Journal of Chromatography, 103 (1975) 396–401

CHROM. 7797

Note

Thin-layer chromatography for the separation of digitoxin, digitoxigenin and related compounds*

G. ZÜLLICH, W. BRAUN and B. P. LISBOA

Pharmakologisches Institut und Frauenklinik des Universitäts-Krankenhauses Hamburg, Hamburg (G.F.R.)

(Received July 17th, 1974)

Ascending one-dimensional thin-layer chromatography (TLC) on silica gel layers has been widely employed for the separation of heart glycosides and cardenolides using either a single development[1,2] or multiple runs[3,4].

During our study on the metabolism of digitoxin in rats[5], we used TLC for the separation and quantitation of the products formed by the cleavage of the three sugar moieties or by metabolism of the aglycone itself. Neither the data reported by Nover and co-workers[1,2] on normal ascending TLC nor the use of multiple developments were adequate for solving our particular problem. Therefore, we have developed a series of systems that have been used satisfactorily in normal and over-run TLC for the separation of cardenolides from their heart glycosides as well as for resolving cardenolides hydroxylated at different positions of the steroid skeleton, including 3-epimeric doublets.

EXPERIMENTAL

Reagents

All the chemicals used were of reagent grade and were purchased from E. Merck (Darmstadt, G.F.R.), if not otherwise stated. The cardenolides and heart glycosides were obtained from E. Merck or Boehringer (Mannheim, G.F.R.) or were kindly provided by Dr. H. Ishii (Japan) and Prof. Ch. Tamm (Switzerland), as indicated in Table I.

Chromatography

For ascending TLC, coated 0.25-mm thick silica gel G layers from E. Merck (20 × 20 cm) were used. In normal TLC, the solvent was allowed to ascend 16 cm from the starting line, which was 2.5 cm above the edge of the plate.

Over-run TLC was carried out in closed tanks after complete saturation. This procedure was employed in order to obtain continuous development over a period of several hours. The layer for over-run TLC was prepared in two different ways: (a) at the top of the plate, a 4-cm wide zone was additionally covered with a slurry

* Taken in part from a dissertation by Gunter Züllich in partial fulfilment of the requirements for the M.D. degree, Medical School, University of Hamburg.

prepared by shaking 12 g of silica gel G–calcium sulphate (70:30) with 20 ml of methanol–water (1:1); the plate was dried at room-temperature overnight; (b) a 20 × 3.5-cm metal container (Fig. 1) with an angle of 45° was fixed at the upper part of the layer and filled with 8–10 g of dry silica gel G. The latter procedure can also be used for self-prepared layers.

Solvent systems. Fourteen solvent systems were used: (I) chloroform–acetone–methanol (56:36:7); (II) chloroform–acetone–methanol (55:35:10); (III) chloroform–acetone–methanol (40:50:10); (IV) chloroform–acetone (65:35); (V) diethyl ether–methanol (90:10); (VI) ethyl acetate (water-saturated); (VII)[6] chloroform–isopropanol (90:10); (VII) chloroform–ethanol (90:10); (IX) chloroform–methanol (92:8); (X)[7] pyridine–chloroform (1:6); (XI) toluene–methanol (80:20); (XII) cyclohexane–acetone–glacial acetic acid (49:49:2); (XIII) ethyl acetate–*n*-hexane–glacial acetic acid (80:10:10); (XIV)[8] ethyl acetate (water-saturated)–*n*-hexane–glacial acetic acid–ethanol (72:13.5:10:4.5); (XV) as XIV, but the ethyl acetate was not water-saturated; (XVI) as XIII, but the ethyl acetate was saturated with water.

Detection of the spots. The spots (5–15 μg) were made visible by the Ekkert reaction[9]. The plates were dried and sprayed with 0.5% anisaldehyde in acetic acid–sulphuric acid (98:2). The colours obtained by heating at 100° for 6–10 min are summarized in Table I.

RESULTS AND DISCUSSION

Table I summarizes the mobility values (hR_F) on silica gel G obtained for 5β-cardenolides and heart glycosides of the digitoxigenin, digoxigenin and gitoxigenin series by ascending TLC using the fourteen solvent systems.

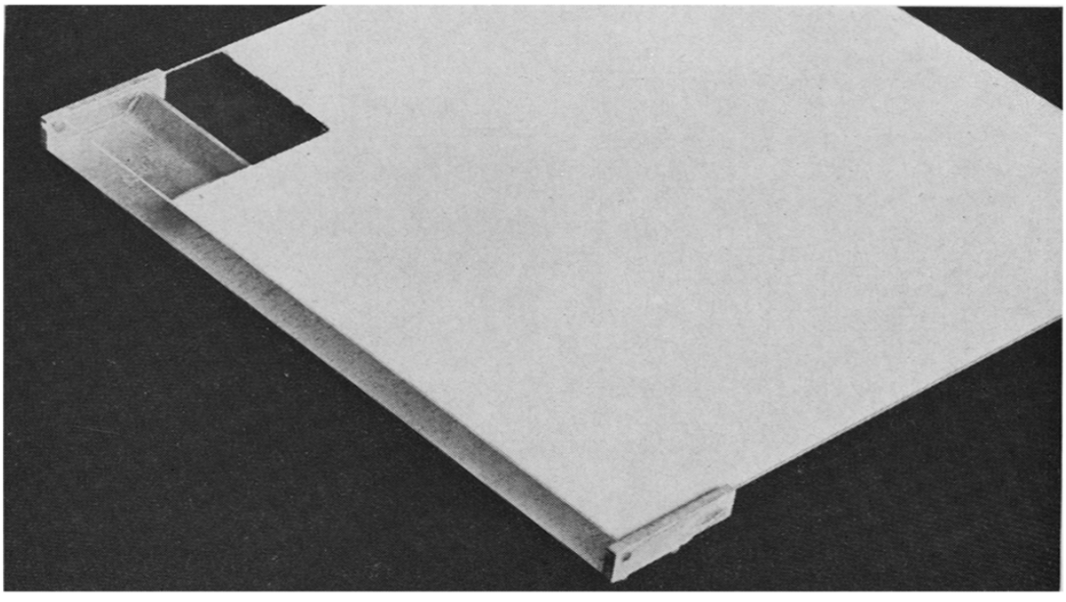

Fig. 1. Metal container (20 × 3.5 cm) attached to the top of the plate during irrigation of the layer by the continuous development procedure. This container is filled with 8–10 g of dry silica gel G.

TABLE I

ASCENDING ONE-DIMENSIONAL TLC OF HEART GLYCOSIDES AND CARDENOLIDES ON SILICA GEL G LAYERS DEVELOPED IN SEVERAL SOLVENT SYSTEMS

The mobilities are expressed as hR_F values; solvent front = 16 cm. The spots were made visible after spraying with anisaldehyde–sulphuric acid.

Heart glycosides and genins[*]	*Source*[**]	*Colour reaction*[***]	*Solvent system*												
			I	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*	*IX*	*X*	*XI*	*XII*	*XIII*	*XIV*
Digitoxigenin (DT)	a	bl-gr→gr	38	44	55	35	43	39	43	48	42	62	38	38	45	61
epi-DT	b	gr	34	43		30	38	33	38	45	39	57	38	34	38	57
DT mono-S	b	bl-v	33			19	34	30	26						33	
DT bis-S	b		30	38	50	15	30	22	22	35	29	39	30	33	21	44
DT tris-S (digitoxin)	a		27	36	48	9	24	16	19	34	29	33	28	27	15	38
Digoxigenin (DG)	b	v	23	33	41	16	26	21	16	26	20	33	22	28	23	41
epi-DG	b	v	19	30	36	11	19	14	10	21	16	24	22	22	17	34
DG mono-S	b	v	21	32	42	11	23	18	12	24	20	29	20	24	19	38
DG bis-S	b	v	18	28	39	7	17	12	10	23	17	23	19	19	11	30
DG tris-S (digoxin)	a		16	28	36	4	14	9	9	23	16	21	20	17	8	25
DG tetra-S	b		11	21	28	2	9	6	6	18	18	18	20	14	6	21
neo-Digoxin	b	v	14	26	34	2	11	5	9	24	16	18	16	11	6	21
Gitoxigenin (GT)	b, c	y→y-gr→bl-gr	25	36	43	18	28	22	20	31	24	34	29	30	23	44
GT mono-S	b	ol→bl-gr	21	33	39	9	22	19	12	25	18	27	26	25	19	39
GT tris-S (gitoxin)	a		17	29	36	4	14	9	9	24	18	20	24	18	8	27
Gitaloxigenin	b	y→y-gr→bl-gr	31	42	48	32	42	40	41	47	40	34	36	40	44	
Acovenosigenin	c	bl				24	31		30	39			33	34	29	46
Periplogenin	d, c	gr				16	29		18	29			33	32	23	40
Sarmentogenin	d	bl								23			24	26	23	39
7β-ol-DT	c					23	37		23				34	36	31	
Strophanthidol	d	bl-gr		30	34					21	18		26	18	16	
Strophanthidin	a	v→bl-v	23	35	41	12	23	17	12	25	21	25	29	26	12	26
g-Strophanthin	a	y	s.l.[§]	s.l.	s.l.	s.l.	s.l.	s.l.	s.l.	s.l.	s.l.	s.l.	3	s.l.	s.l.	

[*] DT = 3β,14β-dihydroxy-5β-cardenolide; *epi*-DT = 3α,14β-dihydroxy-5β-cardenolide; S = digitoxoside; DG = 12β-ol-DT; GT = 16β-ol-DT; gitaloxigenin = 16β-formyloxy-DT; acovenosigenin = 1β-ol-DT; periplogenin = 5β-ol-DT; sarmentogenin = 11α-ol-DT; strophanthidol = 5β,19-dihydroxy-DT; g-strophanthin = 5β-ol-19-oxo-DT; strophanthidin (ouabain) = ouabagenin rhamnoside (1β,3β,5,11α,14β,19-hexahydroxycardenolide rhamnoside).

[**] a, E. Merck, Darmstadt, G.F.R.; b, Boehringer, Mannheim, G.F.R.; c, Dr. Hiroshi Ishii, Shionogi Research Laboratory, Shionogi Co., Osaka, Japan; d, Prof. Ch. Tamm, Institut für Organische Chemie, Universität Basel, Basel, Switzerland.

[***] Anisaldehyde–sulphuric acid reaction, colour developed by heating at 100° for 4–6 min: bl = blue; gr = green; v = violet; y = yellow; ol = olive.

[§] s.l. = starting line.

A series of systems (IV, V, VI and XIII) was used for the separation of digitoxin from its main metabolites (digitoxigenin bisdigitoxoside, digitoxigenin monodigitoxoside and digitoxigenin). Similarly, the heart glycosides from the digoxigenin series (digoxigenin mono-, di-, tri- and tetradigitoxiside) can be separated in several systems, especially system XIV, developed by Lisboa[8,10,11] for the separation of polar steroids of the C_{18}, C_{19} and C_{21} series. A good separation could also be achieved for the cardiac steroids of the gitoxigenin series in systems IV, V, X, XII and XIV.

The separation of the glycoside pair digoxin–gitoxin in system XI (hR_F values 32 and 39) deserves particular mention; these substances could not be separated in the systems proposed by Nover *et al.*[1] and Sjöholm[7] or those of Bulger *et al.*[3] using four developments in cyclohexane–acetone–acetic acid (65:33:2).

An inversion in the polarity was observed during the chromatography of the monodigitoxosides of gitoxigenin and digoxigenin: in systems III and IV, but not in XI, the gitoxigenin derivative was more polar than that of digoxigenin. These systems can be combined in a two-dimensional TLC technique in order to achieve a better separation of the two compounds.

Since the work of Repke and Samuels[12,13] on the conversion of 3β-hydroxycardiotonic steroids into their 3α-epimers in rat liver, the separation of 3-epimeric cardenolides has been of great importance. The epimeric pair digitoxigenin–*epi*-digitoxigenin and digoxigenin–*epi*-digoxigenin can be separated on silica gel layers in several systems, particularly X, XII and XIV. Epimeric cardenolides of the 5α-series (uzarigenin–*epi*-uzarigenin) have already been separated by Tschesche *et al.*[14] on silica gel layers developed in ethyl acetate (system X).

Over-run TLC in the systems XII and XV (Fig. 2) improved the separation of the pair digoxigenin (f)–*epi*-digoxigenin (h). Over-run TLC in these systems did not improve the separation of the pair digitoxigenin–*epi*-digitoxigenin compared with normal development. This could be explained by a migration distance for these compounds about or greater than 7 cm (hR_F greater than 44) during normal development; our experience indicates that an over-run technique should be used only for the separation of compounds with hR_F values lower than 35 in normal developments with the same system.

The data in Table I indicate that complete separation of the monohydroxylated derivatives of digitoxigenin was achieved in system VIII. The sequence of polarity obtained in this system, $11\alpha > 12\beta > 5\beta > 16\beta > 1\beta$, is similar to that reported by Nover *et al.*[15] on silica gel G layers for a series of solvent systems. Periplogenin, a metabolite of digitoxigenin in the rabbit liver[6], can be separated from sarmentogenin in systems XI and XII, whereas the optimal separation of 1β- and 7β-hydroxydigitoxigenins was achieved in system V or VII.

Periplogenin and gitoxigenin, both tentatively identified as polar metabolites of digitoxigenin in the rat[16], can be separated in several solvent systems. Periplogenin is more polar than gitoxigenin in systems IV, VII, VIII and XIV, and less polar in systems XI and XII. Finally, gitoxigenin and digoxigenin are well separated using system XI.

The systems described in this paper have been used in our laboratories in normal and over-run procedures to investigate the metabolites of [^{3}H]digitoxin in rats, and to detect cardenolides and cardiac glycosides in fractions collected in Sephadex gel column chromatography[17].

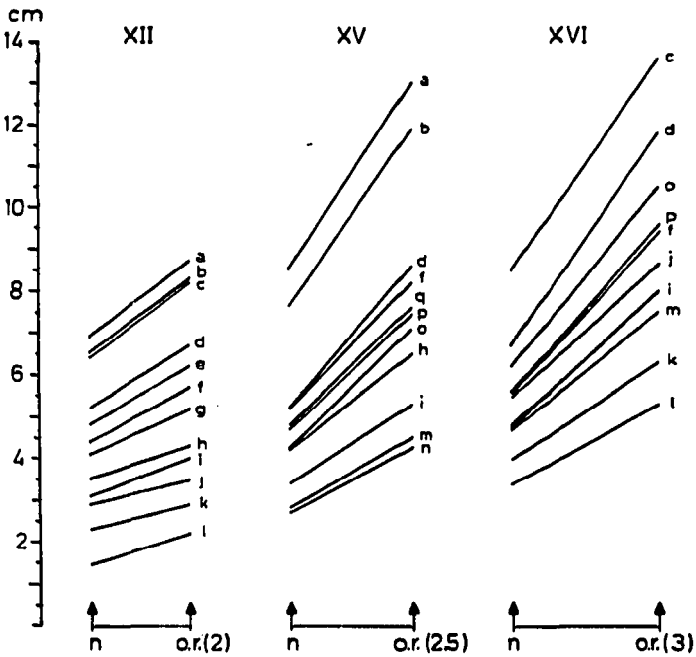

Fig. 2. Examples of the separation of cardiac steroids by over-run TLC in solvent systems XII, XV and XVI. In each case the mobility values obtained by continuous development were compared with those obtained under normal conditions (16-cm front). The mobilities of the following authentic heart glycosides and cardenolides are shown: (a) digitoxigenin (DT); (b) *epi*-DT; (c) 16β-formyloxy-DT; (d) DT bisdigitoxoside; (e) gitoxigenin; (f) digoxigenin (DG); (g) 5β-ol-19-oxo-DT; (h) *epi*-DG; (i) DG bisdigitoxoside; (j) 5β,19-diol-DT; (k) DG tetradigitoxoside; (l) *neo*-digoxin; (m) gitoxin; (n) DG trisdigitoxoside; (o) DT trisdigitoxoside; (p) DG monodigitoxoside; (q) gitoxigenin monodigitoxoside. Solvent systems: XII = cyclohexane–acetone–glacial acetic acid (49:49:2) (over-run: 2 h); XV = ethyl acetate–*n*-hexane–glacial acetic acid–ethanol (72:13.5:10:4.5) (over-run: 2.5 h); XVI = ethyl acetate (water-saturated)–*n*-hexane–glacial acetic acid (80:10:10) (over-run: 3 h). For further details, see text.

ACKNOWLEDGEMENTS

Thanks are due to Dr. Hiroshi Ishii (Shionogi Research Laboratory, Fukushima-ku, Osaka, 533 Japan) and Prof. Christian Tamm (Institut für Organische Chemie der Universität Basel, 4056 Basel, Switzerland) for the generous gift of some of the cardenolides used in this work. This investigation was supported in part by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich-34).

REFERENCES

1 L. Nover, G. Jüttner, S. Noack, G. Baumgarten and M. Luckner, *J. Chromatogr.*, 39 (1969) 419.
2 L. Nover, in K. Macek (Editor), *Pharmaceutical Applications of Thin-Layer and Paper Chromatography*, Elsevier, Amsterdam, 1972, pp. 348–408.
3 W. H. Bulger, R. E. Talcott and S. J. Stohs, *J. Chromatogr.*, 70 (1972) 187.
4 M. L. Carvalhas and M. A. Figueira, *J. Chromatogr.*, 86 (1973) 254.

5 G. Züllich, K. H. Damm, W. Braun and B. P. Lisboa, to be published.
6 W. H. Bulger and S. J. Stohs, *Biochem. Pharmacol.*, 22 (1973) 1745.
7 I. Sjöholm, *Sv. Farm. Tidskr.*, 66 (1962) 321.
8 B. P. Lisboa, *Acta Endocrinol.*, 43 (1963) 47.
9 B. P. Lisboa, *Methods Enzymol.*, 15 (1969) 3.
10 B. P. Lisboa, *Clin. Chim. Acta*, 13 (1966) 179.
11 B. P. Lisboa, *J. Chromatogr.*, 19 (1965) 81.
12 K. Repke and L. T. Samuels, *Biochemistry*, 3 (1964) 685.
13 K. Repke and L. T. Samuels, *Biochemistry*, 3 (1964) 689.
14 R. Tschesche, W. Freytag and G. Snatzke, *Chem. Ber.*, 92 (1959) 3053.
15 L. Nover, G. Baumgarten and M. Luckner, *J. Chromatogr.*, 39 (1969) 450.
16 R. E. Talcott, W. H. Bulger and S. J. Stohs, *Steroids*, 21 (1973) 87.
17 G. Züllich, B. P. Lisboa and K. H. Damm, *Arch. Int. Pharmacodyn. Ther.*, (1975) in press.