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Note

Comparative study of thin-layer chromatographic techniques for separation of digoxin, digitoxin and their main metabolites

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Most of the experimental studies on the metabolism of cardiac glycosides have been carried out with labelled compounds using thin-layer chromatography (TLC)[1–5]. Studies in humans with unlabelled compounds need more sensitive techniques such as that of Watson *et al.*[6], based on gas chromatography.

At the beginning of a project on the metabolism of cardiac glycosides in different animal species, we tried to find TLC techniques that would permit a good separation of digoxin, digitoxin and their metabolites without interferences from the components of the biological materials under study (urine, faeces and bile).

Omitting the description of the results of several techniques that very soon proved to be unsatisfactory[3,7–10], we present in this paper the results obtained with five other TLC techniques that we have been using with variable success to test the purity of batches of [^{3}H]digitoxin and to analyse qualitatively and quantitatively its metabolites in rats.

Three solvent mixtures used in some of the systems described here have already been discussed by Waldi[11] and Wilson and co-workers[9,10]. These workers used them for the separation of cardiac glycosides and made four to six successive runs with the same solvent mixture. In the present work, we matched them one after the other and obtained better separations in a shorter time.

Although special systems have been suggested for the study of polar metabolites of digitoxin, we were able to obtain a good separation of the hydrolysed products with the same techniques used for the parent compounds.

EXPERIMENTAL

Reagents

All solvents were analytical-reagent grade (BDH, Poole, Great Britain). Plates were prepared with silica gel G or GF_{254} (E. Merck, Darmstadt, G.F.R.). [^{3}H]-Digitoxin, obtained from New England Nuclear, Boston, Mass., had a specific activity of 4.5 Ci/mmole. Digitoxin, digoxin and their bis- and mono-digitoxosides and aglycones were purchased from Boehringer Mannheim Corp., Mannheim, G.F.R. Epidigitoxigenin and dehydrodigitoxigenin were prepared in this laboratory from digitoxigenin[3].

Thin-layer chromatography

Freshly prepared solvent mixtures with the following compositions were used under the conditions mentioned:

System 1. Ethyl acetate–chloroform–acetic acid (90:5:5). One development of 15 cm (*ca.* 1 h).

System 1a. Composition as in system 1. Two developments of 15 cm.

System 2. Cyclohexane–acetone–acetic acid (49:49:2). One development of 15 cm (*ca.* 1 h).

System 3. First development (12 cm): cyclohexane–acetone–acetic acid (65:33:2). Second development (15 cm): cyclohexane–acetone–acetic acid (49:49:2).

System 4. First development (15 cm): cyclohexane–acetone–acetic acid (65:33:2). Second development (15 cm): ethyl acetate–chloroform (9:1).

System 5. Chloroform–isopropanol–acetone (80:5:15). Two developments of 17 cm (*ca.* 1 ½ h each).

Standard solutions of the cardiac glycosides and their metabolites (1 mg/ml) were prepared in chloroform–methanol (1:1). The materials to be chromatographed (5–10 μg) were spotted 2 cm from the bottom of the plates, except for the solvent mixture cyclohexane–acetone–acetic acid (49:49:2), for which a distance of 1.5 cm gave better separations. The tanks were allowed to equilibrate for 24 h at 20° before use.

Thin-layers of 0.25 mm thickness were prepared on 5×20 cm and 20×20 cm glass plates, using a Quickfit spreader. The layers were dried at room temperature and activated for 2 h at 120° immediately before use. For better results with systems 1 and 1a, the plates were activated at 105° for 30 min.

The spots on the developed chromatogram were rendered visible under UV light at 254 nm or after spraying with several chromogenic reagents[12]. Chloramine T–trichloroacetic acid, which, under UV light at 360 nm gave a yellow fluorescence for the digitoxin group of compounds and a blue fluorescence for the digoxin group, was used routinely.

Scintillation counting

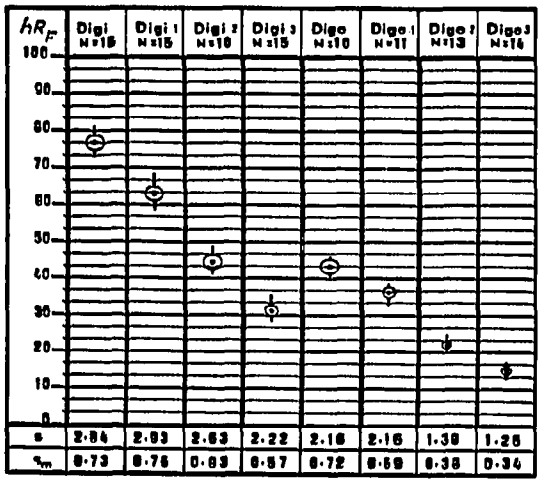
After the development and evaporation of the solvent, the plates were scraped in 2-mm sections with a zonal scraper (Analabs, North Heaven, Conn., U.S.A.) directly into vials. The radioactivity of the samples was measured in a Packard Tri-Carb liquid scintillation spectrometer, Model 3320. To each vial, 5 ml of scintillation fluid was added (100 g of naphthalene, 7 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)benzene per litre of dioxan).

RESULTS AND DISCUSSION

Fig. 1 shows the hR_F values obtained with the TLC systems in comparison with the 95% limits of confidence (circles) and the dispersion of the results (lines) in N experiments.

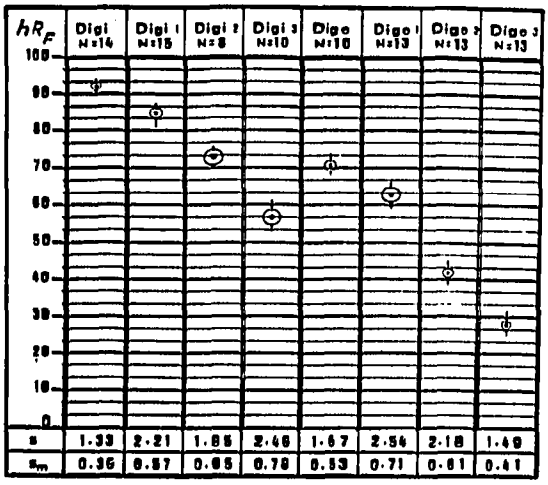
It can be seen that systems 2, 3 and 5 permitted a good separation of the eight compounds. When comparing systems 2 and 3, the latter gave a better separation of the digitoxin group from the digoxin group. System 1, with one or two developments, did not separate digoxigenin from the bisdigitoxoside of digitoxigenin. With

System 1



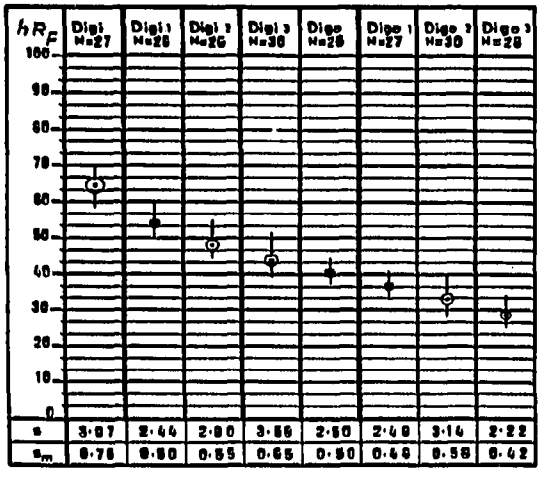
s	2·84	2·83	2·63	2·22	2·16	2·16	1·38	1·26
s_m	0·73	0·76	0·83	0·57	0·72	0·69	0·35	0·34

System 1a



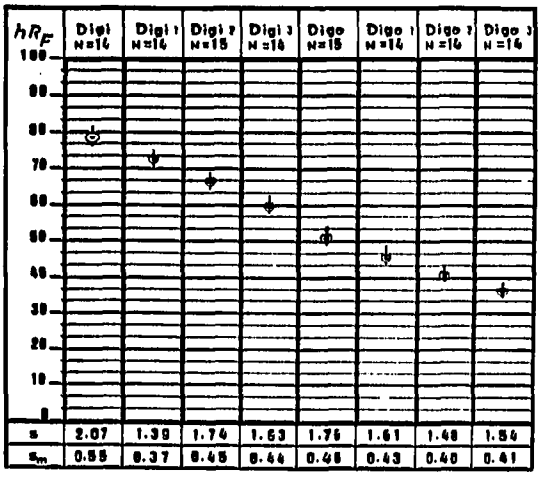
s	1·33	2·21	1·85	2·46	1·67	2·54	2·18	1·49
s_m	0·36	0·57	0·65	0·78	0·53	0·71	0·61	0·41

System 2



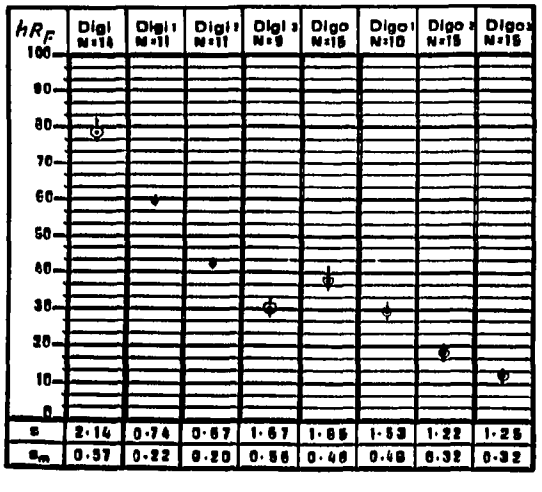
s	3·97	2·44	2·80	3·56	2·50	2·48	3·14	2·22
s_m	0·76	0·60	0·55	0·65	0·50	0·46	0·56	0·42

System 3



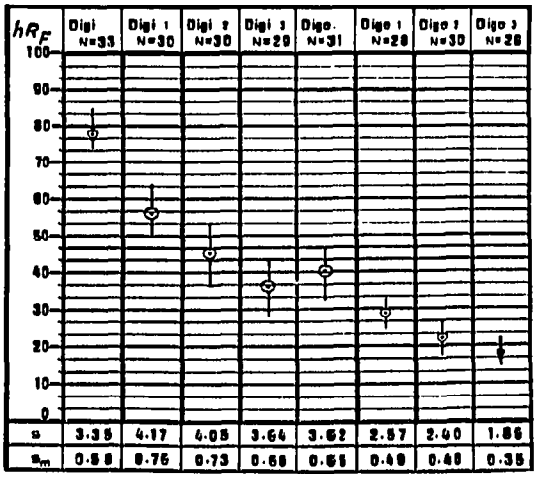
s	2·07	1·39	1·74	1·63	1·75	1·61	1·48	1·54
s_m	0·55	0·37	0·45	0·44	0·48	0·43	0·40	0·41

System 4



s	2·14	0·74	0·67	1·67	1·85	1·53	1·22	1·25
s_m	0·57	0·22	0·20	0·56	0·46	0·48	0·32	0·32

System 5



s	3·35	4·17	4·05	3·64	3·52	2·57	2·40	1·86
s_m	0·58	0·75	0·73	0·68	0·61	0·49	0·48	0·35

system 4, digitoxin and the monodigitoxoside of digoxigenin had the same R_F value (Fig. 2).

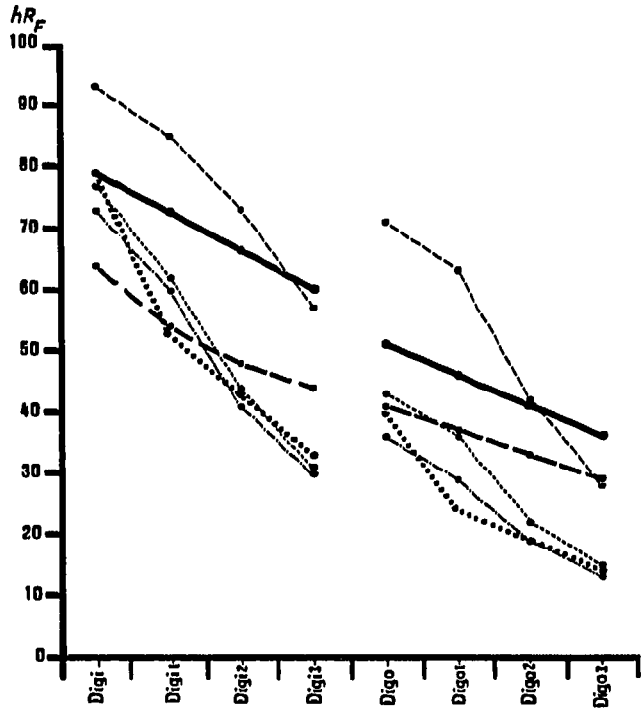


Fig. 2. hR_F values of digitoxin, digoxin and their main metabolites. -----, System 1; — —, system 1a; — —, system 2; ———, system 3; .— · — ·, system 4; · · · · · ·, system 5.

Systems 3, 4 and 5 separated epidigitoxigenin from dehydrodigitoxigenin but only system 5 separated them from digitoxigenin, with the following hR_F values: dehydrodigitoxigenin, 86, 87, 81; epidigitoxigenin, 78, 80, 74. Digitoxose gave hR_F values of 39, 33, 55 and 26 for systems 1a, 3, 4 and 5, respectively.

Comparing our results with data from the literature (Table I), the TLC techniques described here gave better separations with one (systems 1 and 2) or two developments (systems 1a, 3, 4 and 5). System 1 gives a greater distance between mono- and bis-digitoxosides of digoxigenin, which makes it very useful in the study of the metabolism of digoxin and digitoxin. Watson *et al.*[6] needed to separate the metabolites before their conversion into the heptafluorobutyrate of digoxigenin and the system of Grade and Forster[16] which they used gives a good separation but five successive runs, each of 1 h, are necessary. The same applies to the technique of Kuhlmann *et al.*[15].

Fig. 1. Chromatographic behaviour of some digitalis glycosides and aglycones in the TLC systems described. Digi= digitoxigenin; Digo= digoxigenin; Digi 1 and Digo 1= monodigitoxosides; Digi 2 and Digo 2= bisdigitoxosides; Digi 3= digitoxin; Digo 3= digoxin. N= number of determinations; S= standard deviation; s_m= mean deviation.

TABLE I

hR_F VALUES OF CARDIAC GLYCOSIDES AND AGLYCONES

System 6= cyclohexane–acetone–acetic acid (65:33:2): A, four developments[13]; B, six developments[9]; C, six developments[14]. System 7= Diisopropyl ether–methanol (9:1); four developments[6]. System 8= Diisopropyl ether–methanol (9:1); four developments; then methyl ethyl ketone–chloroform (3:1)[6]. System 9= chloroform–pyridine (6:1): D, one development[5]; E, two developments[15]. System 10= chloroform–acetone (1:1); four developments[15].

Compounds	*Systems in this paper*						*Systems from the literature*							
	1	*1a*	*2*	*3*	*4*	*5*	*6*			*7*	*8*	*9*		*10*
							A	*B*	*C*			*D*	*E*	
Digitoxigenin	77	93	64	79	79	78	53	74	74	69	—	—	—	—
Digitoxigenin monodigitoxoside	62	85	54	73	60	56	44	—	62	53	—	—	—	—
Digitoxigenin bisdigitoxoside	44	73	48	67	42	45	35	58	52	37	—	—	—	—
Digitoxin	31	57	44	60	30	36	26	52	44	24	—	—	—	—
Digoxigenin	43	71	41	51	38	40	24	50	40	27	52	52	56	81
Digoxigenin monodigitoxoside	36	63	37	46	30	29	—	40	31	23	44	44	49	75
Digoxigenin bisdigitoxoside	22	42	33	41	19	22	—	29	23	13	34	37	42	63
Digoxin	15	28	29	36	12	18	9	21	17	9	26	30	34	56

TABLE II

CHROMATOGRAPHIC ANALYSIS OF [^{3}H]DIGITOXIN (%)

The values given are the means of five determinations in each system. Relative percentages were calculated from the total radioactivity recovered from the plate.

Compounds	*System 1a*	*System 3*	*System 4*	*System 5*
Digitoxin	79.0	78.5	78.3	80.0
Digitoxigenin bisdigitoxoside	12.5	11.3	12.5	10.9
Digitoxigenin monodigitoxoside	2.5	2.3	2.3	2.4
Digitoxigenin	1.5	2.3	1.8	1.1
Unidentified compounds ($hR_F < hR_F$ digitoxin)	4.0	4.7	4.7	5.1

The purity of one batch of [^{3}H]digitoxin was assayed and Table II presents the relative percentages of the contaminants found, bisdigitoxoside of digitoxigenin being the principal contaminant. These results differ from those presented by Watson *et al.*[6] for [^{3}H]digitoxin of the same origin; this result may be due to the different batches used or to the technique employed. As shown in the graphical representation of the radioactivity corresponding to each of the 2-mm sections of the plates (Fig. 3),

no digoxigenin was present. The high percentage (29.7%) of this compound found by those workers could be due to elution of a zone corresponding to digitoxin itself. The absence of digoxigenin was also confirmed by the chromatographic analysis of the products from the hydrolysis of [^{3}H]digitoxin, in which 95% of the amount applied was recovered from the plates.

Dichloromethane extracts of urine, faeces and bile from rats were evaporated to dryness, dissolved in chloroform–methanol (1 : 1) and aliquots submitted to TLC. The hR_F values of cardiac glycosides and their metabolites applied in the same spot were not significantly modified by the biological material.

ACKNOWLEDGEMENT

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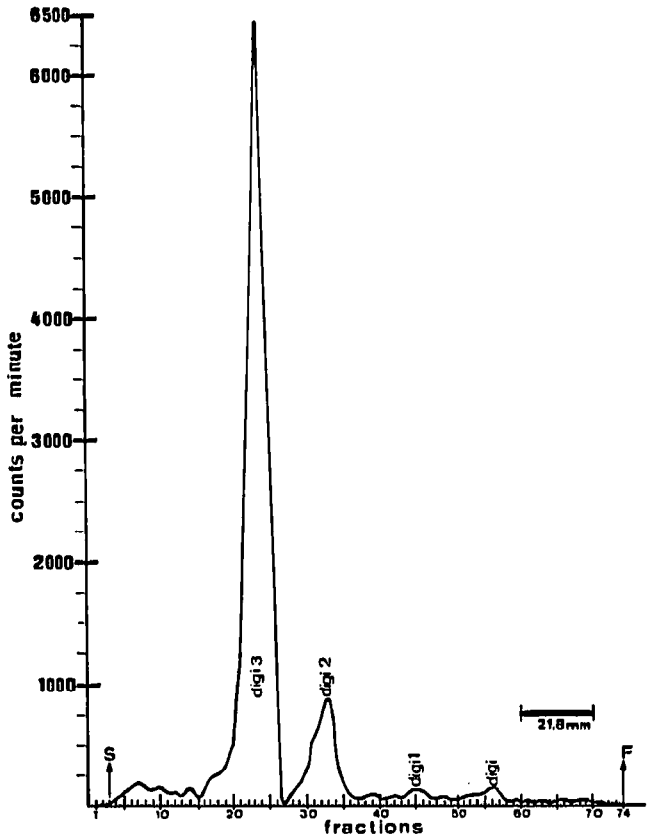

Fig. 3. Zonal scan (2 mm) of [^{3}H]digitoxin (30000 cpm) on silica gel G using system 4.

REFERENCES

1 C. L. Griffin, R. Pendleton and S. Burstein, *Biochem. Pharmacol.*, 20 (1971) 97.
2 K.-D. Kolenda, H. Lüllmann and T. Peters, *Brit. J. Pharmacol.*, 41 (1971) 661.
3 S. J. Stohs, L. A. Reinke and M. M. El-Olemy, *Biochem. Pharmacol.*, 20 (1971) 437.
4 W. Voigtlander, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 272 (1972) 46.

5 B. Beermann, *Eur. J. Clin. Pharmacol.*, 5 (1972) 28.
6 E. Watson, P. Tramell and S. M. Kalman, *J. Chromatogr.*, 69 (1972) 157.
7 R. M. Abel, R. J. Luchi, G. W. Peskin, H. L. Conn and L. D. Miller, *J. Pharmacol. Exp. Ther.*, 150 (1965) 463.
8 E. J. Johnston and A. L. Jacobs, *J. Pharmacol. Sci.*, 55 (1966) 531.
9 W. E. Wilson, S. A. Johnson, W. H. Perkins and R. E. Ripley, *Anal. Chem.*, 39 (1967) 40.
10 W. E. Wilson and J. E. Ripley, *Anal. Chem.*, 41 (1969) 811.
11 D. Waldi, in E. Stahl (Editor), *Thin-Layer Chromatography*, Springer, Heidelberg, 1st ed., 1965, p. 275
12 R. Neher, in E. Stahl (Editor), *Thin-Layer Chromatography*, Springer, Heidelberg, 2nd ed., 1969, p. 323.
13 W. H. Bulger, R. E. Talcott and S. J. Stohs, *J. Chromatogr.*, 70 (1972) 187.
14 M. J. Frey and G. M. Jacobson, *Anal. Biochem.*, 36 (1970) 78.
15 J. Kuhlmann, V. Abshagen and N. Rietbrock, *Naunyn-Schmiedeberg's Arch. Pharmacol.*, 276 (1973) 149.
16 K. Grade and W. Forster, *Biochem. Pharmacol.*, 16 (1967) 1299.