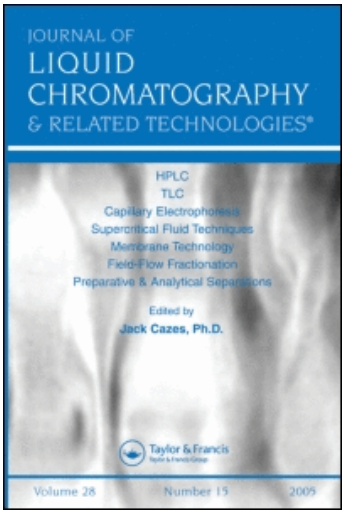


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Quantitative Determination of Digitalis Glycosides in Digitalis Purpurea Leaves by Reversed-Phase Thin-Layer Chromatography

Youichi Fujii[a]; Yukari Ikeda[a]; Mitsuru Yamazaki[a]
[a] School of Pharmacy Hokuriku University, Kanazawa, Japan

To cite this Article Fujii, Youichi , Ikeda, Yukari and Yamazaki, Mitsuru(1990) 'Quantitative Determination of Digitalis Glycosides in Digitalis Purpurea Leaves by Reversed-Phase Thin-Layer Chromatography', Journal of Liquid Chromatography & Related Technologies, 13: 10, 1909 — 1919
To link to this Article: DOI: 10.1080/01483919008049000
URL: http://dx.doi.org/10.1080/01483919008049000

JOURNAL OF LIQUID CHROMATOGRAPHY, 13(10), 1909-1919 (1990)

QUANTITATIVE DETERMINATION OF DIGITALIS GLYCOSIDES IN DIGITALIS PURPUREA LEAVES BY REVERSED-PHASE THIN-LAYER CHROMATOGRAPHY

YOUICHI FUJII, YUKARI IKEDA, AND MITSURU YAMAZAKI
School of Pharmacy
Hokuriku University
3 Ho, Kanagawa-machi
Kanazawa 920-11, Japan

ABSTRACT

A densitometric reversed-phase thin-layer chromatographic(RP-TLC) method for the determination of digitalis glycosides in *Digitalis purpurea* leaves has been developed. The procedure involves extraction of dry leaf powder with ethanol/chloroform (2 : 1) and clean-up by Sep-Pak cartridges prior to RP-TLC analysis. RP-TLC was performed on an octadecylsilyl bonded silica gel plate, using a developing solvent of acetonitrile/0.5 *M* NaCl(1 : 1) for secondary glycosides and acetonitrile/0.5 *M* NaCl(2 : 3)for primary glycosides. The plate was scanned with a reflectance densitometer at 222 nm. Quantitation of these glycosides was carried out by the internal standard method. The amounts of digitoxin, gitoxin, gitaloxin, purpurea glycoside A, purpurea glycoside B, and glucogitaloxin in a leaf powder sample of *Digitalis purpurea* were estimated from the calibration graphs for pure glycosides.

INTRODUCTION

The cardiac glycosides prepared from *Digitalis* plant have been widely used in the therapy of congestive heart failure. *Digitalis*

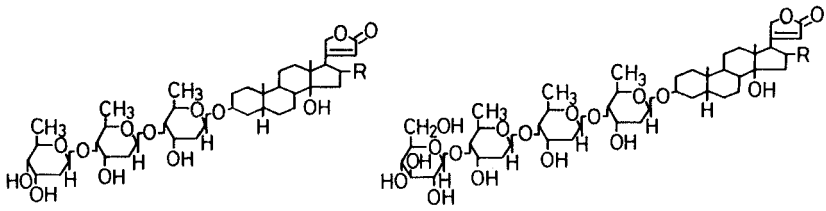
purpurea leaves are known to contain primary glycosides (purpurea glycoside A, purpurea glycoside B, and glucogitaloxin) as well as secondary glycosides (digitoxin, gitoxin, and gitaloxin). Because of these pharmacological importance, many studies of their separation and determination have been made. Although high-performance liquid chromatography has been shown to be an effective method for the quantitation of cardiac glycosides in *Digitalis* leaves (1 - 4), thin-layer chromatography(TLC) remains an invaluable aid for simple, inexpensive, and sensitive analysis. Previously published conventional TLC methods of determining glycosides in *Digitalis purpurea* have utilized measurement of the spot areas (5), *in situ* fluorimetric measurement on a plate (6 , 7), or colorimetric measurement of solutions obtained after spot elution (8). Evans *et al.*(9) have reported a densitometric TLC method for the estimation of A and B series glycosides as their respective aglycones. Recently, Hagiwara *et al.*(10) have proposed a technique for the content uniformity test of lanatoside C in tablets using high-performance TLC followed by densitometry. The usefulness of adapting a reversed-phase system to the TLC analysis of cardiac steroids has been described by Bloch (11). However, no author has reported the reversed-phase TLC(RP-TLC) method for the quantitation of cardiac glycosides in *Digitalis* leaves.

In this paper, we present the RP-TLC procedure for the quantitative determination of digitalis glycosides in *Digitalis purpurea* leaves. The method involves clean-up with Sep-Pak cartridges, TLC separation on ODS bonded silica plates, and subsequent densitometric scan with ultraviolet absorbance (222 nm) based on the butenolide ring.

MATERIALS AND METHODS

Materials

Gitoxin, lanatoside A, and lanatoside B were obtained from E. Merck (Darmstadt, F.R.G.), digitoxin from Wako (Osaka, Japan), gitaloxin from Boehringer Mannheim (Mannheim, F.R.G.), and Sep-Pak



Secondary glycosides
Digitoxin : R=H
Gitoxin : R=OH
Gitaloxin : R=OCHO

Primary glycosides
Purpurea glycoside A : R=H
Purpurea glycoside B : R=OH
Glucogitaloxin : R=OCHO

FIGURE 1. Structures of the cardiac glycosides investigated.

cartridges from Waters (Milford, MA, U.S.A.). Purpurea glycoside A and purpurea glycoside B were synthesized from lanatoside A and lanatoside B, respectively, according to the procedure of Pekić and Miljković(12) and recrystallized repeatedly from dichloromethane/methanol. Glucogitaloxin was prepared from purpurea glycoside B by the method of Haack *et al.* (13). 14α,15α-Epoxy-"β"-anhydrodigitoxin (internal standard I) and 14α,15α-epoxy-"β"-anhydropurpurea glycoside A (internal standard II) were also synthesized in four steps from digitoxin and purpurea glycoside A, respectively, by the method adapted from Sawlewicz *et al.* (14). The chemical structures of secondary glycosides and primary glycosides investigated are given in Figure 1. Standard solutions of the glycosides were prepared by dissolving each compound in ethanol/chloroform (2 : 1).

Sample preparation

Digitalis purpurea L. leaves in the second year were collected in the medicinal botanical garden (Kanazawa, Japan) of Hokuriku University on June 21. The half of harvested leaves were immediately freeze-dried in a Neocool Model DC-55A apparatus (Yamato Scientific, Tokyo, Japan) (Freeze-drying I). The other leaves were stored at

a low temperature for four months and then freeze-dried (Freeze-drying II). The leaf powder was prepared by a previously reported method (4).

Approximately 250 mg of dry leaf powder were accurately weighed and added to ethanol/chloroform (2 : 1) (25 ml) containing internal standards I (44.69 μg) and II (83.93 μg). After ultrasonication for 1 h in an ultrasonic cleaning bath, the extract was filtered and evaporated to dryness using a rotary evaporator. The resulting residue was dissolved in chloroform/acetic acid (100 : 0.1) and subjected to the Sep-Pak silica cartridge. Chloroform/acetic acid (100 : 0.1)(15 ml), chloroform/methanol/acetic acid (100 : 5 : 0.1)(20 ml), and chloroform/methanol/water/acetic acid (80 : 20 : 2.5 : 0.1)(15 ml) were successively passed through the cartridge. After evaporation of the second fraction, the residue was dissolved in methanol/water/acetic acid (10 : 10 : 0.02) and loaded on the Sep-Pak C_{18} cartridge. The cartridge was washed with methanol/water/acetic acid (10 : 10 : 0.02)(30 ml). Secondary glycosides and internal standard I were eluted with methanol/water/acetic acid (20 : 10 : 0.03)(20 ml) (Fr. 1). On the other hand, the third fraction on the silica cartridge was also applied to the C_{18} cartridge. After washing with methanol/water/acetic acid(10 : 10 : 0.02)(20 ml), primary glycosides and internal standard II were eluted with methanol/water/acetic acid (20 : 10 : 0.03)(10 ml)(Fr. 2). Fr. 1 and Fr. 2 were concentrated *in vacuo* and analyzed by RP-TLC. A scheme illustrating the various steps in extraction and clean-up procedures of the leaf powder is outlined in Figure 2.

RP-TLC determination

RP-TLC was performed on 5 x 20 cm Whatman KC_{18} plates (Clifton, NJ, U.S.A.) with a thickness of 0.2 mm. Two mobile phases were used in this study, acetonitrile/0.5 *M* NaCl (1 : 1) for determination of secondary glycosides and acetonitrile/0.5 *M* NaCl (2 : 3) for primary glycosides. Aliquots (4 μl) of the solutions were spotted with Drummond Microcap micropipets on RP-TLC plates. The plates were developed in glass chambers that were lined with filter paper

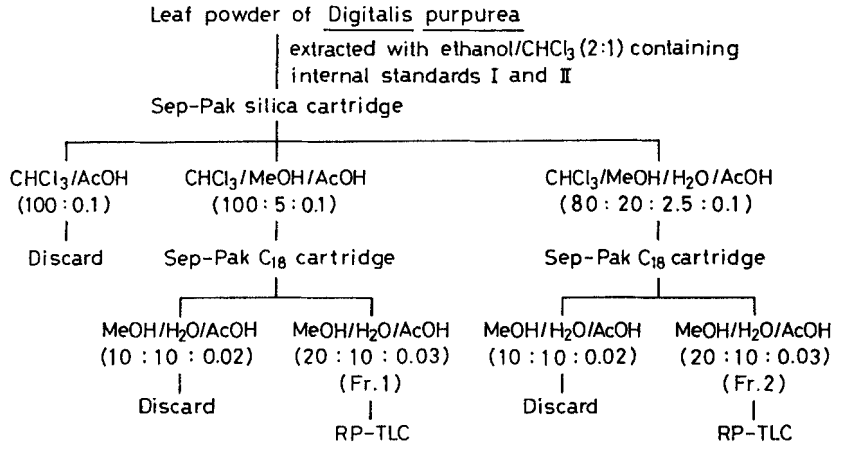


FIGURE 2. Schematic diagram of the sample preparation for the RP-TLC analyses of digitalis glycosides in *Digitalis purpurea* leaves. Fr.1 = secondary glycoside fraction; Fr.2 = primary glycoside fraction. MeOH = methanol; AcOH = acetic acid.

and preequilibrated with mobile phase for 10 min. The development time was about 70 min and the migration distance was 17 cm. After air drying, the plates were scanned with a Shimadzu Model CS-920 high-speed zig-zag TLC scanner(Kyoto, Japan) connected to a Shimadzu Model U-135 recorder. This TLC scanner has the curve linearizer programed according to the Kubelka-Munk equation. All measurements were made in the reflectance mode, scanning range *X* 12 mm and *Y* 180 mm, and wavelength 222 nm. Each sample was usually determined in triplicate on the plate, and the result was the mean of three determinations. Peak areas printed out by the TLC scanner were used for each analysis. Calibration graphs were constructed by plotting the peak area ratios of each secondary glycoside to internal standard I and of each primary glycoside to internal standard II against the amount of each compound.

RESULTS AND DISCUSSION

The RP-TLC separation of cardiac glycosides and the selection of internal standards were carried out. After investigating many substances, 14α,15α-epoxy-"β"-anhydro-digitoxin (internal standard I) and 14α,15α-epoxy-"β"-anhydro-purpurea glycoside A (internal standard II) were found to be the most suitable for the determinations of secondary glycosides and of primary glycosides, respectively. RP-TLC was performed on C_{18} silica plates using the binary solvent mixture of acetonitrile and 0.5 *M* NaCl as the mobile phase. Digitoxin, gitoxin, gitaloxin, and internal standard I were separated into four spots when acetonitrile/0.5 *M* NaCl (1 : 1) was employed. On the other hand, the separation of purpurea glycoside A, purpurea glycoside B, glucogitaloxin, and internal standard II was achieved by using acetonitrile/0.5 *M* NaCl (2 : 3). The chromatograms and R_f values of these compounds are shown in Figure 3. All of the R_f values were within the optimum range of 0.3 to 0.7 for accurate and precise determination.

The separation of digitalis glycosides in *Digitalis purpurea* leaves was then undertaken. Quantitation of these glycosides was performed by incorporation of two internal standards in a leaf powder sample. The dried leaf powder was extracted with ethanol/chloroform (2 : 1) by ultrasonication. For the purpose of removing many coexisting materials in the extract and dividing the glycosides into secondary and primary glycosides, the extract was submitted to a Sep-Pak cartridge packed with silica gel. Furthermore, Sep-Pak C_{18} cartridges were used in a clean-up step prior to RP-TLC. The purified secondary glycoside fraction (Fr.1) and primary glycoside fraction (Fr.2) were subjected individually to RP-TLC using the developing solvents as described above. Figure 3 shows the representative RP-TLC patterns of Fr.1 and Fr.2, compared with pure glycoside mixtures. The R_f values and colors of spots by visualization using concentrated sulfuric acid spray agree well with those of the authentic samples. The separations are of sufficient quality and reproducibility to permit quantitative work.

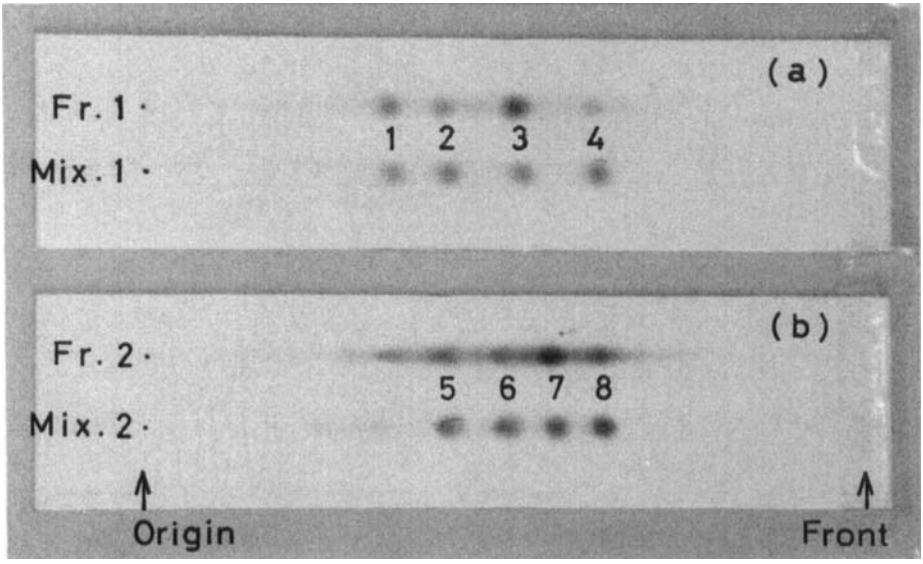


FIGURE 3. RP-TLC separations of digitalis glycosides in *Digitalis purpurea* leaves and mixtures of authentic glycosides on Whatman KC_{18} plates. Fr. 1 and Fr. 2 refer to Figure 2. Mix. 1 = a mixture of secondary glycosides and an internal standard I; Mix. 2 = a mixture of primary glycosides and an internal standard II. Developing solvents : plate (a), acetonitrile/0.5 *M* NaCl (1 : 1); plate (b), acetonitrile/0.5 *M* NaCl (2 : 3). Visualization : spraying with concentrated sulfuric acid followed by heating in an oven at 120 °C for 10 min. Spot identification : 1 = 14α, 15α-epoxy-"β"-anhydro-digitoxin (R_f 0.34); 2 = digitoxin (R_f 0.41); 3 = gitaloxin (R_f 0.51); 4 = gitoxin (R_f 0.62); 5 = 14α,15α-epoxy-"β"-anhydro-purpurea glycoside A (R_f 0.41); 6 = purpurea glycoside A (R_f 0.49); 7 = glucogitaloxin (R_f 0.56); 8 = purpurea glycoside B (R_f 0.63).

The densitometric measurement was achieved by a Shimadzu zig-zag TLC scanner without treating the spot with any reagents. A detection wavelength of 222 nm was employed on the basis of the α,β-unsaturated lactone ring attached at the C-17 position of the steroid nucleus. This maximum wavelength was determined from the absorption spectrum by scanning the spot on the plate. Figure 4 depicts

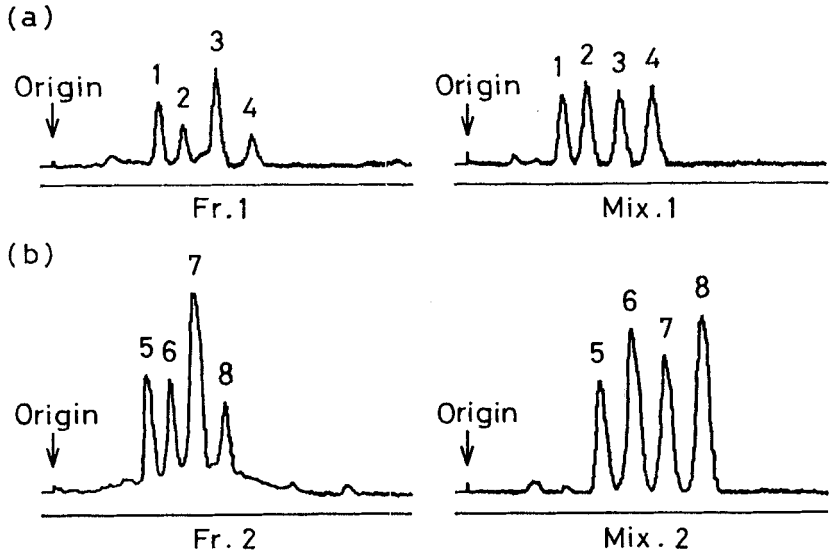


FIGURE 4. Densitometric profiles of digitalis glycosides on Whatman KC_{18} TLC plates using a Shimadzu high-speed zig-zag TLC scanner CS-920. Measuring mode : reflection-absorption photometry at 222 nm; scanning range : *X* 12 mm, *Y* 180 mm; chart speed : 24 mm/min. Peak numbers, samples, and developing solvents are the same as those in Figure 3.

the densitometric profiles obtained from the RP-TLC separations. The peak area for each spot was determined by the integrated absorbance value from the TLC scanner. Linear calibration graphs were constructed by plotting the peak area ratios of each glycoside to internal standards I or II against the amount of each glycoside. The regression equations, ranges of linearities, and correlation coefficients are compiled in Table 1.

The present method was then applied to the quantitation of secondary and primary glycosides in *Digitalis purpurea*. The cut leaves from the living plant were promptly freeze-dried (Freeze-drying I) or freeze-dried after storage for four months (Freeze-drying II). The amounts of digitoxin, gitoxin, gitaloxin, purpurea

TABLE 1

Regression Equations, Ranges of Linearities, and Correlation Coefficients of the Calibration Graphs for Digitalis Glycosides

Glycoside	Equation	Range (μg)	Correlation coefficient
Digitoxin	$Y_1 = 0.0251\,X + 0.208$	20 - 100	0.993
Gitoxin	$Y_1 = 0.0323\,X - 0.137$	10 - 50	0.992
Gitaloxin	$Y_1 = 0.0189\,X + 0.289$	40 - 140	0.982
Purpurea glycoside A	$Y_2 = 0.0160\,X + 0.011$	10 - 90	0.991
Purpurea glycoside B	$Y_2 = 0.0174\,X + 0.015$	10 - 90	0.991
Glucogitaloxin	$Y_2 = 0.0142\,X - 0.052$	50 - 400	0.993

Y_1: peak area ratios of each secondary glycoside to internal standard I; Y_2: peak area ratios of each primary glycoside to internal standard II; X: amount (μg) of each glycoside (number of points = 7).

TABLE 2

Contents* of the Digitalis Glycosides in *Digitalis purpurea* Leaves by the Present Method

Glycoside	Freeze-drying I**		Freeze-drying II***	
	Mean ± S.D.	(n)	Mean ± S.D.	(n)
Digitoxin	14.5 ± 1.3	(5)	25.6 ± 0.84	(5)
Gitoxin	4.3 ± 0.22	(5)	7.1 ± 1.6	(5)
Gitaloxin	25.9 ± 2.4	(5)	46.3 ± 6.3	(5)
Purpurea glycoside A	25.6 ± 0.63	(10)	18.4 ± 1.1	(7)
Purpurea glycoside B	18.2 ± 0.82	(10)	13.8 ± 2.3	(7)
Glucogitaloxin	64.0 ± 3.6	(10)	48.0 ± 2.3	(7)

S.D. : standard deviation; n : number of samples.
*Numerals represent the amount (μg) of cardiac glycosides per 100 mg of a dry leaf powder sample.
**The leaves were immediately freeze-dried after collection.
***The leaves were stored for four months and then freeze-dried.

glycoside A, purpurea glycoside B, and glucogitaloxin per 100 mg of the dry leaf powder are given in Table 2. The percentages of each primary glycoside on Freeze-drying II to that on Freeze-drying I were 72 % for purpurea glycoside A, 76 % for purpurea glycoside B, and 75 % for glucogitaloxin. On the other hand, the contents of secondary glycosides on Freeze-drying II in comparison with those on Freeze-drying I increased. From these data, it was suggested

that part of primary glycosides were enzymatically converted into the corresponding secondary glycosides during the storage of *Digitalis* leaves.

In conclusion, the RP-TLC determination of secondary and primary glycosides in *Digitalis purpurea* leaves was achieved by using the pretreatment with Sep-Pak cartridges and the two internal standards. Gitaloxin and glucogitaloxin readily lose the formyl group at position C-16 giving rise to gitoxin and purpurea glycoside B, respectively, especially under alkaline conditions. For the purpose of preventing deformylation of gitaloxin and glucogitaloxin, a very small amount of acetic acid was added to the eluent for the Sep-Pak cartridges. The present method provides a significant advantage in terms of selectivity, sensitivity, and convenience. It is hoped that this method can be adopted for estimation of the quality of *Digitalis purpurea* leaves.

ACKNOWLEDGEMENT

The authors thank the staff of the medicinal botanical garden of Hokuriku University for providing the leaves of *Digitalis purpurea*.

REFERENCES

1. Cobb, P.H., Application of High-performance Liquid Chromatography to the Separation of Cardenolides and the Assay of Digoxin in *Digitalis lanata* Leaf, Analyst (London), 101, 768, 1976.
2. Wichtl, M., Wichtl-Bleier, W., and Mangkudidjojo, M., Hochleistungs-Flüssigkeits-Chromatographische Analyse von Digitalis-Blattextrakten. II. Quantitative Analyse, J. Chromatogr., 247, 359, 1982.
3. Orosz, F., Nuridsány, M., and Ovádi, J., Isolation and Quantitative Determination of Some Cardioactive Glycosides from *Digitalis lanata* by High-performance Liquid Chromatography, Anal. Biochem., 156, 171, 1986.
4. Fujii, Y., Ikeda, Y., and Yamazaki, M., Separation and Determination of Purpurea Glycosides in *Digitalis purpurea* Leaves by Micro-HPLC, J. High Resolut. Chromatogr. Chromatogr. Commun., 10, 137, 1987.

5. Elkiey, M.A., Ahmed, Z.F., Abdelkader, E.M., and Abdelwahab, S. M., Application of Thin Layer Chromatography to the Separation, Identification, and Estimation of the Glycosides of Digitalis Species Cultivated in Egypt, J. Pharm. Sci. U.A.R., 5, 139, 1964.

6. Lugt, Ch.B., Quantitative Determination of Digitoxin, Gitaloxin, Gitoxin, Verodoxin, and Strospesid in the Leaves of *Digitalis purpurea* by Means of Fluorescence, Planta Med., 23, 176, 1973.

7. Lugt, Ch.B. and Noordhoek-Ananias, L., Quantitative Fluorimetric Determination of the Main Cardiac Glycosides in *Digitalis purpurea* Leaves, Planta Med., 25, 267, 1974.

8. Khafagy, S.M. and Girgis, A.N., A Micro Method for the Estimation of the Individual Glycosides in Digitalis Leaves and Pharmaceutical Preparations, Planta Med., 25, 350, 1974.

9. Evans, F.J., Flemons, P.A., Duignan, C.F., and Cowley, P.S., A New Thin-layer Densitometric Technique for the Assay of Cardenolides from *Digitalis purpurea*, J. Chromatogr., 88, 341, 1974.

10. Hagiwara, T., Shigeoka, S., Uehara, S., Miyatake, N., and Akiyama, K., High Performance Thin Layer Chromatography-Densitometric Method for Content Uniformity Test of Lanatoside C Tablets, J. High Resolut. Chromatogr. Chromatogr. Commun., 7, 161, 1984.

11. Bloch, D.E., Reversed Phase Thin Layer Chromatographic Procedure for Identification of Digoxin and Related Fluorescing Substances, J. Assoc. Off. Anal. Chem., 63, 707, 1980.

12. Pekić, B. and Miljković, D., A New Method of Preparation of Deacetyl-lanatosides, Planta Med., 27, 178, 1975.

13. Haack, E., Gube, M., Kaiser, F., and Spingler, H., Über die Isolierung von Glucogitaloxin aus den Blättern von *Digitalis purpurea*, Chem. Ber., 91, 1758, 1958.

14. Sawlewicz, L., Linde, H.H.A., and Meyer, K., Epoxide von Cardenoliden und Cardenolidglykosiden Partialsynthetische Versuche in der Reihe der Herzgifte, Helv. Chim. Acta, 51, 1353, 1968.