REVIEW ARTICLE

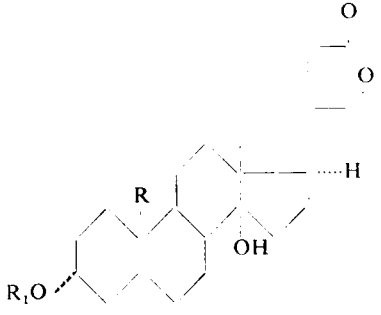
THE CHEMICAL ESTIMATION OF DIGITALIS AND STROPHANTHUS GLYCOSIDES

BY TEODOR CANBÄCK, LEG. APOT., FIL. LIC.

Director of the Pharmaceutical Control Laboratory, Stockholm, Sweden
Member of the Swedish Pharmacopœia Commission

CERTAIN glycosides of plant origin possess valuable cardiotonic properties. The Families Scrophulariaceae and Apocynaceae are the plant orders which contain most members furnishing drugs of therapeutic value. If in this connection the toad poisons are included, the heart-active substances from a chemical viewpoint may be subdivided into three large groups: the digitalis-strophanthus group, the scilla-helleborus group and the nitrogenous venoms secreted by the parotid glands of toads. The chief differences between the groups are: the members of the first group are glycosides, the aglucones of which contain a five-membered lactone ring, the members of the second group are glycosides, the aglucones of which contain a six-membered lactone ring and the members of the last group are suberylarginine derivatives of acetylated hydroxylactones more closely related to the scilla group than to the digitalis group.

Only the first group will be discussed here. The ring system of the cardiac glycosides from the genera *Digitalis* and *Strophanthus* is given below:—



The sugar moiety R_1 may be composed of as many as four sugar molecules. The sugars are for the most part α-desoxy sugars (cymarose, digitoxose and sarmentose) and digitalose, glucose and rhamnose. When an α-desoxy sugar is joined directly to the nucleus, the hydrolysis of the glycoside bond is easily effected; when rhamnose or glucose is the first sugar molecule the union is much firmer, and drastic conditions are necessary for splitting off the sugars. Digitoxin may be mentioned as an example of the first group; ouabain as an example of the second group. In fact, it is only very recently that Mannich has succeeded in

splitting off rhamnose from ouabain without dehydrating the aglucone at the same time. The aglucones are steroids* with a $\Delta\alpha$:β-butenolide C_{17} side chain and they are hydroxylated in positions 3 and 14. Many of the aglucones have additional hydroxyl groups in positions 5, 12 and 16. Summarising, it may be said that the characteristic cardiac-stimulating activity of the glycosides is due to the steroid skeleton of the aglucone, the C_{17} side chain, the C_{14} hydroxyl group and to a minor extent to the structure of the sugar moiety. The glycosides are present in the plants in mixtures. From *Digitalis purpurea* a number of substances have been isolated: the purpurea glycosides, which on partial hydrolysis give digitoxin and gitoxin, gitalin, the inactive glycoside diginin, saponins such as digitonin, tannins and enzymes.

It is about fifty years since the first paper was published on the biological assay of digitalis, and there is still controversy among the biologists as to which method shall be used. In recent publications Gold *et al*[1] advocate a human assay of digitalis to overcome the discrepancy between the potencies of digitalis preparations obtained by the cat method and the actual activity in man, especially by oral administration. This discrepancy has emphasised the necessity of finding a more specific method of analysis than the crude lethal dose method. Thus it is desirable that any assay method of cardiotonic drugs should take account of not only the amount and the distribution of the active glycosides, but also of the absorbability of the active principles from the intestine.

Chemical Testing

It is symptomatic that in the last few years some very interesting papers on the chemical estimation of digitalis and strophanthus preparations have been published. Some of these papers will now be reviewed and commented upon.

The chemical assay of digitalis leaf is a complicated matter. The ideal method should give figures for:—(1) the total amount of active glycosides; (2) the amount of each different glycoside; (3) the amount of aglycones; (4) the absorbability of the glycosides. As the potency of the glycosides varies with the number of sugar molecules attached to the nucleus it is also desirable to have information of the amount of sugars attached.

The structure of the compounds also gives some clue to the solution of the problem. Purely physical methods such as ultra-violet and infra-red spectroscopy would give some information. Colorimetric methods which determine either the sugars or the butenolide group should give reliable figures of the amount of active principles present, but unfortunately no method is known, which differentiates between the glycosides and the aglucones when working on the genin part of the glycoside. However, by combination of a method based on the sugars and a method

* The alkaloids from *Erythrophleum* have cardiotonic activity. They are not steroids.

based on the butenolide group it is easy to calculate the amounts of glycosides and aglucones present.

It should also be possible to separate the glycosides from the aglucones by chromatography on aluminium oxide, or charcoal, or paper strips or by partition chromatography. The separation of the different glycosides (or, after hydrolysis, the aglucones) would also be possible by some kind of chromatography. The chromatographic spectrum of the glycosides could be determined by the aid of pure glycosides.

The extraction of the glycosides from digitalis leaf powder or from galenicals can be done in different ways. Usually an aqueous, methyl alcoholic or ethyl alcoholic extract is prepared. The tannins are removed by shaking with fresh lead oxide or precipitated by lead acetate solution, the precipitate is filtered off and the dissolved lead removed by hydrogen sulphide or sodium phosphate. From this partially purified extract the glycosides and aglucones are extracted with chloroform. Free sugars are left in the aqueous phase. When the final estimation is made on the sugar moiety of the glycosides the latter are hydrolysed and the free sugars estimated.

This route of isolation and purification has some disadvantages, as at all stages large losses are suffered. Extraction with water or with alcohols of different strengths may result in a fractionation of the glycoside complex, as the different glycosides have very different solubilities in these solvents. When the tannins are precipitated by lead acetate, coprecipitation of the glycosides will occur, and it is difficult to wash the precipitate free from them. The extraction of the glycosides and the aglucones from the aqueous or alcoholic solution by chloroform is a very troublesome procedure. The presence of saponins enhances the solubilities of the active principles in the aqueous phase, and even repeated extraction will not give a quantitative yield.

Methylene chloride is a much better solvent than chloroform when extracting pure or purified glycosides from tablets containing lactose because the solubility of lactose in methylene chloride is much less than in chloroform. Lactose disturbs most of the known colour reactions, especially those carried out in alkaline solution, and measuring the transmission at about 500 mμ is unreliable when chloroform is used as the solvent.

Methods Dependent on the Butenolide Side Chain

All glycosides from the digitalis and strophanthus groups show in ethyl alcoholic solution an absorption maximum between 215 and 220 mμ in most cases at 217 mμ with log ε about 4·2. This absorption is very characteristic and is due to the $\Delta^{\alpha:\beta}$-butenolide group. Of course, it is not possible to differentiate between glycosides and aglucones. The method has been used for some years in this laboratory as routine control on injections containing ouabain or digitoxin. When suitable

equipment is available and interfering substances are known to be absent, the method is very rapid.

Extinction plotted against amount of glycoside present gives a straight line when the measurements are made on the peak of the absorption curve. If alkali is added, it is possible to follow the isomerisation of the glycosides as the peak at 217 mμ gradually disappears, which is a good identification of the substance present.

The following methods depend on a reaction with the butenolide group: the Legal reaction with sodium nitroprusside and alkali, the Knudson and Dresbach method with alkaline sodium picrate solution, the Raymond method with *m*-dinitrobenzene in alkaline solution, the Kedde method with alkaline sodium 3 : 5-dinitrobenzoate solution and the Warren, Howland and Green method with sodium β-naphthoquinone-4-sulphonate.

The Legal reaction depends on the formation of a red colour when sodium nitroprusside is added to an alkaline solution of the glycosides. The colour test has been described by Jacobs, Hoffman and Gutus[2] and has been extensively used in research work on the cardiac glycosides. Qualitatively the test is performed so that a relatively large amount (0·01 g.) of the glycoside to be tested is dissolved in pyridine and an equal volume of water is added. A few drops of a 10 per cent. sodium hydroxide solution are added and then 1 ml. of 0·3 per cent. sodium nitroprusside solution. When a heart-active glycoside is present, a bright red colour develops, which slowly fades. The test has been criticised by Elderfield[3], who proposed the use of potassium ferricyanide instead of sodium nitroprusside. Hardegger, Heusser and Blank[4] have shown that the Legal test is not specific for the butenolide group, but that certain related synthetic products also give a positive reaction. The reaction probably involves a condensation of the butenolide group with the reactive NO group (in alkaline solution), and the colour is probably due to salt formation of an isonitroso derivative.

Kedde[5] has developed a quantitative method for the assay of digitalis preparations based on the Legal reaction. He works in a buffered solution of *p*H 11, and obtains a relatively stable colour. The absorption band has a maximum at about 470 mμ.

Some years ago Knudson and Dresbach[6] used the Baljet[7] reaction to estimate digitalis preparations. The Baljet reaction is based on the Jaffe reaction[8]: creatinine gives with alkaline picrate solution a red colour. The glycosides give a red-orange colour when their solutions are treated with alkaline picrate solution. The method has recently been of current interest in the United States of America and Bell and Krantz[9] especially have contributed some interesting investigations. In a series of papers they have shown that the method gives reliable results when the transmission is measured by means of a photoelectric colorimeter. In a collaborative study[10] of the assay of digitalis and its preparations by the Baljet reaction and the cat method it was shown that significant correlation between the two methods existed, showing that both methods

measure the same activity of the drugs. Their method has been made the basis for method I, Digitoxin Colorimetric Controls, in the U.S.P. XIII. For the decolorisation of ethyl alcoholic extracts of digitalis leaf powder they use lead acetate and sodium phosphate. No extraction of the glycosides with chloroform is used, but the reagent, alkaline picrate solution, is added directly to the purified aqueous extract of the drug. After 20 minutes the difference in transmission between the extract and a blank is measured at about 525 mμ. As standards the U.S.P. digitalis reference standard tincture and the same tincture of half strength are used.

U.S.P. XIII uses absolute methyl alcohol to extract digitoxin from tablets.[11] It is necessary to change the composition of the reagent to avoid precipitation of digitoxin and sodium picrate in the final solution. To the methyl alcoholic digitoxin solution containing 0·1 to 1·0 mg. in 10 ml. are added 10 ml. of reagent (2 g. of trinitrophenol dissolved in methyl alcohol to 50 ml., 5 ml. of sodium hydroxide solution (1 in 10) and water to 100 ml.) and after 30 minutes the transmission is measured at about 525 mμ.

The modified Knudson-Dresbach chemical assay has been criticised from different aspects and published results indicate that the values obtained are generally higher than the values obtained by bioassay procedures. The most serious drawback is that glycosides as well as aglucones are determined and reported, as Baljet pointed out. Goldstein[12] has shown that ageing of digitalis tinctures does not apparently affect their potencies according to the chemical assay, which is rather improbable. Elmqvist and Liljestrand,[13] working with infusions and pills, point out that while the bioassays (guinea-pig) indicate a rapid deterioration of the preparation with time, the potencies obtained by the chemical assays are constant or sometimes even increase. Vos and Welsh[14] have reported similar results.

Another disadvantage of the Knudson-Dresbach method is that the difference between the absorption curve of the picrate ion in alkaline solution and the absorption curve of the test solution with alkaline picrate solution is very small, while difficulties of purely technical kinds are present. The Lambert-Beer law is not fulfilled. Lactose interferes as it gives a pronounced colour with the alkaline reagent. An advantage is that the curves obtained by plotting photometer readings against time indicate the presence of interfering substances when the resulting curves differ in shape from those obtained with a standard.

In 1935, Marthoud[15] pointed out that a solution of *m*-dinitrobenzene and heart-active glycosides in ethyl alcohol, on addition of alkali, develops a bright blue-violet colour. This colour test was quantitatively used by Raymond[16] to estimate the amount of ouabain in East African arrow poisons prepared from seeds of *Strophanthus* species and from *Acocanthera* wood. Rasmussen[17] used the method to estimate ouabain in injections, Anderson and Chen[18] to assay digitoxin. Canbäck modified the method and estimated digitalis, strophanthus[19] and uzara

preparations[20]. The reaction is a special case of the general reaction of *m*-dinitrobenzene with active methylene groups in alkaline solution.

Anderson and Chen[18] use the following technique: 150 to 250 μg. of digitoxin is dissolved in 10 ml. of 47·5 per cent. ethyl alcohol and 1 ml. of a 1 per cent. *m*-dinitrobenzene solution in absolute ethyl alcohol is added. The mixture is placed in an ice-bath for 5 to 10 minutes. Then 2 ml. of a 20 per cent. solution of sodium hydroxide is added and the mixture returned to the ice-bath. Exactly 5 minutes after the addition of the sodium hydroxide the transmission is measured in a photoelectric colorimeter equipped with an orange filter. Water is used as a blank. The amount of digitoxin present is read from a standard curve. The colour fades fairly rapidly and it is necessary to read exactly 5 minutes after the addition of the alkali. The colour intensity is dependent on the alcoholic strength and the temperature, both of which must be rigidly controlled.

To overcome most of the difficulties the following technique[19] for the estimation of digitoxin tablets has been used in this laboratory: —

As much of well powdered tablets as corresponds to 800 μg. of digitoxin is weighed into a 15-ml. centrifuge tube and 10·00 ml. of methylene chloride is added. The tube is immediately closed with a cork covered with tinfoil and shaken in a machine for 30 minutes. Then the mixture is centrifuged at 2,000 r.p.m. for some minutes. 5·00 ml. of the clear solution is transferred to a beaker and the methylene chloride evaporated in a vacuum at 50°C. The residue containing the glycoside and any lubricant that may be present (stearic acid), is treated with 5 ml. of 50 per cent. ethyl alcohol and carefully warmed to about 70°C. for a few seconds. This warming is necessary to dissolve the glycosides under the prescribed conditions. The solution is cooled and the separated stearic acid removed by filtration. The filtrate is adjusted to 6·00 ml. with 50 per cent. ethyl alcohol. To this solution 2·00 ml. of 10 N sodium hydroxide solution is added. At the same moment as the alkali is added a stop watch is started and the extinction is determined at about 620 mμ every 30 seconds. The logarithms of the extinctions were plotted against time and a straight line is obtained. Log extinction at zero time is read from the graph. A standard curve is prepared from " zero " extinctions, obtained with weighed amounts (200 to 600 μg.) of digitoxin.

In this modification the Raymond method is unaffected by reasonable variations in alcoholic strength of the final solution and by temperature variations within the range 17° to 23°C. From measurements made on digitoxin, cymarin, strophanthin, ouabain, gitoxin, digitoxigenin, strophanthidin, and on the synthetic analogue β-phenyl-$\Delta^{\alpha:\beta}$-butenolide the extrapolated molar " zero " extinction coefficient has been found to be about 12,000 or $\log \varepsilon = 4{\cdot}08$ at 620 mμ. Lactose interferes, therefore it is preferable to extract digitoxin with methylene chloride, instead of with chloroform, from tablets containing this sugar. Injections, even those containing glycerol, are diluted with ethyl alcohol and directly estimated.

The Raymond method has the following advantages over the Knudson-Dresbach method. The absorption maximum lies far in the

red part of spectrum at 620 mμ, and thus yellow and red colours from impurities have a less serious effect on the measurements than in the Knudson-Dresbach method. The reagent is colourless and stable for some days. The measurements are made very rapidly. Another positive character is that the glycosides from *Scilla* and *Helleborus* do not give colours. A disadvantage is that it is necessary to work with speed in order to obtain sufficient points to construct the curve. The Raymond method, like the Knudson-Dresbach method, makes no distinction between glycosides and aglucones.

The Raymond method has been discussed more in detail than the other methods because in my opinion this method is the most promising of those acting on the lactone side chain. In combination with one of the methods discussed below acting on the sugar moiety, it seems possible to devise a good chemical assay of digitalis preparations.

A new reagent for digitalis glycosides has recently been proposed by Kedde[5]. He uses 3 : 5-dinitrobenzoic acid in alkaline solution to estimate the sum of glycosides and aglucones. The mechanism of the reaction is probably the same as in the Raymond reaction.

Another new reagent is sodium β-naphthoquinone-4-sulphonate used by Warren, Howland and Green[21] for the estimation of digitoxin. Ehrlich and Herter[22] and Feigl and Frehden[23,24] described the use of the reagent for the detection of active methylene groups. The colour produced is probably due to the formation of a quinoid condensation product. The condensation reaction is carried out in alkaline solution and the colour produced is purple. On acidification with acetic acid a stable yellow colour is obtained which may be extracted by dibutyl phthalate.

Warren, Howland and Green[21] assay digitoxin tablets in the following way. Tablets are ground to a fine powder, which is extracted with boiling chloroform. After filtering, the volume is adjusted and an aliquot part, corresponding to 200 μg. of digitoxin, is evaporated in an Erlenmeyer flask on a steam bath. 0·1 ml. of chloroform is added and the flask is shaken to wet all the residue. Then 4 ml. of alcohol and 0·5 ml. of 0·05 N sodium hydroxide solution are added. The flask is placed in a 100°C. bath and after 1 minute 1 ml. of the reagent (containing 0·024 per cent. of sodium β-naphthoquinone-4-sulphonate and 0·024 per cent. of sodium sulphite in distilled water) is rapidly added. One and one-half minutes after the addition of the reagent 0·5 ml. of acetic acid solution (containing 13 per cent. of acetic acid in alcohol) is added with agitation. The flask is removed from the bath, and cooled under the tap. The volume is adjusted to 25 ml. with alcohol and the transmission measured within 2 hours at about 450 mμ. A blank is prepared in the same manner omitting the digitoxin. As aldehydes give a positive reaction with the reagent, aldehyde-free ethyl alcohol must be used.

As expected, the authors report that lanatoside A (digitoxin derivative) and lanatoside C (digoxin derivative) gave a strong positive reaction. Curiously, however, gitoxin and its derivative lanatoside B gave only a weakly positive test. The only structural difference between digitoxin

and gitoxin is the additional hydroxyl group on C_{16}, and it is hard to believe that this hydroxyl group could interfere. The slight solubility of gitoxin may be responsible for the different behaviour of the glycoside. Digitonin gave a negative reaction.

Methods Dependent on the Sugar Moiety of the Glycosides

Many methods for the detection and determination of the sugars in the heart-active glycosides have been reported. All the digitalis glycosides of interest are built up according to the same scheme:—aglucone + 3 digitoxose (+ glucose). The strophanthus glycosides contain other sugars instead of digitoxose, for instance, ouabain contains rhamnose, strophanthin-k β-cymarose and glucose, and cymarin, cymarose.

On the basis of a sugar determination it is for the moment a technically easier task to devise an assay method which is specific for the glycosides and excludes the aglucones, than to construct one on the basis of a determination of the butenolide side chain. In the first case it is only necessary to extract the glycosides and the aglucones from an aqueous or ethyl alcoholic extract and to determine the sugars which are set free in a separate hydrolytic reaction. In the latter case it is necessary to separate glycosides and aglucones by a tedious process and then to estimate the glycosides. When working with a sugar method it is, however, always a good thing to remember that the part of the molecule actually measured is not the specific one. On principle those methods which depend on a reaction with the butenolide side chain should therefore be preferred.

Of the proposed colorimetric sugar methods only two recently described modifications of the Keller-Kiliani test will be discussed. The Keller-Kiliani test depends on the colours obtained when concentrated sulphuric acid is added to digitoxose dissolved in glacial acetic acid containing traces of ferric chloride. As early as 1906 Cloetta and Fischer[25] tried this method for the estimation of heart-active glycosides.

Recently James, Laquer and McIntyre[26] have described a modification that has been introduced in the U.S.P. XIII as Method II, Digitoxin Colorimetric Controls. They assayed digitoxin tablets in the following way. Ground tablets corresponding to 4 mg. of digitoxin were extracted overnight with 50 ml. of chloroform. The supernatant chloroform was filtered and the residue extracted 6 times with 5-ml. quantities of chloroform. The volume was adjusted and an aliquot part of the chloroform solution corresponding to 200 μg. of digitoxin was evaporated to dryness. To the residue were added 3 ml. of glacial acetic acid, 0·10 ml. of 5 per cent. ferric chloride solution ($FeCl_3.6H_2O$) and 0·25 ml. of concentrated sulphuric acid. The transmission was measured at 15-minute intervals until a maximum reading was reached, usually within 15 to 45 minutes. The light filter had a maximum transmission between 500 and 570 mμ. The Lambert-Beer law is fulfilled.

In assaying injections containing glycerol it is necessary to prepare a

special graph from measurements made on solutions containing known amounts of glycerol and digitoxin.

In a comprehensive study Soos[27] has investigated the details of the Keller-Kiliani test when applied to digitalis leaf. He used the reagent recommended by Lindewald[28,29], which consists of 97 ml. of glacial acetic acid, 2 ml. of concentrated sulphuric acid and 1 ml. of a 5 per cent. ferric chloride aqueous solution. Soos assays digitalis leaf in the following way. 1·50 g. of powdered leaf is moistened with a small quantity of water in a mortar and allowed to swell during 15 minutes. Then the mixture is washed into a flask with water and the weight adjusted to 151·5 g. The mixture is shaken now and then during 1 hour. After that 15 ml. of a 15 per cent. lead acetate solution is added. The precipitate is allowed to settle and the clear supernatant solution is filtered. 110 g. of the filtered solution, corresponding to 1·00 g. of the drug, is extracted with 3 × 25 ml. of chloroform. The chloroform solution is dried with sodium sulphate and filtered into an Erlenmeyer flask. The chloroform is distilled off and the residue dissolved in 10 ml. of freshly prepared reagent. The extinction is measured at 30-minute intervals and the maximum value recorded during 5 hours. As the reaction is sensitive to light it is preferable to let the solution stand in the dark during this time. The absorption curve has a maximum at 570 mμ.

Soos points out that unknown impurities in the glacial acetic acid he used had a very pronounced effect on the development of the colour. Thus it is necessary to construct a graph with the glacial acetic acid actually used. As standard substances either digitoxin or digitoxose may be used. Drawbacks of the methods are that the development of the colour takes 2 to 5 hours and that the extraction of the glycosides from the aqueous extract with chloroform is never quantitative. Usually about 80 per cent. is recovered. The presence of saponins in the drug decreases the yield obtained by extraction. Nevertheless, the method probably is the best of the known methods for assaying digitalis leaf. It is the only method that measures the glycosides present in the drug excluding the aglucones. But, as pointed out previously, the method has the defect of not measuring an essential part of the heart-active molecule.

The time seems to be near when the pharmacopœias will have seriously to discuss the problem of the assay of drugs containing heart-active glycosides in the light of the experience gained with chemical methods. A combination of a method assaying the total amount of glycosides and aglucones present, and a method assaying only glycosides would appear to define most of the necessary characteristics of the drugs.

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