THE CHEMICAL ASSAY OF DIGITALIS

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A method suggested by Williams (1958) for the separation of "digitoxin" and "primary glycosides" from digitalis leaf extract, using a column of cellulose powder, has been re-investigated. Two digitalis leaf samples were assayed chemically for their content of total glycosides, "digitoxin" and "primary glycosides", using a solution of 3,5-dinitrobenzoic acid as the reagent. The total activity and the activity of the two fractions, namely "digitoxin" and "primary glycosides", were also determined biologically using rabbit isolated auricles and pigeons (U.S.P. XV method). From the results of the chemical assays it was concluded that in some assays some substance, less biologically active than digitoxin must react with the reagent used, and in others the full biological activity was not indicated chemically. Biological estimations indicated that the two fractions were almost equal in potency, and that they accounted for the total activity. Comparisons of the activities of "pure" digitoxin and desacetyldigilanid A by biological methods confirmed that the primary glycoside was almost three times as active as digitoxin.

ACTIVE glycosides of digitalis give colour reactions by which they can be determined quantitatively. Unfortunately, although they give similar colour reactions, their biological activities vary considerably. Tests in which the glycosides have been determined colorimetrically as a group have, therefore, always given misleading results. Paper partition chromatography has been used by a number of workers to separate the constituents of *Digitalis purpurea* (Brindle, Rigby and Sharma, 1954; Heftman and Levant, 1952; Jensen, 1953; Rigby and Bellis, 1956). Although it is possible by this technique to separate quantitatively, practically all the known active glycosides of digitalis from an extract of the leaf and to estimate the quantities present colorimetrically, the process is very time consuming and the results hitherto have usually been considerably below the figures given by biological methods of determining the activity.

If a chemical assay process is to be satisfactory, it must be reasonably easy to carry out. It appeared possible that a partial separation of the glycosides into two or three groups, each of which could be determined colorimetrically, and each containing glycosides of similar activity, might ultimately lead to a chemical assay which would be satisfactory. Williams (1958) used a system of cellulose powder: formamide as the stationary phase and chloroform as eluant to separate "digitoxin" or secondary glycosides and the primary glycosides. He found that the presence of formamide in the fractions gave rise to moist residues and to interferences in colour development. Furthermore, he found that reversed phase partition chromatography on silane-treated kieselguhr could not be used as the glycosides were insoluble in cyclohexane or n-hexane. However, he found that adsorption chromatography on cellulose powder gave

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qualitative and quantitative results similar to those obtained in the presence of formamide, but with the advantage of clean and dry residues. Williams examined by means of paper chromatography the two fractions thus separated, and his results indicated that the "digitoxin" or secondary glycoside fraction contained no primary glycoside and that the primary glycoside fraction contained no digitoxin. Williams also reported that the two fractions together represented the total activity of the drug in so far as this could be determined by chemical methods. The process appeared to offer promise towards separating active glycosidal fractions from digitalis and, as it had not been published, it was decided to examine its possibilities further.

Experimental

Permission was obtained from Dr. Williams to use his process and to publish details of the original method and any modifications which might be introduced. Dr. Williams also gave details of his method to the Digitalis Panel of the Joint Committee of the Pharmaceutical Society and the Society for Analytical Chemistry on the Methods of Assay of Crude Drugs and permission has been obtained from this committee to publish the method and to use for investigation two samples of powdered digitalis leaf which they had issued.

It was considered desirable first to establish the validity of the method by using it to separate quantitatively the constituent glycosides from a mixture of commercially pure digitoxin and desacetyldigilanid A.

Separation of Digitoxin and Desacetyldigilanid A from a known Mixture

Reagents. Cellulose powder. Whatman's standard grade powder for chromatography.

Chloroform (ethanol free). This was prepared by washing chloroform B.P. with four separate portions of water. Each portion of water was equal to the volume of chloroform being washed. The washed chloroform was dried overnight over anhydrous sodium sulphate, filtered and then distilled. The first few millilitres of the distillate were rejected.

Ethanol. 10 per cent v/v in chloroform.

Solution of digitoxin. 40 mg. of "pure" crystalline digitoxin in 100 ml. of ethanol A.R.

Solution of desacetyldigilanid A. 50 mg. of "pure" desacetyldigilanid A in 100 ml. of ethanol A.R.

Method

Preparation of the column. A cotton-wool plug was placed at the bottom of a glass tube about 35 cm. long and 1 cm. internal diameter, of which one end was drawn out to form a small funnel-shaped opening. A tap was fitted at the other end. About 3·0 g. of the cellulose powder was mixed with ethanol-free chloroform into a slurry and poured into the prepared glass tube. After allowing to stand for a short time, the chloroform was run off until the level fell to the surface of the cellulose powder. The column was washed with 15·0 ml. of ethanol-free chloroform to remove any soluble matter from the column.

Accurately measured volumes of the solutions of digitoxin and desacetyldigilanid A were mixed in an evaporating dish and evaporated to dryness on a boiling water bath, taking care not to overheat the residue. 3·0 ml. of ethanol-free chloroform was added to the residue obtained, warmed gently and mixed well. The mixture was allowed to cool and transferred to the prepared column of cellulose powder with a pipette. Chloroform was run off till the solution fell to the surface of the column. The residue was extracted with another 3·0 ml. of ethanol-free chloroform and the extract was transferred to the column in a similar way. The eluate was collected in an evaporating dish. 2–3 drops/sec. emerged from the column. The residue was further extracted with two 5·0 ml. portions

TABLE I

CHEMICAL ASSAY OF GLYCOSIDES GIVING THE AMOUNT OF EACH GLYCOSIDE AND TOTAL GLYCOSIDES APPLIED TO THE COLUMN AND THE AMOUNT OF EACH GLYCOSIDE RECOVERED, ESTIMATED AS DIGITOXIN, USING 3,5-DINITROBENZOIC ACID AS THE REAGENT WITH LIMITS OF ERROR P = 0·95

Weight of each glycoside applied to the column		Amount of total glycosides calculated as digitoxin, applied to the column as estimated colorimetrically	Estimated amount of separated glycosides recovered from the column	
Digitoxin μg.	Desacetyl-digilanid A μg.	μg.	Digitoxin μg.	Desacetyl-digilanid A calculated as digitoxin μg.
800	500	1,482* (1,478–1,486)	660 (635–690)	456† (426–484)
600	750	1,518* (1,516–1,519)	542 (536–548)	654‡ (632–678)

* Two assays. † Four assays. ‡ Three assays.

of ethanol-free chloroform, the extracts transferred to the column and the eluate collected as before. Elution of the column was continued with further quantities of ethanol-free chloroform till about 40 ml. of the eluate had been collected. The chloroform was removed on a boiling water bath and the digitoxin estimated colorimetrically in the residue by means of an alkaline solution of 3,5-dinitrobenzoic acid (Rowson, 1952).

Desacetyldigilanid A was eluted from the column with ethanol 10 per cent v/v in chloroform, keeping the rate of fall of the drops at 2–3/sec. The eluate was collected directly in the evaporating dish which was to contain the glycoside. The column was eluted until a total of about 40 ml. was collected. The eluate obtained was evaporated to dryness and desacetyldigilanid A was estimated in the residue as above. In each case after 40 ml. of the eluate had been collected no more glycoside could be extracted from the column. The results are given in Table I.

It will be noted that in the assay of the first mixture the recovered glycosides totalled 76 per cent, and in the case of the second mixture 79 per cent.

Assays of Digitalis Leaf Samples

The digitalis leaf extracts were prepared and extracted according to the procedure described by Rowson (1955). Definite volumes of the chloroform extracts were measured and the total glycosides estimated by the

method described above. "Digitoxin" and primary glycosides were separated from the residue obtained from a definite volume of chloroform extract using a cellulose powder column. The method followed was that described above. The results are given in Table II.

TABLE II

CHEMICAL ASSAY OF DIGITALIS LEAF. THE ESTIMATION OF "DIGITOXIN" AND "PRIMARY GLYCOSIDES" AND OF TOTAL GLYCOSIDES ALL CALCULATED AS DIGITOXIN WITH LIMITS OF ERROR (P = 0·95)

Digitalis leaf	Total glycosides calculated as digitoxin per cent w/w	"Digitoxin" per cent w/w	Primary glycosides calculated as digitoxin per cent w/w
A	0·38* (0·37–0·43)	0·19† (0·176–0·201)	0·19† (0·182–0·210)
B	0·33‡ (0·325–0·328)	0·16§ (0·156–0·161)	0·13§ (0·128–0·137)

* Mean of fourteen assays. ‡ Mean of four assays.
† Mean of six assays. § Mean of three assays.
With limits for each.

It was considered that valuable information would result from a biological assay of the separated fractions. Such information is not usually available since it is generally difficult to obtain sufficient material by chromatographic methods for the established methods of biological assay. The isolated auricles method investigated and reported by Bhatt and Macdonald (1960), requires very small amounts of glycosides and enables

TABLE III

BIOLOGICAL ASSAY OF SEPARATED FRACTIONS. ESTIMATED POTENCY IN TERMS OF INTERNATIONAL UNITS OF TOTAL GLYCOSIDES, "DIGITOXIN" AND "PRIMARY GLYCOSIDES" AURICLES AND THE U.S.P. XV METHODS AS DETERMINED BY THE ISOLATED RABBIT WITH LIMITS OF ERROR (P = 0·95)

	Isolated rabbit auricles method			U.S.P. XV method		
Digitalis leaf	Total glycosides I.U./g.	"Digitoxin" I.U./g.	Primary glycosides I.U./g.	Total glycosides I.U./g.	"Digitoxin" I.U./g.	Primary glycosides I.U./g.
A	9·25* (8·41–10·1)	4·53	4·71* (4·68–4·74)	7·02	3·10	4·26
B	—	—	1·94* (1·90–1·98)	—	—	—

* Mean of two assays and figures found.

biological assays to be made on the "digitoxin" and "primary glycosides" fractions separated from digitalis leaf.

Biological Assays of Total Glycosides, "Digitoxin" and "Primary Glycosides"

The total glycosides, "digitoxin" and "primary glycosides" obtained by the procedure described above were assayed by the rabbit isolated auricles method (Bhatt and Macdonald, 1960) and the U.S.P. XV methods. Digitalis leaf extract B was decolorised by passing it through a column of alumina as described by Brindle, Rigby and Sharma (1955), since the total glycosides could not be estimated by the isolated rabbit auricles method after lead acetate treatment. The results are given in Table III.

The potency of the "digitoxin" fraction from leaf B could not be determined directly by the isolated auricle method. It would appear that

some material is extracted from this poor quality specimen which interfered with the beat of the auricle. It was later discovered by microscopical examination that leaf B was adulterated with mullein.

It was not possible to obtain sufficient glycosides from leaf B by the cellulose column method to allow an assay to be carried out by the U.S.P. XV method.

As with the chemical assays of the leaf extracts (Table II), and as distinct from the chemical determinations of the mixed pure glycosides (Table I) the recovery of the glycoside fractions from the leaf extracts would appear to be about complete. It would be reasonable to assume that the "digitoxin" fraction of leaf B would have an activity of about 1·66 I.U./g., the

TABLE IV

POTENCY OF DIGITOXIN AND DESACETYLDIGILANID A OBTAINED BY ISOLATED RABBIT AURICLES AND U.S.P. XV METHODS

Glycoside	Isolated rabbit auricles method I.U./mg.	U.S.P. XV method I.U./mg.
Digitoxin	1·35* (1·35–1·35)	1·38
Desacetyldigilanid A	3·96* (3·92–4·00)	3·55

* Mean of two assays, and figures found.

difference between the total activity and the activity of the primary glycosides. It will be observed that the U.S.P. XV method gave a much lower figure than that given by the isolated auricles method for the total glycosides and "digitoxin" fraction in leaf A.

Biological Comparison of "Pure" Digitoxin and Desacetyldigilanid A

For the isolated rabbit auricles method (Bhatt and Macdonald, 1960), measured volumes of alcoholic solutions of digitoxin and desacetyldigilanid A were diluted to definite volumes with 50 per cent v/v propylene glycol in distilled water. The concentration of alcohol was kept below 10 per cent v/v in the final test dilution, as it was observed that a higher amount was harmful to isolated rabbit auricles. If the dilution of the alcoholic solution was made with water, digitoxin was precipitated, but the 50 per cent propylene glycol mixture was found to be satisfactory; 50 per cent propylene glycol in itself had no perceptible effect on the beat of the auricles. Dispersing agents, like Tween 80 and Crill S.6, were found to be unsuitable. Both digitoxin and desacetyldigilanid A were also assayed for their biological activity by the U.S.P. XV method, and the results are summarised in Table IV.

DISCUSSION

The separation of the "pure" glycosides, digitoxin and desacetyldigilanid A, using a cellulose powder column was found to be satisfactory. Biological estimations of the "digitoxin" and "primary glycoside" fractions from the digitalis leaf extracts showed that they were almost equal in activity and that they accounted for the total biological activity of the extracts. Biological assays of "pure" digitoxin and desacetyldigilanid A showed that desacetyldigilanid A was about three times more

active than digitoxin. This confirmed the previous findings of Brindle, Rigby and Sharma (1955).

Correlation of the Results of the Chemical and Biological Assays

Since "pure" digitoxin and desacetyldigilanid A were found to have activities of about 1·36 and 3·75 I.U./mg. respectively (mean of two biological methods in each case), it is possible to calculate if any relation could exist between the results of the chemical and biological assays of the two samples of digitalis. The "digitoxin" fraction is almost certainly a mixture of secondary glycosides, of which the main portion is digitoxin. If the "digitoxin" fractions had consisted of pure digitoxin, their potencies, calculated by multiplying the biological activities of digitoxin (I.U./mg. rabbit auricle method) by the amount of "digitoxin" estimated chemically (mg./ml.) would be as follows:

Sample A. $1·9 \times 1·35 = 2·56$ "I.U."/g.
Sample B. $1·6 \times 1·35 = 2·10$ "I.U."/g.

The actual figures obtained biologically were:

Sample A. 4·53 I.U./g. (auricle method)
3·10 I.U./g. (U.S.P. method)
Sample B. 1·66 I.U./g. (by difference)

It would appear that this fraction of Sample A contained material which was considerably more active than digitoxin, but in Sample B some less active material was present.

In the case of the primary glycoside fractions, the main constituents are probably desacetyldigilanids A and B. The latter has been stated to have about the same biological activity as digitoxin. Since desacetyldigilanid A has about three times this activity, it is necessary to know the proportions of these two which are present before any attempt at the correlation of the chemical and biological assays can be made.

If these fractions were pure glycoside A, the results would be as follows:

Sample A. $1·9 \times 3·96 = 7·52$ I.U./g.
Sample B. $1·3 \times 3·96 = 5·15$ I.U./g.

If pure glycoside B, the figures would be about:

Sample A. $1·9 \times 1·3 = 2·47$ I.U./g.
Sample B. $1·3 \times 1·3 = 1·69$ I.U./g.

Since the actual figures obtained by the auricle method were:

Sample A. 4·71 I.U./g.
Sample B. 1·94 I.U./g.

the possible activities calculated from the results of the chemical assays are consistent with the primary glycoside fractions being, in the main, a variable mixture of desacetyldigilanids A and B. Actually, if the fraction from Sample A consisted of equal proportions of desacetyldigilanids A and B, the chemical result would agree with the biological figure. In the case of Sample B, the results are consistent with the fraction containing about 10 per cent of desactyldigilanid A and 90 per cent of B.

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