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New aliphatic boronate ligands for affinity chromatography

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ABSTRACT

Aliphatic boronates have not been used as ligands in boronate affinity chromatography, possibly because of their low stability. To fill this void, we have prepared three different boronate esters: 1-chloro-5-(3-dimethylaminophenoxy)pentane-1-boronate, 1-thiourea-5-(3-dimethylaminophenoxy)pentane-1-boronate and 1-acetamido-5-(3-dimethylaminophenoxy)pentane-1-boronate, the latter two of which have a hetero atom coordinated with the boron, creating a tetrahedral boronate. These were coupled to the hydroxylic matrix and their ability to interact with various catechols was tested. Chloroboronate gel bound a number of catechol derivatives, except DL-DOPA, quantitatively, but was rather unstable. Thiourea-boronate gel, on the other hand, was relatively stable, although chromatography using it failed. Acetamido-boronate gel did not bind catechol derivatives quantitatively, but did retard them differentially, a property which could be used for their separation. Acetamido-boronate gel was also more stable than chloro-boronate gel, although it was not stable enough for unlimited application.

INTRODUCTION

The use of boronate chromatography for the separation of nucleic acid components and carbohydrates was first reported by Weith *et al.* in 1970 [1]. Since then immobilized boronates have been used successfully for the separation of many various biomolecules containing vicinal (1,2 or 1,3) diols including nucleic acids [2–4], nucleosides [2,5–8], catechols [8–10], carbohydrates [1,8,11] and glycoproteins [12–15]. Boronate matrices are commonly used for the assay of glycosylated hemoglobin in blood, which is an important element in the diagnosis of diabetes mellitus [16–18].

The most widely used boronate matrix is 3-aminophenyl boronic acid coupled to agarose, which gives the best results in terms of operational pH and minimal secondary interactions. However, many other ligands have been tested to improve the properties of boronates, chiefly, to lower their pK_a and to eliminate hydrophobic adsorption, which are the major limitations to their utilization [8,9,19–21]. These new boronate matrices were based on the introduction of electron withdrawing groups on the aromatic ring of phenylboronates.

Because of their reported instability, aliphatic boronates have, to date, never been utilized for boronate chromatography. However, Matteson and co-workers have prepared a tetrahedral aliphatic boronic ester containing a thiourea moiety as a internal Lewis base and used it to form an ester with catechol [22,23] and an anhydride with malonic acid [24], both of which were stable under acidic conditions. Later, Matteson and Schaunberg synthesized a series of compounds with similar properties [25].

Brown and Vara Prasad [26] have investigated

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aliphatic boronic esters complexed with amino diols. ^{11}B NMR showed that coupling of the boron with the amine function gives a chemical shift of 12–14 ppm. These investigations demonstrated that both electronic and steric factors are influential in forming B–N bonds.

It is known that the natural tendency of boron to accept an electron pair favors the stability of tetrahedral boronic esters in contrast to trigonal forms, particularly in aqueous solution under neutral or acidic conditions. For this reason internally coordinated tetrahedral boronic acids may be very useful for affinity chromatography. Recently, Biedrzycki *et al.* [27] described a series of new aliphatic boronate esters, containing such internal coordinating agents, which were prepared as potential ligands for affinity chromatography. Beyond possessing hetero atoms, which coordinate with the boron to create a more stable tetrahedral structure, these boronate derivatives contained a dimethylaminophenoxypentane functional group for immobilization on a solid phase via a diazonium bond. In this study, we have attempted to utilize these boronate esters for boronate affinity chromatography.

EXPERIMENTAL

Chemicals and materials

Sepharose Cl-4B, 4-aminothiophenol and sodium nitrite were purchased from Sigma. 1,4-Butanediol diglycidyl ether, 1-bromo-4-chlorobutane, butyllithium, 1,1,1,3,3,3-hexamethyldisilazane, trimethylborate, catechol, pyrogallol, DL-DOPA and dopamine were obtained from Aldrich. Azomethine H was a product of Pierce.

The ligands, pinacol 1-chloro-5-(3-dimethylaminophenoxy)pentane-1-boronate, pinacol 1-thioura-5-(3-dimethylaminophenoxy)pentane-1-boronate, and pinacol 1-acetamido-5-(3-dimethylaminophenoxy)pentane-1-boronate were synthesized as described below.

Plastic chromatography columns (2 ml volume, 0.5 cm diameter) from Bio-Rad were used for chromatographic experiments. The concentration of catechol derivatives was spectroscopically measured at 280 nm on a Perkin-Elmer Coleman 124 D double beam spectrophotometer.

Synthesis of pinacol 1-chloro-5-(3-dimethylaminophenoxy)pentane-1-boronate, pinacol 1-thiourea-5-(3-dimethylaminophenoxy)pentane-1-boronate and pinacol 1-acetamido-5-(3-dimethylaminophenoxy)-pentane-1-boronate (see Fig. 1)

Pinacol 1-chloro-5-(3-dimethylaminophenoxy)pentane-1-boronate (CIPP). Dichloromethaneboronic acid (**I**) was synthesized as reported by Ranthke *et al.* [29].

Dichloromethaneboronic acid (170 mmol) was dissolved in 120 ml of dry benzene, then 170 mmol of pinacol were added and the mixture was stirred at room temperature until there was no solid left. After drying over $MgSO_4$, the product was distilled at 25 mmHg. The major fraction, which distilled at 80–82°C, was dichloromethaneboronic acid pinacol ester (**II**). The yield was 52% (16.5 g, 88 mmol).

3-Dimethylaminophenol (7 g, 51 mmol) was dissolved in ethanol, containing 1.17 g (52 mmol) of sodium and refluxed for 30 min. Then 8.75 g of 1-bromo-4-chlorobutane were added and refluxed for 9 h under nitrogen. The dark purple liquid, which was obtained after evaporating the solvent, was further purified by low-pressure silica chromatography (230–400 mesh, 60 Å, 50 × 2.5 cm column) using diethyl ether–light petroleum (b.p. 35–60°C) (1:5) to yield 5.5 g (25 mmol) of **III**. Thin-layer chromatography on Al_2O_3 plates was used to monitor the purification.

III (5.4 g) and 0.576 g of Mg (24 mmol) were added to 40 ml of dry tetrahydrofuran (THF) and refluxed for 3 h. The resulting Grignard reagent was added dropwise to 5.2 g of **II** in 50 ml dry THF at −75°C. The mixture was allowed to come to room temperature and was held overnight under nitrogen. After sequentially adding 100 ml of diethyl ether and 100 ml of hexane to precipitate by-products, the solvent was filtered and evaporated yielding 4.7 g of yellow liquid, which was CIPP (**IV**). *m/z* calculated for $C_{19}H_{31}BO_3NCl$ (M^+): 367.2086; *m/z* found: 367.2070. ^1H NMR (C^2HCl_3) δ [ppm *vs.* tetramethylsilane (TMS)] 1.30 (s, 12H, CH_3C), 1.50–2.10 (m, 6H, CH_2), 2.93 (s, 6H, NCH_3) 3.42 (t, J = 7.2 Hz, 1H, CHB), 3.95 (t, J = 6.2 Hz, 2H, OCH_2) 6.10–7.30 (m, 4H aromatic).

Pinacol 1-acetamido-5-(3-dimethylaminophenoxy)pentane-1-boronate. 1,1,1,3,3,3-Hexamethyldisilazane (2.03 g, 12.6 mmol) in 40 ml of THF was treated with 12.6 mmol of butyllithium in hexane

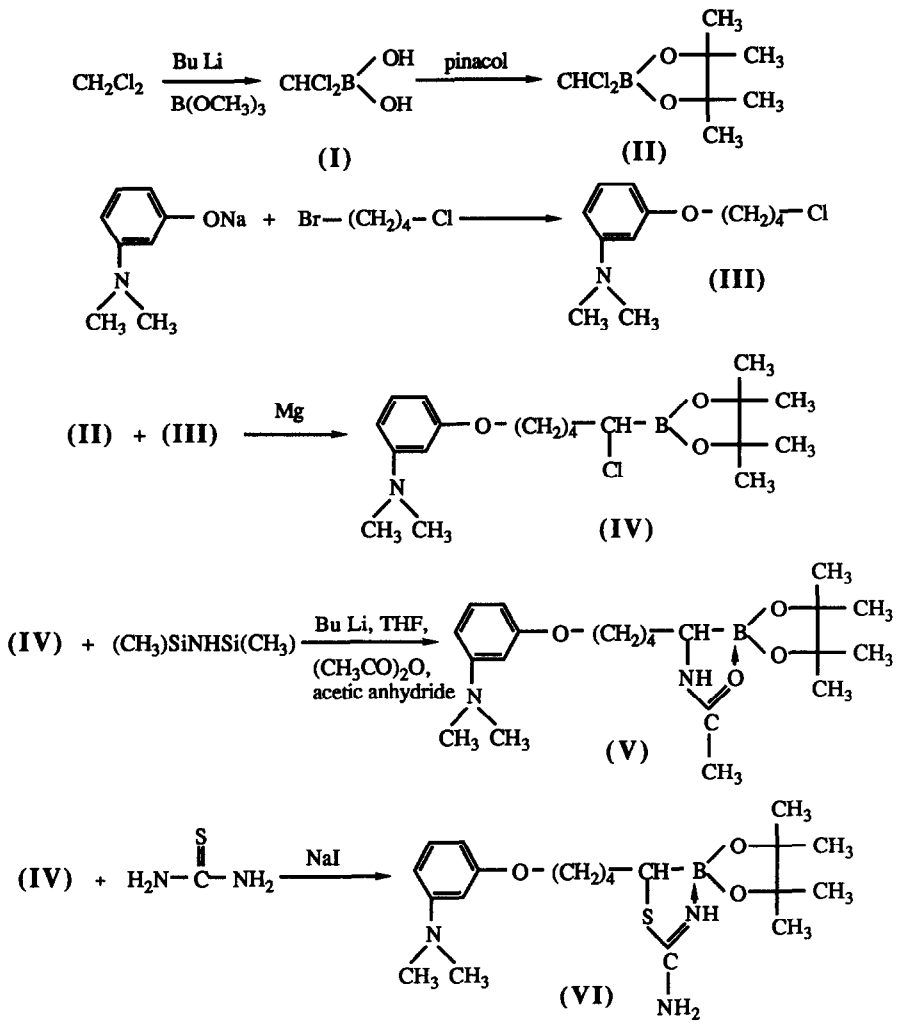

Fig. 1. Synthesis of aliphatic boronate ligands. Pinacol 1-chloro-5-(3-dimethylaminophenoxy)-1-boronate (**IV**), pinacol 1-acetamido-5-(3-dimethylaminophenoxy)-1-boronate (**V**) and pinacol 1-thiourea-5-(3-dimethylaminophenoxy)-1-boronate (**VI**).

(5.04 ml of 2.5 *M* solution) at 0°C. The mixture was cooled to −70°C and 12 mmol of **IV** at 10 ml of THF was added at once. The mixture was allowed to reach room temperature and mixed for 5 h. The solution was cooled to −70°C and 3.86 g (37.8 mmol) of acetic anhydride were added. This was followed by adding 25.2 mmol of acetic acid. The mixture was kept overnight at 25°C and concentrated under vacuum. The residue was dried at 80°C under 0.02 Torr for 1 h and the resulting crude product was extracted three times with 10 ml of hexane. The first fraction was discarded, and the last two fractions were evaporated to yield 1.55 g (33.1%) of **V**. *m/z* calculated for $C_{21}H_{35}BO_4N_2$ (M^+): 390.2690; *m/z* found: 390.2683. ^1H NMR (C^2HCl_3) δ (ppm *vs.* TMS) 1.18 (s, 6H, CH_3C), 1.19 (s, 6H, CH'_3C), 1.31–1.76 (m, 6H, CH_2), 2,06 (s, 1H, CH_3CO) 2.38 (br. m, 1H, CHB), 2.90 (s, 6H, CH_3N) 3.92 (dt, J = 6.2 Hz, 2H, OCH_2), 6.24–6.36 (m, 3H aromatic) 7.10 (t, J = 8.2 Hz, 1H arom.) 9.74 (br. s, 1H, NH).

Pinacol 1-thiourea-5-(3-dimethylaminophenoxy) pentane-1-boronate. Sodium iodide (0.69 g, 4.61 mmol) was added to a solution of 1.69 g (4.61 mmol)

of (IV) in 40 ml of dry acetonitrile. The resulting suspension was mixed for 4 h at room temperature, after which thiourea (0.35 g, 4.61 mmol) was added and mixing was continued at room temperature for 72 h. After this, 2 g of sodium carbonate were added and the resulting suspension was stirred for additional 24 h. This mixture was then filtered and concentrated under vacuum. Extraction with diethyl ether, filtration and precipitation with hexane gave 1.22 g (3.18 mmol, 69%) of **VI**. ^{1}H NMR (C^2HCl_3) δ (ppm *vs.* TMS) 1.09 (s, 3H, CH_3C), 1.12 (s, 3H, CH_3C), 1.13 (s, 3H, CH_3C), 1.15 (s, 3H, CH_3C), 1.37–2.00 (m, 6H, CH_2), 2.38 (m, 1H, SCHB), 2.95 (s, 6H, CH_3N) 3.93 (dt, J = 6.4 Hz, 2H, OCH_2) 6.25 (s NH), 6.86–6.94 (m, 3H aromatic) 7.23–7.28 (m, 2H arom.).

Immobilization of aliphatic boronate ligands

Sepharose CL-4B (10 ml) was washed and then suspended in 7.2 ml of 1.0 *M* sodium hydroxide containing 5.8 mg sodium borohydride per ml and 7.2 ml of 1,4 butandiol diglycidyl ether. The mixture was stirred slowly at room temperature for 11 h [27]. The resulting activated gel was thoroughly washed with 500 ml of water.

The epoxy activated Sepharose CL-4B was added to 23 ml of a 4-aminothiophenol solution in buffer-ethanol (1:1, pH 10.0). The buffer contained 0.2 *M* sodium bicarbonate and 0.5 *M* sodium chloride. The coupling of 4-aminothiophenol to Sepharose CL-4B was performed at 30°C for 8 h [28]. After this, the activated gel was washed sequentially with 500 ml of binding buffer and 500 ml of 1.0 *M* sodium carbonate. To remove unreacted *p*-aminothiophenol, the gel was further washed with 500 ml of distilled water, then 3 × 250 ml of 30:70, 60:40, 80:20 ethanol–water, and finally with 500 ml of ethanol (100%). The Sepharose was subsequently washed with 3 × 250 ml of 80:20, 60:40, 30:70 ethanol–water and then with 500 ml of distilled water and 250 ml of 1 m*M* hydrochloric acid.

Wet 4-aminothiophenol activated Sepharose CL-4B (10.0 ml) was diazotized by reaction with 30 ml of 1.2 *M* hydrochloric acid and 30 ml of 1% sodium nitrite solution at 0°C for 30 min. After this, it was washed with 250 ml of distilled water and incubated in 30 ml of ethanol–water (2:1) containing 0.6 g of pinacol 1-chloro-5-(3-dimethylaminophenoxy)pentane-1-boronate, pinacol 1-thiourea-5-(3-dimethylaminophenoxy)pentane-1-boronate and pinacol 1-acetamido-5-(3-dimethylaminophenoxy)pentane-1-boronate and 2-3 drops of 1 *M* sodium carbonate. The reaction was allowed to proceed at room temperature for 5 h. The activated gel was washed four times with 100 ml each of 70:30, 60:40, 30:70, 20:80 ethanol–water and finally with 200 ml of distilled water [28]. The concentration of boronate was measured in the reaction mixture before and after reaction and in wash solutions using the Azomethine H method [30].

To hydrolyze the resulting pinacol ester, 30 ml of 1% acetic acid were added to 10 ml of wet activated Sepharose CL-4B. The reaction was allowed to continue for 20 min at room temperature after which the gel was washed with 500 ml of distilled water.

The activated gels were stored in 0.05 *M* potassium phthalate, pH 5.0, 0.05 *M* potassium phosphate monobasic, pH 7.0, or 0.05 *M* ammonium acetate, pH 8.5. The resulting gels contain 5–15 μmol of ligand per ml wet agarose.

Affinity chromatography

A 5-ml small plastic column (0.5 cm) was packed with 1 ml of the 1-chloro-5-(3-dimethylaminophenoxy)pentane-1-boronate agarose (chloro-1-boronate gel), 1-thiourea-5-(3-dimethylaminophenoxy) pentane-1-boronate agarose (thiourea-boronate gel) or 1-acetamido-5-(3-dimethylaminophenoxy)pentane-1-boronate agarose (acetamido-boronate gel) and equilibrated with wash buffer (0.25 *M* ammonium acetate, 0.1 *M* NaCl, pH 8.5). After application of 2.5 μmol of catechol, or one of its derivatives, in 50 μl of distilled water, the column was washed with the wash buffer using 1 ml fractions either (a) eight times or (b) until no further catechol derivatives emerged. The elution was accomplished using 1-ml fractions of (a) 0.1 *M* Tris, 0.2 *M* sorbitol, pH 8.5; (b) 1% acetic acid; (c) 0.25 *M* ammonium acetate, 0.1 *M* NaCl, 0.2 *M* sorbitol pH, 8.5; (d) distilled water. If elution was performed with sorbitol, the columns were regenerated before reuse by treatment with 1% acetic acid.

For the separation of DL-DOPA and catechol, the column (0.5 cm) was packed with 3 ml of the acetamido-boronate gel and equilibrated with 0.25 *M* ammonium acetate, 0.1 *M* NaCl, pH 8.5. The 50 μl mixture containing 2.5 μmol of both compounds was applied to the column and washed with the same

buffer until no further DL-DOPA emerged. After that, elution was performed using 1-ml fractions of 1% acetic acid.

Measurement of the stability of chloro-boronate, thiourea-boronate and acetamido-boronate gels under various pH

The freshly prepared gels (1 ml) were suspended in 5 ml of either 0.05 *M* potassium phthalate buffer, pH 5.0, 0.05 *M* potassium phosphate monobasic buffer, pH 7.0, or 0.05 *M* ammonium acetate buffer pH 8.5. The determination of boron [29] was performed in the buffer above the stored gels. The concentration of boron in solution above the gels corresponded with the amount of decomposed ligand. The boron assays were performed until all of the ligand was released from the matrix.

RESULTS AND DISCUSSION

The chromatograms of DL-DOPA, dopamine, pyrogallol and catechol (see Fig. 2) on chloro-boronate gel are depicted in Fig. 3. DL-DOPA was retarded, while the other catechol derivatives were bound tightly to the column. The low affinity of DL-DOPA is probably caused by repulsion by its weakly ionized carboxylic function. Complete elution of catechol, pyrogallol and dopamine was achieved with 0.1 *M* Tris, 0.2 *M* sorbitol (pH 8.5) or 1% acetic acid. Elution with either 0.1 *M* Tris, 0.2 *M* sorbitol (pH 8.5), or 1% acetic acid produced sharp peaks. If elution was performed with sorbitol, the

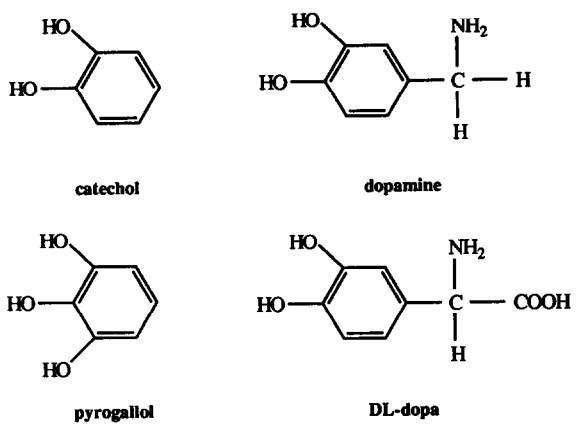

Fig. 2. Structure of catechol derivatives.

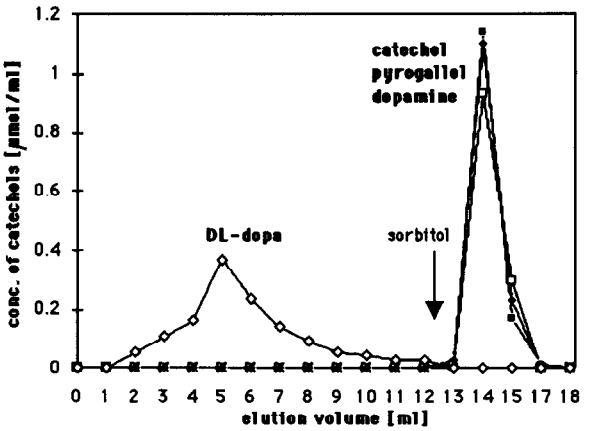

Fig. 3. Chromatography of catechol derivatives on chloro-boronate gel. The column (1 ml volume, 0.5 cm diameter) was packed with 1 ml of gel. Catechol or its derivatives (2.5 μmol in 50 μl of distilled water) was applied and the column was washed with 0.25 *M* ammonium acetate, 0.1 *M* NaCl, pH 8.5 (13 × 1 ml). Final elution was performed using 0.2 *M* sorbitol, 0.1 *M* Tris, pH 8.5.

columns had to be regenerated before reuse by treatment with 1% acetic acid. Elution using distilled water and/or binding buffer containing 0.2 *M* sorbitol wasn't effective and catechol derivatives were removed from the column as broad band.

None of the catechol derivatives were bound to

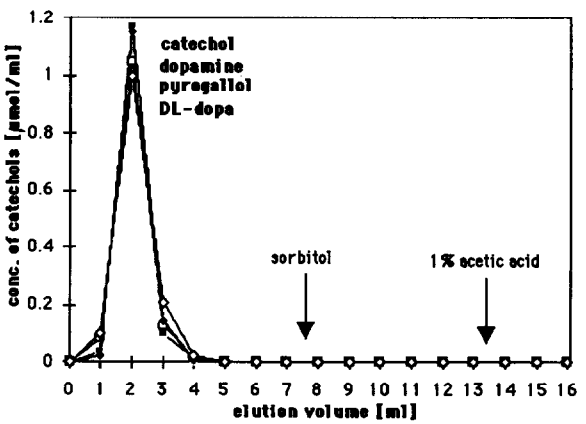

Fig. 4. Chromatography of catechol derivatives on thiourea-boronate gel. The column (1 ml volume, 0.5 cm diameter) was packed with 1 ml of gel. Catechol or its derivatives (2.5 μmol in 50 μl of distilled water) was applied and the column was washed with 0.25 *M* ammonium acetate, 0.1 *M* NaCl, pH 8.5 (7 × 1 ml). Final elution was performed using 0.2 *M* sorbitol, 0.1 *M* Tris, pH 8.5 and 1% acetic acid.

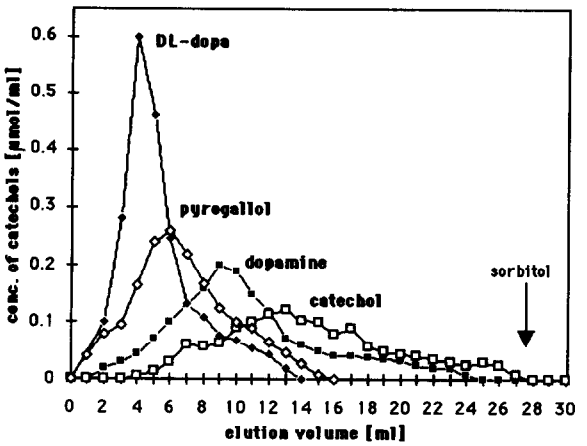

Fig. 5. Isocratic chromatography of catechol derivatives on acetamido-boronate gel. The column (1 ml volume, 0.5 cm diameter) was packed with 1 ml of gel. Catechol or its derivatives (2.5 μmol in 50 μl of distilled water) was applied and the column was washed with 0.25 *M* ammonium acetate, 0.1 *M* NaCl, pH 8.5 (27 × 1 ml). Final elution was performed using 0.2 *M* sorbitol, 0.1 *M* Tris, pH 8.5.

thiourea-boronate gel but instead eluted in the application buffer (see Fig. 4). Comparison of the chromatographic profiles of DL-DOPA on chloro-boronate gel (Fig. 3) and thiourea-boronate gel (Fig. 4) shows the difference between retardation of DL-DOPA on chloro-boronate gel (Fig. 3) and the lack of interaction with thiourea-boronate gel (Fig. 4).

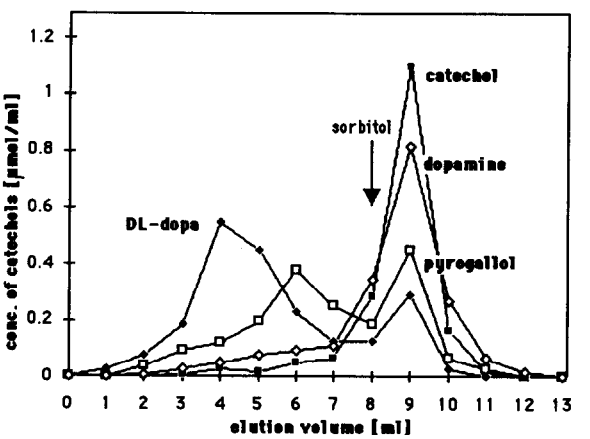

Fig. 6. Affinity elution of catechol derivatives from acetamido-boronate gel. The column (1 ml volume, 0.5 cm diameter) was packed with 1 ml of gel. Catechol or its derivatives (2.5 μmol in 50 μl of distilled water) was applied and the column was washed with 0.25 *M* ammonium acetate, 0.1 *M* NaCl, pH 8.5 (8 × 1 ml). The remaining adsorbed catechol derivatives were simultaneously eluted from the column with the addition of 0.2 *M* sorbitol, 0.1 *M* Tris, pH 8.5.

All catechol derivatives were removed from the column by wash buffer during chromatography on the acetamido-boronate gel and appeared at various retention volumes according their affinity for the sorbent (see Fig. 5). Catechol derivatives were only retarded on the column without elution in order: DL-DOPA, pyrogallol, dopamine and catechol.

Fig. 6 shows chromatograms of catechol derivatives on acetamido-boronate gel. In this experiment, the catechol derivatives were eluted from the column with the addition of sorbitol buffer before being completely eluted with binding buffer, in contrast to the experiments described previously where the catechols were eluted only with binding buffer. These results show that retardation of catechol derivatives is based on the formation of complexes between their hydroxyls and boronate groups of affinity sorbent and not on non-specific interactions based on ionic or hydrophobic effects. After application of a various elution agents (0.1 *M* Tris, 0.2 *M* sorbitol, pH 8.5, or 1% acetic acid), the catechols were released from the column in sharp peak.

Although catechol and dopamine possess different pK_a values (catechol pK_a 9.5; dopamine pK_a 10.5), both have similar affinity for acetamido-boronate gel and are not ionized under these chromatographic conditions. The relatively lower affinity of this gel for dopamine can be due to the cationic charge of primary amine group (pK_a 8.9). The presence of three hydroxylic groups in the molecule decreases affinity of pyrogallol in comparison with catechol, although their hydroxylic groups exhibit the same pK_a. The low affinity for DL-DOPA is apparently caused by the presence of the carboxylic group, which may form an intramolecular hydrogen bond with neighboring hydroxyl groups under alkaline conditions [9]. This chromatographic behavior of DL-DOPA is in agreement with the results obtained on the chloro-1-boronate gel described above (Fig. 3) and with the reports by Singhal *et al.* [8] and Sugurman *et al.* [10], who chromatographed DL-DOPA on various boronate gels.

The stability of boronate gels during storage in aqueous solutions was measured as described under Experimental. All of the gels prepared possess

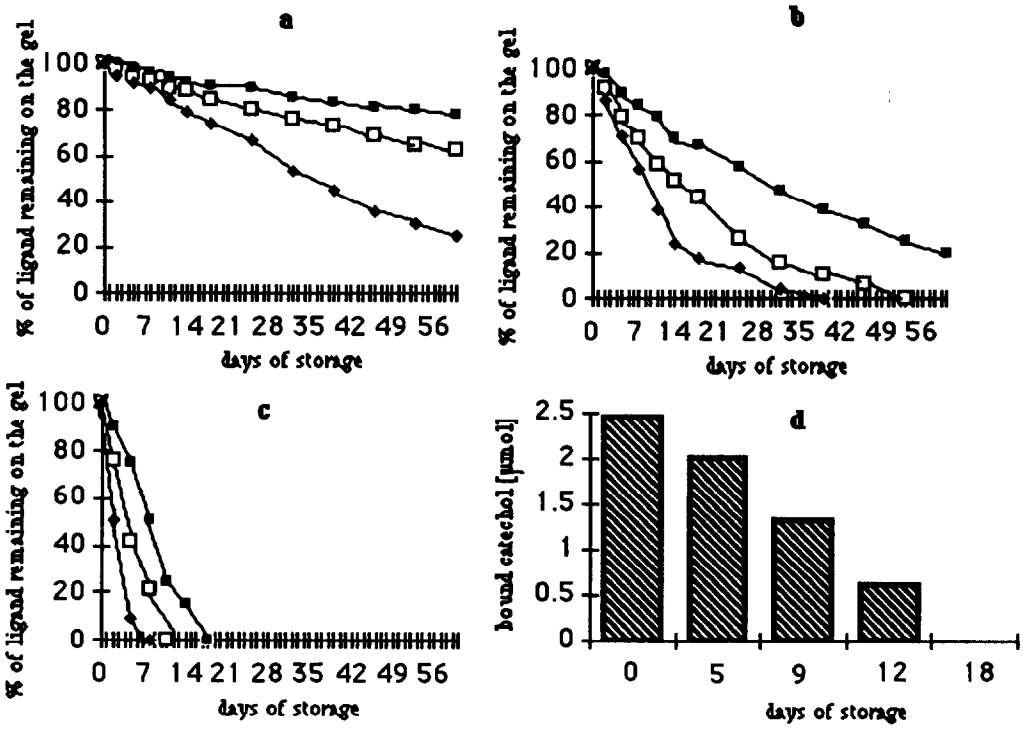

Fig. 7. Stability of aliphatic boronate gels during storage and binding capacity of catechol on the chloro-boronate-gel during storage. Stability of aliphatic boronate gels during storage at various pH; (a) thiourea-boronate gel, (b) acetamido-boronate gel, (c) chloro-boronate-gel. Gels were stored at 4°C in 0.05 *M* potassium phosphate monobasic (pH 7.0) and 0.05 *M* ammonium acetate (pH 8.5) (5 ml of buffer per 1 ml of gel) and the determination of boron was performed on buffer above the stored gels. (d) Binding capacity of catechol on the chloro-boronate-gel during storage in pH 5.0 buffer. Chromatography of catechol was performed in a column packed with 1 ml of gel. Catechol or its derivatives (2.5 μmol in 50 μl of distilled water) was applied and the column was washed with 0.25 *M* ammonium acetate, 0.1 *M* NaCl, pH 8.5 (1-ml fractions). Final elution was performed using 0.2 *M* sorbitol, 0.1 *M* Tris, pH 8.5.

higher storage stability under slightly acidic conditions, rather than neutral or alkaline conditions, as seen in Fig. 7.

Unfortunately, chloro-boronate gel, which possesses the tightest binding to catechols, was unstable even under acidic conditions, and about 50% of bound ligand decomposed after 8 days of storage (see Fig. 7c). The binding capacity for catechol on chloro-boronate gel after storage at pH 5.0 is shown in Fig. 7d. The decrease of binding capacity for catechol (Fig. 7d) corresponds with the rate of decomposition of ligand bound to the agarose (Fig. 7c). This is further evidence that binding catechol to chloro-boronate gel is based on diol interaction with boronate groups and not on non-specific interactions.

In contrast, thiourea coordinates with boron to form a relatively stable thiourea-boronate complex (see Fig. 7a). However, the resulting complex is not able to interact with vicinal diols, as described above (Fig. 4).

Although the acetamido-boronate gel (Fig. 7b) was less stable than thiourea-boronate gel, probably because the effect of the acetamido-boronate complex on ester stabilization is not as significant as in thiourea-boronate complex, the acetamido-boronate complexes were more stable than chloro-boronate.

From these results, it can be seen that the acetamido-boronate gel offers the best compromise between stability and the ability to interact with diols. Separation of DL-DOPA from catechol was chosen as an example of utilization of acetamido-boronate gel (see Fig. 8). While DL-DOPA eluted

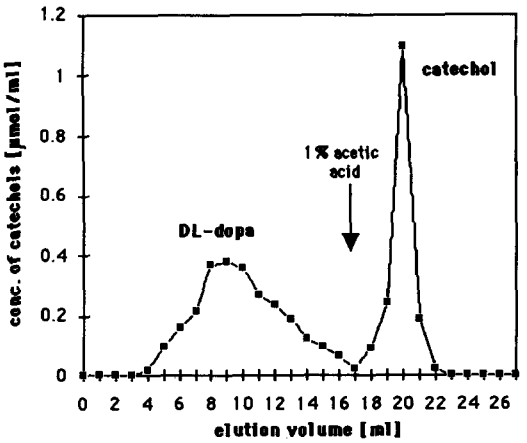

Fig. 8. Separation of DL-DOPA and catechol on acetamido-boronate gel. The column (3 ml volume, 0.5 cm diameter) was packed with 3 ml of gel. The mixture (2.5 μmol of DL-DOPA and 2.5 μmol of catechol in 50 μl of distilled water) was applied and the column was washed with 0.25 *M* ammonium acetate, 0.1 *M* NaCl, pH 8.5 (1-ml fractions). Final elution was performed using 0.2 *M* sorbitol, 0.1 *M* Tris, pH 8.5.

from the column in a low retention volume, catechol was more retarded, and its elution was accelerated using 1% acetic acid, yielding a sharp peak.

CONCLUSIONS

These experiments demonstrate that aliphatic boronate esters can be used as ligands in boronate chromatography and that an adjacent heteroatom, capable of internally coordinating to the boronate, will alter the chromatographic property of the resulting matrix. Acetamido-boronate gel can be used for separation of various catechol derivatives. Thiourea-boronate gel does not interact with catechols, while chloro-boronate gel, which bound catechols quantitatively, is rather unstable. The chief problem is the low stability of aliphatic boronates, which is the major limitation to their further utilization in boronate chromatography. Further investigations may, however, yield aliphatic boronate of sufficient stability for chromatographic application which, in turn, may reduce or eliminate non specific adsorption so often seen with aromatic boronates as ligands.

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