COLORIMETRIC ESTIMATION OF DIGITALIS GLYCOSIDES WITH 2:4-DINITRODIPHENYLSULPHONE

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Received October 25, 1957

The reaction is carried out as follows. The glycoside or aglycone, dissolved in 4 ml. of ethanol, is mixed with 5 ml. of a 0·075 per cent solution of 2:4-dinitrodiphenylsulphone in ethanol followed by 1 ml. of 0·15N sodium hydroxide. Colour density is measured in a 0·5 cm. cell at 20° at a wavelength of 6,000 Å against a blank prepared as the reaction mixture but omitting the glycoside or aglycone. Maximum extinction values of digitoxigenin and of gitoxigenin, boiled with 0·4N acid, are obtained 3 minutes after adding the sodium hydroxide to the reaction mixture. When digitoxin or digitoxigenin are examined without any pre-treatment with boiling acid, maximum colour is obtained 5·5 and 4·5 minutes respectively after addition of the alkali. Molar extinctions of digitoxin and of digitoxigenin are 23,200 and 24,400 respectively: after boiling with 0·4N acid the molar extinctions of digitoxigenin and gitoxigenin are 29,100 and 18,100 respectively. The values are much higher than those obtained with other reagents. A reduction in the ethanol concentration of the reaction mixture only exerts a slight influence on the maximum extinction.

Most of the colour reactions for digitalis glycosides are based on the aglycone part of the molecule, especially the butenolide side chain. A number of reagents such as sodium nitroprusside[1], sodium β-naphthoquinone-4-sulphonate[2], *m*-dinitrobenzene[3], 2:5-dinitrobenzoic acid[4] and trinitrobenzene[5] have been recommended as reagents, but picric acid[6] and 3:5-dinitrobenzoic acid[7] are most frequently used. All of these reactions are carried out in alkaline solution.

In the assay of digitalis leaves the isolated glycosides are always accompanied by a yellow pigment, digitoflavone. This substance reacts with sodium hydroxide to give a yellowish-brown colour and this may interfere with the quantitative estimation. Figure 1 shows the absorption curves of aqueous or diluted alcoholic extracts prepared from the same weights of one sample of digitalis leaf and treated with sodium hydroxide. It will be seen that by the addition of sodium hydroxide to the diluted tincture (curve II) the light absorption increases. This must be due to the digitoflavone. Curve I for diluted tincture is nearly identical with that given by Rowson[8]. Curve III demonstrates that water extracts more pigment from the leaf than does ethanol.

Generally only part of the digitoflavone present in the leaf remains in the reaction mixture during a colorimetric assay. However, it may be seen that if this component is not completely removed, too high extinction values may be obtained, especially when absorption values are measured at about 5,000 Å as in the picric acid reaction. A search was thus made to find a reagent which produces a colour with digitalis glycosides in small amounts and with a maximal light absorption at about 6,000 Å. *m*-Dinitrobenzene gives a blue colour with digitalis glycosides and was

used by Canbäck[9]. The disadvantage of the reagent is that maximum colour intensity is produced at the moment of addition of sodium hydroxide and the procedure proposed by Canbäck is too time consuming for serial work. Moreover the molar extinction for digitoxin is not very high, being 11,100.

Of a number of different reagents investigated 2:4-dinitrodiphenylsulphone gave satisfactory results. The synthetic product had a melting point of 157·4°–158·2°; its solubility in ethanol is not great. The corresponding sulphide and sulphoxide do not react with digitalis glycosides. The reaction was carried out using aldehyde-free ethanol prepared by the method of U.S.P. XIII. The glycoside was dissolved in 4 ml. of ethanol, 5 ml. of solution of 2:4-dinitrodiphenylsulphone in ethanol added followed by 1 ml. of aqueous sodium hydroxide solution. The colour density of the solution was determined in a 0·5 cm. cell at a wavelength of 6,000 Å against a blank of 4 ml. of ethanol, 5 ml. of reagent and 1 ml. of sodium hydroxide solution. The spectral band width was 45 Å and the measurements were made with a spectrophotometer (Bleeker, Holland).

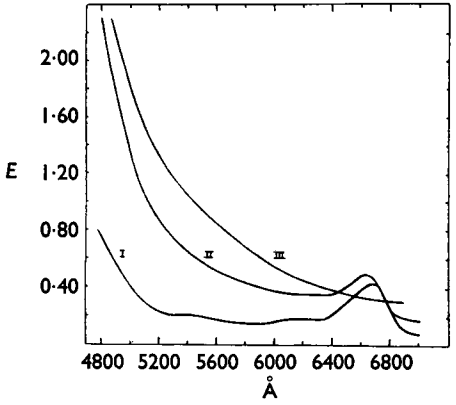

FIG. 1. Absorption curves of digitalis extractions with or without added sodium hydroxide. Curve I. Digitalis tincture (prepared with 70 v/v per cent ethanol), 5 times diluted with water. Curve II. Digitalis tincture, diluted with water and mixed with sodium hydroxide. Curve III. Aqueous digitalis macerate, after filtration, mixed with sodium hydroxide.

Influence of Concentrations of 2:4-Dinitrodiphenylsulphone and Sodium Hydroxide on Maximum Extinction

Solutions were made containing respectively 15, 30, 45, 60, 75 and 90 mg. of 2:4-dinitrodiphenylsulphone per 100 ml. The reaction was performed with 0·400 mg. of digitoxin (Uclaf). The concentration of the sodium hydroxide was 0·1 N (Figure 2) (curve 1), 0·25 N (curve 2) or 0·5 N (curve 3). Figure 2 shows that for each of the three concentrations of sodium hydroxide used the *E* value increases with increase in concentration of 2:4-dinitrodiphenylsulphone up to a level of 75 mg./100 ml., but that only small increases in *E* values are achieved with higher concentrations of the reagent. This is also confirmed by employing 9 ml. of this reagent instead of 4 ml. of ethanol and 5 ml. of reagent when the *E* value is only slightly raised. At a concentration of 100 mg./100 ml. the solution is saturated. In the light of these findings a concentration of 75 mg./100 ml. was selected for this reagent.

It is also seen from Figure 2 that the concentration of sodium hydroxide influences colour density. It also influences the velocity of the reaction

and this was further investigated. An increase in normality above 0·15 does not increase the *E* value but only increases the velocity of reaction as shown in Table I. Thus a concentration of the sodium hydroxide of 0·15 N is recommended.

Absorption Curve

The absorption curve was determined using 0·543 mg. of digitoxin (Uclaf), a concentration of 75 mg./100 ml. of 2 : 4-dinitrodiphenylsulphone

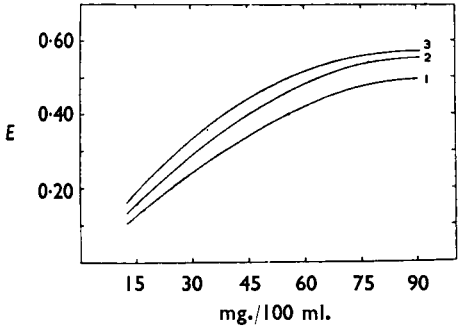

FIG. 2. The influence of the concentrations of 2:4-dinitrodiphenylsulphone and sodium hydroxide on maximum extinction. Curve 1. 0·10 N sodium hydroxide. Curve 2. 0·25 N sodium hydroxide. Curve 3. 0·50 N sodium hydroxide.

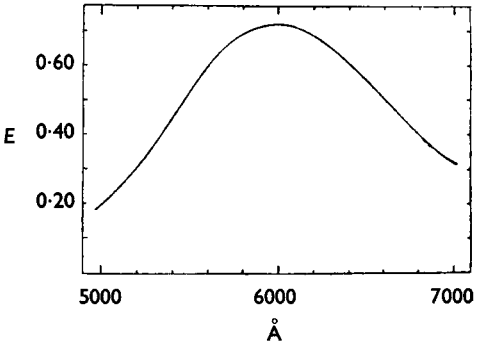

FIG. 3. The absorption spectrum of 0·543 mg. digitoxin. Concentration of 2:4-dinitrodiphenylsulphone 75 mg./100 ml.; 0·15 N sodium hydroxide.

and 0·15 N sodium hydroxide, extinctions were determined each 100 Å or, if necessary, each 50 Å. From Figure 3 it is seen that maximum colour density is obtained at a wavelength of 6,000 Å.

Variations in the Blank

The stability of the blank over a period of 2 hours was determined by measuring the colour density of a mixture of 4 ml. of ethanol, 5 ml. of reagent and 1 ml. of 0·15N sodium hydroxide against a mixture of 9 ml.

TABLE I

THE INFLUENCE OF THE NORMALITY OF SODIUM HYDROXIDE ON THE REACTION BETWEEN 2:4-DINITRODIPHENYLSULPHONE AND DIGITOXIN

Normality of sodium hydroxide	Maximal extinction	Attained after 'minutes'	Remains constant 'minutes'
0·05	0·40[5]	9	2
0·10	0·45[5]	6·5	1
0·15	0·48	5·5	1
0·20	0·48[5]	3·5	0·5
0·25	0·49	2·5	–

of ethanol and 1 ml. of 0·15N sodium hydroxide, at 6,000 Å in a 1·0 cm. cell. Table II shows that the colour of the blank does not change significantly and after 2 hours its value has little influence on results.

Stability of Reagent

From Table III it appears that the reagent can be stored in the dark for several days without deterioration.

Influence of Water

The same amount of digitoxin was dissolved in 17·5, 25·0, 50·0, 75·0 and 96·0 per cent ethanol respectively and the colour reaction was carried out as described. Table IV shows that there is only about a 5 per cent

TABLE II

THE INFLUENCE OF THE TIME ON THE COLOUR OF THE BLANK

Extinctions ($\times 10^3$)	0	0	2	4	5	6	7	9	9	10	10	10
Measured after (minutes)	1	5	10	15	20	25	30	40	60	80	100	120

decrease in value of E when the ethanol level of the glycoside solution is reduced from 96 to 17·5 per cent. At the lower level of alcohol the time required for development of maximum colour is increased by 1 minute.

Influence of Temperature

Solutions were maintained in a thermostat for 45 minutes at temperatures of 15°, 20°, 25° and 30°, the reaction mixture, when prepared, was allowed to stand for 1 minute at the same temperature and the extinction was

TABLE III

THE INFLUENCE OF THE TIME ON THE REAGENT

Extinctions	0·48	0·47[s]	0·47[s]	0·47	0·47	0·48
After days	0	1	3	4	6	11

determined immediately. Results are given in Table V. It will be seen that the maximum E value decreases with increase of temperature, but that the time of attaining that maximum is decreased. It is recommended that the reaction be carried out at a temperature of 20°, and that higher temperatures be avoided.

TABLE IV

THE INFLUENCE OF WATER ON THE EXTINCTION

Ethanol v/v per cent	Extinction	Maximum attained after (minutes)
17·5	0·45[s]	6·5
25·0	0·47	6·5
50·0	0·47[s]	6
75·0	0·47[s]	5·5
96·0	0·48	5·5

Method of Estimation

It was concluded from the foregoing that the estimation of digitalis glycosides should be carried out as follows. The glycoside or aglycone is dissolved in 4 ml. of ethanol, 5 ml. of a 0·075 per cent solution of dinitrodiphenylsulphone in ethanol is added followed by 1 ml. of 0·15N sodium hydroxide. The colour which develops is measured in a 0·5 cm.

cell at a wavelength of 6,000 Å against a blank of 4 ml. of ethanol with 5 ml. of reagent and 1 ml. of 0·15N sodium hydroxide. Spectral band width 45 Å; temperature of reaction must be maintained at 20°. The time required for the development of maximum colour intensity is not the same for some of the digitalis glycosides.

The reaction was applied to the estimation of digitoxin (Merck), digitoxigenin (Hoffmann–La Roche) and gitoxigenin (Sandoz). The same samples were also examined after boiling with acid in the following

TABLE V

THE INFLUENCE OF THE TEMPERATURE ON THE REACTION

Temperature °C.	Extinction	
	Time of development (minutes)	Maximum
15·0	7	0·51[0]
20·0	5·5	0·49[8]
25·0	4·5	0·47[6]
30·0	3·5	0·45[2]

manner; the glycoside or aglycone is dissolved in 5 ml. of ethanol, 2 ml. of 4N HCl and 13 ml. of water added (final acid concentration 0·4N) and the mixture is boiled under reflux for 30 minutes. The contents of the flask are then transferred to a separating funnel, the reflux condenser and flask washed twice with 5 ml. of 25 per cent ethanol which are also transferred to the separating funnel. The acid product and washings are then shaken out with three successive quantities each of 22·5 ml. of chloroform, each shaking being for 1 minute. The combined chloroform extracts are dried with exsiccated sodium sulphate, after which they are filtered, the filter and flask washed with 5 ml. of dry chloroform and the solvent is distilled off.

It was found that the extinction coefficients of the two aglycones were increased by this process of boiling with acid. Smithuis[10] has shown that this increase is connected with the formation of anhydro compounds; gitoxigenin is converted into dianhydrogitoxigenin and digitoxigenin is converted into a monoanhydro compound.

When graphs were plotted of colour extinctions against glycoside or aglycone concentrations of solutions used, they were found to be straight lines for digitoxin and for digitoxigenin whether pre-treated with acid or not. Gitoxigenin, if pre-treated with acid also gave a straight line; but if not so pre-treated the graph was a curve with molar extinctions diminishing from 12,500 to 10,700.

Maximum colour intensity is developed after the addition of the sodium hydroxide in 5·5 minutes for digitoxin, in 4·5 minutes for digitoxigenin and in 3 minutes for these two substances or for gitoxigenin after acid treatment. The maximal colour value is constant for 1 or 1½ minutes.

Table VI records the extinction coefficients and molar extinctions of some digitalis glycosides with this new reagent. These values are also compared with those obtained when using other quantitative reagents, viz. picric acid (Baljet reagent), 3:5-dinitrobenzoic acid (Kedde reagent) and *m*-dinitrobenzene (Raymond reagent). Standard deviations for the new

reagent are also recorded. The Tattje modification of the 3:5-dinitrobenzoic acid process[11] yields a higher value for digitoxin and this is also indicated.

TABLE VI

EXTINCTION COEFFICIENTS AND MOLAR EXTINCTIONS OF SOME DIGITALIS GLYCOSIDES OBTAINED WITH 2:4-DINITRODIPHENYLSULPHONE, COMPARED WITH THOSE OBTAINED WITH PICRIC ACID, 3:5-DINITROBENZOIC ACID AND *m*-DINITROBENZENE

Glycoside of aglycone	Treatment	2:4-Dinitrodiphenylsulphone		Picric acid		3:5-Dinitrobenzoic acid		*m*-Dinitrobenzene
		E (1 per cent, 1 cm.)	Mol. ext.	*E* (1 per cent, 1 cm.)	Mol. ext.	*E* (1 per cent, 1 cm.)	Mol. ext.	Mol. ext.
Digitoxin	none	303 s. d.= 3·22	23,200 s. d. =246	222·5 190[13]	17,000 14,500[13]	111[11] 77.8[12] 77·5[14]	8500[11] 5940[12]	11,110[9] 14,700[12]
Digitoxin	boiled with acid	370 s. d.= 2·73	28,300 s. d.= 209	230	17,600	106·6[12]	8140[12]	
Digitoxigenin ..	none	653 s. d.= 6·33	24,400 s. d.= 237	460	17,200	169·8[12] 255	6360[12] 9540	10,720[14]
Digitoxigenin ..	boiled with acid	778 s. d.= 4·55	29,100 s. d.= 170	486	18,200	230·9[12] 329	8640[12] 12,300	
Gitoxin	boiled with acid	232	18,100	200	15,600	100·1[12]	7810[12]	
Gitoxigenin ..	none	ca. 314		346	13,500	187	7290	3920[9]
Gitoxigenin ..	boiled with acid	464 s. d.= 3·29	18,100 s. d.= 128	379	14,800	286	11,100	

It is seen from Table VI that the 2:4-dinitrodiphenylsulphone reagent is much more sensitive than are the other reagents, especially in the case of digitoxin and digitoxigenin, but also to a lesser extent for gitoxigenin.

Acknowledgements. The author wishes to express his gratitude to Professor Dr. van der Zanden, Groningen, for his help in synthesising the 2:4-dinitrodiphenylsulphone and to acknowledge gratefully gifts of digitoxin, digitoxigenin and gitoxigenin from Uclaf (Paris), Merck (Darmstadt), Sandoz (Basle) and Hoffmann La Roche (Basle) respectively. Thanks are also due to the Netherlands Organisation for Pure Research (Z.W.O.) by whose assistance this work was carried out.

REFERENCES

1. Jacobs, Hoffmann and Gustus, *J. biol. Chem.*, 1926, **70**, 1.
2. Warren, Howland and Green, *J. Amer. pharm. Ass., Sci. Ed.*, 1948, **37**, 186.
3. Morel and Marthoud, *Bull. Soc. chim. Fr.*, 1935, **2**, 949. Raymond, *Analyst*, 1938, **63**, 478; 1939, **64**, 113.
4. Mesnard and Lafargue, *Bull. Soc. pharm. Bordeaux*, 1953, **92**, 160.
5. Kimura, *J. pharm. Soc. Japan*, 1951, **71**, 991; C.A., 1952, **46**, 6325.
6. Baljet, *Pharm. Weekbl.*, 1918, **55**, 457.
7. Kedde, *ibid.*, 1947, **82**, 741.
8. Rowson, *J. Pharm. Pharmacol.*, 1952, **4**, 831.
9. Canbäck, *Svensk Farm. Tidskr.*, 1947, **51**, 261; 1950, **54**, 201.
10. Smithuis, *Pharm. Weekbl.*, 1956, **91**, 253.
11. Tattje, *J. Pharm. Pharmacol.*, 1957, **9**, 29.
12. Langejan, *Pharm. Weekbl.*, 1951, **86**, 593.
13. Rowson, *J. Pharm. Pharmacol.*, 1952, **4**, 814.
14. Rowson, *ibid.*, 1955, **7**, 924.