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Steroidal Sapogenins XL.

Simplified Procedure for the Qualitative Detection of Cardiac Glycosides*

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Plant extracts were partially purified with lead hydroxide and the filtrates subjected to ascending paper chromatography. Those cardiac glycosides characterized by an unsaturated 5-membered lactone ring were detected by color reaction with alkaline 3,5-dinitrobenzoic acid spray. The method was checked by paper chromatography of aglycones formed on acid hydrolysis of positive samples, by infrared spectra of these aglycones, and by bioassay of the glycosides. This simplified procedure is satisfactory for screening plant extracts for cardiac glycosides.

SCREENING PLANT materials for content of cardiac glycosides has, in recent years, consisted primarily of analyses of seeds of species of Strophanthus (1, 2) for sarmentogenin, a potential starting material for synthesis of cortisone. The search for digitalis-like glycosides, the basic therapeutic agents for congestive heart failure (3), in North American plants is an unexplored field. During this laboratory's survey for steroidal sapogenins (4) many plant species were received for which there was no previously reported chemical information. As a contribution to the literature of plant products, a qualitative examination for flavonoids, alkaloids, tannins, and sterols was added to the screening procedure. It seemed desirable to add still another qualitative test, one for cardiac glycosides. For this purpose a procedure was needed which would detect minute amounts of cardiac glycosides yet use a minimum of time and materials. Since a chemical procedure would be preferable to physiological testing, a qualitative colorimetric test to detect microquantities of most cardiac glycosides has been developed.

Color reactions with picric acid (5) and other nitrocompounds (6–8) have been used in qualitative and quantitative determinations of certain cardioactive materials. Raymond (6) reported that *m*-dinitrobenzene reacted with the lactone ring of ouabain and strophanthin and had considerable specificity (9). Schindler and Reichstein (10) adapted the Raymond reaction to the detection of cardiac aglycones on paper chromatograms. They noted that the color reaction

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TABLE I.—RESULTS OF SCREENING PLANT EXTRACTS OR HYDROLYSIS PRODUCTS FOR $\Delta^{20(22)}$–CARDENOLIDES BY PAPER CHROMATOGRAPHY, INFRARED ABSORPTION, AND FROG HEART TESTS

Plant extracted or other cardiac glycoside source	Plant Part[a]	Paper Chrom.[b]	Infrared Regions, cm.$^{-1}$ 1785	1745	1720	1705	Frog Heart
Apocynum cannabinum	Fr	+	×	×	×	..	+
Asclepias humistrata	L	+	×	×	×	..	c
Strophanthidin		+	×	×	×	..	c
Strophanthus hispidus	W	+	×	×	×	..	+
Strophanthus kombe	W	+	×	×	×	..	+
Sarveroside	..	+	×	×	..	×	c
Thevetia sp.	Fr.	+	×	×	..	×	+
Cryptostegia madagascariensis	L, Fr	+	×	×	..	..	+
Digitalis powder (Penick)	L	+	×	×	..	..	c
Nerium oleander	L	+	×	×	..	..	+
Odoroside		+	×	×	..	..	c
Periploca sp.	L	+	..	..	×	×	+
Urginea burkei	B	—[d]	..	..	×	..	+
Scilla biflora	L	—[d]	..	..	..	×	—
Aloe arborescens	L	?	..	..	×	..	—
Agave nelsonii	L	—	..	..	..	×	—
Ambrosia trifida	L, S	—	..	..	..	×	—
Baptisia tinctoria	W	—	..	..	×	..	—
Euonymus phellomana	Fl	—	..	×	..	..	—
Ficus padifolia	L	—	..	..	×	..	—
Yucca gloriosa	L	—	..	..	..	×	—

[a] Code for plant parts: B—bulb, Fl—flower, Fr—fruit, L—leaf, S—stem, W—whole plant.
[b] Color responses on paper chromatograms were the same after hydrolysis as on the partially purified extract.
[c] No test made.
[d] Six-membered lactone ring type.

distinguished between the unsaturated 5-membered butenolide (11, 12) ring of the digitalis–strophanthus-type glucosides and the double unsaturated 6-membered ring lactone of the squill-type glycosides. Bush and Taylor (1) and Tschesche (13) preferred the Kedde reaction (14) because the purple color obtained with 3,5-dinitrobenzoic acid is more stable than the blue obtained with *m*-dinitrobenzene. We found that the reaction of 3,5-dinitrobenzoic acid was equally applicable to the detection of unhydrolyzed glycosides, although on paper chromatograms the appearance of the purple color was sometimes slower in developing for the glycosides than for the aglycones, perhaps due to differences in solubility. The elimination of hydrolysis and subsequent extraction steps were prerequisite to a simple screening test.

Consideration was next given to increasing the sensitivity of the test by separating the cardiac glycosides from most of the broad spectrum of plant constituents present in an aqueous alcohol extract.[1] For most extracts a simple "spot and spray" technique was not applicable. Wetting a spot of dried extract with a drop or two of a suitable solvent, a modified radial chromatogram, was an improvement, but for many extracts the color reaction of the glycoside was obscured or rendered doubtful by the presence of plant pigments. Tschesche (13) resolved water-soluble cardiac glycosides with the solvent system *n*-octanol, *n*-pentanol, water, and formamide (8:2:8:2). On paper impregnated with the organic phase, the aqueous phase ascended at the rate of 2–3 cm. per hour. We discovered that if only the aqueous phase were used, without impregnation with the organic phase, the solvent front ascended 10 cm. or more in 20 minutes on Whatman No. 4. This makes the chromatography step applicable to a rapid screening procedure. Cardiac glycosides moved faster than most of the interfering substances. The chromatography technique was further improved by a preliminary purification of the plant extract with freshly precipitated lead hydroxide (10). Although clarification may not be necessary for all extracts, it was considered a practical step in the standard procedure when improved techniques of preparing and handling the lead hydroxide cut the time factor to a few minutes per sample.

A screening program based on the described procedure has been begun in order to search for new sources of cardiac glycosides. Squill-like glycosides will not be detected (*cf.* Table I, *Urginea burkei*), but this was not considered a serious defect. Frerejacque (8) reported that 1,3-dinitronaphthalene gives a color test for both types of cardiac glycosides, being particularly positive with scillaren A and B. Where this reagent is available, the procedure might be employed to detect all cardiac glycosides.

Confirmatory tests included paper chromatog-

[1] These extracts had been prepared for general screening program. When only cardiac glycosides are desired, a water extraction of the plant material is sufficient, and fewer impurities are present.

raphy of partially purified aglycones, infrared spectra, and bioassay by the frog heart test.

EXPERIMENTAL

Screening Procedure.—Preparation of ethanolic plant extracts has been described previously (15). A 10-ml. aliquot, representing the extract from approximately 1 Gm. dry weight of plant, was pipetted into a 15-ml. centrifuge tube. Two milliliters of an aqueous slurry, containing about 0.75 Gm. of freshly precipitated lead hydroxide, was added. Contents of the tube were mixed by shaking. After centrifuging 20 minutes at 2,500 r. p. m., the clear supernatant was decanted into a 20-ml. beaker, evaporated to dryness, and the residue resuspended in 0.2–0.3 ml. of 80% aqueous ethanol. A drop of the concentrate was spotted about 1 inch from the end of a $7^1/_2$ by $^1/_2$ inch strip of Whatman No. 4 paper. After drying, the strip was inserted as an ascending chromatogram into a 6-inch test tube containing 0.5 ml. of aqueous phase of Tschesche's "I" solvent system (13). The tube was stoppered with a foil-covered cork. When the solvent had ascended to within 1 inch of the stopper, requiring about 20 minutes at room temperature, the paper strip was removed and dried under vacuum at 70° for 30 minutes. The spray for detecting the cardiac glycosides was freshly prepared by combining an equal volume of 2% 3,5-dinitrobenzoic acid in methanol with 0.5 *N* potassium hydroxide in water. Areas of color reaction were outlined in pencil.

Paper Chromatography of Aglycones.—An aliquot equivalent to the alcoholic extract of 5 Gm. dry weight of plant was evaporated to dryness in a 100-ml. beaker. The residue was digested with 20 ml. of water, heating under a watch glass for 30 minutes on a steam bath. The suspension was filtered at room temperature through a thin pad of infusorial earth. The filtrate and water washes of the cake were combined, acidified to pH 1.0 with sulfuric acid, and refluxed 45 minutes in the presence of 20 ml. of benzene. After cooling, the benzene was recovered in a separatory funnel, the aqueous layer extracted with 15 ml. of fresh benzene, and the combined benzene extracts washed three times with 10-ml. portions of water. The benzene was concentrated, transferred to a tared 20-ml. beaker, evaporated to dryness, and weighed. The dried residue was taken up in a sufficient volume of solvent to allow convenient transfer of 100 γ of residue to a paper for chromatography. For example, 10 mg. was dissolved with 0.2 ml. chloroform and 0.002 ml. was spotted with a micropipet. The chromatography paper, Whatman No. 4, had just been dipped in propylene glycol-acetone (6:4) and hand pressed between heavy blotting paper.[2] The chromatogram was developed for 6 hours with the solvent mixture benzene-cyclohexane-methanol (80:15:5). The paper was dried at 100° and sprayed with the 3,5-dinitrobenzoic acid reagent.

Infrared Absorption of Aglycones.—Data were obtained with a Perkin-Elmer model 21 spectrophotometer, using chloroform solutions containing 20 to 25 Gm. per L. of the benzene-extracted solids. For confirmatory tests, spectra of the 1,800 to 1,600 cm.$^{-1}$ region are sufficient to detect and partially characterize digitalis-type cardiac aglycones. Complete spectra were obtained for some of the control aglycones.

Biological Testing.—The biological test used on these extracts was the one-hour frog test described in U. S. P. X. It was used as described, except for fewer animals.

DISCUSSION AND RESULTS

Cardiac glycosides and their hydrolysis products used as controls while developing these procedures were obtained from commercial strophanthin, commercial digitalis leaf powder, and as purified samples provided through the courtesy and generosity of Dr. T. Reichstein.

Comparative studies were made of the purification of extracts of the digitalis leaf powder by activated carbon, lead subacetate, and freshly precipitated lead hydroxide. Lead subacetate treatment did not remove a sufficient amount of impurities and involved extra steps in procedure. Activated carbon removed considerable solids, but the lead hydroxide exhibited the greater specificity in removing materials which, having R_f values similar to the cardiac glycosides, otherwise tended to mask the color reaction when the spray was applied.

Hydrolysis conditions used in the confirmatory tests have been purposely made more rigorous than those employed when isolation techniques are involved (10). As long as the chromophoric butenolide ring is retained, dehydration or other artifacts of the steroid nucleus are immaterial to the success of the screening test, and the removal of all sugars makes the aglycone more benzene soluble. Studies on known positives showed that chloroform extractions following the benzene could be eliminated.

Aliquots equivalent to the extract of 1 Gm. dry weight of plant material appear to be more than adequate for the sensitive qualitative screening test. A larger aliquot was taken for confirmatory testing to provide sufficient weight of benzene extractables for infrared analysis.

Complete results of the screening tests are too lengthy to include in this article. Responses to the qualitative color test have been assigned to three classifications: positive, the characteristic purple color reaction; doubtful, a faint to medium pink color; and negative, only the yellow or brown reaction of other plant constituents which are present in most plant extracts. Samples representative of all three classifications have been included in Table I to illustrate the correlation between color tests, infrared data, and biological tests. Biological and color tests, but not infrared, were made on 13 samples not included in the table. Five samples were positive by both tests and eight were negative by both.

Confirmatory tests have supported the assumption that all samples which give a positive color test contain cardiac glycosides. In most instances infrared spectra in chloroform of the hydrolysis products of these samples have peaks in the neighborhood of 1785 cm.$^{-1}$ and 1745 cm.$^{-1}$. Absorption in these regions is attributed to the γ-lactone group in $\Delta^{20(22)}$-cardenolides such as the digitalis-type cardiac glycosides (17). In addition to confirming the presence of the butenolide ring, the infrared

[2] Patterned after Zaffaroni (16). This system was developed in our laboratory and has not yet been published.

spectrum also furnishes other information concerning the structure of the cardiac aglycone. For instance, an aldehyde group is indicated by peaks near 1715 cm.$^{-1}$ for extracts of several Strophanthus species, and for the aglycone strophanthidin (17). Sarveroside, with absorption at 1702 cm.$^{-1}$, can be postulated to contain a carbonyl function at C-11 or C-12. The aglycone, sarverogenin, has indeed been assigned a 12-ketone (18). Accordingly, from Table I, odoroside or the aglycones from *Nerium oleander* should contain no carbonyl grouping on the steroid nucleus. The respective aglycones, digitoxigenin and oleandrigenin, have hydroxyl groups only on the steroid nucleus.

While all extracts which showed absorption in the 1785 cm.$^{-1}$ and 1745 cm.$^{-1}$ regions had previously given positive color tests, there may be exceptions to the converse of this statement (*cf. Periploca sp.*, Table I). Biological testing, however, has always confirmed cardiac activity in those extracts for which a positive color test was obtained.

Confirmatory tests were applied to all samples which gave any indication of a favorable color reaction during the qualitative screening. A pink color was observed on chromatograms of aloe extracts. After hydrolysis a similar pink color appeared on chromatograms of the benzene extracted solids. Neither infrared data nor physiological tests indicated that these samples contained cardioactive materials.

Negative samples will, in general, receive no further consideration. During development of the procedures samples representing various species and families were given some or all of the confirmatory tests. No exceptions to the negative classification were noted. After a thorough sampling of the agaves, yuccas, and dioscoreas, these sapogenin sources were eliminated from the qualitative test. It is anticipated that most of the plant materials collected for the sapogenin screening program will give negative tests for cardiac glycosides.

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