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Role of Nitrogen Lewis Basicity in Boronate Affinity Chromatography of Nucleosides

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Urinary modified nucleosides have a potential role as cancer biomarkers, and most of the methods used in their study have utilized low-pressure phenylboronate affinity chromatography materials for the purification of the *cis*-diol-containing nucleosides. In this study, a boronate HPLC column was surprisingly shown not to trap the nucleosides as would be expected from experience with the classic Affigel 601 resin but showed only partial selectivity toward *cis*-diol groups while other groups exhibited better retention. In aprotic conditions, trapping of nucleosides was possible; however, the selectivity toward *cis*-diol-containing compounds was lost with the Lewis basicity of available nitrogens being the main determinant of retention. The experimental findings are compared to and confirmed by DFT calculations.

Modified nucleosides are naturally occurring modifications of the "normal" nucleosides. They have various roles within many nucleic acids but are mainly found in transfer RNA. They are excreted from the body via the urine as they cannot be salvaged; moreover, some are toxic when allowed to accumulate. Many past reports have investigated the modified nucleosides as potential cancer biomarkers and indicate considerable promise.[1−5] The methodologies used in these studies are wide ranging; however, since the introduction of boronate affinity chromatography as a ribonucleoside-selective cleanup step, on Affi-Gel 601 (Bio-Rad), utilized by Gehrke et al.,[1,2] most research employed this off-line cleanup step process in the analysis. The subsequent identification/quantification of the ribonucleosides was almost exclusively carried out via RPLC-UV methods. More recently, some CE-UV methods have also been developed.[6−9] The further potential/demand to obtain unambiguous identification via mass spectrometric detection led to the development of some off-line boronate chromatography GC/MS procedures.[3,5,10] However, the most natural choice for the analysis of the prepurified urinary nucleosides analysis is found in LC−MS.[11] Yet, the development of LC−MS procedures for urinary nucleosides only advanced[12] when electrospray mass spectrometry (ESI-MS) became available. Past studies by our group have considered the cleanup samples prior to ESI-MS analysis,[13] the optimization of the detection conditions,[14] comparison of various mass spectrometric methods,[15] and identification of the excreted nucleosides.[16,17] Other groups have taken advantage of mass spectrometry in the study of these compounds. Liebich et al. utilized CE and HPLC with matrix-assisted laser

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desorption/ionization time-of-flight MS[18,19] or ion trap mass spectrometry.[20] Again, these studies utilized the Affi-Gel 601 affinity cleanup step off-line prior to analysis. The Affi-Gel 601 resin binds *cis*-diol groups of the nucleosides selectively at high pH. The early reports on the uses of boronate chromatography were to allow the separation of individual nucleosides without preconcentration or prepurification being achieved as per the original paper,[21] while it has been more recently been utilized to preconcentrate and prepurify nucleosides prior to analysis.[22,23] In both cases (with and without the preconcentration being achieved), the complexation of the 2′,3′-*cis*-diol of the β-D-ribofuranosyl moiety of the nucleosides with the immobilized boronic acid is considered responsible for the observed chromatographic behavior.

The underlying concept of boronate affinity chromatography was recognized by Böeseken: diol moieties in a planar cis configuration form a complex with boronic acid resulting in negatively charged cyclic boronate esters[24] (Figure 1b, third equilibrium). In an aqueous environment, the equilibrium between immobilized boronic acids and *cis*-diol-containing species and the resulting cyclic boronate esters is believed to be established sufficiently quickly to permit chromatography. To shift the equilibrium to the cyclic boronate ester, high-pH conditions are required. This leads to the basal concept, as exploited in the preconcentration of urinary nucleosides,[22,23] that the cyclic boronate esters are selectivity formed at high pH (trapping) and that release of the compounds is achieved by lowering the pH. Recent in silico modeling studies suggested that formation of the cyclic boronic ester happens via a stepwise mechanism involving a bimolecular dehydration followed by a unimolecular H_2O elimination.[25] It is also considered that neutral boronate esters can be formed that hydrolyze quickly in nonbasic aqueous conditions (Figure 1a), though in organic (aprotic) solvents they will persist.[26] Additional insights are found in saccharide sensor research.[27,28] Yan et al.[29] negated some general beliefs concerning the use of boronate material, demonstrating that the chemical interactions involved are not fully understood yet; thus, the chemistry of the stationary phase and its subsequent use in purification of *cis*-diol-containing species requires further investigation.

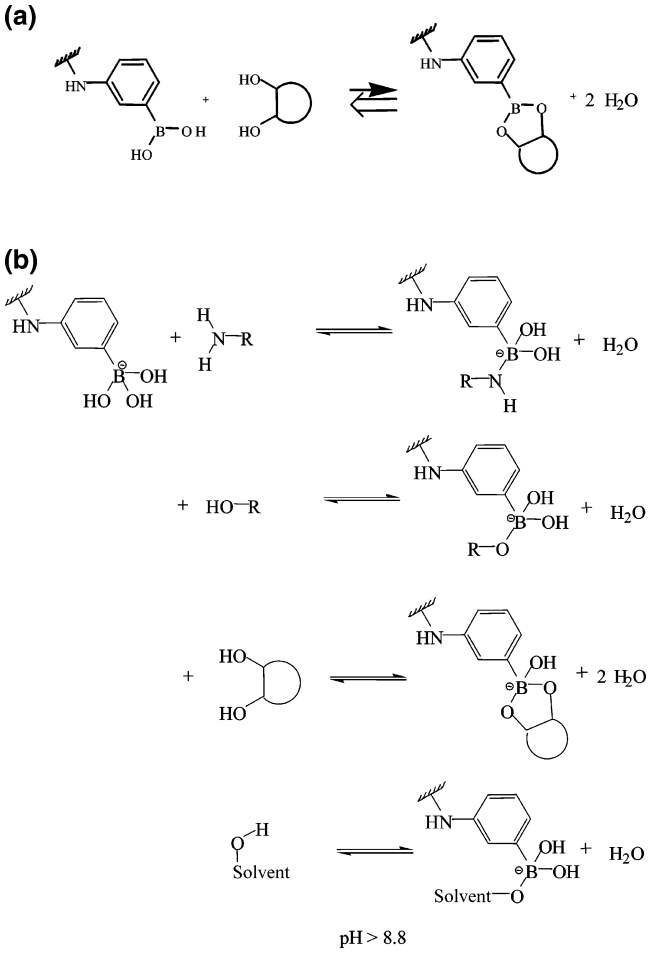

Figure 1. (a) Boronate affinity model for *cis*-diol retention, (b) "Extended" model of boronate affinity chromatography.

HPLC-compatible boronate stationary phases have recently become available (Affi-Gel from BioRad is unsuitable for HPLC due to its limited pressure resistance and change in volume with altered ionic conditions) and offer the potential for on-line purification and thence higher automated throughput of urinary nucleoside analysis. The aim of the work detailed here was to study the properties of these phases in order to investigate whether selective on-line HPLC trapping of nucleosides is practicable. A second aim was to detail the mechanisms important in the retention of nucleosides by the boronate stationary phases in order to determine the chemical criteria that need to be fulfilled when utilizing boronate-based affinity purification methods for nucleoside purification.

EXPERIMENTAL SECTION

Chemicals. All compounds used were purchased from Sigma-Aldrich (Bornem, Belgium) except the following. Thymidine, 2′-deoxyadenosine, and imidazole were from Janssen Chimica (Beerse, Belgium); adenine was from Merck (Darmstadt, Germany); purine was from Fluka (Buchs, Switzerland), and 4-aminopyrimidine, magnesium chloride, ammonium acetate, and NH_4OH 28−30% (w/w) (analytical-reagent grade) were from Acros Organics (Geel, Belgium). Water (HPLC grade) and acetonitrile (ACN, HPLC grade) were respectively obtained from Biosolve (Valkenswaard, The Netherlands) and Acros Organics (Geel, Belgium).

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Sample Preparation. Under protic conditions, the samples were made up in 0.1 M NH_4OAc, pH 8.8 (10^{-4} M). For the aprotic experiments, similar samples were prepared in ACN. When the compounds did not dissolve sufficiently, the oversaturated solutions were sonicated for 5 min and then filtered (13-mm HPLC Syringe Filter, 0.2-μm nylon, Alltech, Lokeren, Belgium). The corresponding saturated solutions were then used. In the case where no or an insufficient UV response was achieved, the samples were made up in 1-methyl-2-pyrrolidinone (NMP). Each compound was injected individually onto the column in order to study specifically their retention without interference caused by alternative mechanisms.

Mobile Phases. The 0.1 M NH_4OAc, pH 8.8, was prepared and NH_4OH was used to adjust the pH. The 0.1 M NH_4OAc/0.1 M $MgCl_2$, pH 8.8, was prepared in the same way. The resulting mobile phases were vacuum-filtered over a 0.2-μm nylon filter (Alltech Associates, Lokeren, Belgium). The 80:20, 90:10, and 95:5 ACN/H_2O mobile phases were prepared by mixing the appropriate volumetric quantities.

Instrumentation and HPLC Conditions. HPLC-UV measurements were performed on a Kontron HPLC system (Kontron Instruments, Milan, Italy) consisting of a 325 pump module, a 465 injector module with a 20-μL loop, and a 332 UV detection module equipped with a conventional flow-through UV cell. UV detection was carried out at 260 nm for the nucleobases, nucleosides, and 2′-deoxynucleosides, and at 220 nm for the simple aromatic species. The boronate column used was a Prosphere Boronate Affinity column with dimensions 75 × 7.5 mm (i.d.), particle size 10 μm (Alltech Associates Inc., Lokeren, Belgium), and capacity factor (k), plate number (N), selectivity (α), and resolution (R) were calculated in order to gauge compound retention using the peak of the solvent as t_0:

$$k = \frac{t_r - t_0}{t_0}; \quad N = 5.54\left(\frac{t_r}{w_{1/2}}\right)^2; \quad \alpha = \frac{k_2}{k_1};$$

$$R_s = 1.18\,\frac{(t_{r_2} - t_0) - (t_{r_1} - t_0)}{(w_{1/2})_1 + (w_{1/2})_2}$$

Chromatographic Conditions. Chromatography is performed at a flow rate of 1 mL/min. Runs were mainly isocratic; however, in the aprotic experiments, a gradient program was used. Following injection, 10 min of 100% ACN was maintained after which a linear gradient to 100% H_2O or 100% 0.1 M NH_4OAc, pH 8.8, in 30 min was used.

Calculations. Density functional theory (DFT) calculations were performed using Gaussian 03,[30] as installed on the computing cluster CalcUA. Equilibrium geometries were obtained at the B3LYP/6-31G(d) level. Additional evidence supporting the nature of the stationary phase was obtained by calculating the corresponding Hessian and by carefully analyzing the number of imaginary vibrational frequencies derived from them. The Cartesian coordinates, charge distributions, and vibrational frequencies for the geometries obtained are available from the authors upon request.

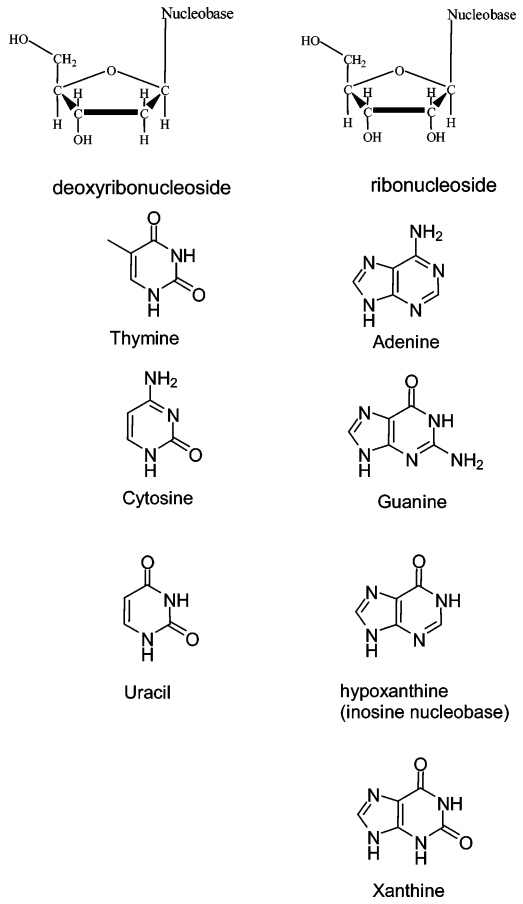

Figure 2. Compounds of test set 1: nucleosides and nucleobase structures.

RESULTS AND DISCUSSION

Protic Conditions. As a first test of the affinity trapping system, the retention behavior of a number of ribonucleosides and 2′-deoxynucleosides (test set 1, Figure 2) in 0.1 M NH_4OAc, pH 8.8, was examined (Table 1); no trapping was achieved under these conditions; however, the 2′-deoxyribonucleosides eluted faster than their corresponding ribonucleosides. The ribonucleosides could also be discriminated from the 2′-deoxyribonucleosides by the N values, as the *cis*-diol-containing species' plate numbers are lower indicating differing retention mechanisms. A pH value of 8.8 was utilized as this replicated the previous experiments for the low-pressure affinity purification of *cis*-diol compounds from urine.[1−5] In order to counter the possibility that the pH was adjusted on the column, the separation was re-examined at pHs between 9.8 and 10.8; again, no group separation of the *cis*-diol-containing compounds was exhibited (data not shown).

The variation in retention among the ribonucleosides clearly indicates that other retention mechanisms are superimposed on the *cis*-diol complexation mechanism. The nucleosides guanosine (Guo) and adenosine (Ado) exhibit higher retention compared to other ribonucleosides, suggesting that the presence of an exocyclic NH_2 group increases retention time. On the other hand, the nucleoside xanthosine (Xao) is not fully resolved from the 2′-deoxyribonucleosides. This behavior can be explained by the presence of the highly acidic N(3)H proton (pK_a 5.7) in Xao,[31]

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Table 1. Summary of the Chromatographic Behavior of Test Sets 1 and 2 under Protic Conditions

compd	k	N		α	R_s
		test Set 1			
dThd	0.18	1391			
dGuo	0.31	1322	dGuo/dThd	1.69	0.94
dAdo	0.42	1132	dAdo/dGuo	1.36	0.99
Xao	1.06	875	Xao/dAdo	2.51	2.47
Urd	1.23	634	Urd/Xao	1.16	0.58
Cyd	3.28	562	Cyd/Urd	2.68	3.96
Ψrd	3.57	543	Ψrd/Cyd	1.09	0.42
Ino	4.19	677	Ino/Ψrd	1.17	0.61
Guo	6.98	620	Guo/Ino	1.66	2.81
Ado	11.54	834	Ado/Guo	1.65	2.81
		Test Set 2			
Hyp	0.22	2105			
dAdo	0.42	1132	dAdo/Hyp	1.90	nc
Pur	0.44	2050	Pur/dAdo	1.04	nc
6-Cl-pur-rib	0.71	742	6-Cl-pur-drib/Pur	1.63	nc
Ade	1.97	2995	Ade/6-Cl-pur-drib	2.77	nc
Neb	7.82	729	Neb/Ade	3.97	nc
Ado	11.54	834	Ado/Neb	1.48	nc

implying Xao is negatively charged under typical boronate chromatographic conditions. Anion exclusion by the stationary phase is anticipated under these conditions. Based on this first set of data, an "extended" hypothesis was formed to try and explain these unexpected retention properties (Figure 1b). This model assumes that other functional groups present in the analytes, such as amino and hydroxyl groups, are also capable of forming covalent bonds with the immobilized boronic acid. The associated equilibria are suspected to compete to some extent with the *cis*-diol complexation. As boronate chromatography for nucleosides is always performed in aqueous conditions, the extended model also considers the solvent as an important competitor, due to its excessive presence.

To further investigate the retention mechanisms of the boronate column, test set 2 (Figure 3) was used to probe the impact on retention of some characteristic functional groups present in nucleosides (Table 1).

The 2′-deoxyadenosine (dAdo) elutes before adenosine and purine (Pur) elutes before nebularine (Neb), indicating that the *cis*-diol moiety plays a role in retention. The extended boronate model predicts no retention for Pur, yet this is contradicted by the experiment. The observation that hypoxanthine (Hyp) and dAdo precede Pur is also unexpected. The contribution of the hydroxyl group of Hyp was thought to aid retention, yet these results suggest that this is not important to retention. The impact of the exocyclic NH_2 is more significant as an associated α-value of 4.48 is found when Pur and adenine (Ade) are compared. This apparent effect is the first indication that competitive mechanisms may limit selectivity toward *cis*-diol moieties exerted by boronic acids. However, this NH_2 effect seems to be suppressed when a 2′-deoxyribofuranosyl moiety is also present, as seen for dAdo. Cummulative effects on retention are clear from comparison of Neb−Ado and *A*de−Ado. The plate numbers (N) determined indicate low N values when reversible cyclic ester formation is involved, thus suggesting a slow equilibration process. It was briefly investigated whether decreasing the flow rate might improve the selectivity of the boronate stationary phase; however, no selective retention enhancement of the *cis*-diol compounds was obtained (data not shown). Previous reports show that the addition of divalent cations, such as Mg^{2+}, positively affects boronate selectivity toward *cis*-diol moieties.[32,33] Therefore, the test compounds were reanalysed using 0.1 M NH_4OAc/0.1 M $MgCl_2$, pH 8.8. The results are summarized in Table 2. A more pronounced separation between dAdo and uridine (Urd) ($\alpha = 3.9$ and $R_s = 4.17$) can be seen, with Xao moving in the elution sequence and now eluting after Urd. The k values of all nucleosides increase by a factor of between 1.88 and 0.29 while non-*cis*-diol compounds are either unaffected or elute earlier; thus, the addition of the Mg^{2+} salt has a major effect on the selectivity of the boronate phase. This phenomenon can be attributed to electrostatic effects of the eluent cations stabilizing the anionic boronate complexes, with divalent cations being the most effective.[33] The increase in k is most pronounced for Xao (× 4.29), which, while hardly retained before, now elutes clearly in the second, *cis*-diol-containing group. In view of its acidic N(3)H, it can be envisioned that the negative charge on the base moiety of Xao is compensated for by Mg^{2+}. Yet boronate ester formation is clearly not the only factor determining retention since Ado is retained almost 10× longer than Urd. Higher amounts of the ion might result in chromatographic behavior suitable for on-line sample cleanup and preconcentration; however, in the context of mass spectrometric detection (the final aim of the on-line detection system), the use of nonvolatile salts is undesirable.

Aprotic Conditions. Theoretically, the aqueous solvent could compete with retention as proposed in the extended model for boronate retention. Therefore, a set of compounds (test set 3, Figure 4) were used in order to investigate whether H_2O is competing with the *cis*-diol moiety. Different ratios of ACN/H_2O were tested, and by lowering the H_2O content, its competition would be expected to diminish. The 80:20, 90:10, and 95:5 ACN/H_2O experiments demonstrated that no compounds showed notable retention, except 4-methoxybenzylamine, which did not elute from the column (Table 3). With 100% ACN, the proposed extended model would predict that non-*cis*-diol hydroxyl groups and amine groups could aid retention (Figure 1b). The observation that anisole is not retained at all is in good agreement with these models; however, from comparison of the k values (Table 3) of anisole, 4-methoxybenzyl alcohol, and phenol, it is clear that the presence of a non-*cis*-diol hydroxyl group does not affect retention. However, catechol is retained under the 100% ACN loading conditions and only released when a H_2O gradient is applied. This demonstrates that the presence of the *cis*-diol causes retention and other hydroxyl groups do not; hence, the assumption made in the extended retention model concerning interactions by non-*cis*-diol hydroxyl groups is not valid, unless high concentrations are present (e.g., H_2O in the mobile phase). When considering the influence of an amine group, a distinct ambiguity appears; the aromatic amine does not experience any substantial retention, whereas the aliphatic amine (4-methoxybenzylamine), differing only in Lewis basicity, is trapped on the column and not eluted.

Differing ACN percentages were utilized for the separation of nucleosides, nucleobases, and related compounds, as previously used in protic conditions. Applying the 95:5 ACN/H_2O conditions

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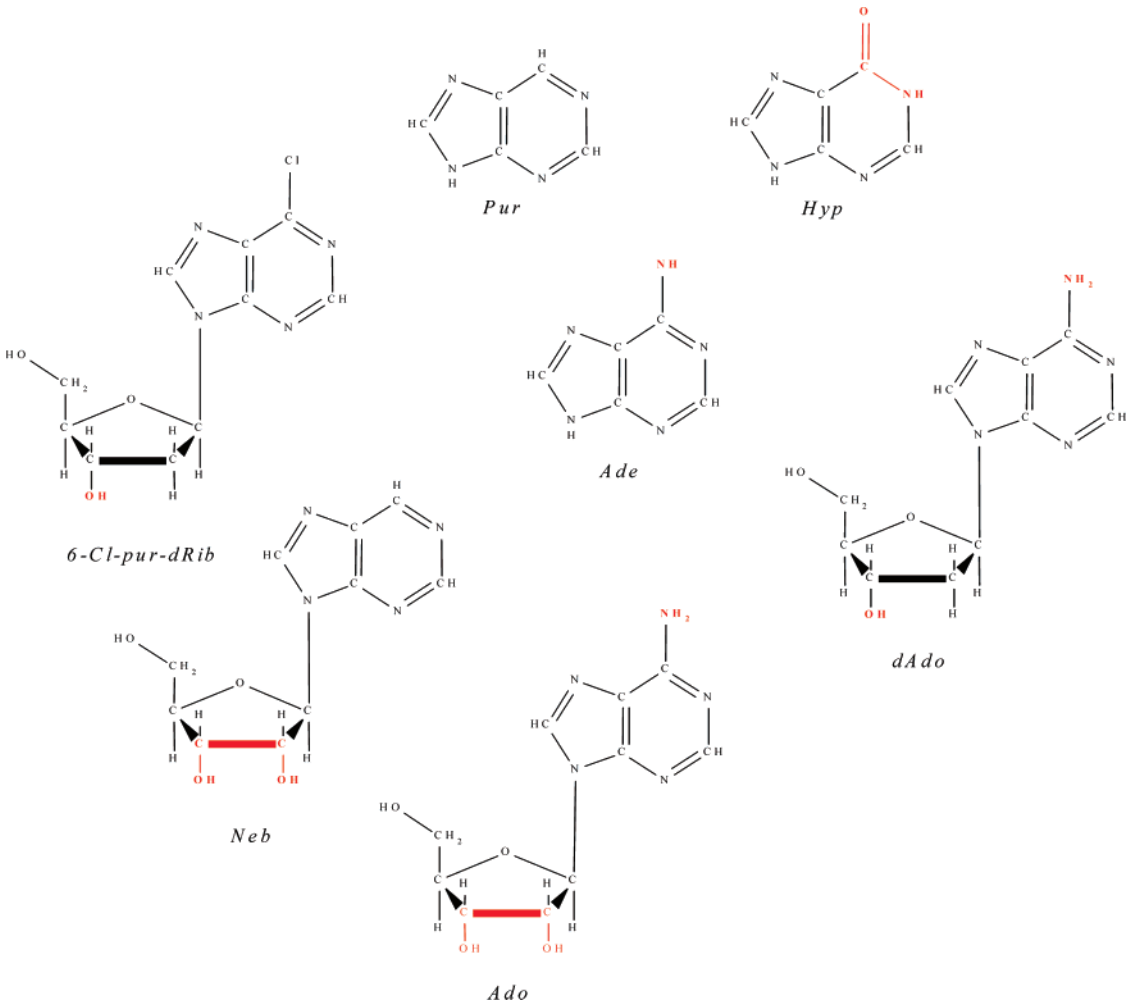

Figure 3. Compounds of test set 2.

Table 2. Effect of Mg^{2+} Ion Addition on the Chromatographic Behavior of the Combined Test Sets 1 and 2

compd	K	N		a	R_s^*	k_{Mg2+}/k_{Ref}
dThd	0.16	1317				0.90
Hyp	0.24	2128	Hpo/dThd	1.45	0.54	1.08
dGuo	0.31	1505	dGuo/Hyp	1.30	0.58	1.00
Pur	0.54	1891	Pur/dGuo	1.73	1.71	1.23
dAdo	0.59	1065	dAdo/Pur	1.11	0.30	1.41
6-Cl-pur-rib	0.73	706	6-Cl-pur-rib/dAdo	1.22	0.59	1.02
Ade	1.24	1305	Ade/6-Cl-pur-rib	1.71	2.06	0.63
Urd	2.30	442	Urd/Ade	1.85	2.42	1.88
Xao	4.54	644	Xao/Urd	1.97	2.97	4.29
Ψrd	7.44	420	Ψrd/Xao	1.64	2.33	2.08
Cyd	7.98	415	Cyd/Ψrd	1.07	0.30	2.43
Ino	9.06	521	Ino/Cyd	1.14	0.62	2.16
Neb	13.57	503	Neb/Ino	1.50	2.07	1.94
Guo	14.90	582	Guo/Neb	1.10	0.54	1.90
Ado	22.48	532	Ado/Guo	1.51	1.99	1.95

caused an increased retention behavior compared to their retention in 80:20 and 90:10 ACN/H_2O (Table 4). The comparison of the retention factors of thymidine (dThd) and Urd under 100% ACN is interesting due to their high structural resemblance and the presence of a *cis*-diol in Urd. This comparison shows that *cis*-diol compounds are retained and are released at low H_2O concentrations, reconfirming the competitive interaction by the aqueous solvent. The data in Table 4 also indicate that in 100% ACN the ribonucleosides and 2′-deoxyribonucleosides are also retained in a manner dependent upon the structure of their base moiety. All compounds, with the exception of dThd, only elute from the column when H_2O is applied. However, the order of release is determined more by the base moiety structure than by the presence of a *cis*-diol group. Provisionally, the contribution to retention can be arranged as follows: exocyclic NH_2 < lactam and pyrimidine < purine. Under the 100% aprotic conditions, Pur is retained on the column (k = 3.40) but elutes just before the H_2O gradient is applied. Its substantial retention is remarkable as it lacks any functional group permitting covalent bond formation and so no boronic ester formation is possible. All other compounds of the test set were retained until the H_2O was added to the mobile phase. The influence of a lactam, an exocyclic NH_2, or a *cis*-diol functionality is supported by the retention behavior of, respectively, Hyp, Ade, and Neb compared to Pur (Table 4). Interestingly, Ade exhibits an increased retention compared to Ado. To probe which structural part of Ade contributes to its retention, imidazole and 4-aminopyrimidine were investigated as these two compounds are identical in structure to the two halves of the Ade molecule. 4-Aminopyrimidine exhibited some retardation yet eluted during the 100% ACN loading (k = 1.67, N = 995), while imidazole is

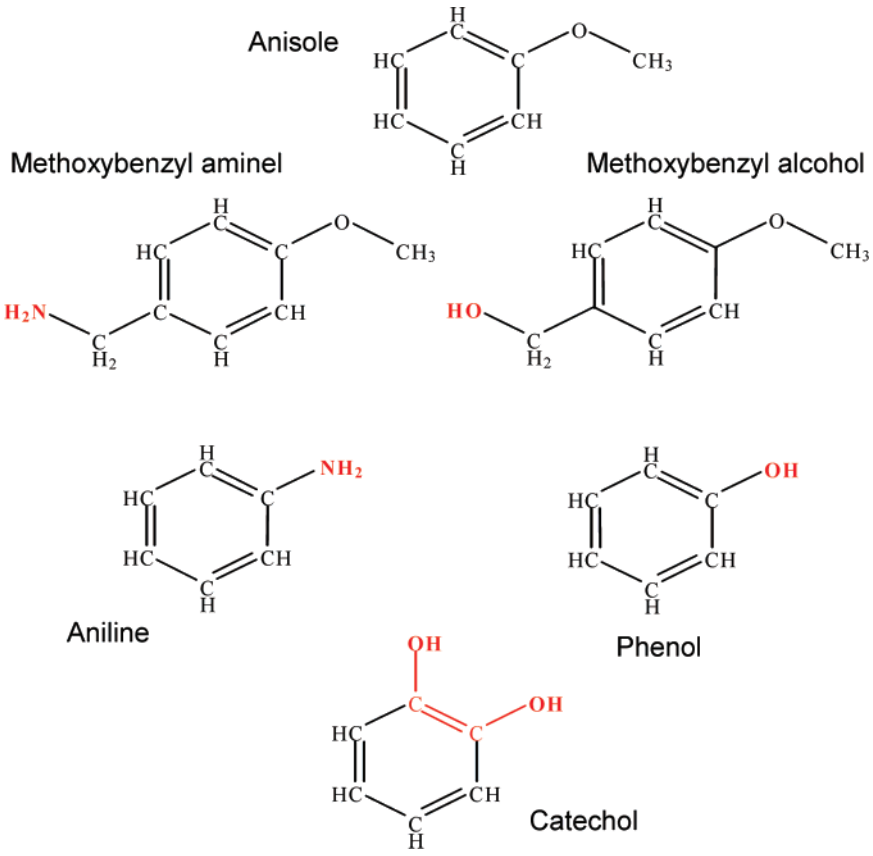

Figure 4. Compounds of test set 3.

Table 3. Summary Chromatographic Behavior of the Test Set 3 under Decreased H_2O Content Conditions

	k values			
compound (order of 95:5)	80:20 (ACN/H_2O)	90:10 (ACN/H_2O)	95:5 (ACN/H_2O)	100% ACN followed by water gradient
anisole	t_0	0.04	0.03	t_0
aniline	0.15	0.10	0.10	0.10
4-methoxybenzyl alcohol	0.03	0.09	0.11	0.26
phenol	0.06	0.12	0.15	0.33
catechol	0.18	0.24	0.68	4.341
4-methoxybenzylamine	trapped	trapped	trapped	trapped

only released when the H_2O gradient is applied (k = 4.1, N = 22 389). These results provide another indication that the Lewis basicity of the nitrogens is involved rather then the possibility to form boramide bonds. In this context, the considerable retention of Pur can be explained, since the N(7) position in the purine nucleobase is known to be a Lewis basic site. The plate numbers show that most of the compounds trapped under 100% ACN are instantaneously released when H_2O is introduced (with the exception of 4-methoxybenzylamine). The H_2O gradient was next replaced with a gradient using 0.1 M NH_4OAc, pH 8.8, but no group selection was obtained (data not shown); however, elution of 4-methoxybenzylamine was finally demonstrated. This suggests that hydroxide ions (OH^-) compete more adequately with the aliphatic amine than H_2O. This behavior can once more be explained in terms of Lewis basicity with the more basic species exhibiting higher affinity toward the stationary phase.

In Silico Modeling Calculations for Boronate Chromatography. In order to obtain further insight into the nature of the different interactions in silico modeling, calculations were performed (Figure 5). A computational study of the mechanism of the cyclic boronic ester formation from a diol and boronic acid has been reported.[25] The current modeling therefore focuses on the interactions between nucleobase moieties and phenylboronic acid. Prior to the DFT calculations, MP2/6-31G(d) calculations where undertaken in order to study the interaction of methylboronic acid with H_2O, NH_3, and HO^- (data not shown). Because the results of the B3LYP and MP2 calculations show very similar results for these systems, the B3LYP method was used for the larger systems. Only the results obtained at the B3LYP level are given. Consistent with the electronic properties of the boron in the boronic acid, it is deduced that the boron is electron deficient, creating an active site for Lewis bases. At the same time, the above results point to interactions involving nitrogen irrespective of its feasibility to form boramides. Therefore, **B—N** interactions are expected to account for the observed retention tendencies. N^9-methyladenine (m^9Ade) and N^9-methylguanine (m^9Gua) were chosen as model compounds to calculate eventual interactions of the nucleobase moieties with the boronic acid functionality. The

Table 4. Summary Chromatographic Behavior of Nucleosides and Related Compounds under Decreased H_2O Content Conditions[a]

	k values			
compound (order of 95:5)	80:20 (ACN/H_2O)	90:10 (ACN/H_2O)	95:5 (ACN/H_2O)	100% ACN followed by water gradient
dThd	nd	nd	nd	1.04
Pur	0.34	0.43$^{(NMP)}$	0.93	3.40
Neb	0.17	0.47	1.14	4.67
Urd	0.17	0.47	1.33	4.59
dAdo	0.26	0.58	1.44	4.34
Hyp	0.38	0.73$^{(NMP)}$	1.92$^{(NMP)}$	4.65
Xao	n.d.	0.70$^{(NMP)}$	2.11$^{(NMP)}$	4.28
Ado	0.35	0.79	2.61$^{(NMP)}$	4.81
Ade	0.54	1.08$^{(NMP)}$	2.82$^{(NMP)}$	5.06
Ino	0.41	0.806	3.1$^{(NMP)}$	4.93
dGuo	0.30	0.98$^{(NMP)}$	3.41$^{(NMP)}$	5.07
ψrd	0.36	1.00	3.59$^{(NMP)}$	4.95
Cyd	0.45	1.31$^{(NMP)}$	nd	5.03
Guo	0.54(?)	1.41	nd	5.21

[a] nd, no data available. Superscript (NMP), compound made up in NMP.

N^9-methyl derivatives are more suited than the common nucleobases adenine and guanine since they exclude any improper interactions via the N(9)H. The use of a methyl group instead of the ribosyl moiety reduces the required computational effort as the optimization of the normally present sugar part is rendered unnecessary. The interactions between methylboronic acid (CH_3–$B(OH)_2$) with the simple Lewis bases H_2O, NH_3, and HO^- were also investigated. For all interactions, geometry optimizations were initiated starting from different relative orientations. The nature of the resulting equilibrium geometries was confirmed by calculating the corresponding Hessian and the vibrational frequencies involved. The obtained equilibrium geometries are summarized in Figure 5. Unexpectedly, only one of the optimized geometries (interaction with HO^-) shows the expected interaction of boron with oxygen; in the other geometries (interaction with H_2O,NH_3), only interactions via the hydroxyls of the boronic acid part are apparent. These results contrast profoundly with the general accepted concept that the boron in the boronic acid is the preferred site of interaction: in agreement with the trigonal

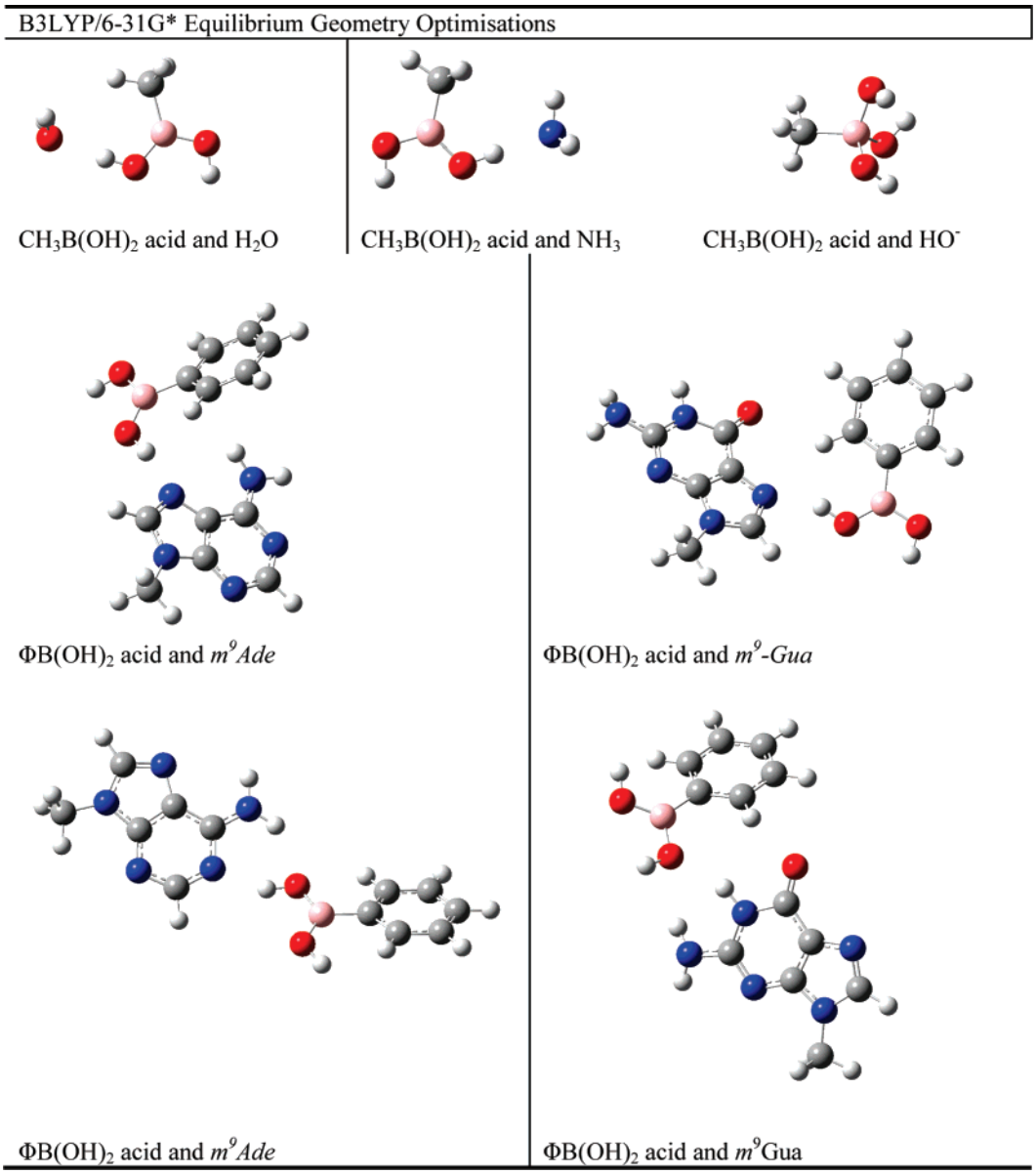

Figure 5. B3LYP/6-31G* derived interactions of interactions between boronic acid functionalities and model compounds.

configuration of boronic acid and the electron deficiency of boron, the Lewis acid properties are thought to prevail. Both H_2O and NH_3 exhibit a strong hydrogen bond with the protons of the boronic acid−H_2O, 1.847 Å, and NH_3, 1.840 Å. Accordingly, the two B**O**−**H** distances in boronic acid differ. For the H_2O adduct, this results in the respective distances of 0.9699 Å for the free B**O**−**H** and 0.9786 Å for the hydrogen-bonded B**O**−**H**EnDash−X. For the NH_3 adduct, this gives 0.9702 (free) and 0.9901 Å (hydrogen bonded). Only hydroxide (HO^-) gives rise to a tetrahedral boron. From the resulting B−O distances, i.e., **B**$^-$-EnDash−**O**H, 0.968 28 Å, and the B(-**O**−**H**)$_3$ distances, i.e., 2.208 Å, it can be concluded that interaction of hydroxide with the boronic acid functionality is driven by the advantageous cyclic stabilization characterized by cooperative effects rather than by the electron-deficient nature of the boron. This favorable complexing of hydroxide with the boronic acid functionality can rationalize the experimental behavior of 4-methoxybenzylamine seen under aprotic loading conditions. Being an aliphatic amine, it will behave similarly to NH_3 resulting in a strong donor−acceptor interaction with boronic acid. Application of the weaker H_2O Lewis base does not result in its elution; only the application of HO^- (0.1 M NH_4OAc, pH 8.8) enables its release. Therefore, with the neutral elution conditions, only intermolecular hydrogen bonds with the **B**−**O**H occur (i.e., B−O**H**−**O**), whereby H_2O is not adequately competitive with the aliphatic amine to induce the latter's elution. However, with the introduction of HO^-, an additional **B**$^-$−**O**H is formed, and all hydroxyl groups are involved in intramolecular hydrogen bonds. At this point, the B−O**H** hydrogens are no longer available for intermolecular H-bonding with 4-methoxybenzylamine, resulting in its elution.

For both m^{9}Ade and m^{9}Gua, two different sites of interaction are derived from the modeling calculations. Both m^{9}Ade and m^9-Gua are involved in a donor−acceptor interaction between a hydrogen of hydroxyborane moiety and the lone electron pair at the N7 of the two purines [B−O**H**−**N**7]. The latter interaction site agrees well with the above conclusions derived from the experimental data. Another favorable interaction site follows from the geometry optimizations: cyclic interaction patterns are seen when the phenylboronic acid interacts via the pyrimidine part of the purine bases. For m^{9}Ade, two distinct hydrogen bonds are found: a hydrogen of the exocyclic NH_2 at C6 interacts with an oxygen of the boronic acid [C(6)-(H)NH−O(H)-B] and a hydrogen of the hydroxyborane shows a hydrogen bond with the N1 of the adenine [B−O**H**−**N**1]. The interactions between the pyrimidine part of m^{9}Gua and phenylboronic acid are slightly different: an oxygen of the boronic acid interacts (donor−acceptor) with both the hydrogen of N1(H) and a hydrogen of the exocyclic NH_2 at C(2). It is important to note that none of these calculated interactions corresponds with the anticipated interaction via the Lewis acidic boron, solely hydrogen bonds are apparent. These computational data support the experimental derived conclusions that in aprotic conditions the interactions between boronic acid functionalities and nucleobase moieties are governed by the Lewis basic characteristics of the latter. Donor−acceptor interactions prevail between the hydroxyl groups of the boronic acid functionality and the nucleobase.

CONCLUSIONS

The aim of this study was to investigate the retention properties of boronate affinity HPLC stationary phases in order to consider their utilization in an on-line trapping system prior to HPLC−ESI MS analysis. The first data showed that *cis*-diol moieties aided retention although electrostatic interactions can adversely affect this retention. The availability of an exocyclic NH_2 group increased while a lactam functionality reduced retention due to the inherent acidity of the lactam group.[33,34] A decreased flow rate aids the separation process, and the addition of Mg^{2+} improves the selectivity toward *cis*-diol-containing compounds without causing definitive trapping. Under aprotic/decreased H_2O conditions, the aqueous solvent has a detrimental effect on nucleoside retention. However, cyclic reversible boronate ester formation is possible when using 100% aprotic conditions and release of the *cis*-diol compounds was easily achieved by protic solvent. Under aprotic conditions, no significant contribution to the retention of hydroxy groups is observed in contrast to amino and imino groups. The apparent Lewis basicity of the functional groups contributes more to the retention mechanism than their aptitude to form boramides. It is deduced that retention of ribonucleosides can either be enhanced or diminished by the nucleobase rather than the presence of a *cis*-diol moiety. The dissonant retention behavior on the HPLC boronate phases compared to the selective retentions achieved with Affi-Gel 601 may be attributed to cumulative effects such as linear flow rates (better equilibrium establishments), active sites (number boronic acids/mL phase), and maybe some nonboronic support effects. Finally, DFT calculations were made in an attempt to gain more insights into the underlying mechanisms, and the computed data fit well with the experimental conclusions.

It is concluded from the experiments outlined that trapping of the nucleosides on the boronate affinity columns is possible only in 100% ACN conditions and this retention is not specific to *cis*-diol-containing compounds. This of course raises further challenges for the materials use in a high-throughput, mass spectrometric, urinary nucleoside detection system.

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