Determination of Lanatoside C and Digoxin in Digitalis lanata by Hplc and Its Application to Analysis of the Fermented Leaf Powder

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DETERMINATION OF LANATOSIDE C AND DIGOXIN IN *DIGITALIS LANATA* BY HPLC AND ITS APPLICATION TO ANALYSIS OF THE FERMENTED LEAF POWDER

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ABSTRACT.—A quantitative hplc method for the simultaneous determination of lanatoside C and digoxin in *Digitalis lanata* was developed. The extract of dry leaf powder with 50% MeOH was applied to a Sep-Pak C_{18} cartridge prior to hplc analysis. The analysis was performed on an octylsilyl bonded silica column, using MeCN-MeOH-H_2O (20:1:50) and uv detection (220 nm). The quantitation was carried out by the internal standard method. The proposed method is sufficiently precise and relatively simple. Application of this hplc analysis to the determination of lanatoside C and digoxin after fermentation of the leaf powder is also described.

Lanatoside C and digoxin, cardioactive glycosides, are therapeutically important substances that are available to medicine today for the treatment of heart disease. *Digitalis lanata* Ehrh. (Scrophulariaceae) is a significant medicinal plant as a source of these compounds. It is well known that lanatoside C is transformed into digoxin by deglucosylation using digilanidase present in the leaves and subsequent deacetylation (1,2). Accordingly, it is necessary to establish a reliable method for the analysis of cardiac glycosides, lanatoside C and digoxin in particular, in *D. lanata*.

A number of tlc procedures have been shown to be useful for the determination of the cardiac glycosides in *D. lanata* leaves (3–6). However, an hplc method appears to be more efficient for the analysis of the plant extract. Previously published hplc methods of separating mixtures of pure cardiac glycosides have utilized a normal-phase silica column (7–10) and a reversed-phase column (10,11). The quantitation of digoxin in *D. lanata* by hplc has been proposed by Cobb (12) and Brugidou *et al.* (13). On the other hand, Orosz *et al.* (14) have reported the determination of lanatoside C in the leaves by using an octadecylsilyl silica column, but a satisfactory chromatogram of the extract has not been obtained. In addition, hplc separations of cardiac glycosides from plant extracts have been accomplished by employing gradient elution (15–17).

We previously reported the hplc analysis of lanatosides and α-acetyldigoxin in *D. lanata* leaves (18,19). This paper describes a convenient method for the simultaneous determination of lanatoside C and digoxin from the extract of such leaves by means of hplc with isocratic elution. The application of this method to the quantitation of these glycosides in the fermented leaf powder is also mentioned.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Instrumentation consisted of a Jasco 880-PU pump, a Rheodyne 7125 loop injector, a Tosoh uv-8010 variable-wavelength detector set at 220 nm and a Shimadzu Chromatopac C-R3A data processor. The hplc separations were performed on a Chemcosorb 5 C_8-U column (4.6 mm × 150 mm) under ambient conditions. This is a reversed-phase column containing 5 μm porous silica particles linked covalently with octylsilyl groups. The mobile phase used for the separation was MeCN-MeOH-H_2O (20:1:50), and the flow rate was adjusted to 0.5 ml/min.

Normal-phase tlc was carried out on a high-performance Si gel 60 F_{254} plate (5 cm × 10 cm, Merck) with a concentrating zone, and reversed-phase tlc on a $KC_{18}F$ plate (5 cm × 20 cm, Whatman). After development and air drying, the plates were checked by uv light around 254 nm, sprayed with concentrated H_2SO_4, and heated at 120° for 10 min.

CHEMICALS.—Lanatosides B and C were purchased from Merck and digoxin from Aldrich. Desacetyllanatoside B was prepared from lanatoside B according to the procedure of Pekić and Miljković (20)

and recrystallized repeatedly from MeOH. All of these compounds were checked for homogeneity by tlc, and solvents used were purified by redistillation prior to use.

PLANT MATERIAL.—*D. lanata* leaves were collected during the flowering stage in June 1988 at the Medicinal Plant Garden (Kanazawa, Japan) of Hokuriku University. The fresh leaves were immediately freeze-dried and then dried using P_2O_5 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 using P_2O_5 under reduced pressure for 5 days.

SAMPLE PREPARATION AND HPLC DETERMINATION.—Leaf powder (ca. 50 mg) was accurately weighed and extracted with 50% MeOH (25 ml) containing desacetyllanatoside B (102.6 μg) as an internal standard. 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 MeCN-H_2O (2:8) (2 ml) and applied to a Sep-Pak C_{18} cartridge (Waters). Then MeCN-H_2O (2:8) (28 ml) and MeCN-H_2O (3:7) (10 ml) were successively passed through the cartridge. After evaporation of the latter fraction using a rotary evaporator, the residue obtained was dissolved in MeCN-MeOH-H_2O (20:1:50) (1 ml), and an aliquot (10 μl) of the sample solution was submitted to hplc. Lanatoside C and digoxin were determined by the internal standard method. Calibration graphs were constructed by plotting the ratio of the peak area of lanatoside C or digoxin to the peak area of the internal standard against the weight of each compound. The average peak areas from three chromatograms were used for the determination.

FERMENTATION OF THE LEAF POWDER.—Leaf powder (50 mg) was suspended in 0.05% $NaHCO_3$ solution (3 ml) and incubated at 40° for 0.25, 0.5, 1, 2, 4, and 6 h. Then HOAc (0.01 ml), MeOH (3 ml), and 50% MeOH (19 ml) containing desacetyllanatoside B (52.34 μg) as an internal standard were added to the suspension. The incubation mixture was extracted by ultrasonication for 1 h. The sample preparation and chromatographic procedure were undertaken in the manner described above.

RESULTS AND DISCUSSION

The quantitation of lanatoside C and digoxin in *D. lanata* was performed by the internal standard method in order to improve the reproducibility of the sample preparation and the chromatographic run. For the selection of an internal standard, a number of compounds were investigated and desacetyllanatoside B was found to be the most suitable. Figure 1A shows the chromatogram of a standard mixture of lanatoside C,

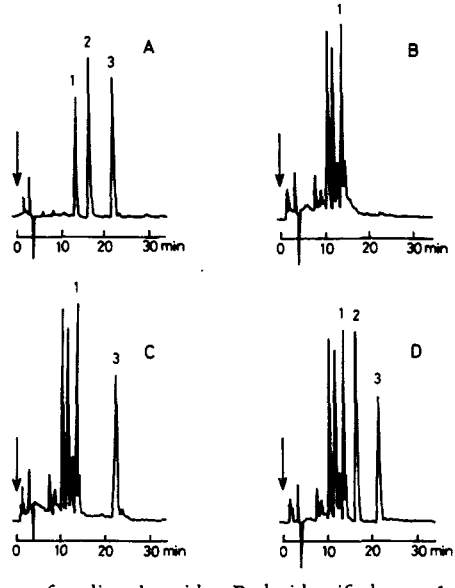

FIGURE 1. Hplc separations of cardiac glycosides. Peaks identified were: 1, lanatoside C; 2, digoxin; 3, desacetyllanatoside B. Chromatographic conditions were: column Chemcosorb 5 C_8-U (4.6 mm × 150 mm); mobile phase MeCN-MeOH-H_2O (20:1:50); flow rate 0.5 ml/min; uv monitor 220 nm; sample volume 10 μl. (A) Mixture of pure compounds. (B) Extract of *Digitalis lanata* leaves without an internal standard. (C) Extract of *D. lanata* leaves with an internal standard. (D) Extract of *D. lanata* leaves after addition of pure digoxin.

digoxin, and the internal standard. Hplc on an octylsilyl bonded silica column with a solvent system consisting of MeCN-MeOH-H_2O (20:1:50) at a flow rate of 0.5 ml/min gave complete separation of these glycosides. A detection wave-length of 220 nm was used, account being taken of the butenolide ring attached at C-17 of the steroid nucleus. The retention times of lanatoside C, digoxin, and desacetyllanatoside B were 13.6, 16.7, and 21.7 min, respectively.

On the basis of these data, the separation of cardiac glycosides in *D. lanata* leaves was carried out. The dry leaf powder was extracted with 50% MeOH containing the internal standard. Many other plant materials in the extract interfered with the hplc separation. Therefore, the extract was submitted to a reversed-phase Sep-Pak cartridge prior to hplc. The purified extract was separated by hplc under the same chromatographic conditions as those described above. Figure 1B illustrates a typical chromatogram of the extract in the absence of the internal standard. A chromatogram of the extract after incorporation of the internal standard is given in Figure 1C. From these chromatograms, the other substances present in the leaves were ascertained not to interfere with the peak of the internal standard. The eluate corresponding to the peak of lanatoside C was collected and evaporated in vacuo, to make sure that the peak was homogeneous. The residue obtained was analyzed by both normal-phase tlc (R_f 0.40) using $CHCl_3$-MeOH-H_2O (80:20:2.5) as a mobile phase and reversed-phase tlc (R_f 0.46) using MeCN-0.5M NaCl (10:13). These analyses did not reveal other components under the peak of lanatoside C. The amount of digoxin in the leaf powder examined was found to be below the detection limit of the determination used. Therefore, pure digoxin was added to the leaf powder and analyzed by the described procedure. Figure 1D shows a representative chromatogram of the extract after addition of digoxin.

For the hplc quantitation, linear calibration graphs were prepared by plotting seven data points in the range of 20–160 μg. The regression equations and correlation coefficients (r) were determined as $Y = 0.00825X + 0.011$ ($r = 0.999$) for lanatoside C and $Y = 0.01478X - 0.007$ ($r = 0.998$) for digoxin, where Y represents the peak area ratio of lanatoside C or digoxin to the internal standard and X the amount (μg) of each compound. The assay results obtained for ten dry leaf powder samples indicated that the average content of lanatoside C per 100 mg of the leaf powder was 212.1 μg with excellent reproducibility (Table 1). The accuracy of the hplc method was confirmed by adding a known amount (47.24 μg) of lanatoside C to five dry leaf powder samples and assaying it by the procedure described. The recoveries ranged from 97.5 to 100% with a mean of 99.1% and a standard deviation of 1.2%. The extraction rate of lanatoside C from the leaf powder versus the number of extraction times was also examined, and the following results were obtained: one time, 219.3 μg per 100 mg of the dry leaf powder;

TABLE 1. Content of Lanatoside C in *Digitalis lanata* Leaves.

Sample number	Found[a] (μg)	Sample number	Found[a] (μg)
1	211.4	7	212.0
2	214.9	8	207.8
3	209.0	9	211.4
4	217.6	10	219.4
5	203.4	Mean ± SD	212.1 ± 4.7
6	214.5		

[a]Values are the amount of lanatoside C per 100 mg of a dry leaf powder sample.

two times, 211.7 μg; three times, 216.1 μg. The proposed procedure using one extraction with 50% MeOH was considered satisfactory for the isolation of lanatoside C from the leaf powder. Furthermore, the determination of digoxin added to the leaf powder was studied. The results indicated that the average recovery from five experiments was 96.1%, with a standard deviation of 3.5% (Table 2). From the above-mentioned data, it is apparent that this quantitative method has sufficient precision and accuracy.

TABLE 2. Determination of Digoxin Added to *Digitalis lanata* Leaf Powder.[a]

Sample number	Found (μg)	Recovery rate (%)
1	86.27	96.7
2	82.21	92.2
3	84.58	94.8
4	85.12	95.4
5	90.67	101.6
Mean ± SD	85.77 ± 3.11	96.1 ± 3.5

[a]Digoxin (89.20 μg) was added to each sample (ca. 50 mg) of *D. lanata* leaf powder.

The analysis of samples obtained by fermentation of *D. lanata* leaf powder as described under Experimental was carried out by the present method. Lanatoside C in the leaves was transformed into digoxin by incubation of the leaf powder in 0.05% $NaHCO_3$ at 40°. In this conversion, the terminal glucose unit of lanatoside C is removed enzymatically by using digilanidase present in the leaves, and then the acetyldigitoxose unit is deacetylated by the mild alkaline incubation medium. As shown in Figure 2, the yield of digoxin in the leaf powder suspension was found to increase continuously during the incubation period, while the content of lanatoside C decreased during the first 2 h. Compared with the previous procedures (6,12) consisting of two separate stages of enzymatic hydrolysis and deacetylation, the fermentation procedure using 0.05% $NaHCO_3$ is favorable for the simple conversion of lanatoside C in the leaves into digoxin.

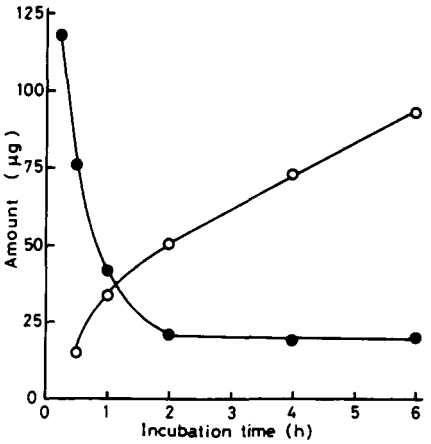

FIGURE 2. Time courses for lanatoside C (●) and digoxin (○) by fermentation of *Digitalis lanata* leaf powder at 40°. The vertical axis represents the amount of lanatoside C or digoxin/100 mg of the leaf powder.

In conclusion, the simultaneous hplc determination of lanatoside C and digoxin in *D. lanata* leaves was achieved under an isocratic elution by using a reversed-phase C_8 column and an internal standard. The clean-up procedure using a Sep-Pak C_{18} cartridge prior to the hplc analysis was of great importance in the elimination of other compounds present in the extract. The proposed method is reliable and relatively simple for the quantitation of these cardiac glycosides. Accordingly, this method can be useful for the quality assurance of *D. lanata* leaves. In addition, the hplc method was shown to have the capability to analyze lanatoside C and digoxin in the fermented leaf powder. We expect that our investigation will serve for the industrial production of digoxin from the leaves of *D. lanata* containing lanatoside C.

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