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

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Introduction

In continuation of our work[1] on the partition chromatography of digitalis glycosides and their biological and chemical assays, we have now made a comparative study of biological and chemical assays of tinctures of digitalis using the 18-hour frog assay method described in the British Pharmacopœia and the Kedde reagent[2] (3 : 5 dinitrobenzoic acid) respectively. Rowson and Dyer[3] (using freshly prepared tinctures from carefully dried leaf) found close correlation between the chemical assay employing 3 : 5-dinitrobenzoic acid and the biological assay using guinea-pigs. Our work shows that the results of the chemical assay are by no means identical with those of the biological assay using frogs when the methods are applied to commercially available tinctures. We indicate the inadvisability of a chemical assay for standardising preparations such as tincture of digitalis which contain several glycosides in varying proportions, unless these proportions can be determined. This investigation is being continued with this object in view.

During the chemical assay of the tinctures the main difficulty experienced was that of decolourisation. Rowson[4] made a comparative study of many decolourisation methods and recommended the use of lead subacetate and sodium sulphate for the decolourisation of tinctures prior to chemical assay. Tinctures decolourised by this method develop an orange-brown to intense brown colour with sodium dinitrobenzoate; this is entirely different from the mauve colour obtained when pure glycosides are similarly treated. An investigation of the decolourisation methods was therefore undertaken and the use of an alumina column for adsorbing the pigments was found suitable.

The constituents of commercial samples of tincture of digitalis were identified by paper partition chromatography using several different phase mixtures, including the formamide system recommended by Heftman and Levant[5] and by Jensen[6]. The decolourised tinctures and the pure glycosides were simultaneously chromatographed on the same sheet of paper and no evidence of the presence of new glycoside-like substances, reported by Ulrix[7] and Jensen[8] was found in the samples of tincture under investigation.

The separation of glycosides on cellulose columns was also achieved, providing a means for the quantitative determination of the constituents in a mixture. The eluate fractions were assayed with the Kedde reagent.

EXPERIMENTAL

A 1 in 10 tincture prepared from the standard preparation of digitalis, and 8 commercial samples of tincture of digitalis obtained from different sources were employed in this work. The tinctures were stored in a refrigerator below 10° C. throughout to minimise deterioration. The authentic samples of glycosides and genins used for the identification of the constituents of the tinctures were very kindly supplied by Professor Arthur Stoll.

BIOLOGICAL ASSAYS

The 8 commercial samples (A-H) were biologically assayed by comparison with a laboratory standard tincture (I), which had been standardised previously against the standard preparation of *Digitalis purpurea* (tincture S).

The 2 and 2 dose, 18-hour frog assay method, as recommended in the British Pharmacopœia 1953, was used for comparison of the potencies of the tinctures. The results were computed from the mortality data by the Pharmacopœial method and that of Gaddum[9]. The results of these biological assays are shown in Table I. It will be noticed that all these commercial samples are considerably below the pharmacopœial standard of 1 unit per ml.

TABLE I

THE BIOLOGICAL ASSAY OF TINCTURES OF DIGITALIS A-H

Tinctures compared 1 : 2	Potency of Tincture 1 expressed as percentage of Tincture 2	Fiducial limits (P = 0·95)	Potency of Tincture 1 expressed as percentage of Tincture S	Potency of Tincture 1 expressed as Standard I.U./ml.
I : S	72·4	64·5–81·3	72·4	0·95
A : I	57·0	46·2–70·7	41·3	0·54
B : I	50·0	42·9–68·3	36·2	0·48
C : I	41·4	31·0–55·2	29·9	0·39
D : I	65·6	54·9–78·3	47·4	0·62
E : I	54·0	42·9–67·9	39·1	0·51
F : I	49·4	40·0–61·1	35·8	0·47
G : I	60·1	48·1–75·9	43·5	0·58
H : I	53·7	42·1–68·6	38·9	0·51

Chemical assays

Prior to performing a colorimetric estimation the tinctures were decolourised by the method recommended by Rowson[4] employing lead subacetate and sodium sulphate. However, the decolourised preparation developed a brown to orange-brown colour with the Kedde reagent, whereas the pure glycosides give a mauve colour. Greater quantities of lead subacetate solution per ml. of tincture were used, but in no case did the decolourised filtrate develop the characteristic mauve colour of the glycosides when mixed with the Kedde reagent. The decolourisation of tinctures of digitalis was therefore investigated as described below.

Chromatographic alumina (B.D.H.) alone, and containing 0·01, 0·05, 0·1 and 0·5 per cent. of activated charcoal were used for adsorbing the pigments. A sample of one of the tinctures was passed through columns

which were packed with each of these adsorbents. The eluate, collected by applying suction to the receiver (or positive pressure at the top of the column), varied in colour from pale yellow to straw, with increasing amounts of activated charcoal. 1 ml. of each of the eluates was transferred to separate small glass-stoppered bottles, and 6 ml. of 30 per cent. ethanol, 2 ml. of a 2 per cent. w/v solution of 3 : 5-dinitrobenzoic acid in 70 per cent. ethanol and 1 ml. of N sodium hydroxide were then added to the contents of each bottle. The colour density was recorded on a Spekker absorptiometer against a blank using 1 ml. of 30 per cent. ethanol in place of 1 ml. of the eluate. The colour produced was very similar to that obtained with pure glycosides and like them gave a maximum Spekker reading when the yellow and green filters were used in combination. The Spekker drum readings for 1 ml. of the tincture decreased with increasing percentage of activated charcoal in the alumina. In subsequent experiments, therefore, columns of alumina only were used and the reproducibility of the colour given by 1 ml. of each of the eluates (decolourised tincture) from 6 columns was investigated. 1 ml. of each of the 6 eluates gave Spekker drum readings of 0·305, 0·301, 0·296, 0·297, 0·300 and 0·295. The deviation of these readings from the mean is about $\pm$ 2 per cent. and therefore the method is reasonably reproducible. Next, successive equal volumes of the eluate from a single tincture—alumina column were assayed in order to decide whether the concentration of "glycoside" (colour active substances) was uniform throughout or altered from fraction to fraction. The results of this experiment are shown in Table II.

TABLE II

SPEKKER DRUM READINGS OF SEPARATE SUCCESSIVE FRACTIONS OF ELUATE OBTAINED BY PASSING A SAMPLE OF TINCTURE G THROUGH AN ALUMINA COLUMN

Fraction number of eluate	Spekker drum readings of successive	
	0·5 ml. volumes of the eulate	1·5 ml. volumes of the eulate
1	0·151	0·440
2	0·155	0·450
3	0·147	0·452
4	0·149	0·455
5	0·151	0·443
6	0·152	—
7	0·150	—
8	0·153	—

No evidence of any variation in concentration of glycosides was found since the successive Spekker drum readings recorded in Table II were reasonably constant in value. In view of this, further experiments were performed to determine the loss, if any, of glycosides, due to adsorption, when a mixture of glycoside solutions is passed through a column of alumina.

Absolute ethanolic solutions of 4 glycosides (purpurea glycoside A, digitoxin, gitoxin and digitoxigenin)were mixed in definite proportions and this solution assayed both before and after passing it through a column of alumina. The loss recorded was about 45 per cent. Attempts to

diminish the loss were made by using a more polar solvent—in this case by reducing the strength of ethanol to about 70 per cent., which is approximately equal to the concentration in tincture of digitalis. The average recovery was about 94 per cent. and the individual readings were reproducible within ± 4 per cent. (Table III).

TABLE III

THE ASSAY OF A SOLUTION IN 70 PER CENT. ETHANOL OF A MIXTURE OF PURE GLYCOSIDES BEFORE AND AFTER PASSING THROUGH A COLUMN OF ALUMINA

Mixed glycoside solution, ml.	Spekker drum readings	
	Before passing through an alumina column	After passing through an alumina column
1	0·120	0·117
1	0·118	0·118
1	0·121	0·108
1	0·117	0·115
1	0·120	0·113
1	0·119	0·113

Further experiments were performed with an artificial tincture prepared by mixing a concentrated ethanolic solution of a mixture of pure glycosides, with an ethanolic extract of fresh grass, containing about 60 per cent. of ethanol. The loss of glycosides on the alumina column was in this case found to be approximately 8 per cent.

It was thought desirable to compare the decolourisation process mentioned above with the lead subacetate sodium sulphate method. Two tinctures (F and B) were used for the comparison. The Spekker drum readings showed a close agreement by the two methods. Green and yellow filters in combination were used in both cases (Table IV).

TABLE IV

COMPARISON OF THE DECOLOURISATION METHODS USING TWO SAMPLES OF TINCTURES

Sample	Average of Spekker drum readings obtained from 6 × 1 ml. volumes of each tincture when assayed after decolourisation by:—	
	Alumina	Lead subacetate
F	0·300 ± 0·005	0·301 ± 0·004
B	0·248 ± 0·001	0·252 ± 0·006

The "glycoside" concentration in 8 commercial samples of tincture of digitalis (A-H) and of the tincture (S) prepared from the standard preparation of digitalis was estimated absorptiometrically by the method, using 3:5-dinitrobenzoic acid, described above using 6 different volumes of each of the tinctures decolourised by the alumina method (Table V). The Spekker drum readings for the 9 tinctures were plotted against the corresponding volumes, and it was found that for the same volume of any 2 tinctures, the ratio between the absorptiometer readings was constant over the range of 0·25 ml. to 1·5 ml. The volume of each unknown tincture which would produce the same Spekker drum reading as that produced by 0·5 ml. of the "standard tincture" was found by reference to

TABLE V

COLOUR DENSITY READINGS FOR TINCTURES A-H AND S

Sample	Spekker drum readings for decolourised tinctures					
	0·25 ml.	0·50 ml.	0·75 ml.	1·00 ml.	1·25 ml.	1·50 ml.
A	0·061	0·120	0·175	0·231	0·293	0·345
B	0·068	0·135	0·192	0·253	0·303	0·357
C	0·052	0·100	0·151	0·199	0·247	0·295
D	0·108	0·213	0·300	0·390	0·482	0·570
E	0·090	0·181	0·265	0·352	0·438	0·520
F	0·078	0·155	0·232	0·300	0·366	0·430
G	0·076	0·152	0·220	0·285	0·345	0·415
H	0·060	0·120	0·176	0·231	0·283	0·340
S	0·092	0·182	0·253	0·320	0·395	0·470

the graphs, and the same repeated for 0·75 ml. and 1·0 ml. volumes of the "standard tincture". From these figures, the ratio

$$\frac{\text{Volume of "standard tincture"}}{\text{Volume of unknown tincture producing the same Spekker drum reading,}}$$

was calculated at the three points corresponding to 0·5 ml., 0·75 ml. and 1·0 ml. of "standard tincture". For all tinctures, these 3 estimates of the above ratio agreed closely and their average was calculated and multiplied by 100. The estimated concentration of "glycoside" in each tincture is thus expressed as a percentage of that in the "standard tincture" and these results along with those of the biological assays are shown in Table VI. These figures indicate that the chemical method of assay yields results which do not agree with the results of the biological method of assay used.

TABLE VI

COMPARISON OF THE BIOLOGICAL AND CHEMICAL ASSAYS

Sample	Potency of tincture sample expressed as a percentage of standard tincture (determined biologically)	Estimated concentration of glycoside in each tincture expressed as a percentage of that in the standard tincture (determined chemically)
A	41·3	68·0
B	36·2	75·0
C	29·9	57·0
D	47·4	122·5
E	39·1	105·0
F	35·8	90·6
G	43·5	86·1
H	38·9	67·2

Partition Chromatography of the Tinctures

The 9 tinctures used in these investigations were chromatographed after decolourisation by alumina on Whatman No. 1 paper. Chloroform: methanol: water (10:5:5), benzene: formamide, and chloroform: formamide were used as phase mixtures. Good separations of the constituents present were obtained, especially when formamide was used as the stationary phase. Pure glycosides were applied on the same sheet as reference spots. The chloramine trichloracetic acid reagent[6] was used for spraying the chromatograms; this reagent produces a yellow-orange and a blue fluorescence respectively with the glycosides of the A and B series.

No constituents other than those commonly known to be present in *D. purpurea* could be detected in the samples of tincture analysed. Relatively large quantities were therefore chromatographed in order to increase the possibility of finding such constituents, Whatman No. 3 MM paper being used for this purpose. Again, no new constituent was found to be present.

TABLE VII

CONSTITUENTS IDENTIFIED BY CHROMATOGRAPHY IN TINCTURES OF DIGITALIS

(P.G.A. = purpurea glycoside A.
P.G.B. = purpurea glycoside B.)

Tincture sample	Constituents detected by paper partition chromatography using chloroform: methanol: Water (10:5:5), Formamide-chloroform, and Formamide-Benzene systems
A	P.G.A., P.G.B. and gitoxigenin.
B	,, ,, digitoxigenin and gitoxigenin.
C	,, ,, (traces) digitoxigenin and gitoxin.
D	,, ,, digitoxin and digitoxigenin.
E	,, (traces) P.G.B. (traces), digitoxin, digitoxigenin and gitoxin.
F	,, (traces) P.G.B., digitoxigenin and gitoxigenin.
G	P.G.B. (traces), digitoxin, digitoxigenin and gitoxin.
H	P.G.A. (traces), digitoxin, gitoxin and gitoxigenin.
S	P.G.A., P.G.B. and gitoxin (traces).

In the tincture S from the standard preparation no digitoxigenin, digitoxin, gitoxigenin and only traces of gitoxin were detected. It is possible that a colorimetric assay may give fairly reliable results when applied to freshly-prepared tinctures from carefully dried leaf. The results would be progressively less satisfactory with increase in the age of the tincture and unsatisfactory methods of drying the leaf or of storage of leaf or tincture would accentuate any discrepancy.

DISCUSSION

As previously stated[1] the chemical evaluation of a tincture of digitalis is not likely to give identical results with those obtained by biological assay, unless the constituents are separated and separately assayed. The genins which of all the constituents, produce, weight for weight, the greatest colour density with the Kedde reagent, possess relatively little potency. The work here described indicates that the results of the frog assay and of the Kedde chemical assay do not agree when commercially available tinctures are examined.

With regard to the decolourisation of tinctures of digitalis, the alumina decolourisation method described, is convenient and simple and gives results comparable with the existing methods. Moreover, using this method, the colour produced when the Kedde reagent is added to the decolourised filtrate has an absorption maximum at $\lambda 540 m\mu$, as has the colour produced in the same way with pure glycoside solutions. On the other hand, tinctures decolourised by lead acetate methods, on mixing with the Kedde reagent give rise to a brown colour which has a maximum absorption at $\lambda 420 m\mu$, and the density of such brown solutions would therefore normally be estimated using light of wavelength 420 $m\mu$. If, however, the intensity of the reddish component in the brown colour be

estimated colorimetrically, using light of wavelength about 540 mμ, the readings agree with those obtained when the same tincture is decolourised by the alumina method and assayed with the same reagent Intense green tinctures present a difficulty in that some chlorophyll passes out in the eluate and causes slight precipitation on dilution with 30 per cent. ethanol; this can be avoided by filtering the diluted decolourised tincture through a sintered pyrex filter.

Summary

1. 8 samples of tincture of digitalis have been compared chemically and biologically with a tincture prepared from the standard preparation of digitalis. The results of the frog assays do not agree with those of the chemical assays using the 3 : 5-dinitrobenzoic acid reagent.

2. A new method for the decolourisation of tinctures of digitalis has been investigated. The tincture is passed through an alumina column which adsorbs the pigments, and the eluate can be directly assayed with the Kedde reagent. The total time taken for decolourisation is at the most 5 minutes and the loss of colour-producing active constituents—presumably due to adsorption on the alumina is less than 10 per cent.

3. The constituents of 9 samples of tincture of digitalis have been identified by paper partition chromatography.

The work described above was part of an investigation undertaken while one of us, S. N. Sharma, was the holder of the Allen and Hanbury Fellowship in Pharmacy, at Manchester University.

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