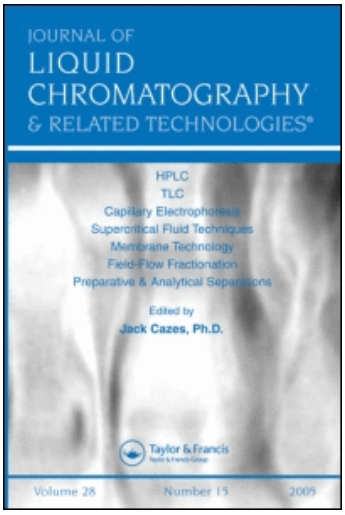

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Csiky, Istvan and Hansson, Lennart(1986) 'High Performance Liquid Affinity Chromatography (HPLAC) of Sterols with Tomatine Chemically Bonded to Microparticulate Silica', Journal of Liquid Chromatography & Related Technologies, 9: 4, 875 — 886
To link to this Article: DOI: 10.1080/01483918608076675
URL: http://dx.doi.org/10.1080/01483918608076675

JOURNAL OF LIQUID CHROMATOGRAPHY, 9(4), 875-886 (1986)

HIGH PERFORMANCE LIQUID AFFINITY CHROMATOGRAPHY (HPLAC) OF STEROLS WITH TOMATINE CHEMICALLY BONDED TO MICROPARTICULATE SILICA

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ABSTRACT

A selective HPLAC stationary phase for the separation of sterols was achieved by the chemical bonding of tomatine to microporous silica. Columns packed with tomatine silica have given promising results indicating their usefulness as well for selective on-column enrichment as for HPLC separations. Good separation was achieved with a mixture of five sterols known as oxidation products of cholesterol.

INTRODUCTION

The steroid saponins are a group of substances known for their ability to form equimolar "sparingly soluble" complexes with cholesterol (1). The determination of the insoluble cholesterol digitonide has been used since the 30's when Schoenheimer et. al

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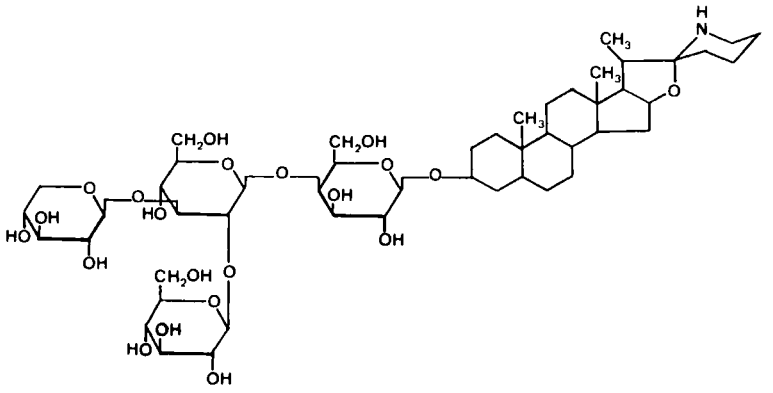

FIGURE 1. Chemical structure of tomatine.

(2) developed a procedure for the precipitation and gravimetric determination of the complex. Other workers investigated the selectivity of the digitonide (3) and tomatide (4,5) formation and found that precipitation can be obtained with a large number of steroids, all structurally equal to the 3β-hydroxy position. The selectivity was also affected by the cis-trans configuration of the 5 : bond. The 3α-hydroxy compounds did not precipitate with the saponins.

Some papers have reported on the use of digitinonin as a selective stationary phase in chromatography. Digitonin-impregnated silica plates were applied at thin layer chromatographic separations of 3-hydroxysterol mixtures (7,8). Sterols were successfully removed from lipid extracts on packed beds of celite coated with digitonin (9).

The aim of this investigation was the preparation and utilization of tomatine chemically bonded to silica gel as a stationary phase for high performance liquid chromatography.

EXPERIMENTAL

Materials

Porous silica (LiChrosorb Si 100, 5 um) was obtained from E. Merck (Darmstadt, GFR), γ-glycidoxpropyltrimethoxysilanole (solane Z-6040) from Dow Chemicals (Midland, MI, USA). Tomatine, cholesterol, cholecalciferol (vimatin D_3) and glyceroltrioleate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Keto-and hydroxysterols were purchased from Steraloids (Pawling, NJ, USA).

All other solvents and chemicals obtained from commercial sources were used without purification or dried as mentioned below. A reference mixture of 4-cholesten-3-one (2.6 mg/ml) choelst-5-ene3β, 4β-diol (2.5 mg/ml), 3β-hydroxycholest-5-ene-7-one (6.8 mg/ml), cholest-5-ene-3β,7β-diol (2.5 mg/ml) and cholesterol (16.3 mg/ml) was prepared by dissolving the substances in hexane/propanol (99.9/0.1) and further diluting in hexane. When the sterols were separated in aqueous eluents, hexane was evaporated from the mixture and the sterols were redissolved in ethanol.

Apparatus

Separations were performed with a Varian Model 5000 liquid chromatograph equipped with a Valco loop injector. The eluent was

monitored at 211 nm with a Pye-Unicam LC-UV-detector. A LDC Constsametric II high pressure pump with a LDC spectromonitor III was used in the breakthrough experiments.

A Carlo Erba Elemental Analyzer-Model 1102 was used for C, H, and N analysis.

Synthesis of Tomatine-substituted Silica

Epoxy-substituted silica was used as starting material for the synthesis. This material has been described previously (10). The elemental analysis of the epoxy-substituted silica dried in vacuum gave C = 7.39%, H = 1.4% and N<0.02%.

Epoxy-substituted silica (3.0 g) and tomatine (1.5 g) were dried at 100°C in vacuum overnight, mixed with 20 ml dioxane (dried with basic alumina) and 1 ml borontrifluorideetherate in a glass ampoule. The coupling reaction was conducted in the glass ampoule at 21°C for 40 h. The tomatine-substituted silica was filtered and washed thoroughly with dioxane, methanol, methanol/water, water, methanol, dioxane, diethylether and finally dried at 100°C in vacuum. The elemental analysis of the gel gave C = 11.1%, H = 2.03% and N = 0.10% (mean of two determinations).

Epoxy-substituted silica (2 g) was hydrolyzed in 40 ml 0.1 M sulphuric acid-sodium hdyroxide buffer (pH 2, 50°C, 5 h) and the diolsilica was used as a blank material to study the influence of matrix in the separations.

Columns

Column A was a 30 mm x 4.5 mm ID glass column containing 0.3 g tomatine silica. The void volume in the column was reduced by inserting a tightly fitting porous PTFE rod with the appropriate length. Column B was a 100 mm x 4.5 mm ID stainless steel column with 2 um frits in both ends and containing 2.7 g tomatine silica. Column C was a 100 mm x 5.0 mm ID Pharmacia glass column with freely adjustable ends containing 3.0 g diol silica. The columns were packed in methanol with the upward slurry technique.

RESULTS

Breakthrough curves

In order to ascertain to what extent the bonded tomatine phase shows affinity to sterol structures, breakthrough experiments, presented in fig. 2, were made on a short column of tomatine silica.

Hexane solutions of cholesterol (0.149 mg/ml), cholecalciferol (0.018 mg/ml) and glyceroltrioleate (0.010 mg/ml) were pumped through the short tomatine column (column A) at a flow rate of 1 ml/min and the eluate was monitored at 210 um. Cholesterol was strongly held by the column. The breakthrough capacity (C/Co = 0.01) was about 19 umole cholesterol/g tomatine silica.

The regeneration of the column from adsorbed cholesterol was tested by washing with 10% hexane solutions of n-propanol,

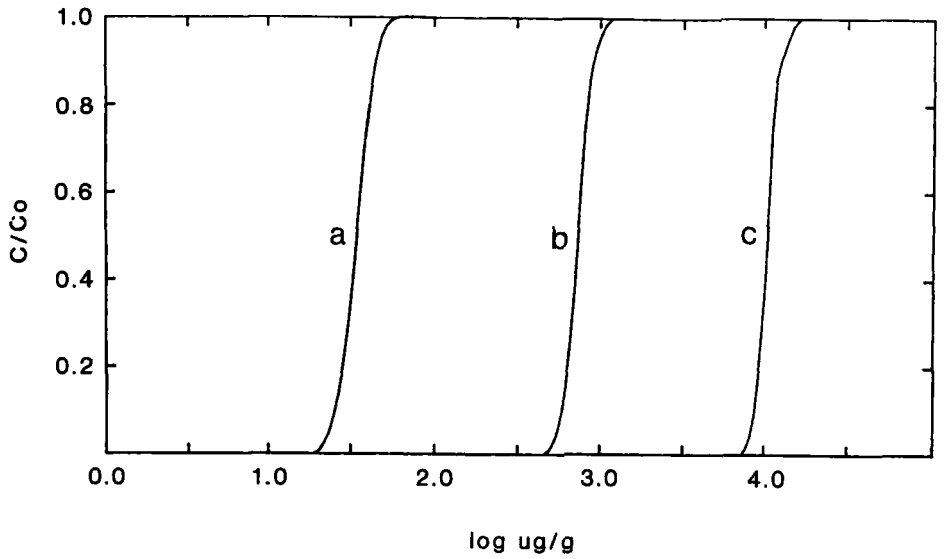

FIGURE 2. Breakthrough capacities of tomatine silica, a = glyceroltrioleate, b = cholecalciferol, c = cholesterol. Observed concentration (C) relative to steady state plateau concentration C_o vs. amount of a, b, c.

tetrahydrofurane, cyclohexane and cyclohexanol. Only the cyclohexanol mixture removed the adsorbed sterol effectively. The propanol solution desorbed the sterol very slowly and the cyclohexane and tetrahydrofurane solutions did not release any sterol.

Chromatographic separation

The chromatogram in fig. 3 demonstrates the separation of 10 ul of a synthetic mixture of five sterols in hexane applied to a column (Column B) with silica-bound tomatine.

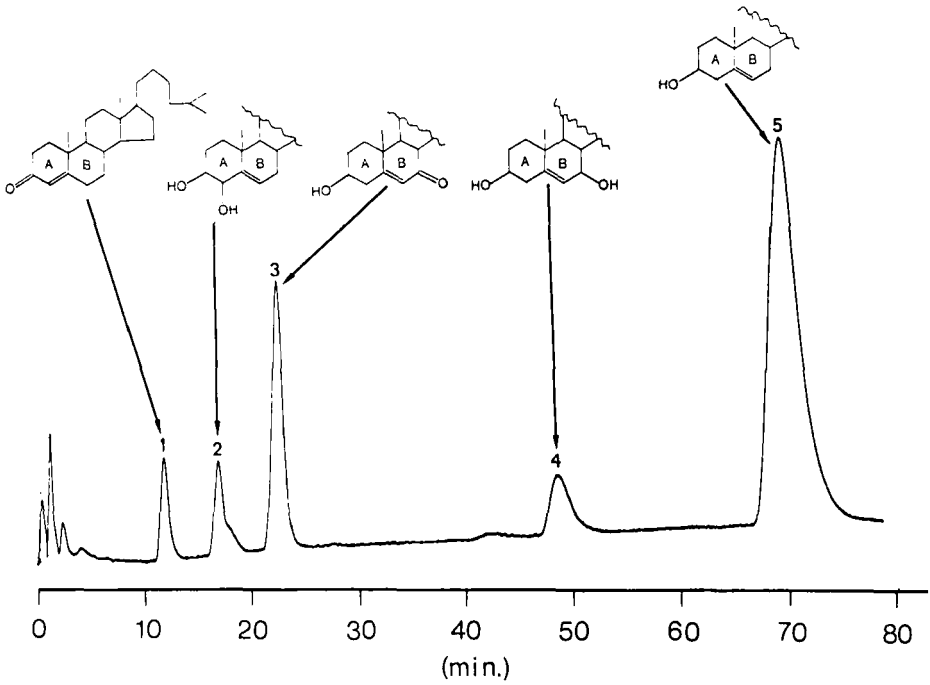

FIGURE 3. Separation of sterols on silica-bound tomatine. Sample: a mixture of 4-cholesten-3-one (1), cholest-5-ene-3β,4β-diol (2), 3β-hydroxycholest-5-ene,7-one (3), cholest-5-ene-3β,7β-diol (4) and cholesterol (5). Eluent: n-hexane-cyclo-hexanol (98.5:1.5, v/v). Flow rate 1.0 ml/min. Detection: A_{211}.

The retention of the sterols could be controlled by changing the amount of cyclohexanol in the mobile phase as it is shown in Table 1.

Ethanol-water was also studied as mobile phase. The ethanol content was varied from 85-100%. The flow rate was 1.5 ml/min and 20 ul of the reference mixture dissolved in ethanol was injected. No separation was obtained.

TABLE 1

K'-Values for Five Sterols Injected on Tomatine Silica Column at Various Amounts Cyclohexanol in the Mobile Phase.

Compound	%Cyclohexanol in n-hexane		
	5.0	3.0	1.5
cholesterol	2.0	22.3	61.1
cholest-5-ene-3,7-diol	0.1	7.9	41.2
5-cholesten-7-one	0.1	1.5	18.8
cholest-5-ene-3,4-diol	0	0.6	13.7
cholest-4-ene-3-one	0	0.3	10.2

To investigate if the silica support itself or the spacer group coupled to the silica could contribute to the separation, the mixutre was applied to a column (Column C) with diol silica. The chromatographic conditions were as in Fig. 3. The sterols were eluted with the solvent front; thus the highly selective separation of sterols on tomatine silica can be ascribed to the chemically bonded tomatine groups.

DISCUSSION

In the reaction between epoxy-substituted silica and tomatine, both the hydroxyl groups of the glycosides and the secondary amine group of the aglycone can be utilized for the coupling to the epoxy groups according to Figure 4.

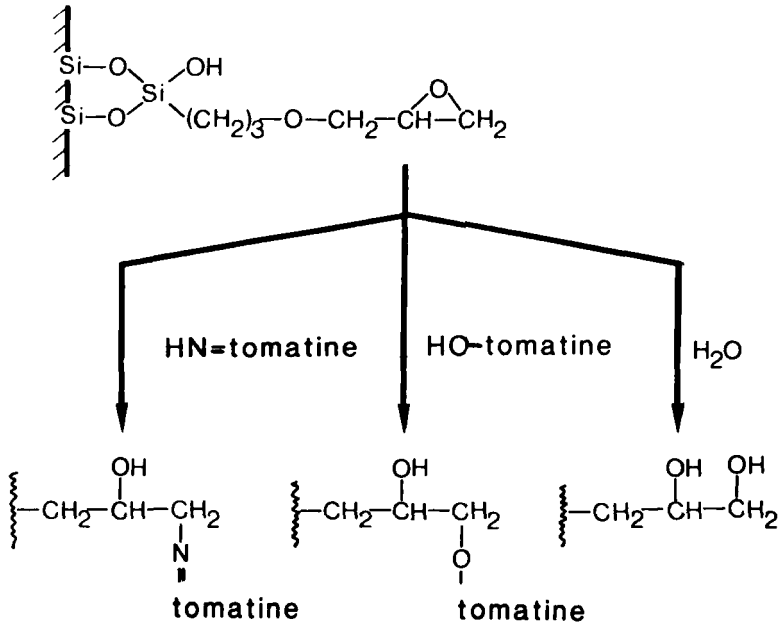

FIGURE 4. Synthetis of tomatine silica and diol-silica.

Borontrifluoride acts as a catalyst and in order to get a good yield, all chemicals have been carefully dried. Calculation based on the carbon elemental analysis of the epoxy-substituted silica estimates the concentration of epoxy groups to be ca 880 umol per gram. The amount of tomatine coupled to the epoxy-substituted silica was calculated from the differences of carbon elemental analysis and estimated to be about 60 umol tomatine per gram gel. Calculations based on the elemental analysis of hydrogen and nitrogen are also in accordance with this value. The relative low yield of the reaction is probably due to the steric hindrance of the bulky tomatine molecules and a slow reaction.

Since point of attachment of tomatine to epoxy silica is unknown the column capacity for cholesterol, 19 umole/g, is in good agreement with the calculated amount of tomatine coupled to the silica.

A promising approach is to use the tomatine-silica column to "selectively sorb" the sterols from a crude sample, followed by direct displacement into another column for the final separation, thus utilizing it only for the isolation of a group of substances. The preference for cholesterol on column A as shown in Fig. 2, indicates that the material can be used for trace enrichment. Trioleine, chosen as a model substance for fat components in lipid extracts, was completely unaffected by the bonded phase. The cholecalciferol was slightly retarded compared to the front trioleine, indicating a very weak interaction with the bonded phase. The presence of an epimethyl group at C-10 in cholecalciferol prevents complex formation with tomatine as it is reported in the case of digitonin (11).

In the literature cited both precipitation and thin layer chromatography are performed in aqueous solvent mixtures thus indicating that a strong interaction between saponin and sterols can be expected in hydrophilic media. Our chromatographic experiments with Column B cannot confirm this idea. When the column was eluted with ethanol-water, the injected sterol mixture eluted with the solvent front and no retention was observed. However, when the mobile phase was n-hexane the injected sterols

were very strongly retarded. As is shown in Table 1, even low amounts of cyclohexanol in the mobile phase modify elution from the column. This could be due to the competitive effect of cyclohexanol for the selective binding sites on the stationary phase. Also, nonspecific hydrophobic interactions are probably minimized by the mobile phase.

The selectivity of the tomatine silica is most sensitive on differences around the 3β-hydroxy position of the sterols, which is in accordance with other reports (4,5) on tomatine selectivity.

ACKNOWLEDGEMENTS

The authors thank Dr. Lars Haraldson for valualbe discussions and continuous support.

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