SECTIONS

BIOLOGICAL ASSAY OF GELSEMIUM.*,[1]

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PART I. INTRODUCTION.

The need for a suitable assay for preparations of gelsemium has been pointed out in a previous work (1) and this paper gives data to further substantiate the belief that the pigeon emesis method, as previously outlined, can be used as a satisfactory method for measuring the activity of this drug.

Several important questions relating to the feasibility of the method have been studied and the data are presented here. By restandardizing some of the preparations used in the previous work an idea of the accuracy of the method could be obtained and the question of sensitiveness of pigeons to repeated injections could be studied, while the standardization of new preparations would add additional data. In order to have material for comparisons, other methods of biological assay were tried and the results are given. Considerable study was devoted to the question as to what principle or principles contained in the drug were responsible for the emetic action. For this it was necessary to isolate the alkaloids as reported in the literature and to give doses of each sufficiently large to allow conclusions to be drawn. It seemed that the knowledge of the cause of the emetic action would aid in judging the plausibility of the method.

A complete bibliography of all work reported in the literature through 1935 is available (2). Since the compilation of that bibliography Ramond-Hamet (3, 4, 5) has given some data concerning the action of the three crystalline alkaloids and Moisset (6) reports some work with the electrocardiograph and the dog.

THE PIGEON EMESIS ASSAYS.

Experimental Procedure and Data.—The procedure for this was described in the previous report (1) and there has been only one change made. A thirty minute period of observation was recommended at first but further study has shown that twenty minutes is long enough to allow before calling a reaction negative, since emesis is generally provoked in ten minutes or less. This reduction in observation time permits the assay to be completed more rapidly.

The Minimum Emetic Dose (M. Em. D.) is defined as that minimum quantity, expressed in cc. per Kg. of total weight of pigeon, which, when injected intravenously, will cause emesis within twenty minutes in seventy-five per cent of the pigeons injected.

Five of the eight tinctures used in this study had been standardized in the previous investigation and three of them (Preparations 110, 114 and 116) had not. The following table gives the source of the three new preparations and the source of the other preparations is given in a previous report (1).

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TABLE I.—TABULATION OF DATA CONCERNING GELSEMIUM PREPARATIONS.

Preparation.	Menstruum.	Source.
110	N. F. VI	From fresh Florida grown drug. 100 cc. represents 10 Gm. dried drug.
114	N. F. VI	From representative sample of 100 pound lot obtained on market.
116	N. F. VI	Same drug as for 110 after careful drying.

Eighty-five pigeons were used, ranging in weight from 250 to 400 Gm., and which had not been used for injections of Gelsemium prior to this investigation. They were fed a diet prepared especially for pigeons by commercial feed producers with some green material at intervals, and were allowed access to gravel.

The M. Em. D. of each preparation was determined following the procedure already described, a second determination was made for three of the preparations, and a third was made for one preparation. Approximately two thousand injections were made in this study but it is possible to show here only the results of the emetic dose and the dose immediately below it, which is presented in Tables II and III.

TABLE II.—MINIMUM EMETIC DOSE OF EACH PREPARATION WITH DOSE IMMEDIATELY BELOW.

Preparation.	Dose Cc./Kg.	Number of Injections.	Results of Injections. Emesis.	No Emesis.
102*	0.50	16	12	4
	0.45	8	4	4
102	0.45	32	25	7
	0.40	20	7	13
104*	0.90	16	13	3
	0.85	12	7	5
104	0.90	12	10	2
	0.85	16	10	6
104	0.75	16	15	1
	0.70	12	6	6
104	0.75	20	16	4
	0.70	20	11	9
106*	0.30	12	11	1
	0.25	8	3	5
106	0.35	20	16	4
	0.30	20	12	8
107*	0.70	12	9	3
	0.65	8	4	4
107	0.95	20	15	5
	0.90	20	11	9
108*	0.75	8	8	
	0.70	8	4	4
108	0.80	20	15	5
	0.75	20	11	9
108	0.80	20	16	4
	0.75	16	10	6
110	0.60	20	18	2
	0.55	20	13	7
110	0.65	18	15	3
	0.60	20	14	6
114	0.85	15	15	
	0.80	20	13	7
116	0.55	16	12	4
	0.50	12	8	4

* These determinations are from the previous report (1).

TABLE III.—THE M. EM. D. (CC./KG.) OF THE EIGHT PREPARATIONS SHOWING THE REDETERMINATIONS.

Preparation.	M. Em. D. Previous (1) Determination.	M. Em. D. This Report.	M. Em. D. Second Determination.	M. Em. D. Third Determination.
102	0.50	0.45	...	...
104	0.90	0.90	0.75	0.75
106	0.30	0.35	...	...
107	0.70	0.95	...	...
108	0.75	0.80	0.80	...
110	...	0.60	0.60	...
114	...	0.85	...	...
116	...	0.55	...	...

Twelve injections for each dose seems to be a sufficient number to determine the action of a specific dose. In most cases twenty injections per dose, within the critical range, were used; however, there was only one preparation (108) in which the results of the first twelve injections would alter the M. Em. D. as determined by twenty injections per dose, and that was true with only one injection. Although Burn (7) recommended that twenty-five pigeons be used for each dose in standardizing digitalis, it is believed that twelve is sufficient to give the reaction of a dose of Gelsemium.

By referring to Table III it may be seen that only one preparation (107) shows enough difference between previous determinations and the present ones to warrant consideration of deterioration. Since the difference of 36 per cent between the original determination and the one two years later is so much larger than that of the other preparations, it is indicated that Preparation 107 has lost part of its activity. The age of the tincture at the original standardization is not known. The fact that this tincture contained a much heavier sediment than any of the other tinctures may further indicate that deterioration took place.

Accuracy of the Method.—The size of the doses given was varied by 0.05 cc. per Kg. This is about as small a difference as can be measured using the dilutions necessary and a syringe graduated to $^1/_{100}$ cc. This difference can be detected easily. In the seventeen standardizations (Table II) the range of positive reactions with the M. Em. D. is from 75 per cent to 100 per cent, with an average of 83 per cent, and with the dose 0.05 cc. per Kg. smaller the range is from 35 per cent to 70 per cent positives, with an average of 55 per cent.

This difference of 0.05 cc. per Kg. makes a difference in size of dose ranging from 5.25 per cent to 16.7 per cent, depending upon the size of the emetic dose. Hence, from the standpoint of variance in size of dose the method proposed measures potency within a range of from 5 to 16 per cent. However, there remained the question as to whether a second determination would give results comparable to the first. In order to settle this several redeterminations where made and all determinations were compared with those of the previous study. Table III shows the M. Em. D.'s of all the determinations and it may be seen that in no case is the difference greater than 16.7 per cent except in Preparation 107 which was deteriorated. Since no difference is in excess of 20 per cent, it may be said that the accuracy of the method is within 5 to 20 per cent.

Preparation 104 has been considered the standard because it was prepared from a mixture of five samples of crude drug, but it would be well to allow 10 per cent plus or minus to allow for the experimental error of the method.

Sensitiveness of Pigeons.—Several workers (8, 9), who have used pigeons in assaying other drugs, have referred to the animals becoming more sensitive when used for several successive administrations and have reported a spontaneous emesis in some birds. Following the usual routine, six instances of spontaneous emesis were noticed. Because of this it was decided to determine whether or not the birds as a group were becoming more sensitive to doses of the drug when allowed the usual resting period of two weeks.

These pigeons were removed from use in assays and were injected with a series of doses of saline and alcohol of the same strength as the dilutions injected for assay work. The rest period was increased to a month. Following the series of saline injections drug was administered and the response was no greater than with other birds. Lieb and Mulinos (9) reported that the pigeon could be desensitized with saline injections and this appears to be true when they become sensitive to Gelsemium.

To test the group for sensitiveness each of the eighty-five birds used was injected with a dose of tincture slightly above half the M. Em. D. There was emesis in fifteen of the birds which is about 17 per cent. These fifteen were removed from routine assay and given several injections of saline-alcohol, then injected with the drug and the response was normal, indicating that sensitiveness can be overcome. These birds were later used for other determinations and their response was normal.

After the removal of these more sensitive birds, several preparations were standardized again, using the remaining ones, to determine whether the presence of these would change the emetic dose. As can be seen by referring to Table III the later determinations show no greater variation than the experimental error. Thus it is seen that even though a few birds do seem to become a little more sensitive the amount of sensitiveness for the group as a whole does not exceed the experimental error of the method, and will not impair its usefulness. Should a bird develop sensitiveness during use, it would be easy for the worker to notice and eliminate it. One point to bear in mind is the fact that pigeons feed their young by regurgitation and even feed each other at times by this method so that this will not be confused with sensitiveness.

It is felt that the two weeks rest period maintained throughout this study is sufficient but other workers (9) have suggested a month to be allowed and this might have served to prevent the development of occasional sensitiveness.

METHODS OTHER THAN PIGEON EMESIS.

Minimum Lethal Dose for Frogs.—Since no data were available showing the M. L. D. for frogs this was determined for comparative purposes. Preliminary investigations were made using three tinctures, the injections being made into the ventral lymph sac. A twenty-four-hour observation period was used because the end-point was difficult to determine, for, even though the frog showed no signs of life, when opened its heart might be found beating and would continue to beat for several hours.

The method was unsatisfactory for use because of the indefinite end-point and the difficulty involved in injecting frogs and holding them twenty-four hours for observation, so no further determinations were made.

Minimum Lethal Dose for Mice.—This method of standardization has been recommended (10) and it was deemed desirable to use it in order that the M. L. D. for mice might be compared with the M. Em. D. for pigeons.

TABLE IV.—MINIMUM LETHAL DOSE FOR MICE OF EACH PREPARATION WITH DOSE IMMEDIATELY BELOW.

Preparation.	Dose Cc./Gm.	Number Injections.	Results of Injections. Recovery.	Death.
102	0.00075	12	3	9
	0.00070	12	5	7
104	0.00155	12	3	9
	0.00150	12	9	3
106	0.00060	8		8
	0.00055	12	7	5
107	0.00230	12	1	11
	0.00225	12	5	7
108	0.00165	12	2	10
	0.00160	12	10	2
110	0.00165	12	1	11
	0.00160	12	5	7
114	0.00230	12	1	11
	0.00225	12	7	5

Injections were made intraperitoneally using dilutions consisting of 1 part tincture and 19 parts physiological saline or 1 part tincture and 9 parts saline solution, depending upon the strength of the tincture. Although many workers use 50 per cent deaths as the lethal dose it was decided to use 75 per cent deaths in this work since this percentage was used in determining the M. Em. D. The Minimum Lethal Dose is defined as that dose, measured in cc. per Gm., which will kill

three-fourths of the injected mice within one hour. Twelve injections per dose within the critical range were made and the dose was varied by 0.00005 cc. per Gm. Table IV shows the M. L. D. and the dose immediately below it.

The mice died in from twenty to thirty minutes after injection. If the dose was not fatal, recovery was rapid, for in no case did death occur after an hour and the mouse seemed completely recovered after this time. Of the approximately four hundred injections made about eighty-five mice were opened after death. The majority of the hearts were in ventricular systole, only fifteen being in ventricular diastole. Death was from respiratory failure as the heart could be found to beat several minutes after respiration ceased.

Table V shows a comparison of the M. Em. D. for pigeons and the M. L. D. for mice and also the comparative potency of each preparation by the two methods using Preparation 104 as 100 per cent in each case. The difference in potency as shown by the M. Em. D. is about the same as the difference shown by the M. L. D. Although this parallelism is not exact it does seem to show that there is some relationship between the toxicity and the emetic action and that this emetic action does measure activity. The average ratio between the M. Em. D. for pigeons and the M. L. D. for mice is 1:2.1, on the basis of Kg. of each.

TABLE V.—A COMPARISON OF THE M. EM. D. FOR PIGEONS AND THE M. L. D. FOR MICE. PREPARATION 104 IS USED AS 100 PER CENT.

Preparation.	M. Em. D.	Comparative Potency, Per Cent.	M. L. D.	Comparative Potency, Per Cent.
102	0.45	189	0.00075	203
104	0.85*	100	0.00155	100
106	0.35	242	0.00060	259
107	0.95	90	0.00230	68
108	0.80	106	0.00165	94
110	0.65	130	0.00165	94
114	0.85	100	0.00230	68

* This figure is taken as the average of the four determinations.

PART II. INVESTIGATION OF THE ALKALOIDS.

Extraction and Isolation.—For this part of the study samples of number 20 powder from a 100 lb. lot of crude drug were used. The moisture content by the toluene method was 7.5 per cent, total ash was 6.4 per cent and the acid-insoluble ash was 4.8 per cent. This acid-insoluble ash content is quite high in view of the fact that the N. F. limit is 2 per cent. A tincture made from a respresentative sample had a M. Em. D. of 0.85.

The procedure given by Sayre and Watson (11) was followed in isolating those alkaloids reported by them. A 10-Kg. sample of the drug was extracted by fractional percolation using 70 per cent alcohol. The powdered drug was moistened with 700 cc. menstruum per Kg. The moistened drug was packed in glass percolators of one and two Kg. capacity and extracted by Process C. for fluidextracts. However, to obtain complete extraction it was necessary to add about twelve liters more menstruum than was required to obtain the specified portions. Extraction was called complete when Mayer's Reagent gave only a faint turbidity. No attempt was made to assay the different portions so no conclusions can be drawn regarding the amount of extractive that was obtained in the different portions before addition of the extra menstruum. However, it seems that the fractional percolation method using 70 per cent alcohol and a number 20 powder does not give complete extraction with this drug, but more study would be necessary to justify definite statements.

The only difficulty experienced in following the procedure given by Sayre and Watson was the formation of emulsions with the chloroform shake outs. These were avoided by gentle shaking, long waiting and increasing the volume of solvent.

Gelsemic Acid (scopoletin) was removed and purified by crystallization from alcohol. The uncorrected melting point of 203° C. seemed to be close enough to the reported (12) melting point of 204° C. to justify considering them the same, since the material met other descriptions such as fluorescence in alcoholic solutions, green color with ferric chloride and needle crystals. No at-

tempt at chemical identification was made. About one gram of the purified material was obtained.

Gelsemine was isolated in the form of the hydrochloride and 7.5 Gm. was obtained. Its uncorrected melting point of 318° to 320° C. is in conformity with the reported melting point of about 300° C. The melting point of Merck's Gelseminine HCl (Gelsemine HCl) was 317° to 318° C. The exact melting point is rather difficult to determine since the material begins to darken well below the melting point and continues darkening until it is quite black at the melting point.

Sempervirine was isolated in the form of the nitrate which crystallized from alcohol in yellow needle-shaped crystals. The uncorrected melting point of 278° to 280° C. is in agreement with the reported (13) melting point of 280° to 282° C. with decomposition. The materials begins to darken at about 250° C. and continues darkening until it is quite black at the melting point. This alkaloid is fluorescent.

Gelsemidine and Gelsemoidine, the two remaining alkaloids reported by Sayre and Watson, were obtained in the form of the hydrochlorides. Since no melting points are available these could not be checked, but their forms, colors, solubilities, reactions, etc., were the same as those published, so for the purposes of this investigation they were accepted as being the same.

An alkaloid, different from those mentioned above, was reported by Chou (15). He agreed with former workers on the identity of gelsemine, reported gelsemicine as the new alkaloid, and further reported that he did not feel that his sempervine was the same as the previously reported sempervirine although their appearance and melting points were in close agreement. Hasenfratz (14) who published the formula for sempervirine said that they seemed to be the same, and later writers seem to consider them identical. Chou also reported an amorphous alkaloid for which he gave no name.

In order that all the alkaloids which have been reported might be studied, an attempt was made to follow the procedure outlined by Chou (15, 16). It was difficult to follow the procedure as given and no gelsemicine was obtained.

Tests for Emetic Action.—Pigeons were injected with solutions of the isolated principles in the same manner as with dilutions of the tinctures so that it might be determined what principle or principles cause the emetic action. The solutions were made either on the day of injection or on the afternoon before.

Gelsemic Acid, in 50 per cent alcohol, was used in strengths varying up to 20 mg. per cc. Doses were administered in increasing size until a dose of 20 mg. per Kg. had been injected into 8 pigeons without any action. Since this dose is several times the quantity that could be expected to be in an emetic dose of the tincture, it is felt that this principle cannot be responsible for any of the emetic action.

Gelsemine Hydrochloride in aqueous solutions was injected in varying doses until 8 pigeons had received 20 mg. per Kg. without emesis. The total alkaloids contained in the tinctures on the market is said to be 0.05 Gm. per 100 cc., which would be 0.5 mg. total alkaloids per cc. tincture. Since in no case has a tincture been found with a M. Em. D. of as much as 1 cc., it may be assumed that 0.5 mg. per Kg. of total alkaloids would produce emesis in all cases. Thus, since a dose of Gelsemine hydrochloride of 20 mg. per Kg., which is forty times the amount of total alkaloids assumed to be able to cause emesis, did not cause it, it seems reasonable to assume that this alkaloid is not responsible to any appreciable extent for the emetic action of the drug.

Sempervirine Nitrate was dissolved in hot alcohol and the solution adjusted to 50 per cent alcohol. Doses ranged from 0.8 mg. to 2.8 mg. per Kg. with results as shown in Table VI.

TABLE VI.—RESULTS OF INJECTIONS OF SEMPERVIRINE NITRATE.

Dose Mg./Kg.	Number of Injections.	Emesis.	No Emesis.
0.8	1	..	1
1.6	3	..	3
2.4	4	1	3
2.6	2	..	2
2.7	12	3	9
2.8	12	10	2

Sempervirine nitrate has a M. Em. D. of 2.8 mg. per Kg. with prompt action that occurs in three to seven minutes. Although the size of this dose is about five times the size of the dose of

total alkaloids considered as sufficient to cause emesis, it can be seen that this alkaloid is partially responsible for the emetic action. Since the alkaloid does not likely occur in the plant in this form no direct quantitative comparison can be drawn. The small quantity of material obtained by following Chou's procedure for isolation of his sempervine had a melting point a little under the one reported by him, so the material is doubtless impure. The dose of 2.8 mg. per Kg. did not produce emesis in four pigeons but nausea was noticeable. However, the number of injections is too small to be of any value, but since the quantity was not sufficient to make more injections no further data could be obtained. It seems reasonable to consider sempervirine and sempervine to be identical since Hasenfratz (14) considered them the same and since subsequent workers (12) appear to accept this.

Gelsemidine Hydrochloride was injected in aqueous solutions in doses ranging from 0.5 to 20 mg. per Kg. without provoking emesis. Since this is several times the quantity that could be expected to exist in an emetic dose of the tincture, it is believed that this alkaloid is not responsible to any appreciable extent for the emetic action.

Gelsemoidine Hydrochloride, in aqueous solution, was injected in doses ranging from 0.6 to 1.2 mg. per Kg. with the results shown in Table VII.

TABLE VII.—RESULTS OF INJECTIONS OF GELSEMOIDINE HCl.

Dose Mg./Kg.	Number of Injections.	Emesis.	No Emesis.
0.6	3	1	2
0.75	3	1	2
0.90	12	8	4
0.93	12	5	7
1.14	4	2	2
1.20	8	7	1

Thus it is seen that the amorphous alkaloid of Sayre and Watson is partially responsible for the emetic action of the drug since it has an emetic dose of about one mg. per Kg. The emetic action was not quite as prompt as for sempervirine since the emesis was produced in ten to fifteen minutes.

Gelsemicine could not be used in these tests for emetic action since none of this alkaloid has thus far been isolated in this investigation. However, it has been reported (17) that gelsemicine does produce emesis in pigeons, but it is not yet possible to say what the emetic dose is.

Discussion of the Emetic Principles.—Very little experimental work has been published giving the action of the various alkaloids, but it seems generally considered that gelsemine is not active as far as mammals are concerned, although Chon (15) has shown that 7 mg. gave a fall in blood pressure in one cat and Raymond-Hamet (4) states that it is active in guinea pigs. It does produce mydriasis locally. The literature indicates that this alkaloid is not responsible to a very great extent for the therapeutic action of the drug, and this study shows that in doses of 20 mg. per Kg. it is not responsible for the emetic action.

Sempervirine is reported as active by Raymond-Hamet (3) and Hou (18). Since this alkaloid does produce emesis in pigeons it appears that this method would serve to measure its activity.

The action of gelsemidine has not been published except for frogs but it does not seem that it is responsible for the therapeutic action to any extent. It does not produce emesis in pigeons in doses of 20 mg. per Kg.

The only physiological action that has been reported for the amorphous alkaloid, gelsemoidine, is its quieting effect on frogs. Gelsemium is used for its quieting action on the human. Gelsemoidine produces emesis in pigeons and thus its activity is measured by this method. Since the identity of this alkaloid is questionable, as previously stated, nothing definite can be said about it for it may contain some of another substance.

Gelsemicine is the most toxic alkaloid in the drug and affects respiration (19). Since this produces emesis (17) in pigeons this method measures its activity.

The quantities of the two alkloids which were found in this investigation to produce emesis is larger than could be responsible for the emetic action of the drug, but gelsemicine also produces

emesis although the dose has not yet been published. There is also a possibility of a synergistic action of these alkaloids which do produce emesis.

The pigeon emesis method does measure activity of those alkaloids which have been demonstrated to give physiological activity. With those alkaloids which do not give emesis the physiological activity has not been sufficiently substantiated. Thus it seems reasonable to assume that emesis in pigeons is a result of the action of those alkaloids which give the therapeutic action of the drug and that the method is suitable for assay.

CONCLUSIONS.

From the further data obtained in this investigation it has been shown that with gelsemium, pigeons do not become more sensitive to repeated injections than the limits of experimental error.

The method has an accuracy of from 5 to 20 per cent.

Four preparations (102, 106, 110 and 116) made from drug grown in Florida have emetic doses smaller than other preparations, indicating that such drug is more potent than that ordinarily found on the market.

Frogs are not a suitable animal for the assay of Gelsemium.

The 75 per cent M. L. D. for mice and the M. Em. D. for pigeons run somewhat parallel, indicating that pigeon emesis does measure activity of the drug.

The alkaloids sempervirine and gelsemoidine have been shown to produce emesis in pigeons and gelsemine and gelsemidine have been shown not to produce emesis in doses of 20 mg. per Kg. It has also been pointed out that another worker has obtained emesis with gelsemicine.

Pigeon emesis serves to measure the activity of Gelsemium more satisfactorily than any other method so far suggested. The method possesses the following desirable qualities: economy, simplicity, rapidity and a definite end-point.

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