Journal of Chromatography, 178 (1979) 453–458

CHROM. 12,104

THIN-LAYER CHROMATOGRAPHY OF CARDENOLIDES IN THE PRESENCE OF BORIC ACID

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(First received February 6th, 1979; revised manuscript received June 11th, 1979)

SUMMARY

The thin-layer chromatographic behaviour of cardenolides and their derivatives was investigated in the presence of boric acid, which forms cyclic derivatives with *cis*-1,2- and -1,3-diols. Boric acid reduces the mobility of cardenolide glycosides containing diol units in their carbohydrate moiety whereas with cardenolides or cardenolide glycosides possessing 1,3-diol units in the genin part it increases the mobility. The formation of boric acid derivatives resulted in an improved separation of certain cardiac steroids and afforded the possibility of detecting cardenolides containing reactive diol units.

A method of impregnation in the vapour phase was developed which ensured mild treatment of the layers and yielded an extremely uniform impregnation.

INTRODUCTION

The quantitative determination of digitalis cardiotonic steroids is usually preceded by thin-layer chromatographic (TLC) separation[1–10]. The resolution of digoxin and gitoxin and of related compounds is, however, poor or not reliable in the numerous solvent mixtures used so far[11–16]. Our aim was therefore to find a TLC developing system in which these cardenolides can be clearly separated. Although they can be well separated on adsorbents impregnated with formamide[4,17–19], *i.e.*, by partition chromatography, the impregnation and the removal of trace amounts of formamide which interfere with the quantitation make this procedure tedious.

The chromatographic separation of polyhydroxy compounds (carbohydrates, fatty acids, polyalcohols etc.) has been carried out successfully in many instances on adsorbents impregnated with boric acid[20–26] if the component to be isolated contains hydroxyl groups in a position and conformation favourable for the formation of a cyclic boric acid derivative, and if the polarity of this derivative is different from that of the free diol. In addition to boric acid, other diol reagents have also been used for the improvement of chromatographic separation.

In cardenolides, such active pairs of hydroxyl groups can be present in the terminal sugar molecule of the carbohydrate chain and/or in the genin.

Owing to the O–O distance in boric acid, six-membered cyclic derivatives

can be formed by participation of the 22′,24′-diol unit of the terminal glucose molecule in the primary glycosides[27]. In the secondary glycosides the terminal sugar is digitoxose, *i.e.*, a 2-deoxyhexose, with which five-membered cyclic derivatives can be formed by the participation of the vicinal *cis*-15′,16′-hydroxyl groups[28]. The 1,3-coaxial hydroxyl groups of genin can react with boric acid to give a six-membered ring.

A coaxial structure of the 1,3-diols is essential for ring formation. No similar reaction occurs between the 12β- and 14β-hydroxyl groups of digoxigenin and boric acid, owing to the nearly double distance between these hydroxyl groups (Fig. 1).

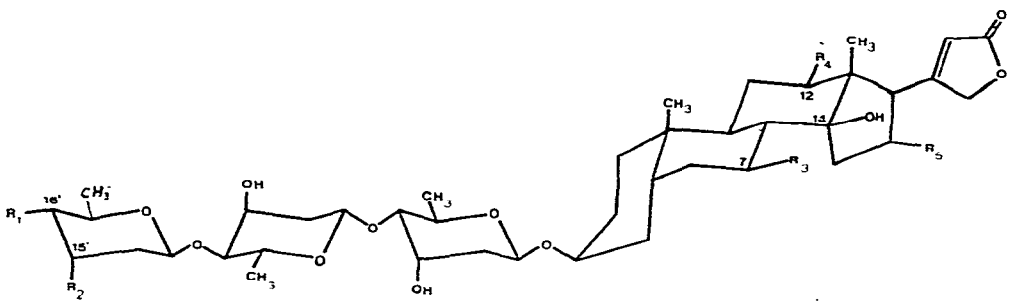
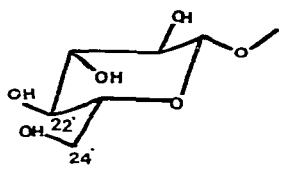


Fig. 1. Structures of cardenolides:

	R_1	R_2	R_3	R_4	R_5
Digoxin	OH	OH	H	OH	H
Lanatoside C		OH	H	OH	H

For steric reasons the six-membered cyclic derivatives of trigonal boric acid are usually more stable than the five-membered derivatives[29,30].

In Table I, the cardenolides examined and their derivatives are listed, with an indication of the position of their hydroxyl groups, which are able to form cyclic esters.

Up till now, the TLC separation of cardenolides in the presence of diol reagents has been reported only by Reichelt and Pitra[31] and later by Lindig *et al.*[32]. While this paper was under in preparation, Megges *et al.* published two detailed studies[33,34] on the chromatographic separation of cardenolides and bufadienolides. However, they carried out the chromatography on formamide-impregnated paper using mostly phenylboric acid and diphenylboric acid as diol reagents.

EXPERIMENTAL

For TLC, DC-Alufolien Kieselgel 60 (Merck, Darmstadt, G.F.R.) ready-made layers were used. A 1 μg/μl solution of each cardenolide sample was applied. The eluent was chloroform–methanol–acetic acid (90:10:1) with and without 0.5% of boric acid. The development was carried out in a saturated Desaga tank and the distance

travelled was 13.5 cm. The chromatograms were detected by spraying with anisaldehyde reagent containing 0.8 ml of anisaldehyde, 100 ml of acetic acid and 0.6 ml of concentrated sulphuric acid, followed by heat treatment.

The adsorbent can also be impregnated with boric acid prior to the actual chromatography. The impregnation of the ready-made layers is carried out by immersion of the plates in the solution of the impregnating agent, by spraying or by previous running of this solution[35]. The disadvantages of these procedures are that the adsorbent is not always uniformly impregnated and the layer may suffer mechanical damages during the procedure.

We have developed a mild method by impregnation in the vapour phase which leads to an extremely uniform impregnation. The plates are placed in the saturated vapour phase of a 1% boric acid solution in methanol for a few minutes. In this solution a volatile methyl borate is formed, which is uniformly adsorbed on the layer. On removal of the plates from the tank the methyl ester is decomposed and boric acid remains on the adsorbent.

The relative mobility of the cardenolides (R_s) was compared to that of a reference dye (4,5-dichlorofluorescein). The change in mobility observed in the presence of boric acid is characterized by the difference between the two R_s values:

$$\Delta R_s = R_{s\ \text{(with boric acid)}} - R_{s\ \text{(without boric acid)}}$$

and by the percentage change in R_s, compared with $R_{s\ \text{(without boric acid)}}$:

$$\Delta R_s\,(\%) = \frac{\Delta R_s \cdot 100}{R_{s\ \text{(without boric acid)}}}$$

RESULTS

As Fig. 2 and Table I demonstrate, digoxin and gitoxin and lanatoside C and B can be separated completely in the presence of boric acid. The polarity of gitoxin borate is low so that it moves ahead even of digitoxin. An analogous inversion in the order of R_s valves can also be observed with the cardenolide pairs lanatoside A and B and digitoxigenin and gitoxigenin.

The R_s values of cardenolides lacking active pairs of hydroxyl groups (9–12, 15–22) are unaffected by boric acid. The relative mobilities of cardenolides containing active hydroxyl groups only on the carbohydrate chain (1, 3–6) are reduced by boric acid. This decrease is especially large when expressed as ΔR_s (%).

Cardenolides with active pairs of hydroxyl groups in the genin part behave in the opposite way. The mobility of these cardenolides (2,7,8,13,14,23) is considerably increased in the presence of boric acid (Fig. 3).

If, for cardenolides containing pairs of hydroxyl groups of both types (7,8), the opposite effect of the pairs of hydroxyl groups in the carbohydrate chain is considered, the mobility-increasing effect of the pairs in their genin part is about the same as that obtained for the genins 13 and 14.

In the cyclic sugar ester the boron atom is presumably tetrahedral, while in the genin ester it is trigonal. This may explain the reverse chromatographic behaviour of the pairs of hydroxyl groups in the carbohydrate chain and in the genin nucleus in the presence of boric acid.

TABLE I

STRUCTURES AND RELATIVE MOBILITIES OF CARDENOLIDES

Cardenolides	*Positions of active hydroxyl groups*		R_s		ΔR_s	ΔR_s (%)
	Sugar chain	*Genin*	*Without boric acid*	*With boric acid*		
Primary glycosides						
1 Lanatoside A	22′,24′	—	0.311	0.252	−0.058	−19
2 Lanatoside B	22′,24′	14β,16β	0.221	0.286	+0.065	+29
3 Lanatoside C	22′,24′	—	0.172	0.126	−0.046	−26
4 Purpureaglycoside A	22′,24′	—	0.107	0.076	−0.031	−29
Secondary glycosides						
5 Digitoxin	15′,16′	—	0.967	0.874	−0.093	−10
6 Digoxin	15′,16′	—	0.672	0.563	−0.109	−16
7 Gitoxin	15′,16′	14β,16β	0.754	0.983	+0.229	+30
8 7β-Hydroxydigoxin	15′,16′	7β,14β	0.590	0.672	+0.082	+14
9 Acetyldigitoxin	—	—	1.311	1.353	+0.043	+ 3
10 Acetyldigoxin	—	—	1.025	1.042	+0.017	+ 2
Genins						
11 Digitoxigenin	—	—	1.147	1.146	−0.001	0
12 Digoxigenin	—	—	0.721	0.706	−0.015	− 2
13 Gitoxigenin	—	14β,16β	0.902	1.294	+0.392	+43
14 7β-Hydroxydigoxigenin	—	7β,14β	0.492	0.706	+0.214	+43
15 14-Anhydrodigitoxigenin	—	—	1.467	1.546	+0.079	+ 5
16 14-Anhydrodigoxigenin	—	—	1.066	1.092	+0.026	+ 2
17 14,16-Dianhydrogitoxigenin	—	—	1.443	1.513	+0.070	+ 5
18 Gitoxigenin 16-acetate	—	—	1.180	1.210	+0.030	+ 2
19 Gitoxigenin 3,16-diacetate	—	—	1.606	1.664	+0.058	+ 4
20 Gitoxigenin 14,16-phenylboronate	—	—	1.434	1.513	+0.079	+ 5
21 7β-Hydroxydigoxigenin 7,14-phenylboronate	—	—	0.975	1.017	+0.042	+ 4
22 7β-Hydroxydigoxigenin 7,14-phenylboronate 3,12-diacetate	—	—	1.787	1.781	−0.006	0
23 7β-Hydroxydigoxigenin 3,12-diacetate	—	7β,14β	1.508	1.723	+0.215	+14

With secondary glycosides the 1,2-diol units in the sugar form strained, planar five-membered rings, while the 1,3-diol units in the genin part form unstrained six-membered rings. The strain in the five-membered ring can be relieved by transformation of the boron atom into the tetrahedral state[29], this explains the increased polarity of the molecule. On the other hand, the 1,3-diol units in the genin are "hidden" by the trigonal boric acid, and so the polarity of molecule decreases.

The 1,3-hydroxyl pairs in the terminal sugars of the primary glycosides also form six-membered rings. Either a trigonal or a tetrahedral boron atom can be present in an unstrained six-membered ring, but the formation of a tetrahedral boron atom is influenced by the polyhydroxy components present in the ester bond[36]. In a study of hexosides, Ferrier[37] found that B–O bond fission occurred easily on addition of water or alcohol in the case of the six-membered cyclic esters. The hydrolysis is preceded by the formation of a tetrahedral boron atom[36].

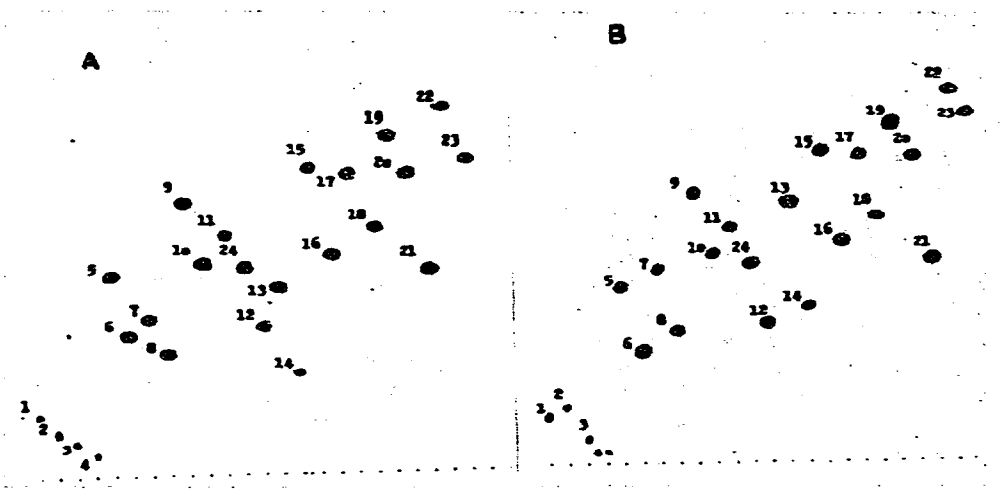


Fig. 2. TLC separation of cardenolides and their derivatives in the system chloroform–methanol–acetic acid (90:10:1) (A) without and (B) with 0.5% of boric acid on DC-Alufolien 60 (Merck) ready-made layers. In (B) we obtained the same results by the method of impregnation in the vapour phase. 1 = Lanatoside A; 2 = lanatoside B; 3 = lanatoside C; 4 = purpureaglycoside A; 5 = digitoxin; 6 = digoxin; 7 = gitoxin; 8 = 7β-hydroxydigoxin; 9 = acetyldigitoxin-β; 10 = acetyldigoxin-β; 11 = digitoxigenin; 12 = digoxigenin; 13 = gitoxigenin; 14 = 7β-hydroxydigoxigenin; 15 = 14-anhydrodigitoxigenin; 16 = 14-anhydrodigoxigenin; 17 = 14,16-dianhydrogitoxigenin; 18 = gitoxigenin 16-acetate; 19 = gitoxigenin 3,16-diacetate; 20 = gitoxigenin 14,16-phenylboronate; 21 = 7β-hydroxydigoxigenin 7,14-phenylboronate; 22 = 7β-hydroxydigoxigenin 7,14-phenylboronate 3,12-diacetate; 23 = 7β-hydroxydigoxigenin 3,12-diacetate; 24 = 4,5-dichlorofluorescein.

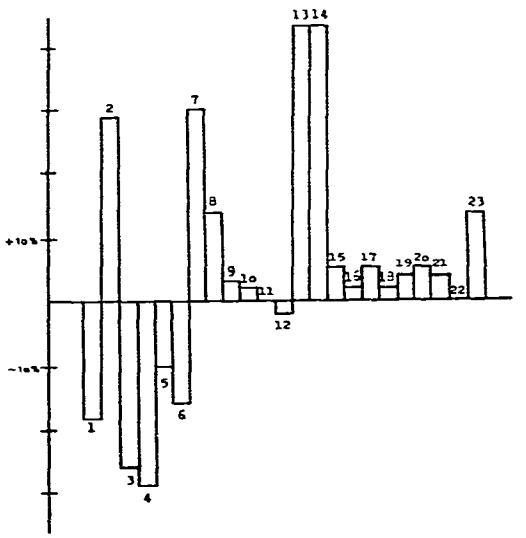


Fig. 3. ΔR_x (%) values of cardenolides and their derivatives. For numbering see Table I.

The boric acid esters of the 1,3-diol units in the genin nuclei are more stable than those in the carbohydrate chains, *i.e.*, in the former instance the boron atom is less able to assume the tetrahedral state; this was confirmed also by our studies (unpublished results).

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