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Note

Separation of digitalis glycosides by micro high-performance liquid chromatography

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The cardiac glycosides are therapeutically important substances which are widely used in the treatment of heart disease. Methods published so far for the determination of digitalis glycosides are based on paper, thin-layer and gas–liquid chromatography. High-performance liquid chromatography (HPLC) has been also employed[1–7] but it is not always satisfactory with respect to the determination of low concentrations of digitalis glycosides. In recent years, micro high-performance liquid chromatography (MHPLC) has been developed for measuring micro-scale amounts by Ishii and co-workers[8–12].

This paper reports the separation and quantitation of digitalis glycosides by MHPLC. The study was concentrated on the investigation of the chromatographic behaviour of nanogram amounts using a reversed-phase system and ultraviolet (UV) detection (220 nm) based on the α,β-unsaturated lactone ring. The MHPLC of digitoxin, gitoxin, digoxin, lanatoside A and lanatoside B was performed on a 5-μm ODS column, using acetonitrile–methanol–water (1:1:1) as the mobile phase. Various mixtures of methanol and water were used as the mobile phase for the separation of digitoxin and its metabolites, gitoxin and its metabolites and digoxin and its metabolites. Detection limits were as low as 1 ng for a 0.1-μl injection and the separation times varied between 30 and 45 min.

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 (0.5 mm I.D.) containing 5-μm porous silica particles covalently linked with ODS groups.

Materials

The chromatographic solvents were of analytical-reagent grade from Wako (Osaka, Japan). The cardiac glycosides were purchased from E. Merck (Darmstadt,

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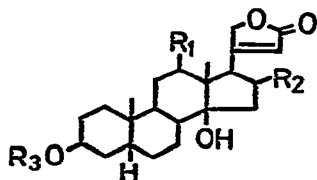
G.F.R.). The mono- and bisdigitoxosides of digitoxigenin, gitoxigenin and digoxigenin were prepared by hydrolysis of the secondary glycosides according to the methods of Kaiser and co-workers[13,14]. All of these materials were checked for homogeneity by thin-layer chromatography and their structures are given in Table I.

TABLE I

STRUCTURES OF THE DIGITALIS GLYCOSIDES AND AGLYCONES INVESTIGATED



Compound	R_1	R_2	R_3*
Digitoxigenin	H	H	H
Digitoxigenin monodigitoxoside	H	H	-D
Digitoxigenin bisdigitoxoside	H	H	-D-D
Digitoxin	H	H	-D-D-D
Gitoxigenin	H	OH	H
Gitoxigenin monodigitoxoside	H	OH	-D
Gitoxigenin bisdigitoxoside	H	OH	-D-D
Gitoxin	H	OH	-D-D-D
Digoxigenin	OH	H	H
Digoxigenin monodigitoxoside	OH	H	-D
Digoxigenin bisdigitoxoside	OH	H	-D-D
Digoxin	OH	H	-D-D-D
Lanatoside A	H	H	-D-D-AcD-G
Lanatoside B	H	OH	-D-D-AcD-G

* D = digitoxose; AcD = acetyldigitoxose; G = glucose.

Procedure

Solvents were prepared immediately before use. The chromatographic conditions for each separation are presented with the chromatograms. Separate solutions of each cardiac glycoside or aglycone were carefully prepared by weighing the compound and dissolving it in methanol. Chromatographic retention times of standards were determined, and compared with peaks with similar retention times in mixture of cardenolides. Calibration graphs were constructed using the average peak areas from three chromatograms.

RESULTS AND DISCUSSION

The chromatographic separation of representative cardiac glycosides was carried out under a variety of conditions. For the reversed-phase chromatography, the selection of a suitable eluent was studied by investigating combinations of the organic solvent and water; the UV absorption of the chromatographic solvents has an influence on the sensitivity of detection at 220 nm.

Initial work was directed towards the separation of digitoxin, gitoxin, digoxin,

lanatoside A and lanatoside B. As shown in Fig. 1, these substances were separated satisfactorily on an ODS bonded silica column (SC-01) when acetonitrile–methanol–water (1:1:1) was employed as the mobile phase at a flow-rate of 4 μl/min. The PTFE column (165 × 0.5 mm I.D.) used in this technique was much smaller than those used in ordinary HPLC. A detection wavelength of 220 nm was employed on the basis of the butenolide ring.

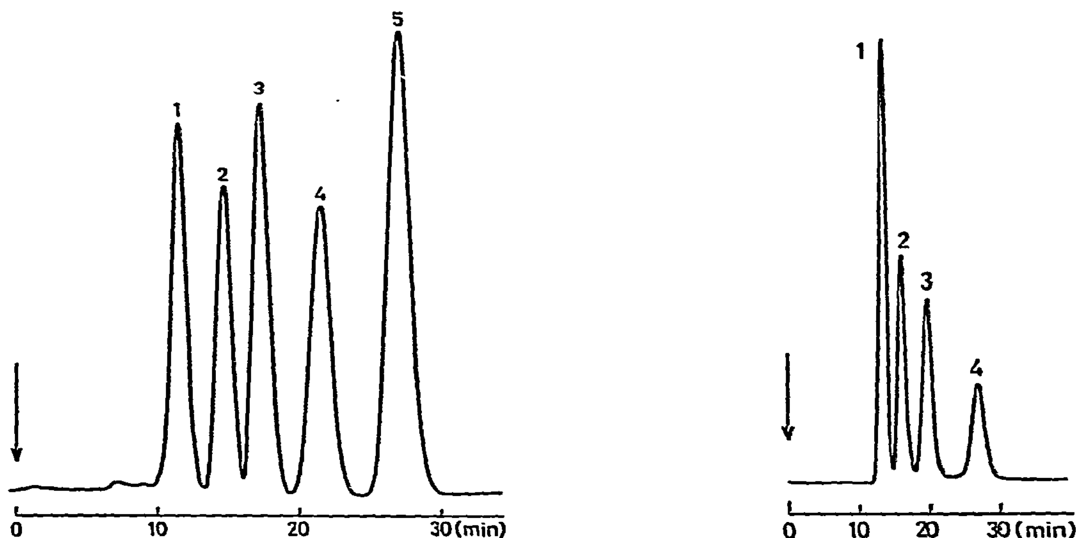


Fig. 1. Separation of a mixture of digitalis glycosides. Peaks: 1 = digoxin; 2 = lanatoside B; 3 = gitoxin; 4 = lanatoside A; 5 = digitoxin. Conditions: Jasco SC-01 column (165 × 0.5 mm I.D.); mobile phase, acetonitrile–methanol–water (1:1:1); flow-rate, 4 μl/min; UV monitor at 220 nm; sample volume, 0.1 μl.

Fig. 2. Separation of digitoxin and its metabolites. Peaks: 1 = digitoxigenin; 2 = digitoxigenin monodigitoxoside; 3 = digitoxigenin bisdigitoxoside; 4 = digitoxin. Conditions: Jasco SC-01 column (151 × 0.5 mm I.D.); mobile phase, methanol–water (5:2); flow-rate, 4 μl/min; UV monitor at 220 nm; sample volume, 0.1 μl.

Fig. 2 shows the separation of a mixture of digitoxin and its metabolites (digitoxigenin bisdigitoxoside, digitoxigenin monodigitoxoside and digitoxigenin) eluted with methanol–water (5:2). Similarly, gitoxigenin, its mono- and bisdigitoxosides and gitoxin were separated into four peaks when methanol–water (2:1) was employed, as illustrated in Fig. 3. Fig. 4 shows the results of the chromatography of a mixture of digoxin and its metabolites using methanol–water (6:5). The separations in all examples are sufficiently good and reproducible to permit quantitative work.

The calibration graphs in Fig. 5 were constructed by plotting the peak areas of digitoxin and gitoxin obtained by MHPLC using acetonitrile–methanol–water (1:1:1) as the mobile phase against amount of sample. Detection with a Uvidec-100 UV spectrophotometer (220 nm) showed a linear response to each cardiac glycoside in the range 5–25 ng. Similarly, Fig. 6 shows the calibration graphs for the MHPLC of digitoxigenin, its mono- and bisdigitoxosides and digitoxin using methanol–water (5:2) as the mobile phase. Peak areas for each compound were measured for various

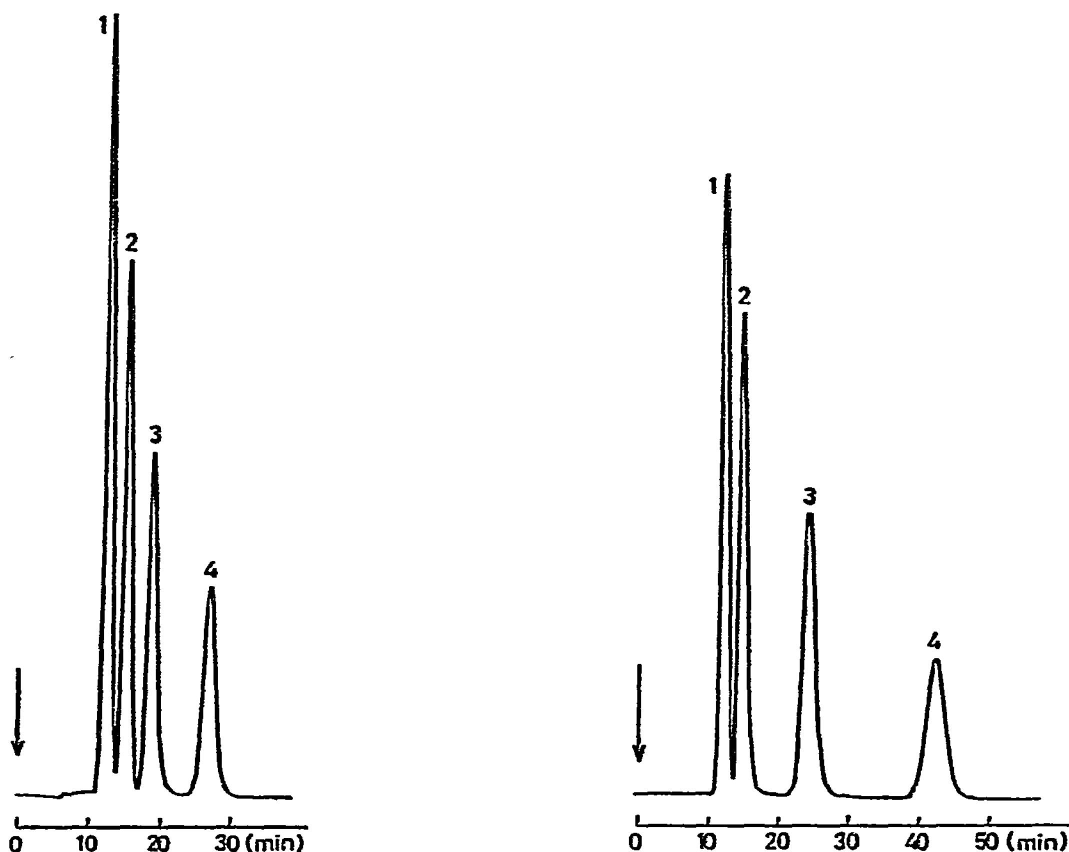
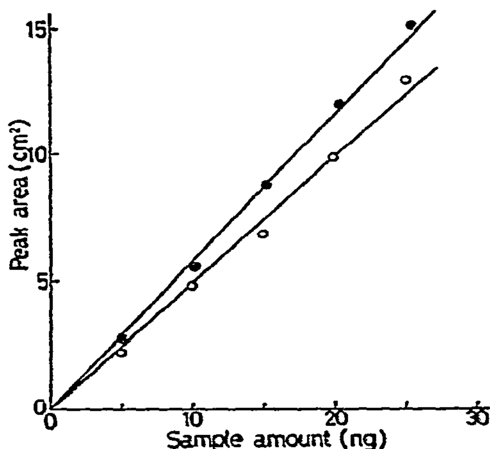


Fig. 3. Separation of gitoxin and its metabolites. Peaks: 1 = gitoxigenin; 2 = gitoxigenin monodigitoxoside; 3 = gitoxigenin bisdigitoxoside; 4 = gitoxin. Conditions: mobile phase, methanol–water (2:1); other conditions as in Fig. 2.

Fig. 4. Separation of digoxin and its metabolites. Peaks: 1 = digoxigenin; 2 = digoxigenin monodigitoxoside; 3 = digoxigenin bisdigitoxoside; 4 = digoxin. Conditions: mobile phase, methanol–water (6:5); other conditions as in Fig. 2.

amounts from 5 to 40 ng by UV detection (220 nm). The limit of accurate quantitative measurement of these steroids was about 5 ng and the detection limit at a signal-to-noise ratio of 3:1 was less than 1 ng.

The method presented appears to be better than previous methods, as MHPLC of digitalis glycosides is more sensitive, convenient and inexpensive than ordinary HPLC. On the basis of the separation efficiency and sensitivity of the proposed procedure, it seems feasible to determine digitalis glycosides and their metabolites in urine and other biological fluids. Moreover, this work suggests that MHPLC can be a valuable method for the direct coupling of liquid chromatography and mass spectrometry. Quantitation of major cardiac glycosides in *Digitalis purpurea* leaf by MHPLC is being conducted and the details will be reported elsewhere.



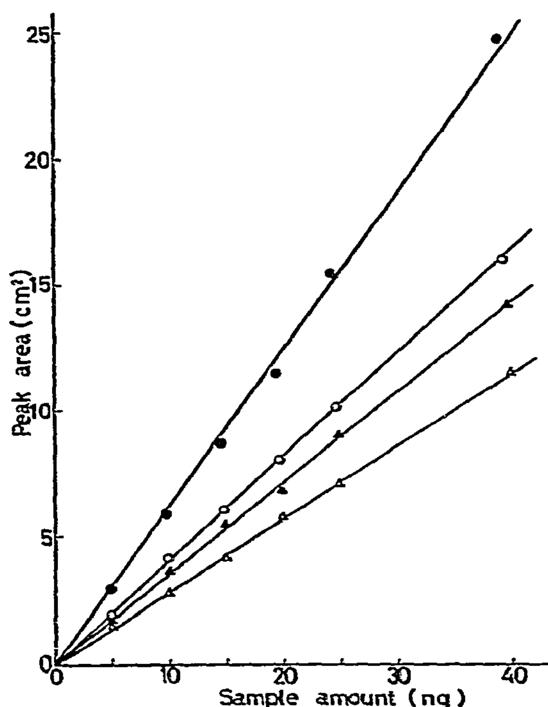


Fig. 5. Calibration graphs for digitoxin (●) and gitoxin (○).

Fig. 6. Calibration graphs for digitoxin and its metabolites. ●, Digitoxigenin; ○, digitoxigenin monodigitoxoside; ▲, digitoxigenin bisdigitoxoside; △, digitoxin.

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