Journal of Chromatography, 479 (1989) 319–325
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 21 684

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF SECONDARY CARDIAC GLYCOSIDES IN *DIGITALIS PURPUREA* LEAVES

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(First received April 6th, 1989; revised manuscript received June 12th, 1989)

SUMMARY

An analytical method for the determination of secondary cardiac glycosides in *Digitalis purpurea* leaves by high-performance liquid chromatography (HPLC) is described. The procedure consisted of extraction of dry leaf powder with ethanol–chloroform (2:1) and clean-up by Sep-Pak cartridges prior to HPLC analysis. HPLC was performed on an octylsilyl bonded silica column, using acetonitrile–methanol–water (4:4:5) for trisdigitoxosides and acetonitrile–methanol–water (8:30:43) for strospeside; the effluent was monitored by ultraviolet detection (at 220 nm). Quantitation of these cardiac glycosides was carried out by the internal standard method. The amounts of digitoxin, gitoxin, gitaloxin and strospeside per 100 mg of dry leaf powder were estimated to be 22.6, 14.0, 54.7 and 1.9 μg, respectively. The method is sufficiently sensitive and reproducible to assay secondary glycosides in *Digitalis purpurea* leaves.

INTRODUCTION

The digitalis glycosides are well known as a group of important medical therapeutic agents for the treatment of heart disease. *Digitalis purpurea* leaves contain secondary glycosides such as digitoxin, gitoxin, gitaloxin and strospeside which are produced by the enzymatic conversion of primary glycosides of purpurea glycoside A, purpurea glycoside B, glucogitaloxin and digitalinum verum, respectively.

Previously published methods of determining secondary glycosides in *Digitalis purpurea* leaves have utilized paper chromatographic[1] and thin-layer chromatographic (TLC)[2–5] techniques, but they did not always yield accurate quantitation of cardiac glycosides in the leaves. High-performance liquid chromatography (HPLC) appears to be more suitable for the determination of secondary glycosides. The separation of various mixtures of these compounds was accomplished by use of a normal-phase silica column[6–8] and a reversed-phase C_{18} column[8]. In addition, Nachtmann *et al.*[9] have proposed the separation of digitalis glycosides by HPLC following precolumn derivatization with 4-nitrobenzoyl chloride. However, there have been few attempts to introduce HPLC for the quantitation of cardiac glycosides in the extract of *Dig-*

0021-9673/89/$03.50

italis purpurea leaves. Wichtl *et al.*[10] have carried out the quantitation of digitoxin and several primary glycosides in the leaves using gradient elution, but not data on gitoxin, gitaloxin and strospeside were given.

In the previous paper of this series we reported the micro-HPLC analysis of purpurea glycoside A, purpurea glycoside B and glucogitaloxin in *Digitalis purpurea* leaves[11]. The present study focuses on the quantitative method for secondary cardiac glycosides from the extract of such leaves, which involves clean-up with Sep-Pak cartridges followed by HPLC on a reversed-phase C_8 column with ultraviolet (UV) detection (at 220 nm).

EXPERIMENTAL

Instruments

The HPLC system consisted of a JASCO 880-PU pump (Japan Spectroscopic, Tokyo, Japan), a Model KHP-UI-130 injector (Kyowa Seimitsu, Tokyo, Japan), a Model UV-8010 detector (Tosoh, Tokyo, Japan) monitoring the absorbance at 220 nm and a Chromatopac C-R3A data processor (Shimadzu, Kyoto, Japan). The stainless-steel column (150 mm × 4.6 mm I.D.) was packed with Chemcosorb 5 C_8-U (Chemco Scientific, Osaka, Japan). This was a reversed-phase column containing 5-μm porous silica particles linked covalently with octylsilyl groups.

Materials

Gitoxin and digoxin were obtained from E. Merck (Darmstadt, F.R.G.), gitaloxin and strospeside from Boehringer Mannheim (Mannheim, F.R.G.), digitoxin from Wako (Osaka, Japan) and Sep-Pak cartridges from Waters (Milford, MA, U.S.A.). The chemical structures of digitoxin, gitoxin, gitaloxin and strospeside are given in Fig. 1. 14α,15α-Epoxy-"β"-anhydrodigitoxin (internal standard I) was synthesized in four steps from digitoxin according to the procedure of Sawlewicz *et al.*[12] and recrystallized repeatedly from methanol. Digoxigenin bisdigitoxoside (internal standard II) was also prepared from digoxin by the method of Haack *et al.*[13]. All of these compounds were checked for homogeneity by TLC, and solvents were purified by redistillation prior to use.

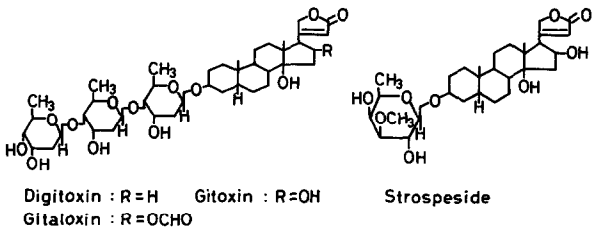


Fig. 1. Structures of the secondary glycosides investigated.

Preparation of the leaf powder

Leaves of *Digitalis purpurea* L. plant in the second year were collected in the medicinal botanical garden (Kanazawa, Japan) of Hokuriku University on June 21. The harvested leaves were quickly washed with water and immediately stored at

−4°C for 17 months. The frozen leaves were freeze-dried in a Neocool Model DC-55A apparatus (Yamato Scientific, Tokyo, Japan), and then dried using phosphorus pentoxide under reduced pressure at room temperature. The dried leaves were pulverized and sifted through a sieve of mesh width 500 μm. The leaf powder obtained was further dried under reduced pressure for 5 days.

Extraction and clean-up procedures

About 50 mg of leaf powder were accurately weighed and added to ethanol–chloroform (2:1) (25 ml) containing 14α,15α-epoxy-"β"-anhydrodigitoxin (44.69 μg) and digoxigenin bisdigitoxoside (1.18 μg) as internal standards. After ultrasonication for 1 h in an ultrasonic cleaning bath, the extract was filtered and evaporated to dryness using a rotary evaporator. The residue was dissolved in chloroform–acetic acid (100:0.1) (2 ml) and applied to the Sep-Pak silica cartridge. Chloroform–acetic acid (100:0.1) (28 ml) and chloroform–methanol–acetic acid (100:5:0.01) (25 ml) were successively passed through the cartridge. After evaporation of the latter fraction, the residue obtained was dissolved in methanol–water–acetic acid (10:30:0.04) (1 ml) and loaded on the Sep-Pak C_{18} cartridge. After washing with methanol–water–acetic acid (10:30:0.04) (9 ml), secondary glycosides and internal standards were eluted with methanol–water–acetic acid (20:10:0.03) (20 ml). The eluate was evaporated to dryness *in vacuo*.

Determination of secondary glycosides

For the simultaneous determination of trisdigitoxosides, the extract pretreated above was dissolved in acetonitrile–methanol–water (4:4:5) (0.5 ml) and analyzed by HPLC. The remaining solution was dried under a gentle stream of nitrogen, and the resulting material was redissolved in acetonitrile–methanol-water (8:30:43) (0.1 ml) and used for the determination of strospeside by HPLC.

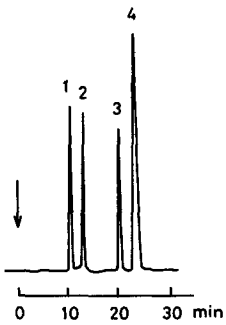
A 10-μl volume of each sample was injected into the liquid chromatograph and the flow-rate was adjusted to 0.5 ml/min. The separations were performed under ambient conditions. The mobile phase for each separation is listed with each chromatogram. Digitoxin, gitoxin, gitaloxin and strospeside in *Digitalis purpurea* were determined by the internal standard method. Calibration graphs were constructed using the average peak areas from three chromatograms.

TLC procedure

Normal-phase and reversed-phase TLC were performed on 5 cm × 10 cm high-performance silica gel 60 F_{254} plates (E. Merck) with a preadsorbent spotting area and 5 cm × 10 cm $KC_{18}F$ plates (Whatman, Clifton, NJ, U.S.A.), respectively. Aliquots (2 μl) of the solutions were spotted with Drummond Microcap micropipettes on TLC plates. The plates were developed in glass chambers that were lined with paper and preequilibrated with mobile phase for 10 min. After air drying, the plates were checked by fluorescence quenching of the layers under UV light around 254 nm, and then sprayed with concentrated sulphuric acid and heated in an oven at 120°C for 5 min.

RESULTS AND DISCUSSION

Our initial effort was directed to the chromatographic separation of secondary glycoside mixtures and the selection of the internal standard. The separation was achieved by using two kinds of isocratic solvent systems, because the polarity of strospeside is much different from that of a group of trisdigitoxosides. 14α,15α-Epoxy-"β"-anhydrodigitoxin (internal standard I) and digoxigenin bisdigitoxoside (internal standard II) were found to be the most suitable for the separations of trisdigitoxosides and strospeside, respectively. HPLC was performed on an octylsilyl bonded silica column using a ternary solvent mixture of acetonitrile, methanol and water. A detection wavelength of 220 nm was employed on the basis of the α,β-unsaturated lactone ring attached at the C-17 position of the steroid nucleus. Fig. 2 shows the chromatogram of a mixture of trisdigitoxosides (digitoxin, gitoxin and gitaloxin) and internal standard I. These compounds were separated into four peaks using acetonitrile–methanol–water (4:4:5) as the eluent. The retention times for gitoxin, gitaloxin, digitoxin and internal standard I were 10.6, 13.2, 20.4 and 23.3 min, respectively. On the other hand, the separation of strospeside and internal standard II was accomplished when acetonitrile–methanol–water (8:30:43) was employed as the mobile phase, as illustrated in Fig. 3.



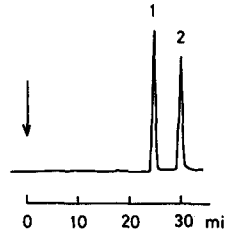
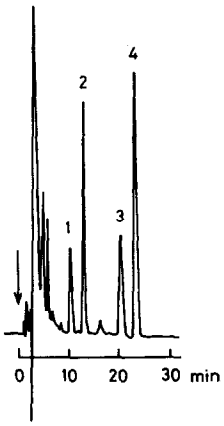


Fig. 2. Separation of a mixture of digitoxin, gitoxin, gitaloxin and internal standard I. Peaks: 1 = gitoxin; 2 = gitaloxin; 3 = digitoxin; 4 = 14α,15α-epoxy-"β"-anhydrodigitoxin. Conditions: Chemcosorb 5 C_8-U column (150 mm × 4.6 mm I.D.); mobile phase, acetonitrile–methanol–water (4:4:5); flow-rate, 0.5 ml/min; UV monitor at 220 nm; sample volume, 10 μl.

Fig. 3. Separation of a mixture of strospeside and internal standard II. Peaks: 1 = strospeside; 2 = digoxigenin bisdigitoxoside. Mobile phase: acetonitrile–methanol–water (8:30:43). Other conditions as in Fig. 2.

On the basis of these data, the determination of secondary glycosides in *Digitalis purpurea* leaves was carried out. The dried leaf powder was extracted with ethanol–chloroform (2:1) by ultrasonication. Many coexisting materials in the extract exert a significant influence on the HPLC separation of these glycosides. Therefore, Sep-Pak cartridges packed with silica gel and ODS-bonded silica gel were used in a clean-up step prior to HPLC. The purified material was subjected to HPLC using the two types of mobile phases as described above. Figs. 4 and 5 depict representative chromatograms of trisdigitoxosides and strospeside, respectively, in the extract after



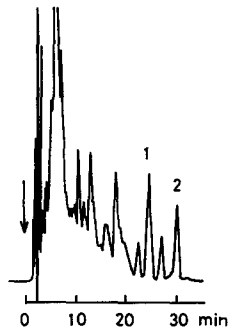


Fig. 4. Chromatogram of the extract of *Digitalis purpurea* leaves for the determination of digitoxin, gitoxin and gitaloxin. Peaks: 1 = gitoxin; 2 = gitaloxin; 3 = digitoxin; 4 = 14α,15α-epoxy-"β"-anhydrodigitoxin. Conditions as in Fig. 2.

Fig. 5. Chromatogram of the extract of *Digitalis purpurea* leaves for the determination of strospeside. Peaks: 1 = strospeside; 2 = digoxigenin bisdigitoxoside. Conditions as in Fig. 3.

incorporation of the internal standards. From chromatograms of the extract in the absence of the internal standards, the coexisting substances present were ascertained not to interfere with the peaks due to internal standards I and II. The eluate corresponding to each peak on the chromatograms was collected and evaporated *in vacuo*. The materials obtained were analyzed by both normal-phase TLC (digitoxin, R_F 0.61; gitaloxin, R_F 0.59; gitoxin, R_F 0.49 and strospeside, R_F 0.38) using cyclohexane–ethyl acetate–ethanol–acetic acid (9:5:4:0.02) as a developing solvent and reversed-phase TLC (strospeside, R_F 0.56; gitoxin, R_F 0.47; gitaloxin, R_F 0.40 and digitoxin, R_F 0.32) using acetonitrile–0.5 *M* NaCl (10:13). No impurities were detected on both TLC plates on exposure to UV light around 254 nm and visualization using concentrated sulphuric acid spray. These results indicated single components in each zone with retention times corresponding to digitoxin, gitoxin, gitaloxin and strospeside. Linear calibration graphs were obtained by plotting the peak area ratios, y, of each trisdigitoxoside to internal standard I and of strospeside to internal standard II against the amount of each compound, x (μg). The regression equations, ranges of linearities, R, and correlation coefficients, r, were $y = 0.0340\,x - 0.0020$ (R 2–20 μg, $r = 0.997$) for digitoxin, $y = 0.0315\,x - 0.0007$ (R 2–16 μg, $r = 0.997$) for gitoxin, $v = 0.0246\,x + 0.0060$ (R 5–40 μg, $r = 0.999$) for gitaloxin and $y = 1.017\,x - 0.002$ (R 0.1–2.0 μg, $r = 0.999$) for strospeside.

The quantitation of trisdigitoxosides and strospeside in *Digitalis purpurea* by the proposed method was then undertaken. The assay results obtained from ten determinations of dry leaf powder samples are compiled in Table I. The data indicate that the average contents of digitoxin, gitoxin, gitaloxin and strospeside per 100 mg of the dry leaf powder were 22.6, 14.0, 54.7 and 1.9 μg, respectively. The amount of strospeside in the leaves was considerably lower, as compared with the other compounds. The coefficients of variation were found to be within 2.8–6.6%. The recovery

TABLE I

CONTENTS OF THE SECONDARY GLYCOSIDES IN *DIGITALIS PURPUREA* LEAVES OBTAINED BY THE PRESENT METHOD

Values are the amounts per 100 mg of a dry leaf powder sample.

Glycoside	*Found (μg)*		*Mean ± S.D. (μg)*	*C.V. (%)*
Digitoxin	21.31	23.73		
	21.38	23.45		
	22.55	23.15		
	23.17	20.80		
	23.47	22.95	22.6 ± 1.0	4.6
Gitoxin	15.43	13.15		
	14.30	14.04		
	13.70	14.06		
	14.78	13.42		
	13.53	13.45	14.0 ± 0.7	5.0
Gitaloxin	53.42	54.35		
	55.17	56.42		
	53.13	53.82		
	53.61	57.57		
	55.97	53.35	54.7 ± 1.5	2.8
Strospeside	1.77	1.91		
	1.83	1.97		
	2.11	1.96		
	2.03	1.71		
	2.05	1.88	1.9 ± 0.1	6.6

was examined by adding pure secondary glycosides to dry leaf powder samples and following the procedure described. Added glycosides equivalent to half of the amount of each glycoside contained in the leaves were recovered sufficiently, more than 96%. The values of the coefficient of variation and recovery indicated the quantitative usefulness of this HPLC method.

In the present study, the HPLC determination of secondary glycosides was achieved by using two different isocratic solvent systems and two internal standards because of the large differences in polarities of trisdigitoxoside and strospeside. The pretreatment with Sep-Pak cartridges was much more efficient and convenient than the previous method[14] consisting of a solvent-partition sequence and preparative TLC. Gitaloxin is known readily to lose the formyl group at position C-16 giving rise to gitoxin, especially under alkaline conditions. Therefore, a very small amount of acetic acid was added to the eluent used with the Sep-Pak cartridges, in order to prevent deformylation of gitaloxin. Our value for gitaloxin in comparison with gitoxin was higher than previous results obtained by paper chromatography[1] or TLC[3,4]. On the other hand, the content of strospeside in the leaves was almost the same as that reported by Lugt and Noordhoek-Ananias[4]. In conclusion, digitoxin, gitoxin, gitaloxin and strospeside in *Digitalis purpurea* leaves can be analyzed with good resolution and reproducibility by the HPLC method described. This technique may be useful for estimation of the quality of *Digitalis* leaves.

ACKNOWLEDGEMENT

The authors thank the staff of the medicinal botanical garden of Hokuriku University for providing the leaves of *Digitalis purpurea*.

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