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SEPARATION AND DETERMINATION OF CARDIAC GLYCOSIDES IN *DIGITALIS PURPUREA* LEAVES BY MICRO HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A quantitative micro high-performance liquid chromatographic (MHPLC) procedure for the determination of gitoxin and digitoxin in *Digitalis purpurea* leaves has been developed. Quantitation of cardiac glycosides was carried out by the incorporation of an internal standard. The extract of dry leaf powder was submitted to a solvent-partition sequence followed by preparative thin-layer chromatography before MHPLC analysis. MHPLC was performed on an ODS micro column, using a mobile phase of acetonitrile–methanol–water (15:15:19) and an ultraviolet detector at 220 nm. The amounts of gitoxin and digitoxin per 100 mg of dry leaf powder were estimated to be 0.1453 and 0.0820 mg, respectively. The proposed method using MHPLC has proved to be precise and reproducible.

INTRODUCTION

Digitalis purpurea is known to contain cardiac glycosides, therapeutically important substances available to medicine today for the treatment of heart disease. The methods for quantitative analyses of cardiac glycosides in the leaves of *D. purpurea* published so far have involved paper chromatographic[1] and thin-layer chromatographic (TLC)[2–5] separation. A number of high-performance liquid chromatographic (HPLC) procedures have also been shown to be effective for separating mixtures of cardiac steroids[6–14]. Cobb[15] has proposed the use of HPLC on silica gel for the assay of digoxin in *D. lanata* leaf, but it seems that no author has reported the HPLC analysis of digitalis glycosides in *D. purpurea*. In recent years Ishii *et al.*[16] have developed micro-HPLC (MHPLC), a technique for measuring micro-scale quantities. Previously we reported[17] the separation of digitalis glycosides by MHPLC; this involved an investigation of the chromatographic behaviour of nanogram amounts using a reversed-phase system.

The present paper describes the separation and determination of gitoxin and digitoxin in *D. purpurea* leaves using MHPLC. Quantitation was achieved using an internal standard method. Solvent partition of the leaf extract and then preparative TLC were employed for the pre-treatment. MHPLC was carried out on a reversed-

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phase micro column with ultraviolet (UV) detection (220 nm) based on the α,β-unsaturated lactone ring.

EXPERIMENTAL

Instruments

The apparatus was a Familic-100 micro high-performance liquid chromatograph (Japan Spectroscopic Co., Tokyo, Japan) equipped with a Uvidec-100 UV spectrophotometer monitoring the absorbance at 220 nm. The micro flow-through cell consisted of a quartz tube with a volume of 0.3 μl. The micro column used throughout was a PTFE tube packed with SC-01 (Japan Spectroscopic Co.). This was a reversed-phase column (162 $\times$ 0.5 mm I.D.) containing 5-μm porous silica particles covalently linked with ODS groups. The column was used under ambient conditions.

Materials

The chromatographic solvents and digitoxin were provided by Wako (Osaka, Japan). Silica gel HF for TLC and gitoxin were purchased from E. Merck (Darmstadt, G.F.R.). 14α,15α-Epoxy-"β"-anhydrodigitoxin, used as an internal standard, was prepared in four steps from digitoxin by the method of Sawlewicz *et al.*[18] and recrystallized repeatedly from methanol. All materials were checked for homogeneity by TLC and solvents were purified by distillation prior to use. All reagents employed were of analytical-reagent grade.

Preparation and extraction of the leaf sample

In October 1980, *D. purpurea* L. leaves in the third year were collected in the medicinal botanical garden (Kanazawa, Japan) of this University. The leaves were quickly washed with water and dried under reduced pressure at room temperature for 2 weeks. The dried leaves were pulverized and then sifted through a sieve of mesh width 500 μm.

Approximately 50 mg of leaf powder were accurately weighed and extracted with 120 ml of ethanol–chloroform (2:1) containing an internal standard (0.09149 mg) in a Soxhlet apparatus for 4 h. The extract obtained was evaporated to dryness using a rotary evaporator and partitioned between chloroform (35 ml) and water (20 ml) saturated with sodium chloride in a separatory funnel. The chloroform fraction was concentrated *in vacuo* and successively partitioned between cyclohexane (40 ml) and methanol–water (4:1) (45 ml). The methanol of the aqueous layer was removed and further re-extracted with chloroform (35 ml). After evaporation of the solvent, the residue was submitted to preparative TLC on silica gel using cyclohexane–chloroform–methanol (3:3:1) as developing solvent. The adsorbent corresponding to R_F 0.15–0.4 was eluted with ethyl acetate and the eluate was then evaporated *in vacuo*. The material was redissolved in the mobile phase (0.1 ml) and 0.3 μl of the solution was injected into the liquid chromatograph.

Determination of gitoxin and digitoxin

Separate solutions of each steroid were carefully prepared by weighing the compound and dissolving it in acetonitrile–methanol–water (15:15:19). The chro-

matographic retention times of the standards were determined and compared with peaks having similar retention times in mixtures of cardenolides.

A 0.3-μl volume of each sample was injected onto the column and acetonitrile–methanol–water (15:15:19) was employed as mobile phase at a flow-rate of 4 μl/min. Gitoxin and digitoxin in *D. purpurea* leaves were determined by the internal standard method. Calibration graphs were constructed by plotting the ratio of the peak height of each cardenolide to the peak height of 14α,15α-epoxy-"β"-anhydrodigitoxin against the weight of each sample, whereby satisfactory linearity was observed. The peak heights were measured from an extrapolated baseline.

Recovery test

To four dry leaf-powder samples were each added gitoxin (0.03611 mg), digitoxin (0.05762 mg) and an internal standard (0.09149 mg), and the mixtures were then extracted in a Soxhlet apparatus. The extract obtained was submitted to the solvent-partition sequence and preparative TLC, followed by MHPLC in the manner described above.

RESULTS AND DISCUSSION

An initial study was focused on the chromatographic separation of gitoxin, digitoxin and 14α,15α-epoxy-"β"-anhydrodigitoxin as internal standard. It is evident from the chromatogram illustrated in Fig. 1 that reversed-phase chromatography on an ODS bonded silica column (SC-01) using acetonitrile–methanol–water (15:15:19) as a mobile phase at a flow-rate of 4 μl/min gave complete separation of the three steroids. A detection wavelength of 220 nm was employed on account of the butenolide ring attached at C-17 of the steroid nucleus. The above three-component solvent system does not have any appreciable UV absorption at 220 nm. The PTFE column size used in this technique was much smaller than those used in ordinary HPLC. The retention times for gitoxin, digitoxin and the internal standard

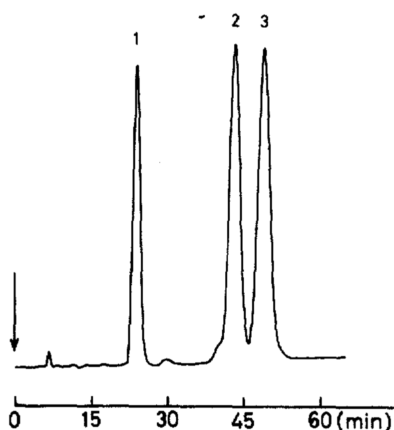


Fig. 1. Separation of a mixture of digitalis glycosides and an internal standard. Peaks: 1 = gitoxin; 2 = digitoxin; 3 = 14α,15α-epoxy-"β"-anhydrodigitoxin. Conditions: Jasco SC-01 column (162 × 0.5 mm I.D.); mobile phase, acetonitrile–methanol–water (15:15:19); flow-rate, 4 μl/min; UV monitor at 220 nm; sample volume, 0.3 μl.

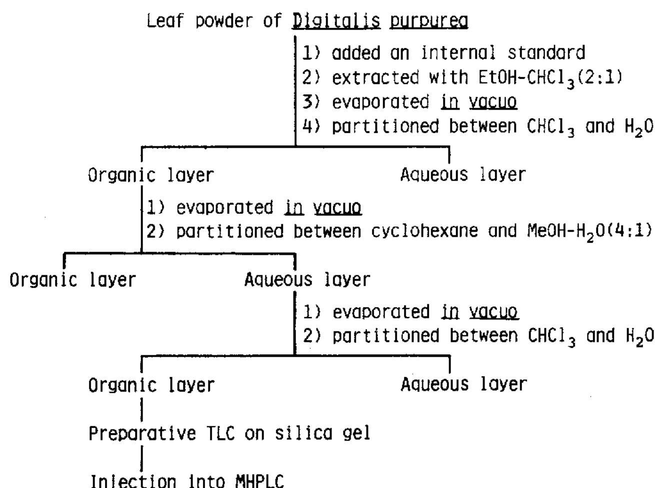
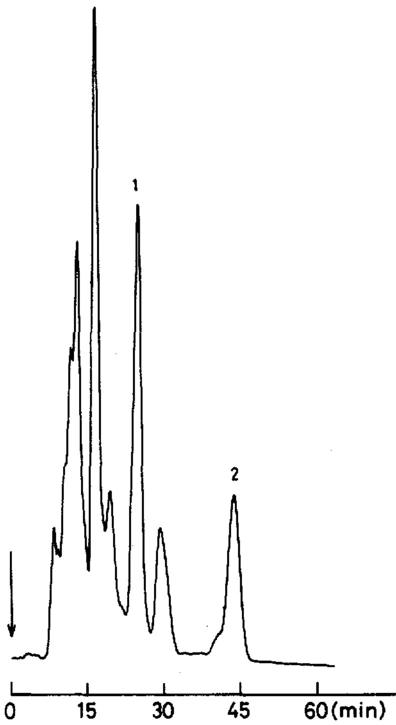


Fig. 2. Schematic diagram of the sample preparation for the MHPLC analysis of gitoxin and digitoxin. EtOH = ethanol; MeOH = methanol.

were 23.7, 43.2 and 48.6 min, respectively. The separation is sufficiently good and reproducible to permit quantitative work.

On the basis of these data, the separation of gitoxin and digitoxin in *D. purpurea* leaves was then undertaken. Quantitation of these cardiac glycosides was performed by incorporation of an internal standard in a leaf-powder sample. A scheme illustrating the various steps in the extraction procedure of the dried leaf powder is outlined in Fig. 2. It is absolutely necessary to remove the many other plant materials such as chlorophyll and lipids prior to submitting the sample to MHPLC. The solvent-partitioning procedure and preparative TLC on silica gel were used to clean up the extract. Gitoxin, digitoxin and an internal standard were located on preparative TLC in the zone of R_F 0.15–0.4, but in the subsequent step these were readily separated from the other components. The MHPLC of the extracted material was carried out under the same conditions as described above and the separation time was less than 1 h. Fig. 3 shows a chromatogram of the extract in the absence of an internal standard. Gitoxin and digitoxin were separated satisfactorily from the impurities of the extract. On the other hand, a typical chromatogram of the extract after addition of an internal standard is illustrated in Fig. 4. No peaks corresponding to the retention time of the internal standard are found in Fig. 3. Fig. 5 shows the calibration graphs of gitoxin and digitoxin using acetonitrile–methanol–water (15:15:19) as eluent. The ratios of gitoxin or digitoxin to peak heights of internal standard against the amount of each compound were linear over the range 0.01–0.15 mg.

The present method was then applied to the simultaneous quantitation of gitoxin and digitoxin, the principal digitalis glycosides in *D. purpurea*. The quantitative analysis of these cardiac glycosides indicated that the *D. purpurea* leaves contained 0.1453 mg of gitoxin and 0.0820 mg of digitoxin per 100 mg (dry weight) of the leaf powder with excellent reproducibility (Table I). The recovery test for cardiac glycosides applied to this procedure was then carried out. Known amounts of pure gitoxin and digitoxin added to four dry leaf-powder samples were recovered sufficiently,



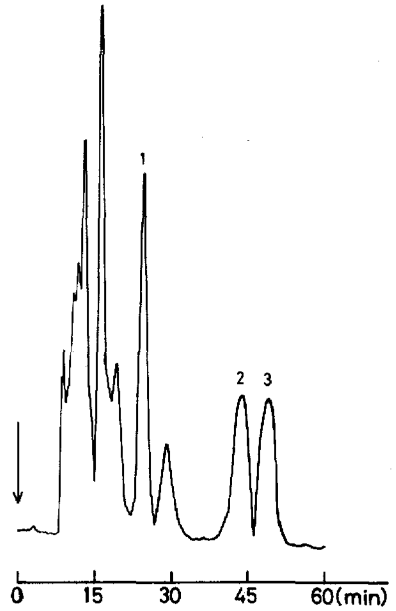


Fig. 3. Chromatogram of the extract of leaves of *D. purpurea* without an internal standard. Peaks: 1 = gitoxin; 2 = digitoxin. Conditions are the same as those in Fig. 1.

Fig. 4. Chromatogram of the extract of leaves of *D. purpurea* with an internal standard. Peaks: 1 = gitoxin; 2 = digitoxin; 3 = 14α,15α-epoxy-"β"-anhydrodigitoxin. Conditions are the same as those in Fig. 1.

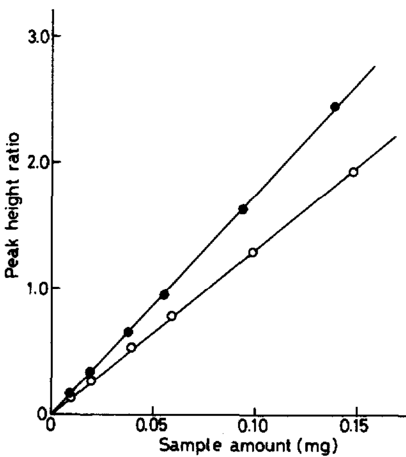


Fig. 5. Calibration graphs prepared by plotting the amount of gitoxin (●) and digitoxin (○) against the peak-height ratio of gitoxin or digitoxin/internal standard.

TABLE I

REPRODUCIBILITY OF THE PRESENT METHOD FOR QUANTITATION OF GITOXIN AND DIGITOXIN IN *DIGITALIS PURPUREA*

Numerals represent the amount of gitoxin or digitoxin per 100 mg of a dry leaf powder sample.

Glycoside	*Found (mg)*		*Mean ± S.D. (mg)*
Gitoxin	0.1352	0.1611	
	0.1430	0.1419	0.1453 ± 0.0111
Digitoxin	0.0812	0.0841	
	0.0790	0.0838	0.0820 ± 0.0024

average recoveries being 97.5% for gitoxin and 99.5% for digitoxin. Standard deviations of 1.2 and 3.8% for gitoxin and digitoxin, respectively, were found, indicating the quantitative usefulness of this technique. The results of this evaluation are compiled in Table II.

The data presented here demonstrate that the internally standardised MHPLC method for the assay of gitoxin and digitoxin in *D. purpurea* leaves appears to be better than previous methods, as these glycosides can be separated, characterized and determined simultaneously under mild conditions without any derivatization. Moreover, MHPLC can be a valuable method for quantitating these compounds and is more sensitive, convenient and inexpensive than ordinary HPLC. It is hoped that the proposed method might prove useful for the analysis of cardiac steroids in the tablets and ampoules used in medical practice.

In order to determine the total amount of the main cardiac glycosides in *D. purpurea* leaves, it is indispensable to determine primary glycosides such as purpurea glucoside A and B as well as digitoxin and gitoxin. Quantitation of these purpurea glucosides in *D. purpurea* by MHPLC is currently in progress.

TABLE II

RECOVERY TEST FOR GITOXIN AND DIGITOXIN ADDED TO *DIGITALIS PURPUREA*

Digitalis glycosides were determined after the addition of known amounts of pure gitoxin and digitoxin to the leaf-powder sample by the present method.

Weight of D. purpurea leaves (mg)	*Calculated after addition* (mg)*		*Found (mg)*		*Recovery rate (%)*	
	Gitoxin	*Digitoxin*	*Gitoxin*	*Digitoxin*	*Gitoxin*	*Digitoxin*
50.4	0.1093	0.0990	0.1051	0.1011	96.2	102.2
52.8	0.1128	0.1009	0.1102	0.0968	97.7	96.0
50.7	0.1098	0.0992	0.1067	0.1025	97.2	103.3
51.8	0.1114	0.1001	0.1103	0.0965	99.0	96.4
Mean					97.5	99.5
± S.D.					±1.2	±3.8

* Gitoxin (0.03611 mg) and digitoxin (0.05762 mg) were added to each sample of *D. purpurea* leaf powder.

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