ESTIMATION OF DIGOXIN AND DIGITOXIN IN *DIGITALIS LANATA*

BY P. TANTIVATANA* AND S. E. WRIGHT

From the Pharmacy Department, University of Sydney, Sydney, Australia

Received December 2, 1957

Digoxin and digitoxin present in extracts of dried leaves of *Digitalis lanata* have been estimated colorimetrically with xanthydrol after paper chromatographic separation on formamide-impregnated paper, using methyl *iso*butyl ketone-*iso*propyl ether-formamide as developing solvent. The method is reasonably rapid, both glycosides may be estimated on the same chromatograph, and it may be used for the analysis of crystalline glycosides.

THE quantitative estimation of digitoxin in *Digitalis purpurea* using paper chromatographic methods of separation followed by fluorimetric or colorimetric analysis has been investigated by Jensen[1] and by Sellwood[2], but no assay of this type for the glycosides present in *Digitalis lanata* has yet been published.

Freshly gathered leaves of *Digitalis lanata* yield negligible amounts of desglucoglycosides if they are extracted without allowing enzymatic action to occur[3,4] and in order to estimate the potential digoxin or digitoxin content of the plant it is necessary to ensure that enzymatic hydrolysis of the lanatoside precursors takes place. To obtain a rapid assessment of the digitoxin content of a crop, Sellwood[2] allowed freshly gathered leaves of *D. purpurea* to undergo fermentation at 37° in the presence of water for 72 hours before extracting the glycosides with propylene dichloride. In this investigation, however, we have estimated the digoxin and digitoxin content of air-dried leaves of *D. lanata* as used in commerce, enzymatic hydrolysis of the lanatosides having apparently occurred during the long period of drying. Maceration of the dried leaves with several quantities of 20 per cent ethanol followed by chloroform extraction of the aqueous-ethanol solution resulted in complete extraction of digitoxin and digoxin accompanied by a minimum amount of pigment which did not interfere with the chromatographic separation.

Formamide-impregnated paper was used for chromatography to permit greater loading of the paper with the plant extract or glycosides and by using the solvent systems methyl *iso*butyl ketone-*iso*propyl ether-formamide[4] for development, clear separation of the glycosides was obtained without interference from pigment. This system of chromatography is not very sensitive to temperature variations because of the relatively high vapour pressures of the solvents and it is possible to estimate both digoxin and digitoxin on the one chromatograph. If temperatures of 20 to 23° are used, the time of development is only four or five hours and reasonably compact glycoside zones are obtained.

For colorimetric assay the xanthydrol reagent of Arreguine and Pasqualis[5] was chosen. This reagent has been used in modified forms by

*Colombo Plan Junior Fellow; present address School of Pharmacy, Bangkok.

by several workers[2,6–8] for the estimation of cardiac glycosides containing 2:6-desoxyhexoses. It is very sensitive and the colour is developed by immersing the zones of paper containing the glycosides directly in the reagent, thus avoiding elution losses.

Experimental Methods

The leaves of *Digitalis lanata* were air-dried on racks protected from the weather for six to eight weeks. The moisture content of the dried powdered leaves averaged from 11 to 14 per cent.

Method of Extraction

Some preliminary experiments were carried out in which the plant material was extracted with (*a*) 70 per cent ethanol followed by treatment with lead subacetate[9], (*b*) 20 per cent ethanol by stirring for 16 hours, precipitating the pigments with lead subacetate and finally extracting with chloroform[10], (*c*) chloroform-methanol followed by removal of some of the pigments with light petroleum[11]. Methods (*a*) and (*b*) gave results which were considered to be somewhat low and method (*c*) resulted in an extract which was too heavily pigmented. It was finally decided to use 20 per cent ethanol followed by chloroform extraction but without lead treatment. The pigments extracted did not interfere with the chromatographic separation and the details of the process are as follows:

2 g. of dried powdered leaf was shaken with 3 × 25 ml. of 20 per cent ethanol for 3 successive periods of 6 hours. The solvent, after each extraction, was decanted through a Buchner funnel and the residue, after the final filtration, washed with about 10 ml. of 20 per cent ethanol. The clear alcoholic extract was shaken with five successive 20 ml. portions of chloroform and dried over anhydrous sodium sulphate. The chloroform was filtered and the flask and sodium sulphate washed with 3 × 10 ml. of chloroform. The chloroform extract was evaporated to dryness under reduced pressure and the residue in the flask dissolved in a small volume of a mixture of chloroform: methanol (1:1), transferred quantitatively to a weighed test tube and evaporated in a boiling water bath to dryness. 1 ml. of a mixture of chloroform:methanol (1:1) was added and the final solution weighed.

Paper chromatograms prepared from a 90 per cent ethanol extract of the exhausted leaves, and of further chloroform extracts of the 20 per cent ethanol solution showed no detectable amounts of digoxin or digitoxin.

Preparation of Chromatograms

Whatman No. 1 filter paper approximately 6 in. × 16 in. was ruled with a starting line 4 in. from one end. Guide lines to assist in applying the solutions to the chromatograph were ruled 1/12 in. on each side of the starting line. The paper was impregnated by drawing it slowly through a 25 per cent solution of formamide in acetone (about 15 seconds was allowed for this procedure) and then allowed to dry at room temperature

for about 5 minutes before applying the solution. The time needed for the preparation of the paper between dipping and developing was about 15 to 20 minutes.

A weighed quantity of the solution of plant extract (about 30 to 40 mg.) delivered from a tared capillary pipette was spotted on to a section of the starting line (approximately 1¼ in. in length) in small quantities at a time so that the solvent did not spread beyond the guide lines to any appreciable extent. Towards the centre of the starting line a secondary quantity

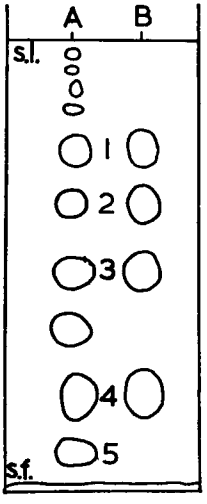

Fig. 1. Paper chromatograph of extract of dried leaves of *Digitalis lanata* on formamide impregnated paper, solvent system: methyl *iso*butyl ketone-*iso*propyl ether-formamide. A, plant extract, B, control glycosides, 1, digoxin, 2, gitoxin, 3, acetyl digoxin, 4, digitoxin, 5, acetyl digitoxin, s.l., starting line, s.f., solvent front.

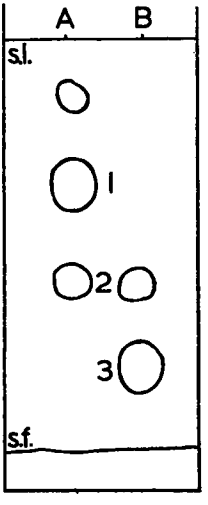

Fig. 2. Paper chromatograph of samples of crystalline digoxin and digitoxin, on formamide impregnated paper, solvent system: methyl *iso*butyl ketone-*iso*propyl ether-formamide. A, digoxin, B, digitoxin, 1, digoxin, 2, gitoxin, 3, digitoxin, s.l., starting line, s.f., solvent front.

of the same extract (approx. 20 mg.) was applied in the same way to a short length of the starting line as a control. Solutions of digoxin and digitoxin (10 to 20 μg. of each) were also spotted on to the line towards the opposite edge of the paper to the extract being estimated.

Development

Methyl *iso*butyl ketone: *iso*propyl ether (100:25 by volume) were shaken with excess formamide and allowed to separate completely, the upper layer being used for development. The atmosphere in the tank was kept saturated by dipping a sheet of filter paper into a layer of the developing solvent on the bottom of the tank. The chromatograms, for quantitative estimations, were developed by the descending method, until

the solvent front reached the end of the paper (about 4 hours at 20° to 23°). At the end of this time the paper was removed and dried in an oven at about 100° for 10 to 15 minutes. This time is not critical and it is not necessary to evaporate all the formamide. The paper was then cut vertically, the portion containing the extract control and the pure glycosides sprayed with 25 per cent antimony trichloride in chloroform and then heated in an oven at about 70° for 2 to 3 minutes only. After this treatment digoxin showed as a greyish-purple band in daylight and as a brownish-orange band under ultra-violet light; gitoxin appeared slightly

TABLE I

ESTIMATION OF CRYSTALLINE GLYCOSIDES ON CHROMATOGRAMS

Glycoside	Per cent recovery	
	Digoxin zone	Combined zones
Digoxin sample A	99·0, 98·3	99·9, 99·8, 99·3
Digoxin sample B	99·6, 99·5 97·1, 97·8 96·6, 96·0	101·0, 99·6, 99·0
	Digitoxin zone	Combined zones
Digitoxin sample A ..	98·8, 98·1, 97·0	99·0, 99·0, 102

yellow in daylight and bright blue under ultra-violet; pure digitoxin appeared rust-red under ultra-violet, but was generally found to be blue in the plant extract. (Fig. 1.)

Two chromatograms were run in the same tank enabling duplicate results to be obtained under the same conditions.

Colorimetric Estimation

Reagents. Xanthydrol 0·125 per cent in glacial acetic acid. This solution was prepared by diluting a 10 per cent methanol solution of xanthydrol with glacial acetic acid. The glacial acetic acid was refluxed with chromic acid for 4 hours, and distilled. The distillate was tested until it did not give a colour with xanthydrol and the subsequent fractions collected and stored in the dark.

Method. The positions of the glycosides being estimated in the unsprayed part of the chromatogram were located from the control and the zones cut out. The rectangular pieces of paper were cut into small strips and placed in a test tube with 10 ml. of the xanthydrol reagent and 0·1 ml. of concentrated hydrochloric acid added and mixed thoroughly. The reaction mixture was then placed in *boiling* water for exactly 1 minute producing a distinct pink colour and immediately cooled in an ice bath for about 5 minutes. The optical density of the solution was then measured using an E.E.L. colorimeter with filter No. 624. Corresponding zones of the blank paper were also cut out and extracted with the reagent as controls in colorimetry.

A standard curve was prepared with pure digitoxose (20, 40, 60, 80 μg.) for each estimation.

RESULTS

Recovery of Glycosides from Chromatograms

Without Development. Known amounts of digoxin and digitoxin were spotted on to filter paper impregnated with formamide. The papers were not chromatographed but dried immediately after spotting and the glycosides in the spots estimated colorimetrically. The recoveries obtained with quantities from 20 to 60 μg. of glycosides were within the range 95 to 100 per cent.

After Development. Weighed amounts of solutions of digoxin an digitoxin (containing quantities of glycosides varying from 20 to 30 mg.) were chromatographed separately according to the method used for plant extracts. Minor glycosidic constituents were present in the samples (Fig. 2) so that colorimetric estimations were made on (*a*) the digoxin or digitoxin zones, and (*b*) on the combined zones present in each chromatogram using pure digitoxose as standard. The results are shown in Table I.

Estimation of Plant Samples

Three batches of leaves were estimated, Sample 1 being taken from a different crop than Samples 2 and 3 which, however, were gathered at

TABLE II

DIGOXIN AND DIGITOXIN CONTENT OF *Digitalis lanata* LEAVES

Sample	Digoxin per cent	Digitoxin per cent
1	0·17, 0·16 0·156, 0·165	0·034, 0·038 0·038, 0·041
2	0·125, 0·127, 0·133	0·047, 0·044, 0·055
3	0·128, 0·131, 0·121	0·046, 0·041, 0·052

different intervals towards the end of the growing season. As shown in Figure 1 a number of glycosides were detected on the chromatograms but only digoxin and digitoxin were estimated. The results are shown in Table II.

Acknowledgements. The Authors wish to thank Burroughs Wellcome and Co. (Australia) Ltd. for generous supplies of digoxin and *Digitalis lanata.*

REFERENCES

1. Jensen, *Acta pharm. tox. Kbh.*, 1956, **12**, 27.
2. Sellwood, *J. Pharm. Pharmacol.*, 1956, **8**, 1061.
3. Stoll and Renz, *Verh. Naturf. Ges. Basel*, 1956, **67**, 392.
4. Gisvold and Wright, *J. Amer. pharm. Ass. Sci. Ed.*, 1957, **46**, 535.
5. Arreguine and Pasqualis, *Rev. univ. natl. Cordoba (Arg.)*, 1945, **32**, 439.
6. Pesez, *Ann. pharm. franc.*, 1952, **10**, 104.
7. Tschesche, Grimmer and Seehofer, *Chem. Ber.*, 1953, **86**, 1235.
8. Tuzson and Vastagh, *Pharm. Acta Helvet.*, 1955, **30**, 444.
9. Rowson, *J. Pharm. Pharmacol.*, 1955, **7**, 924.
10. Bates and Wilkinson, private communication.
11. Silberman and Thorp, *J. Pharm. Pharmacol.*, 1953, **5**, 428.