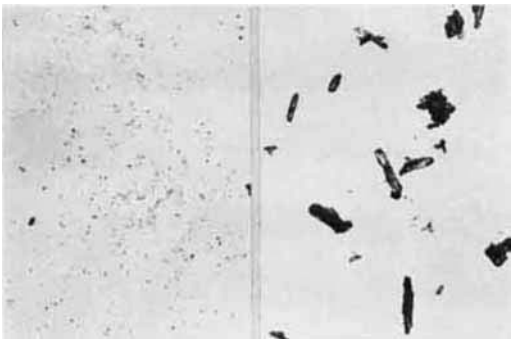

Fig. 4.—Photomicrograph of aluminum acetylsalicylate (left) and aspirin (right) used in this study. (22 × magnification).

used in this investigation was considerably smaller than that of ASA (Fig. 4). Better quantitative correlation between dissolution and absorption rates could possibly be obtained by administering thin, highly compressed, nondisintegrating tablets of the pure drugs so that surface area would be the same in each case. The contact-irritation liability of salicylates (14,15) makes such an experiment unfeasible.

The results of this investigation indicate that Al.ASA is inherently absorbed less rapidly than ASA, and that this difference is probably due to the slow dissolution of Al.ASA as compared with ASA. Administration of Al.ASA in the form used in this study would not bring about a pharmacologic effect as rapidly as administration of ASA. The use of Al.ASA may be limited markedly by its absorption pattern and by the possibility of incomplete biological availability. While it must not be excluded that the use of certain additives could enhance sufficiently the dissolution rate of this drug to overcome the shortcomings outlined above, it is necessary to subject Al.ASA preparations to careful clinical evaluation to assure complete availability of the drug and to ascertain that it is absorbed at a rate consistent with the medicinal use of the particular product.

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Paper Chromatographic Separation and Colorimetric Estimation of the Glycosides of *Digitalis ferruginea* Seeds

By AYHAN ULUBELEN

Digitoxin, gitoxin, digoxin, digilanide A, digilanide B, digilanide C, obtained from *Digitalis ferruginea*, were successfully separated by paper chromatographic techniques and estimated colorimetrically with alkaline 3,5-dinitrobenzoic acid.

A SURVEY of the literature to date would indicate that, the seeds of *Digitalis ferruginea* have not been investigated either qualitatively or quantitatively for their cardioactive glycoside content. Considerably less attention has been given to the phytochemical investigation of the seeds of the digitalis species than of their leaves. The phytochemical investigation of the seeds of digitalis species, therefore, has been initiated as part of a long range program. In these initial studies the air-dried seeds of *Digitalis ferruginea* that were collected from Uludag-Bursa, Turkey, on October 7, 1959, were used.

A number of authors have used different reagents for the colorimetric estimation of various cardiac glycosides. The most important methods are those based on the Baljet reaction (1–6), colors given by the xanthydrol reagent (7–10),

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Keller-Kiliani reaction (11, 12), color reaction with 2,4-dinitrophenylsulfone (13, 14), fluorimetric methods (15, 16), methods based on the Raymond reaction (17), and color reaction with 3,5-dinitrobenzoic acid (18, 19).

An attempt was made to adapt the Baljet reaction to the small amounts of all six glycosides which were found in an extract of the seeds of *Digitalis ferruginea*. Since the intensity of the colors was very weak and not equally uniform with all six glycosides, this method was not used. The Raymond reaction, although a little more promising, was not used for the same reasons.

Finally, the method based on the color reaction given by 3,5-dinitrobenzoic acid was applied to the small amounts of glycosides and good results were obtained.

This method was described as a modification of the Raymond process by Canbäck (20) and used by Kedde (21), Rowson (18), and Lemli (19), in the estimation of digitoxin or total glycoside content of *Digitalis purpurea*.

EXPERIMENTAL

Extraction.—Before extraction, the seeds were crushed with a ball mill using flint stones. A 50-Gm. quantity of the powder, which was an oily mass, was extracted with ether. The ether was removed and the oily content of the seeds was found to be 19.4%. The ether-extracted powder, when percolated with methanol, gave a reddish-brown extract that was concentrated in a vacuum. The solid residue thus obtained was extracted with chloroform-methanol mixture, 2:1 (by volume). This extract was shaken with a 2% aqueous solution of sodium hydroxide until the aqueous layer became colorless (3 × 10 ml. sodium hydroxide was used). The chloroformic solution then was made neutral by shaking with 1% acetic acid, dried with anhydrous sodium sulfate, and evaporated in a vacuum. The residue was dissolved in a chloroform-methanol mixture, 2:1, and diluted to exactly 5 ml.

Paper Chromatography

Whatman No. 1 paper, approximately 11.5 × 36 cm., was used in the descending technique.

System I.—Method of Gisvold (22), methyl isobutyl ketone - isopropyl ether - tetrahydrofuran-formamide, 40:10:15:15 (by volume). The stationary phase formamide was applied to the paper via a 30% solution in acetone, no equilibration being necessary. The atmosphere of the tank was kept saturated by the same solvent system which was placed in a glass dish on the bottom of the tank. Development time was 4 to 5 hours.

Results.—Six very small distinct spots corresponding to digilanide A, digilanide B, digilanide C, digoxin, gitoxin, and digitoxin, respectively, were obtained with a good separation (Fig. 1). The R_f values were: digilanide C, 0.073; digilanide B, 0.126; digilanide A, 0.210; digoxin, 0.300; gitoxin, 0.490; and digitoxin 0.710.

System II.—Method of Tulus and Ulubelen (23),

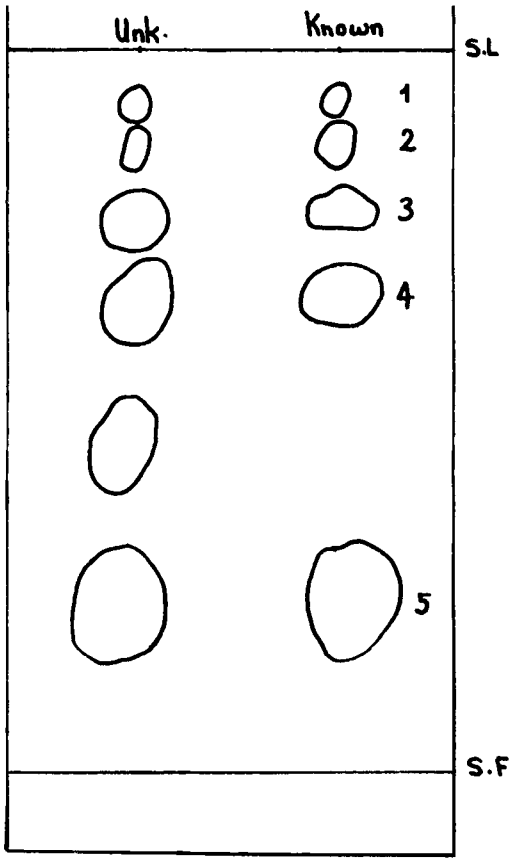

Fig. 1.—Solvent system 1. S.F, solvent front; S.L, starting line; 1, digilanide C; 2, digilanide B; 3, digilanide A; 4, digoxin; 5, digitoxin.

xylene-methyl ethyl ketone-methanol-formamide 10:10:4:0.4 (by volume). The paper was first impregnated with a 1% solution of lithium chloride and dried in the air, then the stationary phase formamide was applied to the paper by means of a 10% solution in acetone. The tank was saturated with the same solvent system; overnight equilibration was necessary. Development time was 1.5 hours.

In both systems the papers were dried 20 minutes at 110° and a sprayed Raymond's reagent was used to locate the glycosides.

Results.—The six glycosides separated very distinctly when solvent system II was used. The spots obtained by this system were somewhat larger than those obtained with solvent system I, and therefore the R_f values are different (Fig. 2). These values are as follows: digilanide C, 0.160; digilanide B, 0.210; digilanide A, 0.310; digoxin, 0.410; gitoxin, 0.610; and digitoxin, 0.780.

Preparation of the Standard Curves

A Coleman universal spectrophotometer was used in the colorimetric assays.

In order to determine the minimum time necessary for full color development and to test the stability of the color, a series of samples containing 50, 300, and 500 mcg. of digitoxin were added to 2 ml. of

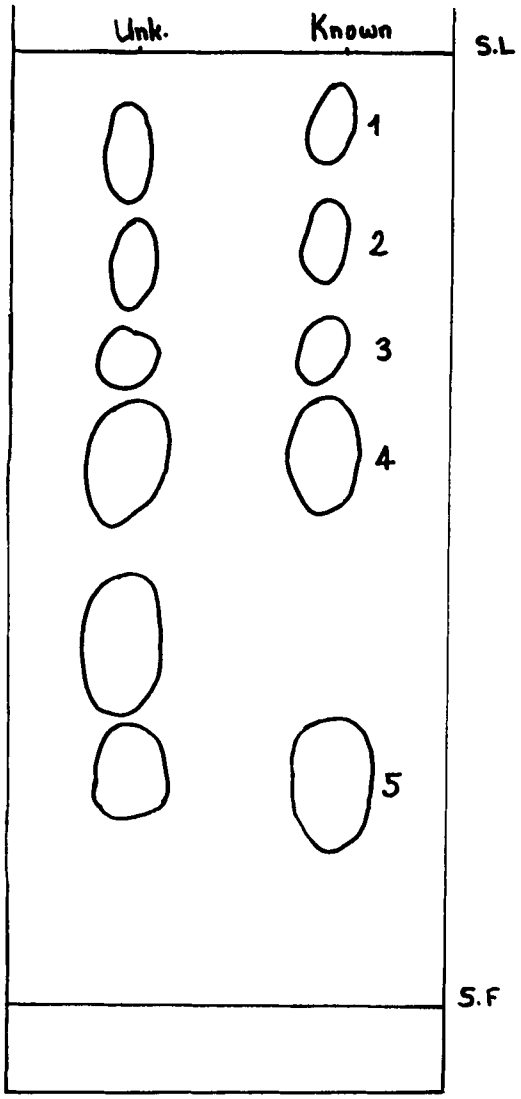

Fig. 2.—Solvent system II. S.F, solvent front; S.L, starting line; 1, digilanide C; 2, digilanide B; 3, digilanide A; 4, digoxin; 5, digitoxin.

2% 3,5-dinitrobenzoic acid (reagent I) and 1 ml. 1 *N* alcoholic solution of sodium hydroxide (reagent II), and diluted to 10 ml. with 50% alcohol. The intensities of the colors were measured at 1, 3, 5, 10, 15, 20, 30, and 40-minute intervals, and it was found that after 3 minutes the maximum color was reached. This color was stable for 30 minutes for all concentrations of digitoxin that were used. The value of color intensity for 50 mcg. was 0.08; for 300 mcg. was 0.300; and for 500 mcg. was 0.45.

The wavelength for maximum absorption was determined with 400 mcg. digitoxin and 535 mμ was found as the best wavelength (Fig. 3, Table I).

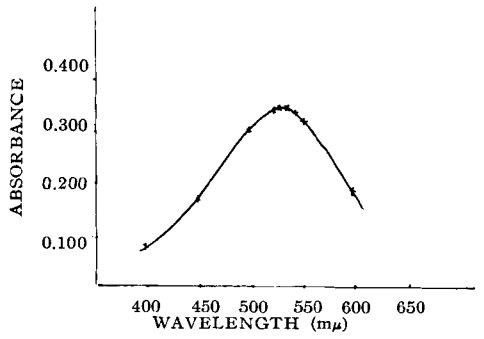

Fig. 3.—Absorption spectrum of the color produced by digitoxin with alkaline Kedde's reagent.

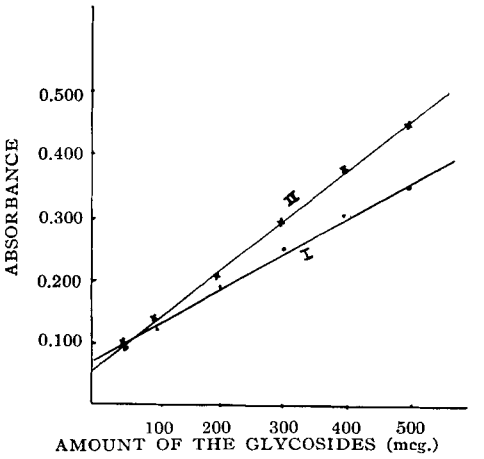

Fig. 4.—Relationship between the concentration of the glycosides and the intensity of the colors when the Kedde reaction was used. Curve I, digilanide A, digilanide B, digilanide C; curve II, digitoxin, digoxin.

TABLE II.—REPRODUCIBILITY OF THE INTENSITY OF COLOR

Sample	Absorbance 100 mcg.	Absorbance 500 mcg.
1	0.135	0.46
2	0.130	0.455
3	0.130	0.45
4	0.130	0.45

Reproducibility of the color was checked and the results are shown in Table II.

The amounts of the reagents I and II were varied, respectively, and it was found that 1.5 ml. of reagent I and 1 ml. of reagent II were sufficient for the development of maximum color when 500 mcg. of digitoxin was used in each case (Tables III and IV).

Standard curves were prepared by plotting various concentrations of digilanide A, digilanide B, digi-

TABLE I.—WAVELENGTH FOR MAXIMUM INTENSITY OF COLOR

Wavelength, mμ	400	450	500	510	520	530	535	540	550	600
Absorbance	0.08	0.17	0.30	0.31	0.33	0.335	0.34	0.32	0.31	0.18

TABLE III.—DEVELOPMENT OF COLOR WITH REAGENT I

Amount used, ml.	0.1	0.3	0.5	1.0	1.5	2.0	3.0	4.0	5.0
Absorbance, mcg.	0.12	0.32	0.40	0.43	0.45	0.45	0.45	0.45	0.45

TABLE IV.—DEVELOPMENT OF COLOR WITH REAGENT II

Amount used, ml.	0.2	0.5	1.0	1.5	2.0	3.0
Absorbance, mcg.	0.12	0.32	0.45	0.45	0.39	0.24

TABLE V.—RELATIONSHIP BETWEEN THE CONCENTRATIONS OF VARIOUS GLYCOSIDES AND INTENSITY OF THE COLORS

	Absorbance				
Glycosides, mcg.	Digilanide C	Digilanide B	Digilanide A	Digoxin	Digitoxin
50	0.09	0.09	0.10	0.09	0.095
100	0.125	0.12	0.12	0.13	0.13
200	0.190	0.185	0.190	0.205	0.205
300	0.250	0.240	0.240	0.290	0.300
400	0.300	0.300	0.300	0.370	0.360
500	0.340	0.340	0.340	0.445	0.450

TABLE VI.—PERCENTAGES OF VARIOUS GLYCOSIDES IN THE SEEDS OF *Digitalis ferruginea*

Glycosides	Absorbance[a]	mcg. in 60 μl.	mg. in 5 ml.	% in Drug[b]
Digilanide C	0.147	140	11.666	0.02333
Digilanide B	0.168	175	14.58	0.02916
Digilanide A	0.168	175	14.58	0.02916
Digoxin	0.147	115	9.583	0.01916
Gitoxin	0.177	155	12.91	0.02582
Digitoxin	0.194	180	16.00	0.03200

[a] Average of 4 determinations. [b] These amounts were calculated from the relationship between the concentration and absorbance which is shown in Fig. 4. Total amount of glycosides is 0.1579%.

lanide C, digitoxin, and digoxin *versus* the intensities of the colors over a range 50–500 mcg. The concentrations were found proportional to the absorptions within this range. Table V gives the results and Fig. 4 shows the curves.

Table V and Fig. 4 show that digilanide A, B, and C give the same curve (curve I) and digitoxin and digoxin give curve II. There is no reason why gitoxin should not also give the same curve.

Analysis of Glycosides of the Seeds

Known amounts of glycosides (150 mcg. each) were applied to the paper and after development according to the methods I and II, respectively, the areas on the paper containing glycosides were cut out and extracted by means of 10 × 2 ml. methanol. The solvent was evaporated and the color was developed as described previously. The amounts of the known glycosides were measured against a blank, which was prepared with the reagents. The amount that was applied to the paper was always recovered. Then exactly 60 μl. of the chloroformic solution of the crude drug, which was previously prepared, was applied to the paper. After development, the areas on the paper containing the glycosides were cut out and extracted by means of 10 × 2 ml. methanol and the colors were developed and measured as described above. Results are shown in Table VI.

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