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Note

Fractionation of steroid digitonides by thin-layer and column chromatography on silica gel

FRANCOIS-X. GARNEAU, JEAN CÔTÉ, ODETTE HARVEY and JEAN-LUC SIMARD
Département des Sciences Pures, Université du Québec à Chicoutimi, Chicoutimi, Québec G7H 2B1 (Canada)

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The quantitative determination of cholesterol has often been accomplished by its isolation as an insoluble complex with digitonin[1,2] followed by fractionation of the digitonide in order to recover the cholesterol. The same complex-forming reaction has been used to selectively precipitate sea cucumber saponins (holothurins) using cholesterol as the complexing reagent[3,4]. In the course of our investigations on the selectivity and efficiency of this complex formation as a means of isolating holothurins, we observed that steroid digitonides could be separated into their constituents by thin-layer (TLC) and column chromatography on silica gel. The digitonides are most often decomposed using pyridine[3,5] or dimethyl sulphoxide (DMSO)[6] under reflux followed by extraction with solvents. Dextran gel column chromatography has been successfully used[7], although digitonin and stigmasterol are eluted fairly close to one other. The TLC method described in this report has the usual advantages associated with this technique, as well as resulting in very different R_F values for the constituents (cholesterol–digitonin and stigmasterol–digitonin) and also in a partial separation of the saponin mixture itself. As expected, column chromatography also permitted a very good fractionation of larger quantities of the digitonides as well as resulting in highly different migration rates for the constituents and a partial separation of the commercial digitonin mixture.

EXPERIMENTAL

Materials and reagents

Digitonin (ICN Pharmaceuticals, Plainview, N.Y., U.S.A.), m.p.: 240–245° (d), stigmasterol (Sigma, St. Louis, Mo., U.S.A.), m.p. 164–167°, and cholesterol (J. T. Baker, Phillispburg, N.J., U.S.A.) m.p. 144–146° were used as received. Benzene, chloroform, methanol and diethyl ether were from Fisher Scientific (certified ACS grade; Fair Lawn, N.J., U.S.A.). The diethyl ether was fractionally distilled. Silica gel 7G (TLC reagent) and silica gel (60–200 mesh, analyzed) were purchased from J. T. Baker. Polygram SIL G (Brinkmann, Westbury, N.Y., U.S.A.) precoated plastic sheets were used for qualitative analysis.

Preparation of digitonides

The method for digitonide formation was that described by Fernholz[5] and involves adding the same volume of a 1% solution of digitonin in ethanol to a hot solution of the sterol in 90% ethanol. The precipitated digitonide was washed several times with diethyl ether (benzene and chloroform with the stigmasterol digitonide) and methanol to remove all traces of uncomplexed sterol and digitonin respectively.

Thin-layer chromatography

The preparative TLC was carried out by spreading a uniform layer (1 mm) of silica gel 7G on 20 × 20 cm glass plates with a Desaga/Brinkmann standard adjustable applicator. The plates were then placed in an oven at 110° for 1 h. Great care was taken in placing the complexes (a suspension in benzene–methanol, 1:1) as a uniform band on the plates. Development was carried out in a glass chamber with the lower phase of the solvent system chloroform–methanol–water (65:35:10) (solvent I). Visualization was achieved on a similar plate using the Liebermann–Buchard detection reagent followed by heating in an oven at 110° for 5 min. Cholesterol and digitonin were extracted exhaustively from the silica gel using diethyl ether and methanol respectively. The purity of the sterols and the digitonin was checked on pre-coated plastic sheets (3 × 8 cm) of silica gel and they were identified by a comparison of their melting points and/or their R_F values with authentic material. Cholesterol ($R_F = 0.79$) and stigmasterol ($R_F = 0.81$) each appeared as a single pink spot and digitonin as two light brown spots ($R_F = 0.19, 0.26$) with the aforementioned detection reagent.

Column chromatography

A 37.5-g amount of silica gel was introduced as a slurry with chloroform–methanol–water (60:15:2) into a glass column (2 × 37.5 cm) fitted with a PTFE stopcock. The complexes were placed on the column as a partially soluble mixture with benzene–methanol (1:1). Elution was achieved with chloroform–methanol–water (60:15:2 and 60:30:4) (solvents II and III). The fractions were examined by TLC and the compounds were identified by comparison of melting points and/or R_F values with authentic material.

RESULTS AND DISCUSSION

The insoluble complex formed from digitonin and cholesterol in alcohol was separated into its constituents when placed on an analytical pre-coated plastic sheet of silica gel and developed with solvent I. It was subsequently observed that the efficiency of fractionation depended on the quantities of digitonide relative to the thickness of the silica gel layer. A too high ratio of amount of digitonide to layer thickness resulted in a black spot at the point of application (presumably intact complex) as well as the spots characteristic of digitonin and the sterol. On a preparative scale, a quantity $\geqslant 16$ mg of digitonide on 1 mm of silica gel resulted in a poor fractionation. An ideal situation was obtained with 10 mg of digitonide on a 1 mm layer of silica gel. The bands resulting from the fractionation of the cholesterol digitonide were extracted and the purity and identity of the two constituents were confirmed by analytical TLC. 90% of the digitonin was recovered based on a 1:1

complex. Although the stigmasterol digitonide was not fractionated on a preparative scale by TLC, a purified sample of the complex was separated into its constituents in the same manner on an analytical plate.

Larger quantities of the digitonides were fractionated by column chromatography on silica gel. Ratios of adsorbent to digitonide of 1000:1 and 1500:1 resulted in complete fractionation whereas the presence of both cholesterol and digitonin was observed (TLC) in the initial fractions when a ratio $\leq 750:1$ was used. In this latter case, the column was prepared and eluted with solvent I. In a typical run, 37.5 mg of the cholesterol digitonide was placed on a column containing 37.5 g of silica gel. Elution was carried out at first with 200 ml of solvent II followed by solvent III (5-ml fractions). Cholesterol was obtained in fractions 13–40 and the digitonin was collected as follows (monitored by TLC): fractions 54–61, component 1 ($R_F = 0.26$); fractions 62–67, components 1 and 2 ($R_F = 0.26, 0.19$); and fractions 68–87, component 2 ($R_F = 0.19$). An essentially quantitative yield was obtained of cholesterol (m.p. 140–143° after one recrystallization) and digitonin (combined fractions) based on a 1:1 complex.

In the same manner, 37.5 mg of the stigmasterol digitonide were fractionated. Stigmasterol was collected in fractions 9–32 (5 ml each) using solvent II. At fraction 37 elution was continued with solvent III (10 ml fractions). The two components of digitonin were again separated as follows: fractions 49–57, component 1; fractions 58–64, components 1 and 2; and fractions 65–86, component 2. Here again an essentially quantitative yield of stigmasterol was obtained as well as a yield of $\approx 85\%$ digitonin.

This method is particularly useful when isolating saponins as insoluble complexes with cholesterol as it allows an efficient fractionation of the complex under conditions which also favor a separation of the constituents of saponin mixtures in a single chromatography operation. The method may be applicable to the quantitative determination of sterols which precipitate with digitonin.

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