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Comparison of the One- and Eighteen-Hour Frog Method for the Assay of Digitalis

*By C. W. Chapman**

At a conference of those interested in the problems involved in the biological assay of digitalis held at the Atlanta meeting of the AMERICAN PHARMACEUTICAL ASSOCIATION, 1939, representatives of several laboratories agreed to take part in a collaborative study. The chairman of the U. S. P. Revision Committee undertook to supply the digitalis required for this work. It was suggested that the potency of every lot of digitalis obtained by him be tested biologically before being mixed or treated in any manner for distribution to the collaborating laboratories. Dr. Lloyd C. Miller, of the Food and Drug Administration, and Dr. C. W. Chapman, of the University of Maryland, volunteered to perform these preliminary assays on samples submitted by the chairman of the Revision Committee. The potency of these samples in terms of International Standard, 1936, and the methods used to determine these values are given in this paper.

EXPERIMENTAL

Methods.—Dr. Miller employed the U. S. P. one-hour frog method designing each assay and calculating results by the procedure of Miller, Bliss and Braun (1). The eighteen-hour or over-night method reported by Chapman and Morrell (2) was used by Dr. Chapman. The essential differences in these two methods are:

(*a*) The absorption period in the one-hour procedure is exactly one hour in contrast to the longer period of eighteen hours or "over-night" absorption time of the other method.

(*b*) The end-point in the one-hour method is the condition of the frog's heart (auricles dilated and in standstill, ventricle in systolic standstill). In the over-night method, all dead animals as determined by tactile stimuli are recorded as reacting positively.

(*c*) The method of Miller, Bliss and Braun (1) requires for each substance injected, "standard" or sample, that three different doses be injected into each of three groups of frogs. In the Chapman and Morrell procedure (2) a single dose of "standard" is injected into one group of frogs and one dose of sample into another group of frogs.

(*d*) In the Miller, Bliss and Braun (1) method the slope of the dosage-effect curve *b* is determined in each assay for "standard" and "unknown." This involves the statistical calculations given by these authors. In the single-dose method the slope of the dosage-effect curve is assumed to be constant or, using Trevan's (3) description, "characteristic" for this particular assay procedure. The characteristic curve referred to in this work is that reported by Chapman and Morrell (4) in 1931, for ouabain, not digitalis. By assuming a constant, *b*, or slope for the dosage-effect regression line the

* School of Pharmacy, University of Maryland, Baltimore, Maryland.

calculation of the relative potency of a sample of digitalis in terms of a reference standard is extremely easy, involving only a simple proportion.

Standard of Reference.—The International Standard digitalis powder, 1936, supplied by the chairman of the U. S. P. Revision Committee was used for each comparison. A solution containing one International Unit per cc. was prepared by macerating the contents of one ampul of standard (approximately 3 Gm.) with U. S. P. menstruum (approximately 76% ethanol) in the proportion of 0.08 Gm. of standard to each cc. of menstruum and centrifuging. This standard was injected simultaneously with each sample.

Results.—The potency of each sample in terms of International Units per Gm. are compiled in Table I for both one-hour and eighteen-hour methods. It should be emphasized that these figures are the result of routine assays and do not involve the use of a prohibitive number of animals.

Table I.—Digitalis Assays

No. of Sample	Description of Sample	International (1936) Units per Gm. One-Hour Method	International (1936) Units per Gm. Eighteen-Hour Method
A 501-11	Powder	16.3	16.4
B 505-12	Powder	14.2	13.4
C 739	Powder	12.0	11.0
D 509-7	U. S. P. pdr.	15.3	15.2
E 503-5	I. S. D. pdr.	13.3	12.5
F 504-14	Leaf	11.2	8.3
G 510-15	Leaf	11.8	15.8

DISCUSSION

The agreement between the potency of the first five samples (*A*, *B*, *C*, *D*, *E*) as determined by both one-hour and eighteen-hour methods is very good. The maximum variation from the official one-hour method is approximately 5%, which is well within the error to be expected from the standard deviation of the results. Samples *F* and *G* were whole- or crushed-leaf specimens. The agreement between the assays for these is not as close as for the other samples. This is quite possibly due to the difficulty in properly sampling a crude drug which consists of whole leaves or parts of large dimensions. It emphasizes the fact that much care must be exercised in taking such samples.

Sample *E* is a sample of the 1936 International Standard which was identified only by a number and included with the other batches to be assayed against another portion of this same standard. The labeled potency of this standard is 12.5 International Units per Gm. (one International Unit = 0.08 Gm.). By the eighteen-hour method the determined potency was exactly 12.5 units, by the one-hour method 13.3 units. These values are identical within the limits of error of the method and are the same as the labeled potency for this standard.

Sample *D*, the current U. S P. Reference powder possesses 15.25 International (1936) Units per Gm. determined by taking the average of both methods, 15.3 and 15.2 units. One gram of the U. S. P. Reference powder is equivalent to 15.25 International Units when the International (1936) Standard is considered to possess one International Unit per 0.08 Gm., or 0.0656 Gm. U. S. P. Reference Standard contains 1 International (1936) Unit. Both one-hour and eighteen-hour methods give this equivalent. This is not in accord with the official factor of 0.0745 which appears on the label of the U. S. P. Reference Standard. Referring to the official one-hour and the "over-night" methods, Edmunds, Moyer and Shaw (5) state that "with different periods of observation the potencies of different powders in relation to a standard vary considerably." The results reported here do not confirm this statement. Both methods give the same relationship within the limits of error for each of the powders assayed (*A*, *B*, *C*, *D*, *E*) in comparison with the International (1936) Standard.

It can be argued that the characteristic curve for the reaction of frogs to digitalis will vary from time to time or from one batch of frogs to another when every precaution is taken to keep the conditions of assay constant. There is also the possibility that samples of digitalis are from different sources, of different age or prepared by different methods. This may result in significantly different values for *b* when tested under identical conditions. Calculations for the one-hour method (1) used in this work include the determination of *b* for every assay in order to eliminate any of these possible variations. However, that the introduction of a predetermined characteristic curve or *b* value does not affect the results appreciably is demonstrated in the eighteen-hour method results. Elimination

of the determination of b greatly simplifies the calculations. If a standard or characteristic curve, such as that employed in this work, can be used in the assay of digitalis (4) it is to be preferred because of the simplicity in performing an assay and calculating results.

CONCLUSION

1. Seven samples of digitalis, powder and leaf, are compared by the one-hour and eighteen-hour methods. Good agreement was observed.

2. The U. S. P. Reference powder possesses 152.5% of the potency of the 1936 International Standard when the latter is considered to possess an activity of one International Unit per 0.08 Gm.

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Progress Report on the U. S. P. (1939-1940) Digitalis Assay Study*

By Lloyd C. Miller

As a result of a conference on the Assay of Digitalis held in connection with the Atlanta Meeting of the AMERICAN PHARMACEUTICAL ASSOCIATION, a collaborative study of the assay of digitalis using frogs has been in progress during the past year. The program is a U. S. P. project made possible by the coöperation of the participating laboratories. As a consequence of my offer to calculate and compile the results of the study, it now falls to my lot to serve as a narrator in presenting a summary of the progress to date.

* Report prepared at the request of Dr. C. W. Chapman, Chairman, A. PH. A. Committee on Physiological Testing, with the approval of Dr. E. E. Nelson, Chairman, Committee on U. S. P. (1939–1940) Digitalis Assay Study.

EXPERIMENTAL

The conference decided that the U. S. P. reference digitalis powder was unsuitable for use in this collaborative program and requested Professor Cook to collect immediately samples of both domestic and imported digitalis leaves of good quality to be composited as study material for this project. As a result 110 lb. were collected from various sources and upon the very prudent suggestion of Dr. C. W. Chapman, who had had considerable experience in compositing the present Canadian digitalis standard powder, each of these samples of leaves was subjected to bioassay. The findings in these assays have just been reported by Dr. Chapman.

It was the consensus of the Atlanta conference that probably the most important issue was the question of the alleged superiority of the so-called "over-night" frog method over the official U. S. P. one-hour method. Of interest also was the most suitable means of preparing an extract of a standard powder prior to assay, *i. e.*, a hot exhaustive extraction as compared with a cold maceration procedure. Then and in subsequent correspondence the cat method was considered, but it was decided finally to seek to perfect one method at a time.

It was felt that the details of preparing a macerate could be specified rigidly enough to insure a preparation sufficiently uniform to enable a valid comparison between the one-hour and eighteen-hour methods. It is to be noted that the term "eighteen-hour" was applied to designate the so-called "over-night" method, a decision which was indicative of the resolve of the conference to make a fresh start. It was freely expressed at the conference that great care should be exercised toward devising a workable and well-defined plan for the assays. It may now be said that this desire was thoroughly justified.

THE FIRST COMPARISON

It was decided that the program should be made up of a series of what may be termed major comparisons, each of which would bear directly on a well-defined and vital phase of the problem. Thus the first comparison was designed to determine (1) the relative merits of the one- and eighteen-hour methods when conducted under as nearly identical conditions as practicable and (2) the practicability of diluting a standard powder with exhausted marc. Toward this end two samples of digitalis powder, Samples 1 and 2, were submitted to nearly 20 laboratories. One of these powders was a dilution of the other, the diluent being thoroughly exhausted marc (the inactivity of which was proved biologically) in a proportion known to none of the collaborators. Each collaborator was requested to determine the relative potency of these two samples by both the one- and eighteen-hour methods under conditions quite rigidly specified.

The data for the one-hour and eighteen-hour methods were compared on the basis of four criteria which were proposed in a paper presented to the