RESEARCH PAPERS

AN INVESTIGATION OF THE CONSTITUENTS OF *DIGITALIS PURPUREA*

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Received October 17, 1957

Increase in the concentration of ethanol or the inclusion of methanol, ethylene glycol or glycerol in the alkaline 3:5-dinitrobenzoic acid assay for digitalis glycosides, progressively reduces the maximum optical density of the reaction mixture. The constituents of mixtures of two digitalis glycosides have been separated by paper partition chromatography and recovered. The percentage recoveries obtained lay between 90 and 110 per cent of the quantity taken. The concentrations of four named glycosides in samples of powdered *D. purpurea* have been estimated by chromatographic separation followed by chemical assay. Estimates of the biological activity represented by the concentrations of these four constituents in each of the leaf samples have been made; the total biological activity of each leaf sample estimated in this way was found to be approximately half of the total biological activity of the whole leaf sample, as estimated by direct biological assay.

THE experimental work described in this paper is presented in two parts. Part I deals with the composition of the reaction mixture in the alkaline 3:5-dinitrobenzoic acid reaction[1] used for the assay of digitalis glycosides and aglycones and Part II with the results of a quantitative chromatographic investigation of *D. purpurea*.

Previously published work[2–5] has dealt with the chemical assay, the chromatographic analysis and the biological assay of some of the constituents of *D. purpurea*. Tattje[6], Rigby[7] and Rowson[8] have investigated the effect of varying the concentrations of the reactants in the assay with alkaline 3:5-dinitrobenzoic acid with similar conclusions. Tattje[6] observed the effect of methanol and *n*-propanol and we now describe the effects of methanol, ethanol, ethylene glycol and glycerol in the assay of digitoxin with alkaline 3:5-dinitrobenzoic acid (Part I). The results presented here agree with and extend some of the work reported by Tattje.

The chromatographic investigation of *D. purpurea* has been continued by the introduction of a new non-aqueous solvent system for use in paper partition work. This system, ethylene glycol:chloroform[5] was used because the two primary glycosides of *D. purpurea* were separated as isolated spots before the solvent front reached the bottom of the chromatogram; no such separation was achieved by the commonly used formamide systems.

The ethylene glycol:chloroform system has now been used for the separation, recovery and assay of the constituents of mixtures of digitalis

* Based upon a thesis for Ph.D. at Manchester University, 1956.

glycosides and the work has been extended to the estimation of the concentrations of four named glycosides in samples of powdered *D. purpurea* leaf.

Experimental Methods

Materials

Ethanol, dehydrated alcohol B.P.C.; ethylene glycol, reagent quality; methanol, A.R.; glycerol, A.R.; chloroform, A.R.; 3:5-dinitrobenzoic acid reagent—2·0 per cent w/v solution of 3:5-dinitrobenzoic acid in ethanol; xanthydrol reagent—xanthydrol 0·125 per cent w/v glacial acetic acid to 100 per cent, add 1·0 per cent v/v of concentrated hydrochloric acid to a suitable volume of the xanthydrol solution immediately before use; chromatographic paper, Whatman No. 3 MM filter paper 3 in. × 22·5 in.

Part I

The effect of ethylene glycol on the reaction between alkaline 3:5-dinitrobenzoic acid in ethanol and digitalis glycosides was first examined.

Method

10 ml. of reaction mixture was prepared by mixing 1·0 ml. of a solution in ethanol of a commercial sample of digitoxin containing 0·3 mg. in 1·0 ml., 2·0 ml. of 3:5-dinitrobenzoic acid reagent, a definite volume of ethylene glycol, 1·0 ml. of N NaOH and sufficient ethanol and distilled water to adjust the volume to 10 ml. and the ethanol concentration to between 65 per cent and 70 per cent v/v.

Triplicate mixtures were prepared and the maximum extinction at 535 mμ and 20° measured using a Uvispek spectrophotometer, by comparison with a blank prepared in exactly the same way but in which the 1·0 ml. of digitoxin solution was replaced by 1·0 ml. of ethanol.

The extinction was measured after five minutes and again at one minute intervals until the maximum extinction had been determined.

The effects of methanol and of glycerol were similarly investigated. In addition, the effect of variation of the ethanol concentration of the reaction mixture was investigated. In this series of experiments, the results of which are set out in Table II, no alcohol other than ethanol was present in the reaction mixtures.

Results

The presence of ethylene glycol, methanol or glycerol in the reaction mixture significantly reduces the extinction (Table I).

The concentration of ethanol was varied from 30 per cent v/v to 70 per cent v/v; the maximum extinction produced by 0·3 mg. of the commercial digitoxin in the alkaline 3:5-dinitrobenzoic acid reaction progressively decreased as the ethanol concentration was increased above 40 per cent v/v (Table II).

PART II

The Separation by Paper Partition Chromatography, Recovery and Assay of Some Constituents of D. purpurea

Four commercial samples (I to IV) of powdered *D. purpurea* leaf were investigated. Sample I was a moderately fine powder; samples II and III were moderately coarse powders and sample IV was a coarse powder.

Extraction of the powdered leaf. From each leaf sample a 1 in 10 tincture was prepared by maceration with continuous mechanical agitation for 24 hours with 80 per cent v/v ethanol.

Treatment of the tincture before application to the chromatogram. 18 ml. of tincture was mixed with 6 ml. of distilled water and the diluted tincture was drawn through a 10 cm. × 1 cm. column of chromatographic

TABLE I

THE EFFECT OF METHANOL, ETHYLENE GLYCOL AND GLYCEROL ON THE MAXIMUM EXTINCTION OF AN ALKALINE 3 : 5-DINITROBENZOIC ACID REACTION MIXTURE CONTAINING 0·003 PER CENT W/V OF COMMERCIAL DIGITOXIN

Concentration of ethanol in the reaction mixture per cent v/v	Concentration of methanol in the reaction mixture per cent v/v	Concentration of ethylene glycol in the reaction mixture per cent v/v	Concentration of glycerol in the reaction mixture per cent v/v	Maximum extinction of the reaction mixture
70				0·180
40	30			0·128
30	40			0·137
70				0·184
67		3·25		0·149
63		7·5		0·130
70				0·184
67			3·25	0·128
63			7·5	0·091

alumina (B.D.H.). This procedure removes a large proportion of the pigments from the tincture and approximately 6 per cent of the glycoside content of the tincture[3].

Four 3 ml. volumes of the partially decolourised eluate were separately evaporated almost to dryness in Quickfit boiling tubes at a temperature not exceeding 70°. The residue in each tube (approximately 0·2 ml.) was then transferred to the starting line of a strip of chromatographic paper. 3 ml. of eluate contains the extractive from 0·225 g. of leaf.

Quantitative transfer of the leaf extractive to the starting line of the prepared chromatographic paper. Each chromatographic paper strip was folded along a line 4 in. from one end to form a crease which facilitated the application of the leaf extract. The strips were passed through a 30 per cent v/v solution of ethylene glycol in methanol and then blotted between a fold of Whatman No. 1 paper.

The residual solution in one of the four tubes was drawn into a very fine capillary pipette fitted with a rubber teat and expelled along the length of the starting line of one of the prepared strips. When all the solution had been applied, the pipette was rinsed with boiling chloroform and methanol 1 : 1, and the rinsings expelled into the boiling tube and evaporated at a

temperature between 65° and 70° to a volume of approximately 0·2 ml. This solution was applied to the starting line of the paper strip, using the same pipette.

The process of rinsing, evaporation and application of the residue to the strip was repeated five times.

Test for incomplete transference of the leaf extractive to the chromatographic paper. Quantitative transference of the leaf extractive to the paper was tested by determining whether or not any glycosidal matter

TABLE II

THE VARIATION OF THE MAXIMUM EXTINCTION WITH THE ETHANOL CONCENTRATION OF AN ALKALINE 3 : 5-DINITROBENZOIC ACID REACTION MIXTURE CONTAINING 0·003 PER CENT W/V OF COMMERCIAL DIGITOXIN

Concentration of ethanol in the reaction mixture per cent v/v	Maximum extinction of the reaction mixture
30	0·215
40	0·232
50	0·214
60	0·188
70	0·182

remained in one of the four Quickfit tubes as follows: 2·0 ml. of xanthydrol reagent was added to the tube, the corresponding pipette was rinsed in this and the tube was placed in a beaker of boiling water for five minutes. The absence of a pink or red colour was taken to indicate that no glycosides remained in the tube. If such a colour developed, the corresponding chromatogram was later used to locate the separated constituents of the leaf, i.e., as a qualitative chromatogram. Rinsing of the three remaining tubes, evaporation of the rinsings and application to the paper strips was repeated twice more and one of the tubes was then tested with the xanthydrol reagent as described above.

Development of the chromatograms. The top portion of each chromatogram was trapped inside a fold of dry Whatman No. 3 MM paper and the four chromatograms were suspended from a single tray supported inside a chromatographic chamber containing at the bottom about half an inch depth of chloroform. The chamber was sealed and left undisturbed overnight. The mobile phase, chloroform saturated with ethylene glycol, was then added to the tray and development allowed to proceed at 20° ± 2°, until the solvent front reached to the bottom end of the chromatograms (approximately two hours).

The fold of paper around the end of the chromatogram inside the tray prevents excessive deposition of ethylene glycol on the chromatogram itself; such deposition slows the development.

Localisation and extraction of the separated digitalis constituents. Before extracting the isolated glycosides, the ethylene glycol was removed from the developed chromatograms by drying them at 60° to 65° for 18 hours, since glycol interferes with the alkaline 3:5-dinitrobenzoic acid assay (Part I).

The chromatogram intended for qualitative examination was cut into longitudinal strips which were treated with the following reagents:

trichloroacetic acid and hydrogen peroxide[9], alkaline 3:5-dinitrobenzoic acid and xanthydrol[10], to locate and where possible identify the separated constituents.

Corresponding pieces of paper, each bearing the same constituent, were cut from the three remaining chromatograms. Each piece was separately extracted by elution with 70 per cent ethanol for 48 hours.

During the later stages of the experimental work, an alternative method of extraction was used; the paper strips cut from the chromatograms were extracted by shaking for 30 minutes with three successive 40 ml. volumes of a mixture of chloroform and methanol 1:1.

Whichever method of extraction was used, the extracts from the three paper strips were separately evaporated to dryness under reduced pressure at a temperature not exceeding 60° in 100 ml. Quickfit flasks.

Assay of the recovered glycosides. The blank was prepared by adding 7·0 ml. of ethanol, 2·0 ml. of distilled water and 1·0 ml of N NaOH to one of the flasks. The flask was shaken to dissolve the recovered glycoside and the solution was filtered through a No. 4 sintered glass filter. 2·0 ml. of distilled water, 5·0 ml. of ethanol and 2·0 ml. of 3:5-dinitrobenzoic acid reagent were added to one of the remaining flasks and the recovered glycoside dissolved by shaking the flask. 1·0 ml. of N NaOH was then added and the mauve coloured solution immediately filtered as described above.

The extinction of this solution at 535 mμ relative to that of the blank was measured immediately and at one minute intervals until the maximum extinction (E_1) had been determined.

The extinction of the blank at 535 mμ relative to that of distilled water was then determined (E_2).

The contents of the remaining flask were similarly dissolved, the solution filtered and the maximum extinction at 535 mμ of the filtrate relative to that of distilled water determined (E_3). Theoretically, the extinction E_1 should be identical with the difference between the extinctions E_3 and E_2.

The 3:5-dinitrobenzoic acid reagent is present in the two assay solutions but is absent from the blank. The action of NaOH on the reagent produces a solution with a small absorption at 535 mμ and this necessitates a correction to the above readings. An estimate of the correction to the extinction readings was obtained by measuring the extinction at 535 mμ of a solution containing 2·0 ml. of 3:5-dinitrobenzoic acid reagent, 5·0 ml. of ethanol, 2·0 ml. of distilled water and 1·0 ml. of N NaOH, relative to that of distilled water. Measurements were made at two minute intervals for 20 minutes. The mean of these readings was 0·010 $\pm$ 0·002 and the readings did not increase with time during the 20 minute period.

The corrected duplicate extinctions were interpreted as mg. of the constituent under assay by reference to a calibration curve.

Results

The constituents separated from the leaf extracts and estimated in this way were the desacetyldigilanids A and B, digitoxin and gitoxin. In addition, unidentified compounds remaining on the starting line were

estimated and their concentration expressed as "equivalent mg." of desacetyldigilanid B.

The term "equivalent mg." was used to express the number of mg. of desacetyldigilanid B which produced the same extinction (when assayed

TABLE III

THE QUANTITY OF CERTAIN DIGITALIS CONSTITUENTS ESTIMATED TO BE PRESENT IN ONE G. OF EACH OF FOUR SAMPLES OF POWDERED *Digitalis purpurea* LEAF. THE CONCENTRATION OF UNIDENTIFIED CONSTITUENTS IS EXPRESSED AS MG. OF EITHER DESACETYLDIGILANID B. OR GITOXIN

Leaf sample	Constituents found on the starting line of the developed chromatogram, expressed as "equivalent mg." of desacetyldigilanid B mg.	Desacetyl digilanid A mg.	Desacetyl digilanid B mg.	Digitoxin mg.	Gitoxin mg.	Unidentified constituents expressed as "equivalent mg." of gitoxin mg. (i)	(ii)
I ..	0·65 0·70	1·1 1·0	0·75 0·90	1·1 1·3	0·85 0·65	0·20 0·15	— —
II ..	2·1 2·0	1·3 1·2	1·1 1·0	1·2 1·3	1·0 0·8	0·20 0·20	0·20 0·10
III ..	1·15 0·95	2·45 1·55	1·3 1·95	1·2 1·2	0·3 0·4	0·90 1·0	— —
IV ..	0·6 0·5	0·25 0·40	0·35 0·35	1·3 1·2	1·2 1·55	0·20 0·35	

by the method described on page 229—omitting the ethylene glycol) as the corrected duplicate extinctions obtained from the unidentified compounds remaining on the starting line.

Other unidentified compounds with R_F values lying between 0·4 and 0·7 were estimated and their concentration similarly expressed as "equivalent mg." of gitoxin.

The concentration of each of the leaf constituents estimated has been expressed as the amount present in one gram of powdered leaf (Table III).

TABLE IV

THE RECOVERY OF DIGITALIS GLYCOSIDES FROM DEVELOPED ETHYLENE GLYCOL-CHLOROFORM CHROMATOGRAMS

Mixture number	Name and weight of each glycoside in the mixture applied to the starting line of the chromatogram	mg.	Estimated weight of each glycoside recovered duplicate assays mg. (i)	mg. (ii)
1	Desacetyldigilanid A	0·4	0·39	0·385
	Desacetyldigilanid B	0·4	0·405	0·41
2	Desacetyldigilanid A	0·4	0·405	0·405
	Digitoxin	0·4	0·38	0·39
3	Desacetyldigilanid B	0·4	0·40	0·395
	Gitoxin	0·3	0·28	0·275
4	Digitoxin	0·4	0·37	0·36
	Gitoxin	0·3	0·32	0·325

To obtain an estimate of the errors involved in the assay described above, the process was first used to separate and assay the constituents of different mixtures of known amounts of two digitalis glycosides. The results of this work suggest that the techniques involved in the assay are associated

with an error of not more than ± 10 per cent (Table IV). This error is probably less than the error associated with the results of the assays of the isolated leaf constituents (Table III), since the latter assays are more difficult to perform and are complicated by the presence of plant pigments and constituents other than glycosides.

Brindle, Rigby and Sharma[4] and Rigby[7] biologically assayed several digitalis glycosides, individually and in mixtures, using the guinea pig and the frog. From their results which are expressed as *international units of*

TABLE V

COMPARATIVE ASSAYS OF EACH OF FOUR SAMPLES OF POWDERED *Digitalis purpurea*

Leaf sample	"Assumed activity" of the leaf sample. Units/g. (Results of duplicate assays)	Biological activity of the leaf sample I.U./g. with 95 per cent fiducial limits
I	4·88 4·81	6·6—7·7—8·9
II	5·84 5·64	8·2—9·4—10·7
III	9·07 7·30	12·2—13·7—15·3
IV	2·39 2·72	3·7—4·8—6·1

activity per mg. of glycoside, the number of units of activity represented by the weight of each of the four glycosides, desacetyldigilanid A, desacetyldigilanid B, digitoxin and gitoxin in one gram of each leaf sample was calculated.

The sum of the number of units of activity per g., represented by each of these four constituents, was calculated for each of the four leaf samples, and has been termed the "assumed activity" of the leaf.

The "assumed activity" of each leaf is shown in Table V along with the results of a biological assay of the leaf. The "assumed activity" has been expressed in units.

The 1 in 10 tinctures prepared from the powdered leaf samples as described on page 230 were biologically assayed by the 18 hour frog lymph sac method as described in the British Pharmacopoeia.

DISCUSSION

It has been shown (Table II) that variation of the ethanol concentration in the alkaline 3 : 5-dinitrobenzoic acid reaction with digitoxin significantly affects the extinction of the mixture. The presence of certain other alcohols in the reaction mixture considerably reduced the maximum extinction (Table I). From these data it was calculated that in the presence of equimolar concentrations of different alcohols, the reduction in maximum extinction was progressively greater as the number of hydroxyl groups in the alcohol molecule increased. The decrease in maximum extinction when 0·1 g. molecule of an alcohol was present in 100 ml. of a reaction mixture containing 0·003 per cent. w/v of commercial digitoxin, was calculated to be approximately 2·5 per cent in the case of the two monohydric alcohols, methanol and ethanol, about 25 per cent in the case of the dihydric alcohol ethylene glycol and about 50 per cent in the case of the trihydric alcohol glycerol.

The figures in Table V show that the sum of the biological activities represented by the concentrations of four specified glycosides in any one of the leaf samples investigated is approximately 50 to 60 per cent only of the total biological activity of the leaf sample as estimated by bioassay. In making this comparison, it has been assumed that not more than 15 per cent potentiation of activity occurs when a mixture of the four constituents investigated is assayed biologically. This assumption is based on previously published work[4]. This 40 to 50 per cent discrepancy may be due to several factors. These may be,

(*a*) the loss of active constituents during the partial decolourisation process,

(*b*) the failure to transfer the "decolourised" extract without loss to the starting line of the chromatogram,

(*c*) the adsorption at the starting line, by the paper, of constituents which would normally move down the paper during development of the chromatogram,

(*d*) the distribution of small amounts of a constituent of high R_F over that part of the chromatogram traversed by the constituent during development,

(*e*) the failure to extract a separated constituent completely from the developed chromatogram,

(*f*) the presence of unidentified constituents in the leaf, one or more of which might have considerable biological activity, and

(*g*) the presence of pigments derived from the leaf, in the assay reaction mixture.

Many of the possible sources of error listed above are associated with the techniques employed in the chromatographic assay. The results of the quantitative separation, recovery and assay of the constituents of mixtures of two glycosides (Table IV) suggest that the techniques employed in the assay may give rise to an error of approximately 10 per cent. However, the presence of pigments and other extractive in the partially decolourised leaf extracts may considerably affect (*a*) the adsorption of the glycosides by the paper, especially at the starting line, (*b*) the distribution of the glycosides over the whole chromatogram, and (*c*) the efficiency of elution of the separated glycosides from the developed chromatogram. These factors would inevitably increase the error of the result. More complete decolourisation might reduce the error.

In addition, loss of active constituents from the leaf extracts occurs during partial decolourisation; this loss may be as high as 6 per cent (Brindle, Rigby and Sharma[3]).

In conclusion, it can be said that the chemical assay of certain constituents separated by paper partition chromatography from an extract of *D. purpurea*, cannot, at present, replace the biological assay of *D. purpurea*.

Furthermore, in view of the numerous sources of error listed above and bearing in mind the large number of constituents identified in samples of *D. purpurea* it is not likely that a chemical assay of this type can be achieved.

The chemical assay following chromatographic separation described in this paper can, however, be applied to the estimation of the concentration of one or more of the constituents of a leaf, provided that the limitations of the assay are recognised; for example, the isolation of certain constituents may necessitate the use of a particular chromatographic solvent system or of a suitable reagent for the chemical assay. The work described by Sellwood[11] may be quoted as an important example in this respect.

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