

BIOLOGICAL AND CHEMICAL EVALUATIONS OF DIGITALIS PURPUREA¹

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The importance of digitalis leaf preparations in one or other form for clinical use may be gauged from their inclusion in various Pharmacopoeias inspite of the availability of pure digitalis glycosides. The drug may vary in its contents of glycosides depending upon the methods of cultivation, collection and preservation of the leaves. Several physiologically active and inactive substances which are closely related and may give similar reactions occur together. This makes it difficult to separate quantitatively the cardiac glycosides in a pure form and assay them chemically. Many attempts have been made, with limited success, for the quantitative separation of the glycosides and their chemical assays. Biological assay of digitalis leaf and its preparations is inevitable till a suitable chemical method of assay is devised. Several biological methods (Knaffl-Lenz, 1926 ; Hanzlik, 1929 ; Hagg and Woodley, 1934 ; Trevan and Boock, 1928) have been used for the assay of digitalis. Stewart (1958) studied the influence of digitalis preparation and of cardiac glycosides on an electrically stimulated isolated outer wall of the right ventricle of the guineapig. He found that log time to zero amplitude gives the highest index of precision in comparison with other metameters he studied. The use of isolated rabbit auricles for the assay of digitalis was suggested by Trevan and Boock (1928) as they showed a reversible increase of amplitude when digitalis was added to the bath and the increase was proportional to the dose added.

The chemical assay of digitalis is a problem by itself since it is based on the reactions which may be applicable to compounds of similar structure. However, a number of workers (Rigby and Bellis, 1956; Jenson, 1953; Brindle *et al.*, 1954) have separated the constituents of *Digitalis purpurea* using paper partition chromatography. Williams (1958, personal communication) used cellulose powder formamide as stationary phase and chloroform as eluent to separate digitoxin and primary glycosides. He experienced that the presence

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of formamide interfered with the development of colour. He also found that reversed phase partition chromatography on silane treated kieselg uhr was not of much use as the glycosides were insoluble in cyclo-hexane or in hexane. However, he found that the absorption chromatography on cellulose powder gave quantitative results. The use of cellulose powder column for the separation of digitoxin and primary glycosides from the total glycosides of digitalis extract was investigated by Bhatt, Macdonald and Brindle (1960). In the present study an attempt has been made to evaluate biologically and chemically the constituents from digitalis.

METHODS

Isolated right ventricle method: Male albino guineapigs weighing between 350 to 450 g were used. Following the usual procedure, the outer wall of the right ventricle of the guineapig heart was removed. The tissue was attached to a platinum electrode (20 S.W.G.) and suspended in well oxygenated Ringer's solution (Stewart, 1958). A spring lever, balanced by a definite load, was used throughout the work. The temperature of the organ bath was maintained at $35^{\circ} \pm 1^{\circ}\text{C}$. The free electrode was kept as near the tissue as possible. The tissue was allowed to settle for at least two and a half hours before the addition of the drug. The tissue was stimulated (50 volts) by square waves of 5 m. secs. at the rate of one per twenty seconds. Three doses of the standard and three doses of a test preparation were tried in a definite dose ratio. Each dose was tried six times. One ventricular strip was used for a single dose only as the used preparation did not respond to stimulation even after repeated washings. Six metameters in relation to the time required were studied, (a) time required for the first increase in amplitude (the first point at which the increase in amplitude was 1 mm. over the initial amplitude), (b) beginning of plateau of maximum contraction (taken as the time required to reach 95 per cent of the maximum amplitude), (c) duration of plateau (time embraced by the 95 per cent limits of maximum contraction), (d) maximum amplitude, (e) half of maximum amplitude, and (f) zero amplitude. Analysis of variance (Burn *et al.*, 1952) showed that log time to maximum amplitude might be taken as a suitable metameter for the assay of digitalis.

The isolated auricles method. An adult rabbit was killed by a blow on the head and the auricles removed taking the usual precautions and suspended in an organ bath containing a highly oxygenated Ringer's solution (Trevan and Boock, 1928) maintained at a temperature of $37^{\circ} \pm 1^{\circ}\text{C}$. A light lever was used and the auricles were allowed to beat rhythmically till an uniform amplitude was obtained. As alcoholic extracts could not be used, definite

volumes of the standard (international) extract and the test extract were taken and alcohol removed on a boiling bath water. The residues were mixed with similar volumes of normal saline and filtered. Two doses of the standard extract and two doses of the test extract which gave a measurable increase in amplitude were selected in a definite dose ratio and added to the organ bath containing the auricles. The speed of the drum was adjusted in such a way that each auricular beat was recorded separately and clearly. The doses were duplicated. The record of the auricular beats was taken first before the addition of the drug, one minute after and finally five minutes later. The actual increase in amplitude over the one minute the record was used for assay. Before the another dose, auricles were allowed to beat for twenty minutes after they were washed four times in the Ringer's solution. The auricles could respond upto sixteen doses of the saline extract of digitalis.

Pure digitoxin and desacetyldigilanid A were assayed by the isolated rabbit auricles method and the U.S.P. XV method.

Chemical Assay. A column of cellulose powder (Whatman's Standard grade for chromatography) was prepared using a glass tube about 35 cm long and 1 cm diameter of which one end was drawn out to form a small funnel and a tap was fitted at the other end. Ethanol free chloroform was used for the preparation and washing of the column.

The chloroform extracts of digitalis leaf were prepared by the procedure described by Rowson (1955) and the volumes adjusted to 200 ml. Measured volumes of the chloroform extracts were taken and the total glycosides estimated by means of an alkaline solution of 3 : 5 dinitrobenzoic acid (Rowson, 1952)

An accurately measured volume of the chloroform extract was evaporated to dryness on a boiling water bath. Care was taken to see that the residue was not overheated. The residue was extracted with 3.0 ml of ethanol free chloroform by warming gently on a water bath and mixing well. The extract was transferred to the cellulose column with the help of a pipette. Chloroform was run off till the solution fell to the surface of the column. The residue was further extracted with 3.0 ml and 5.0 ml (two portions) of ethanol free chloroform. The extracts were transferred to the column in a similar way and the eluate collected. The column was eluted with larger quantity of ethanol free chloroform till about 40 ml of the eluate was collected. The rate of the drops was adjusted to 2 to 3 per second. After removal of the chloroform from the extract "digitoxin" in the residue was estimated using 3:5 dinitrobenzoic acid as the reagent.

"Primary glycosides" were eluted from the column with ethanol 10 per cent v/v in chloroform till about 40 ml of the eluate was collected in the evaporating dish containing the residue. The eluate collected was evaporated to dryness and "primary glycosides" estimated in the residue as above.

RESULTS

The results obtained by the isolated rabbits' auricle an isolated right ventricle of guineapig are tabulated with those obtained by using other standard (B. P. 1958 and U. S. P. XV) techniques (Table I). The auricle method gave fairly comparable results.

TABLE I

Assay of digitalis purpurea by B.P., U.S.P., isolated outer wall of right ventricular and isolated auricular methods

Digitalis purpurea	B. P. I.U./g	U. S. P. I.U./g	Isolated right ventricle I.U./g	Isolated rabbit auricles I.U./g
A	10.79	9.20	9.00	9.55
B	4.60	4.40	5.12	3.20
C		1.04	—	9.50

Biologically, crystalline primary glycoside A was found to be nearly three times as active as crystalline digitoxin (Table II) which was in agreement with the results of Brindle, Rigby and Sharma (1955). This difference was not indicated by the chemical method of assay.

TABLE II

Potency of digitoxin and desacetyldigilanid-A by isolated auricles and pigeon method

	Isolated rabbit auricles	Pigeon method
	I. U./mg	I. U./mg
Digitoxin	1.35	1.38
Desacetyldigilanid-A	3.92	3.55

The biological assays of the total glycosides and the two major fractions, "primary glycosides" and 'digitoxin' showed that the fractions constitute the total activity of the digitalis leaves and that they were equal in activity (Table III).

TABLE III

Biological assays for total glycosides, 'digitoxin' and 'primary glycosides' from digitalis leaf

Digitalis leaf	Total glycosides I. U./g		"Digitoxin" I. U./g.		"Primary glycosides" I. U./g	
	Isolated auricles method	Pigeon method	Isolated auricles method	Pigeon method	Isolated auricles method	Pigeon method
A	10.1	7.00	4.53	3.1	4.74	4.26
B	3.60	—	—	—	1.90	—

Total glycosides, and "digitoxin" and 'primary glycosides' separated from total glycosides using a cellulose column, were also estimated chemically in terms of digitoxin using 3:5 dinitrobenzoic acid as the reagent. Results obtained (Table IV) indicated that a biologically poor sample of digitalis leaf (B) contained same physiologically inactive substances which produced colour reaction with the reagent used.

TABLE IV

The estimates by chemical method of "digitoxin" and "primary glycosides" and of total glycosides calculated as digitoxin from digitalis leaf

Digitalis leaf	Total glycosides calculated as digitoxin % w/w	"Digitoxin" % w/w	"Primary glycosides" calculated as digitoxin % w/w
A	0.370	0.201	0.182
B	0.325	0.157	0.137

DISCUSSION

During the course of standardisation of samples of digitalis leaf preparation against the International Standard preparation it was observed that the pigeon method of U. S. P. XV has some advantages over the guineapig method of B. P. 1958. Pigeons are anaesthetised within ten minutes by ether whereas guineapigs require about two hours to get anaesthetised after the subcutaneous injection of urethane solution. The surgery may impose some strain on the guineapig as compared with the procedure of fixing a hypodermic needle in the alar vein of a pigeon. The most important point

in favour of the pigeon method is the greater ease of determining the end point. The cardiac arrest in pigeons is very sharply marked, as it is accompanied by emesis and convulsions.

Regarding the unofficial methods studied, the question arises whether the use of the isolated ventricle preparations represent an advance over the guineapig method (B. P.), especially when the ventricle like the guineapig could be used to measure only a single response to a single dose of glycoside. The extra time and effort involved might be justified if the assay yielded a better grouping of figures, sharper end point or the assays involved fewer discrepant results. The present study in which hundreds of ventricles were used did not warrant any such conclusions.

The study of the isolated auricle method, however, indicated that this offered a definite advantage over the pigeon, the guineapig and isolated right ventricle (guineapig) methods of assay. It was found that the increase in amplitude was proportional to the dose of glycoside or extract added and that the effect was reversible. It was also found that several doses of saline extract of digitalis could be tried on the same isolated rabbit's auricles and that a test preparation could be satisfactorily compared with a standard preparation on one and the same tissue.

SUMMARY

The use of isolated right ventricle of the guineapig for the assay of digitalis was reinvestigated but the method could not be supported for the assay of digitalis and its preparations.

The use of isolated rabbit's auricles for the assay of digitalis has been found to be economic and time saving and also gave reasonably comparable results.

Cellulose power column for the separation of 'digitoxin' and 'primary glycosides' from total glycosides of digitalis leaf has been described.

Digitalis leaf samples were assayed chemically and biologically for their contents of 'digitoxin', and primary glycosides' and total glycosidal" activities. The results of the chemical assays indicated that they could not be considered for evaluating digitalis in terms of its biological activity.

Pure digitoxin and primary glycoside A were assayed by the isolated rabbit auricles method and the pigeon method. Biologically, primary glycoside A was found to be three times more potent than digitoxin.

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