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Note

Separation and determination of lanatosides in *Digitalis lanata* leaves by high-performance liquid chromatography

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Digitalis lanata leaf is known to contain lanatosides and their secondary glycosides. These compounds are pharmaceutically important drugs for the treatment of congestive heart failure. For the determination of lanatosides in *Digitalis lanata*, thin-layer chromatography (TLC) was applied, utilizing measurement of the spot areas[1], spectrophotometric determination (*in situ*) on a thin layer[2] or spectrophotometric measurement of solutions obtained after spot elution[3,4]. However, high-performance liquid chromatography (HPLC) appears to be the most effective method for the analysis of digitalis glycosides. The determination of digoxin in *Digitalis lanata* by HPLC has been proposed by Cobb[5] and Brugidou *et al.*[6]. Also, the separation of a mixture of pure lanatosides was accomplished employing a normal-phase silica column[7,8] and a reversed-phase column[9]. Orosz *et al.*[10] have determined lanatoside C in *Digitalis lanata* using an ODS column, but no data on lanatoside A and B were given.

Previously, we reported the separation and determination of digitalis glycosides in *Digitalis purpurea* by reversed-phase micro-HPLC[11,12]. This paper describes a method for the simultaneous determination of lanatosides in *Digitalis lanata* leaves, which involves clean-up with Sep-Pak cartridges and subsequent separation by normal-phase partition HPLC.

EXPERIMENTAL

Instruments

The liquid chromatograph consisted of a Model KHP-010 pump (Kyowa Seimitsu, Tokyo, Japan), a Model KHP-UI-130 injector (Kyowa Seimitsu), a Model UVILOG-5 III A variable-wavelength detector (Oyo-bunko Kiki, Tokyo, Japan) and a Chromatopac C-R3A data processor (Shimadzu, Kyoto, Japan). The stainless-steel column (250 × 4.6 mm I.D.) was packed with TSK-gel Amide-80 (Tosoh, Tokyo, Japan). This was a column containing 5-μm porous silica particles derivatized with carbamoyl groups. The detector was set at 220 nm. The separations were performed under ambient conditions.

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Materials

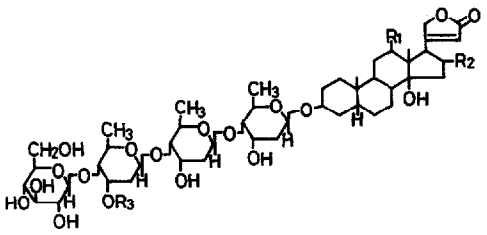
Lanatoside A, B and C were purchased from E. Merck (Darmstadt, F.R.G.), desacetyllanatoside C from the National Institute of Hygienic Sciences (Tokyo, Japan) and Sep-Pak cartridges from Waters Assoc. (Milford, MA, U.S.A.). Desacetyllanatoside A and B were prepared from lanatoside A and B, respectively, according to the procedure of Pekić and Miljković[13] and recrystallized repeatedly from dichloromethane–ethanol. The structures of these cardiac steroids are given in Fig. 1. 14α,15α-Epoxy-"β"-anhydrodesacetyllanatoside A, used as the internal standard, was synthesized in four steps from desacetyllanatoside A by the method adapted from Sawlewicz *et al.*[14]. All of these materials were checked for homogeneity by TLC, and solvents were purified by redistillation prior to use.

Preparation of the leaf powder

Leaves of *Digitalis lanata* EHRH. plant in the second year were collected in the medicinal botanical garden of Hokuriku University (Kanazawa, Japan) on July 29, 1987. The freshly harvested leaves were quickly washed with water, 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

Approximately 50 mg of leaf powder were accurately weighed and extracted with 25 ml of ethanol–chloroform (2:1) containing an internal standard (125.9 μg) in an ultrasonic cleaning bath for 1 h. The extract was filtered and evaporated to dryness at 40°C using a rotary evaporator. The residue was dissolved in 2 ml of ethyl acetate–ethanol (100:1) and applied to the Sep-Pak silica cartridge. Then 18 ml of ethyl acetate–ethanol (100:1) and 10 ml of ethyl acetate–ethanol (5:1) were successively passed through the cartridge. The latter fraction (10 ml) was collected and evaporated at 40°C using a rotary evaporator. The residue obtained was dissolved in 1 ml of methanol–water (2:3) and loaded on the Sep-Pak C_{18} cartridge. After washing with 14 ml of methanol–water (2:3), cardiac glycosides were eluted with 15 ml of methanol–



Lanatoside A	: $R_1=R_2=H$, $R_3=COCH_3$	Desacetyllanatoside A	: $R_1=R_2=R_3=H$
Lanatoside B	: $R_1=H$, $R_2=OH$, $R_3=COCH_3$	Desacetyllanatoside B	: $R_1=R_3=H$, $R_2=OH$
Lanatoside C	: $R_1=OH$, $R_2=H$, $R_3=COCH_3$	Desacetyllanatoside C	: $R_1=OH$, $R_2=R_3=H$

Fig. 1. Structures of lanatosides and desacetyllanatosides.

water (2:1). The eluate was evaporated at 40°C *in vacuo* and the resulting material was dissolved in 0.1 ml of acetonitrile–water (96:4) and analysed by HPLC.

HPLC of lanatosides

A 10-μl volume of the purified sample solution (0.1 ml) was injected into the liquid chromatograph and acetonitrile–water (96:4) was employed as the mobile phase at a flow-rate of 0.8 ml/min. The mobile phase used for the separation of desacetyllanatosides was acetonitrile–water (9:1). The effluent was monitored by UV absorption at 220 nm. Calibration graphs were constructed using the average peak areas from three chromatograms.

TLC procedure

Normal- and reversed-phase TLC were performed on 5 × 10 cm high-performance silica gel 60 F_{254} plates (E. Merck) and 5 × 10 cm $KC_{18}F$ plates (Whatman, Clifton, NJ, U.S.A.), respectively. After development and air drying, the plates were checked by fluorescence quenching of the layers under UV radiation at *ca.* 254 nm, and then sprayed with concentrated sulphuric acid and heated in an oven at 120°C for 10 min.

RESULTS AND DISCUSSION

An initial study was focused on the selection of an internal standard and the chromatographic separation of cardiac glycosides. Many compounds were investigated and 14α,15α-epoxy-"β"-anhydrodesacetyllanatoside A was found to be the most suitable. HPLC was performed on a carbamoyl-bonded silica column using acetonitrile–water as eluent. A detection wavelength of 220 nm was employed, account being taken of the α,β-unsaturated lactone ring attached at the C-17 position of the steroid nucleus. The separation of lanatoside A, B and C and the internal standard was achieved when acetonitrile–water (96:4) was used as the eluent, as illustrated in Fig. 2. The retention times of desacetyllanatoside A, B and C were 80, 129 and 135 min, respectively. Also, Fig. 3 shows the chromatogram of lanatosides, desacetyllanatosides

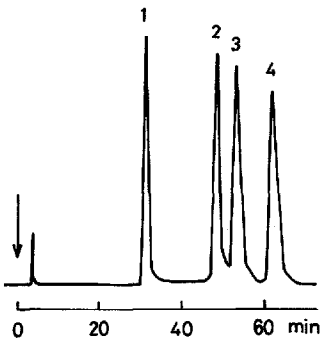


Fig. 2. Separation of a mixture of lanatosides and the internal standard. Peaks: 1 = lanatoside A (0.40 μg); 2 = lanatoside B (0.43 μg); 3 = lanatoside C (0.59 μg); 4 = 14α,15α-epoxy-"β"-anhydrodesacetyllanatoside A (0.84 μg). Conditions: TSK-gel Amide 80 column (250 × 4.6 mm I.D.); mobile phase, acetonitrile–water (96:4); flow-rate, 0.8 ml/min; UV detection at 220 nm; sample volume, 10 μl.

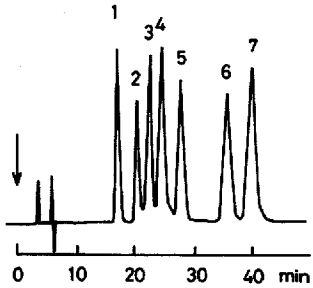


Fig. 3. Separation of a mixture of lanatosides, desacetyllanatosides and the internal standard. Peaks: 1 = lanatoside A (0.15 μg); 2 = lanatoside B (0.15 μg); 3 = lanatoside C (0.20 μg); 4 = 14α,15α-epoxy-"β"-anhydrodesacetyllanatoside A (0.22 μg); 5 = desacetyllanatoside A (0.27 μg); 6 = desacetyllanatoside B (0.45 μg); 7 = desacetyllanatoside C (0.55 μg). Conditions: mobile phase, acetonitrile–water (9:1); flow-rate, 0.5 ml/min; other conditions as in Fig. 2.

and the internal standard. These compounds were separated into seven peaks using acetonitrile–water (9:1) as the eluent. The separations are of sufficient quality and reproducibility to permit quantitative work.

The determination of lanatosides in *Digitalis lanata* leaves was carried out by an internal standard method. The leaves were dried and then extracted with ethanol–chloroform (2:1) by ultrasonication. In order to remove the many other plant materials in the extract, it is necessary to introduce a purification step prior to HPLC. Sep-Pak cartridges packed with silica gel and ODS-bonded gel were used to clean up the extract. For the complete HPLC separation of lanatosides in the purified extract, acetonitrile–water (96:4) was employed as the mobile phase. When acetonitrile–water (9:1) was used, the presence of co-extracted constituents of the leaves interfered with the peak of lanatoside C. Fig. 4 depicts a typical chromatogram of the extract after addition of the internal standard. From the chromatogram of the extract in the absence of the internal standard, the co-existing substances present in the leaves were ascertained not to disturb the internal standard peak. The eluate corresponding to each peak was collected and analysed by TLC. Both normal-phase TLC (lanatoside A, R_F 0.46; lanatoside C, R_F 0.39) using chloroform–methanol–water (80:20:2.5) as developing solvent and reversed-phase TLC (lanatoside A, R_F 0.40; lanatoside C, R_F 0.68) using acetonitrile–0.5 *M* sodium chloride (10:13) indicated single components in each zone with retention times corresponding to lanatoside A and C.

Linear calibration graphs were obtained by plotting the peak-area ratios (y) of lanatosides to an internal standard against the amount of lanatosides (x μg); the regression equations and correlation coefficients (r) were $y = 0.0108\,x - 0.0004$ ($r = 0.997$) for lanatoside A and $y = 0.0101\,x + 0.150$ ($r = 0.998$) for lanatoside C. The plot was from 10 to 70 μg for lanatoside A and over the required range from 40 to 180 μg for lanatoside C. The intra-assay relative standard deviations ($n = 9$) were 0.9–2.5% for lanatoside A (10–70 μg) and 1.0–1.7% for lanatoside C (44–177 μg), and the inter-assay relative standard deviations ($n = 9$) were 2.1–3.0% for lanatoside A and 1.7–2.8% for lanatoside C.

The assay results obtained from ten dry leaf powder samples (mean of three determinations per sample) are collected in Table I. The data indicate that the leaves contained 66.9 ± 4.4 μg (mean ± S.D.) for lanatoside A and 216.3 ± 7.1 μg for

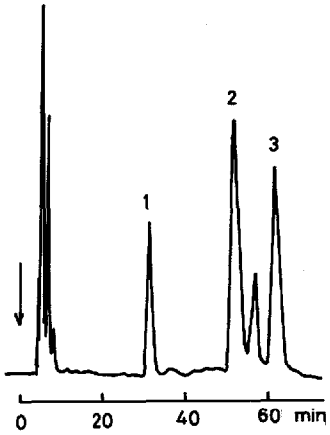


Fig. 4. Chromatogram of the extract of *Digitalis lanata* leaves with the internal standard. Peaks: 1 = lanatoside A; 2 = lanatoside C; 3 = 14α,15α-epoxy-"β"-anhydrodesacetyllanatoside A. Conditions as in Fig. 2.

lanatoside C per 100 mg of the dry leaf powder. The amount of lanatoside C was three times higher than that of lanatoside A. The quantitative usefulness of this technique was confirmed by the relative standard deviations of 6.6% and 3.3% for lanatoside A and C, respectively.

The recovery was examined by adding pure lanatoside A and C equivalent to half of the amounts contained in the leaves and applying the described procedure. Both of the compounds were recovered satisfactorily (>95%). The limits of quantitative measurements of lanatosides and desacetyllanatosides with the present method were 2–4 μg per 50 mg of dry leaf powder, considering the interfering peaks of impurities originating from the leaves. The amounts of lanatoside B and desacetyllanatosides in the leaves examined were below the limits of determination.

Previously, the separation of lanatoside and desacetyllanatoside standards[7,8], the qualitative analysis of cardiac glycosides in *Digitalis lanata*[9] and the determination of digoxin in the leaves[5,6] were reported. However, these studies were not concerned with the quantification of lanatosides in *Digitalis lanata*. For the simultaneous determination of lanatoside A, B and C in the leaves, the elimination of interfering

TABLE I

CONTENTS OF LANATOSIDE A AND LANATOSIDE C IN *DIGITALIS LANATA* LEAVES DETERMINED BY THE PROPOSED METHOD

Glycoside	*Found (μg)*[a]	*Mean ± S.D. (μg)*	*Relative standard deviation (%)*
Lanatoside A	63.4, 72.0, 68.1, 72.1, 66.0, 67.9, 66.2, 72.0, 60.0, 61.1	66.9 ± 4.4	6.6
Lanatoside C	223.4, 209.1, 223.9, 224.8, 209.1, 212.6, 219.4, 222.7, 209.2, 209.1	216.3 ± 7.1	3.3

[a] Amount of lanatoside A or C per 100 mg of a dry leaf powder sample.

peaks by the pretreatment step before the HPLC analysis is of great importance. The described clean-up procedure with the Sep-Pak cartridges was much more efficient and convenient for the determination of cardiac glycosides in *Digitalis* leaves than the previous method[11] involving a solvent-partition sequence and preparative TLC. In fact, the secondary glycosides such as digitoxin and digoxin were eluted with ethyl acetate–ethanol (100:1) and the primary glycosides (lanatosides) were eluted subsequently with ethyl acetate–ethanol (5:1) on a Sep-Pak silica cartridge.

Recently, the TSK-gel Amide-80 column material made of silica gel modified with carbamoyl groups has been developed, which permits the separation of monosaccharides, oligosaccharides and sugar alcohols. On this normal-phase partition column, a reversed-phase type solvent system composed of acetonitrile–water can be used, which has good ultraviolet transparency at a wavelength (220 nm) nearer the λ_{max} of the butenolide ring. Therefore, the use of this column is preferable to the quantification of digitalis glycosides.

In conclusion, the HPLC method described here has been demonstrated to be accurate, precise and selective for the determination of lanatosides in *Digitalis lanata* leaves. This method can be useful for estimation of the quality of the leaves.

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REFERENCES

1 M. A. Elkiey, Z. F. Ahmed, E. M. Abdelkader and S. M. Abdelwahab, *J. Pharm. Sci. U.A.R.*, 5 (1964) 139.
2 E. Bancher, Th. Prey and F. Wurst, *Planta Med.*, 29 (1976) 393.
3 S. M. Khafagy and A. N. Girgis, *Planta Med.*, 25 (1974) 350.
4 P. Horváth, *Acta Pharm. Hung.*, 52 (1982) 133.
5 P. H. Cobb, *Analyst (London)*, 101 (1976) 768.
6 C. Brugidou, M. Jacques, L. Cosson, F. X. Jarreau and T. Ogerau, *Planta Med.*, 54 (1988) 262.
7 W. Lindner and R. W. Frei, *J. Chromatogr.*, 117 (1976) 81.
8 P. A. Linley and A. G. M. Mohamed, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 4 (1981) 239.
9 M. Wichtl, M. Mangkudidjojo and W. Wichtl-Bleier, *J. Chromatogr.*, 234 (1982) 503.
10 F. Orosz, M. Nuridsány and J. Ovádi, *Anal. Biochem.*, 156 (1986) 171.
11 Y. Fujii, H. Fujii and M. Yamazaki, *J. Chromatogr.*, 258 (1983) 147.
12 Y. Fujii, Y. Ikeda and M. Yamazaki, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 10 (1987) 137.
13 B. Pekić and D. Miljković, *Planta Med.*, 27 (1975) 178.
14 L. Sawlewicz, H. H. A. Linde and K. Meyer, *Helv. Chim. Acta*, 51 (1968) 1353.