

STUDIES ON THE BIOASSAY OF DIGITALIS.*¹

II. NEW LEG-VEIN AND INTRAMUSCULAR GUINEA-PIG METHODS.

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Guinea-pig, cat and frog methods for the assay of digitalis are optional in the recent British Pharmacopœia (1). In the United States the one-hour frog method is official (15) and the intravenous cat method (7) is highly recommended by various investigators (6, 8, 13), while the use of the guinea pig for this purpose is rather limited.

The practical value of the guinea pig in toxicological tests seemed to warrant further investigations with this animal in the assay of digitalis. New leg-vein and intramuscular minimum lethal dose methods with simplified technique were devised and checked against the subcutaneous guinea-pig method of Reed and Vanderkleed (10), the U. S. P. X one-hour frog method (15), the modified four-hour U. S. P. X frog method (4), the intravenous frog method of Smith and McClosky (14), and the intramuscular frog method of Dooley and Higley (5). Since some uncertainty seemed to exist as to whether the death of this animal following digitalis intoxication was primarily of cardiac or respiratory origin, this subject also was investigated by observing the relative occurrence of cardiac and respiratory failure.

METHODS.

The Subcutaneous Guinea-Pig Method (10).—Tincture of digitalis, rendered alcohol free by evaporation and diluted with water, is injected subcutaneously in the abdominal region of guinea pigs weighing about 240 Gm., and the minimum lethal dose determined, which is the smallest amount injected causing death within a period of two hours.

The New Leg-Vein Guinea-Pig Method.—Guinea pigs ranging in weight from 250 to 350 Gm. were used. All alcohol was removed from the tincture by evaporation, and the residue diluted with physiological saline to make preparations varying from 3 to 5 per cent in strength, so that the amount of fluid injected ranged from 1 to 2 cc., depending on the dose and size of the animal. The guinea pig was then securely tied, abdomen downward, to the special animal board, and the large superficial vein on the dorsal and inner aspect of the hind leg was exposed according to the procedure of Roth (12). The preparation was then slowly injected at the rate of 1 cc. per minute in order to avoid toxic effects due to concentration of the drug. The symptoms were noted and the time of death recorded, the minimum lethal dose being the smallest amount injected causing death within two hours, in over half the animals.

The New Intramuscular Guinea-Pig Method.—Guinea pigs ranging in weight from 250 to 350 Gm. were used. All alcohol was removed from the tincture by evaporation, and replaced with physiological saline, so that the preparation retained its original drug strength. Dilutions were not made in order to avoid the injection of too large an amount of fluid. The dose was injected in equal amounts into the posterior muscles of each thigh. The minimum lethal dose is the smallest amount injected which caused death within two hours, in over half the animals.

The U. S. P. X Frog Method (15).—This is the official method of the United States Pharmacopœia.

The Modified U. S. P. Four-Hour Frog Method (4).—This method differs from the U. S. P. X method only in that four hours instead of one hour are allowed for the absorption and action of the drug.

The Intravenous Frog Method of Smith and McClosky (14).—The minimum systolic standstill dose is determined one hour following the injection of digitalis into the abdominal vein of the decerebrated frog.

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The Intramuscular Frog Method of Dooley and Higley (5).—The dose of digitalis is injected in equal amounts into the thigh muscles of the frog. The minimum systolic dose is the smallest amount injected causing systolic standstill in one hour.

EXPERIMENTAL PROCEDURES.

Four tinctures of digitalis, designated as "B," "C," "D" and "Dx," were examined. All except "Dx" were defatted tinctures made according to U. S. P. X directions. "B" was made from Gilpin "M" leaves, "C" and "D" from McIlvaine leaves, while "Dx" was made from "D" by dilution, the degree of dilution being unknown to the author until all assays were completed.

In the guinea-pig tests male and female animals weighing from 250 to 350 Gm. were used. They were kept in the animal building at room temperature and fed on a well-balanced ration consisting of a brand of prepared food known as Purina Rabbit Chow, and water, this diet occasionally being supplemented with hay and green leafy vegetables.

Food was withheld from the guinea pigs the night before the experiments and the animals were weighed to within one Gm. immediately preceding the test. Doses were calculated on the basis of these weights. The alcohol-free preparations were not filtered before injection. Death occurring within two hours as the result of respiratory failure was taken as the end-point. Re-injected guinea pigs were not included in the results, as they invariably lost weight and were in poor condition for many days following the first injection, contrary to the observations of Vanderkleed and Pittenger (16).

The following is a typical protocol of the course of events in the new leg-vein method, the symptoms described being identical with those of the subcutaneous and new intramuscular methods, except for their slightly earlier appearance as the result of this method of administration:

Guinea pig, male, weight 326 Gm.

Tincture "B:" Evaporated 15 cc. to 3 cc., and diluted residue with physiological saline to make a 5 per cent solution (1 cc. = 50 mg.).

- 11:20-11:22 Injected 1.3 cc. of above preparation (= 0.2 of a mg. per Gm.) into leg vein at the rate of 1 cc. per minute.
- 11:30 Grinding of teeth, retching attempts (nausea).
- 11:35 Violent trembling, retching and salivation, slow labored respiration.
- 11:40 Gaspings, clonic convulsions.
- 11:42 Violent convulsions; animal falls on side, exhausted from respiratory distress.
- 11:45 Animal remains lying on side in comatose condition; respiration very slow and labored.
- 11:48 Respiration stopped.
- 11:50 Heart still beating.
- 11:55 Chest opened and heart observed; auricles contracting efficiently but slowly; ventricles contracting efficiently but very slowly (36 per minute).
- 12:00 Ventricles stopped; auricles beating slowly.
- 1:00 Auricles still beating.
- 1:30 Auricles stopped.

The results shown in Table I are typical, representing numerous similar observations in all three guinea-pig methods, and indicate that respiratory failure is the primary cause of death. After respiration has stopped the ventricles continue to beat in an efficient manner at a slowed rate, and may continue to do so for several minutes.

TABLE I.—OCCURRENCE OF RESPIRATORY AND CARDIAC FAILURE IN GUINEA PIGS FOLLOWING DIGITALIS INJECTION. (TINCTURE "C.")

Animal Number.	How Injected.	Time to Respiratory Failure, Ventricle,		Time to Cardiac Failure. Auricle.	Time between Respiratory and Ventricular Failure, Minutes.	Time between Respiratory and Auricular Failure.	Dose in Mg. per Gm.
		Minutes.	Minutes.				
4-P	Subcut.	103	106	Not observed	3	Not observed	0.25
5-B	"	30	102	" "	72	" "	0.3
6-I	"	96	143	" "	47	" "	0.3
6-J	"	119	139	" "	20	" "	0.3
6-K	"	96	113	" "	17	" "	0.3
6-L	Intrav.	29	34	" "	5	" "	0.175
6-H	Subcut.	101	110	" "	9	" "	0.3
6-M	"	85	93	" "	8	" "	0.3
6-N	"	67	77	252 min.	10	185 min.	0.35
6-P	"	81	108	251 "	27	170 "	0.35
6-R	"	79	98	249 "	19	170 "	0.35

At this time it should be mentioned that out of 281 guinea pigs injected, using various doses of three tinctures and three methods of assay, 155 animals died within two hours, and only 10 of the surviving 126 died within twenty-four hours. It is therefore apparent that the two hours' limit is entirely satisfactory from a practical standpoint.

TABLE II.—SUMMARY SHOWING RESULTS OF GUINEA PIG AND FROG ASSAYS OF TINCTURES "B," "C," "D" AND "DX."

Tincture.	Guinea-Pig Methods.			Frog Methods.			
	Subcutaneous.	New Leg Vein.	New Intramuscular.	One-Hour U. S. P. X.	Four-Hour Modified U. S. P. X.	Intramuscular (Dooley-Higley).	Intravenous (Smith-McClosky).
"B"	0.25	0.175	0.8	0.8	0.8	0.4
"C"	0.3	0.2
"D"	0.3	0.2	0.225	0.7	0.8	0.8	0.4
"Dx"	0.4	0.9

Table II is a brief summary of the minimum lethal and minimum systolic standstill doses obtained on four tinctures by guinea-pig and frog methods, respectively. The number of animals used in the guinea-pig assays ranged from 17 to 48 per assay method, depending on the number needed to obtain clear-cut results, and the number of animals from which the minimum lethal dose itself was determined never was less than 5, and never more than 12.

Tincture "C" was exhausted before it could be assayed on frogs, so that it affords a comparison only of the subcutaneous and leg-vein guinea-pig methods.

Since tincture "Dx" was a preparation of unknown strength made from tincture "D" by dilution, prepared particularly for further investigation of the new intramuscular guinea-pig method first used in the assay of tincture "D," the guinea-pig results were only checked against the official U. S. P. X one-hour frog method.

Analysis of this table shows that the minimum lethal dose in the leg-vein method is smaller than that in the subcutaneous method, and in the one case where comparison is possible (tincture "D") the intramuscular minimum lethal dose falls between the leg vein and the subcutaneous minimum lethal doses.

While three of the four methods indicate that tinctures "B" and "D" are of identical strength, the two guinea-pig methods indicate that tincture "B" is approximately 15 per cent stronger than "D." These results in themselves are insufficient for the purpose of determining which animal gave the better picture of the relative strengths of the two tinctures.

An interesting comparison of the intramuscular guinea-pig and the U. S. P. X frog methods is furnished in the assay of tinctures "D" and "Dx." According to the new intramuscular guinea-

pig assay tincture "Dx" is 56.3 per cent of the strength of tincture "D," while by the U. S. P. X frog assay "Dx" is 77.7 per cent of the strength of "D." As tincture "Dx" was made by adding two parts of the menstruum to three parts of tincture "D," the former preparation was actually 60 per cent of the strength of tincture "D." It is thus apparent that the intramuscular guinea-pig method gave a much more accurate evaluation than did the official U. S. P. X frog method.

DISCUSSION.

The administration of lethal amounts of digitalis to the guinea pig causes death apparently by its toxic action on the respiration (protocol and Table I). In the case of observations on many other animals, the results of which are not included in Table I, the heart beats could be felt through the chest wall for some time after respiration had stopped.

The question arises as to whether the conclusion of Richaud (11) is sound, that is, whether respiratory failure renders valueless the guinea pig as an assay animal for digitalis, a drug mainly used for its effect on the heart. One of the principles of bioassay which Dale (3) and Burn (2) emphasize is that a test does not have to be identical with therapeutic effect as long as it measures the important active principle. Comparison of the results obtained by four frog and three guinea-pig methods of assay on digitalis, in which artificial respiration was not employed, show a fairly close agreement, the advantage lying slightly on the side of the guinea-pig method. It seems logical to conclude from these results that lethal dose guinea-pig methods, in which death is primarily due to respiratory failure, are reliable measures of the activity of digitalis, when compared with frog heart methods.

The subcutaneous, new leg-vein and new intramuscular guinea-pig methods possess advantages over the intravenous methods of Knaffl-Lenz (9) and that of the recent British Pharmacopœia in that anesthesia is unnecessary, artificial respiration is not employed, and the operative procedure is less severe; as a result they are less time consuming and larger numbers of animals can be used in a given length of time, with a resultant decrease in experimental error.

Preference in the three guinea-pig methods is therefore not dependent on relative bioassay value, as all apparently give satisfactory results, but upon utility as to time and technique. The subcutaneous and new intramuscular methods are very simple and can be quickly performed. The new leg-vein method is more time consuming. The investigations with the new intramuscular method indicate that this method may prove to be of greater practical value in the assay of digitalis than the other methods.

SUMMARY AND CONCLUSIONS.

Male and female guinea pigs varying in weight from 250 to 350 Gm. showed no apparent differences in susceptibility to digitalis.

Guinea pigs once injected with digitalis should not be used again in assaying this drug, except in preliminary tests, as these animals lose weight and often remain in poor condition for many days following its administration.

Evidence is presented which indicates that respiratory failure is the primary cause of death following the parenteral administration of digitalis to guinea pigs.

The subcutaneous, new leg-vein and new intramuscular guinea-pig minimum lethal dose methods compare favorably with the frog heart method for the bioassay of digitalis.

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A COMPARATIVE STUDY OF THE ABSORBABILITY OF SIX
CALCIUM COMPOUNDS.*¹

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INTRODUCTION.

Two preliminary reports of this investigation have been published (1, 2). This paper reports the findings of the completed study, and covers the relatively wide gaps (0.25 mg.) between the quantities of Magnesium Sulphate required to completely neutralize the absorbed Calcium, presented in the previous papers, as well as time intervals above and below the time factor (2 hours) used in the earlier investigations.

The first study concerned itself with Dicalcium Phosphate, Calcium Chloride, Lactate, Glycerophosphate, Gluconate and Hexacalcium Inosite Hexaphosphate. These six calcium compounds and Calcium Lacto-phospho-gluconate were the agents investigated in the second study. The first six calcium combinations were employed in the final investigation.

Since a review of the literature related to these investigations has been included in the reports already published, space will be conserved by omitting the review in this final paper.

THE METHOD.

The technique (6) employed in the final study was the same as that used before, and is based on the antagonism between magnesium and calcium, earlier experiments having shown that animals narcotized by magnesium are awakened by injections of calcium salts, and, conversely, animals which have absorbed increasing quantities of calcium require greater amounts of magnesium for narcosis. Pure-bred albino mice were used. The calcium preparations were administered by stomach tube, and, after time intervals ranging from 30 minutes to 9 hours, magnesium sulphate was injected subcutaneously. Controls were run at the same time.

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