STUDIES IN THE GENUS DIGITALIS

PART III. THE EXTRACTION AND EVALUATION OF *Digitalis purpurea* LEAF

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A RECOMMENDED process for the estimation of powdered digitalis leaf or of digitoxin by means of 3:5-dinitrobenzoic acid has been described in two previous papers of this series[1,2]. This present communication reports more detailed investigations of the precise conditions under which the dinitrobenzoic acid process will yield accurate and reproducible results: the preparation of leaf extracts by different periods of maceration in 70 per cent. ethanol has also been studied and the results obtained when powdered leaf samples of *Digitalis purpurea* were examined by biological methods and by the chemical process of estimation are compared.

EXPERIMENTAL

The recommended process for chemical estimation, using the 25 mg. level of Pb for decolorisation, has been employed throughout the present work[1]. Biological estimations were carried out by Dr. F. J. Dyer, using the intravenous guinea-pig method already described[2].

Sodium Hydroxide Concentration

A purple colour is produced when a mixture of 3:5-dinitrobenzoic acid and either digitoxin or decolorised tincture of digitalis, in the presence of dilute ethanol, is rendered alkaline with N sodium hydroxide; 1 ml. of alkali in 10 ml. of reaction mixture being employed. The theoretical equivalent of the dinitrobenzoic acid used is 1 ml. of 0·19N sodium hydroxide, thus a large excess of alkali is present throughout the reaction. To investigate the influence of this excess of alkali on the course of the reaction, a series of experiments was made in which 1 ml. quantities of sodium hydroxide solutions varying in strength from 0·6 to 3N were employed for the estimation of fixed quantities of both digitoxin and a decolorised tincture of digitalis. Results are expressed in Table I. The colour densities of a fixed concentration of glycosides are seen to increase considerably as the concentration of alkali in the reaction mixture is raised and a corresponding acceleration in the development of the maximum colour is observed. The change in apparent k (1 mg.) value for digitoxin with variation in alkali concentration was linear over the range 0·8 to 2N; below 0·8N the rate of change was still more pronounced, but between 2 and 3N it was less accentuated. Similar results were found for the decolorised tincture although the change at 0·6N and the increase from 2 to 2·5N were both less pronounced than for digitoxin. The time required for maximum colour development in both series of tests was inconveniently long at low concentrations of alkali but at concentrations

greater than 2N this time was too short to allow for accurate spectrophotometric measurement. Within the range of alkali concentration 0·8 to 1·8N there is little obvious advantage at any particular level and the previously recommended concentration of N sodium hydroxide is confirmed as suitable, but this reagent must be adjusted to strength with analytical accuracy.

TABLE I

3 : 5-DINITROBENZOIC ACID REACTION
VARIATION IN CONCENTRATION OF ADDED NaOH SOLUTION (1 ml.)

NaOH solution	Digitoxin		Leaf tincture (decolorised)	
	k (1 mg.)	Time of maximum colour development	k (1 ml.)	Time of maximum colour development
3·0N	1·071	2 minutes		
2·5N			0·456	3 minutes
2·0N	1·040	6 ,,	0·452	3 ,,
1·8N			0·450	5 ,,
1·4N			0·420	6 ,,
1·3N	0·870	9 ,,		
1·2N	0·832	9 ,,	0·375	7 ,,
1·1N	0·821	12 ,,		
1·0N	0·775	12 ,,	0·360	8 ,,
0·9N	0·743	16 ,,		
0·8N	0·718	17 ,,	0·330	8 ,,
0·6N	0·446	22 ,,	0·234	13 ,,

3 : 5-*Dinitrobenzoic Acid Reagent*

An ethanolic solution of this reagent, mixed with a dilute ethanolic solution of the glycosides, is immediately rendered alkaline in the recommended process for estimation. Some preliminary experiments have been made to investigate the possible influence upon the colour intensity of increase in time of interaction between the dinitrobenzoic acid and the glycosides before rendering alkaline. Using fixed quantities of both digitoxin and decolorised tincture this period of reaction was varied between half a minute and 1 hour, after which sodium hydroxide was added and maximum colour determined by the normal process of estimation. No differences in results were found and it was concluded that sodium hydroxide may conveniently be added immediately after shaking together the dinitrobenzoic acid reagent and the glycosides. It has also been shown that the ethanolic solution of dinitrobenzoic acid is stable for periods up to 7 days if stored in the dark.

TABLE II

VARIATION IN CONCENTRATION OF 3 : 5-DINITROBENZOIC ACID

Dinitrobenzoic acid		Digitoxin k (1 mg.)
2 Per cent. reagent, ml.	Concentration in reaction mixture	
0·5	0·1 per cent.	0·395
1·0	0·2 ,, ,,	0·519
1·5	0·3 ,, ,,	0·629
2·0	0·4 ,, ,,	0·770
2·5	0·5 ,, ,,	0·870
3·0	0·6 ,, ,,	0·878
5·0	1·0 ,, ,,	0·934

No chemical explanation for the colour produced in this reaction has as yet been proved and hence the chemical equivalence between digitoxin and dinitrobenzoic acid is not known, although from the disparity in amounts taken in the reaction mixture it is probable that a very large excess of dinitrobenzoic acid is present. The influence of varying the

concentration of dinitrobenzoic acid used in the colorimetric estimation of the same weight of digitoxin has been investigated by employing different quantities of a 2 per cent. solution of this reagent in ethanol, additional ethanol being added to give 9 ml. of reaction mixture before making alkaline. The same amount of sodium hydroxide has been used in each reaction and no attempt was made to maintain the same excess of alkali in each estimation. Apparent k (1 mg.) values are set out in Table II. The results show that there is a linear increase in colour density for the same weight of digitoxin, with increase in dinitrobenzoic acid content of reaction mixture over the range 0·1 to 0·5 per cent. (0·5 to 2·5 ml. of 2 per cent. reagent solution); the increase is less marked for higher levels of dinitrobenzoic acid concentration. Variation in the accuracy of preparing this reagent solution would thus be somewhat less marked at such higher concentrations but at any concentration investigated in this work it is essential that the solution be prepared by accurate weighing. Under these conditions the previously used level of 0·4 per cent. dinitrobenzoic acid in the reaction mixture (i.e., 2 ml. of 2 per cent. solution or 5 ml. of 0·8 per cent. solution) has been considered to be satisfactory.

TABLE III

TEMPERATURE OF 3:5-DINITROBENZOIC ACID REACTION

Temperature	Time of maximum colour development	Digitoxin k (1 mg.)	Decolorised tincture k (1 ml.)
5° C.	14 minutes	1·030	0·462
10°	14 ,,	0·919	0·449
15°	10 ,,	0·845	0·399
20°	7 ,,	0·778	0·355
25°	5 ,,	0·736	0·316
30°	2 ,,	0·580	0·282

Temperature of Reaction

In order to investigate the influence of temperature upon maximum colour density and time of its development in the recommended process of estimation, a temperature-controlled room was employed and the reaction was carried out at temperatures ranging from 5° to 30° C., using both digitoxin and decolorised tincture. The spectrophotometer and all reagents were allowed to stand for one hour at each temperature before carrying out the estimation. Results are given in Table III. It was found that the colour density for the same weight of glycoside decreased in linear proportion with increase in temperature throughout the temperature range. The development of maximum colour was much speeded up at higher temperatures but colour density values, measured at a fixed time interval of 5 minutes after making alkaline, were found to vary for reactions proceeding at different temperatures. The present results, taken at the time of maximum development, are considered to be the correct indication of the behaviour of the reaction at different temperatures. Because of the rapid colour development at 30° C. the k (1 mg.) value for digitoxin is low in relation to the behaviour of the reaction at lower temperatures.

Leaf Extraction

Earlier work has been based upon 1 in 10 tinctures prepared from powdered digitalis leaf using 70 per cent. ethanol and macerating with gentle agitation for a period of 48 hours[2]. A preliminary investigation, using the dinitrobenzoic acid method of estimation, was made to examine the efficacy of 70 per cent. ethanol as an extraction solvent when the periods of maceration were varied. In each experiment 1 in 10 tinctures were prepared by maceration with gentle agitation, employing a powdered leaf sample containing 11·1 I.U./g. of activity, and using the mechanical device previously described[3]. This consists of a suitably mounted cycle wheel carrying wooden racks on which 60 ml. bottles may be clamped, driven through a chain of gears by a low-speed electric motor fitted with a variable resistance. Macerations were carried out for periods of 72, 48, 42, 36, 30, 24, 18, 12 and 6 hours, also for 5, 4, 3 2, and 1 hour; finally, periods of maceration of 50 minutes, 40 minutes, 30 minutes, 20 minutes and 10 minutes were examined. Estimations of these tinctures showed no significant differences between those prepared by 40 minutes and by 72-hour macerations and concordance of duplicates was always obtained.

TABLE IV

EXTRACTION OF LEAF SAMPLES
3:5-DINITROBENZOIC ACID ESTIMATION

Activity compared with Standard Preparation of powdered digitalis

Leaf sample	Equivalent I.U./g.		Leaf sample	Equivalent I.U./g.	
	1-hr. maceration	48-hr. maceration		1-hr. maceration	48-hr. maceration
L.S.	13·2	13·8	33	12·2	12·5
2	13·3	13·3	101	10·3	10·3
5	14·3	15·6	102	10·0	10·1
7	13·5	13·2	103	9·0	9·0
8	12·4	12·4	104	9·7	9·8
26	10·3	11·3	140	11·1	11·0

Values for 30 minute, 20 minute and 10 minute macerations were somewhat more variable between duplicates but their averages showed the presence of 1·02 I.U./ml. of activity. A tincture prepared by maceration for one hour without agitation was shown to contain 1·07 I.U./ml. of activity, which is only slightly below the mean value for all estimations upon tinctures prepared by agitation.

The above results were considered to indicate that a 1-hour period of maceration was as efficient as the 48-hour period prescribed by the British Pharmacopœia for the preparation of Tincture of Digitalis. This was more fully investigated by preparing tinctures from 12 different leaf samples by both the 1-hour and the 48-hour maceration processes and these were estimated chemically, the results being given in Table IV and in Figure 1. Further confirmation was established by means of both chemical and biological methods of estimation applied to 1-hour and 48-hour tinctures prepared from 6 further leaf samples. The results are given in Table V and in Figure 2.

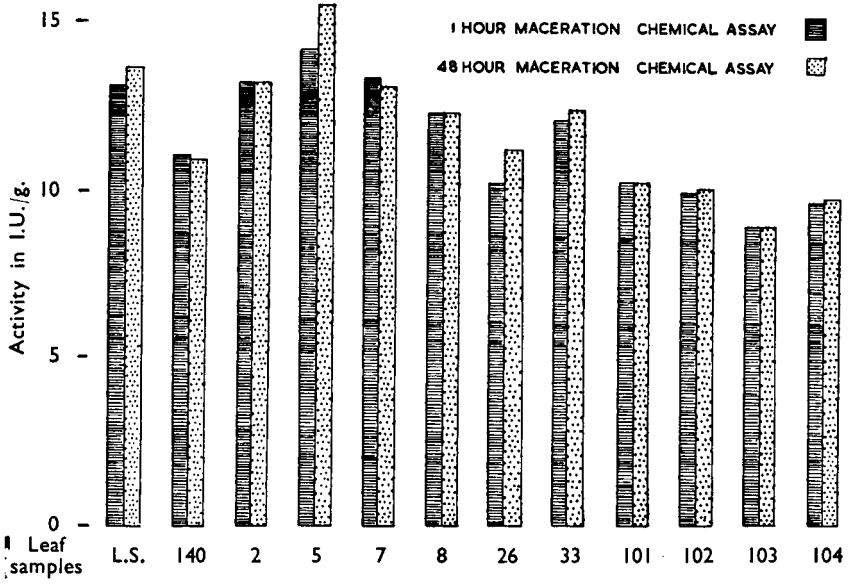

FIG. 1. Leaf extraction with 70 per cent. ethanol. 1 hour and 48 hour maceration.

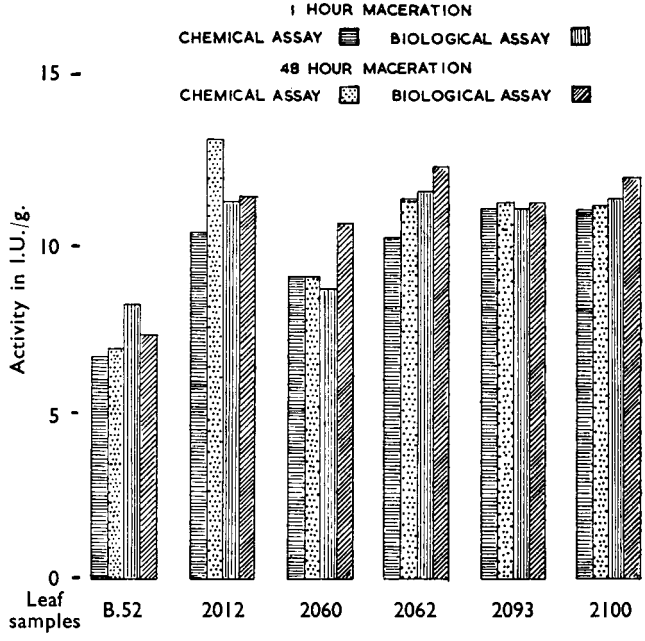

FIG. 2. Leaf extraction with 70 per cent. ethanol. 1 hour and 48 hour maceration.

TABLE V

EXTRACTION OF LEAF SAMPLES
1-hour and 48-hour maceration
Activity by chemical and biological estimations

Method of estimation	Chemical, equivalent I.U./g.		Biological, I.U./g.	
Period of maceration	1 hour	48 hour	1 hour	48 hour
Leaf B.52	6·7	6·9	8·2	7·3
2012	10·4	13·2	11·3	11·5
2060	9·1	9·1	8·7	10·7
2062	10·2	11·4	11·6	12·4
2093	11·1	11·3	11·1	11·3
2100	11·1	11·2	11·4	12·0

Application of Method

Using the 1-hour maceration process for leaf extraction, a number of samples have been investigated by the chemical method outlined in this paper and by the intravenous guinea-pig method in order to determine the comparability of results obtained by the two processes of evaluation. The results are given in Table VI. The leaf samples in column 1 were cultivated on four separate sites and were collected, dried and stored under controlled conditions. Leaf samples in column 4 were commercial specimens: the tincture recorded in this column was prepared from a leaf sample grown, collected and dried under controlled conditions and the purpose of that experiment was to show the behaviour of the tincture after standing for a period of 5 days from the date of its preparation.

TABLE VI

LEAF ESTIMATIONS
(1-hour maceration)
CHEMICAL AND BIOLOGICAL METHODS

	Method of estimation			Method of estimation	
Leaf sample	Chemical, equivalent I.U./g.	Biological, I.U./g.	Leaf sample	Chemical, equivalent I.U./g.	Biological, I.U./g.
A.2026	14·3	13·9	MS.31	15·9	14·6
A.2100	8·3	8·6	,, 32	14·9	15·4
B.2005	10·6	12·2	,, 33	15·1	14·1
B.2063	8·0	9·4	,, 41	16·9	15·4
M.2064	14·7	16·2	,, 42	15·1	14·5
M.2150	9·4	10·7	Tincture (fresh)	1·38	1·19
S.2005	10·0	12·9	,, (5 days)	1·35	1·12
S.2150	14·8	16·7			

DISCUSSION

The present detailed investigation of the behaviour of the dinitrobenzoic acid reaction for the estimation of digitoxin or of decolorised tinctures of digitalis has shown that the method is efficient and accurate when the conditions of its application are fully controlled. The concentration of the dinitrobenzoic acid reagent influences the colour density produced by a given weight of glycoside and in consequence the reagent must be accurately prepared. If stored in the dark the reagent may be

used for 7 days after its preparation. Despite its presence in great excess, the concentration of sodium hydroxide in the reaction mixture also influences the intensity of colour produced by the same weight of glycosides, other conditions in the reaction being the same, and hence the amount of alkali employed must be carefully controlled. Moreover, the rate of reaction is increased at higher temperatures but the intensity of maximum colour produced is decreased with increase of temperature. It is recommended that a temperature of 20° C. be employed for all estimations of digitoxin or of decolorised tincture of digitalis by the dinitrobenzoic acid process and that the exact concentrations and quantities of reagents be those previously described[1].

The extraction studies of powdered leaf using 10 volumes of 70 per cent. ethanol as solvent have demonstrated that a 1-hour period of maceration with gentle agitation is sufficient to extract the total glycosides. This efficiency of extraction has been shown by both chemical and biological methods of estimation upon a considerable number of leaf samples and it is recommended that the 1-hour maceration process be employed for future assay work.

The figures presented in Table VI are a further confirmation of the fact that the dinitrobenzoic acid method of estimation yields results which are similar to those given by the biological method of estimation when applied to leaves that have been cultivated, collected and dried under controlled conditions, also when applied to a further number of commercial samples.

The examination of a tincture immediately after preparation by 1-hour maceration and again after standing in the laboratory at normal temperature for a period of 5 days has shown that there is little variation in tincture potency during this period. The detailed investigation of changes in potency in digitalis tincture will be reported in a later paper. This present work demonstrates, however, a method which may be employed to ensure the availability of Tincture of Digitalis of standard potency for dispensing purposes. Small quantities of this tincture may be prepared extemporaneously in the pharmacy from standardised powdered digitalis leaf using the 1-hour maceration process, and this material will retain its potency at least for a period of 5 days after preparation.

Summary and Conclusions

1. The 3 : 5-dinitrobenzoic acid process for the estimation of digitoxin or of decolorised tinctures of digitalis should be carried out at a controlled temperature; 20° C. is convenient.

2. The reagents employed in the reaction should be standardised analytically.

3. Powdered digitalis leaf is completely extracted by maceration with gentle agitation for a period of 1 hour using 10 volumes of 70 per cent. ethanol.

4. A parallelism between the results obtained by both the 3 : 5-dinitrobenzoic acid and the biological methods of estimation has been

demonstrated, using 14 leaf samples of *Digitalis purpurea* cultivated and dried under controlled conditions and also using 5 commercial leaf samples.

5. Tincture of digitalis prepared by 1-hour maceration has been shown to retain its potency for at least 5 days and it is recommended that such a preparation be made extemporaneously from standardised leaf powder when tincture of digitalis is prescribed.

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References

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