PAPER CHROMATOGRAPHY OF CARDIAC GLYCOSIDES AND AGLYCONES FROM *DIGITALIS LANATA*

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IN recent years a considerable number of papers have treated more or less the subject of paper chromatography of digitalis glycosides[1 to 28]. With the exception of two[14,17], those articles describing experiments in which glycosides and aglycones from *Digitalis lanata* are investigated[1,6,10,11,12,14,15,17,19,21,22,23,25,26], do not include more than a few substances. Chromatography on filter paper impregnated with formamide —a method previously used by us for the separation of the purpurea glycosides[13,16,24,28]—was found suitable for the identification of the components of mixtures of all the lanata-glycosides and aglycones known at the present day. Chromatography on formamide-impregnated filter paper was introduced by Zaffaroni and co-workers[29,30,31] for the analysis of cortical steroids. The method was later employed for the separation of digitaloid substances by Schindler and Reichstein[32], who developed it further for investigation of strophanthus glycosides. By means of this method, too, Heftmann and Levant[33] separated a series of different cardiac glycosides, their acetates and aglycones.

EXPERIMENTAL

Glycosides and aglycones

The glycosides and aglycones used in the present work are presented in Table I. The substances are designated A-, B- or C-substances according to whether aglycone takes the form of digitoxigenin, gitoxigenin or digoxigenin.

Strospeside, desgluco-digitalinum verum, was kindly presented to us by Professor T. Reichstein, Basle. This glycoside, first isolated from *Strophanthus speciosus* by Reichstein and others[34,35,36], is found, according to Tschesche[37], in considerable quantities in *Digitalis purpurea* and *lanata*. One of the glycosides, recently discovered, which was detected in leaves and seeds of *Digitalis purpurea* by a paper chromatographic investigation performed by Briseid Jensen[16], was called substance *a*. On the basis of its behaviour in different fluid mixtures and with various developing reagents[13,16], substance *a* is probably identical with strospeside.

Gitorin and 16-*acetyldigitalinum verum*. A glycoside mixture isolated from *Digitalis lanata* and consisting of gitorin, which is composed of gitoxigenin and glucose[38], and a substance, which according to Tschesche *et al.*[38] is probably 16-acetyldigitalinum verum, was kindly given to us by Professor Tschesche, Hamburg.

Acetyldigitoxin, *acetylgitoxin* and *acetyldigoxin* were produced from lanatoside A, B and C, Sandoz by enzymatic hydrolysis with a digipurpidase preparation. The hydrolysis took place in phosphate buffer, *p*H 5·9,

TABLE I

REAGENTS FOR THE DETECTION OF LANATA GLYCOSIDES AND AGLYCONES AFTER FILTER PAPER CHROMATOGRAPHY. FLUORESCENCE NOTED WITH 5 μg. OF EACH SUBSTANCE

Substance	Trichloroacetic acid	Trichloroacetic acid-chloramine
Lanatoside A Desacetyl-lanatoside A Acetyl-digitoxin Digitoxin Digitoxigenin	—	Yellow
Lanatoside B Desacetyl-lanatoside B Acetyl-gitoxin Gitoxin Gitoxigenin	Blue	Blue
Lanatoside C Desacetyl-lanatoside C Acetyl-digoxin Digoxin Digoxigenin	Whitish-blue (faint)	Whitish-blue
Gitorin	Blue	Blue
16-acetyl-digitalinum verum	Blue	Blue
Strospeside	Blue	Blue

at 40° C.[28]. According to Stoll and others[39,40,41], mixtures of two isomeric secondary glycosides, with one isomer predominant, result from enzymatic decomposition of each of the three lanata glycosides. As a paper chromatographic separation of the isomeric glycosides is not possible under the given conditions, the quantitative ratio is of no interest to us. Neither Kreis[14] nor Tschesche and others[17] succeeded in separating the α- and β-forms by their paper chromatographic methods. The remaining glycosides and aglycones used in the present investigation were Sandoz preparations.

Materials

Acetone, A.R.
Benzene, A.R.
Chloroform, A.R.
Formamide. Merck.
Filter paper, Whatman No. 1.

Apparatus

The chromatographic vessels used were of glass (50 × 20 × 20 cm.) and furnished with a glass lid. Tightness at the lid was ensured by sealing with a soft paste made of starch and glycerol. The solvent troughs were of steel (18 × 2·5 × 2·5 cm.). To obtain a high degree of saturation of the liquids used, saturation vessels were placed not only at the bottom of the chromatographic cabinet, but also under the lid, and sheets of filter paper having the largest possible evaporation surface were suspended between the upper and lower saturation vessels. The ultra-violet lamp (Philips HPW—125 W.) had a maximum transmission at about 365 mμ.

Chromatographic procedure

Formamide-impregnated filter paper: The formamide was mixed with acetone in the proportion 3 + 7 (v/v). The sheets of filter paper were drawn quickly through the liquid and then air-dried for a couple of minutes before the application of the substances for chromatography. The substances to be chromatographed were applied on freshly impregnated filter paper, which was then suspended in a chromatographic vessel "saturated" with the fluids employed. The temperature was about 20° C. After development of the chromatograms the filter paper sheets were dried by heating for half an hour at 115° C.

Reagents

Trichloroacetic acid[13]: A 25 per cent. w/v solution of trichloroacetic acid in ethanol. The dried chromatograms are drawn quickly through a mixture of equal volumes of trichloroacetic acid and ether. After evaporation of the ether, they are heated at 90° C. for 5 minutes, then investigated in ultra-violet light.

Trichloroacetic acid-chloramine[13]: A mixture of trichloroacetic acid reagent and a 3 per cent. w/v solution of chloramine in water (8 + 2 v/v). The mixture should not be more than two days old. The dried chromatograms are drawn quickly through a mixture of equal volumes of trichloroacetic acid-chloramine and ether. After evaporation of the ether they are heated at 120° C. for 10 minutes, then investigated in ultra-violet light.

The fluorescence of about 5 μg. of each substance after treatment with the above-mentioned reagents is shown in Table I.

Fluid mixtures saturated with formamide

	Chloroform	Benzene	(by volume)
Ia	10	0	
Ib	7	3	
Ic	4	6	
	Chloroform	Acetone	(by volume)
II	8	2	

Ia, Ic and II of the above-mentioned fluid mixtures, with formamide, were previously employed for the separation of purpurea glycosides and aglycones[13,16,24,28].

The fluid mixtures and the saturation and development periods most suitable for the identification of the individual substances are obvious from Table II. Substances that can be identified on the same chromatogram are tabulated in groups, the first named having the shortest run.

The examples of chromatograms recorded in Figure 1 illustrate how all the glycosides and aglycones can be detected by means of this experimental method. As previously stressed[13,16], the distance travelled by the individual substances varies, even if the experimental conditions are maintained as constant as possible. Thus the positions indicated for the individual substances in Figure 1 have no claim to be absolute, but give an orientation of the relative placing of the substances and their separation.

TABLE II

CHROMATOGRAPHY ON FILTER PAPER IMPREGNATED WITH FORMAMIDE. EXPERIMENTAL CONDITIONS FOR ISOLATION OF THE INDIVIDUAL GLYCOSIDES AND AGLYCONES

Substance	Fluid mixture	Saturation period	Period of development
1. 16-acetyldigitalinum verum 2. Gitorin 3. Desacetyl-lanatoside C 4. Desacetyl-lanatoside B	II	—	36–48 hours
5. Lanatoside C 6. Desacetyl-lanatoside A 7. Lanatoside B	Ia	—	36–48 hours
8. Strospeside 9. Lanatoside A	Ib	—	24–36 hours
10. Digoxin 11. Digoxigenin 12. Gitoxin 13. Gitoxigenin	Ic	—	48–72 hours
14. Acetyl-digoxin 15. Acetyl-gitoxin 16. Digitoxin 17. Digitoxigenin 18. Acetyl-digitoxin	Ic	10–12 hours	3–4 hours

All the chromatograms illustrated in Figure 1 represent a mixture of all the glycosides and aglycones used, namely 5 μg. of each substance.

DISCUSSION

Chromatography in four different fluid mixtures was deemed necessary for reliable identification of all the individual substances in a mixture of 18 glycosides and aglycones. It is obvious from Figure 1 that the distance between some of the substances, under the chosen conditions, was relatively small. A finer separation of 16-acetyldigitalinum verum, gitorin, desacetyl-lanatoside C and B could thus have been obtained by increasing the period of development; similarly with strospeside and lanatoside A. As the period of development was initially considerable, this procedure was unfavourable and in certain cases would have resulted in the removal of faster travelling substances from the chromatogram. Consequently one more chromatogram would have been necessary for their detection. This applies to acetyldigitoxin, when a finer separation of acetylgitoxin and digitoxin is obtained by increasing the period of development. However, as the fluorescence obtained with the trichloroacetic acid-chloramine-reagent is not the same for the A-, B- and C-substances and as the trichloroacetic acid-reagent gives no fluorescence with the A-substances and only slight fluorescence with the C-substances, combined use of the two reagents in all cases gives a reliable identification of the substances without any increase of the period of development. As the distance travelled by the substances varies from time to time, identification must always be based on a parallel run with a reference substance or in case of doubt with a mixture of reference and test substances.

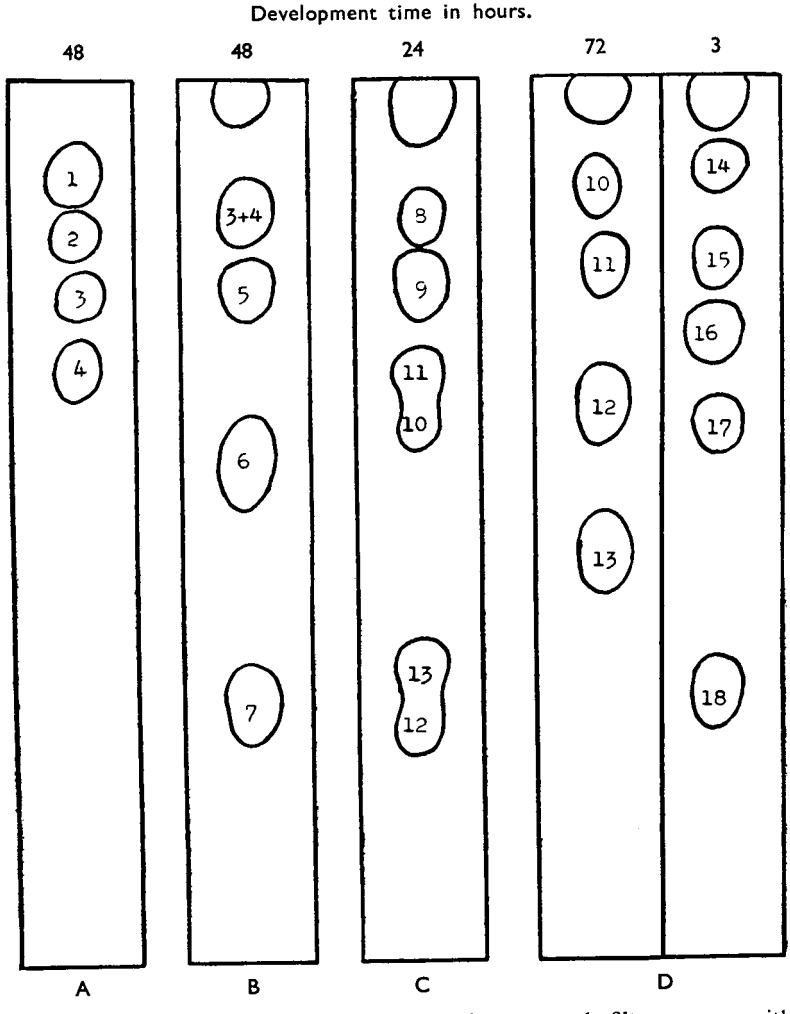

FIG. 1. Chromatography on formamide-impregnated filter paper with different fluid mixtures and periods of development.

A. Fluid mixture II. C. Fluid mixture Ib.
B. „ „ Ia. D. „ „ Ic.

In all the cases a mixture of 5 μg. of each of the substances mentioned in Table II was submitted to chromatography. The designations of the substances in Fig. 1 are the same as in Table II.

Apart from strospeside and gitorin, the fluorescence with the two trichloroacetic acid reagents has been previously discussed for those glycosides and aglycones used in the present experiments which are also found in *Digitalis purpurea*[13]. In other words, the fluorescence of the A- and B-substances but not the C-substances has been described. It was previously noted[1] that the original trichloroacetic acid reagent—a solution of the acid in chloroform—gave a steel-blue fluorescence with

all the C-substances. This solution of trichloroacetic acid in chloroform was, however, unstable[13]—a fact later also observed by Silberman and Thorp[26]—and it was replaced by the two trichloroacetic acid reagents[13] described in the present article. Under the given experimental conditions, both gave fluorescence with all the C-substances, even if the fluorescence was considerably weaker with the reagent without chloramine. On the use of a freshly made solution of trichloroacetic acid in chloroform, Silberman and Thorp[26] obtained fluorescence for lanatoside C but not for digoxin; on addition of a small quantity of hydrogen peroxide to the reagent, both substances gave fluorescence.

According to Table I, the fluorescence for the B- and C-substances is the same for both trichloroacetic acid reagents. This is only an approximation, as the blue fluorescence after use of trichloroacetic acid-chloramine-reagent is tinged with yellow, and this tone becomes more pronounced when the substance is present in quantities greater than the 5 μg. used in the reported data of Table I.

Summary

Data are presented for the separation on paper chromatograms of a series of 18 *Digitalis lanata* glycosides and aglycones in the chloroform-formamide, chloroform-benzene-formamide and chloroform-acetone-formamide systems.

The substances were located on the chromatograms by the fluorescence that arises from heating with trichloroacetic acid and with trichloroacetic acid and chloramine and which differentiates between digitoxigenin, gitoxigenin and digoxigenin and the glycosides derived from these aglycones.

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