ELSEVIER

Journal of Chromatography A, 756 (1996) 287–291

JOURNAL OF CHROMATOGRAPHY A

Short communication

Isolation of cardenolides from a Brazilian cultivar of *Digitalis lanata* by rotation locular counter-current chromatography

Fernão Castro Braga[a], Wolfgang Kreis[b], Alaíde Braga de Oliveira[c,*]

[a]*Departamento de Química, UFMG, Av. Antônio Carlos 6627, CEP 31270-010, Belo Horizonte, Brazil*
[b]*Lehrstul für Pharmazeutische Biologie, Friedrich-Alexander Universität, Staudtstraße 5, D-91058 Erlangen, Germany*
[c]*Faculdade de Farmácia, UFMG, Av. Olegário Maciel 2360, CEP 30180-112, Belo Horizonte, Brazil*

Received 7 February 1996; revised 16 July 1996; accepted 17 July 1996

Abstract

Cardenolides from a Brazilian cultivar of *Digitalis lanata* were isolated by rotation locular countercurrent chromatography (RLCC), employing dichloromethane–methanol–water (5:6:4, v/v) as the solvent system. Highly pure lanatoside C was obtained from the *Digitalis lanata* hydromethanolic extract, pre-purified either by silica gel or reversed-phase chromatography.

Keywords: *Digitalis lanata*; Rotation locular counter-current chromatography; Cardenolides; Glucodigifucoside; Odorobioside G; Purpureaglycoside A; Lanatoside A; Glucoevatromonoside; Lanatoside C; Digoxin; β-Acetyldigoxin; Glucogitoroside

1. Introduction

Digitalis lanata is the industrial source of the cardenolides digoxin and lanatoside C (Fig. 1) that are used for treatment of congestive heart failure. A special task is required to isolate *Digitalis lanata* glycosides, since about 70 different cardenolides have already been reported to occur in this plant species [1]. Most of the described methods for the isolation of these compounds are based on silica gel chromatography [2] or reversed-phase chromatography [3].

Counter-current chromatography is an alternative for the separation of polar compounds and indeed three new cardiac glycosides were isolated from *Digitalis lanata* leaves by droplet counter-current chromatography (DCCC) [4–6].

Rotation locular counter-current chromatography (RLCC) is also an all liquid chromatographic method [7,8]. Its use in the separation of several classes of natural products has been described [9,10], but there is no report on the isolation of *Digitalis* glycosides. This paper describes the isolation of cardenolides from a pre-purified *Digitalis lanata* hydromethanolic extract by RLCC.

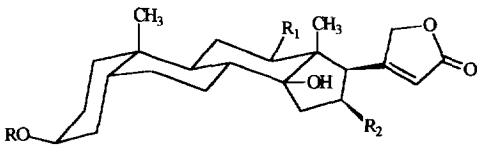
2. Experimental

2.1. Preparation of plant material

The 12-month-old leaves of *Digitalis lanata* were collected from an experimental cultivar at Itatiaia,

* Corresponding author.

0021-9673/96/$15.00
PII S0021-9673(96)00645-0



Compound	Abbreviation	R [a]	R_1	R_2
Glucodigifucoside	Gdf	Glc-β1–4-Fuc-β1–	H	H
Odorobioside G	OG	Glc-β1–4-Dtl-β1–	H	H
Purpureaglycoside A	PA	Glc-β1–4-Dox-β1–4-Dox-β1–4-Dox-β1–	H	H
Lanatoside A	LA	Glc-β1–4-α-AcDox-β1–4-Dox-β1–4-Dox-β1–	H	H
Glucoevatromonoside	Gev	Glc-β1–4-Dox-β1–	H	H
Lanatoside C	LC	Glc-β1–4-α-AcDox-β1–4-Dox-β1–4-Dox-β1–	OH	H
Digoxin	Dg	Dox-β1–4-Dox-β1–4-Dox-β1–	OH	H
β-acetildigoxin	β-AcDg	β-AcDox-β1–4-Dox-β1–4-Dox-β1–	OH	H
Glucogitoroside	Ggr	Glc-β1–4-Dox-β1–	H	OH

[a] Glc = glucose; Dox = digitoxose; Dtl = digitalose; Fuc = fucose; α-AcDox = α-acetildigitoxose β-AcDox = β-acetildigitoxose.

Fig. 1. Chemical structures of cardenolides isolated by RLCC in the present work.

Brazil. The dense rosette leaves (1000 g) were dried at 40°C for 48 h and then percolated with 70% MeOH (3×3 l) at room temperature, for 72 h. The extract was evaporated to dryness under reduced pressure, at 50°C, to give a dark green residue (400 g). Portions of the crude hydromethanolic extract were submitted either to method A or to method B for pre-purification before injection into the RLCC system.

2.2. Method A

A portion of the crude hydromethanolic extract (64 g) was dissolved in 70% MeOH (600 ml) and partitioned with light petroleum (2×300 ml). The hydromethanolic layer was concentrated in a rotatory evaporator under reduced pressure, at 50°C, to ca. 300 ml and partitioned with $CHCl_3$–iso-PrOH (3:1, v/v) (3×300 ml). The residue from the $CHCl_3$–iso-PrOH layer (3.2 g) was chromatographed in a silica gel column (silica gel 60 70–230 mesh, Merck; 310·25 mm I.D.) using a gradient of EtOAc–EtOH as follows: fractions 1–7 (98:2; 2110 ml); fractions 8–11 (96:4; 5310 ml). Fractions 10 and 11 were combined and concentrated in a rotatory evaporator under reduced pressure, at 40°C, to give a residue (343 mg), which was dissolved in 4 ml of the stationary and mobile phase (1:1, v/v) for injection into the RLCC system.

2.3. Method B

A portion of the crude hydromethanolic extract (50 g) was dissolved in 30% MeOH (1500 ml). Pigments were removed by shaking with polyvinylpolypyrrolidone (PVPP) (150 g), following adsorption of the filtrate on Amberlite XAD-7 (150 g). The resin was washed with H_2O (3×900 ml) and 30% MeOH (3×900 ml). Cardenolides were recovered by elution with 70% MeOH (3×900 ml). This extract was concentrated in a rotatory evaporator under reduced pressure, at 40°C, to ca.

300 ml and partitioned with $CHCl_3$–iso-PrOH (3:2) (3×90 ml). The organic layer was concentrated in a rotatory evaporator under reduced pressure, at 40°C and the obtained residue (1.7 g), dissolved in dimethylsulfoxide (2 ml), was submitted to flash chromatography in a reversed-phase silica column (LichroPrep RP-8, 40–63 μm Merck, pressure 1.2 bar) (460×25 mm I.D). Elution was performed with a MeOH–H_2O gradient: fractions 1–18 (50:50; 1200 ml); fraction 19 (60:40; 50 ml); fractions 20–23 (80:20; 220 ml). Combined fractions 15–18 (231 mg) were dissolved in 4 ml of the stationary and mobile phases (1:1, v/v) and injected into the RICC system using dichloromethane–methanol–water (5:6:4) as solvent system.

2.4. RLCC separations

Separations were performed on an RLCC-A apparatus (Tokyo Rikakikai, Tokyo, Japan), which consisted of 16 columns (45 cm×11 mm I.D.) divided by centrally perforated PFTE disks into 37 loculi each. Injections were made manually by filling the RLCC loop volume (3 ml). The RLCC apparatus was equipped with UV detector (Knauer, Germany). The wavelength employed was 225 nm. Dichloromethane–methanol–water (5:6:4, v/v), was used as solvent system. Solvents of commercial grade were employed. The solvent system was prepared in a 2-l separator funnel, before use, and allowed to equilibrate for 2 h, to separate the phases. RLCC was employed in the ascending mode in all the experiments. The fractions were analyzed by HPLC and those with similar profiles were combined.

2.5. HPLC analysis

Analyses were carried out in a Hewlett–Packard 1090 apparatus series II with diode-array detector (Walbronn, Germany). An ODS C_{18} column (100×2.1 mm I.D.) was employed (Hewlett–Packard, Grom, Germany) with temperature of 40°C, flow-rate of 0.2 ml/min and wavelength of 220 nm. A gradient elution of H_2O (A) and 84% CH_3CN (B) was employed: 0–5 min 85% A, 15% B; 5–10 min 80% A, 20% B; 10–12 min 80% A, 20% B; 12–25 min 73% A, 27% B; 25–35 min 55% A, 45% B; 35–50 min 40% A, 60% B; 50–52 min 5% A, 95% B.

2.6. Identification of the isolated cardenolides

The isolated cardenolides were identified by their UV spectra in diode-array detector and by comparison of their retention time with those of standard samples by HPLC analysis.

2.7. Chemicals

Acetonitrile chromatographic grade LiChrosolv was obtained from Merck (Darmstadt, Germany). Water was purified using the Milli-Q^{50} purification system (Millipore, Eschborn, Germany). PVPP was purchased from Sigma (Taufkirchen, Germany). Lanatoside A (LA), lanatoside B (LB) and lanatoside C (LC) were obtained from Roth (Karlsruhe, Germany); β-acetildigoxin, glucoevatromoiioside (Gev), glucogitoroside (Ggr), glucodigifucoside (Gdf), odorobioside G (OG), purpureaglycoside A (PA) were obtained from Boehringer Mannheim (Mannheim, Germany).

3. Results and discussion

HPLC analysis of the combined fractions from the silica gel column (Method A), that were submitted to RLCC, indicated a mixture of 25 cardenolides, with a large range of polarity (Fig. 2). The cardenolides Ggr, Gdf, LC, OG, PA, β-AcDg and LA were separated in this experiment (Fig. 3). LC was the only cardenolide to be obtained with 91% purity.

Besides removing pigments, a pre-purification method should also promote a previous fractionation of the crude extract in groups of cardenolides of similar polarity which would be submitted to further separation by RLCC. The silica gel column chromatography was effective in eliminating pigments from the crude extract, but did not afford the desired pre-fractionation.

Flash chromatography on reversed-phase silica (method B) was chosen as an alternative pre-treatment for the crude *Digitalis lanata* extract. Combined fractions from the RP-8 column contained medium polar cardenolides, as indicated by HPLC analysis, LC being the major component (Fig. 4). This material was submitted to RLCC separation and it led to the isolation of LC, Gev and Dg (Fig. 5).

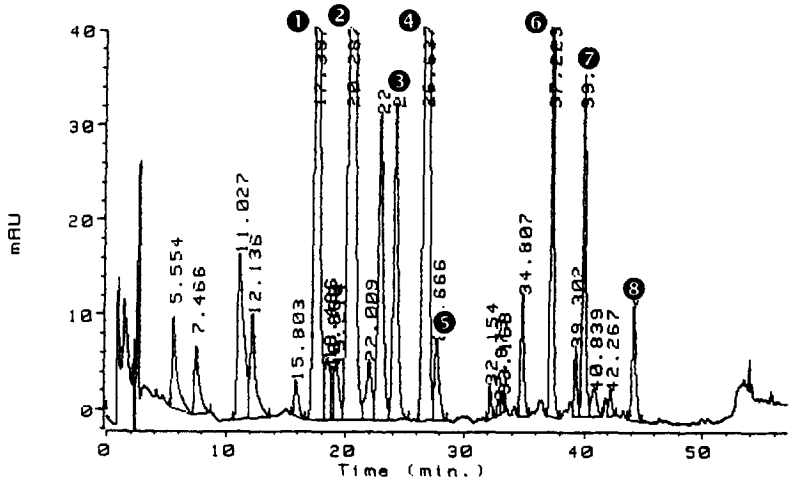


Fig. 2. HPLC chromatogram of the hydromethanolic *Digitalis lanata* leaves extract, prepurified on silica gel column chromatography (method A), prior to fractionation by RLCC. HPLC conditions: see Section 2.5. Identified cardenolides: 1=Ggr; 2=Gdf; 3=OG; 4=LC; 5=β-AcDg; 6=PA; 7=LA.

Again, LC was the only cardenolide isolated in a very pure state (96%).

Other experiments, not reported, were carried out to test dichloromethane–methanol–water (4:4:3) and (7:13:8) as solvent systems for RLCC, aiming to isolate other cardenolides, besides LC, in a pure state. However, such solvent system compositions were not efficient for separating *Digitalis lanata* glycosides.

In conclusion, RLCC has proved to be an efficient method for the isolation of pure LC from a complex *Digitalis lanata* extract pre-purified either by silica gel or reversed-phase chromatography.

Acknowledgments

We thank R.A. Reccio, FUNED, Belo Horizonte, Brazil, for furnishing the plant material and CNPq

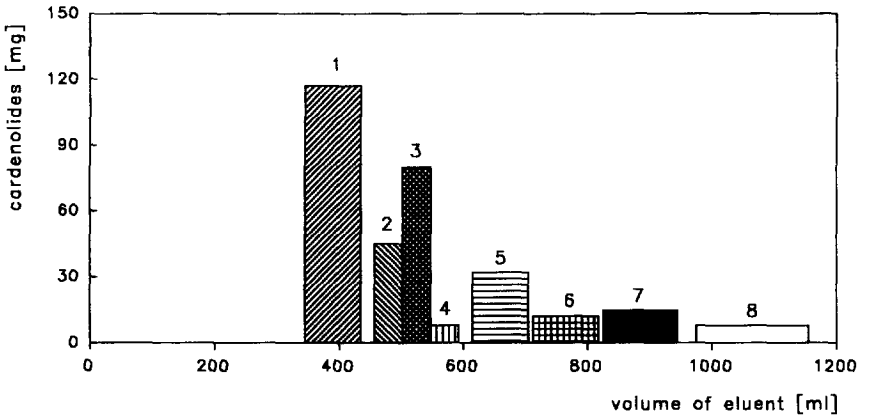


Fig. 3. Plot of the RLCC separation of cardenolides from *Digitalis lanata* leaves extract (method A). Percentile compositions of the fractions: 1, Ggr (53%), Gdf (35%); 2, OG (13%) LC (45%); 3, LC (91%); 4, LC (70%); 5, PA (35%), LA (41%); 6, β-AcDg (39%); 7, β-AcDg (47%), LA (40%); 8, LA (72%). Solvent system: dichloromethane–methanol–water (5:6:4); mobile phase: upper layer; monitoring: UV 225 nm; flow-rate: 0.6 ml/min, 15 ml/tube; recovery: 92%.

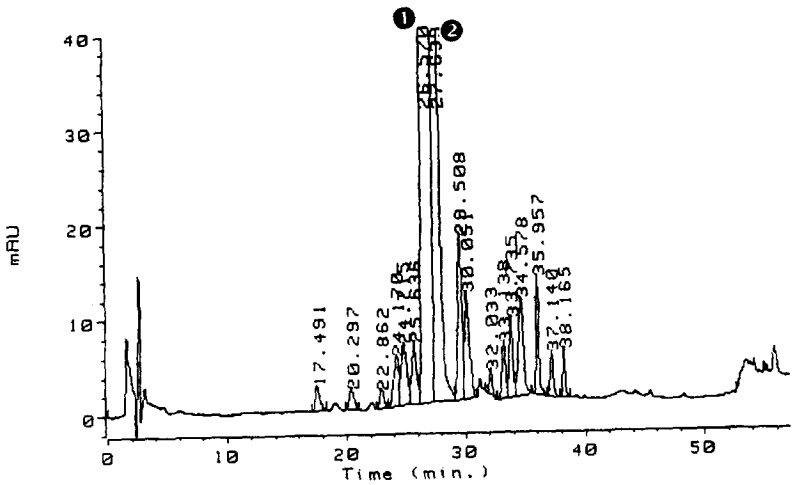


Fig. 4. HPLC chromatogram of the hydromethanolic *Digitalis lanata* leaves extract, prepurified on reversed-phase silica column chromatography (method B), prior to fractionation by RLCC. HPLC conditions: see Section 2.5. Identified cardenolides: 1=LC and Dg; 2=Gev.

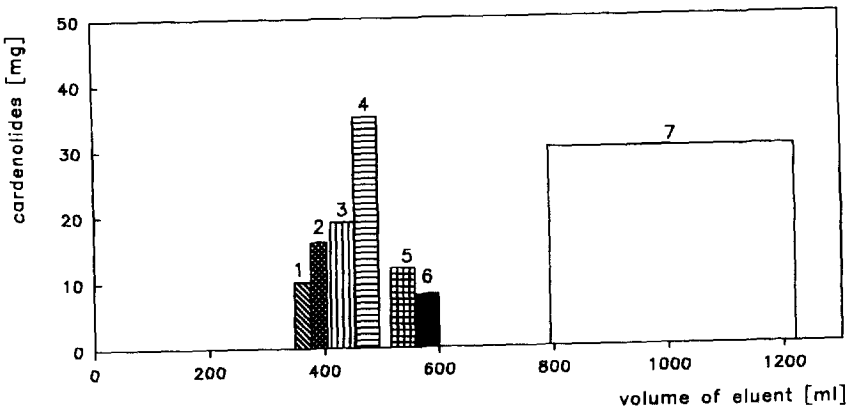


Fig. 5. Plot of the RLCC separation of cardenolides from *Digitalis lanata* leaves extract (method B). Percentile compositions of the fractions: 1, Gev (51%), LC (24%); 2, Gev (31%), LC (52%); 3, LC (96%); 4, LC (85%); 5, LC (81%); 6, LC (38%); 7, Dg (63%). Solvent system: dichloromethane–methanol–water (5:6:4); mobile phase: upper layer; monitoring: UV 225 nm; flow-rate: 0.7 ml/min; 7 ml/tube; recovery: 56%.

(Brazil) for supporting F.C.B. with a RHAE fellowship.

References

[1] M. Wichtl and R. Freier, Dtsch. Apoth. Ztg., 22 (1978) 798.
[2] A. Stoll, E. Angliker, F. Barfuss, W. Kussmaul and J. Renz, Helv. Chim. Acta, 34 (1951) 1460.
[3] C. Theuerer, Doctoral Thesis, University of Tübingen, Tübingen, 1993.
[4] D. Krüger and M. Wichtl, Planta Med., 50 (1984) 168.
[5] D. Krüger and M. Wichtl, Planta Med., 50 (1984) 265.
[6] D. Krüger and M. Wichtl, Planta Med., 50 (1984) 267.
[7] Y. Ito and R. Bowman, J. Chromatogr. Sci., 8 (1970) 315.
[8] Y. Ito and R. Bowman, Anal. Chem., 43 (1971) 13.
[9] J.K. Snyder, K. Nakanishi, K. Hostettmann and M. Hostettmann, J. Liq. Chromatogr., 7 (1984) 243.
[10] A. Marston and K. Hostettmann, J. Chromatogr. A, 658 (1994) 315.