A CHEMICAL METHOD FOR THE ASSAY OF STROPHANTHUS PREPARATIONS.*[1]

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In continuation of work recently reported by us (1) on a chemical method of assaying digitalis preparations, it was thought that it would be of interest to see if a similar method could be applied to the assay of strophanthus preparations. Most of the methods employed at the present time in assaying the latter are biological, identical with the biological methods for the assay of digitalis.

Numerous chemical methods have been reported for the assay of strophanthus preparations, and they are based mainly on the extraction of the glucosides from the preparation and weighing the residue. Some of the chemical methods suggested convert the strophanthins into strophanthidin by acid hydrolysis; the strophanthidin, which is more insoluble and more easily crystallized than the strophanthins, is then weighed. A comparative study of most of these methods and results given by them was made by Lampart and Meuller (2). The objection to all of them is that they are tedious, requiring a number of procedures with chances for large sources of error, especially due to the small amount of active principle separated and to the possibility of other substances being present.

It will be of interest to review briefly the present conditions of strophanthin chemistry. Three varieties of strophanthus are found in commerce—*Kombé*, *hispidus* and *gratus*, two of which, *Kombé* and *hispidus*, are official. From *strophanthus Kombé*, Brauns and Clossen (3) have isolated a crystalline glucoside, known as crystalline Kombé strophanthin. It melts at 178–179°, contains water of crystallization and its composition conforms to the formula $C_{41}H_{56}O_{15}.3H_2O$. This product separates directly when a decolorized 70% alcoholic extract of drug is concentrated. The crude drug contains in larger quantity an amorphous strophanthin. When a water solution of the crystalline Kombé strophanthin is evaporated at moderate heat to a small volume it is converted into acid amorphous strophanthin. The crystalline, amorphous and acid amorphous strophanthin Kombé upon hydrolysis with dilute acid are converted into crystalline strophanthidin ($C_{23}H_{32}O_7$), which is very easily crystallized and isolated. From *strophanthus hispidus* no crystalline glucoside has been isolated, but Heffter and Sachs (4) have obtained an amorphous product called *h*-strophanthin. This product upon hydrolysis with dilute acid yields the same strophanthidin that is obtained from Kombé strophanthins. From *strophanthus gratus* a crystalline glucoside called gratus strophanthin, or ouabain, is obtained (5). It contains water of crystallization and conforms to the formula $C_{30}H_{46}O_{12}.9H_2O$ (water of crystallization may vary from one to nine molecules). Gratus strophanthin has been found present in a number of varieties of seeds (6). It is not converted by hydrolysis into crystalline strophanthidin as are the principles from the other varieties.

EXPERIMENTAL.

In our chemical method for the assay of digitalis principles (1) we made use of the reaction reported by Baljet (7). Baljet observed that cardiotonic glucosides,

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[1] A preliminary report appears in the proceedings of the Society for Experimental Biology and Medicine (A. Knudson and M. Dresbach, *Proc. Soc. Exp. Biol. and Med.*, XIX, 389, 1922). The complete report was presented before the American Society for Pharmacology and Experimental Therapeutics, in Toronto, December 1922.

whether from digitalis or strophanthus, give an orange-red color when added to a dilute alkaline picrate solution. We compared a number of purified strophanthin preparations quantitatively by this color reaction, using a solution of ouabain as a standard. The results of these tests are given in Table I.

TABLE I.—QUANTITATIVE COMPARISON OF DIFFERENT STROPHANTHINS WITH THE ALKALINE PICRATE REAGENT.

Specimen No.	Kind of preparation.	Ouabain used as standard. Mg. = 0.100 mg. Ouabain.
7	Amorphous strophanthin, Merck	0.135
50	Crystalline Kombé strophanthin, P., D. & Co.	0.100
52	Amorphous Kombé strophanthin, P., D. & Co.	0.164
53	*g*-Strophanthin, Merck	0.100
54	Amorphous strophanthin, U. S. P. IX, Merck	0.124
55	*h*-Strophanthin, Merck	0.101
56	Acid amorphous strophanthin (?)	0.149
57	Amorphous strophanthin, U. S. P. IV, Merck	0.131
58	Amorphous strophanthin, U. S. P. IX, P., D. & Co.	0.157

Upon examining this table, it is interesting to note that equal amounts of the *Kombé, gratus* and *hispidus* strophanthins react with the same intensity of color and that the amorphous strophanthins obtained from various sources vary in their reaction. All of these amorphous strophanthins are supposed to be prepared from Kombé seeds, and the fact that they vary in their activity may indicate that they contain some inert impurity and that with our present methods it is not possible to isolate them in a pure crystalline form. In this table it will also be noted that the preparation No. 56, acid amorphous strophanthin, reacts about fifty per cent. weaker than the crystalline Kombé strophanthin. This preparation was made from the crystalline Kombé strophanthin by a method suggested by Brauns and Clossen (3). Due to the fact that our supply of crystalline Kombé strophanthin was very limited, only enough of this material was prepared for our chemical and biological assay. No other tests could be carried out on it and we are therefore hesitant about calling this acid amorphous strophanthin. Since the crystalline Kombé strophanthin, *g*-strophanthin or ouabain, and the *h*-strophanthin react quantitatively the same to the alkaline picrate solution, a method was suggested by which we could quantitatively measure the amount of glucosides present in the strophanthus preparations, using either crystalline Kombé or *g*-strophanthin as a standard. Briefly, the method consists of decolorizing the strophanthus preparations with lead acetate solution, removing the excess lead and treating the decolorized solution with the alkaline picrate reagent. The intensity of color and the amount of strophanthin glucosides in the preparations can then be calculated.

SOLUTIONS NEEDED.

The solutions used in this method are the same as reported for digitalis (1), *viz.*:

a. A 10 per cent. solution of neutral lead acetate.

b. A 10 per cent. solution of Na_2HPO_4.

c. Alkaline picrate solution. This solution is prepared by mixing 95 cc of a 1% purified picric acid solution with 5 cc of 10% sodium hydroxide. The picric acid and sodium hydroxide can be made up as stock solution and the alkaline picrate freshly prepared as needed.

d. As a standard for comparisons, it is convenient to employ the same standard as used for digitalis assay (1). This standard used by us is a solution of crystalline ouabain[1] containing 0.266 mg. per 5 cc of water. Five cubic centimeters of this solution are added to 5 cc of alkaline picrate solution. If testing only strophanthus preparations, a stronger standard can be used containing 0.5 mg. per 5 cc of water. Using the stronger standard simplifies the calculation.

e. The permanent standard as suggested for digitalis assay (1) can also be used in this method. This is prepared by dissolving 3.44 Gm. of pure potassium dichromate in water and making up to a liter. The color of the solution set at 20 mm. in a colorimeter is equal to the color given by 5 cc of ouabain solution containing 0.266 mg. when mixed with 5 cc of the alkaline picrate solution.

PROCEDURE.

Two cubic centimeters of a tincture of strophanthus,[2] or an equivalent amount of drug in the form of an infusion, are measured into a 25-cc volumetric flask and diluted with water to about 15 cc. To this 2.5 cc of lead acetate solution are added, contents mixed, and then diluted with water to the mark. After mixing thoroughly and allowing to stand a minute, the solution is filtered; 12.5 cc of the filtrate are then measured into another 25-cc volumetric flask and 1.25 cc of Na_2HPO_4 solution are added in order to precipitate the excess lead. The contents of the flasks are then diluted to the mark, mixed thoroughly and filtered. The filtrate should be clear and colorless. Transfer from 1 to 5 cc to a 10-cc volumetric flask or tube graduated at 10 cc and at the same time transfer 5 cc of the standard ouabain solution to another volumetric flask. To both of these flasks add 5 cc of the alkaline picrate solution, bring the volume to 10-cc mark if necessary, mix, and allow to stand at least twenty minutes; then make color comparison in colorimeter, setting the standard most conveniently at 20 mm. The color comparison should be made between twenty and thirty-five minutes after the alkaline picrate has been added, so that it is never advisable to develop the color in more than three to five specimens at a time. Instead of the standard ouabain solution the permanent standard of potassium dichromate solution mentioned above can be put in the colorimeter, set at 20 mm. and the unknown specimen compared with it after the color has been allowed to develop in the manner described.

CALCULATION.

The calculations are similar to those which pertain to any colorimetric procedure. The standard contains 0.266 mg. of ouabain. The depth of the standard (in mm.) divided by the reading of the unknown $\times$ 0.266 mg. gives in terms of ouabain the number of mg. of strophanthin in an aliquot part of preparation taken. From this result calculations can be made as to the number of mg. per cc of tincture or other solution that may have been used. If, for example, 1 cc of decolorized filtrate of a tincture were used in the test, 1 cc of filtrate is equal to 0.04 cc of the

[1] We have used throughout our work crystalline ouabain as the standard but would prefer to use crystalline Kombé strophanthin if it could be obtained. The crystalline ouabain may contain from one to nine molecules of water of crystallization and preparations on the market have been shown to contain varying amounts of water. In all of our work we have used a ouabain containing nine molecules of water of crystallization.

[2] For tests on active principles of strophanthin such amounts are taken for the test as would be equivalent in activity to the standard. Also, in the case of pure principles of strophanthin the preliminary procedure of decolorization can be omitted.

original tincture. If the reading of unknown is 21.8 against standard set at 20 mm., then the calculation would work out as follows:

$$\frac{20}{20.8} \times 0.266 = 0.244 \text{ mg. in } 0.04 \text{ cc of tincture.}$$

Then in 1 cc there is 25×0.244 mg., or 6.10 mg. of strophanthin, calculated in terms of the standard, which is ouabain.

RESULTS AND DISCUSSIONS.

Several tinctures and infusions were tested by the above method and the results are given in Table II.

TABLE II.—CHEMICAL ASSAY OF STROPHANTHUS PREPARATIONS.

Spec. No.	Kind of preparation.	Strophanthin in terms of ouabain as standard. Mg. per cc.
34	Tincture, seeds unidentified	1.662
42	Tincture, seeds unidentified	1.495
44	Tincture, seeds unidentified	5.600
45	Tincture, hispidus seeds	5.920
46	Tincture, Kombé seeds	3.540
47	Infusion, 1% hispidus seeds	0.878
47	Infusion, 1%, ten months old	0.715
48	Infusion, 1% Kombé seeds	0.263
49	Infusion, 1% hispidus seeds	0.719
49	Infusion, 1%, ten months old	0.575

Specimens Nos. 34, 42, 44 were prepared from seeds that were not identified as to their variety. The rest of the specimens were prepared from known varieties of seeds. It will be noted that the specimens prepared from *hispidus* seeds contain a larger quantity of strophanthin than similar specimens prepared from *Kombé* seeds. This undoubtedly explains why the hispidus preparations are more toxic than the Kombé.

In order to determine whether or not results obtained by our chemical method would give us any indication as to the biological activity of these preparations, we have assayed them by the Hatcher and Brody cat method (8). Our principal reason for selecting this method rather than other biological methods was that it insures entrance into the circulation of the entire amount of the substance to be tested, so that we obtain an index of the total activity of the preparation. In other biological methods, such as the frog or guinea-pig methods, absorption may not always be complete, a matter of the utmost importance in comparing the toxicity of one substance with that of another. Moreover, we cannot assume that the elimination rate in the frog or guinea pig will be the same as in man. On the other hand, the reactions of the cat in all respects are strikingly similar to those in man. In the series of experiments described in this paper we have used seventy-seven cats and the number discarded as being atypical is only eleven, which is not a greater number of animals than would be thrown out in other biological methods.

From results obtained by the chemical method the biological activity in terms of cat units, as expressed by the Hatcher and Brody method (8), is calculated by dividing by 0.1 mg. In the chemical method the standard we have used is ouabain, of which 0.1 mg. $\times$ Kg. was found by Hatcher and others to be the average fatal dose for the cat. In the case of the specimens of strophanthin given in Table I, the number of mg. (calculated as) equal to a cat unit is the amount of these equiva-

lent to 0.1 mg. of ouabain. These results, together with results obtained by the Hatcher and Brody (8) method at the same time, are given in Table III. In the case of the tincture and infusion listed in Table II, the number of cc calculated as equal to a cat unit is obtained by dividing by 0.1, which gives the cat units per cc of preparation, and then dividing 1.0 by the number obtained gives fraction of a cc equal to a cat unit. These calculations with the determinations by the bio-assay are given in Table IV.

TABLE III.—COMPARATIVE ASSAYS BY CHEMICAL METHOD AND HATCHER AND BRODY METHOD OF PURE PRINCIPLES OF STROPHANTHINS.

Spec. No.	Kind of preparation.	Chemical assay. Mg.=c. u.	Biological. Assay. Mg.=c. u.	No. of Determinations.
7	Strophanthin, amorphous, Merck	0.135	0.126	3
50	Strophanthin, crystalline, Kombé, P., D. & Co.	0.100	0.096	7
52	Strophanthin, amorphous, Kombé, P., D. & Co.	0.164	0.149	3
53	*g*-Strophanthin, ouabain, Merck	0.100	0.081	3
54	Strophanthin, amorphous, U. S. P. IX, Merck	0.124	0.098	5
55	*h*-Strophanthin, Merck	0.101	0.107	4
56	Strophanthin, acid amorphous (?) from Kombé	0.149	0.136	3
57	Strophanthin, amorphous, U. S. P. IX, Merck	0.131	0.124	3
58	Strophanthin, amorphous, U. S. P. IX, P., D. & Co.	0.157	0.157	3

TABLE IV.—COMPARATIVE ASSAYS BY CHEMICAL METHOD AND HATCHER AND BRODY METHOD OF TINCTURES AND INFUSIONS OF STROPHANTHIN.

Spec. No.	Kind of preparation.	Chemical assay. Cc.=c. u.	Biological. Assay. Cc.=c. u.	No. of determinations.
34	Tincture, seeds unidentified	0.062	0.072	4
42	Tincture, seeds unidentified	0.067	0.087	3
44	Tincture, seeds unidentified	0.018	0.018	5
45	Tincture, Hispidus seeds	0.017	0.020	3
46	Tincture, Kombé seeds	0.028	0.028	4
47	Infusion, 1% Hispidus seeds	0.113	0.124	3
47	Infusion, ten months old	0.140	0.144	3
48	Infusion, 1% Kombé seeds	0.380	0.375	3
49	Infusion, 1% Hispidus seeds	0.139	0.151	3
49	Infusion, 1%, 10 months old	0.174	0.180	3

Upon examining Table III it will be noted that the agreement between the chemical assay and the biological assay is very close and in most of the specimens the difference is not more than 10%.[1] It is also important to note that the *Kombé*, *gratus* and *hispidus* strophanthins have the same toxicity by the chemical assay and the assay by the cat method also shows that they have a similar toxicity. Moreover, the figures by the two methods are in good agreement. The amorphous strophanthins are from 25 to 50 or 60% weaker than the crystalline Kombé, *g*-, or *h*-strophanthins. These amorphous strophanthin preparations were obtained at different times and marked as indicated in the table. When specimens were obtained from the same source, each of them had a different lot number. The fact that all the amorphous strophanthins vary in their activity would seem to indicate that they are not pure principles. The preparation No. 56, acid amorphous strophanthin, is about 50% less active than the crystalline Kombé strophanthin from which it was prepared. The agreement between the chemical assay and bio-

[1] Undoubtedly this difference could be reduced by using carefully selected male cats.

assay on this preparation is very good and this is important because, in the preparation of tinctures and infusions of strophanthus, it is believed by some that by the action of water some of the active principles are converted to acid amorphous strophanthin, especially so if heat is used in the procedure. Any change of the active principles to acid amorphous strophanthin in the strophanthus preparations would therefore be detected by the chemical assay just as it would be by the biological assay.

We call attention in Table IV to the remarkably close agreement between the chemical and biological assays. In only one of the ten preparations reported is the difference in results by the two methods more than 20%. In Preparation 42 the difference is about 25%. Specimen 48 gave interesting results by a slow rate of injection as compared with the usual ninety-minute method. Three cats were killed by the latter procedure. The average lethal dose was 0.375 cc × Kg. (the chemical method made the dose 0.380). With five other cats the time of injection was extended about one hour. The average of the five determinations was 0.449 cc. Another injection was prolonged to nearly nine hours. The lethal dose finally amounted to 0.500 cc × Kg. This experiment illustrates the importance of the rate of injection. In this connection, we may state that we have a strophanthin derivative which produces typical cardiac effect and which is practically eliminated from the cat within ten hours.

Deterioration in activity of strophanthus preparations can be detected by the chemical method as shown in the experiments on preparations Nos. 47 and 49, which were tested again after standing in the laboratory for ten months.

SUMMARY AND CONCLUSIONS.

A method for chemically assaying strophanthus preparations by the use of Baljet's alkaline picrate reaction is described.

The method permits of the direct determination of the amounts of strophanthin in terms of a crystalline strophanthin; either *gratus* strophanthin (ouabain) or crystalline *Kombé* strophanthin may be used as a standard. The latter is preferable.

The assays by the chemical method have been compared with those by the cat method of Hatcher and Brody and it is shown that there is a close agreement between them. Thus the chemical method gives an index of the potential cardiac activity of strophanthus preparations, just as it does in the case of digitalis.

It is demonstrated that the crystalline *Kombé* strophanthin, *gratus* strophanthin (ouabain), and *hispidus* strophanthin have the same toxicity in cats.

It is also shown that quantitatively the activity of preparations from *hispidus* and *Kombé* strophanthus seeds is due to the amount of strophanthin they contain. The *hispidus* preparations are more toxic than the *Kombé* because they yield a greater amount of strophanthin.

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FROM THE LABORATORIES OF BIOCHEMISTRY AND PHYSIOLOGY,
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A METHOD FOR DETERMINING STRYCHNINE IN THE PRESENCE OF ARSENIC TRIOXIDE, FERROUS CARBONATE, ALOIN AND CAPSICUM.

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During March the Children's Department of the Minneapolis General Hospital reported receiving an eighteen-month-old girl with severe symptoms of advanced strychnine poisoning. About four hours had elapsed from the time the child ate the tablets, which had been prescribed for her mother, until the initial spasm was evidenced. The remarkable work of the hospital physicians saved the child's life and the patient was dismissed at the end of five days. It was determined that the child had eaten seven of the tablets, each supposed to contain one-thirtieth of a grain or two and two-tenths milligrams of alkaloidal strychnine; a total dose of approximately one-quarter of a grain of strychnine with a four-hour period for absorption. The recovery was so unusual that the remainder of the tablets were sent to the writer in order that the presence as well as the amount of strychnine in the tablets might be verified.

A review of the literature did not disclose a method for the determination of strychnine in the presence of arsenic trioxide, ferrous carbonate, aloin and capsicum. First, the several methods that were used unsuccessfully will be cursorily reviewed and, secondly, the suggested method of analysis that was finally devised and which gave very satisfactory results will be given.

Method A.—Ten tablets were powdered and treated with ten per cent. hydrochloric acid and filtered. The filtrate was heated to boiling and hydrogen sulphide passed into it for ten minutes. The solution was cooled and again treated with hydrogen sulphide for two minutes. The mixture was then filtered and the precipitate carefully washed with ten per cent. hydrochloric acid. The filtrate was made alkaline with ammonia and filtered, the precipitate being well washed with hot water. The filtrate was then extracted with chloroform and the extract filtered through cotton into a tared dish. The precipitated iron was also well washed with chloroform. The chloroform was then evaporated and the residue weighed and subsequently dissolved in $N/10$ sulphuric acid V. S., using $N/50$ KOH V. S. with cochineal indicator to titrate the excess of sulphuric acid.

This method had several very objectionable features. (1) The hydrochloric acid dissolved a colored sticky material from the vegetable drugs and this, passing through all extractions, contaminated the residue making it unsatisfactory from a gravimetric standpoint. (2) The color of the residue, supposed to be a mixture of the color from the aloin and the cholesterin esters of oleic, stearic and palmitic acids from the capsicum, also made it exceedingly difficult to titrate the alkaloid,