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QUANTITATIVE HPLC ANALYSIS OF CARDIAC GLYCOSIDES IN *DIGITALIS PURPUREA* LEAVES

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ABSTRACT.—An analytical method for the determination of cardiac glycosides in *Digitalis purpurea* leaves by hplc was developed. Quantitation was carried out by the incorporation of lanatoside A as an internal standard. The present method is sufficiently precise and relatively simple.

Cardiac glycosides isolated from *Digitalis* spp. have been widely used in the therapy of congestive heart failure and atrial fibrillation. *Digitalis purpurea* L. (Scrophulariaceae) is known to contain primary glycosides including purpurea glycoside A, purpurea glycoside B, and glucogitaloxin. These primary glycosides are transformed into their corresponding secondary glycosides such as digitoxin, gitoxin, and gitaloxin by the plant's own enzyme, digipurpidase, present in the leaves (1,2). Therefore, it is important to include both primary and secondary glycosides in the analysis of *D. purpurea*.

Tlc has been applied in the determination of cardiac glycosides in *D. purpurea* leaves, utilizing fluorimetric measurement on a thin-layer plate (3,4) or by colorimetric measurement of solutions obtained after spot elution (5). However, hplc seems to offer a more convenient mode for the analysis of these glycosides. The separation of various mixtures of pure cardiac glycosides has been accomplished by employing both a normal-phase silica column (6–8) and a reversed-phase column (7,9). As for the analysis of *D. lanata* extract, Cobb (10) and Brugidou *et al.* (11) have used a Si gel column and an octadecylsilyl silica column, respectively, for the quantitation of digoxin by hplc. Orosz *et al.* (12) and Ikeda *et al.* (13) have also determined lanatoside C in *D. lanata* leaves using a reversed-phase column. In addition, the determination of primary glycosides (14) and secondary glycosides (15) in *D. purpurea* leaves has been achieved by micro-hplc and conventional hplc, respectively. Also, the usefulness of adapting gradient elution to hplc analysis of the glycosides from plant extracts has been demonstrated (16–20).

The determination of primary glycosides or secondary glycosides in *D. purpurea* leaves by reversed-phase tlc has been reported in a previous paper (21). The purpose of this study was to develop a convenient method for the simultaneous determination of both primary and secondary glycosides in *D. purpurea* leaves by using hplc with isocratic elution. This paper also describes the application of the method to the analysis of cardiac glycosides after incubation of the leaf powder.

An initial study focused on the selection of an internal standard and the chromatographic separation of cardiac glycosides. Among the many compounds investigated, lanatoside A was found to be the most suitable as an internal standard. Hplc was performed on an octylsilyl-bonded silica column with a three-component solvent system consisting of CH_3CN-MeOH-H_2O (10:15:18) at a flow rate of 0.5 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 1A illustrates the chromatogram of a standard mixture of purpurea glycoside B, glucogitaloxin, gitoxin, purpurea glycoside A, gitaloxin, lanatoside A, and digitoxin, which had retention times of 10.7, 13.6, 19.5, 21.6, 26.2, 33.6, and 44.1 min, respectively. The separation is

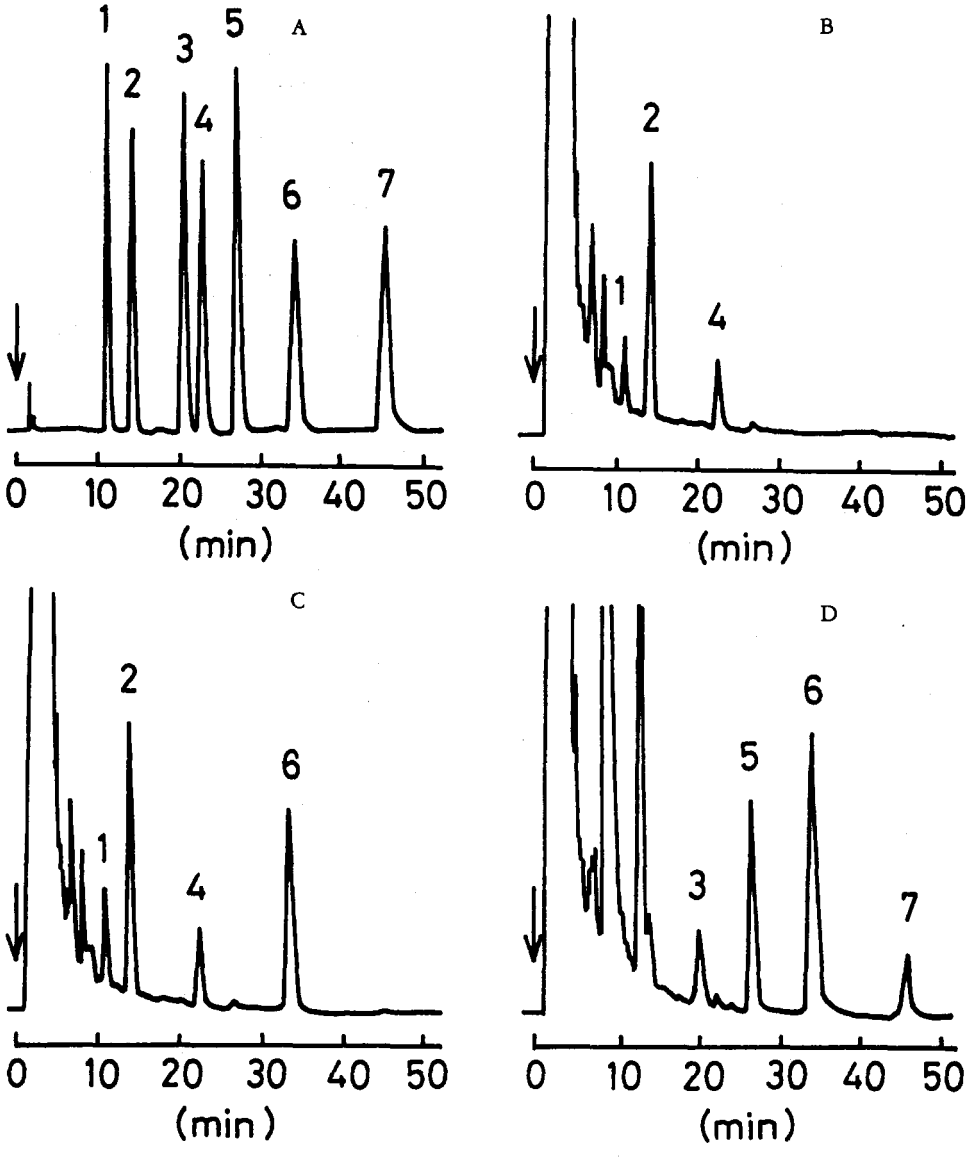

FIGURE 1. Hplc separations of cardiac glycosides: peaks 1, purpurea glycoside B; 2, glucogitaloxin; 3, gitoxin; 4, purpurea glycoside A; 5, gitaloxin; 6, lanatoside A; 7, digitoxin. A. Mixture of pure compounds. B. *Digitalis purpurea* leaf extract. C. *D. purpurea* leaf extract containing an internal standard (lanatoside A). D. Extract of *D. purpurea* leaves after enzymatic hydrolysis at 40° for 3 h.

of sufficient quality and reproducibility to permit quantitative assessment.

The separation of cardiac glycosides of *D. purpurea* leaves was then undertaken on the basis of these data. The dry leaf powder was extracted by ultrasonication with 50% MeOH containing the internal standard. In order to remove interfering substances in the extract, it was necessary to introduce a purification step, using a Sep-Pak C_{18} cartridge prior to hplc sampling. The purified extract was subjected to hplc under the same conditions as the separation of a standard mixture of cardiac glycosides. Typical chromatograms of the extract in both the absence and presence of internal standard are illustrated in Figure 1B and 1C. Car-

diac glycosides were separated satisfactorily from the other constituents of the extract. Peaks corresponding to the retention time of lanatoside A were 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 (purpurea glycoside A, R_f 0.36; glucogitaloxin, R_f 0.33; purpurea glycoside B, R_f 0.29) using $CHCl_3$-MeOH-H_2O (80:20:2.5) as the mobile phase and reversed-phase tlc (purpurea glycoside B, R_f 0.61; glucogitaloxin, R_f 0.59; purpurea glycoside A, R_f 0.49) with MeOH-0.5 M NaCl (4:1). For hplc quantitation, linear calibration graphs were constructed by plotting five data points within the range of 10–300 μg. The regression equations, ranges of linearity, and correlation coefficients are presented in Table 1. The correlation coefficients for all six cardiac glycosides were greater than 0.999.

The quantitation of primary glycosides in *D. purpurea* was carried out as described and the results obtained from ten dried leaf powder samples are given in Table 2. These data indicated that the average content of purpurea glycosides A and B and glucogitaloxin per 100 mg of the leaf powder was 74.4, 39.5, and 192 μg, respectively. The coefficients of variation were found to range between 2.3 and 3.3%. The quantitation of secondary glycosides in these samples was not undertaken, because their concentration levels were found to be below the detection limit. This implied that enzymatic hydrolysis of primary glycosides to secondary glycosides was minimal or did not take place during the drying process.

The present method was then applied to the determination of secondary glycosides following hydrolysis as a result of the incubation of *D. purpurea* leaf samples in H_2O at elevated temperatures. Under these conditions, the terminal glucose unit of primary glycosides is removed by the digipurpidase present in the leaves (2). Figure 1D is a representative chromatogram of the extract obtained from plant materials incubated at 40° for 3 h. From this chromatogram, it is apparent that primary glycosides were quantitatively converted into their corresponding secondary glycosides. The purity of each peak was also checked by tlc of the eluate. Both normal-phase tlc (digitoxin, R_f 0.53; gitaloxin, R_f 0.50; gitoxin,

TABLE 2. Content of Primary Glycosides in *Digitalis purpurea* Leaves.[a]

Glycoside	Mean±SD[b] (μg)	CV (%)
Purpurea glycoside A	74.4±1.7	2.3
Purpurea glycoside B	39.4±1.3	3.3
Glucogitaloxin	191.5±4.8	2.5

[a]Values are the quantity per 100 mg of dried leaf powder sample.
[b]Represent 10 samples.

TABLE 1. Regression Equations, Ranges of Linearity, and Correlation Coefficients of the Calibration Graphs for Cardiac Glycosides.

Glycoside	Equation[a]	Range (μg)	Correlation coefficient (r)
Purpurea glycoside A	Y=0.0074X-0.010	10-300	0.9992
Purpurea glycoside B	Y=0.0082X-0.023	10-250	0.9990
Glucogitaloxin	Y=0.0074X-0.014	10-200	0.9991
Digitoxin	Y=0.0115X-0.010	10-200	0.9993
Gitoxin	Y=0.0111X-0.017	10-200	0.9992
Gitaloxin	Y=0.0090X-0.091	15-300	0.9993

[a]Y: peak area ratios of each cardiac glycoside to an internal standard. X: amount (μg) of each cardiac glycoside (number of points=5).

R_f 0.42) using $CHCl_3$-MeOH-H_2O (85:15:1) and reversed-phase tlc (gitoxin, R_f 0.54; gitaloxin, R_f 0.51; digitoxin, R_f 0.39) using MeOH-0.5 M NaCl (4:1) did not reveal the presence of other components. The hplc determination of digitoxin, gitoxin, and gitaloxin during the period of incubation at various temperatures was then carried out. It was found that the reaction rate increased with elevated temperature and the yield of the secondary glycosides increased continuously with the incubation time, reaching a plateau at 2 h at 40° and 50°, with digitoxin at 46.4 and 45.6, gitoxin at 32.2 and 31.8, and gitaloxin at 130.6 and 133.9 μg/100 mg/leaf sample, respectively. From these data, it seems that the incubation of the leaf powder in H_2O is adequate for the production of secondary glycosides from primary glycosides.

In conclusion, the present hplc procedure is a sensitive, precise, and comparatively simple method for the simultaneous determination of primary and secondary glycosides in *D. purpurea* leaves. The pretreatment procedure using a Sep-Pak C_{18} cartridge before hplc analysis was of great importance in the elimination of interfering substances. The use of lanatoside A as an internal standard enabled the quantitative analysis of six cardiac glycosides. Therefore, the hplc method can easily be applied to the estimation of the quality of *D. purpurea* leaves.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—The hplc system 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 stainless-steel column (150 mm×4.6 mm i.d.) was packed with Chemcosorb $5C_8$-U. This is a reversed-phase column containing 5-μm porous silica particles linked covalently with octylsilyl groups. The separations were performed under ambient conditions.

Normal-phase tlc was carried out on a high-performance Si gel 60 F_{254} plate (10 cm×10 cm, Merck), and reversed-phase tlc on a $KC_{18}F$ plate (5 cm×20 cm, Whatman). After development and air drying, the plates were sprayed with concentrated H_2SO_4 and heated at 120° for 10 min. Gitoxin, lanatoside A, and lanatoside B were obtained from E. Merck, digitoxin from Wako, and gitaloxin from Boehringer Mannheim. Purpurea glycoside A and purpurea glycoside B were synthesized from lanatoside A and lanatoside B, respectively, according to the procedure of Pekić and Miljković (22). Glucogitaloxin was prepared from purpurea glycoside B by the method of Haack *et al.* (23). All solvents were purified by redistillation prior to use.

PLANT MATERIAL.—Leaves of *D. purpurea* were collected during the flowering stage in June 1992, at the Medicinal Plant Garden, Kanazawa, Japan, of Hokuriku University. A voucher specimen has been deposited at the Herbarium of Hokuriku University. The fresh leaves were quickly washed with H_2O and immediately freeze-dried in a Yamato Neocool DC-55A apparatus. The leaf powder was prepared as previously reported (13).

SAMPLE PREPARATION.—Approximately 50 mg of leaf powder was accurately weighed and added to 50% MeOH (25 ml) containing lanatoside A (118.0 μg) as an internal standard. After ultrasonication for 1 h in an ultrasonic cleaning bath, the extract was filtered and evaporated to dryness *in vacuo*. The resulting residue was dissolved in CH_3CN-H_2O (1:9) (2 ml) and passed through a Sep-Pak C_{18} cartridge (Waters). After washing with CH_3CN-H_2O (1:9) (28 ml), primary glycosides, secondary glycosides, and an internal standard were eluted with CH_3CN-H_2O (1:1) (10 ml). The eluate was evaporated to dryness *in vacuo*.

HPLC DETERMINATION.—The mobile phase used for the separation was CH_3CN-MeOH-H_2O (10:15:18) and the flow rate was adjusted to 0.5 ml/min. The pretreated extract described above was dissolved in the mobile phase (1.0 ml), and an aliquot (10 μl) of the sample solution was submitted to hplc. Purpurea glycoside A, purpurea glycoside B, glucogitaloxin, digitoxin, gitoxin, and gitaloxin in *D. purpurea* 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.

ENZYMATIC HYDROLYSIS PROCEDURE.—The dried leaf powder sample (50 mg) was suspended in H_2O (5 ml) and incubated at 30°, 40°, and 50°. After incubation for 0.5, 1, 2, 3, and 4 h, MeOH (5 ml) and 50% MeOH (15 ml) containing lanatoside A (118.0 μg) as an internal standard were added to the suspension. The suspension was treated as described above.

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