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Note

Two-dimensional thin-layer chromatography of *Digitalis* cardenolides using a continuous development technique

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Since Stahl and Kaltenbach[1] first used thin-layer chromatography (TLC) on silica gel to separate cardioactive glycosides, numerous TLC systems have been published for this pharmacologically important group of compounds. Sjöholm[2] recognised that separation of complex mixtures of cardioactive glycosides could not be achieved by conventional TLC, and obtained improved resolution by using two-dimensional TLC. Bennett and Heftmann[3] improved TLC resolution by use of a continuous development technique whereby a trough filled with adsorbent material was fixed to the top edge of the TLC plate to take up the mobile phase. Lisboa and co-workers[4,5] used a similar technique for TLC of steroids, including cardenolides, and found that continuous development gave better resolution of some closely related steroids than multiple developments in the same mobile phase.

The application of TLC to the analysis of cardiac glycosides and their genins has been reviewed by Nover[6]. This article includes a summary of Nover's papers in which the relationship between chemical structure and chromatographic behaviour has been explored.

This paper describes the use of continuous development TLC in two directions to obtain resolution of highly complex mixtures of *Digitalis* cardenolides, including an extract of *Digitalis lanata* leaf. The spots obtained are compact, and the cardenolides can be detected with great sensitivity and specificity. In addition to its use in identifying known components of a mixture, the method described can also be used to furnish structural information on unidentified constituents of natural product extracts.

EXPERIMENTAL

Reagents and reference materials

All solvents used were of analytical grade.

The chromogenic reagent employed was 20% (v/v) aqueous phosphoric acid.

Samples of the mono- and bis-digitoxosides of diginatigenin, digoxigenin, gitoxigenin and digitoxigenin were prepared according to the methods of Kaiser *et al.*[7]. Other compounds were obtained from commercial sources as indicated in Table I.

The particular sample of dried *D. lanata* leaf reported here was obtained from Tabandru (Uden, The Netherlands).

Extraction of D. lanata leaf

The cardenolides were extracted by maceration and percolation of the leaf with 20% aqueous ethanol, and the extract enriched by partition with chloroform as described in an earlier paper[8].

TLC technique

The TLC plates used were Kieselgel 60 DC-Fertigplatten, 20 × 20 cm with a layer thickness of 0.25 mm (Merck, Darmstadt, G.F.R.).

Reference samples were applied as 0.05% solutions in chloroform–methanol (1:1). The extract from 10 g of dried *D. lanata* leaf was dissolved in 0.5 ml of chloroform–methanol (1:1).

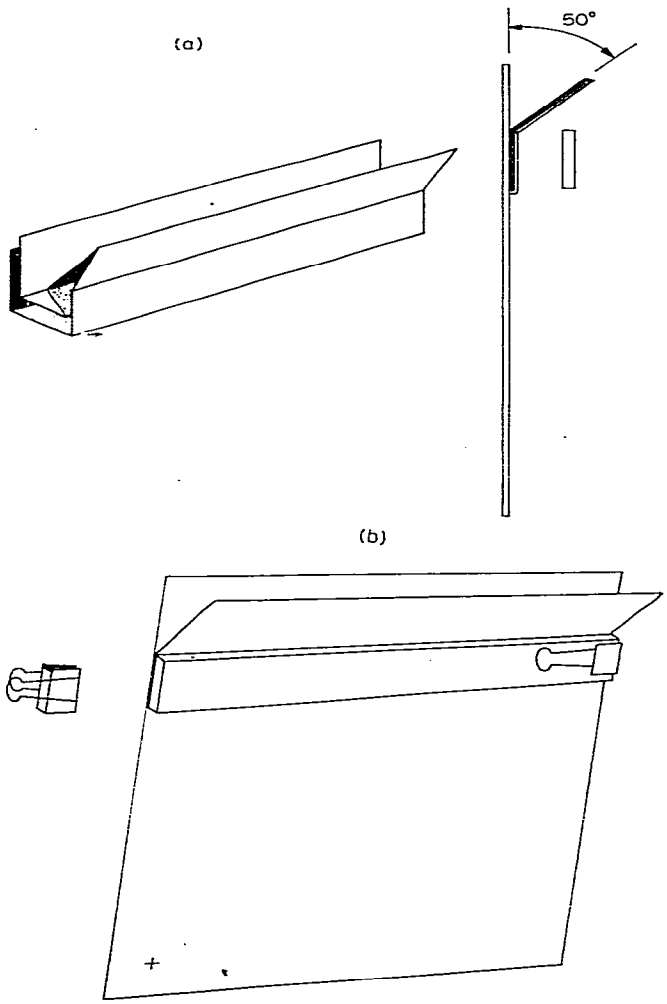


Fig. 1. Preparation of the trough for continuous development TLC. (a) The folded paper is placed against the adsorbent surface; (b) the folded paper is secured to the plate with a glass strip and spring clips.

The sample (0.5 μl) was applied by means of a 7101-SN Point style 3, microliter syringe (Hamilton, The Hague, The Netherlands) to the bottom left-hand side of the plate about 2 cm from the corner on the diagonal.

A trough of adsorbent was fitted to the top edge of the plate in the following manner. A sheet of chromatography paper (20 × 20 cm) was folded three times until its dimensions were (20 × 2.5 cm). The last fold was opened out, and the paper was placed against the adsorbent surface at the top of the plate (Fig. 1a). The bottom half of the folded paper was secured to the plate using a glass strip (20 × 2.5 cm) held by spring clips, and the top half was hinged outwards (Fig. 1b). The open ends of the trough were sealed with adhesive tape and the completed trough was filled with TLC-grade silica gel (20 g).

The plate was developed for 3 h in a conventional TLC chamber using ethyl acetate–dichloromethane–methanol–water (120:72:7:4) as mobile phase. The plate was then removed from the chamber and the trough detached. After drying in air at room temperature the plate was turned through 90° and a new trough attached to the top edge. The plate was then developed for 2 h in the second direction using dichloromethane–methanol (9:1) as mobile phase. After the second development the plate was removed from the chamber and the trough detached. The plate was then dried in air before immersing it in the phosphoric acid reagent for 5 sec (*care:* use gloves). The cardenolides were visualised by heating the plate at 100° for 15 min then viewing under UV light at 366 nm.

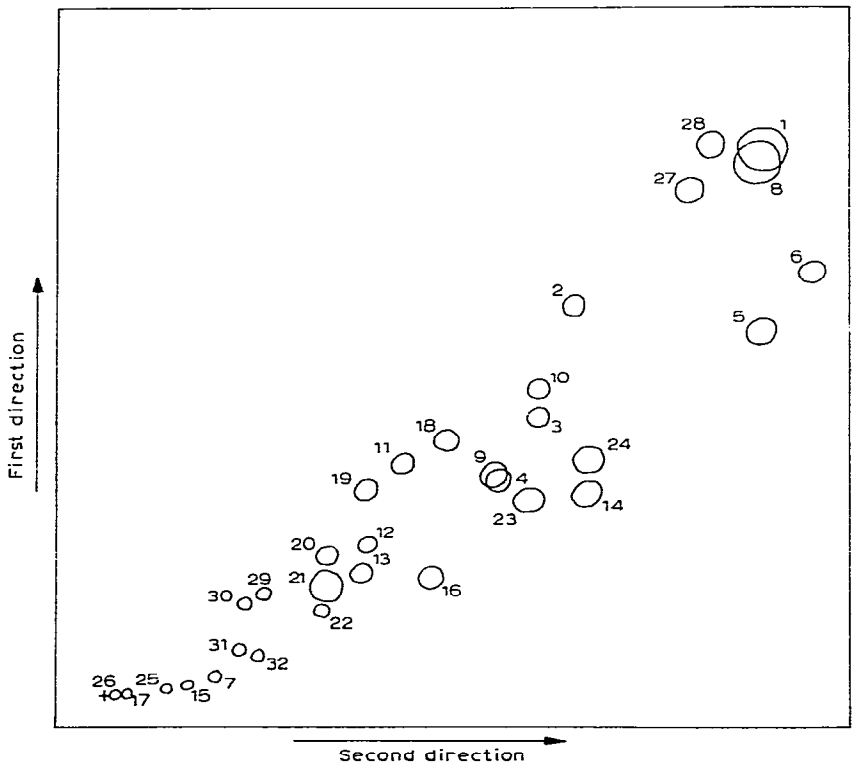
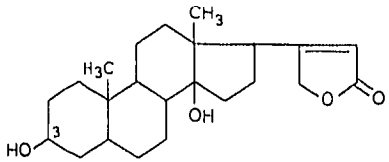
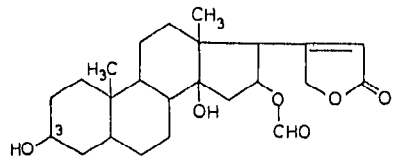
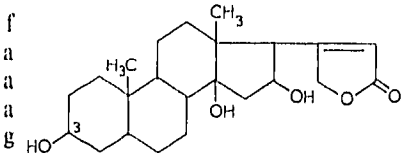


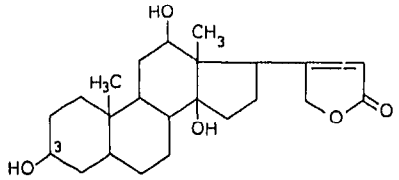
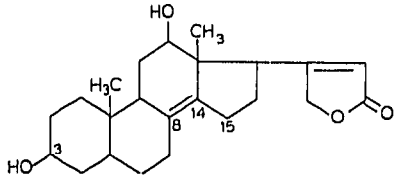
Fig. 2. Chromatogram of a mixture of cardenolide standards. The identity of the spots is given in Table I.

TABLE I

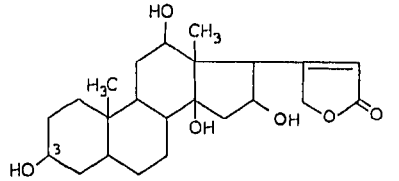
MOBILITIES OF A RANGE OF CARDENOLIDES RELATIVE TO DIGOXIN

Compound	*Symbol*	*Source**	*Aglycone*	*Glycosidically linked residue at C_3***	*UV appearance*	*Mobility relative to digoxin*	
						1st direction	*2nd direction*
Series A							
Digitoxigenin	1	a		-	Yellow/brown	5.09 S.D. ± 0.15	2.82 S.D. ± 0.23
Digitoxigenin mono-digitoxoside	2	a		-D	Yellow/brown	3.59	2.14
Digitoxigenin bis-digitoxoside	3	a		-D-D	Yellow/brown	2.57	1.94
Digitoxin	4	a		-D-D-D	Brown	2.00 S.D. ± 0.03	1.64 S.D. ± 0.07
α-Acetyldigitoxin	5	d		-D-D-D	Brown	3.31	3.11
β-Acetyldigitoxin	6	d		-D-D-D (Ac)	Brown	4.00	3.29
Lanatoside A	7	b		-D-D-D-G			
				-D-D-D-G (Ac)	Brown	0.16	0.46
Series E							
Gitaloxigenin	8	c		-	Brown	4.90	3.06
Gitaloxin	9	c		-D-D-D	Brown	1.97	1.88
Series B							
Gitoxigenin	10	f		-	Blue/white	2.78	1.99
Gitoxigenin mono-digitoxoside	11	a		-D	Blue/white	2.14 S.D. ± 0.06	1.25 S.D. ± 0.05
Gitoxigenin bis-digitoxoside	12	a		-D-D	Blue/white	1.36	1.16
Gitoxin	13	a		-D-D-D	Blue/white	1.12 S.D. ± 0.04	1.14 S.D. ± 0.01
β-Acetylgitoxin	14	g		-D-D-D (Ac)	Yellow	1.98	2.25
Lanatoside B	15	b		-D-D-D-G			
				-D-D-D-G (Ac)	Light brown	0.08	0.29
Strospeside	16	c		-L	Yellow/brown	1.11	1.45
Digitalinum verum	17	b		-L-G	Brown	0.05	0.13

Series C

Digoxigenin	18	a		—	Green	2.36	1.55
Digoxigenin mono-digitoxoside	19	a		-D	Yellow/green	1.93	1.15
Digoxigenin bis-digitoxoside	20	a		-D-D	Yellow/green	1.31	1.02
Digoxin	21	a		-D-D-D	Yellow/brown	1.00	1.00
Digoxoside	22	c		-D-D-D-D	Yellow	0.68	0.95
α-Acetyldigoxin	23	c		-D-D-D (Ac)	Yellow	1.81	1.94
β-Acetyldigoxin	24	c					
Lanatoside C	25	b		-D-D-D-G (Ac)	Brown	0.06	0.23
Deacetyl lanatoside C	26	b		-D-D-D-G	Brown	0.05	0.08
α-Anhydro digoxigenin	27	a			Blue/mauve	4.79	2.65
β-Anhydro digoxigenin***	28	a		—	Yellow	5.10	2.77

Series D

Diginatigenin	29	b		—	Blue/white	0.98	0.68
Diginatigenin mono-digitoxoside	30	a		-D	Blue/white	0.90	0.61
Diginatigenin bis-digitoxoside	31	a		-D-D	Blue/white	0.41	0.57
Diginatin	32	b		-D-D-D	Blue/white	0.33 S.D. = 0.03	0.68 S.D. = 0.02

* a = Burroughs Wellcome (Dartford Great Britain); b = Sandoz (Basle, Switzerland); c = Boehringer (Mannheim, G.F.R.); d = K & K Labs. (Plainview, N.Y., U.S.A.); e = Aldrich (Milwaukee, Wisc., U.S.A.); f = Fluka (Buchs, Switzerland); g = USPC (Rockville, Md., U.S.A.).

** D = digitoxose; G = glucose; L = digitalose; Ac = acetyl.

*** β-Anhydro digoxigenin has a double bond in the 14–15 position.

RESULTS AND DISCUSSION

The cardenolides examined are listed together with their mobilities relative to digoxin in Table I.

A typical chromatogram obtained for a mixture of cardenolides is represented in Fig. 2, and Fig. 3 shows the chromatogram obtained from an extract of *D. lanata* leaf. The spots produced are compact and symmetrical and it is possible to detect 0.015 μg of most of the cardenolides. The reproducibility of the positions of the spots with respect to digoxin was determined using 8 chromatograms developed on separate occasions. Five cardenolides were chosen for this experiment and the standard deviations obtained are shown in Table I. These results demonstrate good reproducibility.

Continuous development is necessary to give the high degree of resolution required in the analysis of complex mixtures of cardenolides. This means that some non-cardenolide components of *Digitalis* extracts run into the trough and are not observed. This represents no disadvantage, however, since conventional two-dimensional TLC can be used if these more mobile compounds are to be studied (Fig. 4).

Some correlation between the chromatographic mobility of the cardenolides and their chemical structure has been observed. The spots due to corresponding cardenolides from each series, *e.g.* diginatin, digoxin, gitoxin and digitoxin lie on straight lines (Fig. 5a). Regular curves can be drawn through the spots from

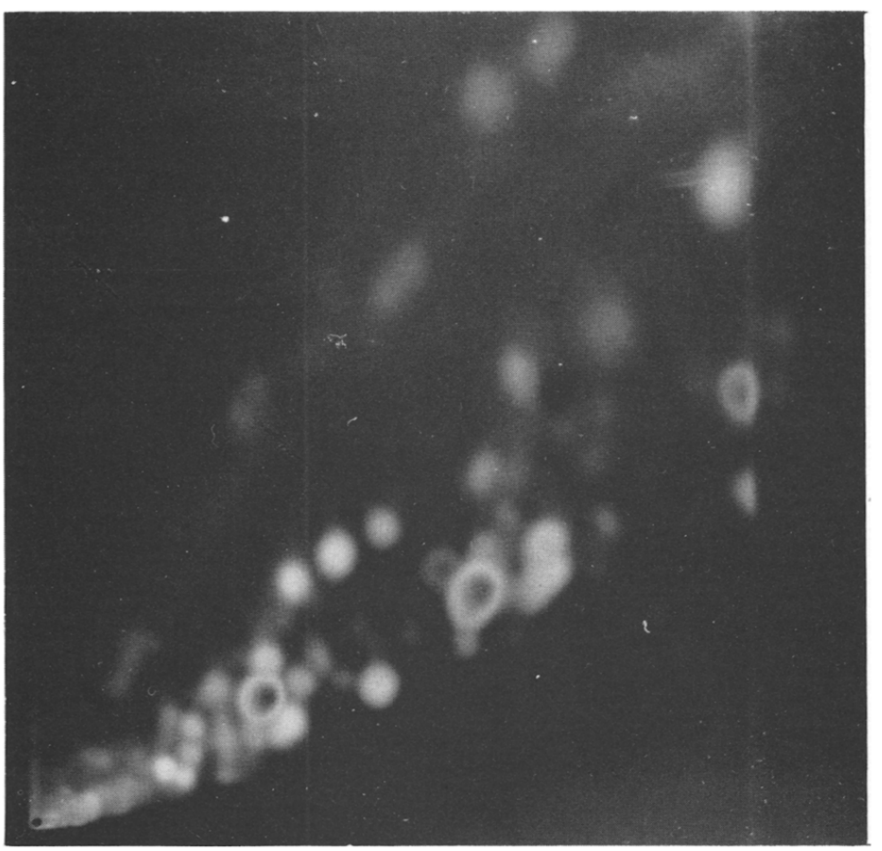


Fig. 3. Typical chromatogram of a *D. lanata* leaf extract.

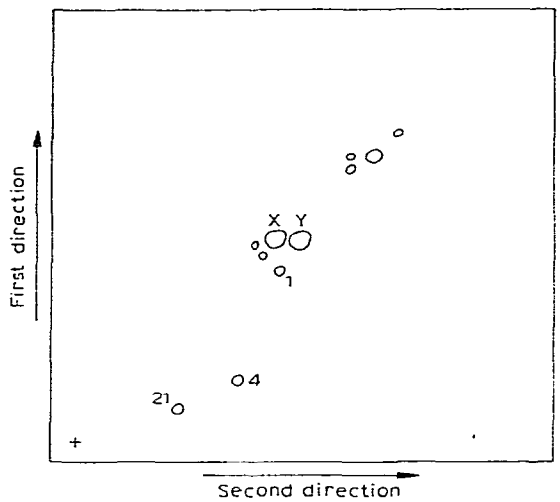


Fig 4. Two-dimensional chromatogram of a *D. lanata* leaf extract developed without using adsorbent troughs. X = 5,7,4′-trihydroxy-3′-methoxyflavone; Y = 5,7,4′-trihydroxy-3′,6-dimethoxyflavone.

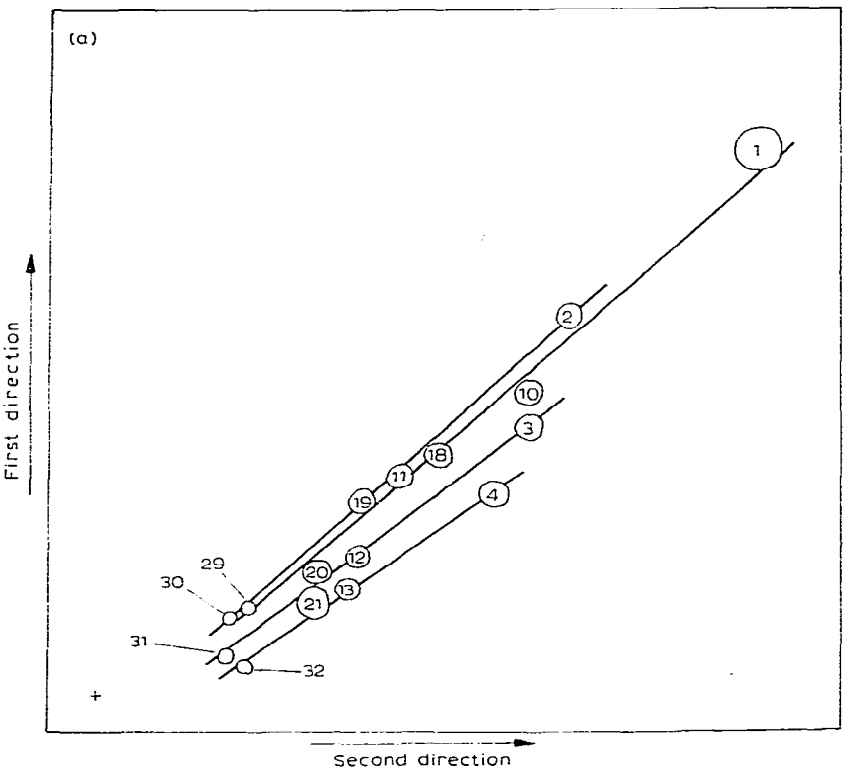


Fig. 5.

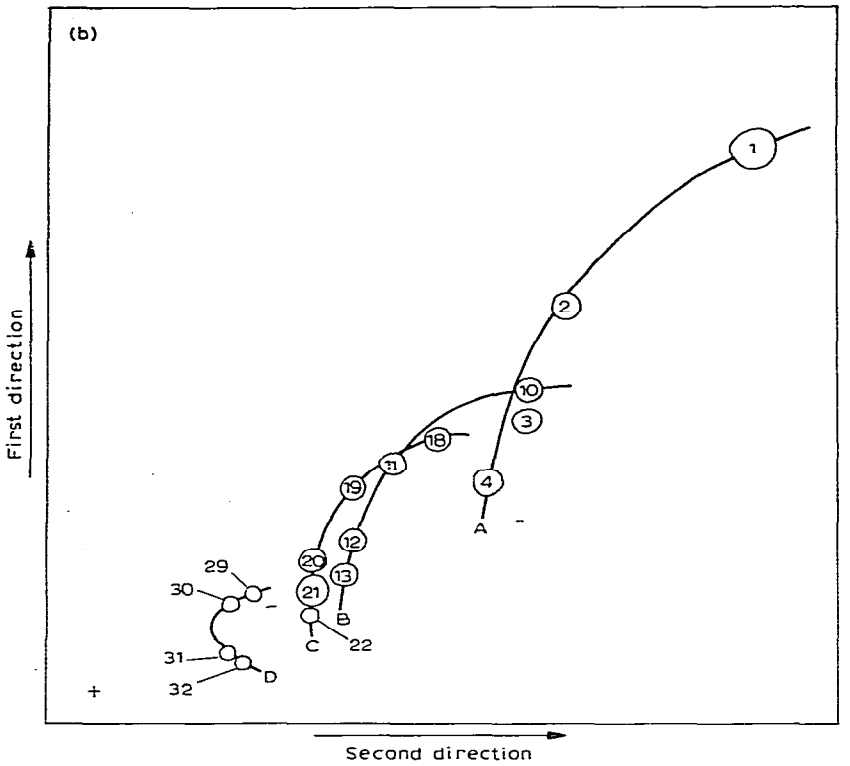


Fig. 5. Relationship between chromatographic mobility and chemical structure. (a) Relationship between corresponding cardenolides from different series; (b) relationship between homologues. The identity of the spots is given in Table I.

homologous compounds, *e.g.* digoxigenin, digoxigenin mono-digitoxoside, digoxigenin bis-digitoxoside and digoxin (Fig. 5b). These empirical relationships were used to identify the positions of digoxoside, diginatigenin mono-digitoxoside and diginatigenin bis-digitoxoside before reference samples became available.

This chromatographic technique has proven extremely useful in the qualitative monitoring of the commercial extraction of digoxin from *D. lanata* leaf. It has also made possible the isolation of previously unknown glycosides from extracts of *D. lanata* and these will be reported in a later paper.

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