THE ESTIMATION OF THE COMPONENT CARDIAC GLYCOSIDES IN DIGITALIS PLANT SAMPLES

PART III. THE SEPARATION AND ESTIMATION OF THE GENINS AND ANHYDROGENINS

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IN two previous communications[1,2] methods were described for the separation of the "genuine" and desglucoglycosides of plants of *Digitalis* species using chromatography on unimpregnated paper sheets. It seemed possible to use these methods to separate the much less polar steroid nucleus of the cardiac glycosides. The genins of the digitalis glycosides, digitoxi-, gitoxi- and digoxigenin, are only very slightly soluble in water and it has been considered by Kaiser[3] and others that separation by partition between two phases one of which is water or mostly water could hardly provide an adequate driving force for their separation.

These considerations have been responsible for the wide and successful application of formamide impregnated paper in the chromatographic separation of the cardiac glycosides by Reichstein and Schindler[4], Jensen[5] and many others. On the other hand Bush[6] had shown conclusively that with an appropriate choice of solvents and careful control of the conditions it is possible to use unimpregnated paper for the separation of many steroids especially of the adrenocortical and sex hormone series which are also almost insoluble in water. The resolution in these instances is sometimes poor owing to a marked tailing, resulting from the strong adsorption of the steroid on the paper. But, with carefully controlled conditions, the genins and anhydrogenins of digitalis can be completely separated; the spots are well defined and separated by colourless intervals so that they can be cut out for elution.

This completes the study of an alternative procedure for the separation of these glycosides and offers an additional system on which their identity and purity may be confirmed. That such non-polar substances can be separated so well is a further indication of the complexity of the factors influencing separation by partition paper chromatography.

For the genins the mobile phase was a mixture of 80 parts of chloroform and 20 parts of benzene equilibrated with 50 parts of water; for the anhydrogenins a mixture of 10 to 20 parts of chloroform with 90 or 80 parts of benzene similarly equilibrated with water was used. The ascending method of irrigation is used most often as described previously.

The separation of the genins of the A series from those of the B and C series did not present difficulty as the R_F value of digitoxigenin is very much greater than that for gitoxigenin or digoxigenin.

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The separation of the last two however is more difficult, especially when digoxigenin is present in high concentration and the use of the descending method of irrigation is necessary to achieve complete separation. If the ascending method is used the spots are more nearly circular whereas in the descending method the length of the spot in the direction of solvent flow may be 1½ to 3 times the width.

This is parallel to the results found in the separation of the desglucoglycosides described previously. It is to be noted that the sequence of the two positional isomers of the aglycones and desglucoglycosides is reversed: whereas gitoxigenin travels faster than digoxigenin and parallels lanatosides B and C, the opposite is the case when comparing the faster moving digoxin which precedes gitoxin.

TABLE I

R_F VALUES OF GENINS AND ANHYDROGENINS OF DIGITALIS GLYCOSIDES

A series	R_F	B series	R_F	C series	R_F
Solvent: Chloroform 80, benzene 20, water 50					
Digitoxigenin	0·82	Gitoxigenin	0·42	Digoxigenin	0·33
Solvent: Chloroform 20, benzene 80, water 50					
Digitoxigenin	0·66	Gitoxigenin	0·15	Digoxigenin	0·09
Digitoxigenin acetate	0·82				
"α + β" Anhydrodigitoxigenin	0·96	Dianhydrogitoxigenin	0·88	"α + β" Anhydrodigoxigenin	0·60
"β" Anhydrodigitoxigenin	0·96			"β" Anhydrodigoxigenin	0·60
"β" Anhydrodigitoxigenin acetate	S.F.				

The anhydrogenins separate much more readily since the R_F values of anhydrodigitoxigenin and anhydrodigoxigenin are different, as can be seen from Table I, and the compound of the β series is dianhydrogitoxigenin (Δ 14,16) which shows an R_F value more remote from the other two than with the genins.

In the formation of the anhydrogenins the two possible isomers corresponding to the (Δ 8:14) and (Δ 14:15) position of the double bond are usually obtained and their chromatographic behaviour has been examined.

The "β" ("β" = Δ 14:15; "α" = Δ 8:14)[7] anhydrodigitoxigenin was prepared by the fractional crystallisation described by Smith[8]. When this substance was compared with a mixture of "α" and "β" anhydrogenins obtained by the usual treatment of digitoxigenin, separately or mixed with the latter, no separation could be achieved by these paper chromatographic methods. It is obvious that the resolution is inadequate to differentiate between compounds of this type differing only in the position of the double bond around the same carbon atom C(14) in the molecule.

Similar results were obtained by comparing "β" anhydrodigoxigenin with a mixture of the "α" and "β" isomers and again no separation could be achieved.

As an example of the efficiency of this chromatographic procedure for analytical work a sample of digoxin (m.p. 265° $[\alpha]_D$ + 12°) was hydrolysed by the method of Smith[9] and the genin thus obtained was twice recrystallised from ethanol-water. The correct melting point (221°) was obtained and the normal specific rotation ($[\alpha]_D$ + 18° ethanol). Upon chromatography using the solvent mixture described herein some anhydrogenin was clearly indicated by the presence of a second spot which was identified by comparison with an authentic sample of anhydrodigoxigenin. Two further recrystallisations reduced the amount of anhydrogenin very considerably although a trace still remained. To achieve complete purity of the genin, alumina column chromatography was necessary. The genin prepared from lanatoside C by the method of Stoll and Kreis[10] behaved in exactly the same way.

The hydrolysis of commercially available digitoxin, by the method of Windaus and Stein[11] resulted in 3 or sometimes 4 spots on the developed chromatogram, two of which corresponded to digitoxigenin and gitoxigenin respectively and the remainder to anhydrodigitoxigenin and dianhydrogitoxigenin when a fourth spot was observed.

When digitoxin was previously purified by partition chromatography as described by Silberman and Thorp[12] and the gitoxin-free material thus obtained was hydrolysed, only the genin and anhydrogenin of digitoxin was detected.

Harrison and Wright[13] of these laboratories have correlated the chromatographic placement and chemical structure of a considerable number of digitalis glycosides and have shown that in "reversed phase" chromatography proposed by Tschesche, Grimmer and Seehofer[14], using paper impregnated with medium molecular weight alcohols (amyl to octyl alcohol) as the stationary phase, changes in the aglycone part of the molecule have a decisive influence in determining the R_F value. These workers found that there was very little difference between the R_F values of the desglucoglycosides and the corresponding aglycones of the A, B and C series. With formamide impregnated paper, although the range of R_F values is much wider, here again the desglucoglycosides were found to run next to the corresponding aglycones.

With the use of unimpregnated paper, saturated with the vapour of the solvent mixture to form the stationary phase, the separation of the glycosides is conditioned strictly by the presence and number of sugar molecules in the compound so that the two systems make use of quite different chemical features of these compounds.

The system at present described is therefore complementary to those previously described and is of particular value in the examination of plant extracts where both "genuine" and desglucoglycosides occur and has also proved very useful in a study of the degradation of cardiac glycosides by ultra-violet irradiation where the progressive disappearance of the starting compound and corresponding formation of secondary products could be followed[15].

The authors regard the chromatographic separation on unimpregnated paper, on formamide paper and on the "reversed phase" system as

complementary in the sense that many compounds which are adjacent or closely placed on one system are widely separated on another and the use of the several methods provides valuable corroborative evidence of their nature or identity.

It has frequently been pointed out that in this field the determination of the R_F value is not a reliable method for the identification of cardenolides since these values are variable and influenced by the composition of the mixture and external conditions; but the application of two or more systems to the problem renders the determination of a definite R_F value much less important.

One disadvantage of the use of unimpregnated paper is the small quantity of material which can be applied to the spot compared with the much greater capacity of the formamide system, but this can be partly overcome by the use of thicker paper such as Whatman 3 MM which gives good resolution with 20 to 30 μg. of each component in a mixture compared with 3 to 5 μg. for Whatman N1 paper. Larger quantities cause streaking and tailing.

Satisfactory fluorescent chromatographic spots are produced by trichloroacetic acid which gives a strong reaction with the genins and anhydrogenins and the colours follow closely those found with the corresponding desglucoglycosides. No colour or fluorescence could be produced with trichloroacetic acid and 14-desoxydigitoxigenin or its acetate or with 14-desoxydigoxigenin although variations in temperature and heating time were tried. Dihydrodigoxin gives the reaction although not as strongly as digoxin itself. The reduced sensitivity in this instance where the lactone ring is hydrogenated and the loss of the reaction in the 14-desoxy-compounds may be of value in future rationalisation of the mechanism of this reaction.

Experimental

The solvents and apparatus were those described in previous communications[1,2]. The spots were applied to the papers and allowed to equilibrate overnight. They were then irrigated for $1\frac{1}{2}$ to 2 hours at a temperature of $23° \pm 3°$. The completeness of equilibration is not as important with the genins as it is with the genuine glycosides neither is the control of temperature. There is similarity in this case with the desglucoglycosides which also do not require such critical conditions. For the quantitative separation of digoxigenin from gitoxigenin paper sheets 4 in. wide by 14 in. long were used in the descending method and the time of irrigation was extended to 8 to 9 hours (23°).

The R_F values for the two solvent mixtures are given in Table I. The compounds used were prepared by the following methods. The genins and dianhydrogitoxigenin were prepared by known methods (*loc. cit.*) and purified by alumina column chromatography.

The mixed "α" and "β" and pure "β" anhydrodigoxigenin was kindly prepared and supplied by Mr. B. T. Brown of this Department. The mixed "α" and "β" anhydrodigitoxigenin was prepared by the method of Cloetta[16], and an attempt to separate the mixture on an alumina column

was unsuccessful, as the different fractions did not show progressive enrichment of one isomer.

A strongly enriched but not completely pure sample of "β" anhydrodigitoxigenin was prepared by fractional crystallisation of the mixture of the isomers. After six alternate recrystallisations from ethyl acetate and acetone a material with a m.p. 197 to 198° (suit 195) and $[\alpha]_D$ — 11° (methanol) was obtained in 10 per cent yield. Smith gives m.p. 202 $[\alpha]_D$ — 13° (methanol).

Pure "β" anhydrodigitoxigenin-acetate was prepared after Hunsiker and Reichstein[17].

A solution of 1·0 g. of digitoxigenin acetate in 12 ml. of pure dry pyridine was prepared, cooled in ice and 3·0 ml. of freshly double distilled phosphorus oxychloride was added dropwise with shaking and the mixture left in ice for a further ½ hour. The mixture was then orange brown and was kept at room temperature for 20 hours and then poured slowly on to 50 g. of ice. The solid white precipitate was repeatedly extracted with a mixture of 3 parts of chloroform and 1 part of ether. The collected extract was then washed free of pyridine with 0·5N hydrochloric acid followed by a 5 per cent solution of sodium bicarbonate and water. The mixture was dried and the solvent removed under reduced pressure. The solid residue was recrystallised twice from ether containing some chloroform. 0·55 g. of "β" anhydrodigitoxigenin acetate was obtained m.p. 187 to 188·5° (lit. 183°) and a further 0·31 g. was separated from the mother liquor showing an extended melting range 160 to 195°.

Summary

1. A chromatographic study of the genins and anhydrogenins of digitalis is described.

2. Complete separation of the anhydrogenins is readily achieved but the separation of gitoxigenin and digoxigenin is less satisfactory although both of these are readily separable from digitoxigenin.

3. The R_F values for these derivatives from the A, B and C series of glycosides are tabulated for several solvent mixtures used for chromatography on unimpregnated paper.

References

1. Silberman and Thorp, *J. Pharm. Pharmacol.*, 1953, **5**, 438.
2. Silberman and Thorp, *ibid.*, 1954, **6**, 546.
3. Kaiser, *Chem. Ber.*, 1955, **88**, 556.
4. Schindler and Reichstein, *Helv. chim. Acta.*, 1951, **34**, 108.
5. Jensen, *Acta pharm. tox. Kbh.*, 1953, **9**, 99.
6. Bush, *Biochem. J.*, 1952, **50**, 370.
7. Smith, *J. chem. Soc.*, 1954, 2012.
8. Smith, *ibid.*, 1935, 1050.
9. Smith, *ibid.*, 1930, 508.
10. Stoll and Kreis, *Helv. chim. Acta*, 1933, **16**, 1390.
11. Windaus and Stein, *Ber.*, 1928, **61**, 2436.
12. Silberman and Thorp, *Australian J. Pharmacy*, 1955, **36**, 1137.
13. Harrison and Wright, *J. Pharm. Pharmacol.*, 1957, **9**, 92.
14. Tschesche, Grimmer and Seehofer, *Chem. Ber.*, 1953, **86**, 1235.
15. Silberman and Thorp, *J. Pharm. Pharmacol.*, 1957, **9**, in the press.
16. Cloetta, *Arch. exp. Path. Pharmak.*, 1920, **88**, 115.
17. Hunziker and Reichstein, *Helv. chim. Acta*, 1945, **28**, 1472.