ELSEVIER

Journal of Chromatography A, 746 (1996) 255–260

JOURNAL OF CHROMATOGRAPHY A

Quantitative determination of cardiac glycosides in *Digitalis lanata* leaves by reversed-phase thin-layer chromatography

Yukari Ikeda*, Youichi Fujii, Megumi Umemura, Tadahiko Hatakeyama, Mayumi Morita, Mitsuru Yamazaki

Faculty of Pharmaceutical Sciences, Hokuriku University, Ho-3, Kanagawa-machi, Kanazawa 920-11, Japan

Received 13 February 1996; revised 10 April 1996; accepted 11 April 1996

Abstract

An analytical method for the determination of cardiac glycosides in *Digitalis lanata* leaves by reversed-phase thin-layer chromatography (RP-TLC) was developed. The procedure consisted of extraction of dry leaf powder with 50% methanol 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–methanol–0.5 *M* NaCl (1:1:1) for primary glycosides and acetonitrile–methanol–0.5 *M* NaCl (12:7:9) for secondary glycosides. The plate was scanned with a reflectance densitometer at 225 nm. The quantitation was carried out by the internal standard method. The present method is reliable and relatively simple for the determination of cardiac glycosides in *Digitalis lanata* leaves.

Keywords: *Digitalis lanata*; Glycosides

1. Introduction

The cardiac glycosides isolated from *Digitalis* leaves are therapeutically important substances that are available for the treatment of congestive heart failure and atrial fibrillation. *D. lanata* is known to contain primary glycosides such as lanatoside A, lanatoside B and lanatoside C. These primary glycosides are transformed into their corresponding secondary glycosides by the plant's own enzyme, digilanidase, present in the leaves [1]. Accordingly, it is extremely important to evaluate the amounts of both primary and secondary glycosides in *D. lanata* leaves. High-performance liquid chromatography has been shown to be an effective method for the quantitation of cardiac glycosides in *D. lanata* leaves [2–6]. However, TLC seems to offer a convenient mode for simple and inexpensive analysis. Hagiwara et al. [7] and Ponder and Steward [8] have used high-performance TLC for the content uniformity test of lanatoside C in tablets and for the assay of digoxin tablets, respectively. A number of TLC procedures have been shown to be useful for the quantitation of cardiac glycosides in *D. lanata* [9–13]. Although the separation of various mixtures of pure glycosides has been accomplished by employing a RP-TLC [8,14], no author has reported the RP-TLC method for *D. lanata* leaves.

We previously reported the usefulness of adapting a reversed-phase system to the TLC analysis of cardiac glycosides in *D. purpurea* leaves [15]. This paper describes the quantitative determination of lanatoside A, lanatoside B, lanatoside C, desac-

*Corresponding author.

0021-9673/96/$15.00
PII S0021-9673(96)00340-8

etyllanatoside C, α-acetyldigitoxin and α-acetyldigoxin in *D. lanata* by using RP-TLC. The method involves clean-up with Sep-Pak cartridges, TLC separation on octadecylsilyl bonded silica gel plates, and subsequent densitometric scan with UV absorption (225 nm).

2. Experimental

2.1. Instrumentation

The apparatus was a Shimadzu Model CS-920 high-speed zig-zag TLC scanner (Kyoto, Japan) connected to a Schimadzu Model U-135 recorder. This TLC scanner has the curve linearizer programmed according to the Kubelka–Munk equation. The measuring modes were scanning range *X* 15 mm and *Y* 180 mm, chart speed 20 mm/min and reflection–absorption photometry at 225 nm.

2.2. Reagents

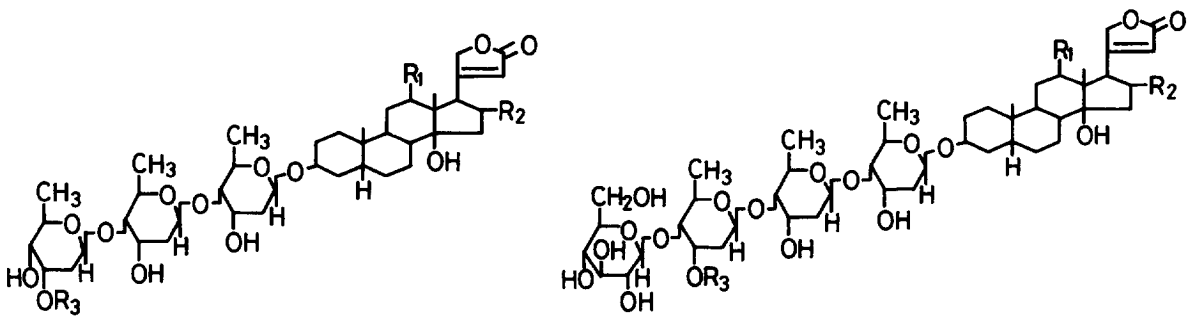
Gitoxin, lanatoside A, lanatoside B and lanatoside C were obtained from E. Merck (Darmstadt, Germany), digitoxin from Wako (Osaka, Japan), digoxin from Aldrich (Milwaukee, WI, USA), α-acetyldigoxin from Boehringer Mannheim (Mannheim, Germany) and desacetyllanatoside C from the National Institute of Hygienic Sciences (Tokyo, Japan). Desacetyllanatoside A and desacetyllanatoside B were synthesized from lanatoside A and lanatoside B, respectively, according to the procedure of Pekić and Miljković [16]. α-Acetyl-digitoxin and α-acetylgitoxin were prepared from digitoxin and gitoxin, respectively, using acetic acid and carbodiimide by the method adapted from Drašar et al. [17] and recrystallized according to the procedure of Stoll et al. [18,19]. β-Anhydrodigitoxin (internal standard I) and β-anhydrodesacetyl-lanatoside A (internal standard II) were synthesized in three steps from digitoxin and desacetyllanatoside A, respectively, by the method of Sawlewicz et al. [20]. The chemical structures of primary and secondary glycosides are given in Fig. 1. All solvents used were purified by redistillation prior to use.

2.3. Preparation of the leaf powder

D. lanata leaves were collected during the flowering stage in June at the Medicinal Plant Garden of Hokuriku University. The fresh leaves were immediately freeze-dried in a Neocool Model DC-55A apparatus (Yamato Scientific, Tokyo, Japan) and then dried using phosphorus pentoxide 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 phosphorus pentoxide under reduced pressure for 5 days.

2.4. Extraction and clean-up procedures

Approximately 250 mg of leaf powder was accurately weighed and added to 50% methanol (25 ml) containing internal standards I (44.4 μg) and II (255 μg). After ultrasonication for 1.5 h in an ultrasonic cleaning bath, the extract was filtered and evaporated to dryness using a rotary evaporator. The resulting residue was dissolved in 3 ml of methanol and then 50 ml of ethyl acetate was added. After filtration of the suspension, the filtrate was evaporated in vacuo. The resulting residue was dissolved in 40 μl of methanol and 40 μl of water, and then 2 ml of chloroform–acetone–acetic acid (70:30:0.05) was added. The solution was applied to the Sep-Pak silica cartridge. Then 30 ml of chloroform–acetone–acetic acid (70:30:0.05) and 40 ml of chloroform–methanol–water–acetic acid (90:10:0.8:0.05) were successively passed through the cartridge. The first fraction (30 ml) was collected and evaporated to dryness using a rotary evaporator. The residue obtained was dissolved in 2 ml of methanol–water–acetic acid (38:62:0.2) and loaded on the Sep-Pak C_{18} cartridge. After washing with 50 ml of methanol–water–acetic acid (38:62:0.2), secondary glycosides and an internal standard I were eluted with 30 ml of methanol–water–acetic acid (70:30:0.2) (Fr. 1). The second fraction (40 ml) on the Sep-Pak silica cartridge was evaporated in vacuo, dissolved in 2 ml of methanol–water–acetic acid (37:63:0.2) and subjected to the Sep-Pak C_{18} cartridge. The cartridge was washed with 50 ml of methanol–water–acetic acid (37:63:0.2), and primary glycosides and an internal standard II were eluted with 30 ml of methanol–



Secondary Glycoside

α-Acetyldigitoxin : R_1=R_2=H, R_3=$COCH_3$

α-Acetylgitoxin : R_1=H, R_2=OH, R_3=$COCH_3$

α-Acetyldigoxin : R_1=OH, R_2=H, R_3=$COCH_3$

Digitoxin : R_1=R_2=R_3=H

Gitoxin : R_1=R_3=H, R_2=OH

Digoxin : R_1=OH, R_2=R_3=H

Primary Glycoside

Lanatoside A : R_1=R_2=H, R_3=$COCH_3$

Lanatoside B : R_1=H, R_2=OH, R_3=$COCH_3$

Lanatoside C : R_1=OH, R_2=H, R_3=$COCH_3$

Desacetyllanatoside A : R_1=R_2=R_3=H

Desacatyllanatoside B : R_1=R_3=H, R_2=OH

Desacetyllanatoside C : R_1=OH, R_2=R_3=H

Fig. 1. Chemical structures of the cardiac glycosides.

water–acetic acid (68:32:0.2) (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 Fig. 2.

2.5. RP-TLC determination

RP-TLC was performed on 5×20 cm Whatman KC_{18} plates (Clifton, NJ, USA) with a thickness of 0.2 mm. Two mobile phases were used in this study, acetonitrile–methanol–0.5 *M* NaCl (12:7:9) for determination of secondary glycosides and acetonitrile–methanol–0.5 *M* NaCl (1:1:1) for primary glycosides. Portions (4 μl) of the sample solutions were spotted with Drummond Microcap micropipets on RP-TLC plates. The plates were developed in glass chambers that were pre-equilibrated with mobile phase for 10 min. After development and air drying, the plates were scanned by the TLC scanner. Each sample was usually determined in triplicate on the plate, and the result was the mean of three determinations. 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.

3. Results and discussion

An initial study was focused on the selection of internal standards and the chromatographic separation of cardiac glycosides. Among the many compounds investigated, β-anhydrodigitoxin (internal standard I) and β-anhydrodesacetyllanatoside A (internal standard II) was found to be the most suitable as internal standards for the determinations of secondary glycosides and primary glycosides, respectively. RP-TLC was performed on an octadecylsilyl bonded silica gel plate using the solvent mixture of acetonitrile, methanol and 0.5 *M* NaCl as the mobile phase. Digitoxin, gitoxin, digoxin, α-acetyldigitoxin, α-acetylgitoxin, α-acetyldigoxin and internal standard were separated when acetonitrile–methanol–0.5 *M* NaCl (12:7:9) was employed. On

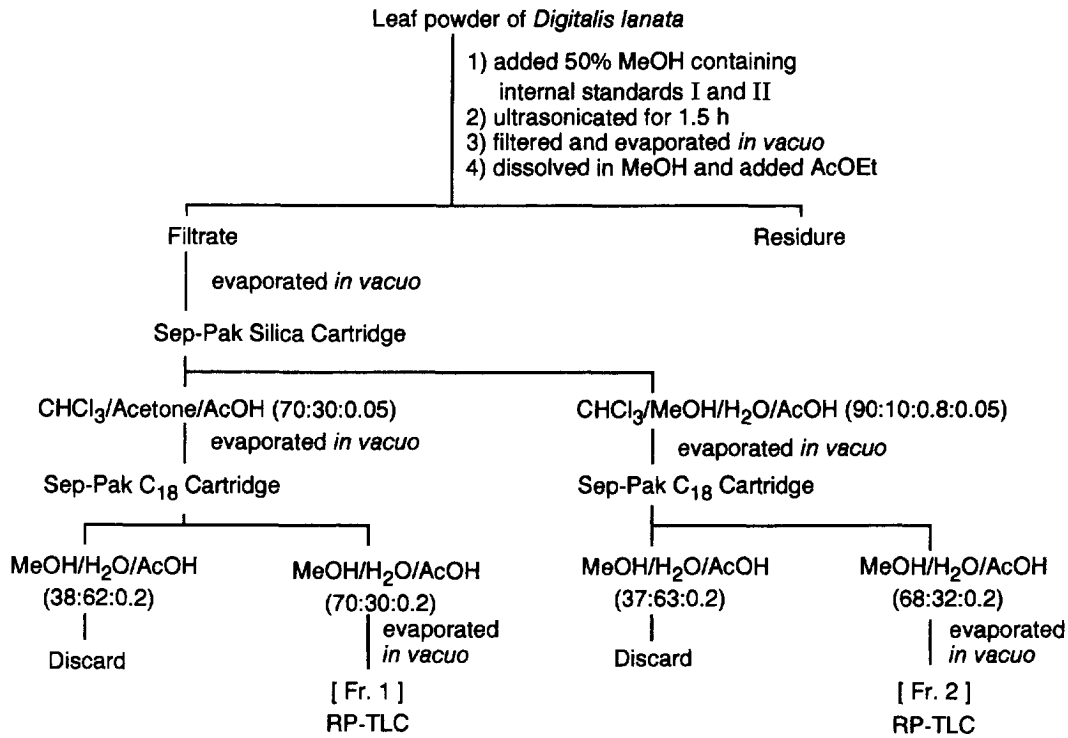


Fig. 2. Sample preparation procedure for the RP-TLC determination of cardiac glycosides in *Digitalis lanata* leaves. Fr. 1=secondary glycoside fraction; Fr. 2=primary glycoside fraction. MeOH=methanol; AcOEt=ethyl acetate; AcOH=acetic acid.

the other hand, the separation of lanatoside A, lanatoside B, lanatoside C, desacetyllanatoside A, desacetyllanatoside B, desacetyllanatoside C and internal standard II was achieved by using acetonitrile–methanol–0.5 *M* NaCl (1:1:1). The chromatograms and R_F values of these compounds are shown in Fig. 3.

The separation of cardiac glycosides of *D. lanata* leaves was then undertaken. The dry leaf powder was extracted by ultrasonication with 50% methanol containing the internal standards. For the purpose of removing many other plant 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, it was necessary to introduce a purification step using a Sep-Pak C_{18} cartridge prior to submitting the sample 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. Fig. 3 shows the representative RP-TLC patterns of Fr. 1 and Fr. 2, compared with pure glycoside mixtures. The R_F values and colours of spots by visualization using concentrated sulphuric acid spray, agree well with those of the authentic samples. The separation is of sufficient quality and reproducibility to permit quantitative assessment.

The densitometric measurement was achieved by a Shimadzu zig-zag TLC scanner without treating the spot with any reagents. A detection wavelength of 225 nm was chosen on the basis of the butenolide 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. The peak area for each spot was determined by the integrated absorbance value from the TLC scanner. Fig. 4 depicts the densitometric profiles obtained from the RP-TLC separations.

From RP-TLC of the extract in the absence of

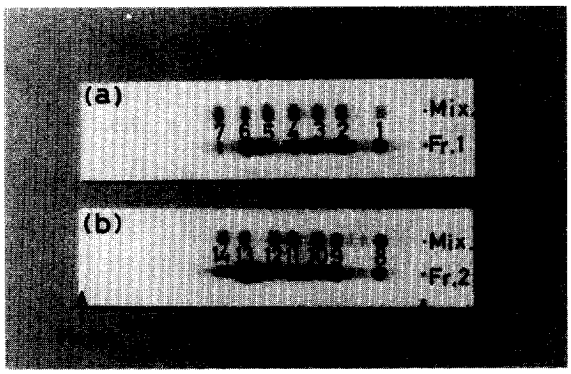


Fig. 3. RP-TLC separations of cardiac glycosides in *Digitalis lanata* leaves and mixtures of authentic samples on Whatman KC_{18} plates. Fr. 1=secondary glycoside fraction; Fr. 2=primary glycoside fraction; Mix. 1=a mixture of secondary glycosides and an internal standard I; Mix. 2=a mixture of primary glycosides and internal standard II. Developing solvents: plate (a) acetonitrile–methanol–0.5 *M* NaCl (12:7:9); plate (b) acetonitrile–methanol–0.5 *M* NaCl (1:1:1). Visualization: spraying with concentrated sulphuric acid followed by heating in an oven at 120°C for 10 min. Spot identification: 1=β-anhydrodigitoxin (R_F 0.13); 2=α-acetyldigitoxin (R_F 0.17); 3=digitoxin (R_F 0.31); 4=α-acetyl-gitoxin (R_F 0.38); 5=gitoxin (RF 0.46); 6=α-acetyldigoxin (R_F 0.52); 7=digoxin (R_F 0.60); 8=β-anhydrodesacetyllanatoside A (R_F 0.13); 9=lanatoside A (R_F 0.26); 10=desacetyllanatoside A (R_F 0.31); 11=lanatoside B (R_F 0.38); 12=desacetyllanatoside B (R_F 0.44); 13=lanatoside C (R_F 0.52); 14=desacetyllanatoside C (R_F 0.59).

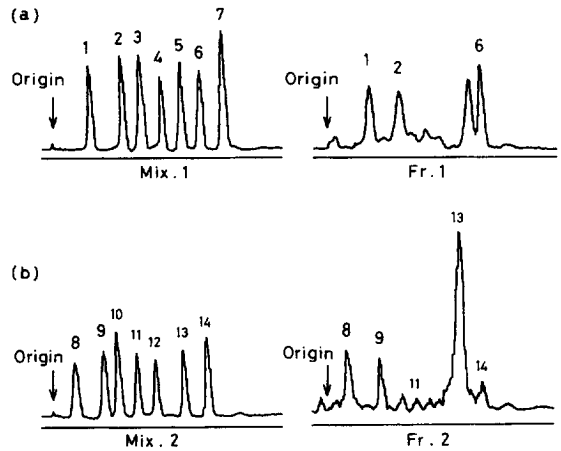


Fig. 4. Densitometric profiles of cardiac glycosides on Whatman KC_{18} TLC plates using a Shimadzu high-speed zig-zag TLC scanner CS-920. Measuring mode: reflection–absorption photometry at 225 nm; scanning range: *X* 15 mm, *Y* 180 mm; chart speed: 20 mm/min. Peak numbers, samples, and developing solvents are the same as those in Fig. 3.

internal standards, the other substances present in the leaves were ascertained not to interfere with the peaks due to internal standards I and II. 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 detection limits of lanatoside A, lanatoside B, lanatoside C, desacetyllanatoside C, α-acetyl-digitoxin and α-acetyldigoxin at a signal-to-noise ratio of 3:1 were approximately 0.5 μg.

The quantitation of primary and secondary glycosides in *D. lanata* was carried out by the present method. The assay results obtained from seven dry leaf powder samples are given in Table 2. The quantitation of desacetyllanatoside A, desacetyllanatoside B, digitoxin, α-acetylgitoxin, gitoxin and digoxin was not undertaken, because their amounts in the leaf powder examined were found to be below the limit of the determination used.

In conclusion, the RP-TLC determination of lanatoside A, lanatoside B, lanatoside C, desacetyl-

Table 1
Regression equations, ranges of linearities, and correlation coefficients of the calibration graphs for cardiac glycosides

Glycoside	Equation	Range (μg)	Correlation coefficient (r^2)
Lanatoside A	$y_1=0.00596x-0.011$	45– 400	0.995
Lanatoside B	$y_1=0.00951x-0.089$	15– 100	0.995
Lanatoside C	$y_1=0.00358x+0.528$	250–2000	0.992
Desacetyllanatoside C	$y_1=0.00631x-0.011$	25– 300	0.996
α-Acetyldigitoxin	$y_2=0.0286x-0.128$	10– 150	0.999
α-Acetyldigoxin	$y_2=0.0350x-0.287$	10– 150	0.991

y_1: peak-area ratios of each primary glycoside to internal standard II; y_2: peak-area ratios of each secondary glycoside to internal standard I; x: amount (μg) of each glycoside (number of points=5).

Table 2
Content of cardiac glycosides in *Digitalis lanata* leaves determined by the present method

Glycoside	Mean±S.D. (μg)
Lanatoside A	57± 2
Lanatoside B	16± 1
Lanatoside C	323±41
Desacetyllanatoside C	27± 4
α-Acetyldigitoxin	21± 3
α-Acetyldigoxin	18± 3

Values are the amounts per 100 mg of dry leaf powder from the same batch ($n=7$).

lanatoside C, α-acetyldigitoxin and α-acetyldigoxin in *D. lanata* leaves was achieved by using octadecylsilyl bonded silica gel TLC plates. The use of two internal standards of β-anhydrodesacetyllanatoside A and β-anhydrodigitoxin enables the quantitative analysis of both primary and secondary glycosides. The pretreatment with a Sep-Pak silica cartridge prior to the TLC analysis was effective for the separation of primary glycosides and secondary glycosides. In addition, the clean-up procedure using a Sep-Pak C_{18} cartridge was of great importance in the elimination of other compounds present in the extract. The proposed method is reliable and relatively simple for the determination of cardiac glycosides in the leaves. Therefore, this method is suitable for the estimation of the quality of *D. lanata* leaves.

Acknowledgments

The authors thank Mr. Masaru Yamamoto of the Medicinal Plant Garden of Hokuriku University for providing the leaves of *D. lanata* used in this study.

References

[1] O. Gisvold and S.E. Wright, J. Am. Pharm. Assoc., 46 (1957) 535.
[2] M. Wichtl, M. Mangkudidjojo and W. Wichtl-Bleier, J. Chromatogr., 234 (1982) 503.
[3] S. Schöner and E. Reinhard, Planta Med., 52 (1986) 478.
[4] Y. Fujii, Y. Ikeda, I. Okamoto and M. Yamazaki, J. Chromatogr., 508 (1990) 241.
[5] Y. Ikeda, Y. Fujii and M. Yamazaki, Anal. Biochem., 196 (1991) 451.
[6] Y. Ikeda, Y. Fujii and M. Yamazaki, J. Nat. Prod., 55 (1992) 748.
[7] T. Hagiwara, S. Shigeoka, S. Uehara, N. Miyatake and K. Akiyama, J. High Resolut. Chromatogr. Chromatogr. Commun., 7 (1984) 161.
[8] G.W. Ponder and J.T. Stewart, J. Chromatogr. A, 659 (1994) 177.
[9] M.A. Elkiey, Z.F. Ahmed, E.M. Abdelkader and S.M. Abdelwahab, J. Pharm. Sci. U.A.R., 5 (1964) 139.
[10] S.M. Khafagy and A.N. Girgis, Planta Med., 25 (1974) 350.
[11] S.I. Balbaa, S.H. Hilal and M.Y. Haggag, Planta Med., 26 (1974) 20.
[12] E. Bancher, T. Prey and F. Wurst, Planta Med., 29 (1976) 393.
[13] P. Horváth, Acta Pharm. Hung., 52 (1982) 133.
[14] D.E. Bloch, J. Assoc. Off. Anal. Chem., 63 (1980) 707.
[15] Y. Fujii, Y. Ikeda and M. Yamazaki, J. Liq. Chromatogr., 13 (1990) 1909.
[16] B. Pekić and D. Miljković, Planta Med., 27 (1975) 178.
[17] P. Drašar, V. Pouzar, I. Černy, M. Havel, F. Tureček, D. Schmiedová and K. Vereš, Collect. Czech. Chem. Commun., 50 (1985) 2760.
[18] A. Stoll and W. Kreis, Helv. Chim. Acta, 35 (1952) 1318.
[19] A. Stoll, A.V. Wartburg and W. Kreis, Helv. Chim. Acta, 35 (1952) 1324.
[20] L. Sawlewicz, H.H.A. Linde and K. Meyer, Helv. Chim. Acta, 51 (1968) 1353.