Preliminary Studies on the Extraction of Glycosides from *Digitalis grandiflora* Seeds

By AYHAN ULUBELEN*

Enzyme-inhibiting and enzyme-favoring extractions were applied to the air-dried seeds of *Digitalis grandiflora*. By paper and thin-layer chromatographic methods, using 70 per cent methanol, native glycosides such as digitalinum verum, purpurea glycoside B, purpurea glycoside A, and strospesid were separated and colorimetrically determined. Digitoxin and gitoxin were obtained when 35 per cent methanolic solution was used.

THE PRIMARY and secondary glycosides found in the air-dried seeds of *Digitalis ferruginea* were reported earlier (1). The glycosides were identified by paper chromatography using two dependable solvent systems. Although the extract of the seeds was obtained by an enzyme-inhibiting technique, investigation showed that secondary glycosides were present in amounts approximately the same as primary glycosides. Early studies on the air-dried seeds of *D. ferruginea* were started only 1 week after their collection; therefore, the enzymatic action could not be attributed to their staying for 1 year, but to some other reason—perhaps the drying conditions.

In this paper air-dried seeds of *D. grandiflora*[1] were used. To eliminate the time factor on the degradation of the glycosides, seeds approximately 10 years old were chosen. As will be seen under *Experimental*, desglucoglycosides were detected when enzyme-favoring conditions were used and native glycosides when enzyme-inhibiting techniques were employed. No secondary glycosides were present in the seeds at the beginning.

After investigating the seeds of some other *Digitalis* species in the near future, a more precise conclusion will be reached about the presence of secondary glycosides in the seeds of *D. ferruginea*.

EXPERIMENTAL

The details of the paper chromatographic technique and the preparation of the standard curves for the colorimetric determination used in this study have been described in a previous paper (1). The extracts were prepared under the enzyme-inhibiting technique—namely, maceration with 70% methanol overnight and percolation the next day. The enzyme-favoring technique was also used and was the same procedure with 35% methanol. Raymond and Jensen reagents (2) were used to detect the glycosides on the paper; 3,5-dinitrobenzoic acid and alcoholic solution of NaOH were used for the colorimetric determination.

Fifty grams of the powdered mass of the seeds of *D. grandiflora* were extracted with petroleum ether; a high yield of oil was obtained (32.7%). The petroleum ether extracted powder was macerated overnight with 100 ml. of a 70% aqueous solution of MeOH, then percolated slowly. When this yellow extract was concentrated under a vacuum, a white precipitate was separated. This precipitate (281.0 mg.) was dissolved in MeOH and purified by recrystallization with ether or isopropyl ether. The melting point of the pure compound was 240–244°, similar to the melting point of digitalinum verum (3), and the amount was 142.7 mg. with a 50.8% yield. This precipitate turned red with the H_2SO_4 solution of *p*-dimethylaminobenzaldehyde—a characteristic reaction for digitaline (4). Paper chromatographic analysis of the crude precipitate showed the presence of two spots using Method *A* (*a*) a yellow pigment (0.02 R_f value) and (*b*) a blue spot with Raymond reagent and an R_f value of 0.083, again similar to that of digitalinum verum (0.082).

This substance was hydrolyzed with 2 *N* HCl and also with 0.1 *N* H_2SO_4, and the aglycon and the sugar parts were analyzed both by paper and thin-layer chromatography.

Gitoxigenin and dianhydrogitoxigenin were found in the aglycon; acid-hydrolyzed products of standard digitalinum verum gave the same compounds with the same R_f values. R_f values obtained from the paper chromatographic separation were: digitalinum verum, 0.08; gitoxigenin, 0.46; and dianhydrogitoxigenin, 0.75. For thin-layer chromatography the R_f values were: 0.81, 0.46, and 0.035, respectively.

Paper chromatography of the sugar of both unknown and the standard digitalinum verum proved the presence of glucose and digitalose. The upper phase of the solvent system of BuOH:H_2O:AcOH (4:5:1) (by volume) was used; the R_f values were: glucose, 0.15 and digitalose, 0.41.

The mother liquid of the precipitate was dried under a vacuum, extracted with a mixture of $CHCl_3$:MeOH (2:1) (by volume), and diluted to exactly 5 ml. When examined by paper chromatography, this extract showed several spots corresponding to purpurea glycoside A, purpurea glycoside B, strospesid, and also digitalinum verum. To obtain a good separation of these glycosides, the solvent systems were overflowed—18 hours for Method *A* and 8 hours for Method *B* (Fig. 1).

Another extract was prepared with a 35% aqueous solution of MeOH. The same precipitate was obtained when MeOH was evaporated; this precipitate proved to be the same compound. Paper chromatography of this enzyme-activated extract revealed that most of the native glycosides had been converted to the desglucoglycosides, such as digitoxin and gitoxin (Fig. 2).

It was possible to lose the secondary glycosides by overrunning the chromatograms. Development times were adjusted accordingly—5 hours for Method *A* and 1.5 hours for Method *B*. R_f values were given for Method *A* and *B*, respectively: digitalinum verum, 0.023 and 0.017; purpurea glycoside B, 0.071 and 0.068; purpurea glycoside A, 0.31 and 0.16; strospesid, 0.57 and 0.34; gitoxin, 0.75 and 0.62; and digitoxin, 0.87 and 0.77.

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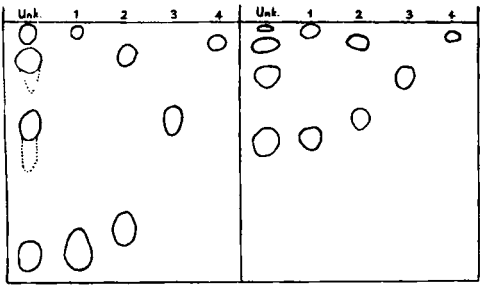

Fig. 1.—Key: *1*, Digitalinum verum, strospesid; *2*, purpurea glycoside B, digilanid A; *3*, purpurea glycoside A; *4*, gitorin; *SL*, starting line.

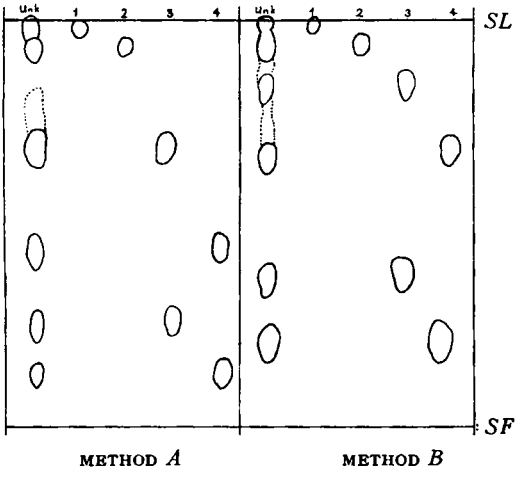

Fig. 2.—Key; *1*, digitalinum verum; *2*, purpurea glycoside B; *3*, purpurea glycoside A; *4*, strospesid, digitoxin; *SL*, starting line; *SF*, solvent front.

Thin-Layer Chromatography.—To establish the paper chromatographic findings and also as a beginning for the future studies, thin-layer chromatographic methods were developed.

Method A.—Smooth glass plates 10 × 20 cm. in size were used. These were coated with an even layer, 0.5 mm. thick, of an adsorbant mixture of silica gel G (Merck). A suspension of 15 Gm. of silica gel G and 50 ml. of water mixed for 1 minute was applied on four to six plates with a simple instrument prepared in our laboratory. After air drying, the plates were activated further by drying at 120° for 1 hour.

The solvent system used by Stahl (5) in the separation of *Digitalis*[2] glycosides was inefficient in this case. The most suitable solvent system was methylisobutyl ketone:isopropyl ether:tetrahydrofuran:methanol (10:5:10:3) (by volume). The total volume of the solvent system depends upon the size of the chromatography jars used. The solvent level should be below the starting line. In this case 20 ml. of solvent was sufficient. After a development time of 30 minutes and air drying, the plates were sprayed with Raymond or Jensen reagents, and a very good separation and small spots were obtained in a short time (Fig. 3). The amounts of standards which were used in this method were about 10 mcg. in each case. R_f values were: purpurea glycoside B, 0.085; strospesid, 0.46; purpurea glycoside A, 0.91; and digitalinum verum, 0.97.

Method B.—The glass plates were coated with a slurry of specially prepared polyamide powder (6). The preparation of the powder is as follows.

One-hundred and fifty grams of Durethan BK 31F (Bayer) is dissolved in 500 ml. of technical HCl over a water bath for 6–7 hours. After cooling, the clear solution is diluted with 250 ml. of MeOH and precipitated with tap water by continuous mixing. The precipitate is filtered and the residue suspended in about 750–800 ml. of MeOH, redissolved with 500 ml. of technical HCl, and reprecipitated as described above. It is then filtered and washed with water until neutral to litmus paper. The neutral residue is suspended in water and stored. At time of use, a part of this suspension is filtered and washed with MeOH. Fifteen grams of the moist residue is suspended in 45 ml. of ethyl acetate which contains 0.5 to 1% Subitogen 2N (Hoest). About six to eight plates can be prepared with this amount. These plates are air-dried; further drying is unnecessary.

Because this polyamide powder was used only recently in the separation of flavon glycosides (6, 7),

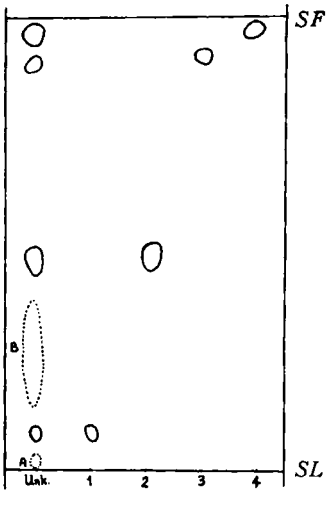

Fig. 3.—Key: *1*, purpurea glycoside B; *2*, strospesid; *3*, purpurea glycoside A; *4*, digitalinum verum; A, yellow pigment; *B*, pink pigment; *SL*, starting line; *SF*, solvent front.

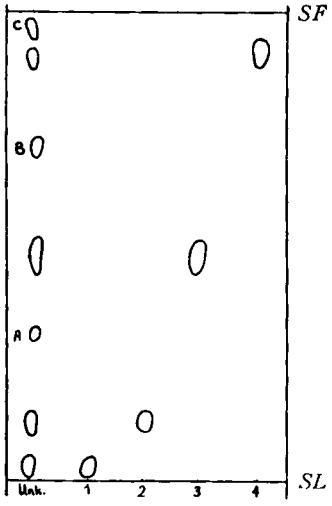

Fig. 4.—Key: *1*, purpurea glycoside B; *2*, purpurea glycoside A; *3*, strospesid; *4*, digitalinum verum; ABC, pink pigments; *SL*, starting line; *SF*, solvent front.

[2] The author thanks Dr. Yoshio Sasakawa for sending the standard digitalis samples.

TABLE I.—PERCENTAGES OF VARIOUS GLYCOSIDES IN THE SEEDS OF *D. grandiflora*

Glycosides	Absorbance[a]	mcg.[b] in 50 µl.	mg. in 5 ml.	Gm. in 100 Gm. Drug
Digitalinum verum	0.115	84	8.4	0.0168
Purpurea glycoside B	0.101	60	6.0	0.0120
Purpurea glycoside A	0.205	230	23.0	0.0460
Strospesid	0.147	134	13.4	0.0268

[a] Average of five determinations. [b] These amounts were calculated from the relationship between the concentration and absorbance which were given in previous paper (1).

it was thought that it could also be used in the separation of digitalis glycosides. After a few experiments with different solvent systems, this material was at least as effective as silica gel G. Figure 4 shows that the pigments were so well separated from the glycosides that it was possible to obtain pure glycosides by using the same powder in a column. The solvent system used was isopropyl ether:methanol (10:3) (by volume). Raymond reagent was sprayed on the plates to develop the spots. The R_f values were: purpurea glycoside B, 0.037; purpurea glycoside A, 0.12; strospesid, 0.48; and digitalinum verum, 0.9.

Quantitative Estimation.—The standard curves were prepared as described previously (1). The same reagents and technique were used.

Fifty microliters of the chloroformic solution of the crude drug was applied to the paper. After development, the areas on the paper corresponding to the native glycosides were cut out and extracted with MeOH. The colors were developed and measured as described previously. The results are shown in Table I.

CONCLUSION

This study showed that only primary glycosides are present in the seeds of *D. grandiflora*. This eliminates the time factor on the degradation of the glycosides and makes the previous findings on the seeds of *D. ferruginea* more interesting. A successful separation was obtained by thin-layer and paper chromatography, the colorimetric determination showed a rather high yield of the total glycosides.

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Effect of Aspartic Acid Salts on Exhaustion Produced by Sleep Deprivation

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Male rats were maintained on a constantly moving wheel in a study of prolonged sleep deprivation. Animals in three age groups were used, and aspartic acid salts were given to half the animals in each age group. The results showed that aspartic acid salts significantly inhibited performance of rats in this situation. Also, the same negative relationship between age and exhaustion time as that found by Webb and Agnew (2) was obtained.

A DELAY IN exhaustion of rats in a swim test as a result of potassium and magnesium aspartate administration has been reported by Rosen *et al.* (1). Webb and Agnew (2) reported a relationship between exhaustion rates and age of rats using a continuous activity wheel. The latter experimenters suggested that sleep deprivation rather than fatigue may have been the critical exhaustion variable involved.

The present investigation duplicated the Webb and Agnew procedure with the addition of aspartate experimental groups.[1] It was reasoned that the more adequately controlled activity output may furnish additional data regarding the action of aspartates. Further, because aspartates are presumably metabolized in the muscle cells, the results may clarify the role of activity *per se* in the exhaustion times reported by Webb and Agnew and hypothesized as sleep deprivation effects.

METHOD

Forty male Lashley-hooded rats from the University of Florida colony were used: Group I, 12 animals, 150 days old (weight range 300–440 Gm.); Group II, 16 animals, 175 days old (weight range 310–460 Gm.); Group III, 12 animals, 235 days old (weight range 370–490 Gm.). Each group was divided into two equal groups matched by weight and assigned to the control or experimental

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[1] The aspartic acid salts used in this study were supplied by Wyeth Laboratories, Inc., Philadelphia, Pa.