PAPER CHROMATOGRAPHIC DETECTION OF NEW CONSTITUENTS OF *DIGITALIS LANATA*

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Since digoxin was first isolated from *Digitalis lanata*[1], a number of new cardiac glycosides derived from this plant have been described. These substances fall into two main groups, the so-called primary glycosides, lanatosides A, B, and C, first isolated by Stoll and Kreis[2], and the secondary glycosides, digitoxin, gitoxin, and digoxin, which are derived from the lanatosides by loss of glucose and an acetyl group. The primary glycosides are more polar and more water-soluble than the secondary glycosides and it is possible to separate the two groups by several methods of paper chromatography. In our experience, the clearest separation has been obtained by using the method of Silberman and Thorp[3], in which the developing solvent is a mixture of ethyl acetate, benzene, and water. If 1 per cent. of ethanol is added to the developing phase, the secondary glycosides travel with the solvent front, but the primary glycosides separate clearly.

When this system was used to study the lanatoside content of *Digitalis lanata*[3,4], it was observed that the chromatograms always showed several distinct spots closer to the starting line than the lanatosides. At first it was thought that one of these apparently highly polar substances was lanatoside C, but this was later shown to be incorrect[5]. We have now examined these substances more fully and have obtained some information about their properties. In addition we have shown that these apparently new constituents are present in very young seedlings before any trace of the lanatosides can be detected, and we have followed the development of the glycosides during the early growing season of a crop of *Digitalis lanata*.

Experimental

Solvents. Ethyl acetate first washed with potassium carbonate and then dried and distilled. Benzene, thiophene free, redistilled. Formamide, Light and Company. Chloroform, redistilled and 1 per cent. ethanol added. Butanol, redistilled (b.pt. 116° C.).

Chromatography. System 1[3]. Ethyl acetate—benzene—water, 84 : 16 : 50 (by volume). The solvents were shaken and allowed to separate clearly. The lower (aqueous) phase was placed in the bottom of the tank (11 × 11 × 17 inches high) and the atmosphere kept saturated by filter paper sheets dipping into the stationary phase. To the upper (organic) phase, 1 per cent. of ethanol was added just before chromatography. This organic phase was then placed in a glass trough resting on the bottom of the tank and used to develop the chromatograms by the upward method for four to five hours after the paper had been equilibrated in the tank

overnight. The temperatures found most suitable for development were between 22–24° C.

A small volume of the extract, usually 0·01 ml., was spotted on to the paper using a micropipette. Whatman No. 1 paper was used placing 7 spots on an 8 inch wide paper. On chromatograms where the developed spots were to be eluted, Whatman No. 3 paper was used, and up to 0·1ml. of the extract spotted on to the paper.

System 2. Strips of Whatman No. 4 paper (11 inches × ½ inch) were dipped into formamide—methanol, 1 : 1, and then exposed to the atmosphere for a short time. The plant extracts were streaked on to the strips 2 inches from the end, and development was carried out in horizontal trays at 32° C. for sixteen to twenty hours. The developing solvent was chloroform—benzene—butanol, 78 : 12 : 5 (by volume), saturated with formamide.

The chromatograms from System 1 were dried at room temperature and sprayed with freshly prepared 33 per cent. trichloroacetic acid in chloroform to which 1 drop of 100 volume hydrogen peroxide per 10 ml. of reagent had been added[7]. The chromatograms were then heated at 110° C. for ten minutes. With this reagent, as with the chloramine-trichloroacetic acid reagent of Jensen[8], glycosides belonging to the A series (digitoxin derivatives) fluoresced yellowish-brown in ultra-violet light. Those of the B series (gitoxin derivatives) fluoresced bright blue, and those of the C series (digoxin derivatives) fluoresced pale blue.

The glycosides were detected on the chromatograms of System 2, by dipping the strips in 5 per cent. *m*-dinitrobenzene in benzene, followed by 20 per cent. aqueous sodium hydroxide. Characteristic blue bands which faded quickly were obtained.

Extraction of plant material[3]

Fresh material. Fresh seedlings 12 g., collected in the early morning, were mashed with 6 g. of ammonium sulphate and shaken for six hours with 60 ml. of ethyl acetate. The mash was then shaken for one hour with 30 ml. of ethyl acetate, decanted and shaken with a further 30 ml. for one hour. The extracts were filtered on a Büchner funnel, and the filtrate was evaporated under reduced pressure to approximately 1 ml. The contents of the flask were absorbed on diatomaceous earth and allowed to stand overnight in an evaporating dish. The diatomaceous earth was then extracted with light petroleum in a Soxhlet extractor for about eight hours and then the constituents were washed out of the diatomaceous earth with chloroform-methanol, 1 : 1. The chloroform-methanol extract was evaporated under reduced pressure, almost to dryness, at a temperature not exceeding 30° C. The extract was then made up to 4 ml. in 80 per cent. ethanol. This extract was used for chromatography.

Dried material. Approximately 2·0 g. of each sample of dried leaf, previously broken up in a mortar, were shaken for three hours with 30 ml. of chloroform-methanol solvent 1 : 1, followed by filtration on a Büchner funnel. This was repeated twice, shaking the residue with 10 ml. of

solvent for one hour. The combined filtrates were evaporated to small bulk under reduced pressure, at a temperature not exceeding 30° C., and then absorbed on diatomaceous earth. Chlorophyll and other impurities were next removed by Soxhlet extraction of the powder with low boiling point light petroleum. The glycosides were redissolved from the diatomaceous earth with chloroform-methanol, 1 : 1, followed by filtration and evaporation to small bulk under reduced pressure. The extract was finally adjusted to 10 ml. in 80 per cent. ethanol.

Elution of glycosides from chromatograms

Whatman No. 3 paper was used as larger volumes of extract could be spotted on to this paper. The substances were localised by spraying a strip of the chromatogram, and the areas were cut up finely and shaken with 30 ml. of chloroform-methanol, 1 : 1, for one hour, decanted and shaken with a further 30 ml. for half an hour. The solution was filtered and a small portion of the filtrate evaporated to dryness and taken up in 0·1 ml. of methanol. This was then used for spotting on System 1 in order to check the purity of the substance. The substance was then run on System 2 as a further check of purity.

Acid hydrolysis of eluted glycosides

The glycoside solution eluted from the paper was evaporated to dryness and 3 ml. of ethanol and 3 ml. of 1 per cent. hydrochloric acid added. The solution was then boiled under reflux in a water bath for thirty minutes. The solution was neutralised with a 1 per cent. sodium hydroxide solution, and extracted with chloroform for one and a half to two hours. This was then evaporated to small volume and used for spotting.

RESULTS

A typical paper chromatogram obtained after running extracts of mature dried leaves on System 1, is shown in Figure 1*e*. Four distinct spots were observed travelling behind lanatoside C. These have been named L1, L2, L5, and L6. Of these, L5 showed a yellowish-brown fluorescence in ultra-violet light after trichloroacetic acid treatment, indicating that it probably belongs to the A series of glycosides. The other three spots fluoresced blue under the same conditions. L5 was eluted from the paper and rechromatographed with deacetyl lanatoside A on System 1. Distinct separation was obtained. Similarly it was found that deacetyl lanatosides B and C did not correspond with L1, L2, or L6, when they were eluted and rechromatographed on both Systems 1 and 2.

The position of these substances on the chromatogram indicated that they might be tannoid complexes and the following test was carried out on the plant extracts[9]: To 4 ml. of the plant extract, 0·6 ml. of a suspension of basic lead acetate in 80 per cent. ethanol was added. The solution was then filtered and centrifuged. To the filtrate 1 ml. of a saturated solution of disodium hydrogen phosphate in water was added and the solution again centrifuged. The decanted liquid was adjusted to 8 ml. with ethanol and then spotted on to paper chromatograms. The position of

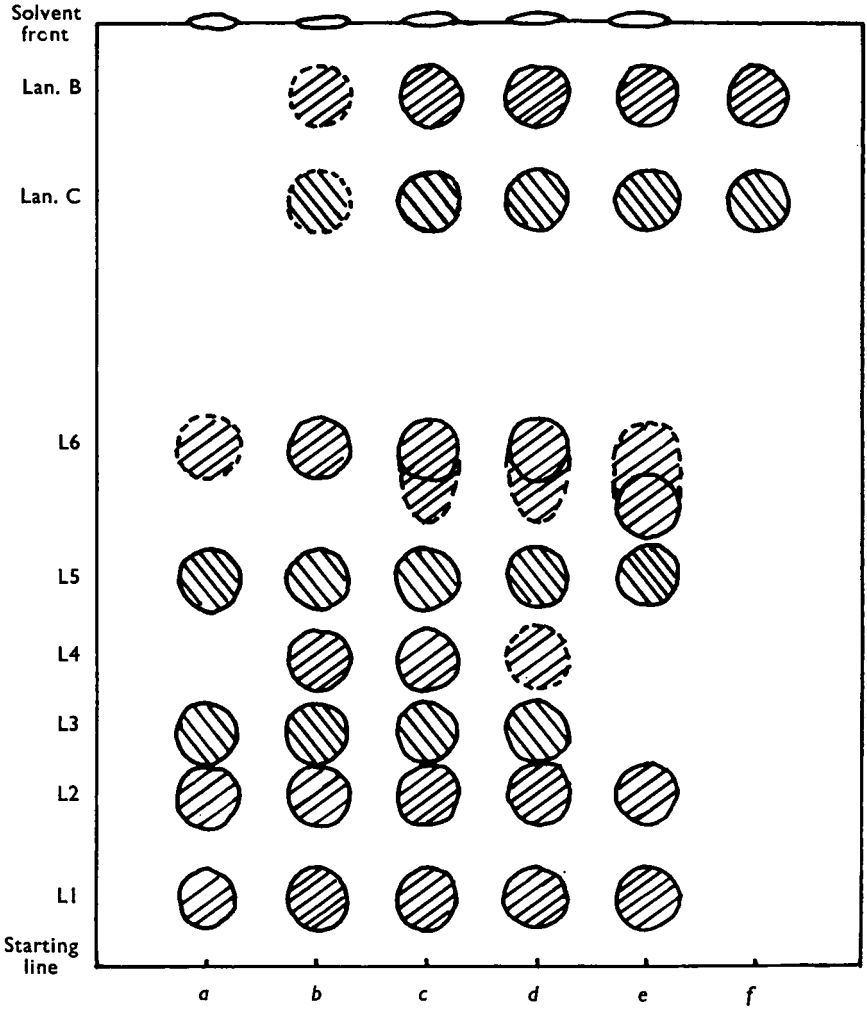

FIG. 1. Chromatography on System 1.

a. Extract of plant 40 days after sowing.
b. ,, 60 ,,
c. ,, 80 ,,
d. ,, 100 ,,
e. ,, approx. 8 months after sowing. (Dried material).
f. Lanatoside B and Lanatoside C.

the spots on the chromatograms after treatment with basic lead acetate was the same as that obtained from the original extract, indicating that the unknown substances are not tannoid complexes.

Investigation of L2 and L6

As chromatograms of many plant extracts showed that L2 and L6 fluoresced more strongly than L1 and L5, only the former two have so far

been investigated. Eluates of L2 and L6 checked for purity by re-chromatography on both systems were evaporated to dryness, redissolved in Ringer solution and tested for toxicity on embryonic chick hearts according to the method of Lehman and Paff[10]. Typical heart blocks were obtained at two dilutions. Both L2 and L6 gave intense blue colours with alkaline *m*-dinitrobenzene and when eluates of L2 and L6 were tested with xanthydrol reagent, pink colours were obtained and persisted for twenty-four hours. A weak Keller-Kiliani test was also obtained with L2.

Hydrolysis of L2

A quantity of L2 obtained from approximately 0·25 g. of air-dried plant by elution from several chromatograms was hydrolysed with dilute acid. The hydrolysed material was chromatographed on System 2 and shown to contain 3 constituents (Fig. 2). One of the bands (B, Fig. 2) appeared to

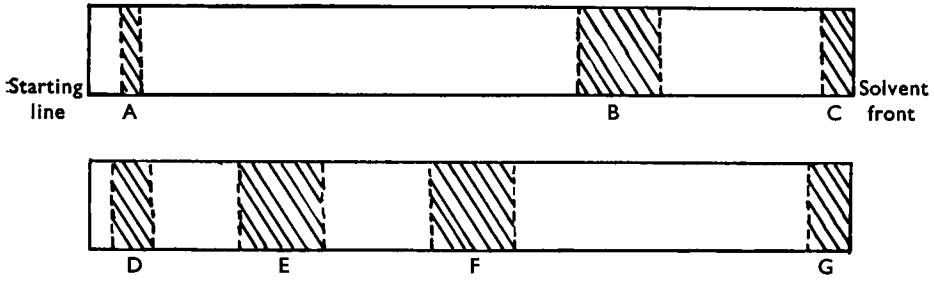

FIG. 2. Chromatograms on System 2.
(1) Hydrolysis of L2 A and C—unknown, B—digoxigenin.
(2) Hydrolysis of L6 D, E, F, G—unknown.

run similarly to digoxigenin and when eluted and re-run with digoxigenin, no separation was obtained on System 2. It did however, separate from gitoxigenin on this sytem. These results were confirmed using the reversed phase system of Tschesche, Grimmer, and Seehofer[11]. The other bands on the chromatogram were not identified, but band A(Fig.2) separated distinctly from the original material on System 1.

Hydrolysis of L6

A quantity of L6 eluted from the same chromatogram as L2, was hydrolysed with dilute acid. In this case, 4 bands of material were detected on chromatograms of the hydrolysate using System 2 (Fig. 2). Of these, band E was the most intense, and was shown to separate from gitoxigenin and digoxigenin. Band F was also shown to separate from gitoxigenin and digoxigenin.

Chromatography of plant extracts obtained from young seedlings

Extracts of freshly picked leaves of *Digitalis lanata* seedlings were taken 40, 60, 80, and 100 days after planting the seeds. Approximately the same quantity of each extract was run on the chromatogram for comparison

as shown in Figure 1. The extract of the youngest seedlings (40 days) showed three spots which on elution and rechromatography could not be separated from L1, L2, and L5 obtained from chromatograms of mature plants. Traces of L6 could also be detected and a new yellow spot appeared travelling just above L2. This has been named L3. There appeared to be no visible trace of the lanatosides. At 60 days, L1, L2, L3, L5 and L6, were visible as well as a further blue spot which travelled between L3 and L5 and has been named L4. A faint trace of the lanatosides B and C could also be detected at this stage. At 80 days the picture was the same with an apparent increase in intensity of L4 and L6 and the lanatosides were beginning to show more prominently. At 100 days after planting, a distinct spot indicated the presence of lanatoside B. Lanatoside C was also visible and L4 fluoresced only faintly. In the two later extracts, L6 which had been increasing in intensity with the age of the plant, appeared to be splitting into two separate constituents.

Discussion

Paper chromatograms of extracts of dried mature plants of *Digitalis lanata* showed clearly the presence of 4 unknown constituents which may be cardiac glycosides. These substances were apparently more water soluble than the lanatosides (digilanids) and separated from them quite clearly. One of these substances (L5) could belong to the A series (digitoxigenin derivatives) but the only evidence for this was the yellow-brown fluorescence in ultra-violet light after treatment with trichloro-acetic acid. Two of the constituents (L2 and L6) appeared to be present in reasonably large amounts. Both were cardiotoxic, gave the characteristic colour reactions of cardiac glycosides, and possibly contained digitoxose or related sugars. Paper chromatographic evidence indicated that one of them (L2) yielded digoxigenin on hydrolysis. The other substances (L6), although showing a blue fluorescence in ultra-violet light after trichloroacetic acid treatment was apparently not a derivative of either digoxigenin or gitoxigenin.

These substances in addition to several others of similar polarity or solubility, were also present in very young seedlings of *Digitalis lanata* before the lanatosides or other glycosides could be detected. It is possible that some of these substances may be precursors of the lanatosides containing for example, more sugar residues, but the present study gives little indication of their chemical nature. It does, however, indicate the complexity of the problem of the constituents of *Digitalis lanata.*

Summary

1. Extracts of mature and young plants of *Digitalis lanata* have been examined by paper chromatography and shown to contain a number of new substances which are apparently cardiac glycosides and more polar in nature than the lanatosides.

2. The eluates of two of these substances obtained from the paper chromatograms have been examined in more detail. Both substances are cardiotoxic and one appears to contain digoxigenin as its aglycone.

3. These new substances are present in very young plants before the lantosides themselves appear and most of them persist throughout the life of the plant.

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DISCUSSION

The paper was presented by DR. S. E. WRIGHT.

The CHAIRMAN asked why the seedlings were collected "early in the morning".

DR. J. M. ROWSON (London) enquired if seedlings collected at other times of the day had been examined to determine whether the content of the new compounds varied with the time of collection. He had harvested at three hourly intervals up to five days and had found some variations. What was the ratio of the content of compounds L1, L2, L5 and L6 to the total glycosides present? Had the authors any information about the cardiac toxicity of the compounds in relation to the better known glycosides, and their possible therapeutic action? How quantitative was the elution of the glycosides from the chromatograms?

PROFESSOR H. BRINDLE (Manchester) said the authors used a very drastic system of extraction. Were they satisfied the new substances were not decomposition products? The substances were very labile, and he asked if simpler extracts of *Digitalis lanata* had been examined, and if so were the same substances obtained?

MR. G. J. RIGBY (Manchester, in a written contribution read by Professor Brindle) said that he had carried out similar work using *Digitalis purpurea* and had found several unidentified substances. Had the developed chromatograms been examined under ultra-violet light before treatment of the paper with trichloroacetic acid? Had the authors determined whether the compounds L1 and L5 reacted with xanthydrol and alkaline reagents used to demonstrate the presence of characteristic lactone rings?

DR. G. E. FOSTER (Dartford) suggested that the use of a large number of solvent systems in chromatographic examination of digitalis was

increasing the complexity of the problem. He asked whether the authors could give any information about the way in which their compounds behaved with solvent systems other than the one described in the paper.

DR. J. W. FAIRBAIRN (London) said that while the authors did not hesitate to label lanatosides B and C on their chromatogram, was it possible that L5 should be labelled lanatoside A?

DR. S. E. WRIGHT, in reply, said the plants were always collected in the morning because it was felt that they should be picked at a constant time. The possibility of variation during the day had not been investigated. It was impossible to say anything about the quantities of L2 and L6 present. They fluoresced brilliantly but that could not be taken as a measure of their concentration. As regards the relevant cardiac toxicity, judging by the colour reaction it fell somewhere on the dose response curve which would be expected for an aglycone. He had no information about the therapeutic effect. In the elution process not less than 90 per cent. recovery had been obtained. The extraction was based on the original method used by Stoll for lanatosides. The temperature did not rise above 40° C., and if decomposition occurred he would expect less polar compounds. They had tried formamide systems, but the substances did not travel very far. L6 did fluoresce before spraying with trichloroacetic acid. It also gave typical cardiac glycoside reactions. They had not tried the reaction of L1 and L5 because the quantities present were very small. Lanatoside A always travelled ahead of lanatoside B.