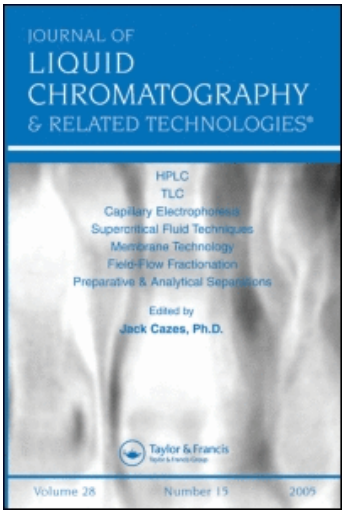

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Quantitative Determination of Lanatosides in the Hybrid *Digitalis ambigua* × *Digitalis lanata* Leaves by HPLC

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Quantitative Determination of Lanatosides in the Hybrid *Digitalis ambigua* × *Digitalis lanata* Leaves by HPLC

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ABSTRACT

The primary glycosides in the hybrid *Digitalis ambigua* × *Digitalis lanata* leaves were determined by HPLC and compared with the measurement of lanatosides in *D. ambigua* and *D. lanata* leaves. The extract of dry leaf powder with chloroform : ethanol (1 : 2, v/v) was submitted to Sep-Pak cartridges prior to HPLC analysis. HPLC was performed on a TSK-GEL AMIDE-80 column using acetonitril : water (96 : 4). The effluent was monitored by ultraviolet (UV) absorption at 220 nm. The quantitation was carried out by the internal standard method. The amount of lanatoside C per 100 mg of dry leaf powder samples of the hybrid *D. ambigua* × *D. lanata* and *D. lanata* were 227.3 μg and 134.4 μg, respectively. The amount of lanatoside C in the hybrid *D. ambigua* × *D. lanata* was higher than in *D. lanata*.

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Key Words: Hybrid *Digitalis ambigua* × *Digitalis lanata*; Cardiac glycoside; Primary glycoside; HPLC.

INTRODUCTION

Digitalis contains cardiac glycosides that are classified into A–E series on the basis of aglycone. The "C" series cardiac glycosides are therapeutically important substances. *Digitalis lanata* is known to contain primary glycosides including lanatosides A, B, and C. On the other hand, *Digitalis ambigua* contains lanatoside A and B, but lanatoside C is not present.[1] Orosz et al. have reported lanatoside C in *D. lanata* extract by employing a reversed-phase column.[2] The usefulness of adapting gradient elution to the HPLC analysis of cardiac glycosides in *D. lanata* or reciprocal cross breedings of *D. lanata* with other *Digitalis* species has also been described by Wichtl et al.[3–6] Recently, we reported the determination of lanatosides in *D. lanata* leaves by HPLC[7,8] and *D. ambigua* by reversed-phase HPLC with isocratic elution.[9] In the present study, we identified lanatosides in the hybrid *D. ambigua* × *D. lanata* leaves by HPLC, and compared its measurement of lanatosides in *D. ambigua* and *D. lanata* leaves.

EXPERIMENTAL

Chemicals

Lanatosides A, B, and C were obtained from E. Merck (Darmstadt, Germany), and their chemical structures are given in Fig. 1. Desacetyllanatoside A was prepared from lanatoside A.[10] 14α, 15α-Epoxy-"β"-anhydrodesacetyllanatoside A used as the internal standard, was synthesized in four steps from desacetyllanatoside A.[11] All of the compounds were checked for homogeneity by TLC, and solvents were purified by redistillation prior to use.

Chromatographic Apparatus

The HPLC system consisted of a Jasco 880-PU pump (Japan Spectroscopic, Tokyo, Japan), a Rheodyne 7125 loop injector (Cotati, CA), a Tosoh UV-8010 variable-wavelength detector set at 220 nm (Tosoh, Tokyo), and a Hitachi D-2500 Chromato-Integrator (Hitachi, Tokyo). The stainless-steel column (250 mm × 4.6 mm I.D.) was packed with TSK-GEL AMIDE-80 (Tosho). This is a column containing 5 μm porous silica particles derivatized

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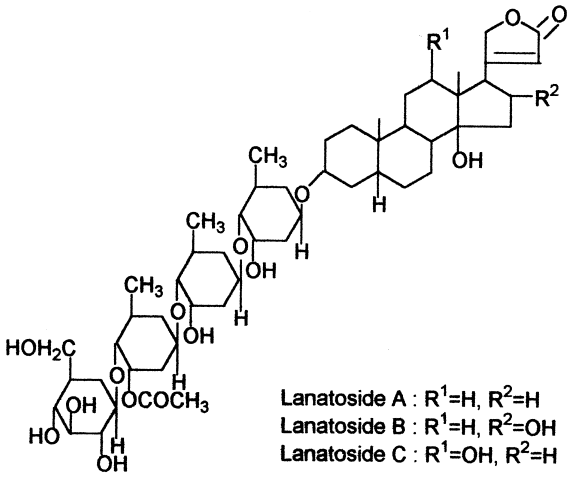

Figure 1. Chemical structures of the lanatosides investigated.

with carbamoyl groups. The separations were performed under ambient conditions.

Preparation of *Digitalis* Leaf Powder

Leaves of *Digitalis* were collected during the flowering stage in June 2000, at the Medicinal Plant Garden (Kanazawa, Japan) of Hokuriku University. A voucher specimen had been deposited at the Herbarium of Hokuriku University. The fresh leaves were quickly washed with water and immediately freeze-dried in a Yamato Neocool DC-55A apparatus (Yamato Scientific, Tokyo). The leaf powder was prepared by a previously reported method.[7]

Sample Preparation for HPLC

Leaf powder (ca. 50 mg) of the hybrid *D. ambigua* × *D. lanata*, *D. ambigua*, or *D. lanata* was accurately weighed and extracted with 5 mL of chloroform : ethanol (1 : 2, v/v) containing 14α, 15α-epoxy-"β"-anhydrodesacetyllanatoside A (90.35 μg) as an internal standard. After ultrasonication for 1 hr in an ultrasonic cleaning bath, the extract was filtered and evaporated using a rotary evaporator. The residue was in 3 mL of ethylacetate : ethanol (100 : 1, v/v) and subjected to the Sep-Pak silica cartridge (Waters, Milford, MA). Then 17 mL of ethylacetate : ethanol (100 : 1, v/v) and 10 mL of

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ethylacetate : ethanol (5 : 1, v/v) were successively passed through the cartridge. The latter fraction (10 mL) was collected and evaporated using a rotary evaporator. The residue obtained was dissolved in 2 mL of methanol : water (2 : 3, v/v) and loaded on the Sep-Pak C_{18} cartridge. After washing with 13 mL of methanol : water (2 : 3, v/v), primary glycosides were eluted with 15 mL methanol : water (2 : 3, v/v). The eluate was evaporated in vacuo and the material obtained was submitted to HPLC.

HPLC Determination

The mobile phase used for the separation was acetonitrile : water (96 : 4, v/v) and the flow rate was adjusted to 1.0 mL/min. The extract pretreated above was dissolved in 1.0 mL of the mobile phase and a 10 μL volume of the sample solution was submitted to HPLC. Lanatosides A, B, and C in *Digitalis* leaf powder were determined by the internal standard method. Calibration graphs were constructed by plotting the ratio of the peak area of the glycoside to the peak area of the internal standard against the weight of each glycoside. The average peak areas from three chromatograms were used for the determination.

TLC Procedure

Normal-phase TLC was carried out on a high-performance silica gel 60 F_{254} plate (5 cm × 10 cm, Merck), and reversed-phase TLC on a $KC_{18}F$ plate (5 cm × 20 cm, Whatman, Clifton, NJ). After development and air drying, the plates were checked by UV light around 254 nm, sprayed with concentrated sulfuric acid, and heated at 120°C for 10 min.

RESULTS AND DISCUSSION

HPLC was performed on a carbamoyl bounded silica column with acetonitrile : water (96 : 4, v/v) at a flow rate of 1.0 mL/min. A detection wavelength of 220 nm was chosen on the basis of the butenolide ring attached at the C-17 position of the steroid nucleus. Figure 2(A) illustrates the chromatogram of a standard mixture of lanatosides A, B, and C, and 14α, 15α-epoxy-"β"-anhydrodesacetyllanatoside A, which have retention times of 24.9, 38.1, 41.5, and 49.4 min, respectively. The separation was of sufficient quality and reproducibility to permit quantitative assessment.

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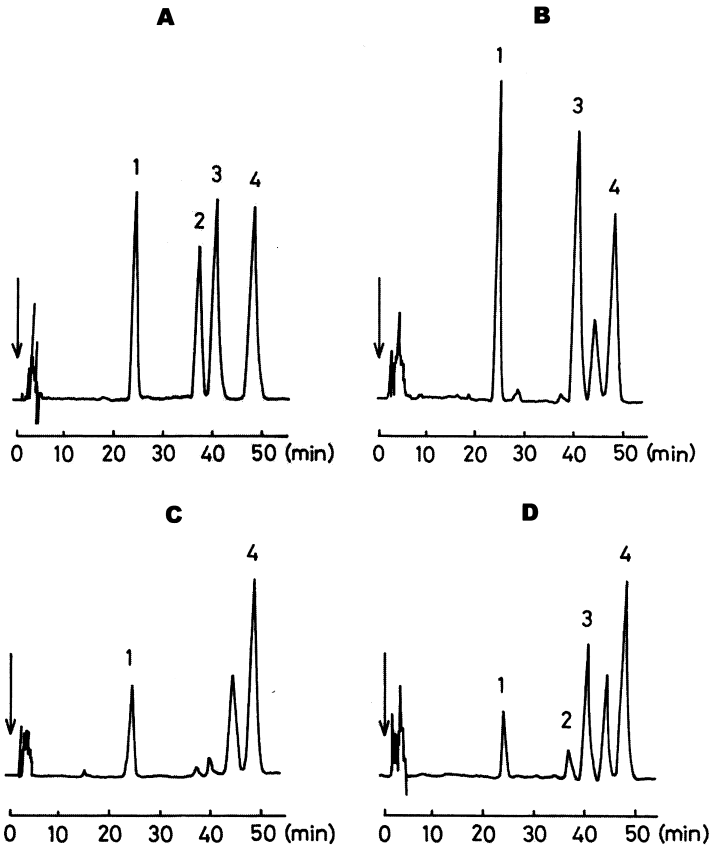

Figure 2. HPLC separation of cardiac glycosides. Peak identification: 1 = lanatoside A; 2 = lanatoside B; 3 = lanatoside C; 4 = internal standard (14α, 15α-epoxy-"β"-anhydrodesacetyllanatoside A). (A) Mixture of pure compounds. (B) *D. ambigua* × *D. lanata* leaf extract containing an internal standard. (C) *D. ambigua* leaf extract containing an internal standard. (D) *D. lanata* leaf extract containing an internal standard.

The determination of lanatosides in *Digitalis* leaves was carried out by an internal standard method. The leaves were dried and then extracted with chloroform : ethanol (1 : 2, v/v) by ultrasonication. In order to remove interfering substances in the extract, it was necessary to introduce a purification step prior to HPLC. Sep-Pak cartridges packed with silica gel and C_{18} were used to clean the extract. The purified extract was subjected to HPLC under the same conditions as the separation of a standard mixture of

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lanatosides. Typical chromatograms of the extract of the hybrid *D. ambigua* × *D. lanata*, *D. ambigua*, and *D. lanata* within the internal standard are illustrated in Fig. 2[(B), (C), (D)]. Lanatosides were separated satisfactorily from the other constituents of the extract. Peaks corresponding to the retention time of 14α, 15α-epoxy-"β"-anhydrodesacetyllanatoside A was found only in the chromatograms of those extracts pretreated with this internal standard. The eluate corresponding to each peak was collected and evaporated in vacuo, to ascertain the peak homogeneity.

The materials obtained were analyzed by both normal-phase TLC (lanatoside A, R_f 0.47; lanatoside B, R_f 0.42; lanatoside C, R_f 0.40) using chloroform : methanol : water (80 : 20 : 2.5, v/v) as the developing solvent and reversed-phase TLC (lanatoside A, R_f 0.43; lanatoside B, R_f 0.55; lanatoside C, R_f 0.70) using acetonitrile : 0.5 M sodium chloride (10 : 13, v/v) indicated single components. For HPLC quantitation, linear calibration graphs were prepared by plotting five data points within the range of 1–250 μg for lanatosides. The regression equations and correlation coefficients (r) were determined as $y = 0.0112x + 0.011$ ($r = 0.999$) for lanatoside A, $y = 0.0138x + 0.036$ ($r = 0.994$) for lanatoside B, and $y = 0.0089x + 0.095$ ($r = 0.999$) for lanatoside C, where y represents the peak area ratio of lanatosides to the internal standard and x the amount (μg) of lanatosides. The quantitation of lanatosides in *Digitalis* was carried out as described and the results obtained from ten dried leaf powder samples are given in Table 1.

These data indicated that the average contents of lanatosides A and C per 100 mg of the hybrid *D. ambigua* × *D. lanata* leaf powder were 103.3 ± 5.4 and 227.3 ± 11.0 μg, respectively. The quantitation of lanatoside B in this sample was not undertaken, due to this content found to be below the detection limit. The amount of lanatoside C is not present in *D. ambigua*, lanatoside A was 38.1 ± 1.5 μg. Also, lanatoside B in this sample was not undertaken, since the content was found to be below the detection limit, the same as with the hybrid *D. ambigua* × *D. lanata*. On the other hand, lanatosides A, B, and C in *D. lanata* were 28.5 ± 1.3 μg, 10.8 ± 0.7, and 134.4 ± 5.3 μg, respectively. The values of lanatosides in the hybrid *D. ambigua* × *D. lanata* were higher than those in *D. ambigua* and *D. lanata*. Especially, the amount of lanatoside C in the hybrid *D. ambigua* × *D. lanata* leaves was about 1.7 times that in *D. lanata*.

CONCLUSIONS

It is well known that lanatoside C is transformed into digoxin by deglucosylation using digilanidase present in the leaves and subsequent

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Table 1. Content of primary glycosides in *Digitalis* leaves.

	D. ambigua × *D. lanata*			*D. ambigua*			*D. lanata*		
Glycoside	Found (mg)		Mean ± SD (μg)	Found (mg)		Mean ± SD (μg)	Found (mg)		Mean ± SD (μg)
Lanatoside A	127.3	134.7		38.8	36.0		29.7	29.4	
	124.3	125.6		36.0	38.0		26.2	29.6	
	124.5	131.9	130.3 ± 5.4	39.0	39.0	38.1 ± 1.5	28.4	27.5	28.5 ± 1.3
	128.0	139.3		39.7	36.6		28.0	27.1	
	130.3	137.5		40.5	37.8		29.7	29.6	
Lanatoside B							10.3	11.4	
							11.6	10.5	
							9.4	10.3	10.8 ± 0.7
							11.2	11.6	
							11.4	10.7	
Lanatoside C	238.4	225.7					132.2	138.3	
	210.4	214.2					139.0	131.7	
	213.4	236.2	227.3 ± 11.0				138.2	133.4	134.4 ± 5.3
	230.1	231.1					127.1	126.9	
	240.5	233.2					143.1	134.4	

Note: Values are the quantity per 100 mg of dried leaf powder sample.

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deacetylation.[8,12] Digoxin is available to medicine today for the therapy of congestive heart failure and atrial fibrillation. We expect that the hybrid *D. ambigua* × *D. lanata* will serve for the industrial production of digoxin.

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