# ASSAY OF DIGITALIS IN PIGEONS BY INTRAPERITONEAL ADMINISTRATION

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A new biological method of assay for digitalis is proposed. Preparations are administered intraperitoneally and the LD50 estimated by means of the "up-and-down" procedure. The LD50 of the preparation examined is compared with the LD50 of the standard preparation, and the result expressed in international units. The proposed method gives more evidence on the loss in potency of the drug than does the intravenous method in pigeons (U.S.P. XVI).

ONE of the main disadvantages of the biological methods for the assay of digitalis is that in cats, guinea-pigs and pigeons the preparations are injected intravenously, whereas clinically they are usually administered by mouth. The activity of glycosides and aglycones introduced directly into the blood is known to differ from that obtained by oral administration. This is because the pathways of absorption by the oral route are by-passed when the drug is given intravenously. Further, some interfering substances from the crude drug, introduced directly into the blood, can influence the activity of glycosides (Hildebrand, 1954) and thus give results different from those obtained by oral administration, where these substances, passing through the gastrointestinal tract do not enter the blood in the same form or in the same quantity.

In the biological assay of digitalis oral administration does not yield constant results as it is greatly influenced by the functional state and content of the digestive tract. Because of the emetic reflex of some species of laboratory animals the oral route is unsatisfactory. Although rats, mice and guinea-pigs do not exhibit this reflex they are unsuitable for this purpose on account of their very high resistance to the digitalis glycosides when given orally. Certain glycosides administered in this way cannot even kill some rodents (for example, strophanthin in rats, Hatcher, 1908). Because of these disadvantages oral administration seems to be generally abandoned in the assay of digitalis (Weese, 1936). Subcutaneous administration of digitalis to warm-blooded laboratory animals appears to be unsuitable since the results obtained are often inconsistent (Weese, 1936).

The overnight frog method, however, partly overcomes the disadvantages of intravenous administration, since the preparations are injected into the lymph sac where conditions for absorption resemble more closely those for oral administration. For this reason the frog method offers more information, with glycoside preparations which have undergone decomposition (Petričić, 1955). However, the main disadvantage of the frog method is that it requires a large number of animals to obtain satisfactory limits of error on the assay (Miles and Perry, 1950).

In order to overcome all these difficulties we chose pigeons as experimental animals because in the intravenous assay of digitalis (Braun and Lusky, 1948), they give results of greater precision than those given by other species of laboratory animals.

In our experiments preparations have been administered intraperitoneally. The LD50 has been determined for both the standard and the test preparations using the procedure of Dixon and Mood (1948). This concentrates testing around the LD50, thereby increasing the accuracy of the estimation. Under these experimental conditions the results obtained have satisfactory limits of error.

## EXPERIMENTAL

Adult pigeons of either sex, showing no evident signs of illness, obesity or emaciation and weighing from 250 to 450 g. are used. The animals are divided into two similar groups, according to their weight. One group is used for testing the standard preparation, and the other the sample under examination. The preparations are administered intraperitoneally. Food, but not water, is withheld for about 12 hr. before the assay.

The approximate LD50 and its standard deviation is previously determined. The tincture or glycoside solution, is diluted with physiological saline, in such a way that the animals get from 1 to 6 ml./kg. weight. The doses selected for the assay have an equal logarithmic interval between them (see Table I). If the animal survives the dose given,

TABLE I

EXAMPLE OF THE "UP-AND-DOWN" PROCEDURE FOR THE ASSAY OF DIGITALIS IN PIGEONS BY THE INTRAPERITONEAL METHOD

| Volume of diluted tincture injected (ml./kg. of body wt.) | Calculated amount of undiluted tincture administered (ml./kg. of body wt.) | Log. ml./kg. of undiluted tincture injected | Results | | | | | | | | | | | | Frequency | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | | | | | x's | o's |
| 3·59 | 0·287 | $\bar{1}$·458 | x | | | | | | | | | | | | 2 | 0 |
| 2·97 | 0·239 | $\bar{1}$·378 | | x | | x | | x | | | o | | x | | 4 | 1 |
| 2·48 | 0·199 | $\bar{1}$·298 | | | o | | o | | x | | | o | | o | 1 | 4 |
| 2·06 | 0·165 | $\bar{1}$·218 | | | | | | | | o | | | | | 0 | 1 |

x = animal died. o = animal survived. Log. dose ratio = 0·08.

the subsequent higher dose is injected into the next pigeon, whereas if the animal dies the subsequent smaller dose is given, according to the procedure described in detail by Kimball, Burnett and Doherty (1957). The result is observed after 24 hr. The LD50 obtained for the test preparation is compared with that of the standard preparation and the potency expressed in international units. For digitalis leaf and its tinctures the International Standard of Digitalis is used, whereas for the assay of digitoxin the Digitoxin Authentic Chemical Substance issued by Apotekens Kontrollaboratorium, Stockholm, is used as the reference standard.

A detailed description of the toxicity test as well as the table of the "G" values is given by Kimball, Burnett and Doherty (1957) and a discussion on the statistical analysis of the method is given by Dixon and Massey (1957).

A suitable dilution for the tincture is between 1 : 8 to 1 : 12 with physiological saline. Digitoxin is dissolved in 6 to 8 ml. of 96 per cent ethanol

and then diluted with saline to give a solution containing about 6 mg./100 ml. The convenient logarithmic interval of the doses was found to be from 0·05 to 0·15. Under these conditions this interval is generally lower than two standard deviations, and $NB-A^2/N^2$ is generally higher than 0·3. This satisfies the criteria for the simplified statistical analysis and gives satisfactory limits of error (Dixon and Massey, 1957).

Generally one preliminary test for each kind of preparation is sufficient to obtain information on the logarithmic dose interval, *d*.

## Results and Discussion

The reproducibility of the procedure has been previously proved by repeating the test on some smaller groups of animals of approximately equal weight. Thereafter, the same concentration of digitoxin (3 mg. per cent) has been administered to eight groups of pigeons each of various

TABLE II

Estimations of the LD50 of one preparation (tincture)

| LD50 ml./kg. | Limits of error per cent (P = 0·95) | Total number of animals used |
|---|---|---|
| 0·24 | 92·1–108·6 | 21 |
| 0·23 | 81·1–123·5 | 10 |
| 0·26 | 85·0–117·4 | 13 |
| 0·23 | 95·9–104·2 | 28 |
| 0·23 | 95·6–104·5 | 24 |

weight to establish whether there was a correlation of dose and body weight. The results obtained showed a statistically significant correlation ($r = 0{\cdot}907$, $P < 0{\cdot}01$). Because of the correlation of LD50 and body weight doses were given in proportion to body weight in all subsequent assays.

The estimation of the LD50 of the same preparation was repeated under the same experimental conditions in five groups of pigeons. The results (Table II) show that good reproducibility and satisfactory limits of error were obtained.

TABLE III

Comparison of the loss in potency of a sample of digitalis as shown by the intravenous and intraperitoneal methods

| | Potency (Intravenous method) | Limits of error per cent (P = 0·95) | No. of animals per group | Potency (Intraperitoneal method) | Limits of error per cent (P = 0·95) | No. of animals per group |
|---|---|---|---|---|---|---|
| Sample of powdered digitalis leaf | 1 | 95·5–104·7 | 12 | 1 | 86·1–116·0 | 21 |
| Above sample mixed with water and dried at room temperature | 0·48 | 92·7–107·9 | 6 | 0·26 | 81·7–122·3 | 19 |

(The toxicity of the sample of digitalis before treatment is calculated as one in both methods used).

Petričić (1955) proved that the frog method as well as the intravenous method in pigeons are satisfactory procedures for determining the loss

in potency of the drug. To examine the proposed intraperitoneal method for this purpose, a sample of powdered digitalis leaf was mixed with two volumes of water and the liquid allowed to evaporate at room temperature (about 2 weeks). From the dry residue obtained a tincture was prepared and assayed by the intravenous and the proposed intraperitoneal methods. The results compared with those obtained for the untreated sample, are shown in Table III.

The ageing of the tincture was studied by both methods and the results obtained are shown in Table IV.

TABLE IV

COMPARISON OF THE LOSS IN POTENCY OF A DIGITALIS TINCTURE AFTER 9 MONTHS STORAGE AS SHOWN BY THE INTRAVENOUS AND INTRAPERITONEAL METHODS

| | Potency (Intravenous method) | Limits of error per cent ($P = 0.95$) | No. of animals per group | Potency (Intraperitoneal method) | Limits of error per cent ($P = 0.95$) | No. of animals per group |
|---|---|---|---|---|---|---|
| Fresh tincture .. .. .. | 1 | 95·5–104·7 | 12 | 1 | 86·1–116·0 | 21 |
| Same tincture after 9 months storage .. .. .. | 0·79 | 93·6–106·8 | 12 | 0·36 | 87·5–114·4 | 13 |

(The toxicity of the fresh tincture is calculated as one in both methods used.)

As shown in Tables III and IV the proposed method illustrates better the extent of the loss in potency of digitalis and its tincture than does the intravenous method. (In all intravenous experiments results were statistically treated according to B.P. 1953.)

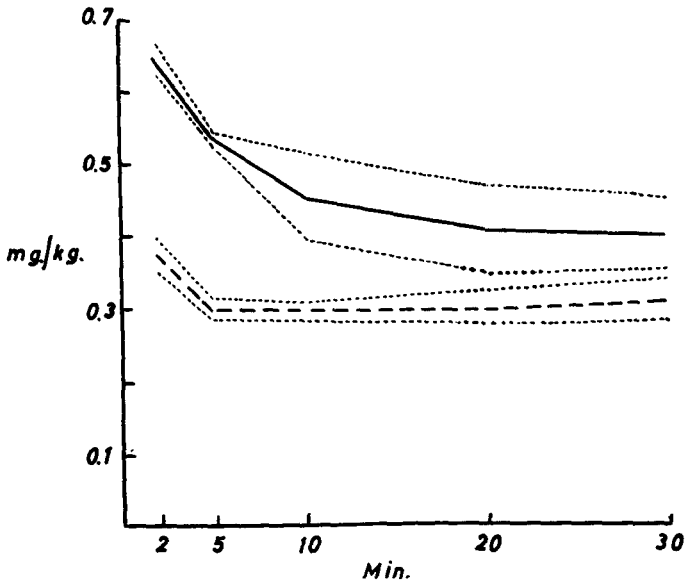


FIG. 1. Relationship of time interval between the intravenous dose of glycoside and the lethal dose in pigeons. Solid line, digitoxin, broken line, lanatoside C. Limits of error are indicated by dotted lines.

The activity of pure glycosides is generally more predictable than that of digitalis which contains a mixture of glycosides and interfering substances. For this reason the activity of two pure glycosides, digitoxin and lanatoside C has been determined by both methods. Digitoxin has marked cumulative properties and acts slowly whereas lanatoside C is only slightly cumulative and acts rapidly.

As can be seen from Table V the same relationship of potency for both glycosides has not been obtained by means of both methods, since the ratio of the lethal dose of digitoxin to that of lanatoside C obtained by the intravenous method is greater than that obtained by the intraperitoneal method. The results shown in Fig. 1 offer an explanation for this effect.

TABLE V

COMPARISON OF RESULTS OBTAINED BY THE INTRAVENOUS AND INTRAPERITONEAL METHODS FOR TWO PURE GLYCOSIDES

| | Intravenous lethal dose mg./kg. | Limits of error per cent (P = 0·95) | Intraperitoneal LD50 mg./kg. | Limits of error per cent (P = 0·95) |
|---|---|---|---|---|
| Digitoxin .. .. .. | 0·54 | 99·1–101·0 | 0·32 | 90·1–111·0 |
| Lanatoside C .. .. | 0·30 | 94·7–105·6 | 0·22 | 91·5–109·4 |

The lethal dose of digitoxin and lanatoside C has been determined using five groups of pigeons for each preparation, each group containing 12 to 24 animals. The intravenous method was used, but the time interval between single doses varied for each group thus: 2, 5, 10, 20 and 30 min.

From Fig. 1 it is evident that the 5-min. interval (U.S.P. XVI) is sufficient for each dose of lanatoside C to exert its full effect, since after increasing the interval between doses up to 30 min. no further change in lethal dose was observed. With digitoxin, increasing the interval between doses produced a gradual decrease of the lethal dose such that at the 30-min. interval the lethal dose was reduced to 0·40 mg./kg.

The ratio of the intravenous lethal dose (5-min. dose-interval) to the intraperitoneal LD50 is 1·7 for digitoxin and 1·4 for lanatoside C (Table V). However in the case of digitoxin this ratio is reduced to 1·3 if the intravenous lethal dose (0·4 mg./kg.) obtained after injecting doses every 30 min. (Fig. 1) is used.

This suggests that with the intravenous method as recommended in the U.S.P. (XVI) the lowest mean lethal dose for some glycosides is not always obtained. The values for preparations exhibiting a slow onset of action, like digitoxin, are too high.

The choice of the method would be of no special importance if the sample under examination were compared with a standard preparation of the same composition, and the relative potency expressed in terms of this standard. However, this is not usually the case. Various crude preparations often show considerable differences in their chemical composition and in the quantities of glycoside present, and consequently great differences in potency and rate of action are found. The greatest differences are observed with *Digitalis purpurea*, the drug most frequently

assayed. If the standard and the test preparations show considerable differences in the composition of glycosides, and particularly if one of them contains a high percentage of digitoxin, and the other, more glycosides having a rapid action (for example, gitaloxin), the results obtained might be unreliable as the potency of the tested sample might be over or underestimated. This is confirmed in Table VI where results of testing six various samples of digitalis leaf (tincture) are given.

TABLE VI

COMPARISON OF RESULTS OBTAINED BY THE INTRAVENOUS AND INTRAPERITONEAL METHODS FOR SIX DIFFERENT SAMPLES OF DIGITALIS LEAF (FRESH TINCTURE)

| Preparation | Intravenous (I.V.) lethal dose ml. tinct./kg. | Limits of error per cent (P = 0·95) | Intraperitoneal (I.P.) LD50 ml. tinct./kg. | Limits of error per cent (P = 0·95) | Ratio LD (I.V.) : LD50 (I.P.) | Relative potency in relation to International Standard (Int. Std. = 1) | |
|---|---|---|---|---|---|---|---|
| | | | | | | I.V. | I.P. |
| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Bilj.236 | 0·69 | 96·5–103·5 | 0·33 | 88·1–113·5 | 2·10 | 1·07 | 0·94 |
| A | 1·37 | 97·4–105·3 | 0·52 | 82·5–121·3 | 2·63 | 0·54 | 0·60 |
| C | 1·07 | 97·6–102·4 | 0·61 | 91·4–109·3 | 1·75 | 0·69 | 0·51 |
| J | 0·95 | 97·8–102·2 | 0·88 | 74·9–133·5 | 1·08 | 0·78 | 0·35 |
| L | 0·70 | 95·7–104·3 | 0·31 | 84·0–119·1 | 2·26 | 1·06 | 1·00 |
| JS | 0·67 | 97·4–102·6 | 0·22 | 83·3–120·1 | 3·05 | 1·11 | 1·41 |

$$\text{Column 7} = \frac{\text{I.V. potency of the Digitalis International Standard}}{\text{I.V. potency of the tested sample}}$$

$$\text{Column 8} = \frac{\text{I.P. potency of the Digitalis International Standard}}{\text{I.P. potency of the tested sample}}$$

The results from assaying several samples of digitalis leaf and tinctures (Table VI) show that there is no constant ratio between the potencies obtained by the intravenous and intraperitoneal methods since the ratio varies between 1·08 and 3·05.

Further, the ratio of the highest and lowest result obtained by the intravenous method is approximately 2, while by the intraperitoneal method it is 4.

Taking into account the limits of error obtained the intravenous method shows significantly smaller differences between the potencies of tinctures than does the proposed intraperitoneal method.

On the basis of the results from the present study it may be concluded that the proposed intraperitoneal method is more suitable than the intravenous method for the assay of most of the preparations used clinically.

The proposed intraperitoneal method, however, requires more animals for each assay, and is more time consuming, because of the sequential design of the assay, than the intravenous method.

As the potency of some tinctures decreases rapidly on storage (Kuševič and Porges, 1956) prolonged assays should be avoided by carrying out several sequences of the test simultaneously and combining their results.

The experimental design described by Brownlee, Hodges and Rosenblatt (1953) can be applied when rapid results are required.

The activity of some digitalis preparations should be determined simultaneously in man and by the proposed intraperitoneal method to asses the suitability of the latter for the routine assay of digitalis.

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