THE REACTION BETWEEN DIGITOXIN AND 3:5-DINITROBENZOIC ACID*

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Received October 21, 1955

3:5-DINITROBENZOIC acid was first used as a reagent by Bollinger[1] and by Benedict and Behre[2] for the determination of creatinine. Since, under certain conditions, the presence of digitoxin could be detected in a similar manner to that of creatinine, Kedde[3] investigated the use of 3:5-dinitrobenzoic acid for the estimation of digitoxin and of other cardiotonic glycosides. Langejan[4] and Rowson[5] have also used this reagent in a similar manner to that of Kedde. Pratt[6] used a quaternary base in place of sodium hydroxide in this reaction.

The reagent in ethanolic solution is mixed with a solution of the glycoside in dilute ethanol, standard solution of sodium hydroxide in fixed amount is then added and the intensity of the colour, which develops after a short time, is measured at a wavelength ranging between 5350 and 5500 Å. The final concentration of 3:5-dinitrobenzoic acid is usually 0·4 per cent, that of sodium hydroxide is 0·4 per cent (= 0·1N) and that of ethanol is 52 to 58 per cent. The majority of workers[3–5] have used the same concentrations of reagents. In general it has been recognised that, in order to obtain good results, it is necessary to control both the temperature of the reaction and the time allowed for colour development, also colour change in the blank has been noted. Precise details of these matters, however, are infrequently presented.

We have considered it desirable to study the optimum concentrations and conditions for carrying out this reaction. We have examined the influence of the solvent, the concentration of 3:5-dinitrobenzoic acid, the concentration of sodium hydroxide, and the amount of water in the reaction mixture. The reaction was always carried out as follows: digitoxin 0·4 mg. was dissolved in 4 ml. of solvent either with or without the addition of water, then 5 ml. of solution of 3:5-dinitrobenzoic acid in the same, undiluted solvent was added. After standing, 1 ml. of standard sodium hydroxide solution was added, the time at which the addition was made being noted. The extinction of the reaction mixture was measured at 5400 Å with a spectrum band width of 40 Å in a Bleeker (Holland) spectrophotometer.

Solvent

We have used methanol, ethanol and *n*-propanol, undiluted, as solvents for the digitoxin and also with which to prepare both 1 and 2 per cent solutions of 3:5-dinitrobenzoic acid; 1, 1·5 and 4N sodium hydroxide

* Paper read at the Medicinal Plants sub-Section of the London meeting of the Fédération Internationale Pharmaceutique on Thursday, September 22, 1955.

solutions were employed. Extinctions were measured one minute after adding the sodium hydroxide and then at half-minute intervals. Results are shown in Figure 1.

From these results it was concluded that *n*-propanol (curve 4) was unsuitable as a solvent, for the maximum extinction in it is low, N sodium hydroxide is adequate and an increase in its concentration greatly reduces the extinction values. Moreover, this solvent does not readily mix with water and the addition of more than 1 ml. of an aqueous solution is difficult.

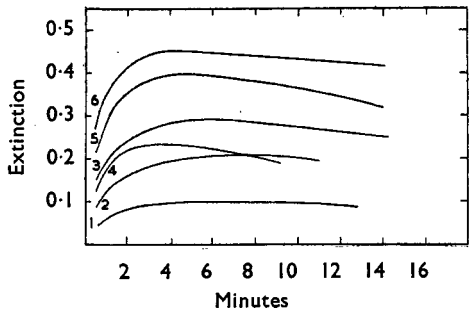

FIG. 1. Influence of solvent on colour development.

Curve 1: methanol, 2 per cent 3:5-dinitrobenzoic acid, 1·5N sodium hydroxide.

Curve 2: methanol, 1 per cent 3:5-dinitrobenzoic acid, 4N sodium hydroxide.

Curve 3: methanol, 2 per cent 3:5-dinitrobenzoic acid, 4N sodium hydroxide.

Curve 4: *n*-propanol, 1 per cent 3:5-dinitrobenzoic acid, 1N sodium hydroxide.

Curve 5: ethanol, 1 per cent 3:5-dinitrobenzoic acid, 1·5N sodium hydroxide.

Curve 6: ethanol, 2 per cent 3:5-dinitrobenzoic acid, 1·5N sodium hydroxide.

Using methanol as solvent the maximum *E* values were much lower than with ethanol; also 4N sodium hydroxide was necessary for optimum reaction (curve 3) and when only 1·5N alkali was used (curve 1) the colour was much less intense.

Ethanol as solvent gave the highest *E* values and for this reason it was used as solvent in the subsequent work.

Concentration of 3:5-Dinitrobenzoic Acid

From Figure 1 it is noted that the concentration of 3:5-dinitrobenzoic acid influences both the maximum extinction value and also the time required for its development. For this reason we have employed this reagent in concentrations of 0·5, 0·75, 1·0, 1·5, 2·0, 2·5, 3·0 and 4·0 per cent in ethanol. At the same time we have varied the normality of the sodium hydroxide solution between 1·0, 1·5 and 2·0N. The curves for maximum extinction values only are shown in Figure 2.

It will be seen that there is a marked increase in maximum values of *E* with increasing concentrations of 3:5-dinitrobenzoic acid reagent up to a 2 per cent reagent. Above that concentration an increase in strength of reagent resulted in maximum *E* values remaining almost the same or decreasing (curves 1 and 4). Since the changes in *E* values are relatively great for small changes in lower concentrations of 3:5-dinitrobenzoic acid, it is desirable to employ as reagent a 2 per cent solution of this acid, giving a final concentration of 1 per cent in the reaction mixture. The influence of 3:5-dinitrobenzoic acid on the time of reaction is not great as shown in Table II.

TABLE I

INFLUENCE OF SODIUM HYDROXIDE CONCENTRATION ON EXTINCTION AND ON RATE OF REACTION

1 per cent 3:5-Dinitrobenzoic acid			2 per cent 3:5-Dinitrobenzoic acid		
	Extinction			Extinction	
Sodium hydroxide N	Maximum	After (minutes)	Sodium hydroxide N	Maximum	After (minutes)
0·5	0·14[5]	7	0·5	0·11	7
1·0	0·32	7	1·0	0·40	7
1·5	0·39[5]	4·5	1·5	0·45	4
2·0	0·42	3	2·0	0·46	2·5
2·5	0·42	2	2·5	0·46[5]	2
3·0	0·42	1·5	3·0	0·46	1·5

Normality of Sodium Hydroxide

From Figure 1, curves 1 and 3, it may be seen that in a methanolic reaction mixture an increase in normality of sodium hydroxide resulted in a marked increase in *E* values. Figure 2 shows similar results and, up to a concentration of 2 per cent of 3:5-dinitrobenzoic acid reagent, the normality of the sodium hydroxide is of considerable significance. This is so when either ethanol (curves 1–3) or diluted ethanol (curves 4–6) are used as solvents. Strengths of 1N or lower must be avoided since small variations produce marked changes in *E* values; such changes in *E* values are much smaller when 1·5 or 2N sodium hydroxide solutions are used.

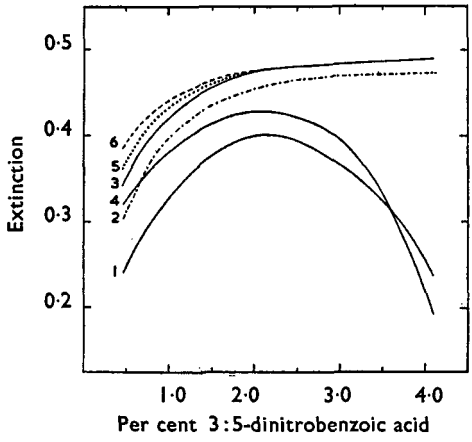

FIG. 2. Influence of concentrations of 3:5-dinitrobenzoic acid, sodium hydroxide and ethanol on maximum extinction.

Curve 1: ethanol 96 per cent, 1N sodium hydroxide.

Curve 2: ethanol 96 per cent, 1·5N sodium hydroxide.

Curve 3: ethanol 96 per cent, 2N sodium hydroxide.

Curve 4: ethanol 17·5 per cent, 1N sodium hydroxide.

Curve 5: ethanol 17·5 per cent, 1·5N sodium hydroxide.

Curve 6: ethanol 17·5 per cent, 2N sodium hydroxide.

Table I records the maximum *E* values and the times taken for their development when using either 1 or 2 per cent 3:5-dinitrobenzoic acid and varying the strength of sodium hydroxide between 0·5 and 3N; ethanol being the solvent for digitoxin. It was concluded that there is an optimum concentration of sodium hydroxide and above 2N the time of colour development is too short for convenience.

In Table II we have recorded, for different concentrations of 3:5-dinitrobenzoic acid and for different normalities of sodium hydroxide,

the times required for development of maximum E values (column A) and the time during which they remain constant (column B); ethanol and diluted ethanol were used as solvents. It will be seen that an increase in normality of sodium hydroxide not only speeds up the development of maximum E values but also reduces the time over which this maximum is maintained.

TABLE II

INFLUENCE OF CONCENTRATIONS OF SODIUM HYDROXIDE AND OF 3 : 5-DINITROBENZOIC ACID ON RATE OF REACTION

Minutes

Solvent	Sodium hydroxide	Concentration of 3 : 5-dinitrobenzoic acid reagent per cent															
		0·5		0·75		1·0		1·5		2·0		2·5		3·0		4·0	
		A	B	A	B	A	B	A	B	A	B	A	B	A	B	A	B
Ethanol 96 per cent	1·0N	8	5	8	2	7	2	7	2	7	2	7	2	7	2	5	1
	1·5N	6	3	5	2	4·5	1·5	4	1	4	1	4	1	4	1	4	1
	2·0N	4·5	1·5	4	1	3	1	3	1	3	1	2·5	1	2·5	1·5	2	1
Ethanol 17·5 per cent	1·0N	11	6	9	5	9	5	9	5	8	5	8	5	8	5	8	5
	1·5N	7	4	6	4	6	3	6	3	6	3	6	3	6	3	6	2
	2·0N	6	3	4·5	2·5	4	2	4	1	4	1	4	1	4	1	4	1

A = Time for development of maximum extinction
B = Time during which maximum extinction is a constant

Influence of Water

When the ethanol concentration of the reaction mixture is reduced, it influences the E values. Thus in Figure 2 we see that E values are greater when using 17·5 per cent ethanol than when using 96 per cent ethanol (curves 1 and 4, 2 and 5, 3 and 6). This effect is more pronounced when the concentration of 3 : 5-dinitrobenzoic acid is low and it is less pronounced when using this reagent in a 2 per cent solution; using the more dilute ethanol the rate of reaction is also slowed down and the maximum extinction is maintained for a longer time also, especially when using the weaker solution of 3 : 5-dinitrobenzoic acid.

In all the variations of the process of estimation described above we have plotted the absorption curve of the colour produced and the maximum extinction was always obtained at a wavelength of 5400 Å.

From the foregoing it was concluded that the following conditions should be observed in the reaction: the 3 : 5-dinitrobenzoic acid reagent should be of 2 per cent concentration (giving a 1 per cent concentration in the final reaction mixture); the sodium hydroxide solution should be 1·5N (0·15N = 0·6 per cent in the final reaction mixture); the digitoxin may be dissolved in either ethanol or diluted ethanol since the concentration of ethanol has almost no influence on results under these conditions. Using this reaction mixture and dissolving the digitoxin in ethanol for reason of convenience, we have investigated the influence of temperature upon this reaction, the stability of the reagent, the variations in the colour of the

blank during the assay and also the possible influence of different concentrations of digitoxin.

Influence of Temperature

Solutions were maintained in a thermostat for 30 minutes at temperatures of 15°, 20°, 25° and 30°; the reaction mixture when prepared was allowed to stand for one minute at the same temperature and the extinction was determined immediately. Results are given in Table III. It is seen that *E* values decrease with increase in temperature, especially at 25 and 30° a change of 10 per cent being recorded. The rate of reaction is less influenced. It is recommended that this reaction be carried out at 20° and that higher temperature be avoided.

TABLE III

INFLUENCE OF TEMPERATURE ON EXTINCTION

Temperature degrees	Extinction	
	Time of development minutes	Maximum
15	4	0·44[3]
20	4	0·44
25	4	0·42[5]
30	3	0·40[5]

Stability of Reagent

Since a divergence of opinion exists as to the necessity of preparing a fresh solution of 3:5-dinitrobenzoic acid, we have investigated the problem. Samples of 0·4 mg. digitoxin were estimated on successive days with the same 2 per cent solution of 3:5-dinitrobenzoic acid in ethanol, the reagent being stored away from light. From Table IV it will be seen that the reagent may be stored for a number of days without exerting any significant variation in the results.

TABLE IV

INFLUENCE OF AGE OF REAGENT ON EXTINCTION

Age in days	0	2	3	4	5	6	7	11	14	18	21
Extinction	0·44[3]	0·44	0·43[7]	0·44	0·44	0·44[5]	0·44[3]	0·45[5]	0·46[3]	0·46[7]	0·46

Variations in the Blank

During these researches it was observed that the colour of the blank tended to darken, for in each assay a freshly prepared blank has been used. The changes in colour of a blank consisting of 5 ml. of reagent, 4 ml. ethanol and 1 ml. of 1·5N sodium hydroxide were measured against a mixture of 9 ml. ethanol and 1 ml. of 1·5N sodium hydroxide, readings being taken every 10 minutes at 5400 Å. From Table V it is seen that the colour of the blank does deepen after some time, thus if one blank were used for successive estimations the results would be too low. Hence the preparation of a fresh blank for each estimation is necessary.

TABLE V

CHANGE IN COLOUR OF BLANK WITH TIME

Time in minutes	1	10	20	30	40	50	60	70	75
Extinction	0·04	0·04[5]	0·05[3]	0·06[1]	0·07	0·07[7]	0·08[3]	0·08[9]	0·09[3]

Influence of different Concentrations of Digitoxin

When the reaction was carried out using 0·2, 0·4, 0·6 and 0·8 mg. digitoxin the time for development of maximum colour remained the same and was four minutes from the addition of the sodium hydroxide. Absorption curves for each quantity of digitoxin are plotted in Figure 3 from which it is seen that maximum E values are always obtained at 5400 Å. The reaction obeys the Beer-Lambert law and E(1 per cent, 1 cm.) = 111 ± 1·5.

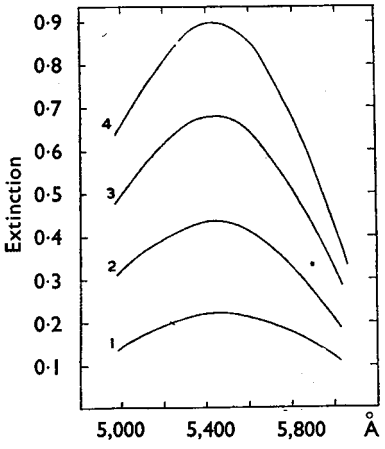

FIG. 3. Absorption curves for digitoxin.

Curve 1: 0·2 mg.; 2: 0·4 mg.; 3: 0·6 mg.; 4: 0·8 mg. digitoxin.

SUMMARY AND CONCLUSIONS

Digitoxin may be estimated by means of 3:5-dinitrobenzoic acid under the following conditions:

1. Digitoxin, up to 0·8 mg., dissolved in 4 ml. ethanol, is mixed with 5 ml. of a 2 per cent solution of 3:5-dinitrobenzoic acid in ethanol, 1 ml. of 1·5N sodium hydroxide is then added and the colour intensity measured in a 1 cm. cell at a wavelength of 5400 Å exactly four minutes after the addition of the alkali.

2. The reaction obeys the Beer-Lambert law: E(1 per cent, 1 cm.) = 111 ± 1·5.

3. The reaction should be carried out at a temperature of 20°, a reduction to 15° exerts no influence, above 25° a diminution in E values results.

4. A fresh blank should be made for each estimation.

5. A diminution in ethanol content of reaction mixture exerts only slight influence on maximum extinction or on rate of reaction.

6. Solutions of 3:5-dinitrobenzoic acid in ethanol may be stored for several days without deterioration.

We wish to thank UCLAF (Paris) for the supply of digitoxin used in these researches. Thanks are also due to the Netherlands Organisation for Pure Research (Z.W.O.) by whose assistance this work was carried out.

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