THE ASSAY OF TINCTURE OF DIGITALIS AND OF THE GLYCOSIDES OF *DIGITALIS PURPUREA*

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PREVIOUS work carried out in this department (Brindle and Rigby[1]), has established that the removal of saponin from a saponin containing tincture of digitalis causes no significant change either in its potency to frogs or in the intensity of its reaction with the Baljet alkaline picrate reagent. This has been confirmed by Neuwald and Zöllner[2]. It was also shown that the Knudson and Dresbach colorimetric assay of tinctures prepared from the same sample of leaf, yields results which are reasonably comparable with those obtained by an 18-hour frog bioassay. Finally, previous work indicated that the Knudson and Dresbach assay of two glycosides—gitoxin and digitoxin—gave a false estimate of their potencies.

Comparative chemical and biological assays have been carried out on several tinctures of different origin, using the frog bioassay, and chemical assays based on the Baljet reaction and on the Kedde reaction.[3] No correlation between the results of the chemical and biological assays was obtained. This led to an investigation of the individual primary and secondary glycosides, and the aglycones of *D. purpurea*, using the 18-hour frog assay and a colorimetric assay based on the Kedde reaction. The results of this work are published below, and show the inadvisability of such a chemical assay in standardising preparations of digitalis containing mixtures of these constituents in unknown concentration. The results also indicate that 3 glycosides have relatively little potency to frogs. The chemical evaluation of tinctures of digitalis should therefore be based on a reagent which estimates only the active constituents. The reaction of each constituent with the reagent should be proportional to its potency, otherwise the active constituents must be isolated and their individual concentrations estimated.

EXPERIMENTAL

The Glycosides and Aglycones of D. purpurea

Small quantities of about 25 mg. of several glycosides were very kindly supplied by Professor Arthur Stoll and standard solutions in ethanol were prepared. Similar solutions of commercial digitoxin and gitoxin were also prepared.

Bioassays. After obtaining approximate values for the LD50 of each glycoside, groups of frogs were injected using from 3 to 5 dose values, and 20 frogs for each dose value where the LD50 was low and 10 frogs where it was high, so that sufficient of each glycoside remained for future work. The large LD50 for gitoxin was determined by using a 100 mg./100 ml. solution in polyethylene glycol, diluted appropriately with saline solution immediately before injection, as a solution of gitoxin in the concentration

required could not be obtained in the usual non-toxic solvents. Polyethylene glycol alone was injected into a control group of frogs. From the results of these bioassays regression equations have been calculated for each glycoside and the experimental data are shown in Table I. Desacetyldigilanids A and B (purpurea glycosides A and B) and digitoxin are the most potent of these 6 compounds. Gitoxin, gitoxigenin and digitoxigenin can be regarded as having a very low potency to frogs. No sample of gitalin was available, but in view of the report by Merz[4] that its potency is slightly less than that of gitoxin, it may also be regarded as having little potency.

TABLE I

DATA COMPUTED FROM THE RESULTS OF THE BIOASSAYS OF SEVERAL OF THE CONSTITUENTS OF *D. purpurea*

Glycoside	Number of frogs used	Number of dose values employed	Regression Equation	Regression coefficient (b) ± σ (b)	Approx. LD50 mg./kg.	Fiducial limits (P=0·05)	X^2
"A" Series Purpurea Glycoside A. ..	100	5	Y = 5·15 + 3·15 (X − 0·386)	3·15 ± 0·93	2·2	1·8 – 2·7	0·88
Digitoxin (Stoll)	80	4	Y = 4·88 + 7·46 (X − 0·556)	7·46 ± 1·51	3·7	3·4 – 4·1	2·34
Digitoxin (commercial) ..	80	4	Y = 4·83 + 4·8 (X − 0·585)	4·8 ± 1·45	4·2	3·6 – 4·8	0·72
Digitoxigenin	40	4	Y = 5·00 + 4·97 (X − 1·241)	4·97 ± 2·23	17·4	14·4–21·0	1·30
"B" Series Purpurea Glycoside B. ..	80	4	Y = 4·69 + 4·74 (X − 0·691)	4·74 ± 1·28	5·7	4·8 – 6·75	1·10
Gitoxin (Stoll)	30	3	Y = 5·25 + 4·82 X − 1·265	4·82 ± 2·7	16·3	12·6 – 21·2	0·152
Gitoxin (commercial) ..	40	4	Y = 5·11 + 5·48 (X − 1·19)	5·48 ± 1·97	13·1	10·7–16·0	0·69
Gitoxigenin	30	5	No deaths were observed with doses up to 40 mg./kg.		40·0		
Polyethylene Glycol 20 ml./kg.	40	No deaths were observed					
Polyethylene Glycol 10 ml./kg. and Saline 10 ml./kg. ..	40	No deaths were observed					

Chemical Assays. Different volumes of each glycoside solution were added to sufficient distilled water or ethanol (30 per cent.) to produce 7·0 ml. To each 7·0 ml. were added 2·0 ml. of a 2·0 per cent. w/v solution of 3:5-dinitrobenzoic acid in ethanol (90 per cent.), and 1·0 ml. of N sodium hydroxide solution. The density of the mauve colour developed, was determined by comparison with a control containing no glycoside, at one-minute intervals. The maximum intensity was usually reached in 9 to 12 minutes. A photoelectric absorptiometer was used to make these determinations. Distilled water was used when the volume of the glycoside solution was 5·0 ml. or more since sodium 3:5-dinitrobenzoate is insoluble in concentrated ethanolic solutions. From the density readings obtained, those for 0·5 mg. of each glycoside in 10 ml. of the final coloured solution have been selected and are recorded in column 3 of Table II.

In column 4 of Table II the potency of each glycoside to frogs is expressed as the number of LD50 doses per g. of glycoside using the data of Table I. Comparison of the figures in columns 3 and 4 shows that the relative potency of each glycoside is not proportional to its Kedde colour density. The Kedde reagent is reported to react with the unsaturated lactone grouping in the aglycone portion of the molecule. Since each molecule

TABLE II

COMPARISON OF THE POTENCY TO FROGS OF THE DIGITALIS GLYCOSIDES, WITH THEIR DENSITY READINGS WHEN ASSAYED BY TWO COLORIMETRIC METHODS

1 Glycoside	2 Molecular weight	3 Density readings for 0·5 mg. of glycoside using the Kedde reagent	4 Number of frog LD50 doses/kg. in 1 g. of glycoside	5 "Density" of glycoside using the Kedde reagent calculated on a molar basis (2xM.W. × reading in column 3)	6 Density reading for 0·3 mg. of glycoside using hydrochloric acid
"A" Series					
Purpurea Glycoside A ..	926	0·270	455	500	0·216
Digitoxin (Stoll) ..	764	0·330	270	504	0·300
Digitoxin (commercial) ..	764	0·311	238	476	0·365
Digitoxigenin	374	0·635	57	475	Nil
"B" Series					
Purpurea Glycoside B ..	942	0·186	175	350	0·245
Gitoxin (Stoll)	780	0·266	61	415	0·307
Gitoxin (commercial) ..	780	0·240	77	374	0·360
Gitoxigenin	390	0·389	<25	303	Nil

contains only one lactone grouping, one would expect that the product of the molecular weight and the colour density per mg. for each glycoside would be constant. Reference to column 5 of Table II, shows that this is approximately so in the case of the "A" series of glycosides, but not in the case of the "B" series, which are unusual in other ways—for example in their reaction with sulphuric acid and the Keller-Kiliani reagent, in their relatively low potency and in the insolubility of the second member, gitoxin, in ethanol.

It is possible that this anomalous behaviour is due to the hydroxyl group in the C_{16} position in the molecule of gitoxigenin and its glycosides, so that in the presence of ethanol and alkali, there is the possibility of isomerisation with the formation of an isogenin containing a 5-membered ring. This ring would be more stable than the 6-membered rings formed by isomerisation involving the hydroxyl group in the C_{14} position, which is present in both digitoxigenin and gitoxigenin and in their glycosides. This theory was put forward by Bell and Krantz[5] in their investigations with the Baljet reagent.

The Chromatographic Analysis of Some of the Constituents of D. purpurea

Ascending and descending paper partition chromatographic techniques have been employed using a variety of solvent mixtures. The spots were detected by spraying the developed chromatograms with a solution of

trichloracetic acid in chloroform or with a solution of antimony trichloride in chloroform, and heating, as described by Heftmann and Levant[6] and by Jaminet.[7]. The R_F values of the glycosides tested are shown in Table III.

The chloroform, methanol and water solvent mixtures used by Svendsen and Jensen[8] were preferred and using these systems, the effect of temperature on the R_F values was investigated. Variation in temperature during development of the chromatograms produced no significant change in the R_F values.

The method was applied to the analysis of tinctures of digitalis. 6 samples of tincture of digitalis were decolourised by standard methods, and volumes of the decolourised products equivalent to 1 ml. of tincture were applied to filter paper sheets and chromatograms were developed using the chloroform, methanol, water system A, of Table III.

TABLE III

THE R_F VALUES OF SEVERAL OF THE CONSTITUENTS OF *D. purpurea*

	R_F values for digitalis glycosides using the following phase systems							
Glycoside	Chloroform 10 Methanol 2 Water 5 A	Chloroform 10 Methanol 4 Water 5	Chloroform 10 Methanol 8 Water 5	Ethyl acetate 2 Pyridine 1 Water 2	Ethyl acetate 1 Water 1	Ether 2 Methanol 1 Water 1	Butanol 1 Water 1	Methyl ethyl Ketone 1 Water 1
Purpurea Glycoside A ..	0·08	0·11	0·13	0·80	0·06	0·12	0·70	0·86
Digitoxin (Stoll) ..	0·91	—	—	0·90	—	—	0·88	—
Digitoxin (commercial)	0·89	0·92	0·91	0·92	0·70	0·88	0·86	0·81
Digitoxigenin	0·92	0·93	0·91	0·90	0·71	0·89	0·86	0·80
Purpurea Glycoside B	0·02	0·04	0·06	0·73	—	0·18	0·62	0·80
Gitoxin (Stoll)	0·78	0·82	0·82	0·90	0·67	0·85	0·81	0·80
Gitoxin (commercial) ..	0·83	—	—	0·86	—	—	0·84	—
Gitoxigenin	0·81	0·84	0·85	0·92	0·66	0·86	0·82	0·82
Digitoxose	0·52	0·55	0·55	0·44	0·09	0·54	0·40	0·78

Chromatograms of all 6 tinctures showed 4 bands with R_F values corresponding with those of purpurea glycosides A and B, digitoxin and gitoxin respectively. Two of the chromatograms showed an extra band, the R_F of which corresponded with that of digitoxose. Other constituents, present in concentrations below the sensitivity of the reagents used, may have been undetected by this method. Furthermore, the R_F values of digitoxin and gitoxin are almost indentical with those of digitoxigenin and gitoxigenin respectively so that it was not possible to determine whether or not these two aglycones were also present. This last point, considered along with the very low potency of these aglycones towards frogs, led to the development of the hydrochloric acid assay, described below, which was found to estimate primary and secondary glycosides but not aglycones.

Colorimetric Assays of Glycosides in the Presence of Aglycones

Such assays are based on reactions involving the pentose portion of the molecule (digitoxose). We have investigated the action of phosphoric

acid, Bial's reagent, phoroglucinol in hydrochloric acid, and 33 per cent. hydrochloric acid. Bellet[9] has shown that phosphoric acid produces a yellow colour with digitoxin and a greenish-yellow colour with gitoxin; using 66 per cent. acid, we have verified this and have employed also 90 per cent. phosphoric acid which produces a greyish-brown colour and a strong mauve colour respectively with these glycosides. All the colours deepened or altered on heating and the results were not reproducible quantitatively. Militzer[10], Fernell and King[11] and others have described the use of Bials' reagent for the quantitative estimation of pentoses. These methods and numerous modifications of Bial's method have been applied by us to the glycosides of digitalis, but the results obtained could not be reproduced very accurately. Phloroglucinol in hydrochloric acid reacted with the glycosides, but again the results obtained were not constant or reproducible. The estimation of the density of the yellow colour which develops when the glycosides are heated with concentrated (33 per cent.) hydrochloric acid has proved the most satisfactory method of assaying the glycosides in the presence of their aglycones. The colour produced is stable and the readings are reproducible.

Varying volumes of the standard alcoholic glycoside solutions were placed in Folin-Wu tubes which were immersed in a boiling water bath until each glycoside solution had been evaporated to dryness. 5 ml. of 33 per cent. hydrochloric acid was added and the tubes immersed in the bath for 3 minutes, cooled and the volume in each adjusted to 10 ml. with 33 per cent. hydrochloric acid. The results obtained for 0·3 mg. of each glycoside are shown in column 6 of Table II, and it is apparent that the relative colour density of each glycoside is not proportional to its potency to frogs. The method would appear to have promise in investigating the activity of tinctures of digitalis if used in conjunction with a method of separating the glycosides quantitatively.

Summary

1. No correlation has been found between the potency to frogs of the glycosides of *D. purpurea*, and the intensity of their reaction with the Kedde 3:5-dinitrobenzoic acid reagent or with hydrochloric acid.

2. Purpurea glycosides A and B, and digitoxin are regarded as being mainly responsible for the potency of tinctures of digitalis.

3. Equimolar concentrations of the glycosides of the "A" series—i.e., purpurea glycoside A, digitoxin, and digitoxigenin have been shown to produce approximately equal colour densities with the Kedde reagent—but the same consideration does not apply in the case of the "B" glycosides.

4. The R_F values of several of the constituents of *D. purpurea* have been determined by paper partition chromatography.

5. The constituents of 6 tinctures of digitalis have been investigated.

6. 33 per cent. hydrochloric acid is shown to be a useful reagent for the estimation of primary and secondary glycosides as it does not react with the aglycones.

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DISCUSSION

The paper was presented by MR. G. RIGBY.

DR. J. M. ROWSON said that the colorimetric assay could not be expected to give a true picture of the glycosidal activity of every tincture of digitalis. Nevertheless, in view of the difficulty and cost of biological estimation, many would entertain some hopes for the chemical assay. It was a pity that Mr. Rigby had not given more details about the tinctures which he mentioned. It was important that there should be some idea of the range of the primary glycosides, secondary glycosides and their degradation products in digitalis preparations. It was only when there was that true pattern in mind that one was in a position really to tell whether the colorimetric method would give the same sort of results as the biological. The author had not presented any comparative figures for colorimetric and biological estimations of any digitalis tinctures. The authors were tying themselves too much to tinctures; they rapidly deteriorated on storage, and in his view it would be preferable if the dried leaf stored in an atmosphere of low moisture content were used, and the tinctures prepared rapidly and examined immediately. Using both the chemical and biological methods of estimation it had been proved conclusively that a sample of digitalis leaf in moderately fine powder was completely extracted by 70 per cent. alcohol after maceration and agitation over a period of one hour. This procedure, by reducing deterioration of tinctures to a minimum, was very useful. He gave results of work as yet unpublished, on the rate of degradation of tincture of digitalis on the shelf and in the refrigerator, taking samples over a period from a few days up to 6 months. The samples were assayed by guinea-pig and chemical methods. By the former method, no change was found in the refrigerated sample of the tincture, but there was a steady decline in the shelf sample. The chemical method showed each tincture to have approximately the same activity with only slight degradation.

DR. S. ROHATGI (India) pointed out that after the publication of their original paper in 1950, Svendsen and Jensen found that the method described therein did not give satisfactory results. He wondered whether the authors obtained consistent results with the use of that method, particularly with the use of trichloroacetic acid.

MR. C. J. EASTLAND (London) drew attention to the authors' statement that gitoxin and digitoxin had very low potency, and to their subsequent

similar remark about gitalin. He wondered whether the low potency recorded was a function of the test animal, because, using guinea-pigs for the assay, he had found that gitoxin possessed something like 50 per cent. of the potency of digitoxin. Of course, there was always the difficulty inherent in the low solubility of gitoxin and the fact also that it was less cumulative than digitoxin. Recent evidence had suggested that gitalin had considerable potency although, again, it was less cumulative than digitalin.

MR. G. RIGBY, in reply, said he recognised Dr. Rowson's preference for dealing with dried leaf and the rapid preparation of tinctures. He agreed with his comments on their rate of deterioration. The one-hour maceration period for the leaf was an interesting suggestion. Although Svendsen and Jensen modified their method in 1952, since then the authors and Silberman in Australia had confirmed Svendsen's original results, so it was a question of choice. The authors' aim had been to compare various chromatographic solvent mixtures to see which was preferable for the work. So far the chloroform-ethanol-water system had been perfectly satisfactory, although it was necessary to modify the method for the separation of certain glycosides. With regard to the use of trichloroacetic acid, it was well known that the reagent did not react very well with aglycones. There was no reaction with digitoxigenin or gitoxigenin and for them antimony trichloride was preferable. There were many reports in the literature that gitoxin and gitalin possessed higher potency than that reported by the authors, but they were usually in respect to the guinea-pig; the frog potency of the glycosides was very low.