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SEMI-PREPARATIVE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY IN THE ISOLATION OF DERIVATIVES OF CARDIOACTIVE STEROIDAL LACTONES

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SUMMARY

Until now, semi-preparative high-performance liquid chromatography has rarely been applied to cardioactive steroidal lactones and their derivatives. The examples given clearly show the usefulness of the technique for this class of compounds. The technique is superior to open-column and preparative-layer chromatography, especially with respect to speed, separation efficiency and ability to handle sparingly soluble and sensitive compounds.

INTRODUCTION

We have been chemically modifying natural cardioactive steroidal lactones in our search for derivatives with improved therapeutic qualities. In this synthetic work the majority of the time is employed in the separation of the reaction mixtures and in the purification of compounds by open-column or preparative-layer chromatography (PLC), far more than for the chemical transformation itself. We were therefore interested in improving our semi-preparative high-performance liquid chromatography (HPLC) separation technique so as to be able to obtain in a short time enough pure substance for structural assignment and biological testing. Despite the successful use of analytical HPLC in the field of cardioactive steroidal lactones (*e.g.*, refs. 1 and 2), to the best of our knowledge there are only two reported examples of the use of semi-preparative HPLC in the isolation of small amounts from plant[3] or toad[4] materials.

EXPERIMENTAL

Equipment

The pump (Micropump MC 706.1, Microtechna, Prague, Czechoslovakia) was improved by attachment of a second plunger and a home-made filter assembly including glass fibre paper (pore diameter 2 μm) at the solvent inlet and a second at the pump exit. Pulse damping was effectively attained by a Halász-pot with a 0.3-mm stainless-steel membrane, filled with *n*-hexane (130 ml), connected to a stainless-steel capillary (20 m × 0.2 mm I.D.). Sample introduction was realized by loops of ad-

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equate volume (0.2–5 ml) and a stop flow technique. Home-made stainless-steel columns (250 × 10 mm I.D.) were filled by the slurry technique (pressure *ca.* 400 atm) with silica gel (15–20 μm) or with SI C18, 7.5 μm (Lachema, Brno, Czechoslovakia). The silica gel was fractionated from Kieselgel HF_{254} Typ 60 (E. Merck, Darmstadt, G.F.R.) with a Labor–Zickzack–Sichter 100 MZR (Alpine, Augsburg, G.F.R.). For filling of the column, the gel was suspended in carbon tetrachloride–dioxane (1:1) and degassed by ultrasonics. If the samples contained strongly polar impurities, silica columns were protected from excessive contamination by pre-columns (length 60 mm, dry-filled with Silasorb 600, 30 μm; Chemapol, Prague, Czechoslovakia). A Dukol UV detector (254 nm) was used together with a K 201 one-line recorder (both VEB Carl Zeiss Jena, Jena, G.D.R.). To avoid gas bubbles being formed in the detector cell (8 μl), the solvent was degassed by ultrasonics (50 W, 5 min, Sonifier B12; Branson Sonic Power, Danbury, CT, U.S.A.). The whole system permitted a maximum flow-rate of 8 ml/min.

Procedure

The solution of the sample was filtered by a syringe through a filter assembly similar to that described for solvent introduction. Fractions corresponding to the peaks of the absorption curve were collected from the column. Aliquots (100 μl) were tested by thin-layer chromatography (TLC) on silica sheets (Silufol UV_{254}; Kavalier, Sklárny, Czechoslovakia) (detection with phosphoric acid). After separation, the silica columns were eluted with ethanol for purification and were then effectively reactivated with 2,2-dimethoxypropane–acetic acid–*n*-hexane (2.5:2.5:95) (*ca.* 20 times the column void volume) followed by *n*-hexane[5].

RESULTS

Digitoxigenin (I) and its 3-acetate (II)*

A 200-mg amount of a mixture containing mainly I and II dissolved in 1 ml of chloroform was injected on to a silica column and eluted (4.2 ml/min, 10 atm) with chloroform for 12 min 45 sec, then with chloroform–4% ethanol. For TLC control, chloroform–10% acetone was used twice. Pure II (96.5 mg) was found in fraction 3 (4 min–12 min 45 sec) and pure I (56.5 mg) in fraction 5 (17 min–26 min 40 sec).

3-Acetyldigitoxigenin-14β-O-(N-trichloroacetyl)carbamate (III)[6]

Compound III is moved in TLC (chloroform–5% ethanol) as a distinct spot, but elution gave only its elimination product, 3-acetyl-14-anhydrodigitoxigenin. Preparative purification of 25 mg of crude III [dissolved in 0.85 ml of acetonitrile–water (7:3)], however, was attained by HPLC using the same solvent as eluent (1.9 ml/min, 15 atm) on a C_{18} silica column (150 mm). TLC control (see above) showed that fraction 4 (11 min 30 sec–17 min 15 sec) contained pure III (12.5 mg).

21-Cyanogitoxigenin 3,16-diacetate (IV)[7]

Purification of IV by repeated PLC (chloroform–5% ethanol) was unsuccesful, because IV was partially degraded. Despite this lability, HPLC of 177 mg of the crude

* For structures see Fig. 1.

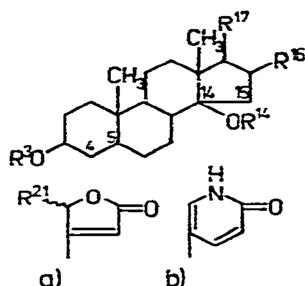

Fig. 1. Structure of investigated compounds. R^n = H if not specified otherwise.

Compound	R^3	R^{14}	R^{16}	R^{17}	R^{21}	*Other*
I				a		
II	CH_3CO-			a		
III	CH_3CO-	OC–CCl_3 | HN–CO–		a		
IV	CH_3CO-		CH_3CO_2-	a	–CN	
A	Acetyl-α-L-rhamnosyl–			b		Δ^4

mixture on a silica column with chloroform–*n*-hexane (1:1) (4.5 ml/min, 25 atm) gave 37 mg of nearly pure IV from 26 min 50 sec to 37 min 50 sec. Repetition under the same conditions removed the last impurities running in TLC above the spot for IV.

Isolation of side products A and C formed during synthesis of proscillaridin A-lactam[8]

Isolation of A and C by PLC (chloroform–20% ethanol) was unsuccessful, because these products do not move owing to their low solubility. This problem, however, was solved by HPLC: 78 mg of crude mixture, dissolved in chloroform–3% ethanol (120 ml), are filled into the apparatus in 26 min 20 sec using the solvent inlet. In this solvent neither A nor C was mobile but was adsorbed on the top of the silica column. Elution (4.1 ml/min, 13 atm) was performed with a stepwise gradient starting with chloroform–3% ethanol and increasing the ethanol concentration by 1% every time at the end of a peak. As TLC control (system as above) shows, pure A (22 mg) was eluted in fraction 3 from 60 min 30 sec to 83 min 30 sec and pure C (16 mg, structure unknown) in fraction 7 from 134 min 50 sec to 177 min 30 sec.

DISCUSSION

The examples reported in this paper, with many others accumulated in our laboratory, show clearly the usefulness of this technique for this class of compounds. It is superior to open-column and preparative-layer chromatography especially with respect to speed, separation efficiency and ability to handle sparingly soluble and sensitive compounds. Furthermore, the results with compounds III and IV show that complete or partial degradation during PLC takes place, when the plates became dry by evaporation of the mobile phase. Avoidance of degradation is therefore not only possible by use of a reversed-phase system (as in the case of III), but also by HPLC on a silica column (as in the case of IV), because there is no contact with dry silica.

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