THE DETERMINATION OF DIGITOXIN IN DIGITAN.

BY L. E. WARREN.

Because of the great value of digitalis in medicine, the chemistry of the drug is a subject of much interest. Although many chemists have devoted their energies to the solution of the problems of digitalis chemistry, the subject, because of its great complexity, still remains only partially solved. A number of proximate principles are reported to have been obtained from digitalis, chief among which are digitoxin, digitalein, digitophyllin, digitonin and gitalin. Since the chemistry of digitalis is in a controversial state, some of these so-called active principles may be shown later to be mixtures or derivatives of more complex substances, originally present in the drug. Alkaloids are not present.

It is generally conceded that the activity of digitalis leaves is due almost entirely to the presence of the glucosides. The physiologic activity of the individual glucosides varies considerably, some being very toxic while others are much less active. The ease with which the glucosides are absorbed by the organism is also an important factor in judging the activity. It seems to be established that the quantities and the proportions in which the individual glucosides exist in digitalis leaves are not constant. This may account for the variable quality of the drug. There has been much controversy as to which of the principles in digitalis is the active therapeutic agent or the chief active therapeutic agent. At one time digitoxin was supposed to have this distinction, but it is now admitted that digitoxin is not the only valuable constituent. At least there are active principles in digitalis other than digitoxin, which have a modifying, additive or synergistic action on the digitoxin.

The chemical assay of digitalis has often been attempted but to date no method satisfactory in all particulars has been evolved. Nearly all of the assay processes proposed endeavor to isolate the digitoxin and either weigh it as such or estimate it by colorimetric methods. One of the earlier methods proposed was that of Keller.[1] Essentially this is as follows:

> The ground leaves (10 Gm.) are percolated with 70 percent alcohol, the solvent removed by evaporation, the residue taken up in water, an excess of lead acetate solution added, the precipitate removed by filtration, the excess of lead in the filtrate precipitated by sodium sulphate, the lead sulphate removed by filtration, the filtrate made alkaline with ammonia water and shaken successively with small portions of chloroform. The solvent is removed by evaporation, the residue taken up in a mixture of chloroform and ether (3 + 7) and the mixture poured with stirring into an excess of petroleum ether. The flaky precipitate is collected, washed, dried and weighed.

The Keller assay method was modified by Fromme[2] who used maceration instead of percolation for extracting the drug and employed aliquot parts in place of the clarified entire extract for the completion of the assay. Fromme determined the digitoxin content of a great many specimens of digitalis at this time and in succeeding years. He and Focke[3] questioned whether the digitoxin assay is a reliable index of the toxicity of digitalis, and they concluded that the digitoxin content and the physiological activity of the drug, as determined on frogs, did not run parallel.

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[1] *Ber d. Pharm. Ges.*, 7, 125, 1897.

[2] Caesar and Loretz, *Geschäfts-Bericht*, 1897, p. 25.

[3] *Deutsch. Aerzt. Ztg.*, 6, 292, 1904; *Hygienic Lab. Bull.*, 48, 9, 1909.

Arnold and Wood[1] appear to have been the first to question whether digitoxin represents the total of the therapeutic activity of digitalis. They determined that the average amount of digitalis to kill a given weight (1 kilo) of dog was 0.150 Gm. Under the same conditions it required about 0.003 Gm. of pure digitoxin to kill. The authors calculated that, since digitalis may be assumed to contain about 0.2 per cent of digitoxin, the fatal dose of the drug for the dog would contain but 0.0003 Gm. of digitoxin or about one-tenth of the amount actually required. In a later report Wood[2] states that, in general, clinical reports indicate that digitoxin has about 25 percent of the therapeutic effect of the digitalis from which it is prepared. Any assay of digitalis, therefore, which is based on digitoxin content discards about three-fourths of the active substance of the drug.

Three specimens of fluid extract of digitalis which had been assayed by the physiological method on frogs, were subjected to the Keller assay by Famulener and Lyons.[3] The results from the few tests made appeared to show that the method does not give very close duplicates and that the results probably do not indicate correctly the relative strength of the samples assayed.

Ziegenbein[4] investigated the reliability of the Keller-Fromme assay. Using frogs for the tests he compared the activity of digitoxin which had been obtained by the Keller-Fromme method from a given weight of dried digitalis leaves with the toxicity of the extract from the same quantity of leaves. He found the activity of the separated digitoxin to be only from 15 to 40 percent of that of the extract. He concludes that the quantity of digitoxin as determined by the assay bears no relation whatever to the toxicity of the drug.

The investigations of Barger and Shaw[5] also showed that the Keller assay is not an index of the strength of galenical digitalis. They assayed commercial specimens of tincture of digitalis by the Keller process, slightly modified, and tested the same preparations by physiological tests on frogs. The separated digitoxin had only about one-half of the toxicity to frogs which was possessed by an equivalent quantity of the original tincture. They prepared an artificial tincture by percolating hay with alcohol and adding a definite weight of digitoxin to it. This tincture was assayed by the Keller method and the resultant substance supposed to be digitoxin was tested on frogs. The activity of this material was only about two-thirds of that expected from the digitoxin taken. The authors concluded that as yet there is no satisfactory method for digitalis assay except the physiological one.

Reed and Vanderkleed[6] assayed nine specimens of digitalis preparations by the Keller method and tested the activity of the same preparations on guinea pigs. They did not determine the activity of the separated digitoxin. They reported a considerable degree of relationship between the findings by the two methods but, as Edmunds and Hale[7] have pointed out, there are serious exceptions to a perfect parallelism.

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[1] *Am. Jour. Med. Sci.*, 120, 165, 1900.
[2] *Am. Jour. Pharm.*, 80, 107, 1908.
[3] *Proc. Am. Pharm. Assoc.*, 50, 415, 1902.
[4] *Arch Pharm.*, 240, 454, 1902.
[5] *Pharm. Jour.*, 73, 249, 1904.
[6] *Am. Jour. Pharm.*, 80, 110, 1908.
[7] *Bulletin, Hygienic Laboratory*, 48, 9, 1909.

Further evidence of the worthlessness of the Keller method has been offered by Tschirch and Walter[1] and by Schmidt and Heyl.[2] Tschirch and Walter tried many methods for the chemical assay of digitalis. The method which they recommend as the best is as follows:

> The leaves are first exhausted with ether by which chlorophyll, fat and resin are removed. They are then treated according to Keller's method but after precipitating with lead acetate, the glucosides are shaken out with acetone, the acetone solution being made to separate by adding sodium chloride. The solvent is drawn off, evaporated on the water-bath and the residue dried and weighed.

By this procedure it is claimed that a mixture of all the active constituents of the leaves is obtained. The physiological activity of this mixture is, however, less than that of the corresponding quantity of the solution before removal of the active principles, although the residue left after the extraction is quite inactive. Nevertheless, the authors claim that the acetone method is a reliable chemical method of assay, inasmuch as by it all of the active constituents, and not the digitoxin alone, are separated and weighed. The work of Tschirch and Walter, apparently, has not been repeated by other workers so that their method is still in the experimental stage.

It will be seen from this brief review that the Keller assay process, with some modifications, is still considered the best method for the chemical assay of digitalis, unless Tschirch and Walter's method be accepted. However, it has been practically demonstrated that digitoxin does not represent the entire therapeutic virtues of digitalis and that the digitoxin content (as obtained by the Keller-Fromme process) does not run parallel with the therapeutic activity of the drug. Consequently a chemical assay of digitalis for its digitoxin content is of little value in evaluating the drug. Most writers believe that the biologic assay is the only criterion by which proper valuation of the drug may be judged. A few like Reed and Vanderkleed[3] maintain that the Keller method, if carefully carried out, is a valuable adjunct to the biologic assay.

Digitan is a digitalis preparation which is claimed to be a mixture of digitalis glucosides in the form of tannates diluted with milk sugar. Weight for weight it is said to be equivalent to digitalis leaves of standard quality. Digitan was originally called *digipuratum* and was introduced into "New and Nonofficial Remedies" under that name. The product is stated to be controlled by the Gottlieb biological method and is standardized so that 0.001 Gm. of the substance will permanently stop the heart of a 30 Gm. frog within an hour in the majority of cases. The method given in "New and Nonofficial Remedies" for the chemical assay of digitan (digipuratum) is an adaptation of a part of the Keller process. It omits clarification of the extractives by lead acetate solution. This method was furnished by Knoll and Company at the time the product (digipuratum) was being considered by the Council on Pharmacy and Chemistry of the American Medical Association for inclusion in "New and Nonofficial Remedies." No control assays were carried out by the A. M. A. Chemical Laboratory at the time.

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[1] *Schweiz. Apoth. Ztg.*, 56, 469, 495 and 512, 1918.

[2] *Am. Jour. Pharm.*, 91, 425, 1919.

[3] *Ibid.*, 80, 110, 1908.

Through a series of biologic tests on extractives obtained from digitan by the "N. N. R." chemical assay method, it seemed doubtful whether the method of chemical assay furnished by Knoll and Company was reliable. This would be expected in view of the generally accepted belief in the unreliability of the Keller-Fromme method. However, since the method given by Knoll and Company is a very marked deviation from the Keller-Fromme process and the product is distinctly different from digitalis, it seemed worth while to investigate the subject further. Accordingly, specimens of digitan were purchased and, after thorough mixing, were assayed for digitoxin by the method described in "New and Non-official Remedies" in the Laboratory of the American Medical Association. The assays were carried out in duplicate. The method used is as follows:

Ten Gm. of digitan are dissolved with moderate heat in 50 cc of water, 5 cc of 10 percent ammonia water are added and the liquid extracted with chloroform. The chloroformic extractions are filtered into a tared vessel and the chloroform removed by distillation. The residue is dissolved in 3 Gm. of chloroform, the solution mixed with 7 Gm. of ether and 50 Gm. of petroleum ether and the mixture allowed to stand over night. The separated flakes are collected on a small filter, the residue on the filter dissolved in absolute alcohol, allowing the solution to run into the tared distilling vessel. The solvent is distilled off and the residue dried to constant weight. The digitoxin thus found should not amount to more than 0.04 Gm.

It will be observed that this method limits the digitoxin obtainable from digitan to 0.4 percent. It should also be noted, as previously mentioned, that the method omits the clarification with lead acetate which is a part of the Keller method.

It had been found in earlier experiments with the assay of digitan that coloring matter (and perhaps other substances) could be obtained apparently almost indefinitely by repeated shaking with chloroform. In these assays, the alkaline solution of digitan was shaken eight consecutive times with 15 cc each of chloroform. This was adopted arbitrarily in lieu of any definite directions concerning the manner in which the shaking is to be carried out and is in agreement with the observations of Tschirch and Walter[1] who found eight shakings to be necessary. In the method originally presented to the Council by Knoll and Company, the directions were to continue the extraction until a portion of the chloroformic extract, after evaporation of the solvent and solution of the residue in glacial acetic acid, would no longer give a greenish blue tint with sulphuric acid containing a trace of ferric iron. This portion of the directions had been omitted from "N. N. R." In the assay, as a matter of record, the chloroformic extracts before purification were evaporated on a gently simmering steam-bath, the residues dried over sulphuric acid and weighed. The chloroform-ether-petroleum-ether solutions, from which the substance supposed to be digitoxin had been precipitated, were evaporated to dryness, the residue dried over sulphuric acid and weighed. These fractions were tested for activity by the biologic method.[2] After extraction by chloroform, the alkaline solution of digitan remaining was submitted to biologic

[1] *Schweiz. Apoth. Ztg.*, 56, 469, 1918.

[2] The several fractions obtained in the digitan assay were tested pharmacologically by Dr. Robert A. Hatcher in the pharmacologic laboratory of Cornell University Medical College. The writer gratefully acknowledges his indebtedness to Dr. Hatcher for the aid rendered and also for many helpful suggestions given.

tests (after removal of the dissolved chloroform by gentle evaporation). The fraction supposed to be digitoxin was also tested for activity by the pharmacologic method.

Duplicate assays of the specimens of digitan gave, respectively, 1.104 and 1.064 percent of substance supposed to be digitoxin, or more than $2^1/_2$ times the quantity permitted by the standards established by the test.

The results of the chemical examination are tabulated herewith.

CHEMICAL ASSAY OF DIGITAN.

Sample.	Weight of Sample.	Chloroform Soluble Residue.	Chloroform-Ether-Petroleum-Ether Residue.	Digitoxin.
A	10.0098 Gm.	0.1837	0.0682	0.1104
B	10.0037 Gm.	0.1822	0.0678	0.1064

The digitoxin residue was dissolved in dilute alcohol and the solution diluted with normal salt solution before testing its activity on cats. The toxicity when injected continuously into cats by the intravenous method was found to average 1.8 mg. × Kg. of body weight. (Full details of the method and protocols of the findings will be published in the *A. M. A. Therapeutic Research Reports.*) A dose of this residue of 1.5 mg. × Kg. of body weight was then injected intravenously into each of three cats, the animals were returned to their cages, after which an interval of three hours was permitted to elapse and the animals were then injected with ouabain solution 1–200,000 until death occurred. The quantity of ouabain required to produce death indicated a toxicity for the residue being tested, of about one-sixth of that of pure digitoxin.

By the same method, the chloroform-insoluble fraction of digitan was found to contain about two-thirds to three-fourths of the total toxicity of the product.

The residue obtained by the evaporation of the chloroform-ether-petroleum-ether solution from which the digitoxin was supposed to have been precipitated was totally inert.

It is evident that the substance obtained from digitan in the digitoxin assay method as described in "New and Nonofficial Remedies" is not pure digitoxin. For the determination of digitoxin the method is, therefore, valueless.

LABORATORY OF THE
AMERICAN MEDICAL ASSOCIATION.

DETECTION OF WOOD SPIRIT IN ALCOHOLIC BEVERAGES.

BY A. B. LYONS.

One of the tests most easily applied for the presence in a liquid of methyl alcohol is that known as Hehner's Test, in which milk plays the part of detective, aided and abetted by a trace of ferric iron. Ordinary milk, however, as a chemical reagent leaves much to be desired. As long ago as 1905 I proposed the substitution for milk of a solution of beef peptone.[1] The suggestion was favorably received, as indicated by a recently published routine method for the detection of methyl alcohol in distilled spirits.[2] It was possibly a retrograde step, but I ventured to suggest in 1920[3] the use in this test of powdered skim milk.

[1] *Proc. Am. Pharm. Assoc.*, 1905, p. 326.

[2] P. Hasse, *Pharm. Zentr.*, 61, 177, 1920 (*J. Soc. Chem. Ind.*, 345A, 39, 1920).

[3] "Practical Standardization of Organic Drugs," p. 84.