (9) was employed.

Many pharmaceutical laboratories do not have polarographic instrumentation available because of the limited applications of polarography in this field. For this reason, a rapid, accurate, and reproducible spectrophotometric method is proposed for laboratory control and stability testing of benzoyl peroxide in pharmaceutical preparations.

## EXPERIMENTAL

### Spectrophotometric Assay

**Apparatus**—Beckman model DU spectrophotometer: 1-cm. matched cells were used.

**Reagents**—Nitrogen, research grade; iodine, A.R.; glacial acetic acid, A.R.; chloroform, A.R.;

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