Boronic Acids as Ligands for Affinity Chromatography

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Abstract A review on the principles and applications of boronic acids as affinity ligands for the chromatographic separation of carbohydrates, nucleic acid components, glycoproteins, and other small biomolecules. The mechanisms of interactions between boronate ligands and analytes are described. Various boronate ligands and supports are discussed. Examples of the use of boronate affinity chromatography for separation of each class of analytes are presented.

Keywords boronic acids, borate, boronate, boronate affinity chromatography, separation of glycoproteins

Boronic acids can be used as ligands for chromatography. This type of chromatography is commonly referred to as boronate affinity chromatography. Retention in this technique is based mainly on the interaction between boronic acids and cis-diol compounds. The binding between borate and cis-diols was discovered more than a hundred years ago. By the 1940’s, the interaction between borate and cis-diols had been employed as a tool in the analysis of carbohydrates. In the 1950’s, borate/cis-diol interactions were used for separations in zone electrophoresis by employing borate buffers and in the 1960’s these same buffers were used with borate/cis-diol systems in ion-exchange chromatography. However, it wasn’t until the 1970’s that researchers developed immobilized boronate columns.

Borinate affinity columns were first employed for the separation of sugars and nucleic acid components by Weith et al. in 1976. Since then this technique has been exploited for the separation of a wide variety of cis-diol compounds, including nucleosides, nucleotides, nucleic acids, carbohydrates, glycoproteins, and enzymes. Earlier reviews of this field are given in Refs. 5–7, while specific experimental protocols used in boronate affinity chromatography are described in Ref. 8. A few examples of recent applications include the purification of arginine containing peptides, the measurement of glycohemoglobin, the purification of glycopeptides, study of the glycation pattern of hemoglobin, study of interaction with antibiotics, temperature controlled isolation of ribonucleic acid RNA, the purification of 5-methyluridine, the separation of mistletoe lectins and neoglycoproteins, the analysis of glycohemoglobin and the determination of hemoglobin HbA1c in diabetic patients. The purification of thymine glycol deoxyribonucleic acid DNA and nucleotides assays for glycated lipoproteins and affinity chromatography of serine proteases using peptide boronic acids as ligands.

Other methods besides affinity chromatography have made use of the interactions between boronates and cis-diols. Examples include an enzyme-linked boronate immunoassay ELBIA for glycated albumin, fluorescent sensors for carbohydrates and photometric sensors for boronic acids. Borate/cis-diol interactions have also been used in molecular imprinting, a technique for synthesizing tailor-made affinity matrices and enzyme mimics.

1 Boronate/analyte interaction

1.1 Primary interactions

The key interaction in boronate chromatography is the esterification that occurs between a boronate ligand and a cis-diol compound. Ideally, this esterification requires that the two hydroxyl groups of the diol be on adjacent carbon atoms and in an approximately coplanar configuration. If these are the esterification groups, the equilibrium will occur as a 1[2-cis-diol]. Interactions between boronates and 1[3-cis-diols] or trident interactions between boronates and cis-inositol or triethanolamine can occur as well but 1[2-cis-diols] give the strongest ester bonds. Since catechol-containing compounds have two...
adjacent and coplanar hydroxyl groups\[ these chemicals can also interact readily with boronate ligands. In addition\( \) trigonal boronates can react directly with \textit{cis}-diols in organic solvents\[ but the rate of esterification is several orders of magnitude lower than it is in an aqueous solution\].

The mechanism of interaction between boronic acids and \textit{cis}-diols is not fully understood. In aqueous solution under basic conditions\[ the boronate is hydroxylated and goes from a trigonal coplanar form to a tetrahedral boronate anion\[ which can then form esters with \textit{cis}-diols. This process is illustrated in Fig. 1. The resulting cyclic diester can be hydrolyzed under acidic conditions\[ thus reversing the reaction. This reaction is unusual in that\[ although two covalent bonds form\[ it is quite reversible in aqueous solution.

![Fig. 1 The interaction between a boronic acid and a \textit{cis}-diol in aqueous solution](image)

The boronate diester bond strength is not well-characterized and only a few dissociation constants have been reported for phenylboronic acid diesters. Those that have been reported include dissociation constants of 2.2 \times 10^{-3} \text{ mol/L} for adonitol\( \) 1.1 \times 10^{-3} \text{ mol/L} for dulcito\( \) 3.3 \times 10^{-3} \text{ mol/L} for mannito\( \) and 5.9 \times 10^{-3} \text{ mol/L} for nicotineamide adenine dinucleotide \( \text{NAD}^+ \) \( \text{H}^+ \). In addition\[ it is known that the dissociation constant for \( N\)-methyl\( N\)-carboxamido-benzenboronic acid with D-fructose diester is 1.2 \times 10^{-4} \text{ mol/L} \). This type of binding is relatively weak compared to that observed for many of the biological ligands used in affinity chromatography and is more typical of the interaction strengths that are used in weak affinity chromatography.

1.2 Secondary interactions

Although boronate/\textit{cis}-diol ester formation is the main basis for boronate affinity chromatography\[ there are several secondary interactions that sometimes play an important role in retention. Four types of secondary processes that can occur on boronate columns are hydrophobic interactions\[ ionic interactions\[ hydrogen bonding\[ and coordination interactions.

1.2.1 Hydrophobic interactions

Most boronate ligands used in affinity columns are aromatic boronates. These contain a phenyl ring\[ which can give rise to hydrophobic interactions or aromatic \( \pi-\pi \) interactions. Hydrophobic interactions can\[ in certain cases\[ provide additional selectivity to the column. But they can also lead to the nonspecific adsorption of analytes like proteins. As a result\[ there have been many efforts to synthesize aliphatic boronate ligands to overcome such adsorption\]. Another way to reduce hydrophobic effects in boronate columns is to keep the ion strength of the mobile phase low. For reasons given in the next section\[ ionic strengths down to 50 mmol/L are generally recommended for such columns\].

1.2.2 Ionic interactions

The negative charge of the active tetrahedral boronate can produce coulombic attraction or repulsion for ionic analytes. In general\[ this effect is weaker than boronate/\textit{cis}-diol ester formation. As was true for hydrophobic effects\[ there are some cases in which ionic interactions can provide additional selectivity for boronate columns. But in other cases these secondary interactions are undesirable. To decrease ionic effects\[ the ionic strength of the mobile phase should be kept high\[ however\[ this will also lead to stronger hydrophobic interactions. A good compromise for minimizing both hydrophobic and ionic interactions is to use mobile phases with ionic strengths between 50 and 500 mmol/L \].

1.2.3 Hydrogen bonding

Since a boronate acid has two hydroxyls or three in the active tetrahedral form\[ it has several possible sites for hydrogen bonding. Although the effect of this added interaction is usually small\[ in special circumstances hydrogen bonding can be strong enough to be the main mechanism for retention on a boronate column. An example of this occurs during the isolation of serine proteases by boronate affinity chromatography \].

1.2.4 Coordination interactions

Because a trigonal uncharged boronate contains a boron atom with an empty orbital\[ this can serve as an electron receptor for a coordination interaction. Unprotonated amines are good electron donors and when an amine donates a pair of electrons to boron\[ the boron atom becomes tet-
rahedral] i.e. the more active form] this explains why amines may serve to promote boronate/cis-diol esterification. However, if there is a hydroxyl group adjacent to the amine[ this hydroxyl group can also interact with the boronate] thus blocking esterification between the boronate ligand and analyte. It is for this reason that Tris and ethanolamine derivatives should be avoided in buffers used for boronate affinity chromatography. Furthermore, carboxyl groups can serve as electron donors for these coordination interactions. Together with \( \alpha \)-hydroxyl groups carboxyls can form stable complexes with boronates[ as demonstrated by the esterification of lactic acid or salicylic acid with boronate affinity columns [30].

2 Boronate ligands and supports

2.1 Boronate ligands

The most common ligand used in boronate affinity chromatography is 3-aminophenylboronic acid[ also known as 3aPBA] see Fig. 2-a[. The anilino group on this ligand is used to couple it to solid supports. The use of a \( \text{meta} \)-amino substitution also lowers this ligand’s \( pK_a \). The \( pK_a \) of 3aPBA is 8.8[40] so for optimum binding to analytes the pH of the mobile phase should be as high as reasonably possible. In all current applications using 3aPBA this mobile pH is greater than 8.0. However, in many cases analytes may lose their biological activities at such high pH values. The pH stability of the support that contains the boronate ligand must also be considered. Thus, the current need for relatively high pH buffers is a major limitation to the use of 3aPBA as an affinity ligand.

A number of efforts have been made to produce better boronate ligands. For instance, Yurkevich et al prepared dextran and cellulose containing bipolar 2[4] 4-boronophenyl \( \text{meth-}

yl[\text{ethylammonio}] \text{ethyl and 2[4] 4-borophenyl[\text{methyl[\text{diethylammonio}] \text{ethyl][41]. These supports were used for separating ribonucleosides such as adenosine, cytidine, guanosine and uridine at pH 8.0. These matrices were found to bind adenosine in a nearly pH-independent manner from pH 2.5 to pH 8.2. However, the most likely mechanism for this adsorption was ionic interactions and not boronate/cis-diol esterification.

Akparov et al. coupled \( \psi \) \( \alpha \)-aminophenyl[phenyl-boronate to CH-Sepharose for performing chromatography with serine proteases at pH 7.0[34]. However, retention in this system was due to coordinated hydrogen bonding in the active site where the boronate served as a transition state intermediate[42]. A similar phenomenon was observed with porcine pancreatic lipase[43]. In another study, Elliger et al prepared poly(\( p \)-vinylbenzenoeboronic acid[coated porous polystyrene beads to separate vicinal diols near pH 8[44]. All analytes considered were small organic compounds. As expected, the observed interaction between \( \text{cis} \)-diols and these boronate matrices were highly pH-dependent.

Wulff et al. synthesized 2-dimethylaminomethyl phenyl-boronic acid[ in which the intra molecular B-N bond was established by \( ^{11} \text{B} \) NMR[34]. This ligand was shown to be highly specific for aliphatic \( \text{cis} \)-diols[ but bound poorly to aromatic \( \text{cis} \)-diols[ amines and monoalcohols. No data has been reported on the interactions of this ligand with large molecules such as glycoproteins or carbohydrates.

A number of researchers have attempted to introduce a strong electron withdrawing group into the phenyl ring of aromatic boronates[ thus lowering the \( pK_a \) of these ligands. Myohanen et al. coupled 3-nitro-4-carboxamidobenzene boronic

\[ \text{Fig. 2 Structures of various boronate ligands: a) 3-aminophenylboronic acid[ b) } NCI \text{ 4-nitro-3-dihydroxyborylphenyl[ succinamic acid[ c) } N\text{-methyl[ carboxamidobenzenoeboronic acid[ and d) a new type of boronate ligand with an internal coordination bond.} \]
acid to Sepharose-CL 6B and found that the glycoprotein α-glucosidase was retained by this support at pH 7.4 [45]. However at pH 8.5 and 6.5 the chromatographic behavior of this material was totally different. This was thought to have been caused by the unstable amide linkage between the ligand and support which would have been rapidly hydrolyzed at an alkaline pH [34].

Johnson coupled a mixture of 2-nitro-3-succinamido-benzenearboronic acid and 3-succinamido-4-nitro-benzenearboronic acid to aminopropyl poly acrylamide beads and used these to separate transfer ribonucleic acid tRNAs at pH 7.4 [46]. Unfortunately it appears that 2-nitro-3-succinamido-benzenearboronic acid gradually hydrolyzes and the freed amine groups can create strong ionic interactions. A report by Singhal et al described a similar approach in which N 4-nitro-3-dihydroxy-borylphenyl succinic acid was coupled to a porous semirigid spherical gel of vinyl polymer see Fig. 2-b [47]. They found this matrix offers enhanced binding of many cis-diol compounds.

Hageman and co-workers synthesized 4 N-methyl carboxamidobenzenearboronic acid see Fig. 2-c and determined that it had a lower pKs 7.86 than 3aPBA [36]. This gave strong cis-diol ester formation with D-fructose with a binding constant of 8 600 L/mol. They suggested that this ligand should provide sufficient acidity binding capacity and hydrolytic stability to make it an excellent boronate ligand for affinity supports. However this ligand has not yet been coupled to such a support and no further publications have appeared using it.

Liu and Scouten reported another new type of boronate ligand with the general structure shown in Fig. 2-d [46]. X-ray crystallography showed that an internal coordination bond was formed between atom X and the boron atom in this ligand [49]. This means the boron is in a tetrahedral conformation which is favorable for esterification between a boronate and cis-diol. In solution this type of ligand can esterify catechol at neutral pH as demonstrated by 1B NMR studies. Subsequently when this ligand is immobilized on a solid matrix chromatographic analysis of catechol and horseradish peroxidase can be performed at neutral pH. Aliphatic boronate ligands were studied by this research group [37]. Their results indicate that although these ligands can be used in affinity chromatography for a short period of time their lack of stability is an obstacle to prolonged use.

2.2 Solid supports

As in other types of affinity chromatography the support material can play an important role in boronate-based separations. Materials that have been used in boronate affinity chromatography include dextran [41] cellulose [42] agarose [43-45] poly acrylamide [46] silica [51] poly styrene [47] and polymethacrylate [52]. Boronate affinity matrices are commercially available from several major chemical and biochemical suppliers. The list given in Table 1 is representative of the current market. The ligand for all these products is 3-amino phenylboronic acid. Users can choose their own products based upon properties such as ligand capacity mechanical stability hydrophilicity hydropobicity porosity and cost.

### Table 1 Commercially available boronate matrices

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Name of boronate affinity gel</th>
<th>Support material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldrich</td>
<td>boric acid gel</td>
<td>polymethacrylate</td>
</tr>
<tr>
<td>BioRad</td>
<td>Affi-Gel 601 gel</td>
<td>polycrylamide</td>
</tr>
<tr>
<td>Pierce</td>
<td>immobilized boronic acid gel</td>
<td>polycrylamide</td>
</tr>
<tr>
<td>Sigma</td>
<td>m-aminophenylboronic acid matrix</td>
<td>polymethacrylate</td>
</tr>
<tr>
<td>Biosep</td>
<td>TSK gel Boronate-5PW column</td>
<td>polycrylamide</td>
</tr>
</tbody>
</table>

3 Applications of boronate affinity chromatography

Boronate affinity chromatography has been employed in the separation of several types of molecules. Specific categories of analytes for which this method has been used include [1] carbohydrates [2] nucleosides [3] nucleotides and nucleic acids [3] glycoproteins and enzymes [3] and [4] miscellaneous small molecules. Further details on each of these applications are given in the following sections.

3.1 Carbohydrates

Carbohydrates containing cis-diols can bind to boronate ligands with a strength that is proportional to the number of cis-diol groups on the analyte. In the 1960’s Bourne et al found that adding phenylboronic acid to developing solvents in paper chromatography enhanced the mobility of sugars that had cis-diol or cis-triol groups [53]. This technique is also suitable for separating free sugars that have cis-diols from their reduced polyol counterparts since the reduced sugars may not have the cis-diols needed to esterify with boronate.

Boronate chromatography has been used for
separating monosaccharides and oligosaccharides with compounds containing a 1,2-cis-diol binding the most strongly to boronate gels. Because of their strong binding to boronates carbohydrates such as sorbitol or mannitol are often used as competing agents for the elution of other analytes from boronate columns. In addition isomeric pentose phosphates have been separated by boronate affinity chromatography. Resolution of D- and L-mannopyranoside racemates using boronate affinity chromatography was reported by Wulff et al using a matrix that was synthesized by molecular imprinting or templated polymerization technology. Fig. 3 shows a chromatogram obtained for a racemic mixture of D/L-mannopyranoside in such an application.

Since there is no 2'-hydroxyl in DNA it doesn’t esterify with boronate matrices. Thus boronate affinity chromatography can easily separate RNA from DNA. Since 3'-phosphorylated ribonucleotides don’t bind to boronate gels for the same reason these also can be readily separated from RNA. Oligonucleotides of RNA and large RNA molecules only have a cis-diol at their 3'-end so their binding to boronate gels is relatively weak. In this case longer RNA chain lengths correlate with weaker binding.

mRNA can be isolated using boronate affinity chromatography. Furthermore boronate affinity chromatography can be used for separating aminoacylated tRNA from nonaminoacylated tRNA. Unfortunately the high pH required in boronate chromatography may hydrolyze the amino acid-tRNA bond. Since dinucleotide cofactors such as NAD, H² and flavin adenine dinucleotide FAD have more than one accessible cis-diol they bind more strongly to boronate chromatography gels than mononucleotides or oligonucleotides.

Secondary interactions are important in the binding between nucleic acid components and boronates. The negatively-charged phosphate groups on these analytes cause strong ionic repulsion with the boronate ligand which may weaken or prevent binding of cis-diols on other parts of the analyte. To minimize this effect divalent cations such as Mg²⁺ are commonly used in buffers for these analytes in boronate affinity chromatography. The nature of the base in the nucleic acid is
also important since purine bases adenine and guanine have stronger binding than pyrimidine bases cytosine and thymine. This is probably due to differences in the hydrophobic interactions and/or hydrogen bonding between aromatic boronates and these bases.

3.3 Glycoproteins and enzymes

One of the most important uses of boronate affinity chromatography is in the separation of glycated hemoglobin from non-glycated hemoglobin. In humans glucose and other aldoses can glycosylate hemoglobin at its amino terminal end and certain lysine residues. The concentration of glycated hemoglobin that is present in blood is known to reflect the blood glucose concentration over the past three months. Thus measurement of glycated hemoglobin levels is an important tool in the clinical management of diabetes. Boronate affinity chromatography is a rapid and accurate means for assaying glycated hemoglobin for this purpose. In addition glycated albumin which can also be used for evaluation of diabetic states can be determined by boronate chromatography. Clinical applications of affinity chromatography including boronate chromatography were recently reviewed by Hage. An example of the clinical use of boronate affinity chromatography in glycated hemoglobin analysis is provided in Fig. 5.

![Fig. 5](image_url)

**Fig. 5** Boronate affinity chromatography for determination of glycated hemoglobin in human blood using Primus ultra2 HPLC system

Conditions: column size 4.5 mm i. d. x 50 mm binding buffer 0.25 mol/L ammonium acetate pH 8.8 elution buffer 0.1 mol/L mannitol and 0.14 mol/L NaCl in binding buffer flow-rate 2.0 mL/min the injected sample 20 μL of 1:200 mixed whole blood to DI water. The hemoglobin absorption was measured at 415 nm. Courtesy of Primus Corporation Kansas City, MO, USA.

The first peak 0.48 min is non-glycated hemoglobin and the second peak 1.04 min is glycated hemoglobin.

Other glycosylated proteins have also been isolated by boronate affinity chromatography. These include human immunoglobulins γ-glutamyltransferase human platelet glycocalcin α-glucosidase from yeast 3,4-dihydroxyphenylalanine-containing proteins membrane glycoproteins from human lymphocytes horseradish peroxidase and glucose oxidase.

Boronic acid derivatives are potent transition-state inhibitors of serine proteases. This means these ligands can also be used for isolation and purification of serine proteases. Examples of such applications include separations that have been reported for α-chymotrypsin trypsin and subtilisin as well as for human neutrophil elastase human cathepsin G and proline pancreatic elastase.

When using boronate affinity chromatography withglycoproteins secondary interactions especially hydrophobic and ionic interactions must be considered. Since hydrophobic interactions can cause the non-specific binding of undesired proteins detergents are sometimes added to the mobile phase buffer to decrease such binding. Ionic interactions involving the negatively-charged boronate ligand can prevent binding by anionic proteins or cause nonspecific adsorption by cationic proteins. Divalent cations such as Mg are often added in low concentrations to the mobile phase to reduce these ionic effects without also enhancing hydrophobic interactions.

Proteins that don’t normally interact with boronate can be isolated by ligand-mediated chromatography. In this process the boronate column is first saturated with a second affinity ligand that contains a cis-diol. Note in this scheme that the second affinity ligand is not boronate but rather a binding agent that has been selected to have specific interactions with the target proteins. The function of the boronate in this situation is merely to provide a means for coupling the secondary ligand to the matrix through the formation of a boronate/cis-diol ester. After the secondary ligand has been adsorbed to the column a sample containing the target protein is applied. This allows the target protein to be retained while other proteins are washed through the column. The bound target can be then eluted by washing with an appropriate buffer or by applying a mobile phase that contains a soluble affinity ligand or competing cis-diol compound.

There are several examples of protein separations based on the boronate columns and the ligand-mediated approach. For example canancavalin A has been isolated using methyl α-D-glucopyranoside as the secondary ligand and glucose-6-phosphate dehydrogenase from yeast.
has been isolated using nicotinamide adenine dinucleotide phosphate cation- NADP⁺ adsorbed to boronate see Fig. 6 [80]. Other examples include the isolation of yeast hexokinase using adenosine triphosphatase ATP as the secondary ligand [80] lactate dehydrogenase using NAD⁺ as the secondary ligand [81] and uridine diphosphate UDP-glucose pyrophosphorylase using adsorbed uridine triphosphatase UTP [81]. Affinity ligands that don’t normally have diols can also be used in this format if they are first derivatized to contain such a group within their structure [82].

Fig. 6 Boronate affinity chromatography of yeast glucose-6-phosphate dehydrogenase G-6-PDH by NADP⁺ on a presaturated matrix gel containing phenylboronic acid

Yeast enzyme concentration [7.5 mg in 0.5 mL] in buffer ligand concentration [41 μmol/mL] was applied to the boronate column [1 mL] that had been pre-equilibrated with NADP⁺. The concentrate contained 19.4 units of G-6-PDH. One mL fractions were collected and assayed for enzyme activity [•] and their optical density [O. D. at 260 nm] [•] and 280 nm [o]. Elution was achieved by applying 2 mmol/L NADP⁺ at the point indicated by the arrow. Reproduced with permission from Ref. [80].

3.4 Miscellaneous small molecules

Other groups of small biological molecules can also be isolated by boronate affinity chromatography. Examples include catechol-containing compounds such as D/L-dopa 5-S-cysteinyl- dopa epinephrine and noradrenaline. Additional small molecules that bind to boronate columns are α-hydroxycarboxylic acids like lactic acid and salicylic acid [85]. Such columns have also been used for separation of pyridoxal [86] quercetin [87] and ecdysteroids [87].

4 Summary & conclusions

In summary boronic acid and its derivatives are versatile ligands that allow for selective binding of a variety of biological molecules. This article has examined the way in which these ligands bind to other agents and the various interactions that lead to retention on boronate affinity columns. The types of ligands and supports that are used in boronate affinity chromatography were also considered as well as the various applications of this method. It was shown that the unique and reversible interactions of boronates with cis-diol compounds offer many advantages for affinity chromatography. Further work with boronates in both this and related fields is expected to continue including work with these ligands in the oriented reversible immobilization of enzymes the creation of molecular imprints and the development of synthetic receptors and probes.

References

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Brief Biography of the Author

Dr. LIU Xiao-Chuan earned his BS and MS from Shandong University in China in 1985 and 1988 respectively, and PhD from Baylor University in USA in 1995. He then completed post-doctoral fellowships in Lund University in Sweden and University of Iowa in USA before moving on to his independent academic career. His research interests include boronate affinity chromatography, molecular imprinting, and enzyme-mimics. He has published more than 20 papers in these areas and served as a consultant for biotech companies.