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IMPROVED THIN-LAYER CHROMATOGRAPHIC METHOD FOR SUGAR SEPARATIONS

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SUMMARY

A method is described for the utilization of pre-coated, non-impregnated silica gel thin layers in one- and two-dimensional separations of carbohydrates and related compounds. Boric and phenylboronic acids were added to the organic elution systems in different concentrations and their interactions with the sugar molecules during the chromatographic process were studied. A comparison was made between solvent systems containing boric or phenylboronic acid and systems devoid of both acids as eluents.

With boric acid-containing solvents the migration of some sugars was considerably inhibited, whereas phenylboronic acid produced an increase in the R_F values of certain sugars.

The combination of these two types of solvent in two-dimensional development resulted in the clear separation of a group of mono- and disaccharides of bioclinical interest.

INTRODUCTION

The separation of carbohydrates on silica gel thin layers usually requires impregnation of the layer with some inorganic salts such as sodium phosphate, borate, tungstate, hydrogen sulphite or acetate or boric acid. These compounds, in the form of aqueous solutions, are loaded on the silica matrix and are intended to interact with the solute molecules and so increase the selectivity of the stationary phase for the sugars. Such additives have been extensively utilized in the thin-layer chromatographic (TLC) separation of carbohydrates and their derivatives[1,2].

Recently, Jones[3] described a TLC system for the rapid separation of fructose and glucose. He used a boric acid-containing eluent on a non-impregnated silica gel thin layer supported on woven glass-fibre, and achieved a reasonable separation of the two hexoses, demonstrating that a sugar–borate complex formation equilibrium occurs with the complexing agent present as a solute in the solvent system instead of being loaded on the silica matrix.

Although the separation of more complex sugar mixtures by such a chromatographic system is, in our opinion, difficult to achieve, nevertheless other more appropriate boric acid-containing mobile phases would enable one to use pre-coated thin layers directly without any loading pre-treatment, giving considerable advantages in comparison with the TLC of sugars on home-made or on pre-coated home-loaded layers, *viz.*, (a) better reproducibility of the results; (b) higher sensitivity of the examined compounds to the detecting reagents; and (c) better tractability of the chromatograms, *i.e.*, after single or multiple elution and even after several applications of the spray reagents the chromatographic layer does not become friable and retains a firmness similar to that of the paper.

Selectivity based on a particular type of solute–solvent interaction also occurs when phenylboronic acid-containing mobile phases are used for the separation of polyhydroxy compounds, as Bourne *et al.*[4] have demonstrated in their work on the paper chromatography of sugars and poly-alcohols.

Substances with polyhydroxy groups in appropriate steric arrangements react with phenylboronic acid to give esters whose solubility in organic solvents is probably increased by the presence of the phenyl group. Phenylboronic esters of several carbohydrates have been obtained in crystalline form, and the molecular structures of some of them have been elucidated[5–7]. One of the properties of these esters is their instability in water-containing organic solvents[4]. The formation of sugar phenylboronates and their aqueous hydrolysis also seem to occur during the chromatographic process[4]. In this instance, not only the solubility of the compounds under examination in the mobile phase but also their hydrolysis equilibrium constants will affect the chromatographic separation of the sugars. Therefore, the study of new solvent systems with different amounts of phenyldihydroxyboric acid, suitable for the separation of carbohydrates and related compounds on silica or cellulose thin layers, is of interest. In addition, such mobile phases should permit the direct use of pre-coated thin layers with the above-mentioned advantages.

We have therefore examined various concentrations of boric acid and phenyldihydroxyboronic acid in different eluent systems which, in a chromatographic study of sugars of bio-clinical interest, should give good separations by a simple technique on pre-coated silica gel thin layers.

In this paper we present the results of studies on (a) quantitative aspects of sugar–boric acid interactions that can occur during the chromatographic process when this acid is present in the solvent system, (b) the effect of phenylboronic acid on the chromatographic behaviour of sugars when it is present in the mobile phase in various concentrations, and (c) the capabilities of eluents containing boric or phenylboronic acid for the one-dimensional TLC separation of sugars.

Also, we have examined the combined use of boric acid- and phenylboronic acid-containing solvent systems in the two-diemensional TLC of carbohydrates and related compounds.

EXPERIMENTAL

Materials and apparatus

All chemicals were of analytical-reagent grade and were used without further purification.

Phenylboronic acid (PHEBA) was purchased from Sigma (St. Louis, Mo., U.S.A.) and silica gel 60 thin layers pre-coated on aluminium sheets from E. Merck (Darmstadt, G.F.R.).

Aqueous boric acid solutions were, in some instances, made alkaline by the addition of monoethanolamine (mETOLA), isopropylamine (IPA) or tri-*n*-propylamine (nTPA). Isopropylammonium benzoate (IPAB) and *n*-propylammonium benzoate (nTPAB) were prepared before use by mixing equimolar amounts of benzoic acid and the corresponding basic species to give an aqueous saline solution of a given molarity and pH.

The following mixtures were used as eluents:

(1) ethyl methyl ketone–2-propanol–acetonitrile–0.5 *M* boric acid + 0.25 *M* IPA/acetic acid (40:30:20:15:0.4).

(2) ethyl methyl ketone–2-propanol–acetone–0.2 *M* boric acid (35:30:15:20).

(3) ethyl methyl ketone–*tert.* butanol–acetone–0.2 *M* boric acid + 0.1 *M* IPA (40:30:15:15).

(4) ethyl methyl ketone–*tert.* butanol–acetone–0.2 *M* boric acid + 0.1 *M* TPAB + 0.05 *M* TPA (40:30:15:15).

(5) acetone–*tert.*-butanol–0.2 *M* boric acid + 0.1 *M* ETOLA (50:30:20).

(6) acetone–*tert.*-butanol–0.2 *M* boric acid + 0.1 *M* TPAB + 0.05 *M* TPA (50:30:20).

(7) diisopropyl ether–methanol–acetic acid–water (88:12:2.0:2.0).

(8) ethyl methyl ketone–diisopropyl ether–2-propanol–pyridine–water–PHEBA (40:10:30:10:10:1.2).

TLC was effected in standard separating chambers at room temperature (18–20°).

Detection of the compounds was accomplished with the following reagents:

(A) 0.2% naphthalene-1,3-diol in 95% ethanol + concentrated sulphuric acid (1:0.5). Diphenylamine (0.4%) can be added to the ethanolic solution in order to reduce the background coloration of the chromatograms;

(B) 2% diphenylamine and aniline in acetone + 85% H_3PO_4 (5:1).

Procedure

Aqueous isopropanol solutions of standard sugars were prepared by dissolving 0.05–2.0 mg of each dry compound in 1 ml of solvent. Volumes of 2.0–2.5 μl of these solutions were spotted on the silica gel thin layers as 1.5-cm streaks at a distance of 1.0 cm from the edge to be dipped in the eluent.

The chromatograms were developed at room temperature in a saturated chamber with a run length of 10 cm.

A double run was effected when required, the layer after the first run being dried under a stream of warm air for 5–10 min. In two-dimensional TLC, the first run was performed in a phenylboronic acid-containing solvent system and the second, at right-angles to the first, in a boric acid-containing system. A rapid intermediate run with solvent system 7, in the direction of the second development, was necessary in order to wash the phenylboronic acid to the front, as this acid has proved to affect adversely the separation of sugars in the second run with boric acid-containing eluents.

After the final development was completed the TLC plates were placed in an

TABLE I

R_F VALUES OF SOME SUGARS CHROMATOGRAPHED ON SILICA GEL THIN LAYERS

Samples were spotted as streaks placed at different distances from the lower edge of the plate. Data obtained from chromatograms developed in solvent systems (b) ethyl methyl ketone–2-propanol–acetone–water (40:30:14:16) and (b_1) ethyl methyl ketone–2-propanol–acetone–0.5 *M* H_3BO_3 (40:30:14:16). Results are R_F values ± standard deviation ($n = 12$).

Sugar	*Solvent system*	*Distance from edge of plate (cm)*				
		1	*2*	*3*	*4.5*	*6*
Fructose	b	0.518 ± 0.019	0.519 ± 0.017	0.518 ± 0.018	0.510 ± 0.020	0.495 ± 0.018
Galactose		0.435 ± 0.017	0.437 ± 0.015	0.433 ± 0.016	0.429 ± 0.018	0.408 ± 0.013
Fucose		0.689 ± 0.006	0.682 ± 0.007	0.675 ± 0.010	0.651 ± 0.010	0.625 ± 0.011
2-Deoxygalactose		0.767 ± 0.006	0.757 ± 0.006	0.744 ± 0.010	0.730 ± 0.010	0.699 ± 0.018
2-Deoxyribose		0.827 ± 0.006	0.820 ± 0.007	0.806 ± 0.007	0.785 ± 0.007	0.754 ± 0.014
Fructose	b_1	0.182 ± 0.004	0.201 ± 0.004	0.233 ± 0.008	0.281 ± 0.022	0.381 ± 0.021
Galactose		0.365 ± 0.007	0.374 ± 0.008	0.388 ± 0.008	0.413 ± 0.017	0.424 ± 0.013
Fucose		0.603 ± 0.007	0.609 ± 0.009	0.623 ± 0.014	0.647 ± 0.023	0.640 ± 0.014
2-Deoxygalactose		0.724 ± 0.006	0.729 ± 0.010	0.738 ± 0.010	0.759 ± 0.022	0.707 ± 0.012
2-Deoxyribose		0.808 ± 0.008	0.811 ± 0.009	0.810 ± 0.007	0.811 ± 0.017	0.760 ± 0.015

oven at 90–100° for 10 min. The sugars were revealed as coloured bands after 2–4 min at 90°.

After marking the spots, the chromatograms could be preserved for many weeks by sandwiching them between two glass plates or two transparent plastic sheets whose borders were heat-sealed.

RESULTS

The results in Tables I–IV were obtained by spotting a mixture of fructose, galactose, fucose, 2-deoxygalactose and 2-deoxyribose as streaks at increasing distances from the lower edge of the plate. The compounds were then subjected alternately to two series of elutions: in the first series boric acid-devoid solvent systems were used, and in the second the sugars were developed with eluents of same composition as those in the first series but containing boric acid.

The effect of interactions between boric acid and the sugars is represented by $Dx_{1,2}$, which is the difference between the R_F value of a given sugar (R_Fx_1) obtained from the first elution series and the R_F value of the same sugar from the second series (R_Fx_2):

$$R_Fx_1 - R_Fx_2 = Dx_{1,2} \quad (1)$$

The effect of boric acid concentration on the migration of fructose is shown in Fig. 1. On increasing the distance of the origin from the lower edge, fructose displays significantly increased $Dx_{1,2}$ values with all of the solvents tested, as shown in Fig. 2. Galactose behaves in a similar manner to fructose, whereas fucose, 2-deoxygalactose and 2-deoxyribose show decreasing $Dx_{1,2}$ values with increase in the spotting distance.

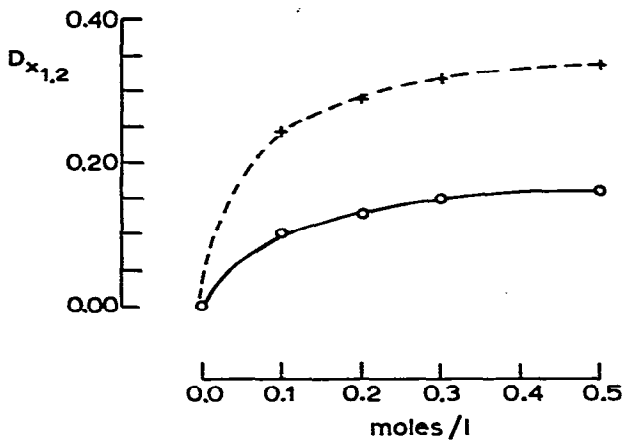

Fig. 1. Effect of boric acid concentration on the chromatographic migration of fructose. Boric acid molarities (abscissa) of the aqueous solutions being added to a given organic solvent system are plotted against the fructose $Dx_{1,2}$ values (ordinate). The curves were traced by plotting the data obtained from chromatograms developed in solvent system a (○—○) and b (+ – – – +), whose boric acid content ranged from 0.0 to 0.5 mole/l.

TABLE II

R_F VALUES OF SOME SUGARS CHROMATOGRAPHED ON SILICA GEL THIN LAYERS

Data obtained from chromatograms developed in solvent systems (a) diisopropyl ether–ethyl methyl ketone–2-propanol–water (40:4:45:15) and (a_1) diisopropyl ether–ethyl methyl ketone–2-propanol–0.5 *M* H_3BO_3 (40:4:45:15). Other details as in Table I.

Sugar	*Solvent system*	*Distance from edge of plate (cm)*				
		1	*2*	*3*	*4.5*	*6*
Fructose	a	0.230 ± 0.007	0.238 ± 0.007	0.237 ± 0.006	0.222 ± 0.011	0.213 ± 0.005
Galactose		0.179 ± 0.005	0.184 ± 0.005	0.178 ± 0.006	0.165 ± 0.008	0.155 ± 0.012
Fucose		0.339 ± 0.006	0.348 ± 0.007	0.350 ± 0.006	0.335 ± 0.011	0 322 ± 0 007
2-Deoxygalactose		0 412 ± 0.007	0.423 ± 0.007	0.427 ± 0.003	0.418 ± 0.011	0.396 ± 0.008
2-Deoxyribose		0.542 ± 0.010	0.550 ± 0.008	0.548 ± 0.004	0.532 ± 0.014	0.498 ± 0.008
Fructose	a_1	0.068 ± 0.003	0.079 ± 0.004	0.088 ± 0.003	0.110 ± 0.007	0.154 ± 0.015
Galactose		0.151 ± 0.005	0.156 ± 0.007	0.155 ± 0.004	0.148 ± 0.006	0.148 ± 0.008
Fucose		0.293 ± 0.010	0.302 ± 0.012	0.308 ± 0.005	0.315 ± 0.010	0.318 ± 0.013
2-Deoxygalactose		0.397 ± 0.009	0.397 ± 0.030	0.406 ± 0.006	0.409 ± 0.010	0.400 ± 0.012
2-Deoxyribose		0.534 ± 0.012	0.540 ± 0.011	0.542 ± 0.009	0.535 ± 0.015	0.502 ± 0.014

TABLE III

R_F VALUES OF SOME SUGARS CHROMATOGRAPHED ON SILICA GEL THIN LAYERS

Data obtained from chromatograms developed in solvent systems (c), acetone–*n*-butanol–water (50:35:15) and (c_1), acetone–*n*-butanol–0.5 *M* H_3BO_3 (50:35:15). Other details as in Table I.

Sugar	*Solvent system*	*Distance from edge of plate (cm)*				
		1	*2*	*3*	*4.5*	*6*
Fructose	c	0.488 ± 0.007	0.476 ± 0.010	0.458 ± 0.011	0.422 ± 0.015	0.359 ± 0.015
Galactose		0.389 ± 0.005	0.380 ± 0.009	0.364 ± 0.009	0.334 ± 0.013	0.275 ± 0.013
Fucose		0.646 ± 0.012	0.623 ± 0.010	0.598 ± 0.013	0.556 ± 0.017	0.501 ± 0.020
2-Deoxygalactose		0.703 ± 0.012	0.683 ± 0.012	0.659 ± 0.014	0.622 ± 0.018	0.573 ± 0.018
2-Deoxyribose		0.766 ± 0.010	0.747 ± 0.012	0.723 ± 0.012	0.707 ± 0.018	0.680 ± 0.018
Fructose	c_1	0.137 ± 0.002	0.144 ± 0.004	0.152 ± 0.002	0.162 ± 0.003	0.189 ± 0.009
Galactose		0.287 ± 0.004	0.286 ± 0.006	0.280 ± 0.004	0.269 ± 0.007	0.247 ± 0.012
Fucose		0.542 ± 0.008	0.534 ± 0.009	0.516 ± 0.008	0.498 ± 0.013	0.478 ± 0.013
2-Deoxygalactose		0.648 ± 0.010	0.635 ± 0.011	0.616 ± 0.008	0.594 ± 0.012	0.579 ± 0.018
2-Deoxyribose		0.738 ± 0.011	0.724 ± 0.013	0.708 ± 0.009	0.697 ± 0.016	0.681 ± 0.016

TABLE IV

R_F VALUES OF SOME SUGARS CHROMATOGRAPHED ON SILICA GEL THIN LAYERS

Data obtained from chromatograms developed in solvent systems (d), acetone–water (85:15) and (d_1), acetone–0.5 *M* H_3BO_3. Other details as in Table I.

Sugar	*Solvent system*	*Distance from edge of plate (cm)*				
		1	*2*	*3*	*4.5*	*6*
Fructose	d	0.508 ± 0.008	0.529 ± 0.016	0.556 ± 0.020	0.615 ± 0.022	0.624 ± 0.033
Galactose		0.379 ± 0.006	0.379 ± 0.010	0.427 ± 0.011	0.491 ± 0.020	0.537 ± 0.035
Fucose		d*	d	d	d	d
2-Deoxygalactose		d	d	d	d	d
2-Deoxyribose		0.887 ± 0.009	0.880 ± 0.009	0.875 ± 0.010	0.876 ± 0.009	0.837 ± 0.017
Fructose	d_1	0.130 ± 0.003	0.154 ± 0.003	0.205 ± 0.008	0.367 ± 0.018	0.546 ± 0.016
Galactose		0.334 ± 0.004	0.368 ± 0.006	0.430 ± 0.010	0.598 ± 0.024	0.635 ± 0.058
Fucose		d	d	d	d	d
2-Deoxygalactose		d	d	d	d	d
2-Deoxyribose		0.906 ± 0.008	0.911 ± 0.013	0.901 ± 0.015	0.913 ± 0.016	0.876 ± 0.021

* d = too diffuse bands.

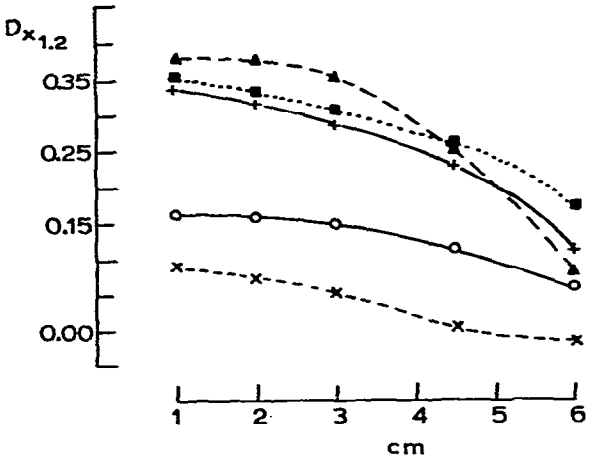

Fig. 2. Gradient concentration of boric acid formed in the adsorbent layer through the eluent flow direction, as revealed by the inhibition effect of boric acid on the fructose migration. Fructose $Dx_{1,2}$ values are plotted *versus* the distances in (abscissa) of sample application streaks from the lower edge of the plate. Curves were obtained by plotting the data of chromatograms developed in solvent systems a_1 (○—○), b_1 (+—+), c_1 (■....■) and d_1 (▲---▲). All solvent systems contained 0.5 mol/l of boric acid. The plot of the fucose $Dx_{1,2}$ values *versus* the distance from the origin is also shown (×.....×).

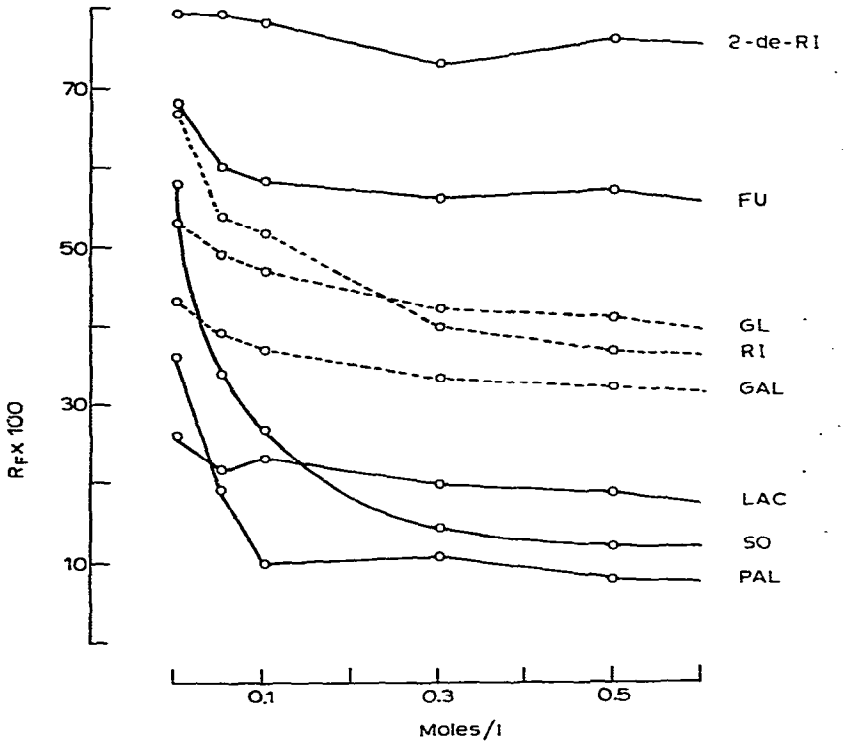

Fig. 3. Effect of boric acid concentration on the chromatographic migration of some sugars. Boric acid molarities (abscissa) of the aqueous solutions being added to the eluting solvent system are plotted against the $R_F \times 100$ values coordinate of each sugar. The solvent system employed as eluent was isopropanol–ethyl methyl ketone–acetonitrile–0.5 *M* H_3BO_3 + 0.25 *M* IPA (30:40:10:20).

TABLE V

R_F VALUES OF SOME SUGARS IN PHENYLBORONIC ACID-DEVOID AND PHENYLBORONIC ACID-CONTAINING SOLVENT SYSTEMS

*Solvent system**	*1A*	*1B*	*1C*	*2A*	*2B*	*3A*	*3B*	*Solvent system**	*4A*	*4B*	*5A*	*5B*	*6A*	*6B*
Ethyl acetate	60.0	60.0	60.0	60.0	60.0	60.0	60.0	Ethyl acetate	55.0	55.0	50.0	50.0	60.0	60.0
2-Propanol	30.0	30.0	30.0	—	—	20.0	20.0	2-Propanol	35.0	35.0	30.0	30.0	30.0	30.0
Methanol	—	—	—	15.0	15.0	—	—	0.5 *M* CH_3COONa	10.0	10.0	—	—	—	—
Pyridine	—	—	—	—	—	10.0	10.0	1% 2-Propylamine	—	—	—	—	10.0	10.0
Acetic acid	—	—	—	15.0	15.0	—	—	0.5 *M* 2-Propyl-ammonium acetate	—	—	15.0	15.0	—	—
Water	10.0	10.0	10.0	10.0	10.0	10.0	10.0	Methanol	2.0	2.0	5.0	5.0	—	—
PHEBA**	—	0.5	2.0	—	1.0	—	1.0	PHEBA**	—	1.0	—	1.0	—	1.0
Sugar	*R_F value*													
α-Methyl glucoside	0.36	0.37	0.38	0.57	0.53	0.58	0.56		0.51	0.47	0.61	0.57	0.46	0.46
Xylose	0.37	0.40	0.48	0.55	0.58	0.54	0.55		0.50	0.53	0.56	0.53	0.43	0.51
Ribose	0.37	0.83	0.89	0.53	0.88	0.55	0.88		0.47	0.87	0.56	0.79	0.43	0.81
Arabinose	0.24	0.24	0.27	0.44	0.43	0.40	0.38		0.34	0.32	0.41	0.40	0.28	0.32
2-Deoxyribose	0.48	0.54	0.60	0.64	0.63	0.72	0.71		0.68	0.67	0.77	0.73	0 62	0.67
Glucose	0.18	0.18	0.21	0.40	0.40	0.32	0.30		0.26	0.25	0.35	0.34	0.21	0.25
Galactose	0.14	0.18	0.26	0.34	0.45	0.24	0.28		0.18	0.26	0.29	0.32	0.16	0.25
Fructose	0.21	0.26	0.34	0.41	0.47	0.36	0.40		0.29	0.35	0.39	0.42	0.25	0.35
Sorbose	0.24	0.35	0.72	0.47	0.72	0.40	0.54		0.34	0.54	0.43	0.48	0.28	0.52
Tagatose	0.31	0.48	0.66	0.48	0.67	0.48	0.60		0 41	0 63	0.50	0.58	0.35	0.41
6-Deoxygalactose	0.33	0.32	0.38	0.51	0.50	0.53	0.50		0.45	0.45	0.54	0.52	0.38	0.43
Sedoheptulose	0.18	0.19	0.22	0.37	0.37	0.34	0.33		0.32	0.28	0.36	0.34	0.21	0.25
Mannoheptulose	0.20	0.24	0.33	0.40	0.48	0.33	0.38		0 28	0 36	0.37	0.40	0.23	0.33
Sucrose	0.03	0.10	0.11	0.28	0.27	0.24	0.21		0.17	0.16	0.27	0.25	0.13	0.14
Lactose	0.03	0.04	0.04	0.17	0.20	0.10	0.10		0.04	0.06	0.09	0.12	0.04	0.05
Palatinose	0.07	0.07	0.08	0.25	0.28	0.17	0.17		0.11	0.11	0.19	0.20	0.09	0.10
Raffinose	0.01	0.03	0.03	0.14	0.22	0.05	0.07		0.02	0.03	0.05	0.08	0.02	0.04

* The amounts of organic solvents are expressed as volumes (ml). 0.5 *M* CH_3COONa, 1% 2-propylamine and 0.5 *M* 2-propylammonium acetate consist of aqueous solutions of pH 8.45, 11.58 and 10.85, respectively.

** PHEBA (phenylboronic acid) is expressed in % (w/v).

The migration of sugars in the presence of phenylboronic acid was tested using several solvent systems containing the acid in different concentrations. The results are reported in Tables V and VI.

The variation of the R_F values of some sugars with boric acid concentration in the eluent is shown in Fig. 3. Figs. 4–6 show the chromatograms of one-dimensional separations of carbohydrates obtained with boric acid- or PHEBA-containing eluents.

The two-dimensional separation shown in Fig. 7 was obtained with a boric acid- and a PHEBA-containing solvent.

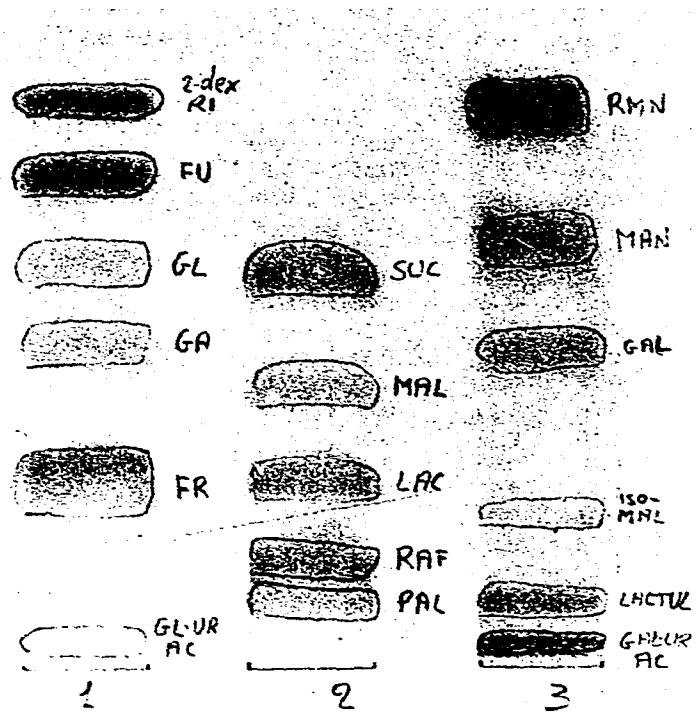

Fig. 4. One-dimensional, single-elution chromatography of some sugars. The separation was performed with solvent system 1. Development for 60–70 min. Abbreviations: LAC = lactulose; RAF = raffinose; PAL = palatinose; iso-MAL = isomaltose; RMN = rhamnose; 2-dex RI = 2-deoxgribose; FU = fulose; GL = glucose; GA = galactose;; FR = fructose; GL-UR-AC = glucuronic acid; GAL-UR-AC = galacturonic acid; SUC = sucrose; MAL = maltose;

DISCUSSION

Boric acid

In order to exploit the sugar–borate complexing reaction in the TLC of carbohydrates, boric acid should be present in adequate concentrations all over the adsorbent layer, as is usually attained by the impregnation of silica gel with an inorganic salt.

In this regard, when boric acid is prepared by dissolving it in the eluent, the flow of the mobile phase can form a concentration gradient along the plate, which varies according to the composition of the eluent.

TABLE VI

R_F VALUES OF SOME SUGARS IN PHENYLBORONIC ACID-DEVOID AND PHENYLBORONIC ACID-CONTAINING SOLVENT SYSTEMS

*Solvent system**	*7A*	*7B*	*8A*	*8B*	*9A*	*9B*	*Solvent system**
Diisopropyl ether	—	—	—	—	20.0	20.0	Diisopropyl ether
Benzene	20.0	20.0	20.0	20.0	—	—	Benzene
Acetone	40.0	40.0	40.0	40.0	40.0	40.0	*tert.*-Butanol
n-Butanol	30.0	30.0	30.0	30.0	30.0	30.0	Methanol
Water	15.0	15.0	—	—	—	—	Acetic acid
1.5 *M* IPAB	—	—	15.0	15.0	15.0	15.0	Water
PHEBA**	—	2.0	—	2.0	—	2.0	PHEBA**
Sugar	*R_F value*						
α-Methyl glucoside	0.42	0.40	0.63	0.60	0.59	0.56	
Xylose	0.42	0.54	0.61	0.65	0.59	0.55	
Ribose	0.44	0.91	0.60	0.91	0.59	0.88	
Arabinose	0.31	0.29	0.54	0.47	0.52	0.46	
2-Deoxyribose	0.61	0.64	0.64	0.58	0.65	0.64	
Glucose	0.23	0.21	0.48	0.43	0.48	0.40	
Galactose	0.19	0.28	0.42	0.48	0.40	0.45	
Fructose	0.28	0.36	0.51	0.50	0.50	0.47	
Sorbose	0.29	0.73	0.53	0.81	0.53	0.67	
Tagatose	0.34	0.69	0.57	0.71	0.57	0.67	
6-Deoxygalactose	0.41	0.40	0.60	0.59	0.64	0.57	
Sedoheptulose	0.24	0.25	0.50	0.41	0.50	0.38	
Mannoheptulose	0.24	0.32	0.48	0.45	0.49	0.43	
Sucrose	0.15	0.12	0.43	0.37	0.44	0.37	
Lactose	0.05	0.06	0.25	0.23	0.26	0.22	
Palatinose	0.11	0.10	0.38	0.27	0.38	0.26	
Raffinose	0.04	0.05	0.20	0.23	0.19	0.20	

* See Table V.
** See Table V.

For detecting the boric acid distribution along the silica gel layer, we estimated the inhibitory effect of the acid on the migration rate of fructose, expressed by $Dx_{1,2}$ (see eqn. 1), relative to the increasing distance of the sample application streak from the lower edge of the plate.

Fructose proved to be suitable for this type of study because it gives a linear graph of R_F *versus* borate concentration (see Fig. 2), so that it is possible to determine, although only approximately, the amount of boric acid that, at a given distance on the plate, causes a certain decrease in the R_F or $Dx_{1,2}$ value.

Using such a criterion, we found that most of the solvent systems tested, from binary to ternary systems in which the major constituent was an organic solvent that can dissolve boric acid appreciably, as well as these composed mainly of a solvent that does not dissolve the acid, display a gradient distribution of boric acid along the adsorbent layer, as shown in Fig. 1, where the plots are derived from data in Tables I–IV. Therefore, whichever eluent is used, the boric acid concentration along the whole layer is sufficient to produce the complexing of sugars and to enhance the separation selectivity of the chromatographic system.

10A	*10B*	*Solvent system**	*11A*	*11B*	*12A*	*12B*	*13A*	*13B*
20.0	20.0	Ethyl methyl ketone	30.0	30.0	35.0	35.0	35.0	35.0
20.0	20.0	Benzene	20.0	20.0	15.0	15.0	15.0	15.0
30.0	30.0	2-Propanol	40.0	40.0	35.0	35.0	35.0	35.0
10.0	10.0	Acetone	—	—	—	—	5.0	5.0
5.0	5.0	Methanol	—	—	5.0	5.0	—	—
15.0	15.0	0.5 *M* IPAB	15.0	15.0	15.0	15.0	16.0	16.0
—	2.0	PHEBA**	—	2.0	—	2.0	—	2.0
0.60	0.60			0.70	0.76	0.70	0.70	0.66
0.58	0.88		0.74	0.74	0.74	0.73	0.69	0.69
0.56	0.96		0.72	0.93	0.70	0.92	0.68	0.94
0.49	0.52		0.57	0.51	0.57	0.55	0.54	0.50
0.65	0.72		0.90	0.81	0.85	0.81	0.83	0.77
0.48	0.48		0.51	0.47	0.50	0.50	0.48	0.43
0.43	0.64		0.42	0.54	0.43	0.55	0.42	0.50
0.49	0.84		0.53	0.63	0.53	0.63	0.52	0.55
0.50	0.98		0.56	0.84	0.57	0.83	0.54	0.76
0.52	0.96		0.63	0.86	0.64	0.81	0.61	0.82
0.56	0.56		0.70	0.66	0.70	0.67	0.65	0.60
0.45	0.48		0.50	0.47	0.50	0.50	0.47	0.44
0.47	0.79		0.51	0.64	0.50	0.64	0.48	0.56
0.38	0.36		0.44	0.38	0.45	0.42	0.45	0.37
0.26	0.29		0.20	0.19	0.23	0.23	0.22	0.20
0.34	0.39		0.34	0.26	0.35	0.31	0.37	0.24
0.24	0.33		0.17	0.22	0.16	0.23	0.19	0.19

Phenylboronic acid

In accordance with the possibility of the formation of sugar phenylboronate esters during TLC, the data in Tables V and VI show significant changes in the R_F values of several carbohydrates according to the amount of phenylboronic acid in the eluent, suggesting that an interaction between the sugar molecules and phenylboronic acid does occur in the mobile phase.

More specifically, of the carbohydrates and related compounds examined, ribose, galactose, fructose, sorbose, tagatose and mannoheptulose exhibit considerable increase in their R_F values according to the content of phenylboronic acid in the eluent. On the other hand, other sugars, such as methyl glucoside, glucose, arabinose, sedoheptulose, fucose and 2-deoxyribose, generally are only slightly affected by the presence of phenylboronic acid, as their R_F values are not appreciably altered (Tables I–IV).

It seems reasonable to assume that sugar–benzeneboronate formation equilibria are probably set up during chromatography with differing stability constants for the different sugars. Of the chemical and physical factors that are likely to influence

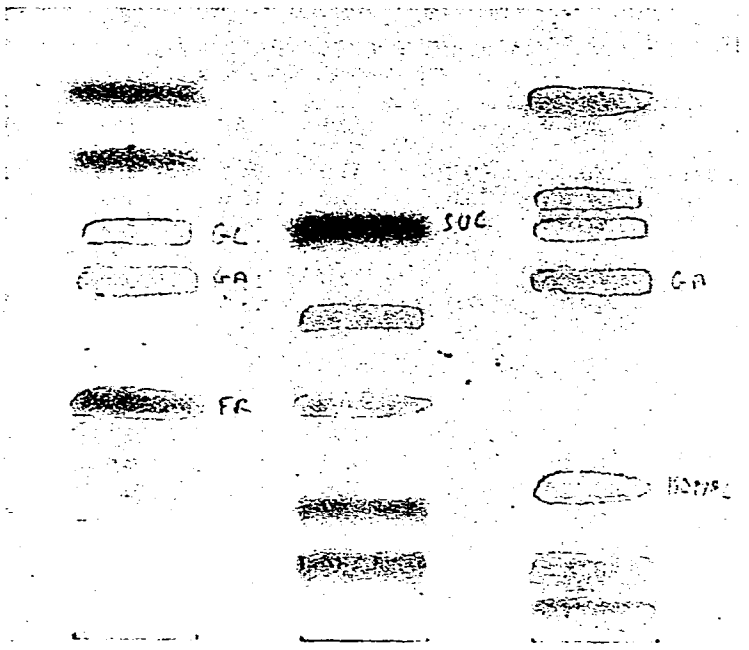

Fig. 5. One-dimensional, dual-elution chromatography of some sugars. The first development was performed with solvent system 1 for 20 min and the second development, after having dried the layer for 5 min under a warm stream of air, was performed with the same solvent system for 60–70 min.

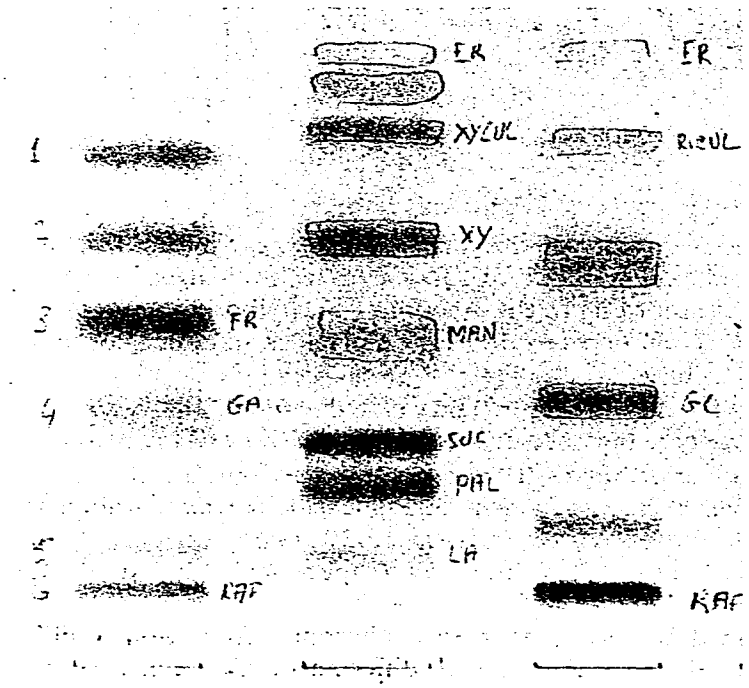

Fig. 6. One-dimensional, single-elution chromatography of some sugars in solvent system 8. Development for 70–75 min.

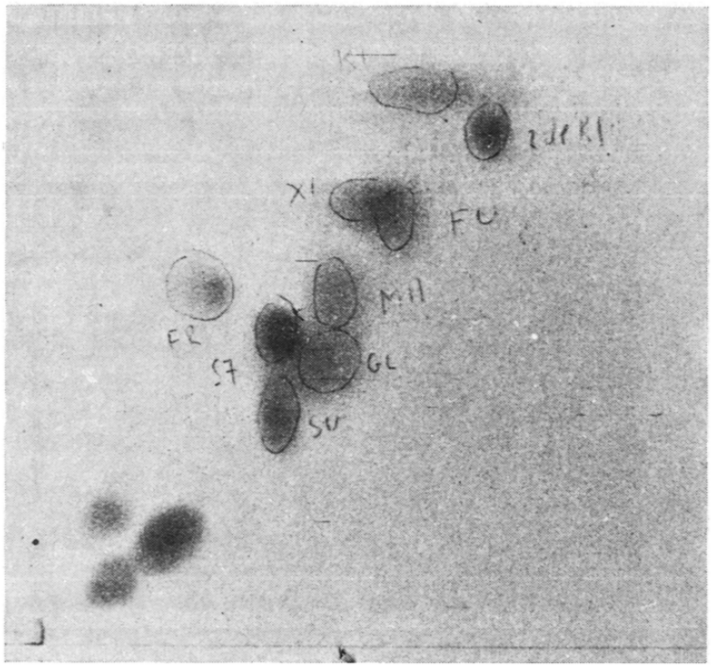

Fig. 7. Two-dimensional, three-fold elution chromatography of a mixture of standard sugars. The first and second elution carried out with solvent systems 8 and 7, respectively, were performed in the same direction, while the third elution (with solvent system 1) was carried out at right-angles to the first two. Total development time, 180–190 min.

such constants, *e.g.*, steric conformation of the sugar molecules and pH of the eluting solvent systems, the composition of the mobile phase has a considerable effect (Tables I–VI). In this regard, it is worth noting that benzene-containing eluents give the greatest differences in the R_F values of the sugars, probably because the stability of certain sugar–benzene boronates is enhanced by the presence of this solvent.

Separation

The capability of the method described for the separation of sugars has been considered with respect to our specific interest in the chromatography of carbohydrates in clinical biochemistry. From this point of view the clear resolution of some groups of sugars, such as glucose–galactose–fructose, glucose–lactose–isomaltose, lactose–galactose and glucose–xylulose, is essential in the analysis of the cases of pentosurias, hexosurias and disaccharidurias where one or more of these sugars are involved.

In the method we have presented, the addition of boric acid to the eluting system makes it possible to separate simultaneously, in a one-dimensional, single-elution mode, most of these sugars, as is shown in Figs. 4 and 5. It is also worth noting that boric acid-devoid solvent systems yield a discrete separation of some mono- and disaccharides.

The use of PHEBA does not always give satisfactory separations, mainly because of adverse tailing of the spots of some sugars, especially when benzene is present in the eluent. In an attempt to eliminate or reduce this tailing, we found some eluent

compositions in which benzoate play a special role. Eluents in which both benzene and 2-propylammonium benzoate, or an other benzoate salt, were present not only gave undiffused spots but also yielded sufficiently different R_F values (see Table VI). Fig. 6 depicts the sugars that can be resolved by PHEBA-containing eluents.

More complex mixtures of carbohydrates are well separated by two-dimensional TLC with PHEBA- and boric acid-containing eluents.

We conclude that this technique is an improvement over previous TLC methods for sugars, inasmuch as the use of pre-coated, unimpregnated thin layers, in comparison with layers that have been subjected to pre-treatment procedures, *i.e.*, impregnation[1,2] or loading of the adsorbent with inorganic salts,[8,9] permits one to obtain (a) good reproducibility, (b) high sensitivity towards the compounds of interest and (c) good tractability of the chromatograms.

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