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A detailed examination of boronic acid–diol complexation

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Abstract—Boronic acids bind with compounds containing diol moieties with high affinity through reversible boronate formation. However, the conditions that foster tight binding between the diol and the boronic acid are not well understood. Also, due to the multiple ionic states of both the boronic acid and boronate ester, the equilibrium constants reported in the literature have not always been strictly defined, and therefore there is a lack of 'comparability' between the reported values. To address these issues, we have developed a method for examining boronate ester stability using the fluorescent reporter Alizarin Red S. We have used this system to determine the binding constants of a series of diols, and as a basis from which to derive a number of relationships that correlate the various equilibrium constants in the literature.
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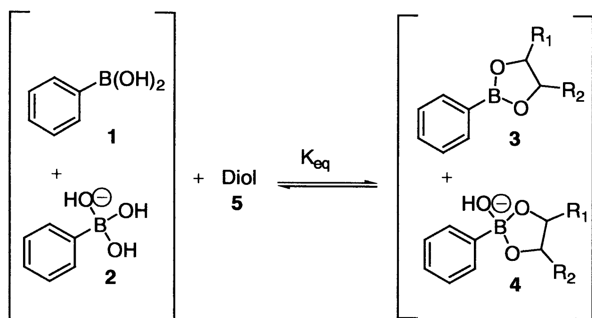
1. Introduction

Understanding intermolecular interactions between different functional groups forms the basis for molecular recognition and is essential for the proper design of selective receptors. Recently, there has been a great deal of interest in studying the interactions between boronic acids and diol-containing compounds. Boronic acids are known to bind with compounds containing diol moieties with high affinity through reversible ester formation (Scheme 1). Such tight binding allows boronic acids to be used as the recognition moiety in the construction of sensors for saccharides,[1–10] as nucleotide and carbohydrate transporters,[11–18] and as affinity ligands for the separation of carbohydrates and glycoproteins.[19–26] Appropriately designed boronic acid compounds also have shown potential as antibody mimics targeted on cell-surface carbohydrates.[27–36]



Scheme 1. Boronate ester formation.

The stability of the boronate ester is pH- and solvent-dependent,[37–39] but the factors that govern these processes are not well understood. A general method for measuring association constants of diol–boronic acid complexes under a variety of conditions would greatly assist in understanding the underlying factors behind boronate ester stability. There are numerous examples in which fluorescence or UV spectroscopy was used for the determination of the binding constants,[8,9,40–44] but these cases are limited to fluorescent or strongly chromophoric boronic acid compounds whose spectroscopic properties are sensitive to the binding event. Due to our interest in developing antibody mimics for cell surface carbohydrates, quite often we need to use boronic acid compounds that are not fluorescent and are only weakly chromophoric. Under such a circumstance, spectroscopic determination of binding constants relying on the intrinsic spectroscopic property changes upon binding becomes very difficult. Therefore, we set out to develop a system that would allow us to easily determine the binding constants regardless of whether the boronic acid compound is fluorescent or not. Using a three component competitive assay containing the fluorescent compound Alizarin Red S. (ARS), phenylboronic acid (PBA), and a diol-containing compound[45] (Scheme 2), we were able to study the stability of a series of boronate esters. Since we were using a fluorescent reporter as our measuring tool, ester stabilities could be monitored under a variety of conditions that included changes in pH, buffer and solvent. This is in direct contrast to the commonly used pH-depression methods used under such circumstances.[46–48] The pH-depression method measures the increase in acidity seen when a diol is titrated into a solution of boronic acid. It is based on the assumption that the boronate ester **3** (Scheme 3) is far more acidic than the boronic acid **1**. The method requires the boronic acid to be used as the buffer, and measures association over a 'floating pH' (see Section 3). The three-component approach

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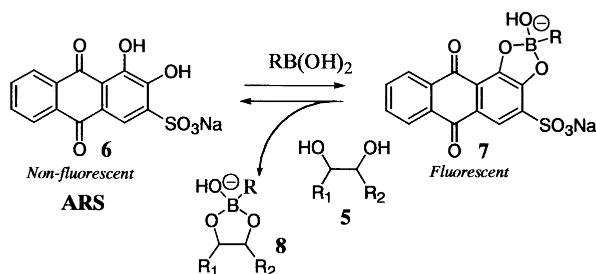
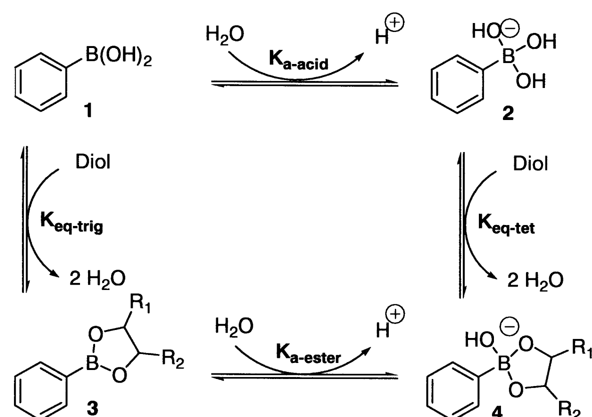


Scheme 2. Competitive binding of a boronic acid with Alizarin Red S. and a 1,2-diol.



Scheme 3. The relationships between phenylboronic acid and its diol ester.

developed in our lab allows us to examine the details of the equilibrium formation and clarify the relationships between association constants reported in the literature. The pH and buffer can be varied, and there are no intrinsic assumptions about the abundance of boronate ester species (see Section 3). In a recent publication, the ARS system was used by others to determine the binding constants of a D-glucose selective diboronic acid.[49] It should be noted that Anslyn and co-workers have also developed three-component spectroscopic methods for the determination of carbohydrate binding with boronic acid,[8] and non-boronic acid artificial receptors.[50] Interestingly, such methods have been used for the determination of the aging of whiskey.[51]

In the boronic acid literature, there are several commonly held beliefs. First, the optimal pH for the binding of boronic acid compounds to diols is above the pK_a of the boronic acid species. Therefore, lowering the pK_a of a boronic acid is known to increase the binding constants of boronic acids.[37,52,53] Second, buffer has no effect on the binding constants between boronic acids and diols.[47] Third, neutral boronate ester species of 1,2 diols do not exist to a significant extent, which forms the basis for the binding constants determination using the pH-depression method and ^{11}B NMR method.[37,46,54,55] Fourth, the binding constants determined using the pH titration,[46,47,56,57] ^{11}B NMR,[37,48,54,58] and spectroscopic methods,[3,9,40–43] have the same physical meaning. Fifth, conversion of the boronic acid to the ester results in a decrease of the pK_a.[37,46] Results reported in this paper clearly demonstrate that all these commonly held beliefs are not always correct. The mechanistic understanding of the binding process inferred from the results presented herein should be of great significance to the further design of boronic acid-based carbohydrate sensors and artificial receptors.

2. Design and methods

It is known that an excited state proton transfer from the phenol hydroxyl group of ARS (**6**, Scheme 2) to the ketone oxygen results in the fluorescence quenching of free ARS.[59] Therefore, it was reasonable to expect that boronate ester (**7**) formation would increase the fluorescence of the system through the removal of the fluorescence quenching mechanism. By taking advantage of such features, a three-component ARS assay system was designed. This system has two competing equilibria. The first equilibrium, between the boronic acid and the fluorescent reporter compound (**6**), can be directly measured. The addition of a carbohydrate (**5**) sets up a second equilibrium between the boronic acid the carbohydrate, to give complex **8**. This perturbs the ARS/boronic acid equilibrium, resulting in a change in the fluorescence intensity of the solution.[45]

There are literature precedents for the use of photometric changes in a three-component system for the determination of binding constants.[8,60,61] Consequently, there are also well-established mathematical models for the determination of the respective equilibrium constants. Two experiments were done to measure the equilibrium constants of the competitive system. First, the association constant for the ARS–boronic acid complex (K_{eq1}) was determined. This was accomplished by making a solution of ARS (9.0×10^{-6} M) in a 0.10 M phosphate buffer solution. Then boronic acid was added to give solutions with a range of concentrations of boronic acid (10–200 equiv.). The fluorescence intensities were measured with an excitation wavelength of 468 nm and an emission wavelength of 572 nm. The relationship between fluorescence intensity changes and the equilibrium constant can be expressed using Eq. (1) (Fig. 1).[61] The double reciprocal of Eq. (1) yields the Benesi–Hildebrand equation (Eq. (2), Fig. 1). The association constant for the ARS–boronic acid complex (K_{eq1}) is the quotient of the intercept and the slope in a plot of 1/[PBA] vs. $1/\Delta F$.[61] In such an experiment, the boronic acid species needs to be in excess (at least 10-fold) compared with the diol.

The association constant for the boronic acid–diol complex (K_{eq}) is found by titrating a boronic acid–ARS solution with the target diol compound. This titration perturbs the first equilibrium (K_{eq1}) and therefore results in a change of the fluorescence intensity of the solution. The extent to which the diol moiety changes the fluorescence intensity depends on the binding affinity between boronic acid and diol. The concentration of boronic acid and ARS were fixed at 2.0×10^{-3} and 9.0×10^{-6} M, respectively in a 0.10 M phosphate buffer solution. Then, the diol compound was added to give solutions with a range of concentrations that covered as much of the binding curve as possible. The K_{eq} is determined by plotting $1/P$ vs. Q, where P is defined as $P=[L_o]-1/QK_{eq1}-[I_o]/(Q+1)$ (Eqs. (3) and (4), Fig. 1). L_o is the total amount of boronic acid, I_o, the total amount of ARS, and K_{eq1} is the association constant of the ARS/

$$\Delta I_f = \frac{(\Delta k p_o K_{eq1})[L][I_o]}{1+K_{eq1}[L]} \qquad (1)$$

$$\frac{1}{\Delta I_f} = \left(\Delta k\ p_o I_o K_{eq1}\right)^{-1}\frac{1}{[L]} + \left(\Delta k\ p_o I_o\right)^{-1} \qquad (2)$$

$$\frac{[S_o]}{P} = \frac{K_{eq1}}{K_{eq}}Q + 1 \qquad (3)$$

$$P = [L_o] - \frac{1}{QK_{eq1}} - \frac{[I_o]}{Q+1} \qquad (4)$$

$$Q = \frac{[I]}{[IL]} \qquad (5)$$

$$K_{eq\text{-}tet} = \frac{1+\frac{[H^+]}{K_{a\text{-}acid}}}{1+\frac{[H^+]}{K_{a\text{-}ester}}} \times K_{eq} \qquad (6)$$

$$K_{eq\text{-}trig} = \frac{1+\frac{[H^+]}{K_{a\text{-}acid}}}{1+\frac{[H^+]}{K_{a\text{-}ester}}} \times K_{eq}\frac{K_{a\text{-}acid}}{K_{a\text{-}ester}} \qquad (7)$$

$$K_{eq} = \%\,acid \times K_{eq-trig} + \%\,ester \times K_{eq-tet} \qquad (8)$$

Figure 1. Equations for association constant determinations. I=indicator (ARS), $[I_o]$=total indicator concentration (ARS), L=ligand (PBA), S=substrate (diol), K_{eq1}=association constant of the ARS–PBA complex. K_{eq}=association constant of the diol–PBA complex, Δkp_o is a constant derived from the intrinsic fluorescence and the laser power, I_f=fluorescent intensity. % Acid is the percentage of total boron that is the free acid. % Ester is amount in the complexed ester form.

boronic acid complex. Q is a ratio of the concentration of free ARS to complexed ARS, (Eq. (5), Fig. 1) and can be determined by the change in fluorescence of the solution. The K_{eq} of the boronic acid–diol complex can then be calculated by dividing K_{eq1} by the slope of the plot (Eq. (3), Fig. 1).

In addition to K_{eq}, we also wished to determine the equilibrium constants of the trigonal ($K_{eq\text{-}trig}$) and tetrahedral ($K_{eq\text{-}tet}$) forms of the boronic acid as shown in Scheme 3. Their relationship to the overall K_{eq} is shown in Eqs. (6)–(8) (Fig. 1). The values of the acidity constants of the boronic

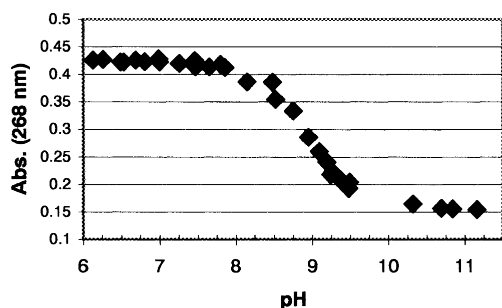


Figure 2. The pK_a of phenylboronic acids can be determined by the absorbance change at 268 nm that occurs upon conversion from the trigonal form (low pH) to the tetrahedral form (high pH). ◆—Phenylboronic acid at 1×10^{-3} M in 0.10 M phosphate buffer.

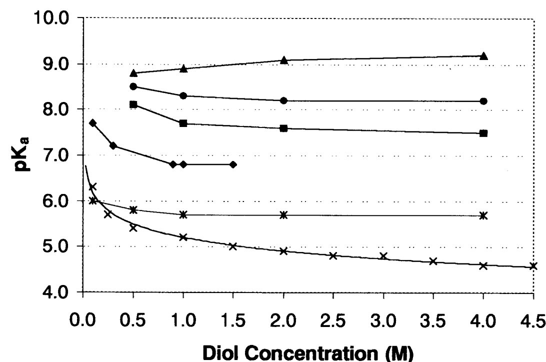


Figure 3. The pK_a determination of arylboronic esters: ▲—1,3 propane diol, ●—ethylene glycol, ■—sucrose, ◆—glucose, ★—sorbitol, ×—fructose. 0.0010 M PBA, and 0.10 M phosphate buffer were used in all studies.

acid ($K_{a\text{-}acid}$) and ester ($K_{a\text{-}ester}$) are required to solve these equations. The pK_a of a boronic acid can be determined based on the change in UV absorption that occurs when the boronic acid converts from the trigonal to the tetrahedral form.[40] The pH titration curve for PBA is shown in Fig. 2. For the determination of the equilibrium constants, we also need the pK_a of the boronate ester. However, the boronate ester always exists in equilibrium with the free acid (**1**, Scheme 3) depending on the concentration of the diol and the K_{eq}. This rapid equilibrium precludes the possibility of determining the pK_a of the pure ester (**3**) by titrating an ester dissolved in aqueous solution as was done with the acid. The presence of boronic acid would shift the apparent pK_a of the solution towards its own pK_a. However, the percentage of the boronic acid that is in the ester form can be increased by adding more diol, which in turn moves the apparent pK_a closer to the pK_a of the ester. When the diol is present in large excess, the apparent pK_a change approaches a plateau indicating the complete conversion of the boronic acid species to the ester (**3**). This allows us to determine the true pK_a of the corresponding ester species by estimating the value of the 'infinite sugar concentration' asymptote (Fig. 3). It should also be noted that we have calculated the ratio of the corresponding ester and boronic acid at different pHs based on the K_{eq} determined and the results are consistent with the complete conversion to the ester where the plateau was reached.

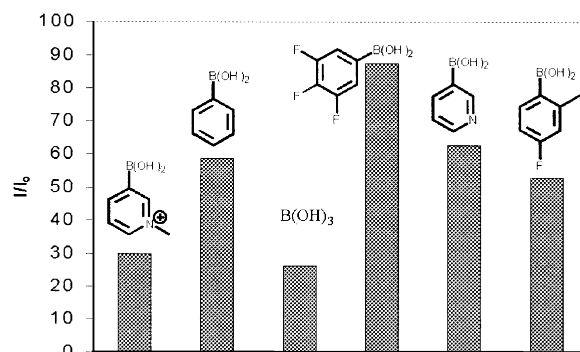


Figure 4. I=Fluorescence of ARS (10^{-4} M) in the presence of boronic/boric acid (10^{-2} M) in pH 7.4 aqueous solution, 0.1 M phosphate buffer. Em. λ=565 nm, Exc. λ=495 nm. I_o=Fluorescence of ARS (10^{-4} M) without boronic acid.

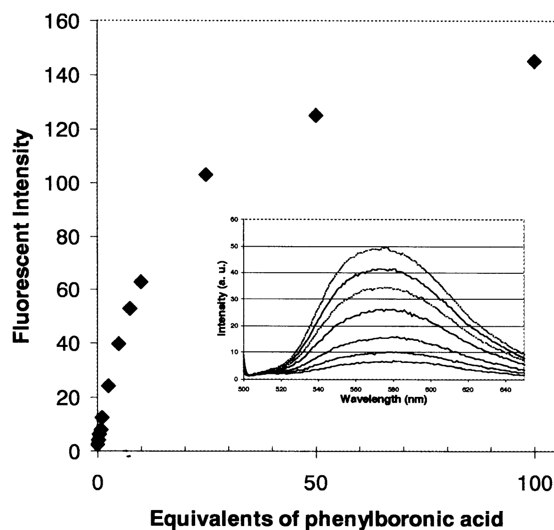


Figure 5. Fluorescent intensity increases (Exc. λ=468 nm, Em. λ=572 nm) of ARS (1.0×10^{-4} M) in the presence of phenylboronic acid (pH 7.4, 0.10 M phosphate buffer). Inset—ARS fluorescent profile with increasing concentration of ARS–PBA complex (0.25, 0.75, 1.0, 2.5, 4.0, 5.0, 7.5 equiv. of PBA).

3. Results and discussion

3.1. The ARS system and its optimal pH

As expected, the addition of boronic acid to an ARS solution increases its fluorescence intensity, presumably through the removal of the fluorescence quenching mechanism. We have examined five boronic acids and found that the fluorescence intensity increases ranged from 30 to 90-fold (Fig. 4). A typical set of fluorescence spectra, which reflect the large changes in fluorescence intensity seen in an ARS solution upon addition of a boronic acid, is shown in Fig. 5. It is worth noting that the solution also shows a λ_{max} change and a corresponding visible color change from deep red to yellow upon addition of boronic acid (Fig. 6). To understand the optimal pH for the ARS system, pH profiling was conducted with PBA and it was found that the maximum fluorescence intensity changes were observed at neutral pH (Fig. 7). This is an ideal situation because we are most interested in searching for sensors that are functional at physiological pH. The reason for the optimal sensitivity at neutral pH is presumably due to pH-dependent binding strength of the ARS–diol complex and ionization state changes. It is known that the affinity of boronic acids with diols at low pH is small and the large increase in fluorescence while raising the pH from 4 to 7 is consistent with an increase in the binding constants in this pH range. This is further substantiated by our own experiments in determining the binding constants at different pHs (Table 2, see Section 3.2). At high pH (7–12), however, we expected a continuation of the leveling off of the fluorescence since it was commonly believed that the binding constants do not reach a maximum until the pH was higher than the pK_a of the boronic acid species. Instead, the results showed a dramatic drop-off in intensity in the pH range of 7–10 (Fig. 7). This indicates that the binding constants reached their maximum at around pH 7, and any further increase in pH results in a decrease of the binding affinity. Because the binding constant of ARS with boronic acid forms the basis for the subsequent determinations of other binding constants, it was critical for us to ascertain that the drop in fluorescence intensity at pH above 7 was due to a decrease in the binding affinity. An alternative explanation is that at high pH the ARS–PBA complex may ionize from its trigonal (**7a**) to its tetrahedral form (**7b**, Scheme 4), and the tetrahedral form may be non-fluorescent. To further probe this issue, we conducted pH titration studies of the ARS solution alone and ARS (10^{-4} M) with PBA added in

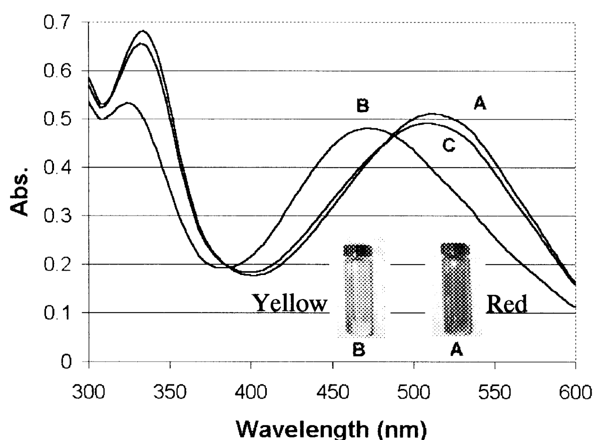


Figure 6. (A) Absorbance of ARS at 10^{-4} M in pH 7.4, 0.1 M phosphate buffer. (B) ARS at 10^{-4} M with PBA at 10^{-3} M. (C) ARS at 10^{-4} M with PBA at 10^{-3} M and fructose at 10^{-1} M.

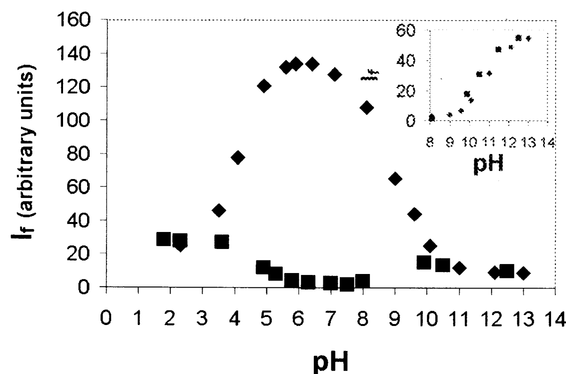
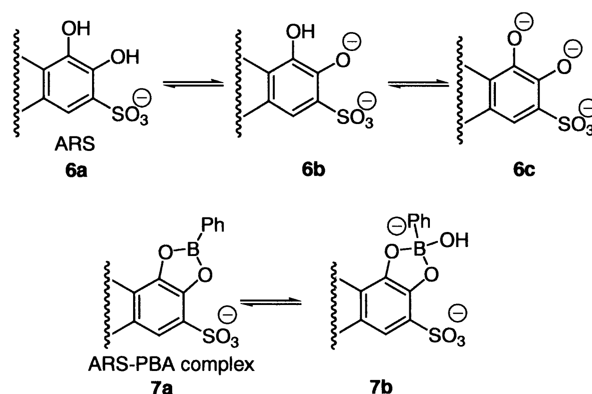


Figure 7. (■)—pH titration of the fluorescence intensity (I_f) of ARS (10^{-4} M). (◆)—ARS (10^{-4} M) in the presence of PBA (10^{-2} M). Em. λ=565 nm, Exc. λ=495 nm, 0.1 M phosphate buffer. Inset—Em. λ=633 nm, Exc. λ=600 nm.



Scheme 4. Ionization states of ARS (**6a–6c**) and its PBA ester (**7a**, **7b**).

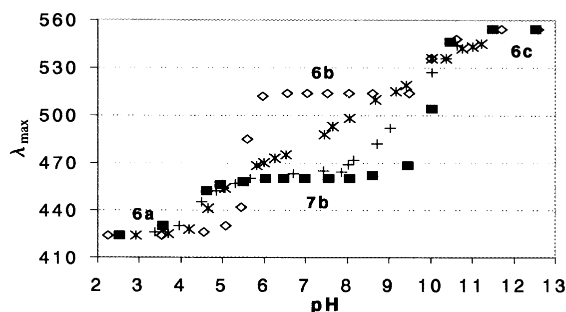


Figure 8. λ_{max} of solutions of ARS and PBA. (◇) λ_{max} of 1.0×10^{-4} M ARS by itself, (*) with 1.0×10^{-3} M PBA, (+) with 5.0×10^{-3} M PBA, (■) with 1.0×10^{-2} M PBA. See Scheme 4 for structures **6a–c**, and **7b**.

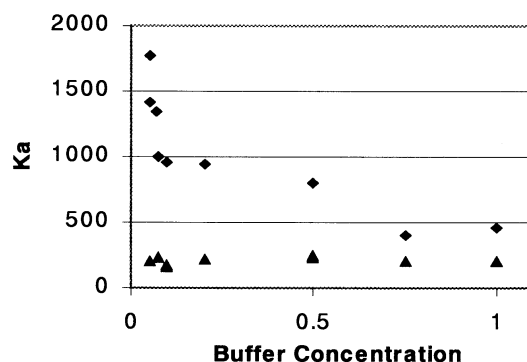


Figure 9. Association constants determined at different buffer concentrations. ◆—ARS/PBA complex in phosphate buffer, pH 7.4. ▲—Fructose/PBA complex in phosphate buffer, pH 7.4.

different ratios (0, 10, 50, and 100 equiv.). In the absence of any PBA, a shift of the UV λ_{max} from 425 to 560 nm was observed when pH was increased from 2 to 13 (Fig. 8), presumably due to the ionization state changes (**6a–6c**, Scheme 4). Two pK_as were observed at about 5.5 and 10.0 corresponding to the removal of the first phenol proton (**6b**, Scheme 4) and the second phenol proton (**6c**). Increasing amounts of PBA shift the λ_{max} in the region of pH 4.5–10 (Fig. 8) indicating the formation of the boronate ester (**7b**, Scheme 4). However, regardless of the amount of PBA added, the λ_{maxs} were the same at high pH which is consistent with the final species at high pH being the same in the presence or absence of the PBA. If the lack of fluorescence at high pH seen in Fig. 7 was due to a non-fluorescent tetrahedral species (**7b**), one would expect a UV λ_{max} in the presence of PBA that was different from the uncomplexed ARS. On the other hand, if the fluorescent decrease were due to the instability of the ester at high pH, results similar to Fig. 8 would be expected. The K_{eq} determination at different pHs further substantiates this argument (see the following sections and Table 2). Although the pK_a of the ARS–PBA ester could not be determined due to solubility problems, it was found that the catechol–PBA ester has a pK_a of 5.5. Due to the electron withdrawing groups on ARS it is likely that the ARS–PBA ester has an even lower pK_a, which would mean that the loss of fluorescence that occurs at pH 7 could not be due to the conversion of **7a** to **7b** (Scheme 4). Instead, it was due to the decreased binding constants at pH's above 7. In the literature, it is often assumed that the optimal pH for boronate esterification is above the pK_a of the boronic acid species, although it has been shown that the pH maximum of the boronate ester may be partially dependent upon the pK_a of the diol (ARS, pK_a=5.5).[37,39] Since the pK_a of ARS is much lower than the conventionally studied diols (i.e. fructose pK_a=12.1[62]), an optimum pH of 7 does appear reasonable. Therefore, the results presented here demonstrate that the optimal pH for the binding of a boronic acid can be much lower than the pK_a of the boronic acid species. The general statement that the optimal pH is above the pK_a of the boronic acid is incorrect. One needs to analyze each situation individually.

3.2. Buffer effects

In an effort to determine the best conditions to use for this study, we have also examined the buffer effect on the equilibrium constants. It has been suggested in the literature that binding constants of boronic acid–carbohydrate complexes are buffer-independent,[47] although there have been reports of buffer effects in boronic acid based affinity chromatography.[22] We tested two buffer systems, phosphate buffer and HEPES buffer. It was found that the K_{eq} in phosphate buffer for the ARS/PBA complex dropped dramatically with increasing buffer concentrations, while the K_{eq} for the fructose/PBA complex stayed fairly constant (Fig. 9). In HEPES buffer the PBA/ARS complex, K_{eq} was independent of the buffer concentration over the tested range (0–0.1 M, data not shown). However, the K_{eq} obtained at a given pH was different for these two buffers (Fig. 9). Therefore, if one is designing an experiment or comparing data from different experiments, the buffer composition and concentration needs to be considered.

3.3. The overall affinities (K_{eq}) of different diols for PBA

Using the ARS method, the K_{eq} values of a series of 22 diol-containing compounds were determined at pH 7.4 (Table 1). The results are in agreement with literature reports that 1,2-dihydroxyphenyl containing compounds, such as ARS and catechol, have very high affinities for PBA with K_{eq} values of 1300 and 830 M^{-1}, respectively. This is followed by sorbitol, fructose, tagatose, mannitol, sorbose, and 1,4-anhydroerythritol with K_{eq} values in the range of 110–370 M^{-1}. Compounds such as erythronic-γ-lactone, arabinose, ribose, sialic acid, *cis*-1,2-cyclopentanediol, glucoronic acid, galactose, xylose, and mannose have moderate affinities for PBA with K_{eq} values in the range of 13–30 M^{-1}. D-Glucose, diethyl tartrate, maltose, lactose, and sucrose only have weak affinities for PBA with K_{eq} values in the range of 0.67–4.6 M^{-1}. These results are in qualitative agreement with the literature rankings of diol affinities for boronic acid.[37,46,63,64]

It is known that pH affects the affinities of boronic acids toward diols. We have examined the pH profiles for the boronate esters formed from D-fructose, D-glucose, catechol, D-sorbitol, D-galactose, and ARS, and found that the K_{eq} increases with increasing pH, within a certain range (Table 2). With the esters of D-fructose, D-glucose, catechol, D-sorbitol and D-galactose, K_{eq} values continue to rise through at least pH 8.5. For example, the K_{eq} values for the D-fructose ester at pH 5.8, 7.4, and 8.5 are 4.6, 160, and 560 M^{-1}, respectively, and the K_{eq} of D-galactose ester increased from 2.1 at pH 6.5 to 80 M^{-1} at pH 8.5.

Table 1. Association constants (K_{eq}) with phenylboronic acid at pH 7.4, 0.10 M phosphate buffer. Values are the average of triplicate runs rounded to two significant figures

Diol	K_{eq} (M^{-1})	Diol	K_{eq} (M^{-1})
Alizarin Red S.	1300	Sialic acid	21
Catechol	830	*cis*-1,2-Cyclopentane diol	20
D-sorbitol	370	Glucoronic acid	16
D-fructose	160	D-galactose	15
D-tagatose	130	D-xylose	14
D-mannitol	120	D-mannose	13
L-sorbose	120	D-glucose	4.6
1,4-Anhydroerythritol	110	Diethyl tartrate	3.7
D-erythronic-γ-lactone	30	Maltose	2.5
L-arabinose	25	Lactose	1.6
D-ribose	24	Sucrose	0.67

Table 2. Association constants (K_{eq}) of the ester formed with PBA at various pHs, in 0.10 M phosphate buffer. Values are the average of triplicate runs rounded to two significant figures

pH	K_{eq} (M^{-1}) of the complex with PBA					
	Fructose	Catechol	Glucose	Galactose	Sorbitol	ARS
4.6						190
5.8	4.6	31				990
6.5	29	150	0.84	2.1	47	1200
6.6	35	160				1500
7.0	92	500	2.0	8.4	160	1500
7.4	160	830	4.6		370	1300
7.5	210			17		1100
8.0	310	2900	7.2	38	840	670
8.5	560	3300	11	80	1000	450

However, the situation with ARS is quite different. For ARS, the K_{eq} reaches a maximum at about pH 7. It is commonly believed that the affinity between a diol and boronic acid is the highest when the pH is above the pK_a of the boronic acid species. The pH profile of the binding between PBA and ARS indicates that such a general conclusion is not correct. In fact, there are earlier literature reports that also indicate that the optimal pH is not necessarily always above the pK_a of the boronic acid, but depends on the pK_a of both the diols and the boronic acid.[37,39] Therefore, one needs to analyze specific situations individually in the search for the optimal binding pH for a particular boronic acid and diol. It also needs to be noted that the affinity of PBA for D-fructose and catechol is very high with a K_{eq} of 160 and 830 M^{-1}, respectively at pH 7.4 (Table 2), which is 1 pH unit lower than the pK_a of PBA. This does indicate that fairly high affinities are achievable at a pH far below the pK_a of a boronic acid species. The results also seem to indicate that a low pK_a for the boronate ester corresponds to high affinity for a diol to bind to PBA, although the relationship is not necessarily linear (Fig. 3, Table 1). For example, D-fructose binds to PBA much better than D-glucose (Table 1) and the pK_a of the D-fructose boronate ester is about 2 pK_a units lower than that of D-glucose (Table 3).

Table 3. The pK_a of phenylboronic acid and six of its boronate esters

Boronic acid or ester	pK_a
Phenylboronic acid	8.8
Fructose ester	4.6
Sorbitol ester	5.7
Glucose ester	6.8
Sucrose ester	7.5
Ethylene glycol ester	8.2
1,3-propane-diol ester	9.2

3.4. Comparison with literature values and the mechanistic implications

There have already been many reports of the binding constants between PBA and the many of the diol compounds listed in Tables 1 and 2.[40,46,65] Most notable among these reports is the paper by Lorand published in 1959, which systematically examined the binding of phenylboronic acid with different diols.[46] Since its publication, this article has been cited more than 160 times, approximately 120 of which were since 1990, reflecting the importance of this paper and the binding constants reported therein. Other than this, there is no other systematic examination of boronic acid bindings with different diols. In the Lorand report, a pH-depression method was used for the determination of the binding constants. The experimental design was based on the fact that upon the formation of the boronate ester, the pK_a decreases compared with the boronic acid itself. This results in a decrease in the pH of the solution. The magnitude of the pH depression under certain conditions is directly proportional to the binding constants. This method has been used by many as a way to determine the binding constants and as a validation of other methods used for such determination.[46,66–70] There were two important assumptions of the pH depression method. First, it is assumed that only the tetrahedral boronate (**2**, Scheme 1) reacts with the diol to form the boronate ester, and secondly, none of the trigonal boronic ester (**3**, Scheme 1) exists in solution. When our binding constants were compared with that of the Lorand's, very significant discrepancies were observed. A careful examination of the mathematical equations (particularly Eqs. (5) and (12) of the cited paper)[46] indicate that the binding constants determined using the Lorand pH-depression method are actually K_{tet}s, not K_{eq}s and therefore cannot be compared with the binding constants determined using other spectroscopic method. This is intuitive from examination of Scheme 1. If the trigonal ester did not exist and only the tetrahedral boronate **2**, as proposed by Lorand, could react with diols, then K_{tet} would describe the equilibrium. In order to compare our data with that of Lorand's, we needed to calculate the values of $K_{eq\text{-}trig}$ and $K_{eq\text{-}tet}$ from the overall K_{eq}. Using the K_{eq}s and pK_a's listed in Tables 1,–3, the $K_{eq\text{-}tet}$s and $K_{eq\text{-}trig}$s at different pHs were calculated for D-fructose, D-sorbitol

Table 4. $K_{eq\text{-}trig}$ (M^{-1}) and $K_{eq\text{-}tet}$ (M^{-1}) values for fructose, sorbitol and glucose at different pH values. Values are the average of triplicate runs rounded to two significant figures

pH	$K_{eq\text{-}trig}$ (M^{-1}) and $K_{eq\text{-}tet}$ (M^{-1}) of the complex with PBA					
	Fructose		Sorbitol		Glucose	
	$K_{eq\text{-}trig}$	$K_{eq\text{-}tet}$	$K_{eq\text{-}trig}$	$K_{eq\text{-}tet}$	$K_{eq\text{-}trig}$	$K_{eq\text{-}tet}$
6.0	0.33	4200	5.0	6300		
6.5	0.42	5300	6.5	8100	0.54	54
7.0	0.47	5900	7.7	9600	0.71	71
7.5	0.43	5400	7.5	9500	0.77	77
8.0	0.20	2600	4.9	6100	0.50	50
8.5	0.12	1500	2.4	3000	0.33	33
9.0	0.072	910	2.3	2900	0.26	26

and D-glucose (Table 4, also Eqs. (6) and (7), Fig. 1). This represents the first time that a $K_{eq\text{-}trig}$ has ever been determined. It had been previously thought that no trigonal ester existed in such reactions.[46] However, our calculations (Fig. 10) of the relative ratios of different species based on their binding constants at different pHs clearly shows that the effect of the neutral ester on the equilibrium cannot be ignored in assessing the overall affinity of boronic acid to a particular diol. It is also noteworthy that the pK_a of the ester is not always lower than that of the boronic acid.[66] For example, with 1,3-propane diol (Fig. 3), and *cis* and *trans*-cyclohexane diol (data not shown), the ester pK_a is higher than that of the boronic acid by itself. Therefore, it seems that with diols that bind to boronic acid strongly, the pH depression effect is most significant. However, with weak binders, the pH depression effect is small or non-existent. For some extremely weak binders, the pK_a of the ester is even higher than the boronic acid.

Several things are obvious after examining the data in Table 4. First, as expected, both $K_{eq\text{-}trig}$ and $K_{eq\text{-}tet}$ are pH-dependent. It is interesting to note that both $K_{eq\text{-}tet}$ and $K_{eq\text{-}trig}$ are related to the K_{eq} with the same dependency on the proton concentration and pK_as, and the ratio of $K_{eq\text{-}tet}/K_{eq\text{-}trig}$ is a constant for a particular diol, equal to K_{ester}/K_{acid} (see Eqs. (6) and (7), Fig. 1). Since the pK_a of the boronate ester of many of the monosaccharides is 2–4 units lower than that of boronic acid, $K_{eq\text{-}tet}$ is expected to be about 10^2–10^4 times higher than $K_{eq\text{-}trig}$. As a result, the $K_{eq\text{-}tet}/K_{eq\text{-}trig}$ ratio for D-fructose is about 12,589, and the same ratio for D-glucose is 100, regardless of the pH. Second, the optimal pH for both $K_{eq\text{-}tet}$ and $K_{eq\text{-}trig}$ is different from that of the overall K_{eq}. For

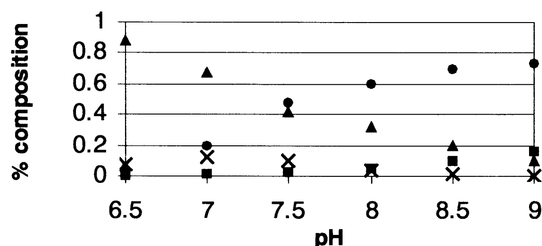


Figure 10. Relative proportions of the trigonal and tetrahedral forms of phenylboronic acid and ester in the presence of 0.0050 M glucose. (0.0020 M PBA, and 9.0×10^{-6} M ARS) calculated from experimentally derived values of the acid and ester pK_as and the glucose association constants over the pH range. ▲—Trigonal boronic acid, ■—tetrahedral boronic acid, ×—trigonal boronate ester, ●—tetrahedral boronate ester.

example, while the optimal pH for the fructose $K_{eq\text{-}tet}$ and $K_{eq\text{-}trig}$ is at about pH 7, there is no leveling off of the overall K_{eq} up to pH 8.5 for fructose (Table 2). The same thing is true for sorbitol and glucose. This is easy to understand since the overall K_{eq} is a function of many parameters including $K_{eq\text{-}tet}$, $K_{eq\text{-}trig}$, pK_as, and the pH. This correlation is not linear with respect to the pK_a and pH. Since $K_{eq\text{-}tet}$ is far greater than $K_{eq\text{-}trig}$ and the concentration of the tetrahedral boronate (**2**, Scheme 1) increases with increasing pH, the concentration of the tetrahedral 'starting material' is greater at higher pH. This increased concentration of the tetrahedral boronate can compensate for the slight decrease in $K_{eq\text{-}tet}$ with increasing pH (Table 4) within the pH range examined, which therefore results in an increase in the overall affinity (K_{eq}) with increasing pH for D-fructose, D-sorbitol and D-glucose.

It is known that boronic acid–diol affinity is pH-dependent, whereas the $K_{eq\text{-}tet}$s obtained with the pH depression method were determined over a pH range of several units (pH not fixed).[46] Most likely, these results closely reflect the $K_{eq\text{-}tet}$ at the end point, which was said to be 2–3 pH units lower than the pK_a of boronic acid (8.8). For example, the $K_{eq\text{-}tet}$ for the glucose–PBA complex calculated using the pH depression method was 110 M^{-1}. With the ARS method, the $K_{eq\text{-}tet}$ for glucose at pH 8.5 was 33 and increased to 77 M^{-1} at pH 7.5, which is close to the likely end pH that one would have obtained using the pH depression method. Similarly, for D-fructose, the $K_{eq\text{-}tet}$ obtained using the pH depression method was 4370 M^{-1}, and with the ARS method, the $K_{eq\text{-}tet}$ for D-fructose at pH 8.5 and 6.0 was 1500 and 4200 M^{-1}, respectively. These results show a somewhat qualitative agreement between these two methods. However, the pH changes in the pH depression experiments are dependent upon the amount of diol added. The binding constants were determined by varying the amount of diol added, and therefore, conceivably varying the pH. This would give numbers that should be intrinsically different because the final pH's would not be the same. In the Lorand paper, the diol concentrations ranged from 0.2 to 1.0 M, and the pH varied by about 2–3 pH units. In our own studies, we have observed that changes in the pH of the solution by 2 pH units in this region could change the binding constants by several-fold (Tables 2 and 4). Therefore, the binding constants obtained with the pH depression method can only be used as a general estimate of the affinity over certain pH range.

Another limitation of the pH depression method is the requirement that the boronic acid be used as the buffer. It is highly unlikely, in the preparation of sophisticated boronic acid-based antibody mimics, that we would have such a large quantity of the boronic acid compound that we could use it as the buffer. Finally, the mathematical equations used in the pH depression method assume that no neutral boronate ester species exists and that the concentration of free trigonal boronic acid remains constant (see Eq. (12) of Lorand's paper). In most cases, the starting pH was about 8.8. Assuming a 3-unit drop in the pH of the solution, this would give a pH of about 5.8. It is known that the pK_a of the ester is often about 2–4 units lower than that of the acid (Figs. 2 and 3, and Table 3). Therefore, it is realistic to expect that a significant portion of the ester

may exist in the neutral form at high diol concentrations (Fig. 10). This consequently invalidates the assumption that no neutral ester exists. Intuitively, this is also easy to understand. The pK_a of the boronate ester of glucose has been determined as 6.8. Therefore, at pH 6.8, half of the ester exists in the neutral form, while half exist in the tetrahedral ionic form. Even at neutral pH (7.0), there should be 39% of the ester in the neutral form. In the pH-depression method, mannitol showed 'abnormal' behaviors.[46] This was explained by assuming that neutral esters were formed which resulted in a deviation from the 'normal' behaviors. However, in our own studies, mannitol behaved the same way as any other sugar, giving linear curve fittings. Therefore, the ARS method overcomes many of the problems associated with the commonly used pH depression method and allows for the determination of the overall affinity, K_{eq}, and the equilibrium constants for each step, $K_{eq\text{-}trig}$ and $K_{eq\text{-}tet}$.

Another commonly used method for detecting boronate esters is the ^{11}B NMR method. When the distinct species can be directly detected, this offers a direct and excellent approach to the determination of the binding constants. However, the ^{11}B NMR method suffers from low sensitivity, difficulties with peak resolution, and the requirement for high concentration of the sensor compound. Such restrictions make the ^{11}B NMR method less useful in the development of boronic acid sensors. As discussed before, fluorescent methods have been used, however, only with those boronic acid compounds that are fluorescent themselves. The ARS method imposes no such limitations. Shinkai and co-workers have also used CD for the determination of the binding constants with much success.[71] However, this again is restricted by the requirement of having chiral substrates, and the sensitivity is not as high as the fluorescent method. Therefore, our approach offers the advantages of: (1) high sensitivity, (2) general applicability, (3) reproducibility, and (4) flexibility with regard to the pH and buffer used.

4. Conclusion

The design of the next generation of boronic acid based sensor will require knowledge of the intrinsic binding affinities between boronic acid and diol moieties. The ARS system can be used to rapidly compare the affinities of a large number of boronic acid bearing compounds. Furthermore, this method provides a mechanism to study ester formation under physiological conditions (aqueous pH 7.4, etc.) without the limitations of some of the previous methods, which included solvent, buffer, and pH constraints as well assumptions that may lead to erroneous values. Our method also allows for the determination of the overall affinity, K_{eq}, and the equilibrium constants for each step, $K_{eq\text{-}tet}$ and $K_{eq\text{-}trig}$. The latter has never been determined before. Our results also correct many literature mistakes or misperceptions. First, the optimal binding pH is not always above the pK_a of the boronic acid species. Second, the nature of the buffer and its concentration do affect the boronic acid binding affinity, sometimes quite dramatically. Third, the neutral boronate species does exist with a non-negligible concentration, even at neutral pH with some esters. Fourth, the binding constants determined using the pH depression method are not overall equilibrium constants. They only represent one step of the equilibrium, and there may be significant experimental errors due to incorrect assumptions. Therefore, the data determined using the pH depression method cannot be directly compared with many binding constants reported in the literature, which were determined using spectroscopic methods. Fifth, the assumption that the pK_a of the ester is always lower than that of the acid is incorrect. The greater understanding of boronate ester formation achieved through this study will aid the effort to develop boronic acid-based antibody mimics and will enhance the potential for future discoveries in the fields of carbohydrate-based labeling, imaging, analysis, and drug delivery systems.

5. Experimental

5.1. General methods

Alizarin Red S. and phenylboronic acid were purchased from Acros and used as received. Sugars, buffers, and diols were bought from Aldrich and Acros and were used as received. The water used for the binding studies was double distilled and further purified with a Milli-Q filtration system. Quartz cuvettes were used in all studies. All data was plotted on Microsoft Excel.

5.2. Fluorescence and absorbance binding studies

A Shimadzu RF-5301PC fluorometer was used for all fluorescent studies. A Shimadzu UV-1601 UV–visible spectrophotometer was used for all absorbance studies. For a typical ARS–boronic acid fluorescent measurement, a 9.0×10^{-5} M stock solution of ARS in 0.10 M sodium phosphate monobasic buffer, made within the last 7 days and stored in the refrigerator, was diluted 10-fold with 0.10 M sodium phosphate monobasic buffer and brought to the correct pH (within.01 units, pH was measured with an Accumet portable pH meter) with 4N NaOH resulting in a 9.0×10^{-6} M solution of ARS with 0.10 M phosphate buffer at the appropriate pH (solution A). This concentration of ARS was chosen because it was within the range of linear response (fluorescence versus concentration, data not shown) and also gave a strong fluorescence profile. PBA was added to a portion of solution A to make a 9.0×10^{-6} ARS, 2.0×10^{-3} M PBA solution (Solution B). The pH was again checked and corrected if necessary. Solution B was titrated into solution A in order to make mixtures with a constant concentration of ARS and a range of concentrations of PBA. In general, eight different concentrations were made in order to cover as much of the binding curve as possible. Each mixture was allowed to stand for at least 5 min, although absorbance and fluorescence studies showed that equilibrium was reached within 30 s and solution measurements were stable for hours. Then 3.5 mL of the mixture was transferred into a cuvette for fluorescence measurement. The intensity of the emission was recorded at 572 nm. The excitation wavelength was set at 468 nm for all quantitative experiments. The experiments were carried out in triplicate. Absorbance studies were performed in a similar

manner except that higher concentrations were used, ARS 1.0×10^{-4} M, and PBA 1.0×10^{-2} M.

5.3. Competitive studies

Competitive studies were run in a similar manner to the ARS–boronic acid studies. A solution of 9.0×10^{-6} M ARS and 2.0×10^{-3} M PBA was brought to the correct pH in 0.10 M phosphate buffer (Solution B). Enough diol was added to a portion of solution B so that 65–80% of the ARS was in free form (Solution C) (measured by fluorescence, see Q, Eq. (5), Fig. 1). Solution C was titrated into solution B in order to make mixtures with a constant concentration of ARS and PBA and a range of concentrations of diol. In general, eight different concentrations were made in order to cover as much of the binding curve as possible.

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