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Short Communication

High-performance liquid chromatographic determination of cardenolides in Digitalis leaves after solid-phase extraction

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ABSTRACT

For HPLC analysis of cardenolide glycosides of ***Digitalis lanata*** the separation of the main compounds from other constituents is useful. An improved method for doing this, based on solid-phase extraction on a C_{18} modified poly(styrene–divinylbenzene) polymer, is described. The presented assay permits quantitative estimation of more than 50 cardenolides in about 2 mg of dried leaf powder of ***Digitalis lanata*** with high speed and accuracy.

INTRODUCTION

The analysis of cardenolides in *Digitalis* leaves has been the subject of a large number of publications during the last 25 years. By implementing more and more efficient chromatographic methods in order to separate the different cardenolides, the analysis has been simplified and sensitivity improved.

After the first successful determination of the whole cardenolide pattern, using a combined column and paper chromatographic method [1], high-performance liquid chromatography (HPLC) is today considered to be the standard method for analysis of cardenolides in *Digitalis* leaves [2–6]. For HPLC analysis, the cardenolides are extracted with aqueous-alcoholic mixtures and preconcentrated by extraction with organic solvents. The extraction of cardenolides from the leaf extract with chloroform-isopropanol mixtures can be replaced by using Extrelut extraction columns for sample preparation [7]. Both methods are based on liquid–liquid extraction and lead to similar results. A disadvantage of the former methods is the loss of cardenolides of up to 10%, and a disadvantage of Extrelut extraction is a partial decomposition of gitaloxigenin glycosides to the corresponding gitoxigenin glycosides [8].

In this paper we present an improved method of cardenolide determination by HPLC, based on solid-phase extraction using an extraction column filled with RP- 18 modified polystyrene (Adsorbex-Polyspher RP- 18).

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EXPERIMENTAL

Chemicals and solid-phase extraction columns

Methanol LiChrosolv was used for extraction and acetonitrile gradient-grade LiChrosolv was used for chromatography (both from Merck, Darmstadt, Germany). Most of the cardenolides are products of previous isolations [8–10]. The internal standard β-methyldigoxin (β-MDg) was donated by Dr. W. Kreis (Tübingen, Germany) and some of the cardenolides were gifts from Boehringer Mannheim (Germany). Adsorbex extraction columns filled with silica-based materials and Adsorbex-Polyspher RP- 18 columns were obtained from Merck.

HPLC conditions and apparatus

Chromatography was carried out at a flow-rate of 1.2 ml/min and a column temperature of 40°C on a high-performance liquid chromatograph consisting of a Model 600 E solvent-delivery system, a Model U6K injector, a Model 481 variable-wavelength UV absorbance detector operating at 225 nm (analytical cell) and a Model 745 integrator (all from Millipore-Waters, Milford, MA, USA).

A LiChroCART 250-4 cartridge column was used in combination with a LiChroCART 4-4 guard cartridge, both filled with LiChrospher 100 RP-18 (5 μm) packing material (all from Merck).

Cardenolides were eluted with an acetonitrile–water gradient as follows: initial = 20% acetonitrile (A), 35 min = 32% A, 45 min = 40% A, 55 min = 50% A, 59 min = 55% A, 61 min = 20% A. The composition of the mobile phase changes linearly.

Extraction procedures

About 300 mg of dried leaf powder were accurately weighed and treated with 20 ml of methanol (70%) containing 1 ml of a methanolic solution of β-methyldigoxin as internal standard at a concentration of 1.2 mg/ml. This solution was refluxed on a boiling water bath for 10 min and then rapidly cooled to room temperature. After addition of 5 ml of a solution of lead acetate (15%), the extract was mixed well, 5 ml of a solution of monosodium phosphate (4%) were added and the extract was mixed again. The extract was diluted with water to 50 ml and centrifuged for 5 min at 3300 g (solution A).

Before use, the Adsorbex-Polyspher RP-18 extraction columns were treated by washing with 2 × 2 ml of methanol, followed by 2 × 2 ml of water. A vacuum of 300 mbar was applied to the end of the column at each stage of the extraction procedure. An aliquot of 10 ml of solution A was passed through the column, followed by washing with 2 ml of water. The columns were eluted with 2 × 1 ml of methanol and 20 μl of this solution were used for HPLC.

For estimation of cardenolides in smaller quantities of drug powder, the method was modified as follows: 1.5-2 mg of leaf powder were treated with 4 ml of methanol (70%) containing 10 μl of the internal standard solution and extracted as described above. Cleaning procedures were carried out with reduced volumes of the above-mentioned solutions of lead acetate and monosodium phosphate. The extract was diluted with water to a volume of 10 ml, and after centrifugation the whole supernatant was passed through the Adsorbex-Polyspher RP-18 column. The eluate was evaporated to dryness and the residue dissolved in 100 μl of methanol. A 20-μl aliquot of this solution was used for HPLC.

RESULTS AND DISCUSSION

Sample preparation by solid-phase extraction

Different materials were tested to determine the most suitable adsorbent for solid-phase extraction of cardenolides. While all modified silica-based adsorbents were unable to retain cardenolides from aqueous solutions, Adsorbex-Polyspher RP- 18 extraction columns as adsorbent yielded a cardenolide-free aqueous eluate.

According to the information supplied by the manufacturer, these columns contain 100 mg of a macroporous, C_{18} modified poly(styrene–divinylbenzene) polymer. The spherical polymer particles have a size of 35 ± 5 μm.

The optimum retention of cardenolides passing the extraction column is achieved at a methanol concentration of the leaf extract of about 30%. Lower concentrations of methanol lead to small losses of all cardenolides as a result of a poor surface contact of the hydrophobic adsorbent with the aqueous solution being extracted. Methanol concentrations greater than 40% lead to losses of polar cardenolides. To remove polar substances like sug-

TABLE I

RECOVERY OF SELECTED CARDENOLIDES AFTER SOLID-PHASE EXTRACTION

A 10-ml volume of a mixture of some cardenolides in methanol (30%) was passed through an Adsorbex-Polyspher RP-18 extraction column and eluted with 2 × 1 ml of methanol (sample B). As reference the same solution was injected without being passed through the column (sample A). The concentration of the cardenolides in the sample mixture was about 0.1–0.2 mg/10 ml, corresponding to the cardenolide concentration in a leaf extract obtained in the described way.

Glycoside (retention time)	Sample A		Sample B		Recovery (%)
	Area (mean of n = 3)	R.S.D. (%)	Area (mean of n = 3)	R.S.D. (%)	
Digitalinum verum (13.9 min)	3768	6.8	3590	2.7	95.3
Digoxigenin mono-digitoxoside (15.1 min)	3266	3.7	3193	2.9	97.8
Strospeside (23.2 min)	5589	2.7	5729	1.9	101.0
Lanatoside B (47.6 min)	867	2.7	871	1.8	100.5
Digitoxin (58.6 min)	2998	2.0	3000	2.3	100.1

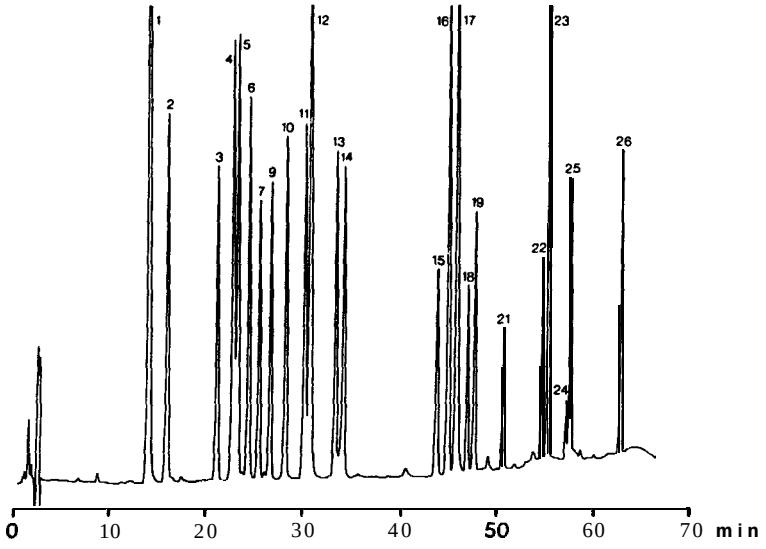

Fig. 1. Chromatogram of a mixture of different cardenolides. A 20-µl aliquot of a sample solution containing 0.05–1.5 µg of each cardenolide was injected. HPLC conditions were as described in the text. Peaks: 1 = digitalinum verum; 2 = GI-Dx-Dx-C; 3 = glucoverodoxin; 4 = strospeside; 5 = glucogitoroside; 6 = desacetyllanatoside C; 7 = Glum-Dx-Dx-C; 8 = neo-glucodigifucoside; 9 = glucodigifucoside; 10 = neo-odorobioside G; 11 = odorobioside G; 12 = verodoxin; 13 = lanatoside C; 14 = glucoevatromonoside; 15 = GI-Dx-Dx-A; 16 = cc-acetyldigoxin; 17 = β-methyldigoxin; 18 = lanatoside B; 19 = β-acetyldigoxin; 20 = purpurea glucoside A; 21 = lanatoside E; 22 = lanatoside A; 23 = a-acetylgitoxin; 24 = β-acetylgitoxin; 25 = digitoxin; 26 = cc-acetyldigitoxin.

TABLE II

RETENTION TIME (t_R) OF DIFFERENT CARDENOLIDES FROM *DIGITALIS* LEAVES

Aglycones: A = digitoxigenin; B = gitoxigenin; C = digoxigenin; D = diginatigenin; E = gitaloxigenin; F = oleandrigenin. Sugars: AcDx = acetyldigitoxose; Didesgl = 2,6-didesoxyglucose; Dtl = digitalose; Dx = digitoxose; Fuc = fucose; Gl = glucose; Glum = glucomethylose.

Common name	Abbreviated form (according to ref. 11)	t_R (min)	Relative t_R (β-methyldigoxin = 1)
	Dtl-D	4.51	0.099
Digoxigenin	C	8.23	0.178
	Dtl-C	12.97	0.280
Digitalinum verum	Gl-Dtl-B	13.88	0.300
Subalpinoside	Gl-Dtl-F	14.49	0.313
	Dx-C	15.08	0.326
	Glum-Dx-C	15.45	0.334
	Gl-Dx-Dx-C	15.69	0.339
	Gl-A	19.09	0.412
	Xyl-Dx-Dx-C	20.26	0.437
Glucoverodoxin	Gl-Dtl-E	21.06	0.455
	Didesgl-Dx-C	21.38	0.462
Strospeside	Dtl-B	22.62	0.488
Glucogitoroside	Gl-Dx-B	23.15	0.500
Gitoxigenin	B	23.71	0.513
Desacetyllanatoside C	Gl-Dx-Dx-Dx-C	24.15	0.521
	Gl-AcDx-C	24.61	0.531
	Dx-Dx-C	24.90	0.538
Diginatin	Dx-Dx-Dx-D	24.92	0.538
	Glum-Dx-Dx-C	25.51	0.551
Neo-glucodigifucoside	N-Gl-Fuc-A	26.04	0.562
Lanatoside D	Gl-AcDx-Dx-Dx-D	26.06	0.563
	Gl-Glum-A	26.57	0.574
Glucodigifucoside	Gl-Fuc-A	26.71	0.577
Glucolanadoxin	Gl-Dx-E	28.33	0.612
Neo-odorobioside G	N-Gl-Dtl-A	28.33	0.612
Odorobioside G	Gl-Dtl-A	30.28	0.654
Verodoxin	Dtl-E	30.80	0.665
	Didesgl-Dx-Dx-C	30.98	0.669
Glucodigoxoside	Gl-Dx-Dx-Dx-Dx-C	32.68	0.706
Lanatoside C	Gl-AcDx-Dx-Dx-C	33.43	0.722
Digoxin	Dx-Dx-Dx-C	33.51	0.724
Glucoevatromonoside	Gl-Dx-A	34.31	0.741
	Glum-A	36.00	0.777
	AcDx-Dx-C	37.33	0.806
	Xyl-Dx-A	39.93	0.862
Purpurea glycoside B	Gl-Dx-Dx-Dx-B	40.69	0.879
Lanadoxin	Dx-E	40.87	0.883
Digitoxigenin	A	41.15	0.889
Digoxoside	Dx-Dx-Dx-Dx-C	41.53	0.897
	Gl-Dx-Dx-A	44.23	0.955
a-Acetyldigoxin	a-AcDx-Dx-Dx-C	45.48	0.982
β-Methyldigoxin	β-MethylDx-Dx-Dx-C	46.31	1.000
Glucogitaloxin	Gl-Dx-Dx-Dx-E	46.34	1.001
Lanatoside B	Gl-AcDx-Dx-Dx-B	47.62	1.028
Evatromonoside	Dx-A	48.45	1.046
β-Acetyldigoxin	β-AcDx-Dx-Dx-C	48.47	1.047
Gitoxin	Dx-Dx-Dx-B	49.80	1.075
Purpurea glycoside A	Gl-Dx-Dx-Dx-A	51.27	1.107
Lanatoside E	Gl-AcDx-Dx-Dx-E	51.48	1.112

TABLE II *(continued)*

Common name	Abbreviated form (according to ref. 11)	t_R (min)	Relative t_R (β-methyldigoxin = 1)
Lanatoside F	Gl-AcDx-Dx-Dx-F	52.49	1.133
	Dx-Dx-A	54.15	1.169
Gitaloxin	Dx-Dx-Dx-E	54.15	1.169
Lanatoside A	Gl-AcDx-Dx-Dx-A	55.62	1.201
cc-Acetylgitoxin	a-AcDx-Dx-Dx-B	56.26	1.215
β-Acetylgitoxin	β-AcDx-Dx-Dx-B	58.31	1.259
Digitoxin	Dx-Dx-Dx-A	58.57	1.265
a-Acetylgitaloxin	a-AcDx-Dx-Dx-E	59.88	1.294
α-Acetyldigitoxin	a-AcDx-Dx-Dx-A	63.94	1.381
β-Acetyldigitoxin	β-AcDx-Dx-Dx-A	66.29	1.431

ars, the column was washed after adsorption of the cardenolides with 2 ml of water. The recoveries shown in Table I demonstrate the elution of the selected cardenolides using methanol as eluent. The methanolic eluate served as sample solution for HPLC analysis. If this sample solution is stored at low temperatures (4°C) no decomposition of cardenolides is detectable. For extraction and sample preparation only about 45 min are needed. Moreover, the Adsorbex-Polyspher RP- 18 extraction columns can be used several times without loss of performance.

HPLC

Chromatograms of a mixture of cardenolides (Fig. 1) and of a *Digitalis lanata* leaf extract (Fig. 2) demonstrate the performance of the HPLC method described in this paper. Using β-methyldigoxin as internal standard the different cardenolides can be identified by their relative retention times (Table II).

The limit of detection for quantitative determination of cardenolides with this method is about 10 pmol (corresponding to approximately 11 ng of lanatoside C).

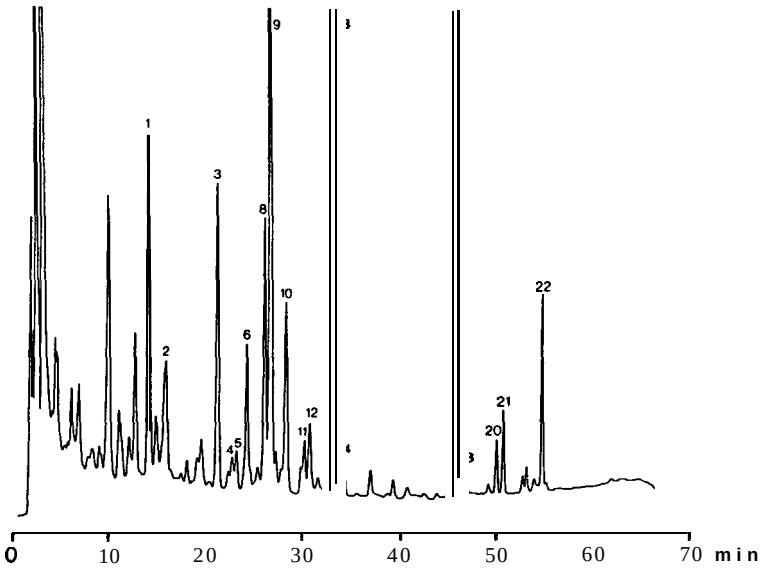

Fig. 2. Chromatogram of a *Digitalis lanata* leaf extract. For preparation of the leaf powder the leaves were collected and immediately frozen to − 196°C in liquid nitrogen, then freeze-dried at a product temperature of ~ 5°C. The dried leaves were powdered and analysed as described in the text. Acetyltridigitoxosides as products of fermentations could not be detected in the leaf extract. Peaks as in Fig. 1.

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