Chromatography on Lipophilic Dextran Gels for Fractionation of Low Molecular Weight Compounds II: Separation of Cardiac Glycosides from Cardiac Aglycones

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Abstract □ A simple method is presented for the separation of cardiac glycosides from cardiac aglycones using highly cross-linked lipophilic dextran gel chromatography with 95% ethanol as the solvent.

Keyphrases □ Dextran gel chromatography—separation of cardiac glycosides from cardiac aglycones □ Cardiac glycosides—separation from cardiac aglycones using dextran gel chromatography □ Chromatography—separation of cardiac glycosides from cardiac aglycones

The successful use of lipophilic dextran gel chromatography for the separation of cardiac glycosides from cardiac genins in a simple and reproducible manner is reported. The force responsible for the separation appears primarily to be a molecular sieving effect.

DISCUSSION

Several chromatographic techniques for the separation and purification of various cardiac glycosides have been extensively used. The techniques most often employed include paper chromatography (1) and TLC (2). Column chromatography (3–5) and countercurrent separation (6, 7) have also been used.

The use of dextran gel chromatography for the separation of compounds of varying molecular weight has been well documented. The separation of various classes of steroids and lipids was attempted using the lipophilic dextran gel[1] (8–11) or partition chromatography on hydrophilic dextran gels of the G type (9, 12, 13). Recently, a hydroxycyclohexyl derivative (14) and four hydroxyalicyclic derivatives of the lipophilic dextran gel[1] (15) were prepared, and standard elution volumes for a model set of steroidal compounds were reported for columns prepared for these modified materials (14, 15). Lipophilic dextran gels, however, have not been used for the separation of cardenolides, although column chromatography on another dextran gel[2] was used in the purification of cardiac glycosides and cardiac genins extracted from cardiac tissues of experimental animals (16).

The chromatographic method reported here was initially applied to artificial mixtures of two or more of the following compounds: digitoxin (mol. wt. 764.92), gitoxin (mol. wt. 780.92), digitoxigenin (mol. wt. 374.50), and gitoxigenin (mol. wt. 390.50). The procedure was then successfully used for the separation of a mixture of ^{3}H-digitoxigenin and ^{3}H-digitoxin prepared by the incomplete acid hydrolysis of ^{3}H-digitoxin. Since radioactive digitoxigenin is not commercially available, while ^{3}H-(G)-digitoxin can be purchased, it was desired to prepare ^{3}H-digitoxigenin from ^{3}H-(G)-digitoxin by acid hydrolysis and subsequent purification. The isolated ^{3}H-digitoxigenin is being used in a series of experiments designed to determine the metabolism of digitoxigenin by tissue homogenates from various experimental animals.

Several solvent systems of 95% ethanol, 70% ethanol, and methanol–chloroform (1:1) were used for eluting the columns[1]. The best separation was obtained using 95% ethanol.

The method provides an effective separation of the glycosides digitoxin and gitoxin from the aglycones digitoxigenin and gitoxigenin. However, there was no separation of the two aglycones from one another, or of the two glycosides from each other under the conditions employed. Apparently, the difference of only 16 molecular weight units is not sufficient to make these separations possible.

This method can be utilized for the preparative separation of the cardiac glycosides from their aglycones.

EXPERIMENTAL

Hydrolysis of ^{3}H-Digitoxin—To 10 μCi of uniformly labeled ^{3}H-digitoxin[3] (specific activity 1 mCi/μmole) was added 5 mg of nonradioactive digitoxin. The sample was dissolved in 10 ml of methanol–water (7:3), 60 μl of concentrated sulfuric acid was added, and the mixture was refluxed for 4 min. The reaction mixture was subsequently extracted with chloroform. The chloroform was then evaporated to dryness, and the residue was dissolved in dry benzene until ready for chromatography.

The residue was then evaporated to dryness and dissolved in 2 ml of 95% ethanol, an aliquot of which was applied to the column. Acid decomposition of the liberated digitoxigenin began to occur before complete hydrolysis of the sugar moiety of digitoxin was achieved, so a method for separating the digitoxigenin from the incompletely hydrolyzed digitoxin was needed.

Separation of Hydrolysis Mixture on Lipophilic Dextran Gels—An aliquot (1 ml) of the ethanolic solution obtained was applied to a column, 2.5 cm in diameter and containing 60 g of the gel in 95% ethanol. Fractions (1 ml) were collected at a flow rate of 0.5 ml/min under a pressure head of 50 cm. Separation of cardenolides from the glycosides was possible with flow rates from 0.3 to 0.7 ml/min.

Fractions were tested by TLC on silica gel H[4] plates with either methylene chloride–methanol (85:15) (17) or cyclohexane–acetone–acetic acid (65:33:2) (18) as the developing solvent. After development, the plates were sprayed with ferric chloride in acetic acid (2 ml of 5% $FeCl_3$ in 20 ml of acetic acid), heated at 100° for 5 min, and cooled. The plates were then sprayed with 50% H_2SO_4 and heated again for 10 min.

The cardiac glycosides and aglycones give yellow to green or blue colors. The relative concentrations of the fractions were determined according to the size and density of the color of the spot of TLC and/or by liquid scintillation counting for the tritium-labeled samples (19). Both digitoxin and gitoxin were eluted in fractions 81–90 with a peak at fraction 84, while digitoxigenin and gitoxigenin were eluted in fractions 94–107 with a peak at fraction 102.

The fractions containing each compound were combined and evaporated to dryness *in vacuo* to obtain chromatographically pure cardiac glycosides and cardenolides as determined by TLC. Approximately 50% of the radioactivity was recovered as digitoxigenin following the hydrolysis of ^{3}H-(G)-digitoxin.

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Antidermatophytic Activity of 2-Thiotetrahydro-1,3,5-thiadiazines and Isothiocyanates

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Abstract □ Benzyl, furfuryl, tetrahydronaphthyl, α-picolyl, and α-homopicolyl derivatives of 2-thiotetrahydro-1,3,5-thiadiazines were studied and found to be more active than β-picolyl and pyridyl analogs on dermatophytes, including the more resistant *Microsporum canis*.

Keyphrases □ 2-Thiotetrahydro-1,3,5-thiadiazines—antidermatophytic activity □ Isothiocyanates—antidermatophytic activity □ Antidermatophytic activity—2-thiotetrahydro-1,3,5-thiadiazines and isothiocyanates

It has been demonstrated that pterigospermin and its fission product benzyl isothiocyanate are potent antifungal agents (1–7). Phytopathogenic and saprophytic fungi, especially the yeasts, show sensitivity to isothiocyanates and their producers (8). However, inadequate dermal absorption, low solubility, and vesication are but a few of the factors that limit their topical application in the treatment of dermoid infections.

It was thought that substitution of the benzyl moiety by other isosteric groups, particularly those with basic residues, would make the compounds more water soluble to overcome the limitations in their use. This report presents the results of studies to test this hypothesis.

EXPERIMENTAL

Organisms—*Trichophyton mentagrophytes*[1] (HM115), *T. rubrum*[1] (HM186), *T. violaceum*[1] (HM164), *Epidermophyton floccosum*[1] (HM78), *Microsporum canis*[1] (I73), and *Candida albicans*[2] (Z248) were used. All dermatophytes and yeasts were maintained on Sabouraud's glucose agar slants.

Inocula—Spore suspensions from 15-day agar slant cultures in sterile saline were prepared, and 0.1 ml of the suspension or of an 18-hr broth culture of *C. albicans* was used as the inoculum in the serial dilution method.

Compounds—Solutions (1 mg/ml) were made in slightly acidified 50% ethanol (basic compounds), 20% dimethylformamide (griseofulvin), or acetone.

Antifungal Activity—Tubes in duplicate containing log concentrations, 0, 0.1, 1.0, 10.0, and 100 μg/ml, of the test compounds in 5.0 ml of Sabouraud's glucose broth were inoculated and incubated at 30° for 14 days (dermatophytes) or for 2 days (yeast). Tubes containing solvents at corresponding concentration levels were also included as blanks. The minimum inhibitory concentration (MIC) required for complete inhibition of growth was scored based on at least two independent experiments.

RESULTS AND DISCUSSION

The results of initial screening of antifungal activity of a few isothiocyanates and their producers, substituted 2-thiotetrahydro-1,3,5-thiadiazines, and other substances are reported in Table I. Benzyl isothiocyanate (XXI) inhibited the dermatophytes and *C. albicans* in the concentration range of 0.1–5.0 μg/ml, while the corresponding thiadiazine (X) did so at a level of 1.0–10.0 μg/ml. Similar levels of activity were displayed by furfuryl and tetrahydronaphthyl analogs (XIII and XIV, respectively). α-Picolyl and α-homopicolyl derivatives (III and VI, respectively) were distinctly more active then the β- and γ-isomers (IV and V, respectively) and far more potent than the pyridyl compounds (I and II, respectively).

[1] Isolated from clinical materials and characterized.

[2] Obtained from the School of Tropical Medicine and Hygiene, London, England.