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Short Communication

Influence of eluent anions in boronate affinity chromatography

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Abstract

The influence of various anions on the retention of nucleosides and nucleotides to a boronate affinity chromatography column was examined. At pH 7.0, F^- ions were found to produce the strongest affinity and the best separation of nucleosides from their 2'-deoxy analogues. ^{11}B NMR studies indicated this was due in part to the propensity of F^- to form tetrahedral fluoroboronate complexes. Using an eluent of 50 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and 500 m*M* KF at pH 7.0, the nucleotides nicotinamide–adenine dinucleotide and flavin–adenine dinucleotide were found to be strongly retained on a boronate column. These nucleotide-saturated columns were examined for their ability to retain the nucleotide-binding enzymes, glucose-6-phosphate dehydrogenase, lactate dehydrogenase and galactose oxidase. No retention of the enzymes was observed.

Introduction

Immobilized boronic acids are used as affinity adsorption ligands for the separation of a wide range of biological molecules, especially compounds containing vicinal diols such as saccharide derivatives [1–4]. The most important factors known to effect retention on a boronate column are: (i) the diol configuration, (ii) other substituents within the diol compound, (iii) the acidity of the immobilized boronic acid, (iv) the pH of the eluent and (v) the ionic strength and valency of the cations in the eluent. Commercially available boronate affinity columns utilize immobilized derivatives of 3-aminophenylboronic acid which has a pK_a value of 8.75 [1]. Consequently, standard binding conditions usually require an eluent of pH 8.0–9.0 and high ionic strength. Since many biological samples are unstable under these conditions, there is a need to develop boronate chromatography systems that can bind effectively at neutral pH. One approach has been to develop matrices containing more acidic boronic acid derivatives and recent attempts have made significant progress [1,2,5]. However, none of these new ligands are readily available and their preparations are difficult for the non-specialist.

The major equilibrium responsible for the diol-selective binding in boronate chromatography is thought to be formation of the anionic tetrahedral boronate complex, **1**, X = OH, as shown in Fig. 1. Two mechanisms have been postulated for boronate–diol complex formation; (i) condensation of the diol with the anionic boronate, **2**, X = OH, as depicted in pathway A in Fig. 1 [6,7] and (ii) direct complexation of the diol with the neutral trigonal boronic acid as

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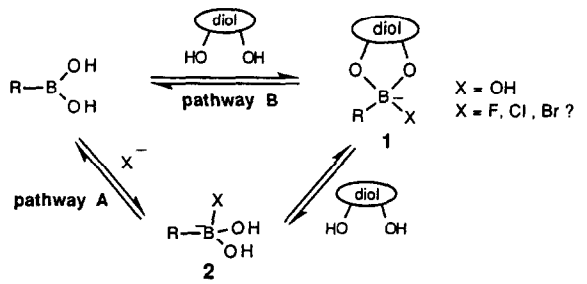

Fig. 1. Reversible complexation of a diol and a boronic acid mediated by an anion X^-.

shown in pathway B [1,8]. Recently, we reported indirect evidence for the formation of the fluoroboronate structure, **1**, X = F, which we believe is formed via the fluoroboronate, **2**, X = F [9]. Accordingly, we were interested if the effects of this complexation would have any significance in boronate chromatography. In this report, we correlate observed nucleoside and nucleotide retention volumes on a commercially available boronate affinity column with the nature of the eluent anions. In agreement with our initial premise, we find that at pH 7.0, F^- ions enhance nucleoside binding more effectively than any other anion examined. This finding has potential practical value as a simple method of increasing diol-specific binding while maintaining near physiological conditions, as well as providing insight into the mechanism of boronate–diol complexation.

2. Experimental

2.1. Materials

AffinityPak polyacrylamide–boronate columns (1 ml, catalogue No. 20368) were purchased from Pierce. The enzymes and reagents were obtained from Sigma and Aldrich, and used without further purification. ^{11}B NMR spectra were acquired on a GE NT-300 spectrometer, and UV spectra were collected using a Perkin-Elmer Lambda 2 instrument.

2.2. Nucleoside and nucleotides binding

Nucleoside and nucleotides (100 μg) were loaded onto a 1-ml boronate column that was presaturated with the appropriate eluent [10]. Fractions (0.5 ml) were collected and monitored for absorption at 260 nm except for reduced nicotinamide–adenine dinucleotide phosphate (NADPH) (338 nm) and nicotinamide–adenine dinucleotide (NAD^+) (259 nm). The columns were re-used after they were washed with water, two column volumes of 1 *M* guanidine (enzyme denaturant), and two column volumes of 0.02% sodium azide (a preservative for column storage).

2.3. Enzyme binding

A 1-ml boronate column was presaturated with a solution of nucleotide cofactor in the appropriate buffer. An aliquot of pure enzyme (100 μl) was then loaded and eluted. The eluent was collected in 0.5-ml fractions, and the protein concentration and enzymatic activity determined by Bradford's reagent and enzymatic assay. Glucose-6-phosphate dehydrogenase [11] and lactate dehydrogenase [12] were monitored for their abilities to convert nicotinamide–adenine dinucleotide phosphate (NADP) to NADPH, the formation of which can be monitored by the change in absorbance at 340 nm. The activity of galactose oxidase was determined via the standard coupled assay with *o*-tolidine and peroxidase to produce a chromophore at 420 nm [11].

3. Results and discussion

A technique which preceded boronate chromatography, combined borate sugar complexation with ion exchange to separate mixtures of sugars, and phosphorylated sugars [13]. During this work the effects of ionic strength were noted; however, since retention was a function of multiple equilibria, a detailed interpretation could not be made. In boronate chromatography, where retention is a function of a single binding equilibrium, the effects of eluent cations

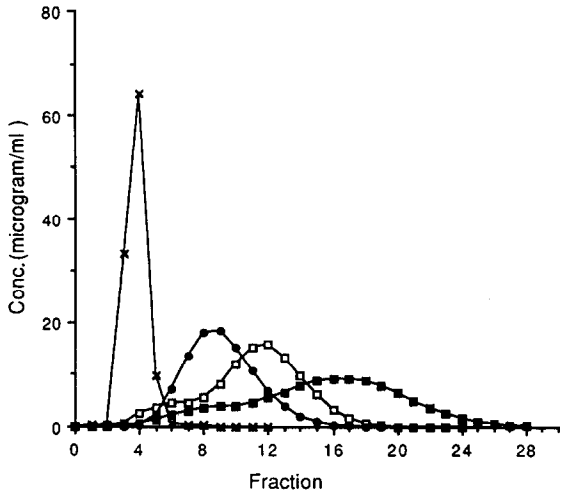

Fig. 2. Chromatographic profile of adenosine on a 1-ml polyacrylamide–boronate column. The column was pre-equilibrated and then eluted with 50 m*M* HEPES, pH 7.0 containing 500 m*M* of (■) KF, (□) KCl, (●) KBr, × no salt. Fractions of 0.5 ml were collected and monitored for absorbance at 260 nm.

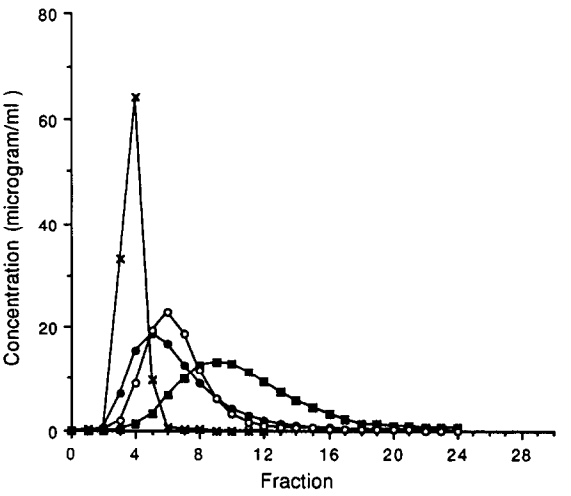

Fig. 3. Chromatographic profile of adenosine on a 1-ml polyacrylamide–boronate column. The column was pre-equilibrated and then eluted with 50 m*M* HEPES, pH 7.0 containing 500 m*M* of tetamethylammonium (■) fluoride, (○) chloride, (●) bromide, × no salt. Fractions of 0.5 ml were collected and monitored for absorbance at 260 nm.

on retention volumes have been summarized, but to our knowledge there has been no systematic study of eluent anions [2,3]. Fig. 2 shows the effect of different potassium halide salts on the retention of adenosine on a commercially available, polyacrylamide-based, low-pressure boronate column at pH 7.0. One of the reasons we chose this column support is that polyacrylamide matrices are known to slowly degenerate under alkaline conditions, so the need to work under mild conditions is emphasized [2]. In the event, the observed order of binding effectiveness was KF > KCl > KBr. We also examined other potassium salts such as phosphate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and nitrate; none were as effective as KF. To minimize the electrostatic contributions of the cation, the elution experiments were repeated with the eluent containing added tetramethylammonium halide salts. Again the retention volumes increased in the order $F^- > Cl^- > Br^-$ (Fig. 3). These results strongly suggest the anions are providing a stabilizing influence on boronate–diol complexation and that the stabilization increases with the charge to surface ratio of the anion.

Fig. 4 shows the separation of a mixture of adenosine and 2′-deoxyadenosine using an eluent of 50 m*M* HEPES buffer at pH 7.0 and (i) no added salt, (ii) added 500 m*M* KCl or (iii) added 500 m*M* KF. Only in the presence of added salt could a successful separation be achieved at pH 7.0 and the best resolution was obtained with added KF. Similar experiments were run with other nucleoside–deoxynucleoside mixtures (uridine, cytidine and inosine) and in each case KF provided the best separation.

Fig. 5 compares the ability of added KF and KCl to retain the nucleotide coenzyme NAD^+ on a boronate column at pH 7.0. In the presence of 500 m*M* KF the NAD^+ was strongly retained on the column and only when the eluent was changed to a "no salt" buffer system did the NAD^+ emerge. With the same amount of added KCl the binding capacity was clearly lower, as about half of the NAD^+ had leaked from the column by the time the "no salt" buffer was added. Under identical conditions, the cofactor

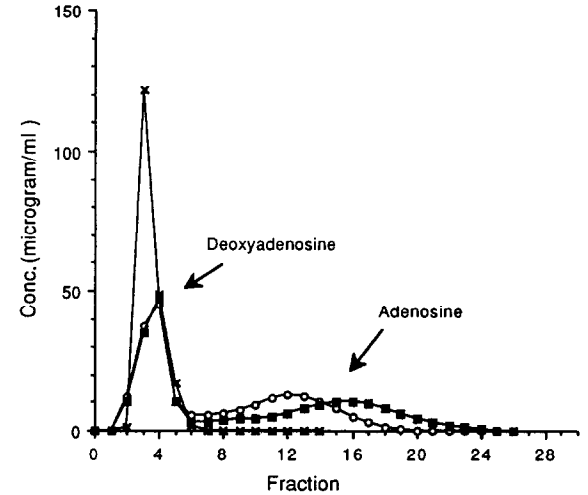

Fig. 4. Separation of adenosine from 2'-deoxyadenosine on a 1-ml immobilized polyacrylamide–boronate column. The column was pre-equilibrated and then eluted with 0.05 *M* HEPES, pH 7.0 containing 500 m*M* of (■) KF, (○) KCl, × no salt. Fractions of 0.5 ml were collected and monitored for absorbance at 260 nm.

flavin–adenine dinucleotide (FAD) displayed very similar binding characteristics, whereas other nucleotide derivatives such as ATP, UMP and NADPH did not bind to the column and were collected in the void volume. The higher affinities of NAD^+ and FAD relative to the other nucleotides have been noted and discussed by previous workers [3,10].

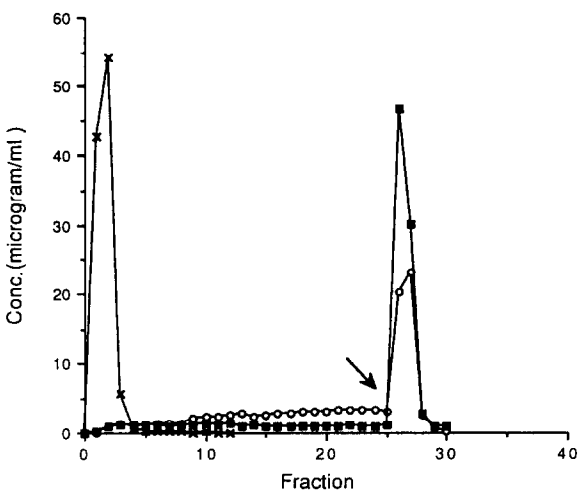

Fig. 5. Chromatographic profile of NAD^+ on a 1-ml polyacrylamide–boronate column. The column was pre-equilibrated and then eluted with 50 m*M* HEPES, pH 7.0 containing 500 m*M* of (■) KF, (○) KCl, × no salt. The arrow indicates a change of eluent to 50 m*M* HEPES, pH 7.0 with no added salt. Fractions of 0.5 ml were collected and monitored for absorbance at 260 nm.

It is known from the literature that retention on a boronate column increases with ionic strength. This phenomenon has been attributed to the electrostatic effects of the eluent cations stabilizing the anionic boronate complexes, divalent cations being the most effective [3]. Our observation that retention is quite sensitive to the nature of the eluent anions is difficult to rationalize using this electrostatic argument. Anions would be expected to destabilize the anionic boronate complexes and reduce retention, with the "harder" anions (anions with a higher charge to surface ratio) having the strongest effect. This is the opposite to that observed (for the halides the order was $F^- > Cl^- > Br^-$) and consequently an alternative explanation is needed. One possible mechanism for complex stabilization by an anion, X, involves the tetrahedral boronate, **2**, and pathway A in Fig. 1. In an attempt to confirm this mechanism, we examined the ability of various anions to form structures analogous to **2** using ^{11}B NMR. It is well known that the ^{11}B chemical shift of a tetrahedral boronate is approximately 20 ppm upfield from a trigonal boronic acid signal [1,14]. The rate of exchange between trigonal and non-chelated tetrahedral boron species is fast on the NMR time scale such that a single resonance is observed whose chemical shift is a measure of the fraction of tetrahedral boron species present. Table 1 shows the results of adding various salts to a solution of phenylboronic acid, buffered at pH 7.0. Only in the case of added KF was there a measurable upfield shift in the ^{11}B signal, indicative of the presence of tetrahedral phenylfluoroboronate anions. From these NMR studies we conclude that F^- is the only anion other than OH^- that can measurably complex with trigonal boronic acid derivatives in aqueous solution and form tetrahedral boronates. Thus, only in the case of X = F is there NMR evidence that the increased retention may be due to the participation of pathway A in Fig. 1.

Table 1
^{11}B NMR chemical shifts for phenylboronic acid as a function of added salt

Added salt (500 m*M*)[a]	^{11}B chemical shift[b] (ppm)
KF	23.9
KCl	28.5
KBr	28.6
Potassium phosphate	28.0
No added salt	28.9
KOH, pH 12	8.0

[a] Salt added to a solution of phenylboronic acid in 50 m*M* HEPES, pH 7.0.
[b] Referenced to external borontrifluoride etherate at $\delta = 0.0$ ppm.

Since diol retention at pH 7.0 is increased by the presence of divalent cations and F^- anions, the logical additive to maximize diol-specific retention at pH 7.0 would be salts such as MgF_2 and CaF_2. Unfortunately, the low solubility of these salts limits their practical utility. As a useful substitute we found that a freshly prepared eluent containing 25 m*M* $MgCl_2$ and 25 m*M* KF provided a maximal adenosine retention volume at pH 7.0 (data not shown).

A potentially attractive application of boronate chromatography is the technique of "exchangeable-ligand chromatography" or "piggyback chromatography" where the boronate column is presaturated with a diol-containing ligand such as a nucleotide cofactor, and the enzyme-binding affinity of the ligand is used to selectively retain enzymes [10,15]. The technique has been reported only a few times with boronate columns, and mixed results have been obtained. A successful example is the purification of UDP-glucose pyrophosphorylase from slime mold using a UTP-saturated boronate matrix [15]. We briefly examined the ability of boronate columns presaturated with NAD^+ (pH 7.0, 50 m*M* HEPES and 500 m*M* KF) to retain the enzymes, glucose-6-phosphate dehydrogenase and lactate dehydrogenase [10]. In addition, boronate–FAD columns were examined under similar conditions for their ability to retain galactose oxidase. In each case, no significant retention of the enzyme was observed. The lack of enzyme binding may be due to a number of reasons. Two possibilities are; (i) the dinucleotide diols that are needed for binding to the column are also required for binding to the enzymes, if this is the case then the generality of the method is severely limited [16]; (ii) the enzyme active site may be too deep for the boronate-bound dinucleotides to form stabilizing interactions, the use of longer spacers connecting the boronate ligands to the column support should, in principle, alleviate this problem [2,17].

In conclusion, we have provided evidence that eluent anions increase diol retention on a boronate affinity column by stabilizing boronate–diol complexation. Although we cannot unambiguously assign the stabilization to the mechanism of pathway A in Fig. 1, we believe pathway A is a reasonable explanation. In any case, it is clear the identity and concentration of the eluent anions are parameters that should be considered when optimizing separations by this chromatographic method.

4. Acknowledgements

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5. References

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