THE ESTIMATION OF THE COMPONENT CARDIAC GLYCOSIDES IN DIGITALIS PLANT SAMPLES

PART II. THE ESTIMATION OF THE DESGLUCO-GLYCOSIDES AND SOME OBSERVATIONS ON THE PRODUCTION OF ULTRA-VIOLET FLUORESCENCE WITH TRICHLORACETIC ACID

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IN a previous communication[1] a method was described for the separation of the naturally occurring lanatosides of *Digitalis lanata.* Using paper chromatography with a solvent mixture of 84 parts of ethyl acetate and 16 parts of benzene, saturated with water and containing 0·5 to 3 per cent. of ethanol, a satisfactory separation of the 3 lanatosides was obtained but these conditions were unsuitable for the desglucoglycosides which travel too near the solvent front and merge too closely together for a convenient separation. In view of the importance of the desgluco-glycosides as the first breakdown products in the naturally occurring enzymatic hydrolysis and the therapeutic and commercial status of digoxin and digitoxin a comprehensive study was made of possible methods for achieving more complete separation.

The present paper describes a method permitting reliable and reproducible separation and estimation of digoxin, digitoxin and gitoxin in the presence of the original lanatosides and other glycosides present in crude plant extracts.

In the previous paper it was mentioned that a mixture of chloroform, benzene and water in the proportions 65 : 15 : 50 and containing 5 per cent. of methanol will separate digitoxin from gitoxin and digoxin but that this procedure does not allow the separation of gitoxin and digoxin from each other.

As the result of trying a variety of solvents (chloroform, carbon tetrachloride, benzene, *cyclo*hexane, ethyl acetate, acetone, *n*-butanol, ethanol and methanol) in different mixtures it was found that a mixture of chloroform, benzene, ethyl acetate and water in the proportions of 60 : 20 : 20 : 50 by volume was suitable for the estimation of digitoxin using the ascending method of irrigation. Digitoxin gives an R_F value of 0·7 under these conditions whereas no other glycoside travels very near and the estimation of this material can readily be performed in this way. Digoxin and gitoxin however give R_F values of 0·25 and 0·15 respectively which are too similar for an unambiguous separation unless advantage is taken of the different behaviour of these two substances when treated with trichloracetic acid mixtures as described later in this paper. But in this case only one of these two glycosides can be determined at a time. By using the descending method of irrigation and

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extending the time of running the chromatogram to 18 to 22 hours the same or a slightly modified mixture of solvents is very suitable for the separation of the closely related isomers digoxin and gitoxin, which differ only in the position of the hydroxyl group in positions 12 and 16 respectively. Under these conditions the digitoxin and all the glycosides travelling in front of it are washed away with the effluent solvent. Digoxin then becomes well separated in front of gitoxin. There is no marked tailing on the developed chromatogram provided that the quantities applied are less than 5 μg. and this should preferably be in the range 0·5 to 3 μg. The digoxin spot is usually an elongated elipsoid with an axis ratio 1 : 2 to 3. The working details and solvent modifications are given in the experimental account below.

The trichloracetic acid reagent used to render the glycoside spots visible under ultra-violet irradiation was usually that described by Svendsen and Jensen[2]. It was found however during an extensive use of this elegant and sensitive reagent that some seemingly unexplained difficulties may be experienced which were at first very difficult to understand and necessitated a complete study of this reaction. It was found that the reagent prepared from the same stock of chemicals behaves differently toward different glycosides depending on the age of the solution and the conditions under which it has been stored. It has been found possible to produce an artificial ageing of the reagent by shaking with air, exposure to light, or by the addition of hydrogen peroxide[3].

Since this work was done Jensen[4] published his experiences with the use of trichloracetic acid and he recommends the use of ethanol as a solvent for the acid, claiming that the reagent so prepared is more stable. He proposes also the use of a trichloracetic acid solution with chloramine in aqueous ethanol for the differentiation of the purpurea glycosides. From the table given by this author it can be seen that purpurea glycosides of the B series react with "stabilised" trichloracetic acid solution with a blue fluorescence and give a negative test with the glycosides of the A series. Further, the chloramine-containing reagent gives a yellow fluorescence with the A series, and the B series again fluoresce blue. Our observations generally confirm those of Jensen but extend these findings to the C series of glycosides.

The reagents used in this work comprised (a) a freshly prepared 25 per cent. solution of trichloracetic acid in chloroform containing 1 per cent. of ethanol, (b) the same solution with the addition of 1 or 2 drops of 100 volume hydrogen peroxide to 10 ml. of the reagent. The hydrogen peroxide can be replaced by 1 to 3 per cent. of benzoyl peroxide. The reagent (a) was also "aged" by storage in a half-filled conical flask at room temperature exposed to daylight. For some purposes a stronger reagent was prepared containing 4 drops of hydrogen peroxide in 10 ml of solution. The results of experiments with the reagents are given in Table I. It will be seen that digoxin is quite invisible when the fresh reagent is used but fluoresces with a bright blue colour with the "aged" solution with an additional intensity with the stronger reagent mentioned

above. With the latter solution lanatoside B and gitoxin appear as greenish spots which are easily distinguished from the intense blue colour of digoxin even when these spots occur in proximity upon a chromatogram. The absence of fluorescence of digoxin with a freshly prepared solution has been used routinely by Ferry and Murphy[3] to test this drug for freedom from gitoxin.

When lanatoside A occurs on chromatograms from plant materials it usually shows a faint greenish fluorescence whether sprayed with fresh or aged trichloracetic acid reagent. This is probably due to trace impurities in the chromatogram which are sufficient to change the colour reaction of this glycoside; digitoxin, however, separated from crude plant extracts behaves as indicated in Table I and does not show this aberrant colour.

TABLE I

Glycosides	Freshly prepared reagent	"Aged" reagent (or with hydrogen peroxide)
Lanatoside A ..	no fluorescence	yellow
Digitoxin	,, ,,	yellow/orange
Lanatoside B	bright blue	greyish blue
Gitoxin	,, ,,	greenish blue
Lanatoside C ..	bright grey-blue	blue
Digoxin	no fluorescence	blue

Jensen[4] in discussing the changes which take place on keeping the trichloroacetic acid solution in chloroform assumes that the difference of action of a freshly prepared and old reagent is due to "free" chlorine which is present in an "aged" solution. To obtain similar results this author recommends the addition of chloramine as a source of free chlorine. Since no molecular chlorine can be generated from chloramine in the absence of hydrochloric acid by a simple reaction, we assume that Jensen understood by free chlorine a source of active chlorine atoms and since similar results can be obtained by adding hydrogen peroxide, benzoyl peroxide, or shaking with air we would like tentatively to point out that all these reagents and procedures have in common processes which are assumed to involve free radicals in their reaction mechanism. This is also the prevailing view with regard to the decomposition of chloroform and its instability on keeping. It seems to us therefore not improbable that changes in the colour of the fluorescence reaction proposed by Svendsen and Jensen are influenced by processes involving a homolytic type of reaction mechanism; a point which merits further experimental investigation.

EXPERIMENTAL

Solvents: The chloroform and ethyl acetate, B.P. grade, used for the chromatography were washed with a 10 per cent. potassium carbonate then with water, dried over anhydrous potassium carbonate and distilled through a short column rejecting the first 5 to 10 per cent. as a fore-run and 5 per cent. as the residue.

The benzene was a commercial nitrating grade reagent, benzene

crystallisable, which was distilled through an 18-in. Vigreux type column; 15 per cent. was rejected as fore-run and 5 per cent. left as residue.

The trichloracetic acid was of A.R. grade.

Apparatus: The tank for the upward method was a square glass jar 6 in. × 8 in. × 14 in. high. For the downward chromatography a round jar 6½ in. in diameter and 15 in. high was used. The tanks were covered by glass plates carrying a rubber stopper through which a bent glass rod could be moved allowing the paper to be dipped into the solvent without opening the container and disturbing the phase equilibrium.

Chromatography: Whatman filter paper No. 1 was used throughout. The "spotted" paper is placed in the tank for 12 hours to equilibrate with the solvent-saturated gas phase and then irrigated for 2½ to 4 hours for the upward method (solvent mixture I) or 18 to 22 hours for the downward method (solvent mixture I); 24 to 28 hours solvent mixture II, 16 to 20 hours solvent mixture III. After removing from the tank the paper was dried by hanging in an airy room for 1 to 2 hours and sprayed with a 25 per cent. trichloracetic solution either freshly prepared or "aged" according to the problem under investigation as explained earlier. Satisfactory results were obtained at temperature 22° to 24° C. Solvent mixtures: I chloroform, 60, ethyl acetate 20, benzene 20, water 50.
II chloroform 40, ethyl acetate 30, benzene 30, water 50.
III chloroform 50, benzene 40, water 50; to the separated organic layer 10 to 15 parts of methanol are added.
The developed fluorescing chromatograms can be photographed as described previously[1] for a permanent record.

Note: Based on a very comprehensive experience covering the determination of the lanatosides by the previously described process some further details may be useful to ensure satisfactory results. The maintenance and the achievement of a complete saturation of the paper sheet before irrigation is of vital importance for a good separation and to avoid tailing. Satisfactory equilibration is more readily obtained in medium sized tanks by hanging papers, dipping into the aqueous layer on the bottom, down the side walls. Good results can also be obtained in a larger tank, 10 in. × 10 in. × 18 in., but it is better in this case to extend the equilibration time to 24 or 36 hours before irrigation. Much poorer results were obtained when the working temperature dropped below 22° C. or was raised over 28° C. The temperature during the irrigation period (2½ to 4 hrs.) should be kept constant to ± 1° and uniform throughout the tank. By adhering to these precautions, reliable and reproducible results were obtained in screening tests with a variety of plant samples and in storage tests performed in these laboratories by other workers[5]. In the case of the desgluco-glycosides separation described in this paper, the conditions of equilibration are less critical and a period of 8 to 12 hours is found to be sufficient in most instances.

Discussion

The procedures described above were applied to fractions collected from chromatographic columns and to follow up fermentation studies of

the transformation of the lanatosides in the leaf material to the desgluco derivatives, also for analysis of different plant samples of *Digitalis lanata*. In all these cases it is important to compare the corresponding unknown spots with known purified compounds and not to rely on the previously determined R_F values even if the experiments are performed under exactly identical conditions. This point was elaborated in our previous communication and was also stressed by Schindler and Reichstein[6] and Jensen[4]. This is especially the case when working with crude extracts of plant material which contain a great variety of glycoside materials. In addition to the known lanatosides, their desgluco-derivatives and aglycones there may also be intermediate compounds such as acetyl desgluco-glycosides described by Stoll and Kreis[7], which we have not had available for comparison. There may also be other glycosides as yet unidentified and in such instances it is helpful if on a certain chromatogram only a limited number of spots are present, as is obtained by using different solvent mixtures for different groups of glycosides, or by extending the time of irrigation in the downward chromatography thus removing the faster travelling components into the effluent solvent. It may be of interest to mention that in all the analysed samples of *Digitalis lanata* we have noticed a very intense spot placed between the lanatosides C and B which we could not identify with any of the glycosides at our disposal. This same compound was also present in the mixtures of the crude lanatosides which were prepared according to the method described by Stoll and Kreis, and as far as it was possible to judge from the colour intensity the concentration of this substance was very considerable.

In some cases it may be a disadvantage compared with the chromatography on formamide-soaked paper that in the case of the desgluco-glycosides only rather small quantities of the order of 0·5 to 5 μg. of a single component can be applied for separation; this is not the case with the lanatosides where higher concentrations (1 to 15 μg.) with the A and C component give the best results. On the other hand sheets of paper up to 8 to 9 in. in width with up to 8 spots on the same starting line can be run at the same time thus making possible the comparison of a greater number of samples under identical conditions. Furthermore in applying the method to the semiquantitative estimation of certain glycosides it is possible to place a larger number of the known test substances at different levels of concentration and so lower the limit of error involved in the method of comparison of colour intensities.

In view of the great complexity of the mixtures of glycosides often present and at the same time, of the great similarity of the physical and chemical properties, it was often found by the formamide method, that two components travel at the same rate and cannot be sufficiently separated. This is also the case with some substances with the method used in our chromatograms and a more limited number of compounds have been compared by this method than in the case of formamide-soaked paper, and therefore it may prove advantageous to confirm the identity and homogeneity of a glycoside on several different systems since

there is then less likelihood that two compounds having similar or identical fastness on one system will behave in a completely parallel manner on another system of chromatography.

We should like to point out that the use of the term "lanatoside" is more in accord with the nomenclature of the cardiac glycosides than is the older term "digilanid" and the former has been used in this paper in place of the latter synonym used previously.

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Correction.

MORPHINE DERIVATIVES WITH ANTIANALGESIC ACTION

By A. F. GREEN, G. K. RUFFELL and E. WALTON

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Page 393, line 3. For R′ read R.
Page 395, line 5. For R′ read R″ and for R″ read R′.
Page 396, line 30. For R′ read R″ and for R″ read R′.
line 32. For R^1 read R, and for R^2 read R′.