COUNTER-CURRENT SEPARATION OF CONSTITUENTS OF *DIGITALIS PURPUREA*

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Mixtures of some glycosides of *Digitalis purpurea* have been partitioned in a mixture of ethyl acetate, benzene and water by counter-current distribution. The "A" and "B" series of primary glycosides were separated from the corresponding secondary glycosides. The partition ratio of each of the glycosides has been determined and used to calculate the theoretical distribution curve: this closely resembled the experimentally determined curve. Deviation of the "experimental" from the "theoretical" curve for digitoxin has been attributed to the presence of gitoxin in the sample.

COUNTER-CURRENT distribution has been used for the separation of many mixtures of closely related compounds, for example, antimalarial drugs[1], fatty acids[2], penicillins[3], phospholipids[4] and alkaloids of rauwolfia[5]. We have now used it to separate mixtures of digitalis glycosides.

EXPERIMENTAL METHODS

Materials. The apparatus used was a modification of the automatic apparatus originally designed by Craig and others[6]. It carried 50 tubes, allowing 49 stages and could conveniently be modified so that recycling of the contents of the tubes could be carried out making an infinite number of stages possible. The glycosides investigated were the desacetyl-digilanids A and B, kindly supplied by Professor A. Stoll and commercial samples of digitoxin and gitoxin. The solvents used were ethyl acetate, distilling between 76° and 77°, benzene, distilling between 79° and 81°, and ethanol, dehydrated alcohol, B.P.C. The 3:5-dinitrobenzoic acid reagent was a 2 per cent w/v solution of 3:5-dinitrobenzoic acid in ethanol.

Methods

Preliminary. Before selecting the solvent system described below, a series of paper chromatograms was prepared using several of the solvent systems commonly employed for the separation of digitalis constituents. A list of some of these systems is included in the paper by Brindle and others[7]. Whatman No. 3 MM paper was used.

Silberman and Thorp[8] in their paper partition experiments used ethyl acetate 86 volumes, benzene 14 volumes and distilled water 50 volumes, to the separated organic phase of which they added up to 7·5 per cent v/v of ethanol.

The use of ethanol in this way cannot be simulated in the counter-current experiments and for this reason ethanol was omitted from the solvent system in our paper partition chromatograms. The approximate R_F values of the four digitalis glycosides when chromatographed on paper

at 20° ± 2° with the above solvent system containing no added ethanol, are desacetyldigilanid B 0·10, desacetyldigilanid A 0·26, gitoxin 0·84, and digitoxin 0·92. This solvent system was also used in the counter-current apparatus.

Counter-current distributions. The appropriate volumes of the solvents were mixed in 2 litre glass-stoppered bottles and shaken occasionally during 48 hours. The upper organic phase was then separated from the lower aqueous phase.

1st experiment. Approximately 10 mg. of each of the three glycosides desacetyldigilanid A, desacetyldigilanid B and digitoxin were shaken for 2 hours with a mixture of 30 ml. of the upper phase and 30 ml. of the lower phase of the solvent system, in a 100 ml. glass-stoppered measuring cylinder.

The mixture was passed through a No. 4 sintered glass filter and the volume of the upper phase adjusted to 40 ml. and that of the lower phase to 50 ml. by the addition of the corresponding phase of the solvent system.

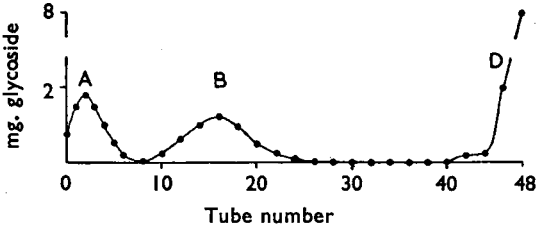

FIG. 1. Experimental distribution curve for desacetyldigilanids (A) and (B) and digitoxin (D). Forty-eight stages completed.

The whole 90 ml. was then placed in the first tube of the distribution apparatus. 50 ml. of the lower phase of the solvent system was placed in each of the remaining 49 tubes and the reservoir flasks were filled with the upper phase. The shaking period was 5 minutes and the settling time 20 minutes. Separation of the two phases in the tubes was fairly rapid and emulsions did not form.

The extraction was allowed to proceed until 48 transfers had been completed when each of the two phases in each of the 49 tubes was assayed as described below.

Assay of tube contents. After separation of the constituents of a mixture of digitalis glycosides in the automatic counter-current distribution apparatus the contents of the tubes were assayed. 10 ml. samples of each phase were separately evaporated to dryness on a steam bath. To each dish was added 4 ml. of ethanol and 2 ml. of distilled water, the residue in the dish was dissolved and the solution transferred to a 10 ml. standard flask. 2·0 ml. of dinitrobenzoic acid reagent was added and the flask transferred to a water bath at 20° for 15 minutes; 1 ml. of N NaOH was then added and the volume adjusted to 10 ml. with ethanol. After immediately filtering through sintered glass, the solution was transferred to an absorptiometer cup and the maximum extinction determined at approximately 535 mμ by comparison with a blank. The blank solution contained 2·0 ml. of dinitrobenzoic acid reagent, 2·0 ml. of distilled water, 1·0 ml. of 1·0N NaOH and sufficient ethanol to adjust the volume to 10 ml.

The weight of glycoside in each tube was calculated from the volume of each phase and by reference to an extinction calibration curve for the particular glycoside identified.

From these results, the distribution curve, Figure 1, showing the weight of glycoside in successive tubes was plotted.

Second experiment. Gitoxin was included in the primary solution in addition to the three glycosides employed in the first experiment. Forty-nine transfers were completed, the solvent system, shaking and settling times being the same as in the first experiment. The 100 assays were carried out as described above and the distribution curve, Figure 2, was constructed from the results.

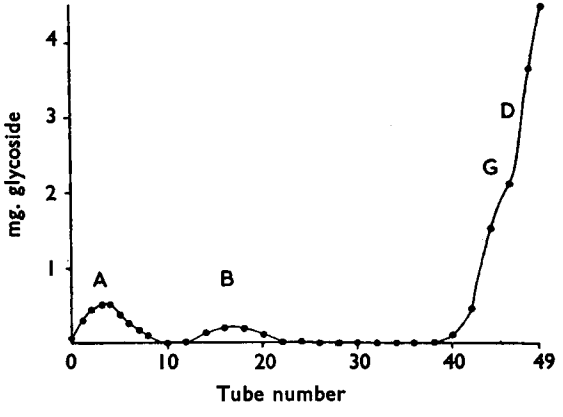

FIG. 2. Experimental distribution curve for desacetyldigilanids (A) and (B), digitoxin (D) and gitoxin (G). Forty-nine stages completed.

The construction of theoretical distribution curves. Theoretical distribution curves for the digitalis glycosides were calculated after determining the partition ratio of the digitalis glycosides in the ethyl acetate 86: benzene 14: water 50 solvent.

Approximately 5 mg. of glycoside was shaken with a mixture of 20 ml. of each phase of the solvent system in a stoppered glass tube. The solution was filtered to remove any undissolved glycoside and 5 ml. of each phase assayed as described on page 238. The estimated partition ratio for each of the four glycosides is, desacetyldigilanid A 0·68, desacetyldigilanid B 0·12, digitoxin 84·8, and gitoxin 14·1.

Calculation of Theoretical Distribution Curves

Martin and Synge[9] showed that the process of counter-current distribution could be expressed mathematically as a binomial expansion:

$$\left[\frac{1}{1+kx}+\frac{kx}{1+kx}\right]^n \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where k = partition ratio, x = ratio of the volumes of the two phases of solvent and n is the number of stages effected. The general term of the expansion of this is:

$$T_r = \frac{n!}{r!(n-r)!} \cdot \frac{k^r x}{(1+kx)^n} \quad .. \quad .. \quad .. \quad .. \quad .. \quad (2)$$

where T_r = the fraction present in the rth tube, of the total material distributed through n tubes.

A simplified method of calculating the theoretical distribution in this way was devised by Liebermann[10]. On dividing T_r by T_{r-1} the expression

$$T_r = \frac{T_{r-1}\, kx(n + 1 - r)}{r} \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (3)$$

is obtained. This may be expressed as

$$\log T_r = \log T_{r-1} + \log kx + \log\left(\frac{n + 1 - r}{r}\right) .. \quad .. \quad .. \quad (4)$$

The logarithm of the zero term is obtained by substituting $r = o$ in equation (2) and expressing logarithmically to give

$$\log T_o = -n \log (1 + kx) \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (5)$$

From equations (4) and (5) and using the partition ratios a theoretical distribution curve for the glycosides desacetyldigilanids A and B, digitoxin and gitoxin was calculated for 49 stages. The curve is shown in Figure 3.

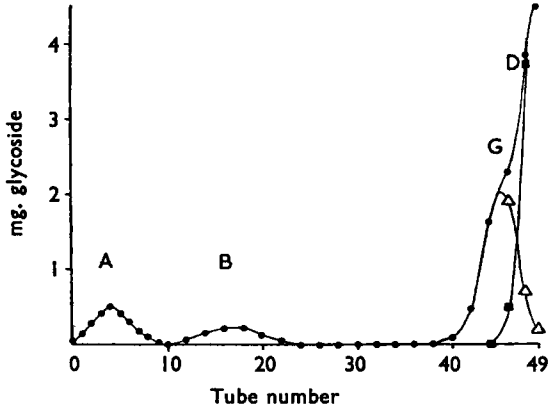

FIG. 3. Theoretical distribution curve for desacetyldigilanids (A) and (B), digitoxin (D) and gitoxin (G). Forty-nine stages calculated. ● total glycoside; ■ digitoxin; △ gitoxin.

RESULTS

In the first experiment (Fig. 1), digitoxin was completely separated from the two primary glycosides which were themselves well separated. A qualitative paper chromatographic examination was unnecessary.

In the second experiment, gitoxin was not separated from digitoxin (Fig. 2). The total amount of glycoside in each of the tubes 38 to 49 inclusive was therefore calculated as "equivalent mg." of digitoxin in order to construct Figure 2. The contents of several of these tubes were examined qualitatively by evaporating 25 ml. of the upper phase in the tube to about 0·25 ml. volume and applying this to the starting line of a formamide:chloroform chromatogram. Both gitoxin and digitoxin were detected in most of the tubes. An approximate estimate of the proportions of the two glycosides in each tube was obtained by comparison of the intensities of the reactions of the isolated glycosides with an alkaline solution of the dinitrobenzoic acid reagent on the developed paper chromatograms (Table I).

DISCUSSION

The primary purpurea glycosides desacetyldigilanids A and B could be separated from one another and from secondary glycosides by

discontinuous counter-current distribution in only 49 stages, in the ethyl acetate: benzene: water solvent (Figs. 1 and 2). Under the same conditions, gitoxin cannot be separated from digitoxin (Fig. 2). The four

TABLE I

QUALITATIVE PAPER CHROMATOGRAPHIC ANALYSIS OF THE CONTENTS OF SELECTED FRACTIONS FROM THE COUNTER-CURRENT SEPARATION OF A MIXTURE OF FOUR DIGITALIS GLYCOSIDES. AN APPROXIMATE ASSESSMENT OF THE CONCENTRATION OF GITOXIN AND DIGITOXIN IN THE LATER FRACTIONS IS INDICATED

Number of the tube in the Craig apparatus	Glycosides found to be present in the contents of the Craig tube
4	Desacetyldigilanid B
17	Desacetyldigilanid A
40	Gitoxin +
42	Gitoxin ++
44	Gitoxin +++
46	Gitoxin +++ and Digitoxin +
48	Gitoxin ++ and Digitoxin ++
49	Gitoxin + and Digitoxin +++

glycosides are distributed in the counter-current experiments in the same order as that in which they are separated by paper partition chromatography with the same solvent.

The similarity of Figures 1, 2 and 3 shows that the theoretical and the experimental distribution of the glycosides are also similar.

Larger amounts of solutes can be investigated by counter-current distribution than is possible by paper partition chromatography. Furthermore, deviations of the experimental from the theoretical distribution curve may be an indication that a hitherto undetected solute may be present in the primary mixture of solutes. The presence of an impurity in a solute might also be recognised in the same way; for example, in Figure 1 the experimental curve for digitoxin exhibits a kink which, by reference to Figure 3, was thought to mean that some gitoxin was present in the commercial sample of digitoxin. This was confirmed by running a large amount (approximately 0·2 mg.) of the commercial sample of digitoxin, on a formamide: chloroform chromatogram, when a spot corresponding in R_F value to gitoxin was detected. This spot produced a blue fluorescence with the trichloroacetic acid: hydrogen peroxide reagent[11].

Attempts are at present being made to effect a complete separation of the two secondary glycosides either by using a different solvent system or by recycling, using the ethyl acetate: benzene: water solvent system.

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