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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATIONS ON DIHYDROXYBORYL-AGAROSE

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SUMMARY

This paper (which is part of a series of articles on high-performance liquid chromatographic separations on agarose columns) describes the chromatographic behaviour of catecholamines, nucleosides, aminoacylated and non-aminoacylated tRNA, and glycosylated and non-glycosylated hemoglobins on agarose matrices derivatized with *m*-aminophenylboronic acid. These matrices primarily bind molecules with two vicinal hydroxy groups in the *cis*-configuration. The possible existence of secondary electrostatic and hydrophobic interactions has been studied with the aid of diagrams showing retention time as a function of pH and buffer concentration.

INTRODUCTION

Under mild, reversible conditions, boronates readily form complexes with molecules containing two vicinal hydroxy groups in the *cis* configuration (*cis*-1,2-diols). In borate buffers, many neutral sugars and polyalcohols therefore become negatively charged and can be separated by electrophoresis[1,2] and ion-exchange chromatography[3–5]. By immobilizing boronates on chromatographic matrices of cellulose[6], polyacrylamide[7,8], agarose[9–11], dextran[12], polystyrene[13] and silica[14,15], or by coating polychlorotrifluoroethylene with hydrophobic boronates[11], both low- and high-molecular-weight substances, such as nucleosides, nucleotides, catecholamines, tRNAs, and glycoproteins, have been separated[16].

Recently we have shown that small, rigid agarose beads can be used as a high-performance liquid chromatographic (HPLC) matrix for molecular-sieve, hydrophobic-interaction, affinity, and ion-exchange chromatography[17–23]. In this paper we show that such beads are also effective for boronate HPLC. We chose catecholamines, nucleosides, tRNA, and glycosylated hemoglobins as model substances.

EXPERIMENTAL

Agarose (EEO = −0.17) was from IBF Villeneuve, La Garenne, France;

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1,4-butanediol diglycidyl ether from EGA-Chemie, Steinheim, F.R.G.; 1,1'-carbonyldiimidazole from Merck-Schuchardt, Darmstadt, F.R.G.; divinylsulphone and *m*-aminophenylboronic hemisulphate from Fluka, Buchs, Switzerland; adenosine, cytidine, guanosine, uridine and HEPES (N-(2-hydroxyethyl)piperazine-N'-2-ethanesulphonic acid) from Sigma, St. Louis, MO, U.S.A.; thymidine from Schwarz Bio-Research, New York, U.S.A.; L-tyrosine from Kebo, Stockholm, Sweden; noradrenaline from Apoteket Kronan, Uppsala, Sweden; L-3,4-dihydroxyphenylalanine (dopa) from ICN Nutritional Biochemicals, Cleveland, OH, U.S.A.

Agarose beads were prepared by a technique described in ref. 24, and were cross-linked with divinylsulphone under conditions[25] that give gels with better chromatographic properties than those prepared by the previous method[26].

tRNA from *Escherichia coli* was labelled with [^{14}C]leucine, and the radioactivity was determined in the chromatographic fractions according to the methods of Patel *et al.*[27]. Aminophenylboronic acid was prepared from the hemisulphate as described by Singhal *et al.*[11].

Coupling of m-aminophenylboronic acid to agarose with 1,1'-carbonyldiimidazole

The procedure was essentially that described by Bethell *et al.*[28]. Agarose beads (12%, cross-linked) with diameters ranging from 3 to 10 μm, were centrifuged at 400 *g* for 10 min. Then 3 g of the sedimented agarose were washed with dioxane to remove water and then activated at room temperature for 15 min with 0.12 g of 1,1'-carbonyldiimidazole, dissolved in 5 ml of dioxane. The agarose was then washed three times with 20-ml portions of dioxane, and 5 ml of 1 *M* sodium carbonate (pH 10) containing 400 mg of *m*-aminophenylboronic acid were added to the sedimented agarose. The suspension was stirred overnight at room temperature, and washed first with a solution of 1 *M* sodium chloride and then with water. The product obtained is referred to as dihydroxyboryl-agarose I. Washing in this and the following procedure was performed by centrifugations at 400 *g* for 5–10 min.

Coupling of m-aminophenylboronic acid to agarose by 1,4-butanediol diglycidyl ether

The coupling method was based on the oxirane method introduced by Sundberg and Porath[29]. First, 3 g of cross-linked 15% agarose beads (diameter *ca.* 15 μm), sedimented as described above, were suspended in 2.5 ml of 1 *M* sodium hydroxide and 10 mg of sodium borohydride. Then 1 ml of 1,4-butanediol diglycidyl ether was added dropwise. The suspension was stirred at room temperature for 10 h. After the epoxy-activated gel was washed with water, 400 mg of *m*-aminophenylboronic acid, dissolved in 3 ml of a 1 *M* sodium carbonate solution (pH 10) was added. The gel suspension was stirred for 48 h at room temperature and then deactivated by 0.1 ml of 2-mercaptoethanol overnight at 4°C. The gel was washed first with 1 *M* sodium chloride and then with water. The agarose derivative thus obtained is referred to as dihydroxyboryl-agarose II.

We have also used another method for coupling *m*-aminophenylboronic acid to agarose, which will be described elsewhere[30]. The product obtained is referred to as dihydroxyboryl-agarose III.

The UV-monitor (2158 SD), HPLC pump (2150) and HPLC controller (2152) were from LKB Produkter, Bromma, Sweden; the integrators C-RIA and 3390 A from Shimadzu, Kyoto, Japan, and Hewlett-Packard, Stockholm, Sweden, respectively.

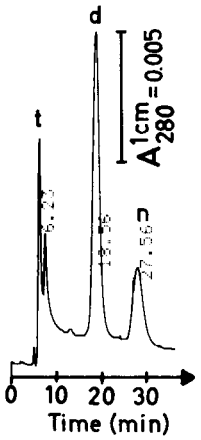

Fig. 1. A test mixture run on an HPLC column of dihydroxyboryl-agarose I. Peaks: t = tyrosine; d = dopa; n = noradrenaline.

Determination of the capacity of the dihydroxyboryl-agarose

We chose adrenaline as a test substance on the basis of preliminary experiments, which showed that it could be adsorbed on dihydroxyboryl-agarose at pH 8 and desorbed at pH 6. Adrenaline was dissolved in 0.1 *M* sodium phosphate buffer (pH 8.0) at a concentration of 0.33 mg/ml and pumped into a column (8.5 × 0.6 cm I.D.) of dihydroxyboryl-agarose I, equilibrated with the same buffer. When the eluate showed constant absorption at 280 nm, *i.e.*, when the column had become saturated with adrenaline, the non-adsorbed adrenaline was washed out with the buffer. The adsorbed adrenaline was then released by 0.1 *M* sodium phosphate buffer (pH 6.0), and the amount determined spectrophotometrically by measuring the volume and the absorption at 280 nm of the eluate. The amount of adrenaline (equal to the capacity of the column for adrenaline) thus calculated was 3 μmol of adrenaline per millilitre of packed gel.

In the same way, the capacities of dihydroxyboryl-agarose II and dihydroxyboryl-agarose III were determined to be 5 and 1 μmol of adrenaline per millilitre of gel, respectively.

A preliminary test of the chromatographic behaviour of the dihydroxyboryl-agarose

A 2-μl volume of a test sample, containing *ca.* 1.5 μg of tyrosine, 1.0 μg of dopa and 1.0 μg of noradrenaline, was applied to a dihydroxyboryl-agarose I column (6 × 0.6 cm I.D.; agarose concentration, 12%; bead diameters, 3–10 μm), equilibrated with 0.1 *M* sodium phosphate buffer (pH 8.0)*. Elution was effected with a 0.1 *M* sodium phosphate buffer (pH 6.5) at a flow-rate of 0.3 ml/min (pressure, 25 bar). The material distribution in the chromatogram in Fig. 1 was determined by absorbance measurements at 280 nm.

Separation of nucleosides

Chromatography was performed isocratically in 0.1 *M* sodium phosphate buf-

* The reasons for the use of this test mixture are given in the discussion section.

fer (pH 8.0) at a flow-rate of 0.3 ml/min on the same matrix as used in the experiment described above. The inner diameter of the chromatographic tube was 0.6 cm and the matrix height 8 cm. The 2-μl sample contained *ca.* 0.5 μg of adenosine, 0.4 μg of cytidine, 0.4 μg of guanosine, 0.2 μg of thymidine, and 0.3 μg of uridine. The material distribution was determined by absorbance measurements at 260 nm (Fig. 2a). The nucleosides could not be separated in a control experiment on non-derivatized agarose.

A similar experiment was performed on dihydroxyboryl-agarose II. The column (8.6 × 0.6 cm I.D.) consisted of beads of 15% agarose with a diameter of *ca.* 15 μm. The sample volume was 5 μl and the flow-rates were 0.8 (Fig. 2b) and 1.5 ml/min (Fig. 2c).

The three chromatograms presented in Fig. 2 show that rapid analyses of nucleosides can be obtained on these HPLC columns.

The experiment on dihydroxyboryl-agarose II was repeated at pH 7.4, 7.8, and 8.6 (0.1 *M* sodium phosphate buffers). The retention times determined for each nucleoside were plotted against pH (Fig. 3a). In another set of experiments the retention times were determined at different concentrations of the sodium phosphate buffer at constant pH 8.0 (for solubility reasons the 0.5 *M* buffer was prepared from potassium phosphate). The result is given in Fig. 3b.

Separation of aminoacylated and non-aminoacylated tRNA

For this experiment, we packed a column (0.6 cm I.D.) to a height of 8.8 cm with 15% cross-linked beads (diameter *ca.* 15 μm) of dihydroxyboryl-agarose II. After equilibration of the column with 0.05 *M* HEPES (pH 8.5), containing 0.05 *M* magnesium chloride and 20% 2-propanol, 2 μl of ^{14}C-Leu-tRNA was injected. The same buffer was passed through the column for 25 min at a flow-rate of 0.2 ml/min (pressure, 2 atm), followed by 0.1 *M* sodium acetate buffer (pH 4.5) containing 0.03 *M* magnesium chloride and 20% 2-propanol. Fractions of 0.4 ml were collected and immediately frozen. After they had thawed, the radioactivity was determined in the acid-precipitable material. As expected, only the non-aminoacylated tRNA (which contains a terminal 2'-,3'-diol) was adsorbed at pH 8.5 and could be released at pH 4.5 (Fig. 4).

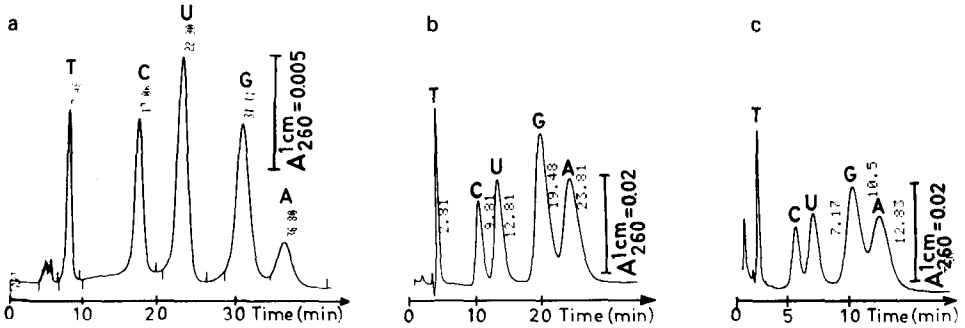

Fig. 2. Separation of nucleosides by HPLC on dihydroxyboryl-agarose. Peaks: T = thymidine; C = cytidine; U = uridine; G = guanosine; A = adenosine. Experiment a was performed on a column (8.0 × 0.6 cm I.D.) of dihydroxyboryl-agarose I; bead diameter, 3–10 μm; flow-rate, 0.3 ml/min; pressure 22 bar. Experiments b and c were performed at flow-rates of 0.8 and 1.5 ml/min, respectively, on a column (8.6 × 0.6 cm I.D.) of dihydroxyboryl-agarose II; pressure, 6 and 12 bar; bead diameter, *ca.* 15 μm.

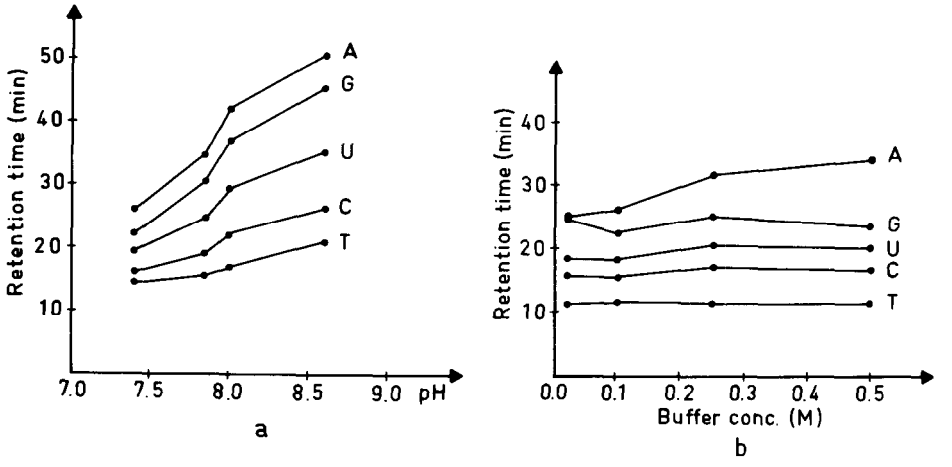

Fig. 3. The retention times of nucleosides plotted against pH (a) and the concentration (b) of the eluent.

Separation of glycosylated from non-glycosylated hemoglobin

Centrifuged erythrocytes from a diabetic patient were washed three times with ten volumes of isotonic saline and were then lysed in five volumes of distilled water. A 2-μl volume of the hemolysate was applied to a 11 × 0.6 cm I.D. column of

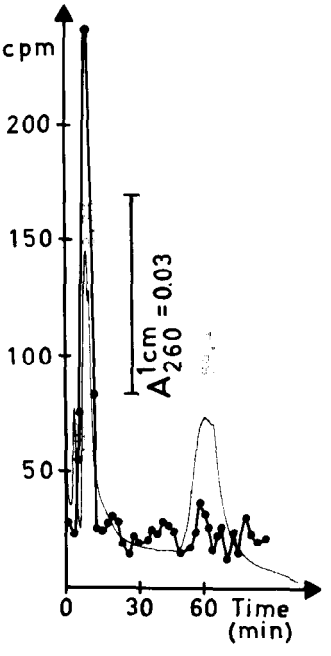

Fig. 4. HPLC separation of aminoacylated and non-aminoacylated tRNA on dihydroxyboryl-agarose II. The first non-retarted peak corresponds to aminoacylated tRNA (^{14}C-Leu-tRNA) and the second to non-aminoacylated tRNA, the terminal 2',3'-hydroxy groups of which interact with the hydroxy groups of the boryl ligand. The continuous line represents the absorbance of the effluent at 260 nm, and the points indicate the radioactivity of collected fractions.

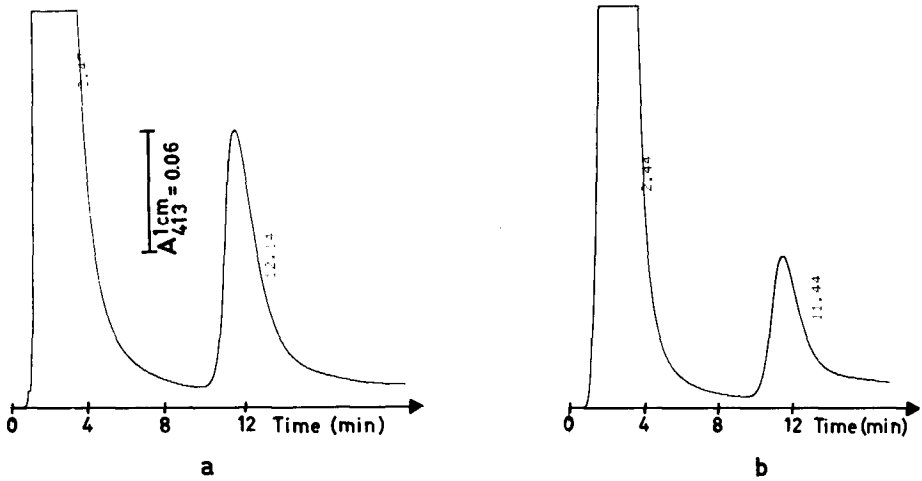

Fig. 5. HPLC separation of non-glycosylated (the first peak) from glycosylated (the second peak) hemoglobin on dihydroxyboryl-agarose III. (a) Hemoglobin from a diabetic individual; (b) hemoglobin from a non-diabetic individual.

dihydroxyboryl-agarose III beads with diameters ranging from 15 to 20 μm. Non-adsorbed material was washed out during 5 min at a flow-rate of 1 ml/min (pressure, 8 atm) with 0.05 *M* HEPES (pH 8.5) containing 0.05 *M* magnesium chloride, the same buffer being used for equilibration of the column. The glycosylated hemoglobin was displaced from the column with the same buffer, containing 0.1 *M* sorbitol. The chromatogram is shown in Fig. 5a. When the experiment was repeated with a hemoglobin sample from a non-diabetic patient, the area of the second peak was considerably lower (Fig. 5b). In fact, a Shimadzu integrator C-R1A showed that the ratio between the amount of hemoglobin in the second peak and that in the first peak was in Fig. 5b about 50% smaller than in Fig. 5a. The same result was obtained in an analysis based on ion-exchange chromatography and performed at the University Hospital.

DISCUSSION

The only difference in structure between tyrosine and dopa is that tyrosine contains one hydroxy group and dopa two vicinal hydroxy groups. The latter compound —but not the former— should, accordingly, interact with dihydroxyboryl-agarose. A mixture of these substances should therefore be suitable to reveal whether the *m*-aminophenylboronic acid has reacted with agarose to give a product with the separation mechanism expected. For this reason, we used a test sample containing these two compounds together with noradrenaline, since the latter catecholamine is known to be more strongly adsorbed on boronate columns than dopa[32]. The chromatogram in Fig. 1 shows that tyrosine, dopa and noradrenaline are eluted in the expected order.

An increase in pH to increase the concentration of the anionic form of the dihydroxyboryl group (p*K ca.* 9) should increase the retention times, since the anionic

form is active in the formation of complexes with *cis*-diols[16]. The result in Fig. 3a is as expected, except in the case of thymidine, which is not a *cis*-diol and should, accordingly, not be affected by a pH change. The pH effect observed for this deoxyribonucleoside thus reflects interactions other than those based on diol reactions. (Perhaps it is caused by the known weak interaction between monoalcohols and boronate[16].) One can therefore expect such secondary interactions to play a role also in the adsorption of cytidine, uridine, guanosine and adenosine in Fig. 2, and they are superimposed on the diol complex with the boronate ion. However, the secondary interactions are not electrostatic in nature (*i.e.* in the form of an attraction between the positively charged nucleosides and the negative boronate ion), since the retention times should in that case decrease with an increase in ionic strength, and Fig. 3b does not show this effect. This figure also indicates that hydrophobic interaction is not an important parameter in the separation of nucleosides (adenosine possibly excepted), since this type of interaction is enhanced by an increase in the salt concentration[33]. (The possibility still exists that electrostatic and hydrophobic interactions balance each other to a zero effect, but this is very unlikely to occur over the whole range of salt concentrations studied.)

The above considerations indicate that the mechanism for the separation of nucleosides is to a very large extent based on the formation of diol complexes of different strengths. (The possible contribution of hydrogen bonds and charge-transfer reactions is difficult to establish experimentally, but is probably small.) This conclusion is certainly also valid for many other low molecular weight substances, but perhaps not for macromolecules, where the secondary interactions can be of importance in multi-point attachment. The relevance of this type of attachment can be illustrated by the fact that proteins, but not amino acids or small peptides, are adsorbed by hydrophobic interaction to octyl-agarose with a normal degree of substitution[33].

The matrix to which the phenylboronic acid is coupled can affect the separations. Cytidine and uridine separate on dihydroxyboryl-substituted gels of agarose (Fig. 2), cellulose[6], and polyacrylamide[34], but not of silica[15]. Some of the differences between the chromatograms obtained with these adsorbents may, perhaps, be ascribed to the fact that different methods for coupling of the ligand give spacer arms which differ in length and chemical structure.

It has been suggested that an increase in the salt concentration should affect the pK value of the boronate group sufficiently to increase the concentration of boronate anions, thereby increasing the retention volume[16]. If this explanation were correct all *cis*-diol compounds should increase their retention volumes with an increase in buffer concentration. Fig. 3b shows that this is not the case.

Columns capable of separating aminoacylated tRNA from non-acylated species can be employed for the purification of isoacceptor tRNAs[35]. Fig. 4 shows that *m*-aminophenylboronic acid, immobilized on HPLC matrices of agarose, has this property. The column was packed with dihydroxyboryl-agarose II, since the boronate group in this matrix is attached to agarose via a longer spacer arm than in dihydroxyboryl-agarose I and should therefore be more accessible for macromolecules and, accordingly, give a higher capacity for non-acylated tRNA. (This expected difference in capacity between these agarose derivatives was confirmed by experiments.) When higher capacities are required, for instance in preparative runs, the ligand

concentration should be increased, by increasing the concentration of *m*-aminophenylboronic acid in the coupling reaction. Propanol was used in the buffer in the tRNA experiment to suppress possible hydrophobic interactions[36], which may be more severe during chromatography of macromolecules than with low-molecular-weight compounds, as discussed above. An obvious advantage of the HPLC technique described here is that the aminoacylated tRNA is exposed to pH 8.5 for only a short time, since it is eluted in less than 10 min, which means negligible hydrolysis of the amino acid/tRNA bond. (If so desired, the elution time can be shortened considerably by increasing the flow-rate.) Another elegant way to suppress the hydrolysis reaction is to use a boronic acid derivative that ionizes at a pH compatible with the stability of the aminoacyl bond[35].

It is interesting to note that previous attempts to adsorb adenosine and uncharged tRNA to dihydroxyboryl-agarose failed[11], whereas the gels described here show a strong interaction.

Thymidine shows very little retention in boronate chromatography (Fig. 2), as would be expected for a deoxyribonucleoside lacking the required vicinal diol structure. In reversed-phase chromatography, however, it is strongly retarded[37]. The ribonucleosides (cytidine, uridine, guanosine, adenosine) on the other hand, are eluted in the same order in reversed phase and boronate chromatography (see Fig. 2 and refs. 37 and 38). Furthermore, the correspondig bases are eluted from the reversed-phase column in the same order as are the nucleosides[39]. Since in this technique the retention increases with increasing hydrophobicity of the solute, one can conclude that the strength of the diol complexes in boronate chromatography also increases with an increase in hydrophobicity of the bases. We have no ready explanation for this: maybe it is only a coincidence (observe, however, that the separations of the nucleosides are not due to hydrophobic interactions with the support, as pointed out above in connection with the discussion of Fig. 3b).

Our finding that the hemoglobin peak eluted with 0.1 *M* sorbitol (the second peak in Fig. 5) is larger for diabetic (Fig. 5a) than non-diabetic (Fig. 5b) hemolysate confirms the results of earlier experiments, which showed that this peak corresponds to glycosylated hemoglobins[31,40,41]. (The adsorbent used in those experiments was a commercial phenylboronate gel, Matrex Gel PBA, available from Amicon, Lexington, MA, U.S.A., and intended for conventional low-pressure chromatography.) The HPLC technique outlined here probably has some advantages and is therefore being explored further.

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