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STUDIES IN BIOASSAYS: "THE PROPOSED INTERNATIONAL STANDARD FOR DIGITALIS."*,[1]

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In U. S. Pharmacopœia IX biological assays were first adopted as an optional method of evaluating certain drugs for which chemical means were not available. In this group of drugs was the digitalis series including digitalis itself, strophanthus and squills. As a standard for these drugs, crystalline gratus-strophanthin or ouabain was adopted and the strength of each preparation was adjusted to a certain equivalent of ouabain as measured by its effect on frogs when tested by the prescribed method. Thus ouabain served as a check upon the frogs, the susceptibility of which to digitalis is known to vary at different seasons of the year.

During the years which followed the publication of this Pharmacopœia (IX), the standard ouabain apparently proved satisfactory because in the various discussions incident to the recent revision (X), no objection was made from any source to its continuance as a standard for the digitalis group, although it was suggested that a strophanthin isolated from Kombé seeds might be substituted for the *g*-strophanthin. This, however, was not done and ouabain was continued as the official standard.

On the other hand at the conference on Biological Standardization held in Edinburgh in 1923, it was recommended that a preparation of powdered digitalis leaves should be used as a standard for digitalis itself and that ouabain should be

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used as a standard for strophanthus. At the Geneva Conference, held in 1925, it was decided to use as a standard for digitalis a powder made by mixing ten different specimens of digitalis which had been dried at 55° to 60° C. the final mixture being carefully assayed by the cat method. The product was to be sealed in brown glass ampuls and distributed to different countries, thus serving as an international standard. Such a powder was prepared and distributed early in 1927, and it is this powder which served as the basis for the present study, the object of which was to ascertain its activity in relation to the present U. S. P. standard.

For a comparative study of the international powder, we used the U. S. P. ouabain and two specimens of powdered digitalis leaf which were obtained on the open market and which were said by the importers to be of German origin. The 1927 specimen was said to assay 110% U. S. P. standard, while the 1928 powder was presumably of U. S. P. strength.

In our study of the question, we utilized the same methods we had employed in our earlier work (which was recently published in THIS JOURNAL, 18 (1929), 338, *viz.*, the official frog method, the intravenous frog method, the 4-hour frog method and the cat method. The drugs were prepared for use in two forms. For the various frog methods we used tinctures which were prepared by taking equal weights of the international standard powder and of the laboratory powders and making tinctures of each as directed in the U. S. P. For the assays by the cat method, 0.5% infusions were prepared as directed by the Geneva Conference. The required amounts of powder and of water were heated for 15 minutes with constant stirring on a water-bath at a temperature of 90° C. The infusion was then filtered and sufficient water added to bring it to the original volume and sodium chloride to render it isotonic. The infusions thus prepared were cooled to body temperature and assayed in the manner described in the earlier paper. The ouabain was used in dilute solution in saline made by adding the required amount of stock solution of ouabain in 70% alcohol to physiological salt solution. The results of our assays are given in Table I.

TABLE I.—ASSAY OF THE PROPOSED INTERNATIONAL STANDARD POWDER OF DIGITALIS AND OF TWO COMMERCIAL POWDERS OF DIGITALIS IN COMPARISON WITH THE U. S. P. STANDARD OUABAIN.

	Methods and Ouabain Equivalents.			
	Official method.	Ouabain equivalent.	Intravenous frog method.	Ouabain equivalent. Mg.
Ouabain	0.00050 mg.		0.00035 mg.	
International powder as a tincture	0.0045 cc.	1 cc. = 0.11 mg.	0.0025 cc.	0.14
Laboratory powder (1927)	0.0045 cc.	1 cc. = 0.11 mg.	0.0030 cc.	0.117
Laboratory powder (1928)	0.0045 cc.	1 cc. = 0.11 mg.	0.0030 cc.	0.117
	4-hour frog method.	Ouabain equivalent. Mg.	Cat method mg. per Kg.	Ouabain equivalent 1 cc. tincture. Mg.
Ouabain	0.00040 mg.		0.103	
International powder	0.0045 cc.	0.089	83.7	0.123
Laboratory powder (1927)	0.0045 cc.	0.089	96.37	0.1068
Laboratory powder (1928)	0.0045 cc.	0.089	88.9	0.116

In order to make comparisons clearer the results given in Table I have been recalculated to show the ouabain value of one gram of each powder in relation to the international standard powder, the latter being considered as 100. These results are given in Table II.

TABLE II.—OUABAIN EQUIVALENTS (IN MILLIGRAMS) OF ONE GRAM OF EACH OF THE THREE DIGITALIS POWDERS.

	Methods.					Ratio.				
	Official	Intravenous.	4-hour.	Cat.	Average.	Official.	Intravenous.	4-hour.	Cat.	Average.
International powder	1.10	1.4	0.89	1.23	1.15	100	100	100	100	100
Lab. powder 1927	1.10	1.17	0.89	1.068	1.06	100	83	100	86	92
Lab. powder 1928	1.10	1.17	0.89	1.16	1.08	100	83	100	94	94

It is interesting to note in the tables that by the two methods requiring absorption (the 1-hour and the 4-hour frog methods) all three powders appear to be of the same strength but the 1-hour method gives a higher value than does the 4-hour method. However, it will be seen (Table I) that this difference in ouabain value is dependent upon the M. S. D. of ouabain itself, as the doses of the digitalis tinctures are the same in all cases, *viz.*, 0.0045 cc. no matter whether the period of observation was one hour or four hours. If it is true, as has been claimed, that one hour does not allow sufficient time for absorption of the active principles of the drug, the 4-hour method should show a higher value, but as a matter of fact it does not do so, and this fact is still further emphasized by the comparative study given later in this paper. Both intravenous methods (frog and cat) yield higher values than the methods requiring absorption. In this connection it is interesting to compare the curves of ouabain equivalents of the digitalis powders with the curves obtained with the strophanthus series. With digitalis the 4-hour frog curve is the lowest of all, while with strophanthus it is the highest. With strophanthus the cat method gave the lowest figures; with digitalis, on the other hand, it yielded high results. These differences are puzzling to explain. That the relative values are in general correct is probable as the curves are with only one or two exceptions parallel. It would seem that they must illustrate again the inherent differences between the two drugs when the question of absorbability is concerned.

With the data available it is interesting to compare the strength of the international powder with the present U. S. P. standard. This standard for digitalis requires that 0.006 cc. of the tincture shall be equivalent to 0.0005 mg. of ouabain or in other words, that 1 Gm. of digitalis shall equal 0.833 mg. of ouabain when tested by the official frog lymph sac method. One gram of the international standard powder, however, equals 1.1 mg. ouabain, when tested by the official method, and it is therefore about 130% of the U. S. Standard. This figure we believe may be accepted as being approximately correct, as the M. S. D. for the powder and for the ouabain standard were ascertained in 1927 and both were verified by another worker (B) a year later, using in the case of the digitalis powder ampuls from an entirely new supply of the standard kindly forwarded for the work by Dr. H. H. Dale.

This value is further confirmed by the fact that the international powder was assayed by the cat method in 1926 in the laboratory of the late Professor Magnus at the University of Utrecht, and as a result of 23 determinations it was reported to have a fatal dose of 89.7 mg. per Kg. cat, an ouabain equivalent of 1.115 mg. per Gm. of powder. Our assays carried out in December 1927, gave a toxicity for cats of 83.7 mg. per Kg., or an ouabain value of 1.23 mg. per Gm. of powder, a difference of only 0.12 mg. (or 10 per cent) per Gm. of powder between the two

findings. This difference could doubtless be reduced had a larger number of cats been employed. An average of these two figures yields a value of 86.7 mg. per Kg. of cat weight. Hatcher considers 100 mg. per Kg. of cat to be about the average value for U. S. P. tincture of digitalis. Based upon these "average" figures the value of the international standard powder would be 116% U. S. P. or 119% U. S. P. if the calculation is based upon our figures alone. These figures form an interesting comparison and confirmation of the value 130% found by the official frog method.

While our results agree so closely with the Utrecht figures, those obtained by Burn and his co-workers differ considerably, as they report that they found that 1 cc. of the tincture made from the international powder was equivalent to only 0.804 mg. U. S. P. ouabain. The average value obtained by Magnus and by ourselves for the digitalis powder was 1.17 mg. ouabain as against a value of 0.804 mg. found by the English workers, a difference of 0.366 mg. ouabain per Gm. of powder, or 32%. This surprising discrepancy is doubtless to be explained by the Burn method of carrying out the assay, reference to which has been made in the earlier sections of this paper. This variation in technique in carrying out the assay by the cat method has given the English workers a value for U. S. P. ouabain of 0.06115 mg. as compared with the Hatcher figures of 0.1 mg. and our value of 0.103 mg.

The 1927 commercial specimen of digitalis powder obtained direct from the importer was accompanied by a statement of assay made according to the official method, using 9 frogs for the digitalis and 8 for the ouabain control. In spite of the small number of frogs employed, an activity of 110% of the U. S. standard was reported. This figure compares very favorably with the results of our assay of this powder by the official method, giving it an activity of about 120% of the U. S. standard. The 1928 powder appeared to be slightly stronger than the 1927 product, but both are about 8% weaker than the proposed international standard. (See Table II.)

The fact that the powder proposed as an international standard has a value somewhere about 130% of the U. S. standard is of considerable interest and importance. That it is not a powder of exceptional strength is shown by the fact that it is a mixture from ten different specimens, four of these being from the 1923 crop, the other six being collected in 1925. Four specimens were from German sources, two from Holland, one from Belgium, two from the United States and one from Canada. It was therefore an "average" powder, and yet it is considerably stronger than the U. S. standard. Possibly this standard is too low. It is certainly true that it is very common to get a digitalis powder of a potency considerably above this figure. Our experience coincides with that of one of the manufacturers who writes that they not infrequently get digitalis leaves of double U. S. P. strength. We have assayed leaves gathered in Oregon which had more than twice the activity of the U. S. P. standard. Leaves from plants raised in the fields near Ann Arbor have shown even higher activity.

It is certainly true that the activity of this powder which is proposed as an international standard will, when the next revision of the Pharmacopœia is undertaken, naturally bring up the question as to whether the U. S. standard should not be raised. *Apropos* of the same question, may we not find here perhaps one

explanation of the demand of the older practitioners for powdered digitalis of English or German origin? With the present careful selection of the crude drug and wide use of standardized preparations together with large dosage, the demand is not so frequently heard to-day.

DISCUSSION.

A comparative study of the data which we have obtained in the course of our assays is of considerable interest, shedding as it does some light upon the problem of absorption—a matter which is of fundamental importance in certain of the assay methods. In order to simplify the figures and eliminate individual variations, we have refigured our results in the cases where the data is complete on a ratio basis, using the frog intravenous doses as 100, choosing these as a basis of comparison because with them the question of absorption does not arise as it does in the other frog methods. The results are shown in Table III.

TABLE III.—RELATIVE DOSAGE OBTAINED BY THE DIFFERENT METHODS OF ASSAY USING FROGS.

	Results of Assays.			Ratio.		
	Intravenous.	4-hour.	1-hour.	Intravenous.	4-hour.	1-hour.
Ouabains.						
U. S. Standard[1]	35[1]	35	45	100	100	128
Merck-10182, Old Standard	35	35	45	100	100	128
Merck-186450	35	35	50	100	100	143
Brauns-Closson	35	40	50	100	114	143
Merck-10977	35	35	45	100	100	128
Merck-21974		35				
		Average		100	103	134
Strophanthins.						
Schuchardt	65	60	100	100		154
Abbott	65	70	100	100	108	154
Merck-28427	65	60	100	100		154
		Average		100	108	154
Tincture Strophanthus.						
Tincture 1	60	65	105	100	108	175
Tincture 2	50	55	100	100	110	200
		Average		100	109	188
Tincture Digitalis.						
Laboratory powder (1927)	30	45	45	100	150	150
Laboratory powder (1928)	30	45	45	100	150	150
International powder	25	45	45	100	180	180
		Average		100	160	160

[1] The figures on Ouabain and Strophanthin have all been taken from Section One of this study (THIS JOURNAL, XVIII (1929), 338); while figures on the Tincture of Strophanthus are from Section Two (THIS JOURNAL, XVIII (1929), 568). For the sake of brevity and simplicity in giving the doses the ciphers and decimal point are dropped. Thus, "35" means a dose of 0.00035 mg. per Gm. of frog; "65" equal 0.00065 mg. per Gm. of frog, etc.

Inasmuch as the individual differences in results are not completely eliminated and they would obscure the general picture, it would seem profitable for the present study to discuss average results only. As the table shows, the

relative doses for the ouabains, for the strophanthins, and for the tinctures of strophanthus agree in that a dose less than 10% larger is necessary to kill by the 4-hour method than by the intravenous method. The total active principle must, therefore, have been absorbed in this 4-hour period and the slightly larger dose is necessary because the drug reaches the heart more slowly. The fate of the tincture of strophanthus is in complete agreement with that of the active principles in that only 9% more is necessary to kill in the 4-hour period than in 1-hour when drug is given intravenously.

By the 1-hour frog method both gratus and Kombé strophanthins require a dose from a third to one-half larger than when given by the intravenous route. This fact is shown quite clearly when the ratio averages are thus studied, and its correctness would seem to be definitely established by the complete agreement between the figures of the individual preparations.

Tincture of strophanthus, however, is apparently very slowly absorbed from the lymph sac, as almost double the dose is needed to kill in one hour when it is given by the lymph sac as when it is given by the intravenous route. It is evident that absorption of strophanthus is far from complete in one hour, but that fact does not in the least interfere with the value of the method for the assay of this tincture provided the absorption of different tinctures is a fairly uniform process. The fate of these two tinctures would seem to indicate that that was a fact but the exact ratio of the 1-hour lymph sac dose to the intravenous dose would need to be established by the examination of a larger number of specimens. Especially is this true as those studied here are the two which were discussed in an earlier section of this paper as displaying an erratic relationship to the other methods of assay. That there was some peculiarity about these two tinctures—doubtless connected in some way with their mode of manufacture—which interfered with their absorption from the frog's lymph sac is shown also by a study of the relationship of the 1-hour dose to the 4-hour dose. (Unfortunately we had figures by the intravenous method on only these two tinctures, XV and XVI, so we could not compare them by this method with the remaining tinctures. However, we had found on these two tinctures that the intravenous route and the 4-hour method gave practically identical figures so that an indirect comparison is possible.) For a comparison of the 4-hour and the 1-hour method, the figures and calculated ratios are given in Table IV.

TABLE IV.—RATIOS OF 1-HOUR DOSE TO 4-HOUR DOSE FOR FIVE TINCTURES OF STROPHANTHUS.

Tincture.	4-hour method cc. per Gm. of frog.	1-hour method cc. per Gm. of frog.	Ratios. 4-hour.	Ratios. 1-hour.	Average.
XV	0.065	0.105	100	162	
XVI	0.055	0.100	100	182	172
XVII	0.050	0.070	100	140	
XVIII	0.100	0.130	100	130	
XIX	0.065	0.100		154	141

These figures again clearly show the difference in behavior between Tinctures XV and XVI and the others of the group. While the latter tinctures require a dose only 40% larger by the 1-hour method than they do by the 4-hour period of

observation, the first two require a dose 72% larger. That this is not accidental is shown by the fact that the figures are so clear cut and have appeared earlier in different relationships. Absorption, however, slow as it is, is complete by the fourth hour and is fairly uniform for different tinctures, as is shown by the individual figures. It would seem that this feature of slow absorption from the lymph sac of the frog as shown by these tinctures is another example of the same difficulty which is exhibited by the strophanthus preparations when they are given by mouth to the higher animals.

The digitalis tinctures differ considerably from the strophanthus tinctures in details of absorption (Table III), although they require, as is to be expected, a larger dose by both lymph sac methods than by the intravenous route. Absorption, however, is apparently as complete in one hour as in four, as the ratio of dosage is the same for four hours as it is for one hour. These results are in interesting contrast to those obtained with strophanthus and they emphasize the relative ease of digitalis absorption as compared with strophanthus. It must be remembered that only three tinctures are being considered and these relations might be altered were a larger number of digitalis powders examined. This criticism or warning is offset somewhat by the fact to which attention was called earlier, *viz.*, that these assays were carried out very carefully by two different workers in two different years and in the rare cases when slightly discordant results were obtained which could not be reconciled by repeated assays, an average of the results was taken. We feel, therefore, that the figures and conclusions drawn therefrom are entitled to considerable respect.

CONCLUSIONS.

The powdered digitalis which is proposed as an International standard is about 30% stronger than the U. S. P. X standard.

With the strophanthins and with the tinctures of strophanthus, a dose 10% larger is necessary to cause systolic standstill of the frog's heart in four hours when it is given into the lymph sac than when the drug is given intravenously and one hour allowed for observation.

A dose of the strophanthins 40–50% larger is required in the 1-hour lymph sac method than in the 1-hour intravenous method.

For tinctures of strophanthus the dose when it is given into the lymph sac and one hour allowed for observation, must be almost double the intravenous dose.

For tinctures of digitalis, the dose, whether for a 1-hour or for a 4-hour period of observation, must be about 65% above the intravenous dose, indicating greater ease of absorption of digitalis from the lymph sac as compared with strophanthus, as absorption is practically complete in one hour.

ORGANIC ADDITION COMPOUNDS OF CALCIUM CHLORIDE AND CALCIUM IODIDE.*

BY FREDERICK R. GREENBAUM.

It is a well-established fact, that calcium chloride forms a number of addition compounds with organic substances. Thus ethyl alcohol and methyl alcohol

* Scientific Section A. PH. A., Portland meeting, 1929.