5. A sufficient number of assays should be made on any unknown sample to definitely determine its potency.

CONCLUSION.

By careful attention to details, ergot and its preparations may be satisfactorily assayed by the cockscomb method outlined in U. S. P. X.

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THE COLORIMETRIC ASSAY OF DIGITALIS.*

BY L. W. ROWE.

Several years have passed since Knudson and Dresbach (1) suggested the use of the Baljet (2) reaction for the colorimetric assay of digitalis preparations. Their work on about 30 preparations of digitalis indicates that the colorimetric method is about as accurate as the Hatcher cat method (3) and checks with the cat method very closely. Only two of the preparations were tested by the U. S. P. frog method. Kruse (4) has reported on the subject and finds the colorimetric method suitable for the determination of total activity but not for activity developed after absorption. He believes that the frog method is most suitable for the standardization of digitalis.

Although the M. L. D. frog heart method of Houghton (5) has been successfully used in our laboratory for standardizing digitalis preparations for more than thirty years, it seemed advisable to try out the colorimetric method because if it is sufficiently accurate it would reduce the time and expense of each assay.

Accordingly experiments were begun and direct comparisons were made between results obtained using the technique of Knudson with a Klett constant light colorimeter and those obtained at about the same time with the M. L. D. frog method. The first series was based on the Hatcher standard since it was known from previous comparisons that 8 heart tonic units of the frog test were about equal to 1 cat unit.

TABLE I.

Preparation and number.	Color test.	Frog test.	Color error.
Tr. Digitalis 768526	200%	105%	48% high
Tr. Digitalis 765576	220%	90%	60% high
Tr. Digitalis 766998	210%	90%	57% high
Tr. Digitalis 157585	200%	100%	50% high
Tr. Digitalis 767577	240%	110%	54% high
Tr. Digitalis 767577 (fortified)	250%	150%	40% high

In this series results were calculated from readings around 10 against 20 for the standard and also in some cases using 2 cc. of the sample instead of the usual 5 cc. It was found that results were particularly high by the color method when 2, 3 and 4 cc. of samples of tinctures were used with proportionate amounts of purifying reagents so this feature was abandoned.

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The artificial standard suggested by Knudson (a solution of pure potassium dichromate 3.44 Gm. per liter) was tried out very thoroughly and was discarded because the quality of color was appreciably different from that developed in the purified digitalis solutions and was consequently more difficult to match.

In the second series of tests the Knudson ouabain standard (0.266 mg. per 5 cc.) was used against our bio-assay standard established many years ago of 65 H.T.U. per Gm. of digitalis leaves and results were more comparable though quite erratic.

TABLE II.

Preparation and number.	Color test.	Frog test.	Color error.
F. E. Digitalis 158274	130%	120%	10% high
Tr. Digitalis 770221,	130%	150%	15% low
Digitalis Sol. Experiment	87%	150%	72% low
Tr. Digitalis 761298	136%	110%	25% high
Tr. Digitalis 763831	140%	130%	7% high
Tr. Digitalis 766793	185%	180%	3% high
Tr. Digitalis 768303	100%	165%	65% low
Tr. Digitalis 157036	162%	165%	2% low
Tr. Digitalis 768138	62%	110%	77% low
Tr. Digitalis from drug	154%	200%	30% low
Tr. Digitalis 764044 (with)	140%	110%	20% high
Tr. Digitalis 764044 (without)	120%	110%	10% high
Tr. Digitalis Composite (five)	119%	129%	8% low
Tr. Digitalis Composite (five)	130%	126%	3% high
Tr. Digitalis 158542	106%	100%	6% high
Tr. Digitalis 772170	187%	165%	12% high
Digitalone Experiment	123%	110%	12% high
Tr. Digitalis 158545	106%	100%	6% high
Digitalin 301470	46%	44%	4% high
Tr. Digitalis C-71	560%	500%	10% high
F. E. Digitalis 131845	118%	88%	25% high
F. E. Digitalis 772944	157%	90%	42% high
Digitalone Experiment	154%	150%	3% high

In this second series the average colorimetric test error was lower largely because of the inconsistency of the two standards. The color standard of 0.266 mg. ouabain per 5 cc. equivalent to the Hatcher cat method standard was used against the M. L. D. frog method standard although it was known from previous comparisons of the two bio-assay methods and standards that the frog method standard was about 25% lower than the Hatcher standard. The results in this series were erratic as shown by the fact that the extremes were from 77% low to 42% high. The average variation was 20% while if the 5 results where the variation was 30% or over are left out the average variation is only 10% which would be very good considering the experimental error of the bio-assay method if it were not for the known discrepancy between the two standards. Variations in the technique were used in this series including the use of the potassium dichromate standard which was found unsatisfactory.

In the third series the technique was standardized to the point where comparative readings of the standard and sample were always made 20 minutes after the color of the final mixture started to develop since this was found to be the better procedure. The color will change appreciably after 20 minutes but the

change between the standard and sample is not as nearly proportional as it is up to 20 minutes. Also in this series each assay was checked once or twice by diluting the sample on the basis of the first assay and testing the dilutions until one was found which averaged between 19 and 21 for three readings with the standard set at 20. Work on a suitable standard was continued by using solutions of digitalin and digitoxin. It was found that a particular lot of German digitalin which was 50% of standard by the frog method was equal to the 1 to 25,000 solution of standard ouabain when dissolved 1 part to 2500 in distilled water. A standard digitalin could therefore be diluted 1 to 5000 and ouabain would only be 5 times as active as digitalin by the color method. By the frog method the ratio is 100 to 1, so this indicates that the digitalin is unsuitable as a color standard but because of its solubility in water and its highly purified condition it was used in this series. Digitoxin, the chief active principle of digitalis leaves was also used as a standard. Two different lots were used and they were found to be quite inconsistent by the frog and color methods. One sample No. 45,125 was found to contain 11,000 H.T.U. of activity per Gm. by the frog test and was consequently eleven times as active as the digitalin with which it was compared. By the color test a 1 to 16,000 dilution was equal to the 1 to 2500 dilution of digitalin showing that it was but 6.4 times as active. Another sample No. 45,805 was found to contain 9000 H.T.U. per Gm. and was thus nine times as active as the digitalin. By the color test a 1 to 22,500 dilution was equal to the 1 to 2500 dilution of digitalin being thus nine times as active and checking with the frog test. Digitoxin should be about 5 times as active as standard digitalin or 10 times as active as the 50% digitalin which was being used. The insolubility of digitoxin in water, the inconsistency of different samples in activity by the same and by different methods, and the fact that it is but one of the active principles of digitalis leaves (even though the most important) are sufficient reasons for its being unsuitable as a standard.

TABLE III.

Preparation and number.		Standard.	Color test.	Frog test.	Color error.
Tr. Digitalis Rx	12284	Digitalin	220%	180%	18% high
Tr. Digitalis	776431	Digitalin	200%	150%	25% high
F. E. Digitalis	766757 (Stock)	Digitalin	115%	110%	4% high
Digitalin Tabs.	777570	Digitalin	125%	75%	40% high
Tr. Digitalis	776113	Digitalin	200%	150%	25% high
Tr. Digitalis	777274	Digitalin	200%	150%	25% high
S. E. Digitalis	778267	Digitalin	135%	165%	15% low
Tr. Digitalis	159949	Digitalin	220%	175%	16% high
Digitalin	304090	Digitalin	70%	35%	50% high
Tr. Digitalis	773516	Digitalin	220%	150%	32% high
Tr. Digitalis	777275	Digitalin	200%	150%	25% high
Tr. Digitalis	777699	Digitalin	240%	165%	31% high
Tr. Digitalis	2748123	Digitalin	200%	165%	18% high
Digitalone	075566	Digitalin	160%	140%	12% high
Digitalin	49929	Digitalin	30%	35%	16% low
Digitalin	49921	Tr. Stroph.	40%	30%	25% high
F. E. Digitalis	777259	Digitalin	135%	95%	30% high
Tr. Digitalis	776558	Digitalin	200%	200%	.....
Tr. Digitalis	12630	Digitalin	250%	225%	10% high

Table III gives the comparative results on this series showing that with the lower standard the results by the color method are invariably high.

The average variation on these 19 tests was 23% including the two where the color test was low. If these two are excluded the average color test error is 24% high or nearly one fourth. It was decided on the basis of this series that digitalin was not a satisfactory standard, though the fact that it was soluble in water, could be used in so dilute a solution that it did not need to be purified and was more representative of complete digitalis activity than any one principle such as digitoxin, appealed strongly in its favor.

The fourth and final series of tests of samples of regular manufactured lots was more extensive than any others and differed from the third series only in the standard used. A carefully standardized tincture of strophanthus (5%, U. S. P. 1890) which had been used as the standard in testing by the frog method was care-

TABLE IV.

Preparation and number.		Standard.	Color test.	Frog test.	Color error.
Digitalone	078634	Tr. Strophanthus	250%	125%	50% high
Tr. Digitalis	779837	Tr. Strophanthus	225%	100%	56% high
Digitalin	20786	Tr. Strophanthus	80%	40%	50% high
Tr. Digitalis	779788	Tr. Strophanthus	200%	150%	25% high
Digitalin	305382	Tr. Strophanthus	40%	30%	25% high
F. E. Digitalis	777259	Tr. Strophanthus	200%	135%	33% high
Tr. Digitalis	160483	Tr. Strophanthus	175%	140%	20% high
Tr. Digitalis	780725	Tr. Strophanthus	200%	120%	40% high
S. E. Digitalis	781724	Tr. Strophanthus	175%	155%	11% high
F. E. Digitalis	133524	Tr. Strophanthus	160%	150%	6% high
Tr. Digitalis	160482	Tr. Strophanthus	250%	175%	30% high
Tr. Digitalis	2709495	Tr. Strophanthus	250%	100%	60% high
Tr. Digitalis	781918	Tr. Strophanthus	220%	150%	32% high
Digitalone	079076	Tr. Strophanthus	120%	70%	40% high
F. E. Digitalis	160569	Tr. Strophanthus	235%	165%	30% high
Digitalin "A"	.....	Tr. Strophanthus	110%	65%	40% high
Digitalone	079076	Tr. Strophanthus	330%	120%	64% high
Tr. Digitalis	781277-8	Tr. Strophanthus	175%	120%	31% high
Tr. Digitalis	779652	Tr. Strophanthus	150%	110%	27% high
Digitalin "B"	.....	Tr. Strophanthus	90%	40%	55% high
Tr. Digitalis	160481	Tr. Strophanthus	175%	165%	5% high
F. E. Digitalis	780569	Tr. Strophanthus	180%	120%	33% high
Tr. Digitalis	4969	Tr. Strophanthus	160%	135%	16% high
Tr. Digitalis	781829	Tr. Strophanthus	165%	120%	27% high
Tr. Digitalis	783423	Tr. Strophanthus	220%	155%	30% high
S. E. Digitalis	783370	Tr. Strophanthus	220%	200%	10% high
S. E. Digitalis	783718	Tr. Strophanthus	160%	145%	10% high
Tr. Digitalis	161033	Tr. Strophanthus	135%	120%	11% high
Tr. Digitalis Crude drug (stab.)		Tr. Strophanthus	220%	165%	25% high
Digitalin "C"	.....	Tr. Strophanthus	100%	38%	62% high
Tr. Digitalis	161028	Tr. Strophanthus	220%	110%	50% high
Digitalone	079836	Tr. Strophanthus	330%	150%	55% high
Digitalis leaves	784345	Tr. Strophanthus	230%	120%	48% high
Dig. Powd.	783718	Tr. Strophanthus	250%	220%	12% high
Tr. Digitalis	782166	Tr. Strophanthus	300%	110%	63% high
P. E. Digitalis	2760232	Tr. Strophanthus	135%	100%	26% high
Tr. Digitalis Crude drug		Tr. Strophanthus	280%	190%	32% high

fully compared with the 1 to 25,000 solution of U. S. P. ouabain and the 1 to 2500 solution of 50% digitalin and found to be equal to them when diluted 1 to 70. This Tr. Strophanthus 1 to 70 was used as the standard without purification in this entire series.

The average color test variation in this series of 37 tests was 32% high with the extremes ranging from 5% to 64%. If the average variation was much more uniform a correction factor could be used but there are about as many samples that are 5% to 20% high as there are that are 45% to 60% high. No further variations in the technique suggested themselves so it was decided that the total activity and that absorbed by the frog in the frog method might not be proportional in different samples.

The accuracy of the color method was further tested, however, by making up dilutions of various preparations that had previously been tested by both methods and the dilutions of which were unknown to the technician making the test. These results were also erratic as shown by the following short table:

TABLE V.

Sample.	Found.	Actually.	Error.
Digitalin, 50%	Dil. 1 to 575	1 to 520	10% high
Tr. Digitalis 176% of Standard	110%	88%	20% high
Digitalin 50%, 1 to 500 sol.	Dil. 1 to 2.27	1 to 2.67	15% low
Tr. Digitalis 200% of Standard	Dil. 1 to 1.9	1 to 1.78	7% high
Digitalin 50%, 1 to 500	Dil. 1 to 2.16	1 to 2.67	19% low

Variations from 15% low to 20% high are too great to compare favorably with the accuracy of the M. L. D. frog method as frequently determined in this laboratory by tests of unknowns, some results being published in 1919 (6) in connection with a consideration of the cat and frog methods of assay.

SUMMARY.

The picric acid colorimetric method for digitalis assay proposed by Knudson was used on a series of nearly 100 samples of digitalis preparations including several dilutions that were unknown to the person making the tests. The original technique and standards as suggested were used and comparative results with those of the M. L. D. frog method were entirely too high. The potassium dichromate standard was dropped because of a distinct difference between the quality of its color and that developed in the samples. The ouabain standard was lowered from 0.266 mg. per 5 cc. to 0.20 mg. per 5 cc. and the average error was considerably reduced but the extremes were still too far apart. German digitalin and samples of digitoxin were also tried as standards and discarded because the color tests of different lots did not correspond with the activity by the frog method. Ouabain solutions were rather unsatisfactory because the rate of development of color was not proportional to that for digitalis. Readings at 20 minutes after mixture with the picric acid reagent seemed to be best but readings of standard and sample at 15 and 25 minutes after gave different results. A carefully stand-

ardized tincture of strophanthus representing a 1 to 20 extract of average drug was thoroughly tried as a standard, a 1 to 70 dilution being equivalent to the 1 to 25,000 dilution of U. S. P. ouabain. This was the most satisfactory of all the standards employed as it was a stable solution which did not need to be purified as digitalis preparations must be and the color seemed to develop a little more nearly in proportion with digitalis solutions. Comparative results even with this standard were very erratic and extremes were too variable to consider the method successful. Several samples of known dilution showed too large an experimental error when compared with the standards used above.

The chief objection to the color method for digitalis even if a suitable standard could be found and more consistent results obtained, would be, as Kruse has suggested, that, like the Hatcher cat method, it tests for total activity and fails to consider the absorption factor which is so important since most digitalis is given orally. Total activity and alimentary absorbable activity are often not proportional in different digitalis preparations. This is clearly shown by the fact that cat method assays of old tinctures of digitalis frequently show them to be highly potent while these same samples, if used clinically show little or no reaction indicating the deterioration which has occurred with aging. The frog method in which the rate of absorption of injected drug is a very important factor has been found to vary proportionally with actual therapeutic value.

CONCLUSIONS.

1. The picric acid colorimetric assay of digitalis preparations is not satisfactory for determining their total activity since it yields high and erratic results.

2. The various standards used such as digitalin, digitoxin, potassium dichromate, ouabain and tincture of strophanthus, as well as minor variations in the technique of the test, failed to make it possible to obtain consistent results with the colorimetric method.

3. The color test for digitalis is certainly not satisfactory for determining comparative therapeutic value since its results do not check with those obtained with the M. L. D. frog method on the same sample.

4. The M. L. D. frog method has been previously shown to be relatively accurate in the test of dilutions of preparations of known activity, has been shown to give results proportional to therapeutic value because the rate of absorption is an important factor in the frog method, and should therefore be the method of choice over the cat method and the colorimetric method for digitalis assay.

ABSTRACT OF DISCUSSION.

Chairman Berg commended the effort in making the comparisons and for simplification and reducing the costs of assays.

James C. Munch referred to the time and work represented by this report. He hoped that the work would be extended; he did not always feel satisfied with the results of the methods employed—there is apt to be variance in the hands of different operators, due to experience with the method employed.

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THE POSSIBLE INFLUENCE OF ETHER ANESTHESIA ON THE ACCURACY OF THE CAT METHOD OF DIGITALIS ASSAY.*

BY H. B. HAAG.

Assays of tincture of digitalis were carried out on decerebrated cats to determine whether ether anesthesia affected the accuracy of the cat method in the assay of digitalis.

Of the many objections raised to the cat method of digitalis assay one frequently brought forward is the possible influence of the anesthetic used. Berardi has recently called attention to the fact that etherized dogs manifest toxic symptoms following doses of digitalis which control animals tolerate with impunity. Here in our laboratory results have been obtained which have led us to believe that digitalis somewhat lowers the resistance of animals to poisoning by ethyl alcohol. Since ethyl alcohol and ether are related both chemically and pharmacologically the question arose in our minds as to the possible influence which ether might exert in the cat method of Hatcher. In an attempt to determine this digitalis was assayed on several series of decerebrate cats, and results here obtained compared with those in which etherized animals were employed.

The method used in preparing the decerebrate beasts was, with the exception of a few modifications, that described by Pollock and Davis of Chicago, the efficacy of which depends on the production of cerebral anemia following ligation of both carotids and the basilar artery. To avoid the use of ether, the animals were anesthetized with a mixture of nitrous oxide and oxygen, and were then tied to the operating table, the head being placed in a head holder. Both carotids were next exposed and ligated and a cannula inserted into the trachea. The gas mask was then removed, the anesthetic being continued by way of the cannula. The mouth was held opened by a suitable mouth-gag, and the tongue brought outside the oral cavity and held in this position by a cord. A median line incision was made in the soft palate from the posterior edge of the hard palate, thence caudad to the free border of the soft palate. The resulting flaps were retracted so as to better expose the underlying field. Next the mucous membrane and muscles of the base of the skull were dissected laterally in such a way as to bring into view the anterior border of the foramen magnum and the tympanic bullae. By means of a curette the bony field was cleared of adhering shreds of mucous membrane and muscle. A trephine opening was then made at a point midway between the tympanic bullae, about one centimeter anterior to the anterior border of the foramen magnum. For this procedure a foot-driven dental burr, of the Vulcanite variety, was used. This operation exposed the underlying *dura mater* which was then punctured and removed; this was to allow escape of the

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